The Role of Endocannabinoids in Early Pregnancy

A thesis submitted for the degree of
Doctor of Medicine

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Abstract

The endocannabinoid N-arachidonoylethanolamine (anandamide; AEA) adversely affects early pregnancy initiation and maintenance in both animals and humans. Anandamide acting through the cannabinoid receptors CB_1 and CB_2 affects many aspects of reproduction and is formed on demand by the hydrolysis of N-arachidonoyl-phosphatidylethanolamine (NAPE), by a specific phospholipase D (PLD) NAPE-PLD. Furthermore, AEA is catabolised by Fatty Acid Amide Hydrolase (FAAH). The aim of this thesis was to investigate how variations in the various components of the endocannabinoid system might lead to different early pregnancy outcomes.

Using a newly developed UPLC-MS/MS method, plasma AEA levels were measured in women throughout pregnancy, compared to previous data and shown to be comparable. Moreover, women presenting with a viable pregnancy (on USS) with a threatened miscarriage (bleeding), who went on to miscarry and women presenting with a confirmed miscarriage (on USS) when compared with women with a viable pregnancy at the time of USS in whom there were no symptoms of miscarriage or those that went to term, all had increased plasma AEA levels. Median plasma AEA levels in the miscarriage group were 3-fold ($p<0.001$) higher than the levels in the live birth group. Using an AEA level of 2.0nM as cut-off for predicting subsequent miscarriage gave a sensitivity of 100% and a specificity of 94%, (negative predictive value 100%; positive predictive value 82%). Although other markers of pregnancy success (progesterone, hCG and PAPP-A) all gave the expected concentrations in on-going, failed and failing pregnancies, there was no statistically significant correlation between these factors and plasma AEA levels.

Immunohistochemical evaluation of CB_1, CB_2, FAAH and NAPE-PLD expression in first trimester pregnancy tissues (trophoblast and decidua) showed that FAAH staining in the miscarriage/non-viable group was significantly lower than that in the surgical termination of pregnancy group. In women undergoing medical termination of pregnancy, using an anti-progesterone (mifepristone), FAAH staining was actually increased compared with that from the surgical termination of pregnancy group; whilst CB_2 had increased staining and CB_1 had reduced staining in the miscarriage and the medical termination groups when compared with the surgical termination of pregnancy group. There was no significant difference in NAPE-PLD staining between all three groups.

These data provide new evidence that the endocannabinoid system plays a significant role in early pregnancy failure/miscarriage and that plasma AEA may be used as a future predictive marker for miscarriage. The lack of correlation between AEA and progesterone and the finding of elevated FAAH in the medical miscarriage (anti-progesterone) group, but low FAAH in spontaneous miscarriage suggest that factors other than progesterone are responsible for increased AEA levels in cases of miscarriage.

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1. Early Pregnancy Failure is Characterised by Elevated Plasma Levels of Anandamide and Inhibition of Trophoblast Cell Growth
Blair Bell Competition Meeting, Royal College of Obstetricians and Gynaecologists, London, December 2007.

2. Plasma levels of Anandamide are Elevated in Early Pregnancy Failure
Annual Scientific Meeting of the Society for Gynecologic Investigations, San Diego USA, March 2008.

CHAPTER 1

Introduction

1.1 Reproduction and Miscarriage

Miscarriage is the most common adverse outcome of pregnancy, and poses a huge burden on society with associated pain, suffering and medical costs (Kline et al., 1989). Consequently a better understanding of the causes and attempts to reduce the rate would be of great benefit.

Miscarriage is defined as pregnancy loss before viability is reached. The World Health Organisation defines viability, 'as 22nd week of gestation or more, or when the fetus weighs more than 500g', however in the UK 24 weeks is generally taken as the limit of viability. Miscarriage is divided into two distinct entities, i.e. first trimester (before 12 weeks), and second trimester (12-viability), mainly due to their different aetiologies. First trimester miscarriage is by far the most common, accounting for the vast majority of the 10-20% of clinically recognised pregnancies that will fail. The actual number of pregnancies lost is substantially higher, as around 75% of all losses occurr prior to implantation (Wilcox et al., 1988, Norwitz et al., 2001a) and therefore before the expected menses, i.e. going unrecognised. The statistics above show how common a problem miscarriage is, and how it is likely to impact either directly or indirectly on the lives of most people at some point.

Any attempt to study miscarriages and how they maybe prevented must firstly start with a thorough the understanding of early pregnancy.

1.1.1 Early Pregnancy

Successful pregnancy and childbirth is essential for the survival of the Human Race. Although it is difficult to accurately estimate the number of pregnancies, due to miscarriages and abortions. The annual worldwide birth rate is estimated to be 133,121,000 and for England and Wales it is 639,721 (Nat Office Statistics 2004). It is therefore surprising that for such a frequent event, the processes involved in early human pregnancy are not yet fully understood, with many questions remaining unanswered. These include how the 'foreign' embryo evades the maternal immune system and how the fertilised ovum interacts with the maternal endometrium prior to and during implantation. One reason for the unanswered questions is that much of our understanding comes from animal work and models, as practical and ethical issues limit research using human pregnancy tissues. The fact that different species have very different processes of implantation and embryonic development means that only a limited amount of information that can be extrapolated to humans can be gained from these sources.

1.1.2 Implantation

1.1.2.1 Physical Process

Pregnancy begins with the fertilisation of an egg by a sperm, which in humans normally occurs in the ampulla of the Fallopian tube 24 to 48 hours after ovulation (Eddy and Pauerstein, 1980). The egg may be held for up to 38 hours prior to fertilisation in the ampulla, meaning that intercourse need not be exactly synchronised with ovulation. The fertilised ovum is then moved towards the uterine cavity by muscular and ciliated activity in the walls of the Fallopian tube, entering the uterine cavity at 4-5 days post-

ovulation. During this time it undergoes several cell divisions, becoming the morula. (Figure 1) (Dean, 1983).

This consists of 16-32 cells closely packed so that it is not possible to discern individual cell outlines. The cells of the morula subsequently separate into two layers, an inner and an outer cell mass. The inner cell mass goes on to form the fetus, whilst the outer epithelial layer of cells, known as the trophoectoderm, goes on to form the placenta and extra-embryonic membranes. When the ovum has differentiated into these two different cell types it becomes known as the blastocyst, and it is this that undergoes implantation in the uterus (Dean, 1983).

Implantation starts with apposition, when the blastocyst lies against the endometrium, the inner cell mass orientated towards its surface. At this point the embryo can no longer move around the uterine cavity, but physical implantation has not begun. This initial contact is thought to be aided by bulbous apical protrusions from the luminal epithelium of the endometrium, called pinopods, which interdigitate with microvilli on the trophoblast (Norwitz et al., 2001a).

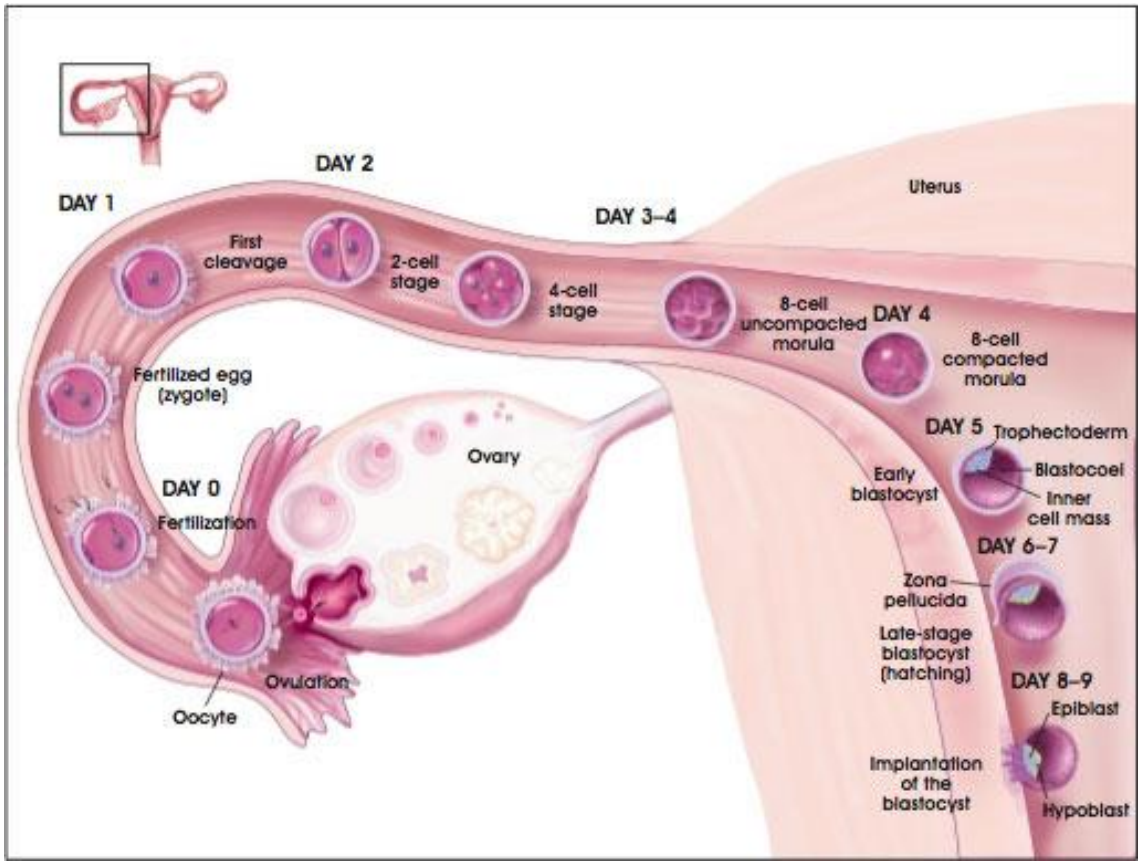

Figure 1.1 Early embryonic development, showing the series of divisions from the fertilised egg to the morula as the embryo travels from the ampulla to the uterine cavity.

[From: Development of the Preimplantation Blastocyst in Humans. National Institutes of Health, U.S. Department of Health and Human Services (© 2001 Terese Winslow)]

After apposition, the next stage is for the blastocyst to gain stable adhesion to the endometrium and then implant, which normally occurs on the 6-7th day after ovulation. To aid invasion into the endometrium, the trophoblast produces proteolytic enzymes and the basal trophoectoderm, known as the cytotrophoblast, divides rapidly forming a more superficial layer called the syncytiotrophoblast, a true syncitium which invades the endometrium, 'eating' into the uterine wall, and an inner layer of larger mono-nuclear cells with well defined limiting membranes, known as the cytotrophoblast. By the 10th day after ovulation the embryo has completely burrowed into the endometrium, with a fibrous plug closing the 'defect' left. The embryo now begins to gain nutrients from the surrounding endometrium, and is already producing human chorionic gonadotrophin (hCG).

From day 10-13 post-ovulation a series of intercommunicating clefts, called lacunae, develop within the trophoblast layer. These become partially confluent, forming the precursor of the intervillous space, progressively eroding the maternal vessels to fill the spaces with maternal blood. Between days 14-21 the lacunae become radially orientated, with a central cellular core produced by the proliferation of the cytotrophoblast from the chorionic base into the syncytiotrophoblast. These cytotrophoblast columns act as the basis for the formation of the villous tree. The columns become vascularised with mesenchymal vessels that extend from the chorioallantoic arteries, which go on to become primary, secondary and tertiary villi. The distal part of these columns does not become vascularised, but becomes anchored to the decidua of the basal plate. By day 21, the placenta is a vascularised villous organ.

Once formed the chorionic sac is composed of (from in to out) (Figure 1.2):

1. Extra-embryonic somatopleural mesoderm – chorionic plate
2. Cytotrophoblast
3. Syncytiotrophoblast through which maternal blood flows
4. Maternal tissue – decidua

The success of implantation described above is the end result of complex molecular interactions between the hormonally primed uterus and a mature blastocyst, while multiple signals synchronise the division of the blastocyst and the preparation of the uterus for implantation at the most appropriate time (implantation window).

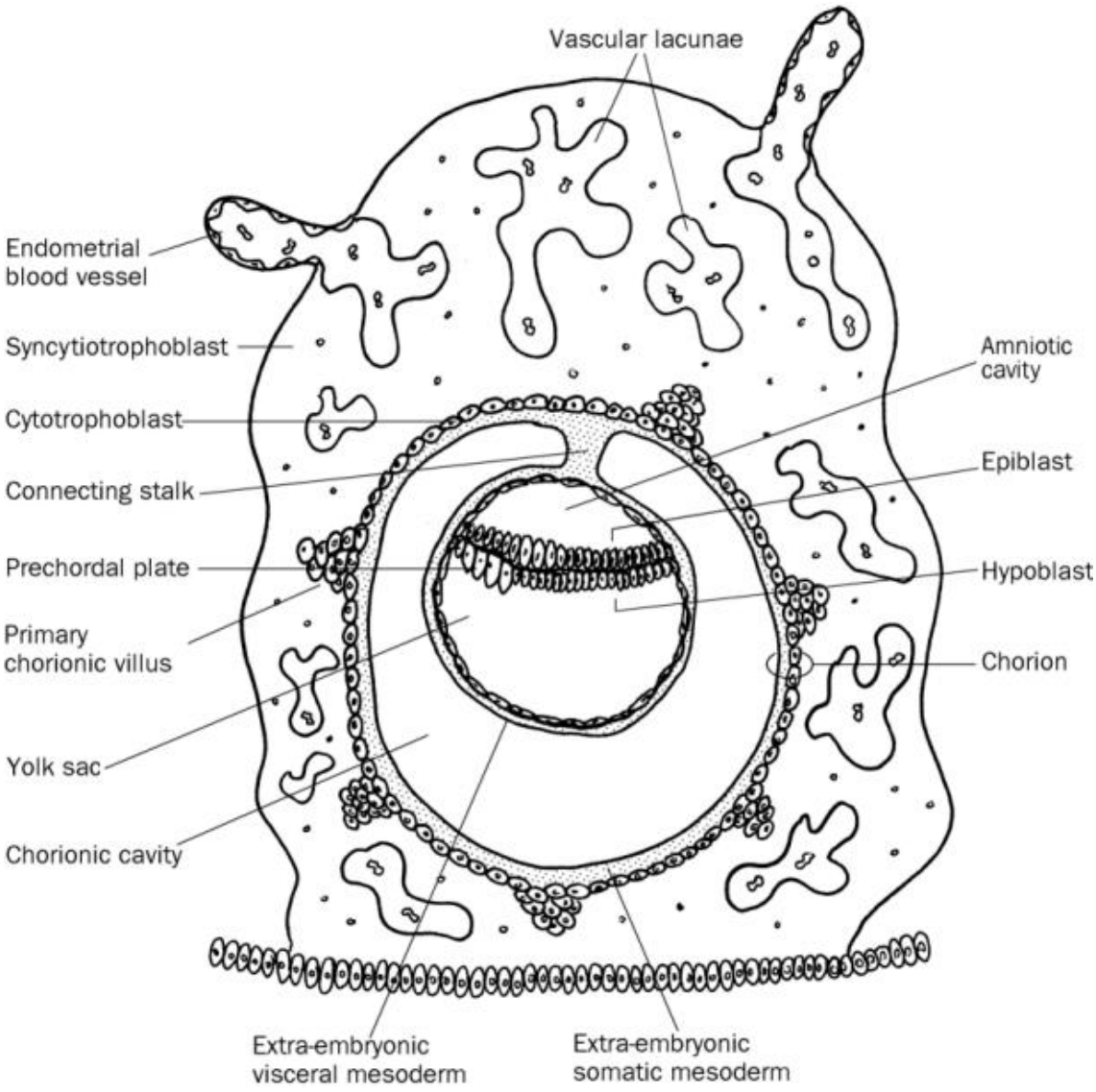

Figure 1.2. Day 14 blastocyst showing structure of the developing placenta. The chorionic sac from in to out is composed of: extra-embryonic somatopleural mesoderm – chorionic plate; cytotrophoblast; syncytiotrophoblast through which maternal blood flows; maternal tissue – decidua

1.1.2.2. Hormonal Process during Early Pregnancy

The need for an 'implantation window' is to ensure that implantation occurs when conditions are optimal for success; usually between days 20 to 24 of a regular 28 day menstrual cycle. Although the role of steroids in the synchronisation process has been extensively investigated, it is clear that this signalling cascade between the blastocyst and uterus involves several biological messengers, which are yet to be thoroughly investigated. It is this area, which the research undertaken hopes to shed new light on. As detailed later, it appears that the endocannabinoid system plays a vital role in the embryo – maternal signalling process, with disruption of this leading to failure of the pregnancy. However, although important, endocannabinoids are not alone in regulating this process and a wide spectrum of messengers and systems appear to take part.

The role of hCG in early pregnancy is the most widely studied, leading to its use in clinical practice to monitor early pregnancies. hCG is secreted by the embryonic trophoblast, helping to maintain the corpus luteum. Abnormal hCG production could be a result of a reduced trophoblast cell number, inhibited growth rate of the blastocyst, ectopic implantation, corpus luteum insufficiency, failure or inadequate penetration of the trophoblast into the decidua or an inadequate villous structure (Wilcox et al., 1988). However, although hCG is vital to early pregnancy development and maintenance of an ongoing pregnancy, it does not synchronise or facilitate implantation (Norwitz et al., 2001a). The steroid hormones oestrogen and progesterone, are well known to play an important role in ovulation, and subsequently help prepare the endometrium to receive the blastocyst during the second half of the menstrual cycle. They exert their actions via nuclear receptors, which when absent in mutant mice lead to infertility. Progesterone, produced by the corpus luteum, promotes proliferation and differentiation of stromal

and epithelial cells within the endometrium. It also plays a role in maintaining myometrial quiescence, possibly by up regulating nitric oxide synthesis, with progesterone antagonists inducing abortion, as does the removal of the corpus luteum (Norwitz et al., 2001a).

Integrins, carbohydrate moieties and their receptors, and other cells surface molecules participate in an adhesion cascade to anchor the blastocyst to the implantation site (Sharkey, 1998, Carson et al., 2000).

1.1.2.3 Growth Factors and Cytokines

Numerous growth factors and cytokines have been implicated as having a role in implantation, including leukaemia inhibitory factor (LIF) and interleukin ll, LIF being one of the most widely studied. LIF levels are maximal during the mid-secretory phase of the menstrual cycle, i.e. the 'implantation window', and low levels have been found in women with recurrent miscarriages. Also mice deficient in LIF will ovulate, but all have implantation failures (Paria et al., 2002a). Interleukin ll is thought to be primarily involved in decidualisation, because this process fails in mice deficient in the interleukin ll receptor (Paria et al., 2002a).

Epidermal growth factors (EGF) and EGF-like factors appear to play a role in the process of implantation, with heparin-binding EGF-like factor (HB-EGF) being the earliest marker of implantation in mice. It is expressed in the uterine luminal epithelium around the pre-attachment embryo in the mouse, and has also been isolated in the human uterus, and stimulates growth in IVF embryos (Paria et al., 2002a).

Prostaglandins, derivatives of arachidonic acid, may act as a common downstream pathway for many substances, via the action of the enzymes cyclo-oxygenase 1 and 2 (COX 1 and COX 2) that are the rate-limiting step in their synthesis. In fact genetic models suggest that COX 2 acts as a common pathway for LIF and HB-EGF. Homozygous COX 2 knockout [COX 2 (-/-)] females have multiple pregnancy failures; including ovulation, fertilisation and implantation failure, whilst when present COX 2 appears to be restricted to implantation sites within the endometrium. However, the concentration of prostaglandins found in the decidua is less than that found in the endometrium during the menstrual cycle, and exogenous prostaglandins induce abortion at any point in gestation. It is thought that to maintain a pregnancy, prostaglandin levels need to be tonically suppressed (Giudice, 1999, Wang and Dey, 2005).

Different studies have demonstrated that a dialogue occurs between the implanting blastocyst and the endometrium and this involves several factors whose exact role is not fully understood e.g. steroid hormones, lectins, integrins, cytokines and growth factors (Giudice, 1999).

1.1.2.4. Endocannabinoids

Most of the messengers/chemicals discussed above are directly involved in either the development of the endometrium or the blastocyst. Precisely what modulates a dialogue between the blastocyst and the uterus to instigate the changes that regulate the timing of implantation is uncertain. The endocannabinoid system has been suggested as perhaps one of the most important signalling pathways between the blastocyst and uterus. Work done in early human pregnancy looking at anandamide [AEA], the main

endocannabinoid, have indicated that elevated levels of this endocannabinoid appear to correlate with subsequent early pregnancy failure (Maccarrone et al., 2000b, Maccarrone et al., 2002a, Habayeb et al., 2008b). Most of the work on endocannabinoids has been in animals, and has also pointed to the endocannabinoid system being important in the development and maintenance of early pregnancy [see section 1.4.2].

Irrespective of the controlling mechanism or factors modulating for early pregnancy success and maintenance, what is certain is that disruption in these processes at any early stage results in early pregnancy failure or miscarriage.

1.2 Miscarriage

1.2.1 Aetiology of Miscarriage

In the majority of cases there is no obvious cause for a miscarriage occurring, and it is often put down to chance or 'bad luck'. However, there must have been a problem present to prevent the normal development of the pregnancy and subsequent miscarriage. These possible problems are not routinely investigated, because the high incidence of miscarriage would necessitate the investment of huge amounts of time and money to implement in all women presenting with miscarriage. Further investigations are therefore commonly offered to those with recurrent miscarriages (RMC). RMC is traditionally defined as 3 or more consecutive pregnancies lost before viability, although increasingly women with 2 miscarriages are being investigated (Potdar and Konje, 2005b). Using 3 miscarriages as the cut off means around 1% of women are affected (Potdar and Konje, 2005b). Causes include implantation problems, ovofetal problems, and maternal problems. Despite these extensive investigations, around 50% of the causes remain unexplained (Potdar and Konje, 2005b).

1.2.1.1 Implantation problems

Implantation of the embryo into the uterine endometrium occurs 8-10 days after ovulation, and requires interaction between the embryo and the endometrium (see above). This interaction appears to be complex involving numerous signals, the exact mechanisms not being fully understood (the possible nature of these interactions and signals will be explored in Chapter 4). It appears that any disruption in the interaction,

or delay in the timing of the implantation beyond the 8-10 day 'implantation window' could lead to miscarriage early in the pregnancy.

1.2.1.2 Ovofetal Factors

Chromosomal abnormalities are the commonest cause for miscarriage, with around 60% of miscarried fetuses having abnormalities present (Garcia-Enguidanos et al., 2002). The commonest genetic problems encountered are autosomal trisomies, which, in order of frequency, are trisomy 16, 22, 21 (Garcia-Enguidanos et al., 2002). The occurrences of these abnormalities are usually random events, which do not have a bearing on the success of subsequent pregnancies. The main factor influencing the incidence of chromosomal abnormalities is maternal age, and to a much lesser degree, paternal age, the risk increasing with age. In some cases, fetal chromosomal abnormalities may be due to structural abnormalities in the parental chromosomes, although the incidence of carrier status after one miscarriage is only around 2.2% (De Braekeleer and Dao, 1990, Garcia-Enguidanos et al., 2002).

1.2.1.3 Maternal Factors

The umbrella 'maternal factors' covers many conditions, but in combination these only account for the cause of a small proportion of miscarriages. Structural defects such as uterine fibroids and uterine septum are known to increase the risk of miscarriage. These tend, however, to be problems of the 2nd trimester, where as the pregnancy expands, the uterine cavity and compromised uterine space becomes an issue (Garcia-Enguidanos et al., 2002).

The other main area encompassed by maternal factors is systemic [autoimmune] diseases such as Systemic Lupus Erythromatosus (SLE) and anti-phospholipid syndrome, which has been associated with miscarriage rates of 70% (Pattison et al., 1993, Jauniaux et al., 2006). Maternal factors also include drug use, both legal and illegal, and others such as cigarette smoking which is known to significantly increase miscarriage rates (Gibson et al., 1983, Makrydimas et al., 2003).

1.2.2 Mechanisms of miscarriage

The majority of miscarriages occur within a few weeks after the death of the embryo. The immediate cause is haemorrhage into the decidua basalis, with necrosis and inflammation in the region of implantation. The gestational sac then becomes partially or completely detached, followed by vaginal bleeding, uterine contractions, and dilation of the cervix. The pregnancy may be expelled *en mass* with the embryo covered by villi and decidua, or the membranes may rupture and the embryo and placenta passed separately. Following expulsion of the products of conception, the uterine contractions and bleeding gradually subside and the cervix closes.

1.2.3 Varieties of Miscarriage

1.2.3.1 Threatened Miscarriage

This is defined as vaginal bleeding, with or without pain, but with an ongoing pregnancy. The bleeding that occurs varies from a few brown spots to heavy fresh bleeding, but is usually relatively light. When these women are examined they have a

bulky uterus consistent with early pregnancy, and a closed cervical os. The definitive diagnosis is only made by ultrasound scan (USS) when a viable fetus with a visible heartbeat is seen. Once viability has been confirmed, the outlook is good with over 90% of pregnancies continuing (Choong et al., 2003). Problems often arise in these patients when they present with symptoms early in the first trimester, i.e. from 4 to 5 weeks gestation. At this time elements of the pregnancy, such as the gestation sac, yolk sac, fetal pole, can be visualised by USS but often no fetal heartbeat can be seen. This is due to the fact that the embryo is too small at this point, and a repeat USS is needed, usually after 1 week. Human chorionic gonadotrophin (hCG) can also be used to assess whether the pregnancy is likely to be viable in the absence of USS reassurance, with levels in viable pregnancies expected to double every 48 hours (Ho et al., 1997). However, it is only USS that can give a definitive answer. In some cases the inability to confirm viability by USS may be indicative of failure of the pregnancy. In this case the diagnosis would not be of a threatened miscarriage, but would instead fit into one of the categories below.

1.2.3.2 Inevitable, Incomplete and Complete Miscarriage

These three types of miscarriages all have the same endpoint of pregnancy loss, and are associated with vaginal bleeding and abdominal pain. The bleeding experienced again varies, but in general is relatively heavier and the pain more severe than that associated with a threatened miscarriage. Inevitable miscarriage encompasses women who on presentation are found to have an open cervical os, often with some products of conception (POC) protruding through. In this case the pregnancy is still '*in utero*' but due to the open cervix will inevitably miscarry, and supportive measures are all that can

be offered. If the bleeding is profuse, medical or surgical intervention to remove the POC may be needed (see later). Some products may be passed spontaneously, with some retained in the uterus, resulting in an incomplete miscarriage. The remaining products may then either be expelled spontaneously or may require medical or surgical intervention to remove them (see later). Just like inevitable and incomplete miscarriage, complete miscarriage presents with pain and bleeding, but this wound usually have peaked and consequently be less severe at the time of presentation. A pelvic examination is characterised by a closed cervical os, and USS shows an empty uterine cavity, as all of the products would have been expelled.

1.2.3.3 Missed Miscarriage

Missed miscarriage describes the scenario when USS shows a non-viable embryo that has a significantly lower gestational age than expected, i.e. the embryo died some time before the scan. These women may present with a small brown/red vaginal loss, or diminishing early pregnancy symptoms, such as 'morning sickness'. The reason why expulsion does not occur spontaneously is unclear, but these women often need medical intervention, as leaving the fetus *in-situ* poses the risk of developing infection and theoretically coagulation defects after more than 2 weeks.

1.2.4 Management of Miscarriage

There are three options for the management of inevitable, incomplete and missed miscarriage. Which of these options is offered to the patient depends upon her clinical condition and her wishes.

1. Conservative Management – this is often seen as ‘letting nature take its course’, and involves giving time for the POC to be expelled spontaneously by the patient. If the patient is bleeding heavily then this method is not an option, but otherwise supportive care is given with a repeat USS after 2 weeks to confirm that the POC have been expelled. If some remain, heavy bleeding occurs in the interim or the clinical condition of the patient changes, 2 other options are available (see below).
2. Medical Management – this entails using medication to bring about passage of the remaining POC. Mifepristone, a progesterone antagonist is given orally to the women, followed 48 hours later by misoprostol, a synthetic prostaglandin, which stimulates uterine contractions and expulsion of the POC.
3. Surgical Management – surgical management is referred to as ‘evacuation of retained products of conception’ or ERPOC. This involves the use of a suction curette passed through the cervix with aspiration of the POC from the uterine cavity. It is used both as an elective procedure at the patients request, and in emergency situations were bleeding is posing a risk to health.

(Sotiriadis et al., 2004, Chen and Creinin, 2007)

1.2.5 Recurrent Miscarriage

A woman is said to suffer recurrent miscarriage (RMC) when she has had three consecutive pregnancy losses before 24 weeks gestation (Habayeb and Konje, 2004). This distressing condition affects around 1% of women, a figure higher than would be expected by chance alone, given a miscarriage rate 15% of clinically recognised pregnancies should give a risk of 0.34% due to chance alone (Jauniaux et al., 2006).

Some of these women will therefore have an underlying condition that leads to a higher rate of miscarriage. As detailed previously, there are a number of possible causes for miscarriage, but these are only usually looked for in women with recurrent miscarriage. In those with recurrent miscarriage, only around 50% will have an identifiable cause found for their problem (Potdar and Konje, 2005b).

Women who suffer with recurrent miscarriage undergo a series of investigations to try to elicit a possible cause for their pregnancy losses. The evidence for some of the investigations is lacking, but as there is a wide variation between Units as to which investigations are performed, it is worth mentioning the main ones (Habayeb and Konje, 2004).

Coagulation Investigations

Acquired maternal thrombophilia is a recognised cause of recurrent miscarriage. Screening involves testing for lupus anticoagulant and anti-cardiolipin antibody, known together as anti-phospholipid antibodies, looking for the presence of antiphospholipid syndrome (Jauniaux et al., 2006). The role of inherited thrombophilias (e.g. Factor V Leiden deficiency, protein S, C deficiency) is unclear due to conflicting evidence from studies of RMC patients (Jauniaux et al., 2006).

Endocrine Investigations

The role of testing for thyroid dysfunction and diabetes mellitus in women with RMC remains unclear, and these are not recommended as routine investigations by the RCOG, due to lack of evidence (Mannavola et al., 2004). Having said that, Obesity, known to be associated with various endocrinopathies is associated with a statistically

significant increase in miscarriage and recurrent miscarriage (Metwally et al., 2007, Metwally et al., 2008).

Immunological Investigations

Excessive maternal immune response to paternal antigens on the embryo is thought to be a contributor to recurrent miscarriage (Quenby and Farquharson, 2006). In this regard much interest focused on the role of natural killer (NK) cells. However, routine investigation of immunological cells including peripheral NK cells has not been of proven benefit, and at present remains in the realms of research (Quenby and Farquharson, 2006).

Cytogenetic Investigations

These involve testing both parents, looking for structural chromosome abnormalities, usually balanced translocations. The chances of finding genetic abnormalities in the parents increase with low maternal age at the time of second miscarriage, and with a history of two or more miscarriages, in siblings or parents. Cytogenetic analysis of subsequent pregnancy losses is also undertaken. If the embryo is found to have a chromosomal abnormality, it actually improves the chance of subsequent pregnancies being successful, as the vast majority of abnormalities will be a one off event that is considered, “down to chance” (Jauniaux et al., 2006).

Anatomical Investigation

USS of the pelvis allows uterine malformations to be identified that have been associated with RMC.

1.2.6 Psychological Effects of Miscarriage

For most women and their partners miscarriage is a distressing event that provokes a grief reaction in 90% of cases. This has been shown to last more than a month in 20% of women, and indeed there is a significant association between baseline depressive symptoms and subsequent miscarriage (Sugiura-Ogasawara et al., 2002)

1.2.7 Conclusion

It is clear that the mechanisms behind the establishment and maintenance of pregnancy in humans are poorly understood, as is what fails or changes during this process to lead to miscarriage. Different factors appear to be involved including the hormones human chorionic gonadotrophin (hCG) and progesterone; cytokines such as Leukemia inhibitory factor (LIF); growth factors such as Insulin-like Growth Factor (IGF) and Epithelial Growth Factor (EGF), and Natural Killer (NK) cells (Piccinni et al., 1998, Sharkey, 1998, Paria et al., 2002a, Quenby and Farquharson, 2006). These may be part of an interwoven signalling process between the embryo and maternal tissues to ensure the success of early pregnancy. However despite extensive investigation into these factors, and may others, there is still a large gap in our understanding of the processes involved, and no reliable way to predict which pregnancies will fail and miscarry.

As mentioned in Section 1.1.2.4, endocannabinoids have been investigated as potentially playing a significant role in this process, and may prove to be a vital piece of the puzzle. Maccarrone and colleagues investigated the endocannabinoid system during early human pregnancy (Maccarrone et al., 2000b, Maccarrone et al., 2002a) and showed that the activity of the system is significantly altered in pregnancies that

subsequently miscarried. Endocannabinoids may therefore be the elusive marker to predict subsequent pregnancy outcome. The next section will therefore review the evidence for this proposition with the hope that such a review will help with the understanding of how the endocannabinoid system may be intimately associated with aspects of early reproduction.

1.3 Endocannabinoids: Biochemistry

1.3.1 'The Beginning'

Cannabis has been used in medicine for over 1000 years for treating various conditions, by different civilisations (Russo E, 2005), including the ancient Egyptians who used "hemp in honey" to induce abortions. Despite this knowledge, the nature of how cannabis produces its effects remained unclear, and it was not until 1964 that the active constituent of plant cannabis, delta-9 tetrahydrocannabinol (Δ^9-THC), was first isolated and its structure elucidated (Mechoulam and Gaoni, 1965). Initially it was thought that exogenous cannabinoids exerted their effects *via* non-specific interactions with cell membranes, due to their lipophilic nature (Roth SH, 1979, Mechoulam et al., 1998). However, the discovery of receptors specific for cannabinoids changed these views, and also raised the possibility of the existence of an endogenous ligand for these receptors, aptly termed 'endogenous cannabinoids' or 'endocannabinoids'. Although the existence of such an endogenous ligand had been suspected after the discovery of the receptor, it was almost a decade later that anandamide, the prototype endocannabinoid, was discovered (Devane et al., 1992).

Endocannabinoids can be defined as '…endogenous cannabimimetic substances capable of binding to and activating one or both of the cannabinoid receptor subtypes characterised so far…' (Di Marzo, 1998). To date, the 'Endocannabinoid System' has been considered to consist of the cannabinoid receptors, the two known subtypes being CB1 and CB2, several endogenous cannabinoids such as *N*-arachidonoylethanolamine (anandamide/AEA), N-oleoyl ethanolamine (OEA), 2-Arachidonoylglycerol 2-AG, of which anandamide was the first discovered and is the most widely studied, and the

enzymes that synthesise (N-acyl phosphatidylethanolamine phospholipase D [NAPE-PLD]) and degrade endocannabinoids (Fatty acid amide hydrolase [FAAH]).

Since their discovery, a great deal of work has been undertaken investigating the role of the endocannabinoid system in numerous physiological and pathological processes. Along with a role in reproduction, endocannabinoids have also been shown to regulate numerous neurobehavioral processes including pain, motility, cognition and feeding (Ledent et al., 1999, Zimmer et al., 1999), with potential therapeutic activity in nausea and vomiting, appetite loss, diarrhoea, asthma, autoimmune disease, fever, and glaucoma (Di Marzo, 1998, Piomelli et al., 2000, Cravatt and Lichtman, 2003). It is believed that in contrast to transient conditions, progressive or chronic disorders can result in a permanent up regulation of the endocannabinoid system (Di Marzo V and De Petrocellis L, 2006).

1.3.2 The Endocannabinoid Receptors: CB1 and CB2

The first direct evidence of a cannabinoid receptor, was its discovery in the rodent brain in 1988 (Devane et al., 1988). Subsequent work showed that the distribution of this receptor within the brain was heterogeneous, the greatest concentrations being found in the basal ganglia, hippocampus and the cerebellum (Herkenham et al., 1990). Subsequently, two subtypes of the receptor were identified, and named CB1 and CB2.

CB1 was first isolated in the cerebral cortex of rat brain (Matsuda et al., 1990), whilst CB2 was initially found in splenic tissue (Munro et al., 1993). Rat CB1 shares 97.3% sequence identity with human CB1 and 100% identity in the transmembrane region (Munro et al., 1993). CB2 shares 68% identity to CB1 within the transmembrane region

and 44% throughout the whole protein (Munro et al., 1993). Both CB1 and CB2 are G-protein coupled, seven trans-membrane spanning receptors (Munro et al., 1993). Of all known neurotransmitters and hormone receptors, the CB1 receptor is by far the most abundant in the mammalian brain (Begg et al., 2005).

Following their initial discovery, both CB1 and CB2 were subsequently identified in many different tissues throughout the body. CB1 has been isolated in the cerebral cortex, hippocampus, basal ganglia, cerebellum, cervical ganglion, peripheral autonomic fibres innervating the vas deferens, bladder and heart, the prostate, uterus, testes, small intestine, spleen and lymphocytes (Paria et al., 1995, Felder et al., 1996, Tsou et al., 1998, Pagotto et al., 2001, Park et al., 2003, Begg et al., 2005, El-Talatini et al., 2009a). CB2 has a different tissue distribution, and seems to be confined mainly to immune tissues, such as the spleen, thymus, tonsils, immunocompetent blood cells, with the highest concentrations seen in B cells, NK cells, mast cells and monocytes (Bouaboula et al., 1993, Pertwee, 1997)

In the rat fetus CB1 mRNA is found to be expressed in the neural tube , retina, autonomic ganglia, enteric ganglia, thyroid and adrenal glands (Buckley et al., 1998). CB2 mRNA is expressed exclusively in the liver.

The activation of the cannabinoid receptors, CB1 and CB2 causes several different pathways to be triggered, leading to:

- Inhibition of stimulus-induced adenylate cyclase (Matsuda et al., 1990) in a dose-dependent, stereo-selective, pertussis-toxin sensitive manner, and

subsequent impairment of cAMP / protein kinase-A mediated short and long term effects.

- Stimulation of mitogen-activated protein kinase (MAPK) signalling (De Petrocellis et al., 2004).
- Stimulation of extracellular signal-regulated kinases type 1 & 2, c-JUN N-terminal kinase, focal adhesion kinase, and protein kinase B/Akt (Bouaboula et al., 1995, Derkinderen et al., 1996, Gomez del Pulgar et al., 2000, Rueda et al., 2000, Derkinderen et al., 2001, Derkinderen et al., 2003).

In addition, activation of CB1 alone causes:

- inhibition of voltage-gated P, Q and N-type Ca^{2+} channels, and stimulation of inwardly rectifying G-protein coupled K^{+} channels (De Petrocellis et al., 2004).
- stimulation of phosphatidylinositol 3-kinase and of intracellular Ca^{2+} mobilization, seemingly through activation of Phospholipase C-γ1 (PLC–γ) (De Petrocellis et al., 2004).

CB2 may act in several different ways to CB1, because CB2 activation does not seem to cause inhibition of voltage-gated P, Q and N-type Ca^{2+} channels, and stimulation of inwardly rectifying G-protein coupled K^{+} channels.

CB1 and CB2 have also been shown to have a high level of ligand-independent activation (i.e. constitutive activity / activity independent of stimulus). It was estimated that in the population of wild-type CB1 receptors only 30% exist in the activated state, while 70% are inactive (Kearn et al., 1999).

The discovery of the endocannabinoid system reignited interest in the use of cannabinoids in the treatment of various medical conditions. Along with the cannabinoids themselves, extensive research is being undertaken, investigating ways of manipulating the endocannabinoid system as a whole. CB1 receptor antagonists have received the most attention and are the furthest along in clinical studies, with Acomplia / Rimonabant being licensed to treat obesity (Jonsson et al., 2006). Many other diseases and conditions are the subject of investigation with regards to the potential benefits of drugs which manipulate the endocannabinoid system in various ways, using products/chemicals such as Δ^9-THC, cannabis extracts, CB1 and CB2 agonists, CB1 antagonists, reuptake inhibitors and FAAH inhibitors, all being investigated (Di Marzo V and De Petrocellis L, 2006).

There has been speculation about the existence of other cannabinoids receptors, although as yet, none have been confirmed. Recently, two patents have been filed (Brown, 2001, Drmota, 2004) which indicate that the orphan G-protein coupled receptor, GPR55, may act as a cannabinoid receptor, and has been loosely labelled 'CB3', although much more work is needed for this to be confirmed. The possible existence of a non-CB1/non-CB2 receptor arose from research using receptor-specific antagonists such as SR141716A (Rinaldi-Carmona et al., 1994) , which was the first reversible CB1 antagonist discovered (Rinaldi-Carmona et al., 1994) that does not act on CB2. Rimonabant is based on SR141716A.

1.3.3 The Endocannabinoid: Anandamide

The following section briefly summarises what is known about anandamide. There are, however, several other endocannabinoids which will not be discussed in detail as they were not investigated.

1.3.3.1 Anandamide

Anandamide (*N*-arachidonoyl-ethanolamine / AEA), an amide of arachidonic acid, was the first endocannabinoid to be discovered in 1992 (Devane et al., 1992) (Figure3). The name anandamide comes from the Sanskrit word 'anand' which means bliss.

AEA is a member of the N-acylethanolamine family, a large class of endogenous fatty acid amide signalling molecules. These compounds are biosynthesised *via* a phospholipid-dependent pathway that involves the enzyme driven hydrolysis of the corresponding N-acyl-phosphatidylethanolamine, which in the case of anandamide is N-arachidonoyl-phosphatidylethanolamine (NAPE) (De Petrocellis et al., 2004). Catalysing this reaction is a phospholipase D (PLD) selective for NAPE (NAPE-PLD) with a low affinity for other membrane phospholipids. (Figure 4)

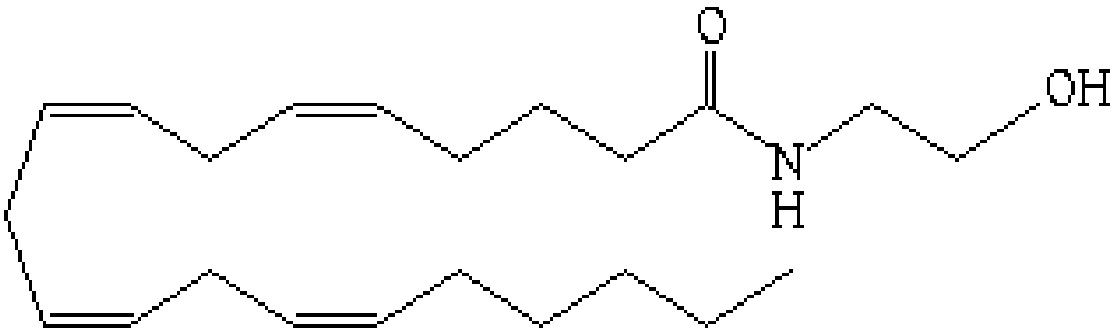

Figure 3: The chemical structure of Anandamide (*N*-arachidonoyl-ethanolamine / AEA).

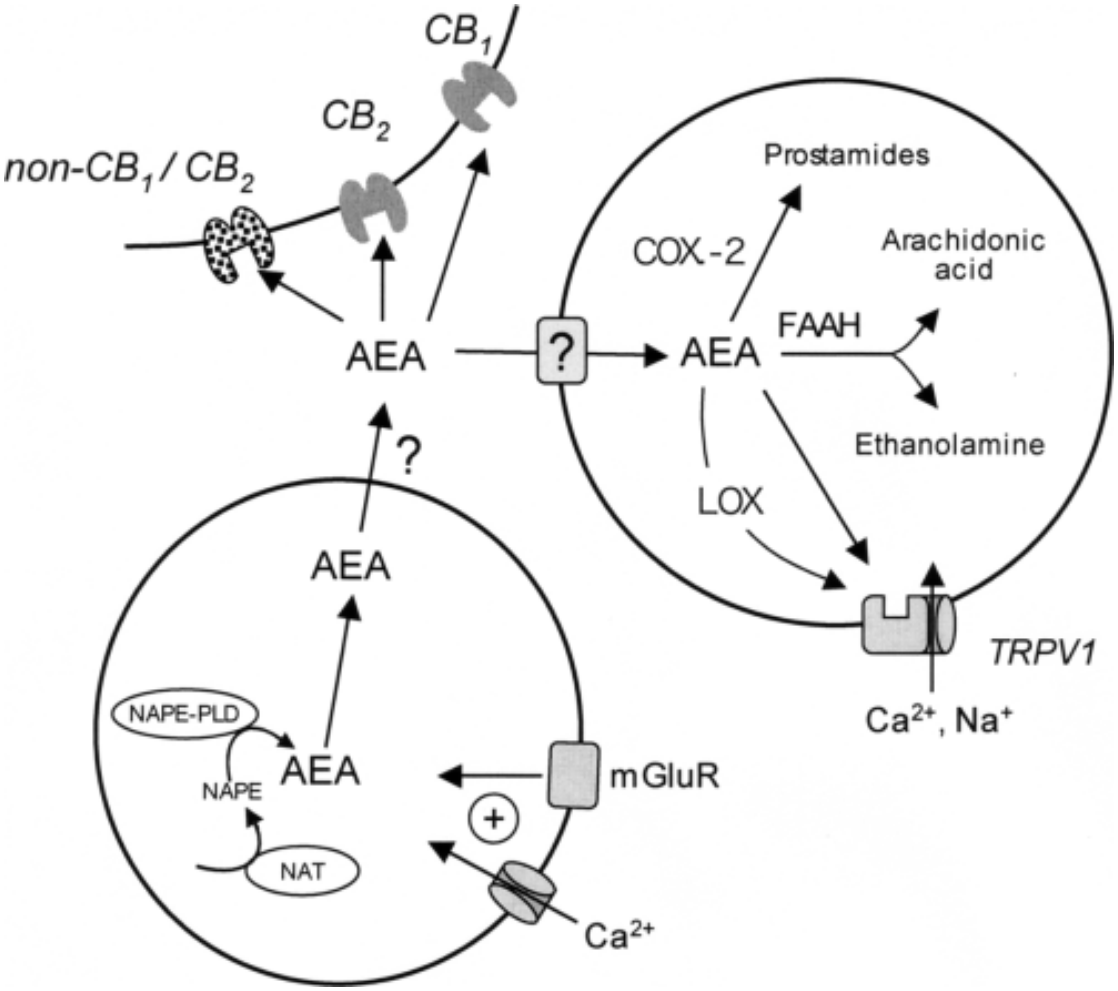

Figure 4: The 'life cycle' of anandamide from the enzyme driven hydrolysis of the corresponding N-acyl-phosphatidylethanolamine, NAPE, catalysed by NAPE-PLD. AEA then acts via CB1, CB2, or non-CB1/2 receptors, and is then hydrolysed by FAAH.

As stated above, the effects produced by exogenous cannabinoids are caused by the interaction of the active constituents of the plant *cannabis sativa*, (such as Δ^9-THC) with CB1 and CB2. Since AEA also binds to CB1 and CB2, it mimics several of the effects of Δ^9-THC, including hypothermia, motor effects (Smith et al., 1994), inhibition of memory consolidation (Castellano et al., 1997), impairment of working memory, exhibition of analgesic properties (Mechoulam et al., 1995), anxiety-like responses, and decreases arousal (Di Marzo et al., 1998). The magnitude and duration of AEA's effects are vastly inferior when compared to those of Δ^9-THC, and may be due to the rapid degradation of the molecule. AEA is a low efficacy agonist for CB1 and CB2 and is functionally more selective for CB1 than CB2 (Breivogel and Childers, 2000, Gonsiorek et al., 2000).

AEA is not stored in 'resting' cells, but is instead synthesised and released 'on-demand' following physiological and pathological stimuli (Di Marzo et al., 1998). After biosynthesis AEA is released into the extracellular milieu *via* unknown mechanisms (De Petrocellis et al., 2004), to reach its targets.

Recent work has also pointed to a pathway for AEA synthesis from NAPE that does not involve NAPE-PLD (Liu et al., 2006, Leung et al., 2006). In NAPE-PLD (-/-) mice there was no reduction in brain levels of AEA under non-stress conditions. It is thought that a distinct alternative two stage pathway may exist, involving phospholipase C (PLC) catalysed cleavage of NAPE to phosphoanandamide (pAEA), which is dephosphorylated by phosphatase to AEA (Liu et al., 2006).

Anandamide has been isolated in numerous different tissues including the brain, kidney, testis, skin, spleen, blood plasma, heart, breast cancer cells and the mouse uterus (Facci et al., 1995, Felder et al., 1996, Cadas et al., 1997, Deutsch et al., 1997, Koga et al., 1997, Schmid et al., 1997a, Stella et al., 1997, Kempe et al., 1996). In all tissues where AEA has been isolated, levels are at least 10 times lower than those of classical neurotransmitters. AEA levels have been shown to increase significantly in post-mortem tissue (Mechoulam et al., 1998) .

Once synthesised Anandamide is able to exert its actions via several different pathways, both CB1 / CB2 mediated and non-CB1 / CB2 (Mechoulam et al., 1998):

CB1 /2 mediated – Activation of receptors leads to modulation of forskolin induced adenylate cyclase, inhibition of N or P/Q type Ca^{2+} channels, activation of inwardly rectifying K+ channels, and activation of mitogen activated protein kinase (MAPK) (Mechoulam et al., 1998).

Non-CB1 / CB2 mediated actions - Activation of arachidonate release, and inhibition of gap junction mediated Ca^{2+} signalling in astrocytes (Felder et al., 1992). AEA also appears to share non-receptor mediated effects on the release of intracellular Ca^{2+} and arachidonic acid with synthetic cannabinoids(Ishac et al., 1996, Stefano et al., 1997, Romero et al., 1998). Endogenous cannabinoids appear to regulate the pre-synaptic release and/or re-uptake of noradrenaline, γ-aminobutyric acid (GABA) and dopamine (Mechoulam et al., 1998). AEA also acts on intracellular sites of some ion channels, such as those of vanilloid receptors (TRPV1) and L/T-type Ca^{2+} channels (Di Marzo et al., 2001a). TRPV1 is a Ca^{2+} permeable ion channel transient receptor potential vanilloid type 1 receptor (Di Marzo et al., 2001a). TRPV1 is a non-selective cation

channel that is present on sensory neurons in tissues such as skin, heart, blood vessels and lung. TRPV1 is sensitive to activation by noxious stimuli e.g. heat, protons, the vanilloid capsaicin, and to a lesser extent AEA (Huang et al., 2002). The best-known vanilloid is capsaicin, extracted from chilli peppers (Pertwee, 2005). An important consequence of its activation is the release of sensory neuropeptides that then produce effects such as pain, tachycardia, vasodilation and bronchial constriction. Whether AEA acts as a TRPV 1 agonist *in-vivo* is still to be resolved (Begg et al., 2005).

Anandamide interacts to differing degrees with the above targets. Its strongest interaction is with CB1, whilst weaker interactions exist with TRPV1, and with the non-CB1/CB2 endothelial receptors (Wilson and Nicoll, 2001, Gerdeman et al., 2002). It has been speculated that endocannabinoids typically do not travel far from their source, usually < 1µm. although distances of up to 10µm are possible (Cabral et al., 1995), suggesting that endocannabinoids act locally to exert their effects.

As well as cannabimimetic actions, AEA has been shown to inhibit macrophage-mediated tumorcidal activity (Wenger et al., 1995), inhibit pituitary hormone secretion (Facci et al., 1995), and modulate mass cell activation (Mechoulam et al., 1998).

Once it has acted, AEA is degraded into arachidonic acid and ethanolamine largely by the action of an enzyme known as fatty acid amide hydrolase (FAAH) (Glaser et al., 2003, Hillard and Jarrahian, 2003), which will be described in Section 1.3.3.3.

1.3.3.2 Transport of Anandamide

Once AEA has performed its action in the cells, it must be removed from the system and degraded by FAAH. However, the process by which AEA enters the cells remains unknown, and the subject of controversy (Di Marzo V, 1994, Maccarrone et al., 1998). Although there is no direct evidence of the mechanism involved, the following information, which has lead to several theories being developed, is known.

AEA is first taken up into cells *via* a selective, saturable, temperature-dependant and Na^+ independent facilitated transport mechanism, with a putative transporter called by some the anandamide membrane transporter (AMT) (Glaser et al., 2003). This transporter has however not yet been isolated. Some suggest that hydrolysis of AEA by FAAH intracellularly could be sufficient alone to drive facilitated diffusion of AEA from the extracellular space into cells(McFarland MJ and Barker EL, 2004).

Because no definite evidence of a transporter of endocannabinoids exists, there could in theory be 4 different ways in which the uptake of AEA occurs, and indeed more than one may exist in practice (McFarland MJ and Barker EL, 2004) .

These four possible ways of AEA uptake include:

1. AEA transported across plasma membrane by a carrier protein / carrier mediated process (McFarland MJ and Barker EL, 2004).
2. FAAH mediated hydrolysis maintaining an inward concentration gradient of AEA that drives simple diffusion of the molecule across the plasma membrane (McFarland MJ and Barker EL, 2004).
3. AEA sequestration in a saturable membrane compartment (McFarland MJ and Barker EL, 2004).

4. AEA uptake by an endocytic process / AEA reuptake occurring via a caveolae related endocytic process (Di Marzo V, 1994, Maccarrone et al., 1998).

Several 'indirect' observations have been reported to support the existence of a dedicated transporter, AMT (Di Marzo et al., 2001a). These include:

1. Several cell types can be found that can rapidly take up AEA from the extracellular medium even though they do not express FAAH.
2. Several compounds have been developed capable of inhibiting AEA cellular uptake without inhibiting AEA hydrolysis by FAAH (Schuel et al., 2002).
3. Substances that can inhibit AMT enhance the effect of AEA that are exerted at extracellular sites (CB1) and inhibit those that are exerted at intracellular targets – if these compounds were acting by inhibiting FAAH they should enhance the effect of AEA in both cases.
4. Lipopolysaccharide inhibits FAAH expression without affecting AEA cellular uptake
5. Plots of initial rates of AEA uptake in rat brain neurons and astrocytes yield apparent Michaelis-Menten constant (Beltramo et al., 1997, Hillard et al., 1997) consistent with a saturable process (Beltramo et al., 1997, Lopez-Rodriguez et al., 2001).
6. AEA uptake is inhibited by certain arachidonic acid derivatives (Piomelli et al., 1999).
7. Neuronal astrocytes in culture internalize AEA but not structurally related fatty-acid ethanolamides.

8. Chiral analogues of AEA compete with AEA for transport in a stereoselective manner, suggesting the compounds may interact with a common macromolecular target (Di Marzo V, 1994, Beltramo et al., 1997, Hillard et al., 1997, Maccarrone et al., 1998, Maccarrone et al., 2000a, Rakhshan et al., 2000).
9. AEA transport can be inhibited by selective fatty acid amide derivatives or specific AEA analogous in a dose-dependant manner
10. AEA transport is temperature dependant, occurs rapidly (t1/2 = 2.5 mins), is regulated by second messengers and signal transduction pathways, and is saturable at 37C (Di Marzo V, 1994, Beltramo et al., 1997, Hillard et al., 1997, Piomelli et al., 1999, Jarrahian et al., 2000).
11. Although AEA can diffuse passively through lipid membranes, this process appears to be accelerated by an energy independent, but highly selective transport system (Fegley et al., 2004).
12. FAAH (-/-) neurons internalise anandamide as efficiently as do wild type cells (Glaser et al., 2003).

Because of the lack of definite evidence and the level of speculation, others have argued against the existence of AMT, citing FAAH driven diffusion as the route of AEA uptake. In support of this view are the facts that:

1. Temperature dependency has been cited as an indicator of carrier-mediated AEA transport, however, hydrolysis of AEA by FAAH is temperature dependent (Glaser et al., 2003).
2. Saturability is the 2nd hallmark of carrier-mediated transport. Previous work used time points > 1 min to determine kinetics. As a result the saturation of uptake in these studies could not only be due to the presence of a transporter

protein, but could also be due to downstream processes such as hydrolysis by FAAH. Here, shorter time points that resulted in unsaturable AEA accumulation were used (Glaser et al., 2003).

3. Substrate specificity is a 3rd indicator. In this work it was confirmed that AMT inhibitors are able to inhibit AEA uptake by cells expressing FAAH when time points are long enough to allow hydrolysis, but also demonstrated that at time points before significant hydrolysis, no inhibition was observed. This suggests that the compounds do not inhibit a transporter, but rather an event downstream. This inhibition leads to a loss of the concentration gradient across the plasma membrane necessary for AEA uptake (Di Marzo V, 1994, Cravatt et al., 1996).

Although these arguments do carry some weight, the consensus view is that a dedicated transporter (AMT) does exist, and that getting direct evidence is 'coming soon.'

1.3.3.3 Fatty Acid Amide Hydrolase / FAAH

The second step in the termination of AEA signalling is its enzymatic hydrolysis by FAAH to arachidonic acid and ethanolamine (Cravatt et al., 1996).

FAAH was originally purified and cloned from rat liver microsomes (Cravatt et al., 1996), and has since been isolated in many tissues, often in association with areas containing CB1 and/or CB2. FAAH levels are highest in liver, brain and kidneys, measurable in the testes and lung, low in the spleen, and undetectable in skeletal muscle and the heart (Mechoulam et al., 1998).

FAAH is primarily responsible for hydrolysis of AEA, but it can also degrade other endocannabinoids, and is recognised by several non-endocannabinoid fatty acid derivatives, for example oleomide (Mechoulam et al., 1998). As a consequence these compounds may inhibit AEA hydrolysis, giving an entourage effect and prolonging the action of AEA (De Petrocellis et al., 2004). FAAH is optimally active at alkaline pH 8.5-10, and is found in microsomal membranes (Cravatt et al., 2001).

The importance of FAAH in the degradation of AEA is demonstrated by the fact that in FAAH -/- mice, AEA levels are 15 times higher than in wild type mice (Maccarrone et al., 2003a, Maccarrone et al., 2003c). When FAAH is present, its activity is not static, but can be up- and down-regulated, mainly by hormones linked to reproduction. Leptin and progesterone, both important in reproduction, both up-regulate FAAH activity (Waleh et al., 2002), whereas oestrogens and glucocorticoids down-regulate FAAH at least in rodents (Puffenbarger et al., 2001). These actions are carried out by acting directly upon the promoter of the FAAH gene (Ortega-Gutierrez et al., 2004). It has been estimated that FAAH, CB1 and simple diffusion account for about 70% of AEA uptake into cells (Persaud and Ellington, 1967, Geber and Schramm, 1969).

1.4 Endocannabinoids: Animal & Human

1.4.1 Exocannabinoids / THC Work

Although there has been much interest on the role endocannabinoids play in reproduction, it is important not to forget the large body of evidence on the effects of exocannabinoids i.e. THC, mainly prior to the discovery of endocannabinoids. As exo- and endo-cannabinoids share in their biological actions, experiments with THC may possibly be extrapolated to endocannabinoids. However the levels of THC used in these studies are likely to far exceed those of endocannabinoids found physiologically, which may possibly lead to exaggerated effects. This means that extrapolating THC results onto endocannabinoids should be with caution.

Early studies indicated that embryotoxicity and specific tetralogical malformations in rats correlated with exposure to natural cannabis extracts during pregnancy (Sassenrath et al., 1978).

Exposure to THC during early pregnancy in rhesus monkeys led to increased miscarriage, whilst the placenta of rhesus monkeys chronically exposed to THC during pregnancy showed gross morphological abnormalities and vascular infarction (Sassenrath et al., 1978). Rhesus monkeys chronically exposed to THC for 5 years had an increased reproductive loss, including miscarriages, resorptions, intra-uterine fetal death, and neonatal death (Mereu et al., 2003). As mentioned earlier, the levels needed to produce gross abnormalities are far above those found in human cannabis users.

In rats, exposure to THC leads to reduced birth weight, and impaired exploratory behaviour and memory in offspring (Rosenkrantz et al., 1986), whilst marijuana

exposure in rabbits is associated with an increased rate of embryo resorption (Sherwood et al., 1999).

In humans, there has been a marked increase in cannabis use during pregnancy over the last 20 years, with estimates of cannabis use by pregnant women varying between 10-20% (Fergusson et al., 2002). Evidence suggests that frequent / regular use of cannabis is more hazardous than occasional use (Mendelson et al., 1986). Acute administration of THC in humans causes suppression of LH; however, the extent of this depends on the timing in the menstrual cycle. If given during the luteal phase there is a 30% suppression, however there is no change in plasma LH if exposure is during the follicular phase or in post-menopausal women (Mendelson et al., 1986). In chronic users it shortens the menstrual cycle, the effects being predominantly due to a short luteal phase leading to menstrual irregularities and anovulation (Gibson et al., 1983, Fergusson et al., 2002).

In humans, marijuana smoking during pregnancy is associated with an increased risk of preterm labour (Zuckerman et al., 1989, Sherwood et al., 1999), fetal growth restriction (Sherwood et al., 1999, Fergusson et al., 2002), low birth weight (Gibson et al., 1983), perinatal death and delay in the commencement of respiration (Fried et al., 2003), as well as subtle cognitive defects in childhood and adolescence (Kolodny et al., 1974).

Chronic marijuana also affects males with decreased plasma testosterone levels, reduced sperm counts and impotency (Wenger et al., 1999).

Human gestational choriocarcinoma cells exposed to THC at concentrations found in cannabis users experienced inhibited proliferation in a dose-dependant manner (Khare et al., 2006).

The work done on THC clearly showed that exposure, both acute and chronic, has adverse effects upon reproduction. With the discovery of the endocannabinoid system the question then was whether these endogenous cannabinoids have a similar adverse effect, and if so, how are these induced.

1.4.2 Animal Work

Following the discovery of the endocannabinoid system there was great interest in what its role is in both physiological processes and pathological conditions. Given the effects of cannabis/THC, the role of endocannabinoids in reproduction has understandably come under investigation. The hope is that this may provide some answers to the numerous questions about the control and regulation of reproduction.

The evidence from several studies support the fact the endocannabinoid system has effects throughout reproduction with a major influence prior to pregnancy. During the menstrual cycle, exposure to AEA decreases serum LH and prolactin levels in rats, but does not effect serum FSH levels (Gonzalez et al., 2000). Also, expression of CB1 in the anterior pituitary is regulated by sex steroids (Gonzalez et al., 2000); the amount of CB1 mRNA fluctuating through the ovarian cycle in rats, with the highest level in diestrus and the lowest in oestrus. The concentration of AEA in the hypothalamus shows a reversed pattern (Wenger et al., 1997).

Anandamide administered chronically to rats, prolonged the duration of pregnancy and increased the stillbirth rate (Wenger et al., 1997). It also reduced levels of serum LH, pituitary prolactin, serum prolactin, serum progesterone and prostaglandins F1 and F2, but there was no effect on FSH or GnRH levels (Schmid et al., 1997a).

Along with the effects on the ovarian-pituitary-hypothalamic axis, it is clear that endocannabinoids have actions at a local level in reproductive tissues. Before becoming receptive to implantation, the mouse uterus contains very high levels of AEA, indeed the highest levels recorded (Schmid et al., 1997a). As the uterus becomes receptive for implantation the AEA concentration drops at the implantation sites, suggesting that a lower 'anandamide tone' is required for success of the process (Paria et al., 1996, Habayeb et al., 2002). Conversely, FAAH activity is higher at implantation sites than at the inter-implantation sites (Das et al., 1995). In addition, activation of trophoblastic cannabinoid receptors inhibits blastocyst development and implantation (Wang et al., 2003). If the only role for AEA is to inhibit implantation, then it follows that CB1/CB2 (-/-) mice should be more fertile than wild type mice, but mutant mice actually have 40% fewer pregnancies, suggestive that its role is likely to be more complex. Explanation for this phenomenon comes from work that showed AEA activates cannabinoid receptors in the trophoectoderm to initiate two distinctive signalling cascades, in a dose-dependant manner. At low concentrations (7nM) AEA stimulated the extra-cellular signal-regulated protein kinase (ERK). At higher concentrations (28nM) AEA reduced calcium entry by closing voltage operated N-type calcium channels. These events had opposite functional consequences, such that blastocysts treated with 7nm AEA became competent for implantation; whereas those treated with 28nm AEA did not. It may be possible that the uterus can titrate AEA levels to either

promote or inhibit implantation (Maccarrone and Finazzi-Agro, 2004). The differences in the effect of AEA may point to dual activity: binding of AEA to CB1 on blastocysts leads to cell death and prevents implantation, whereas its binding to CB1 on uterine epithelium inhibits gap junctions, thus facilitating uterine changes needed for normal implantation (Paria et al., 1996)

These different levels of AEA seem at a local level to be associated with reciprocal levels of both COX 2 and FAAH at the same sites (Liu et al., 2002). *In-vitro* work using uterine epithelium confirmed that low levels of anandamide (14nM) can significantly promote blastocyst attachment and outgrowth, whereas higher levels (56nM) delay attachment and inhibit outgrowth of blastocysts (Xiao et al., 2002).

As mentioned, AEA and FAAH seem to have a reciprocal arrangement, which fits with the role of FAAH as the main enzyme responsible for the degradation of AEA. FAAH mRNA is localised to uterine epithelial cells and circular myometrium during the oestrous cycle. In ovariectomised rats, oestrogen plus progesterone increased FAAH levels in both cells types (Xiao et al., 2002). During the implantation period FAAH mRNA is found not only in epithelial cells and the circular myometrium but also in the primary decidual zone surrounding the implanting embryo. Levels in stromal cells are markedly higher at implantation than at inter-implantation sites (Paria et al., 1996, Paria et al., 1999); the opposite to AEA. FAAH expression and activity are detectable in mouse and human uterine epithelium during the peri-implantation and non-pregnant period, and in pre-implantation and implanting embryos (Das et al., 1995, Paria et al., 1999, Paria et al., 1995).

In mice, CB1, CB2 and FAAH mRNA are expressed in pre-implantation embryos, while only CB1 and FAAH mRNA are present in the uterus (Paria and Dey, 2000). CB1 mRNA was primarily detected from the 4 cell stage through to the blastocyst stages, whereas CB2 was present from the one cell through to the blastocyst stage (Paria et al., 1995, Paria et al., 1999, Wang et al., 1999, MacCarrone et al., 2000). As FAAH is present in the uterus and embryo, this suggests that the embryo may be able to use this to modulate local concentrations of AEA to those conducive for successful implantation and development.

AEA inhibits 2-cell embryo development to the blastocyst stage and reduces the rate of zona hatching of blastocysts *in-vitro*. A synthetic cannabinoid (CP 55940) prevents implantation. All these effects are mediated via CB1 in embryos and the uterus (Paria et al., 1995). The addition of synthetic or endogenous cannabinoids arrested the development of 2-cell embryos into blastocysts in culture (Paria and Dey, 2000). These adverse effects were reversed by the simultaneous addition of selective antagonists to CB1 but not CB2. Furthermore, infusion of synthetic cannabinoid during implantation prevents the process, which is reversed by giving a CB1 antagonist (Paria and Dey, 2000).

AEA also has adverse effects on reproductive functions in mice, including retarded embryo development, fetal loss and pregnancy failure (Paria et al., 2001). In embryos deficient in CB1 and/or CB2, development is asynchronous with uterine development. On the morning of day 4, 98% of wild-type embryos are at the blastocyst stage compared to 62% CB1 (-/-), 71% of CB2 (-/-), 61% of CB1/2 (-/-) (Wang et al., 2004).

Whereas embryonic CB1 appears to contribute to normal development, it appears that oviductal CB1 may play a role in embryo transportation prior to implantation (Wang et al., 2004). CB1 (-/-) mice show retention of embryos within the oviduct, with the embryos appearing morphologically normal. Transferring wild-type mice embryos into wild-type or CB2 (-/-) mice leads to normal implantation, whereas 30% of wild-type embryos were retained in CB1 (-/-) mice (Ishac et al., 1996). CB1 (-/-) and CB2 (-/-) embryos implanted normally in wild-type mice. This effect of oviductal CB1 may be explained by CB1 regulating presynaptic release of noradrenaline that coordinates smooth muscle contraction and relaxation waves for normal embryo transport (Meccariello et al., 2006).

Most of the work with endocannabinoids has focused on the female side of reproduction, but a few studies have shown that the endocannabinoid system may also play a role in spermatogenesis. For example, in frogs, CB1 expression was increased in the testis during times of increased spermatogenesis and decreased during winter stasis (Meccariello et al., 2006). The highest levels of CB1 were detected in the gonads (both) (Chang et al., 1993, Schuel et al., 1994); and THC and AEA reduced the sperm capacity of sea urchins by inhibiting the acrosome reaction, an effect mediated by CB1 (Schuel et al., 2002).

1.4.3 Human Work

Since the discovery of endocannabinoids, studies have been undertaken in both animals and humans to try to elicit what roles they fulfil in physiological and pathological processes within the body. However, some of the animal work described would be

impossible to replicate in humans due to practical and ethical issues, and therefore a number of conclusions can only be extrapolated from that work, rather than replicated. Therefore, as much work as possible had been replicated, but where this is not feasible, novel work has been carried out to elicit the role of the endocannabinoid system in reproduction.

It is unlikely that the endocannabinoid system only exerts its influences on one aspect of reproduction, but instead influences the whole process. This seems likely given the wide distribution of anandamide found in human reproductive fluids, it being present in seminal plasma, mid-cycle oviductal fluid, follicular fluid, and amniotic fluid (Lazzarin et al., 2004).

1.4.3.1 Change in quantified endocannabinoids

The influence of endocannabinoids appears to begin during the menstrual cycle, levels of FAAH and AEA in peripheral lymphocytes undergoing specific changes during the cycle. During the menstrual cycle levels of FAAH in peripheral blood mononuclear cells, particularly the T-lymphocyte fluctuate, falling by a 3rd from preovulation levels to periovulation levels, followed by a rise to approximately double the preovulatory levels in the post ovulation phase (Lazzarin et al., 2004). The highest FAAH and lowest AEA levels are found on day 21 of the normal menstrual cycle, coinciding with the 'implantation window'. There was, however, no change in AMT activity, CB1 binding or NAPE-PLD activity throughout the menstrual cycle (Maccarrone et al., 2001b). These changes during the menstrual cycle could be explained by the fact that progesterone acts to upregulate lymphocytic FAAH activity in a dose-dependant manner (Szekeres-Bartho et al., 1996). Progesterone is essential for the maintenance of

pregnancy and is known to modulate immune function and to elicit an immunological response critical for normal gestation (Maccarrone et al., 2001b). However, progesterone does not change the activity of AMT, NAT, NAPE-PLD or CB1 expression in human lymphocytes, the overall effect being to decrease AEA levels (Maccarrone et al., 2003a). Progesterone exerts this effect by enhancing levels of the transcription factor Ikaros, which in turn increases FAAH gene expression (Maccarrone et al., 2003a, Gasperi et al., 2005).

Leptin, another hormone thought to be essential for successful reproduction, also enhances FAAH gene transcription and therefore activity (Matarese et al., 2002). Leptin is a 16-KDa non-glycosylated hormone secreted and released by adipose cells into the circulation, and is known to regulate energy homeostasis and also serves in the regulation of fertility and modulation of the immune response (Di Marzo et al., 2001b). Defective leptin signalling, which causes sterility in leptin-deficient mice, has been associated with elevated levels of hypothalamic endocannabinoids in the same animals and leptin treatment restores fertility and reduces the levels of the hypothalamic endocannabinoids (Gasperi et al., 2005). As with progesterone, leptin has no effect on AMT, NAT, and NAPE-PLD activities, therefore leading to a fall in AEA levels (Piccinni et al., 1995, Piccinni and Romagnani, 1996).

Leptin and progesterone also alter the production of Th1 and Th2 cytokines, favouring the development of human T lymphocytes producing Th2 cytokines, while inhibiting Th1 cytokines, thereby allowing survival of the fetal allograft (Maccarrone M and al., 2001). What is interesting is that Th2 cytokines up-regulate and Th1 cytokines down-regulate FAAH activity (Gasperi et al., 2005). It is also interesting to note that treatment

of the lymphocytes with AEA reduced production of LIF to 33% compared with controls (*via* a CB1-dependant mechanism) and that 2-AG reduced LIF production by lymphocytes to 42% of controls when used at the same concentration as AEA (El-Talatini et al., 2009b).

Throughout the reproductive cycle, AEA levels appear to fluctuate. Prior to pregnancy, levels in the follicular phase of the menstrual cycle are double that of those during the luteal phase (Habayeb et al., 2004). These luteal phase levels are similar to those in the first trimester of pregnancy, suggesting that conditions reached during the 'implantation window' remain similar to those ideal for maintenance of pregnancy (Habayeb et al., 2004). Levels in the 2nd and 3rd trimesters are about half those in the 1st trimester, followed by a small rise just before labour, and then a dramatic rise during labour to 3.9 times those in the first trimester (Habayeb et al., 2004). The exact reason why plasma AEA levels increase so dramatically during labour is unknown, however it may be that AEA acts as a reservoir of arachidonic acid for the generation of prostaglandins, which act on the myometrium. Alternatively, since progesterone induces FAAH expression in peripheral mononuclear cells and the number of these cells increase during gestation, then the 'functional progesterone withdrawal' that occurs during labour may also act on the peripheral mononuclear cells leading to reduced FAAH and hence raised plasma AEA levels (Dennedy et al., 2004).

1.4.3.2 Localisation of Endocannabinoid System within Reproductive Tissue

Different components of the endocannabinoid system have been isolated / localised throughout reproductive tissues, suggesting the possibility of several mechanisms by which they influence human reproduction. Both CB1 and CB2 have been localised in the human myometrium (Dennedy et al., 2004). It has also been demonstrated that AEA and THC exert a significant concentration-dependent relaxant effect on human myometrial contractility *in vitro*, which appears to be mediated through CB1(Helliwell et al., 2004).

In first trimester tissues, FAAH and CB2, but not CB1, have been isolated (Park et al., 2003), CB1, on the other hand, has been isolated in term placenta (Park et al., 2003). There is strong localisation of FAAH in villous cytotrophoblasts and extravillous trophoblast columns, with weaker localisation in syncytiotrophoblast. CB2 has only been found in a subset of cells within the villous stroma. CB2 and FAAH have been found on fetal macrophages in placental villi.

In the term placenta, CB1 has been identified in most major cell types throughout the membranes, as well as in the placental villi (MacCarrone et al., 2000). FAAH activity has been demonstrated in human uterine epithelial cells (Park et al., 2003) and in the epithelial and decidual layer of the human placenta (Park et al., 2003). CB1 has a strong expression in amniotic epithelium, reticular cells and in cells of maternally derived decidua. Moderate CB1 expression is found in chorionic cytotrophoblast. FAAH expression is high in amniotic epithelial cells and trophoblasts of the chorion and maternal decidua (Maccarrone et al., 2000b, Maccarrone et al., 2002a).

1.4.3.3 What happens when the ECS malfunctions?

As mentioned previously, plasma anandamide has been shown to vary during both the menstrual cycle and pregnancy. It has been suggested from pilot studies that variations in plasma AEA and lymphocytic FAAH could help predict outcomes in early pregnancies (Maccarrone et al., 2000b, Maccarrone et al., 2002a). Women with low FAAH levels, and reciprocally high AEA levels for example, have been shown to mostly miscarry, whereas those with high FAAH and low AEA had viable pregnancies (Piccinni et al., 1998). The FAAH in lymphocytes is thought to be important, as lymphocytes are believed to play a critical role in successful implantation and pregnancy maintenance (Szekeres-Bartho et al., 1996). Lymphocytes produce LIF and immunomodulatory proteins which favour implantation (Maccarrone et al., 2002a). Therefore, the endocannabinoid system may modulate the tight regulation of lymphocyte-dependant networks critical to successful human pregnancy. Although FAAH activity was defective in those with miscarriage, AEA transporter activity, CB1 receptors and hCG levels were the same in both groups, whilst 2-AG levels were undetectable (Maccarrone et al., 2002a). Non-pregnant controls had the same FAAH activity and content as those with on-going pregnancies, suggesting that down-regulation of FAAH occurred in the lymphocytes of patients who failed to achieve a pregnancy (Maccarrone et al., 2002a). This may be explained by the following:

1. the network of maternal signals which keep FAAH high during normal pregnancy is impaired
2. ‘defective’ blastocysts release an inhibitor of FAAH expression – this FAAH expresser may act as a suicide factor favouring elimination of blastocysts committed to die, in keeping with the pro-apoptotic potential of AEA demonstrated in mouse blastocysts

Lymphocytic FAAH was found to peak at around 8-9 weeks prior to dropping by week 12 .

These differences between those with subsequently normal pregnancies and those who miscarry appear to show that whilst the endocannabinoid system is a vital part of early pregnancy establishment and maintenance, if ‘normal’ levels are not present the pregnancy will fail. This needs to be further investigated at both peripheral levels and also at the embryo-maternal interface to try to further establish the role of endocannabinoids, and also to determine whether they could play a role in the prediction of pregnancy outcome and also whether manipulating the system may improve outcome.

1.5 Conclusions

During early pregnancy there is an interaction between the mother and the embryo by way of signalling to ensure that optimal conditions are available to allow normal development of the pregnancy. Failure of this interaction appears to cause these conditions not to be reached and therefore failure of the pregnancy. Because early pregnancy loss is such a common problem, affecting 15% of clinically recognised pregnancies (Maccarrone et al., 2002a, Maccarrone et al., 2000b), and is associated with a great deal of distress for those affected, much effort has directed at attempting to elicit the nature of these interactions, and to discover the dysfunctions associated with miscarriage where there are no karotypic abnormalities. The discovery that endocannabinoids may play an important role in early pregnancy is exciting, as it may be possible to screen women and detect those at high risk of miscarriage, and possibly through modifying the endocannabinoid system, prevent it. However, there is still much that needs to be done before that point is reached.

With regards a failure of the endocannabinoid system, when measured systemically plasma anandamide and lymphocytic FAAH levels appear to be able to predict early pregnancy outcomes (Habayeb et al., 2004), however, more evidence needs to be generated to support these pilot observations . More importantly, as yet we do not know whether these systemic variations reflect what happens at a local level, i.e. at the embryo-endometrial interface, and whether they correlate with each other. If a correlation is established, it would allow the measurement of AEA and FAAH peripherally to be directly linked to their local actions, thus forming the basis of a predictive test for possible miscarriage. The summation of these data has led to the following hypotheses:

1.5.1 Hypotheses

1. Anandamide and FAAH fulfil critical roles in the establishment and maintenance of early human pregnancy and that these roles are mediated locally at the endometrial and embryo levels, by changes in the endocannabinoid system (including a fall in local levels of AEA and up regulation of the activity and level of FAAH). Changes in systemic AEA levels will reflect the local changes.
2. Abnormalities in the local and systemic endocannabinoid system are associated with early pregnancy failure and measurement of AEA levels in plasma may provide an early indicator of impending pregnancy failure and expression / measurement of the endocannabinoid system at the fetal-maternal interface will provide important clues as to the potential mechanism.

These hypotheses will be tested in a series of investigations.

1.5.2 Plan of Investigation

A: the development of an assay to measure AEA.

To allow investigations and to further understand the role endocannabinoids play in reproduction, it is necessary to have a reliable method for their measurement. AEA measurement using single quadrapole HPLC-MS has been the most widely used method, and a variation of this method was employed previously by our group (Devane et al., 1992, Fontana et al., 1995, Koga et al., 1995, Felder et al., 1996, Giuffrida A and al., 2000, Maccarrone et al., 2001a, Habayeb et al., 2004, Vogeser et al., 2004, Schmidt et al., 2006). However, despite giving reliable and reproducible results, the major constraint was the inability to process more than a few samples at a time, due to lengthy AEA retention times, with total run times from 35 min to over 60 min/sample

(Fernandez-Rodriguez et al., 2004). Although plasma AEA concentrations have been determined in a number of human studies, the mean AEA concentrations reported have varied considerably between studies, from 0.37 nM (Vogeser et al., 2006) to 3.73 nM (Fernandez-Rodriguez et al., 2004). This 10-fold variability between studies suggests that plasma AEA concentrations can be difficult to determine routinely and at the time of starting this project a robust, validated, method to measure AEA was not available. More recent work using high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) in the measurement of endocannabinoids in rat brain and human plasma, produced a reduced analysis time to between 7 and 15 min. However, these studies had either not been validated (Richardson et al., 2007), were only used for brain tissues and not plasma (Vogeser et al., 2006) or had comparatively poor limits of detection (Habayeb et al., 2004). It was therefore decided to set out to produce an improved method of plasma AEA measurement employing HPLC-MS/MS, using the previous method used by the group (Habayeb et al., 2004) as an initial template.

B: validation of the assay through pregnancy.

Using work previously undertaken by our group (Devane et al., 1992, Fontana et al., 1995, Koga et al., 1995, Felder et al., 1996, Giuffrida et al., 2000, Maccarrone et al., 2001a, Habayeb et al., 2004, Vogeser et al., 2004, Schmidt et al., 2006) as a known starting point, this section will attempt to replicate those results employing the new method of AEA measurement to further validate the method.

C: application to clinically relevant studies and hormonal correlates

This section will investigate if there is a relationship between systemic plasma anandamide levels and pregnancy loss, and the data obtained used to explore the possibility of these levels being used to predict pregnancy outcome. In addition,

hormone levels (hCG and progesterone) will be quantified and any correlation with AEA and FAAH levels determined.

D: expression of the ECS in first trimester tissues at the fetal maternal interface

This section will investigate the expression of FAAH, CB1, CB2 and NAPE-PLD in maternal and gestational-related tissues during the first trimester of pregnancy, their cellular localisation and levels in relation to ongoing and failing pregnancies. These will be compared with the values obtained in plasma.

CHAPTER 2

Methodology for the measurement of anandamide

2.1. Introduction

Investigating and hence enabling further understanding of the role endocannabinoids play in reproduction, requires a reliable method for their measurement. The endocannabinoid, anandamide (AEA), has been identified in numerous animal and human tissues, including in human blood/plasma, along with other bodily fluids (Lazzarin et al., 2004). Many different methods have been employed to attempt to quantify AEA levels, including High Performance Liquid Chromatography and Mass Spectrometry [HPLC-MS], and Gas Chromatography with MS (Devane et al., 1992, Fontana et al., 1995, Koga et al., 1995, Felder et al., 1996, Giuffrida A and al., 2000, Maccarrone et al., 2001a, Habayeb et al., 2004, Vogeser et al., 2004, Schmidt et al., 2006).

AEA measurement using single quadrapole HPLC-MS has been the most widely used method (Fontana et al., 1995, Giuffrida et al., 2000, Habayeb et al., 2004, Johnson et al., 1993, Koga et al., 1995, Schmidt et al., 2006, Schuel et al., 2002) , and a variation of this method was employed previously by our group (Habayeb et al., 2004). However, despite the reliable and reproducible results obtained with this method, the major constraint has been the inability to process more than a few samples at a time, due to lengthy AEA retention times on the HPLC column, with total run times that vary from 35 min to over 60 min/sample (Devane et al., 1992, Fontana et al., 1995, Koga et al., 1995, Felder et al., 1996, Giuffrida A and al., 2000, Maccarrone et al., 2001a, Habayeb et al., 2004, Vogeser et al., 2004, Schmidt et al., 2006). Although plasma AEA concentrations have been determined in a number of human studies, the mean AEA concentrations reported have varied considerably between studies, from 0.37 nM (Fernandez-Rodriguez et al., 2004) to 3.73 nM (Vogeser et al., 2006). This 10-fold

variability between studies suggests that plasma AEA concentrations can be difficult to determine routinely and at the time of starting this project a robust, validated, method to measure AEA was not available.

More recent work using high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) (Fig 2.1.) in the measurement of endocannabinoids in rat brain and human plasma, produced a reduced analysis time to between 7 and 15 min. However, these studies had either not been validated (Fernandez-Rodriguez et al., 2004), were only used for brain tissues and not plasma (Richardson et al., 2007) or had comparatively poor limits of detection (Vogeser et al., 2006).

It was therefore decided at the onset to produce an improved method of plasma AEA measurement employing HPLC-MS/MS, using the previous method in use by the group (Habayeb et al., 2004) as an initial template.

2.2 Methods

The starting point was the purchase of improved chromatography and mass spectrometry equipment: a Quattro Premier tandem mass spectrometer and an Acquity Ultra-Performance Liquid Chromatography [UPLC] system (Waters Ltd., Manchester, UK), with an Acquity UPLC BEH C_{18} (2.1 x 50 mm) column, as shown in figure 2.2. ULPC is a modification of HPLC that uses narrow, small pore sized columns which theoretically should allow shorter run times and better elution/separation of the AEA. The Quattro Premier mass spectrometer allows two mass spectrometers to be used in tandem (MS-MS), which improves the sensitivity and specificity of the method.

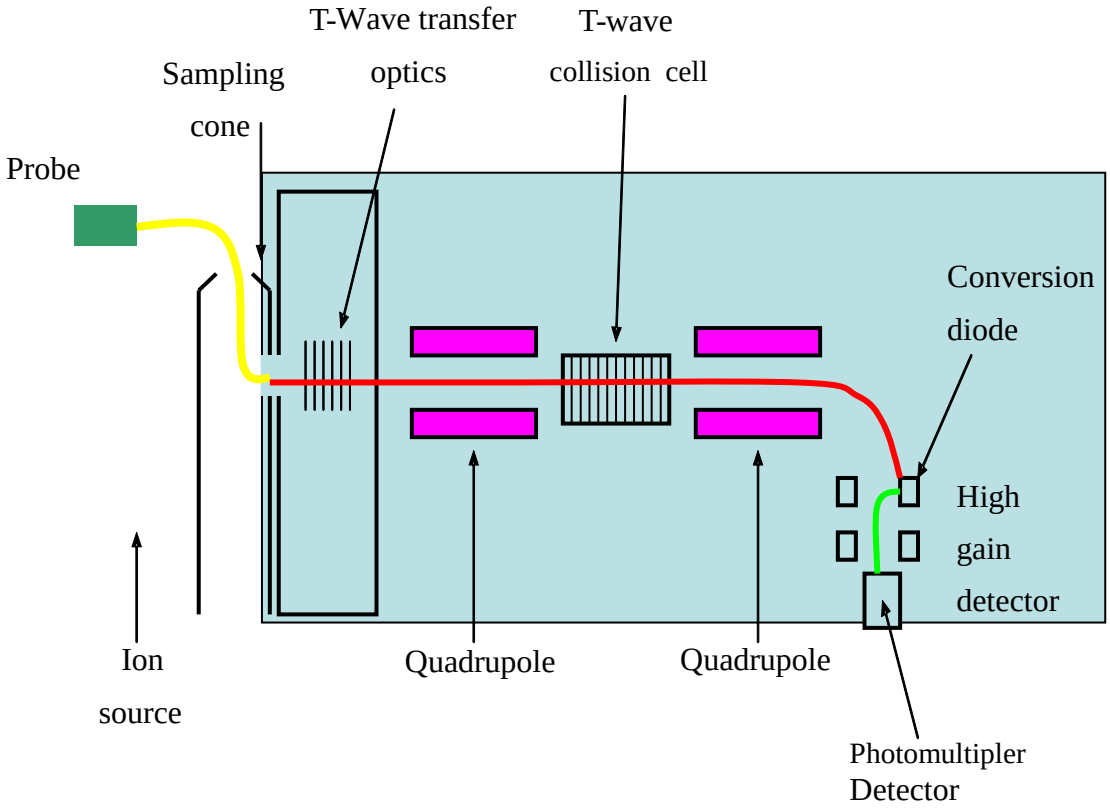

Fig 2.1. Diagram of Mass Spectrometer (MS). The separated sample from the UPLC enters the MS, and undergoes electrospray ionisation. The ionised particles then pass through the first quadropole where an electrical field acts to only allow the desired particle through. These are then fragmented in the collision cell into daughter particles, which pass through the second quadropole. This only allows the desired particles through. These then pass to the detector and separated according to their mass (m) to charge (z) ratio. This is then presented according to the abundance of each particle according in the format of m/z spectrum.

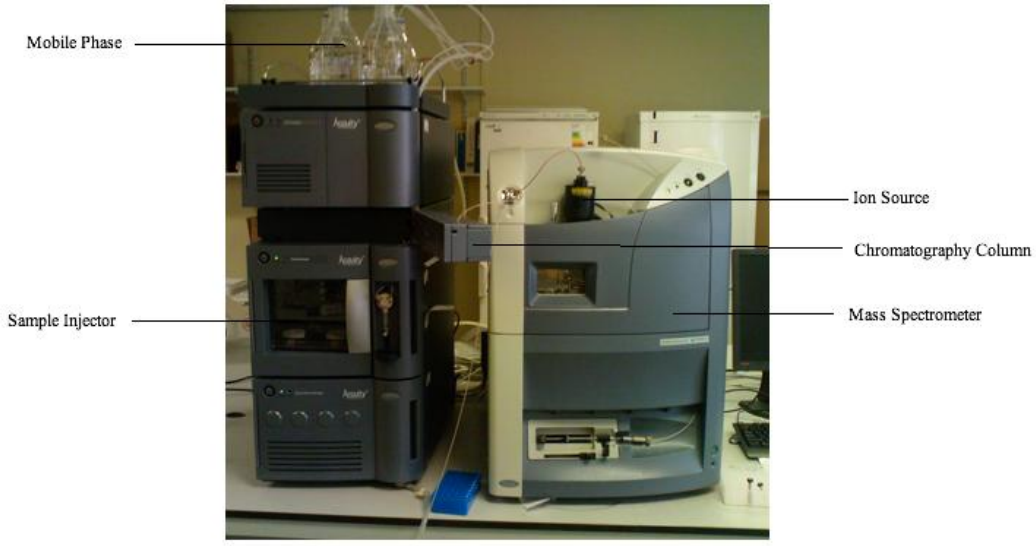

Fig 2.2. Quattro Premier mass spectrometer and an Acquity Ultra-Performance Liquid Chromatography [UPLC] system (Waters Ltd., Manchester, UK)

2.2.1 Chemicals

Anandamide (AEA) and octa-deuterated anandamide (AEA-d8) were obtained from Cayman Chemicals (Immunodiagnostics Systems Limited, Tyne and Wear, UK). Acetonitrile, chloroform, formic acid and methanol, all of HPLC grade and analytical grade ammonium acetate were purchased from Fisher Scientific (Loughborough, UK). HPLC grade water was obtained using a water purification system (Maxima ELGA, ELGA, High Wycombe, UK). All mobile phases were filtered through 47mm diameter, 0.2μm, PTFE filters (Waters Ltd, Elstree, UK) prior to use. Saline was obtained from Fannin UK Ltd (Reading, UK).

2.2.2 Preparation of standards

AEA and AEA-d8 stock solutions were prepared by drying supplied stocks under nitrogen gas and reconstituting the residue in acetonitrile at 5mg/ml and 100μg/ml, respectively. Stocks were aliquoted for single use and stored at -20°C. Further dilutions were made in acetonitrile, on ice, on the day of analysis.

For calculation of the limits of detection and quantification and to overcome the presence of variable AEA concentrations in plasma, both AEA and AEA-d8 were extracted from isotonic saline. To observe the effect of the plasma matrix, the limits of detection and quantification were also determined for plasma 'spiked' with AEA-d8.

2.2.3 Extraction of AEA from human plasma and isotonic saline

Extractions were performed as described by Habayeb *et al*., (2004) (Habayeb et al., 2004), with minor changes. Whole blood (4 mL) was collected in EDTA tubes (Sarstedt

Ltd, Leicester, UK) and then immediately transported on ice to the analytical laboratory for centrifugation at 1200 x *g* for 30 min at 4°C to separate plasma from cells.

Plasma (2mL) was transferred to a 7mL Kimble scintillation vial (Kinesis, St Neotts, Cambridge, UK). For the purpose of determining extraction efficiency, 2.5pmol of AEA-d8 internal standard were added to 2mL of plasma or saline prior to thorough mixing with a desktop vortexer. Protein was precipitated by adding 2mL of ice-cold acetone followed by centrifugation at 1200 x *g* for 10min at 4°C. The supernatant was transferred to a fresh Kimble scintillation vial and the acetone evaporated under a gentle stream of nitrogen gas. Lipid extraction was then performed on the remaining mixture by the addition of 2mL methanol:chloroform (1:2 v/v) followed by gentle mixing by repeated inversion. The sample was centrifuged at 1200 x g for 10 min at 4ºC and the lower chloroform layer was recovered into a fresh Kimble vial and then dried under a gentle stream of nitrogen before reconstitution in acetonitrile (80µl).

To estimate the efficiency of the lipid extraction procedure, peak areas obtained for 2.5pmol AEA-d8 extracted from plasma were compared with 2.5pmol AEA-d8 standard in 80µl acetonitrile.

2.3 Methodology development process

Having chosen UPLC-MS/MS to measure AEA, the first step was to run some 'blank' AEA standard, AEA-d0, through the mass spectrometers to allow tuning of the machine for optimal detection. The aim was to first detect AEA itself [parent ion, MS] and then

by manipulation of collision gas to produce fragments [daughter ions, MS-MS] which were then measured.

The following parameters were manipulated to allow optimal detection:

a. Cone voltage – this was used to add charge to the particles from the UPLC (see Figure 2.8.)
b. Source gas / desolvation gas temperature – this evaporates the solvent.
c. Cone gas / desolvation gas flow – this removes neutral, non-charged, particles from the cone.
d. Collision energy – this causes fragmentation of the parent ion into daughters.

All these produced for AEA a measurable peak on the MS with a parent ion of 348.4 and two daughter ions of 62.3 and 44.5 [as seen in figure 2.3].

The next step was to optimise the chromatography conditions. To do this an initial gradient for the UPLC was devised, taking into account both the previous methodology (Bifulco M and Di Marzo V, 2002, Giuffrida A and al., 2000, Habayeb et al., 2004) and pilot studies performed by Waters Ltd. on my behalf. The mobile phases used were:

A – Water

B – Methanol

and the UPLC run was a total of 2 minutes using the parameters as shown in table 2.1

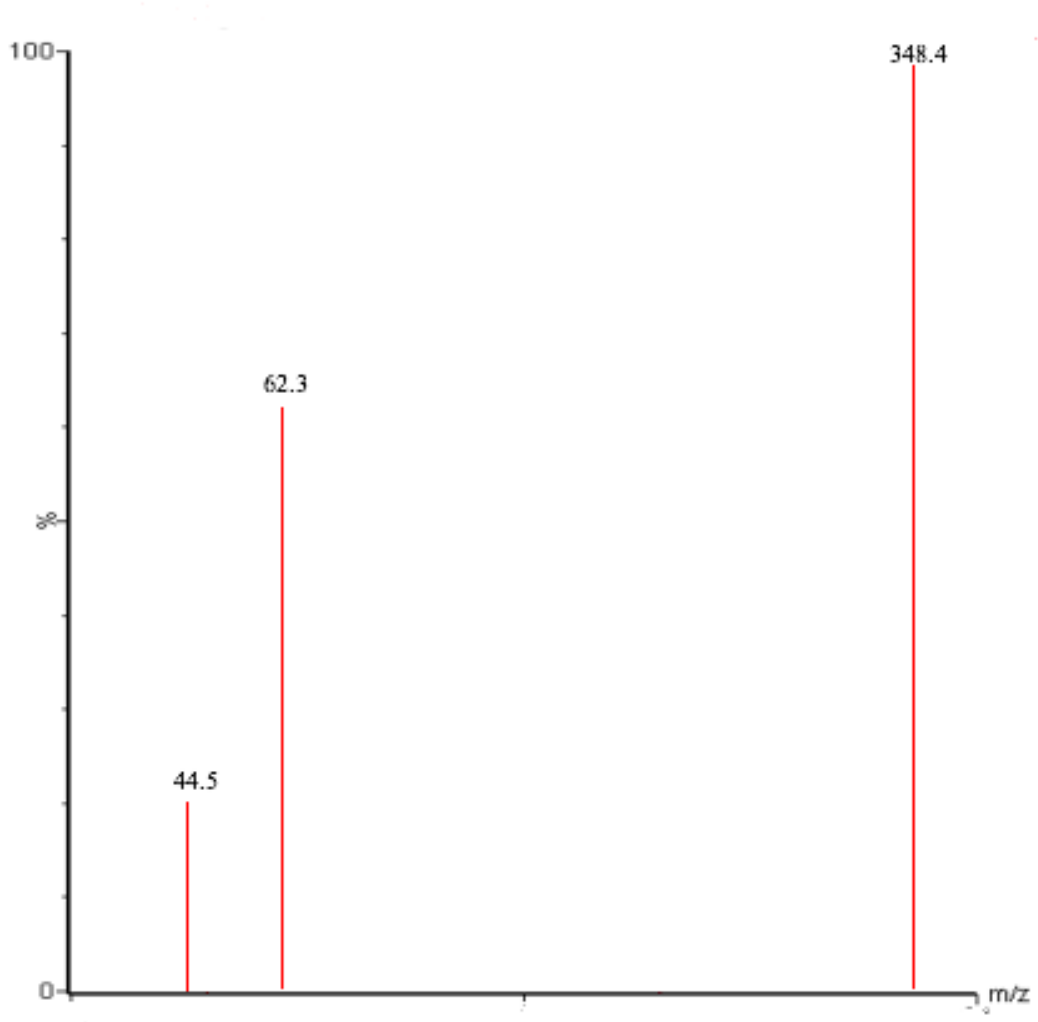

Fig 2.3. Peak for AEA at 348.8, and its daughters at 62.3 and 44.5.

TIME [min]	MOBILE PHASE A %	MOBILE PHASE B %
0	25	75
0.5	0	100
1	0	100
1.1	25	75

Table 2.1. Initial mobile phase run. A = Water, B = Methanol.

Previous methods using HPLC-MS to measure AEA had used the sodium [Na+] adduct as the target ion for measurement as this gave the highest peaks (Giuffrida et al., 2000, Habayeb et al., 2004). As well as the 348.4 parent, which is the H+ adduct, the Na+ adduct also used gave the highest peak on initial tuning. However, it was not possible to fragment the Na+ adduct into measurable daughter compounds, as the Na+ parent appears to fragment into numerous small pieces which cannot be measured (data not shown). The Na+ adduct itself is produced usually from Na ions that are already within the system, for example as a contaminant from glass wear. To reduce the effect of the Na+ adduct two steps were taken. Firstly the glassware was cleaned with HLPC grade methanol and water to avoid contamination, and secondly 0.1% formic acid and 2mM ammonium acetate were added to the mobile phases, to promote protonation of the AEA to the H+ adduct.

Previous methods had used chloroform:methanol mixture to reconstitute the dried AEA for injection into the UPLC, however, it was strongly advised not to use chloroform through the UPLC-MS/MS as it would damage the tubing. Therefore, methanol was used to re-suspend the AEA and to use as solvent for the production of calibration standards because AEA is soluble in methanol and because it matched the original mobile phase.

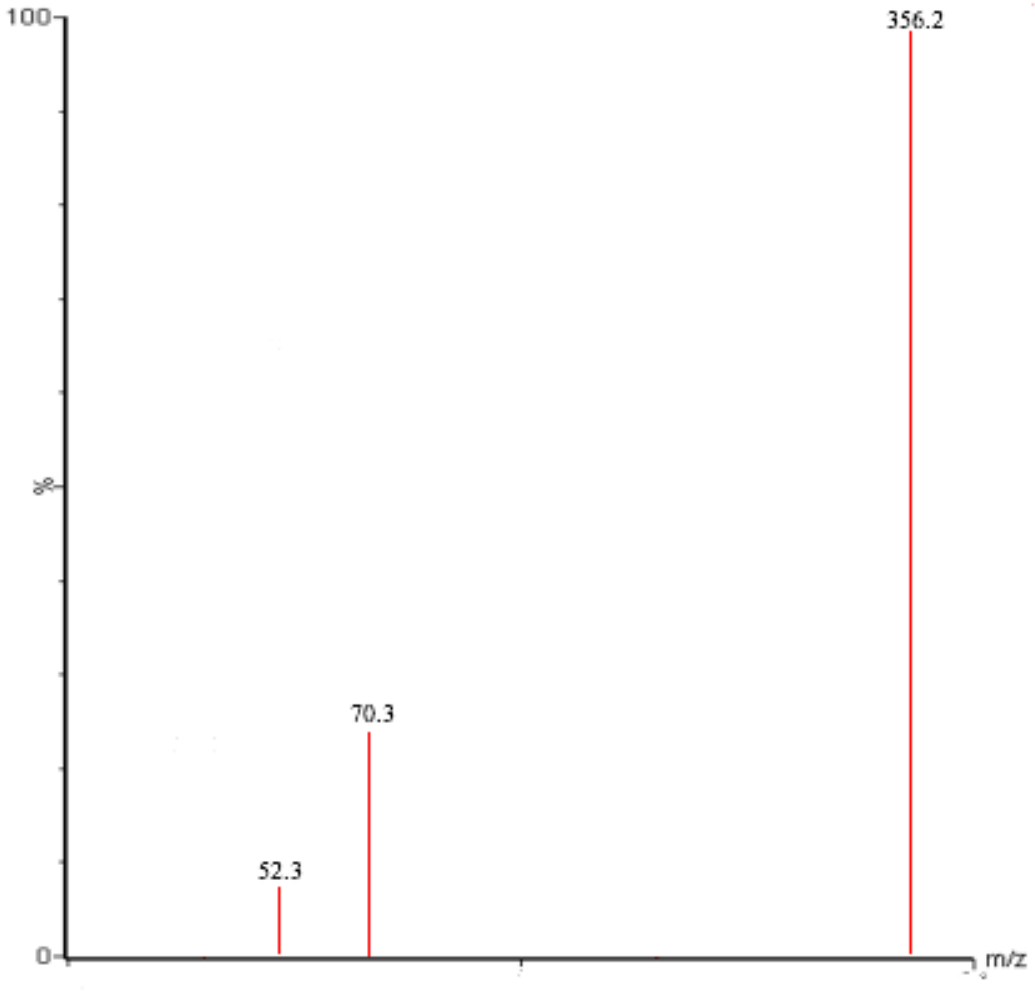

Fig 2.4. Peak for AEAd8 at 356.2 and its daughters at 52.3 and 70.3

Plasma samples were spiked to enable AEA quantifications with a known amount of standard AEA-d8. The MS-MS was tuned in the same way as had been for AEA-d0. From this process, the parent ion produced a peak at m/z of 356.2 and the daughters produced two peaks at m/z of 70.3 and 52.3, as seen in Figure 2.4. The 70.3 peak was used to measure the absolute concentrations of AEA-d8.

25 pmoles of AEAd8 were added to the plasma prior to processing to allow quantification of the endogenous AEA levels. This concentration was used as this had previously been employed by our group in the quantification of AEA (Habayeb et al., 2004)

Solvent standards were also prepared using AEA and AEAd8, and from this calibration curves were produced. The generated curves were then used to quantify AEA in plasma. (see section **2.5.).** Unfortunately calibration tests/runs revealed some areas of concern that could affect the inter- and intra-assay variability.

The three main issues identified were:

1. inconsistent and irreproducible standard calibration curves, both individually and between different members of the group.
2. significant 'background noise' on the MS seen.
3. The chromatography peak seen for AEA tailed off, again signaling interference from other compounds / signals.

Despite using similar concentrations of AEA-d0 and AEA-d8 in the solvent standards, the peak detected for AEAd8 was $1/10^{th}$ that seen for AEA-d0, and the m/z 52.3 daughter peak of AEA-d8 only gave a small peak below the limit of detection.

There was also a concern of carryover of AEA between samples that had necessitated a 6 minute 'wash' of the system with ethanol in between injections of AEA.

Because of these concerns it was necessary to re-evaluate the method that had been developed to look for areas that could account for the errors, and also in which improvements or changes could be made.

2.4 Changes made to method

2.4.1 Dwell Time

To improve the peak shape seen during chromatography the dwell time was reduced from 0.1 to 0.05 seconds. This increased the number of scans that the MS undertook in a given time, which during the peak for AEA increased from 12 to 20 scans. This helped to improve the shape of the peak and reduce any tailing seen.

2.4.2 Flow rate

The flow of the mobile phase was altered stepwise from 0.5 to 1.0ml/min to see if any differences were present. A flow of 0.7ml/min was found to give the best response/ peak, as seen in Figure 2.5.

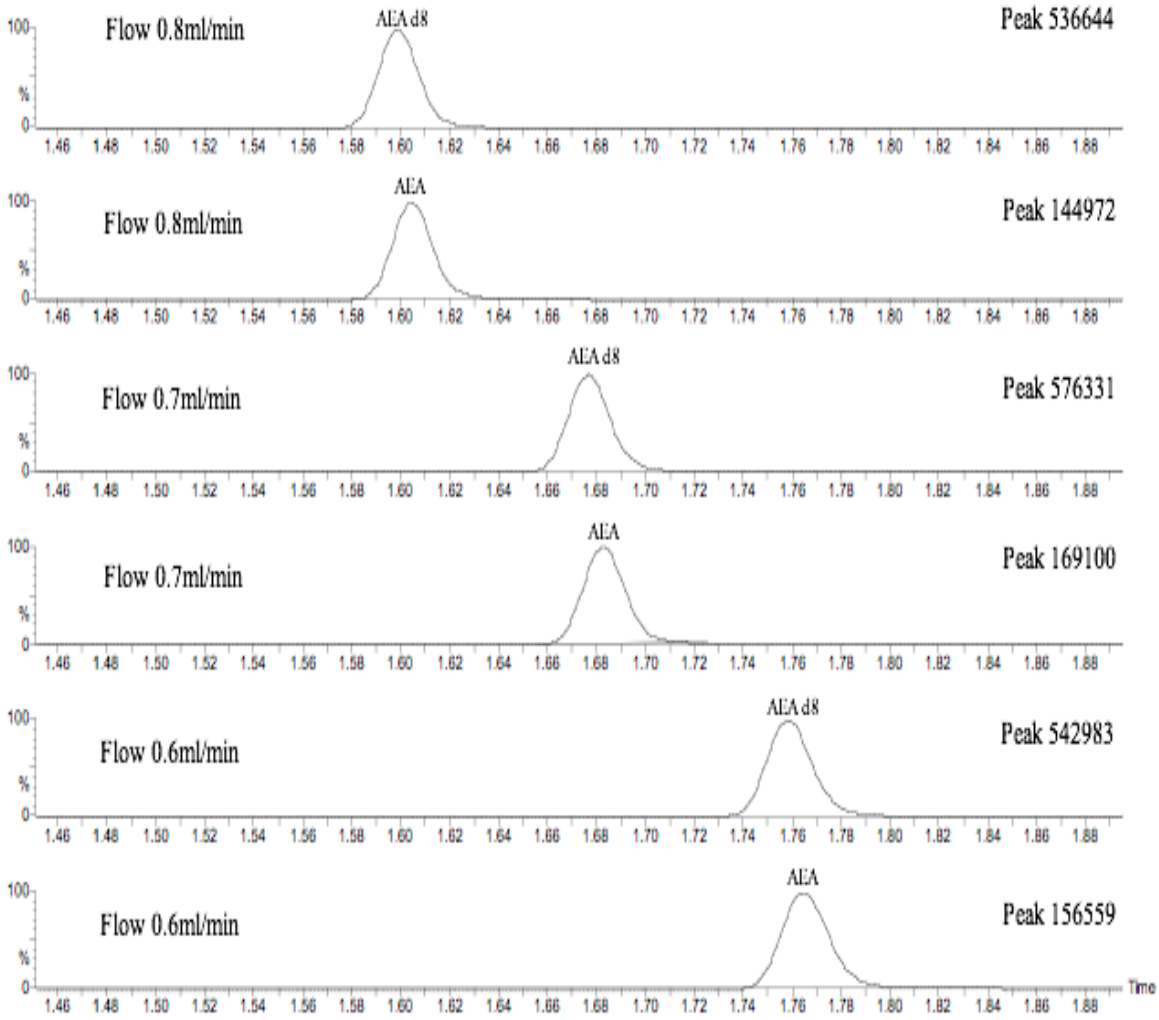

Fig 2.5. Outputs from the UPLC-MS/MS **s**howing the effect of flow rate on peak detected for AEA d0, and AEA d8. Maximal peaks were observed at a flow rate of 0.7ml/min.

2.4.3 Solvent and buffer composition

The anandamide standards had at various times through the development process been suspended in methanol, ethanol and acetonitrile [ACN]. All three were mentioned on the data sheet for AEA-d0 / AEA-d8 as being suitable, but it was unclear, which, if any, had an advantage regarding detection or reproducibility. Therefore, a known amount of standard was made in each solvent and the detector response observed. Acetonitrile gave the most consistently reproducible results with the lowest inter and intra assay variability. (Table 2.2) Therefore all solvents were subsequently prepared in ACN.

ACN was used in place of methanol, with 0.1% formic acid present, ensuring that the solvents injected matched the mobile phase and that protonation was maintained. It was not possible to add ammonium acetate due to solubility problems, but this did not affect the results gained.

The gradient and timing of the mobile phases was modified to produce the best response using the new solvents. Mobile phases were A (water, 2 mM ammonium acetate, and 0.1% formic acid) and B (acetonitrile and 0.1% formic acid).

Solvent	Ethanol	Ethanol	Methanol	Methanol	Acetonitrile	Acetonitrile
Compound	AEA d0	AEA d8	AEA d0	AEA d8	AEA d0	AEA d8
Mean Response	6547	2244	628	2356	5785	1818
Standard Deviation	1387	532	1217	338	844	258
Percentage Deviation	21	24	19	15	14	14

Table 2.2 The response detected by UPLC-MS/MS for both AEA d0 and AEA d8 when suspended in ethanol, methanol or acetonitrile. Each injection was repeated 15 times and the mean and standard deviation calculated. Acetonitrile has the best reproducibility.

TIME [min]	MOBILE PHASE A %	MOBILE PHASE B %
0	80	20
0.5	20	80
1.5	0	100
3.5	80	20

Table 2.3. Revised mobile phase for UPLC. Mobile phase A = 2 mM ammonium acetate and 0.1% formic acid in water, and mobile phase B = acetonitrile with 0.1% formic acid.

The total run time was 4 minutes, with a retention time of 1.67 minutes for AEA-d0. The UPLC gradient time was increased to allow the column to re-equilibrate. A flow divert was added for 1.5 minutes, which meant that during this time nothing ran through the column, reducing contamination on the column.

2.4.4 Injection volume

Previously a 10µl injection loop was used, with a setting on the UPLC of 'partial loop with overfill' with a 10µl injection. However, after consultation with Waters it became apparent that this may have caused a large amount of the problems with reproducibility between different samples and also between repeat injections from one sample. The reason for this is that the 'partial loop with overfill' setting allows injection volumes of 10-70% of the loop to be analysed [1-7µl with a 10µl loop] and takes 2-3µl either side. This meant that with our injections of 10µl there would likely be a variation in the amount analysed. On a 'Full Loop' setting the full size of the loop is analysed but 4 times the volume of the loop is taken [40µl for a 10µl loop]. Due to the limited amount of material available for each sample, 80µl, and the need for 3 injections per sample, it was decided to continue using the 'Partial loop with overfill' setting, but with only 7µl injections to fit with the parameters of this method and loop size.

2.4.5 Re-tuning of the second MS for AEA-d8 measurement

Due to the large difference seen between the response for AEA-d8 and AEA-d0 at the same concentrations, the tuning for AEA-d8 was re-examined to try to address this

difference. Daughters for AEA-d8 were found at m/z of 62.3 and 63.3 (Figure 2.6 and 2.7.), and the response of these compared with that of the previously used daughter at M/z of 70.2. For a given amount of AEA-d8 the responses were:

	70.2 daughter	62.3 daughter	63.3 daughter
Response	4845	72194	102785

The results for the m/z 63.3 daughter gave a response around the same levels as that found in unlabelled AEA-d0 at the same concentration. This daughter / transition was subsequently used, and improved reproducibility and reduced the level of variability.

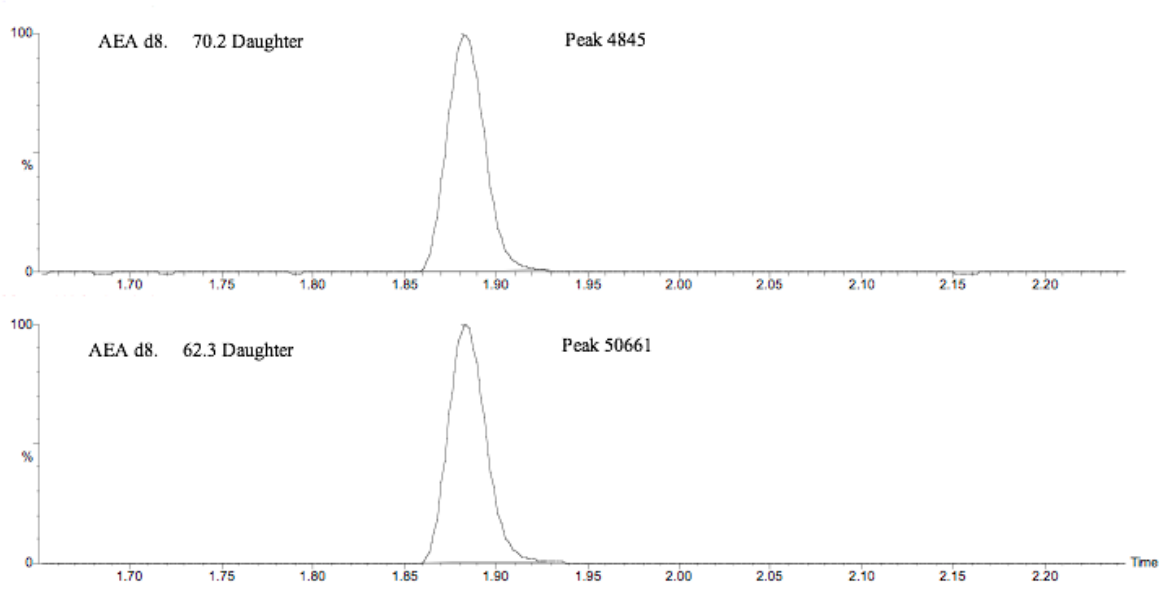

Fig 2.6. Difference in response between the original daughter of AEAd8 produced at m/z = 70.2 and that of the daughter ion at produced at m/z = 62.3.

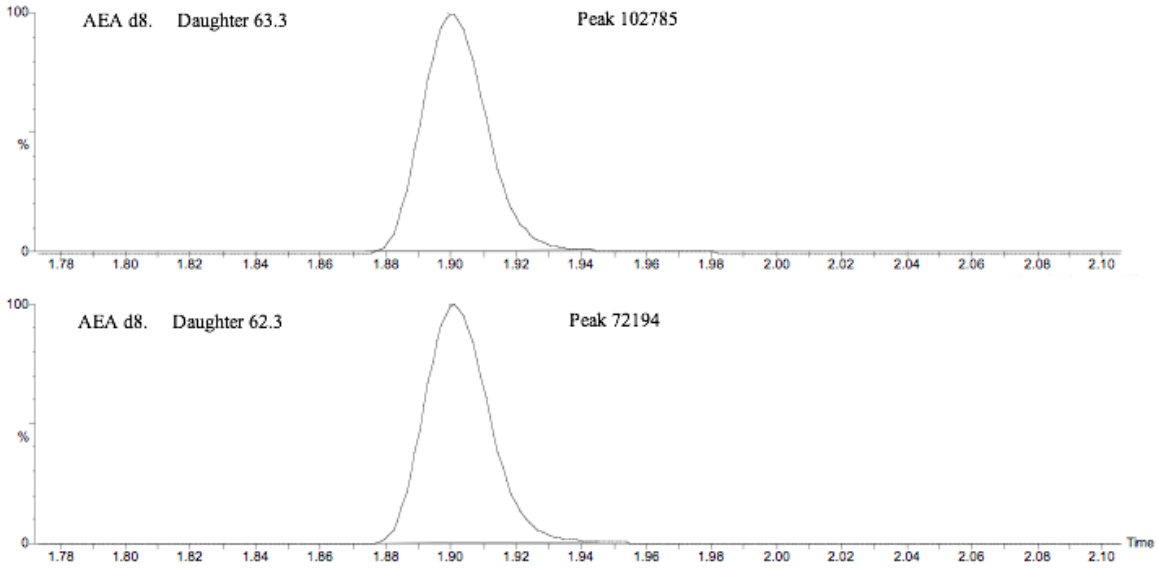

Fig 2.7. Difference in response between the daughter ions of AEAd8 at m/z = 63.3 and m/z = 62.3.

The lower response to AEA-d8 that was present originally necessitated a higher concentration of AEA-d8 to be added to spike plasma samples to ensure that it could be detected. The improvement gained by using the m/z 63.3 transition meant that lower concentration of AEA-d8 could be added, which reduced errors that may have been caused by a small percentage of AEA-d8 that had auto-converted to AEA-d0. At the same time the volume of AEA-d8 added to plasma was increased from 4µl to 40µl, reducing pipetting errors and improving reproducibility. Therefore 2.5 pmoles, rather than 25 pmoles, was added to each sample.

As part of the re-tuning, the capillary voltage was altered and the response noted. 0.6, 1, 2, and 3 volts [the previous setting] were tried and the response noted. 0.6volts gave the best response, as seen in Figure 2.8.

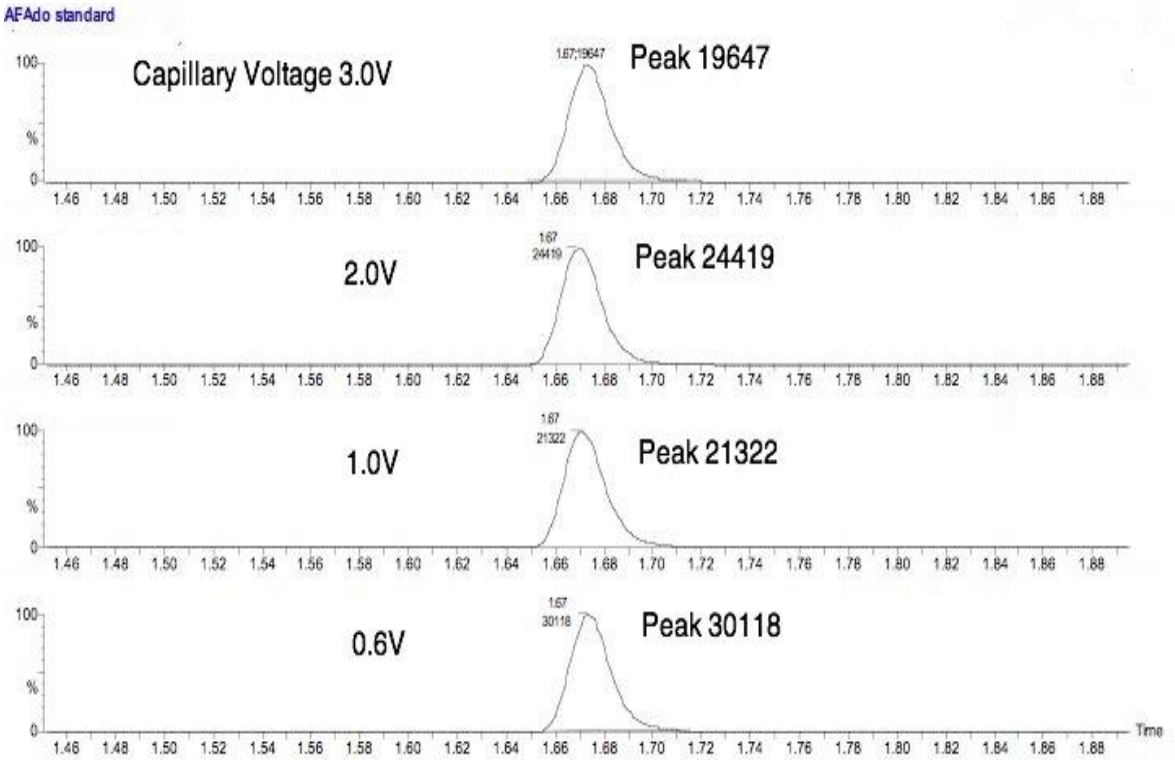

Fig 2.8. The effect of changing cone voltage on size of the AEAd0 peak. Maximal peak height and area was achieved at a cone voltage of 0.6V.

To maintain sensitivity a series of measures were implemented:

1. Regular flushing with solvent to clean the HPLC/MS equipment
2. Regular water washes to eliminate salts which can build up, crystallise and block tubing and column
3. Regular (weekly) washing of MS parts in methanol and water
4. Increased HPLC gradient time to allow column to re-equilibrate

These additional measures gave a much improved method, with reproducible results and standard curves. The next step was to validate the method.

2.5 Validation of UPLC-MS/MS analysis of AEA

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

Consistency of the retention time was investigated after 20 injections of 133 fmol of AEA. Accuracy, defined as the deviation of observed concentration from the expected concentrations, was calculated for solvent containing three concentrations of AEA; 3.33, 6.65 and 133 fmol on the column (20 samples). Assay precision was calculated after 20 repeat injections of 19 nM, 0.95 nM and 0.237 nM AEA standards in acetonitrile (133 fmol, 6.65 fmol and 1.66 fmol AEA on the column).

Limits of quantification and detection were defined as AEA responses which yielded a signal to noise ratio, without smoothing, of greater than 10 and greater than 3, respectively and were calculated for extraction of AEA-d8 from plasma and from saline.

The limits of quantification for AEA-d0 were determined only from saline because of the presence of endogenous AEA present in all plasma samples.

AEA and AEA-d8 were both eluted from the UPLC and detected by MS-MS at 1.67 ± 0.0009 min after injection (Figure 2.9) and had an observed relative standard deviation (RSD) of 0.05 % for the retention time after 20 injections.

Retention time was consistent over the lifetime of the column which represented in excess of 9000 injections. A very minor peak, eluting prior to AEA (1.65 min) was routinely observed in samples extracted from plasma (Figure 2.10). The identity of this peak was thought to be related to the endocannabinoid virodhamine (Figure 2.10) which has an identical molecular weight to AEA and fragments to the same daughter ion (Figure 2.10) [19]. Using authentic virodhamine standards we demonstrate that virodhamine did not contribute to the AEA peak in our analyses. Co-injection of virodhamine with AEA demonstrated two clearly separated peaks with virodhamine eluting 1.52 min after injection (Figure 2.10).

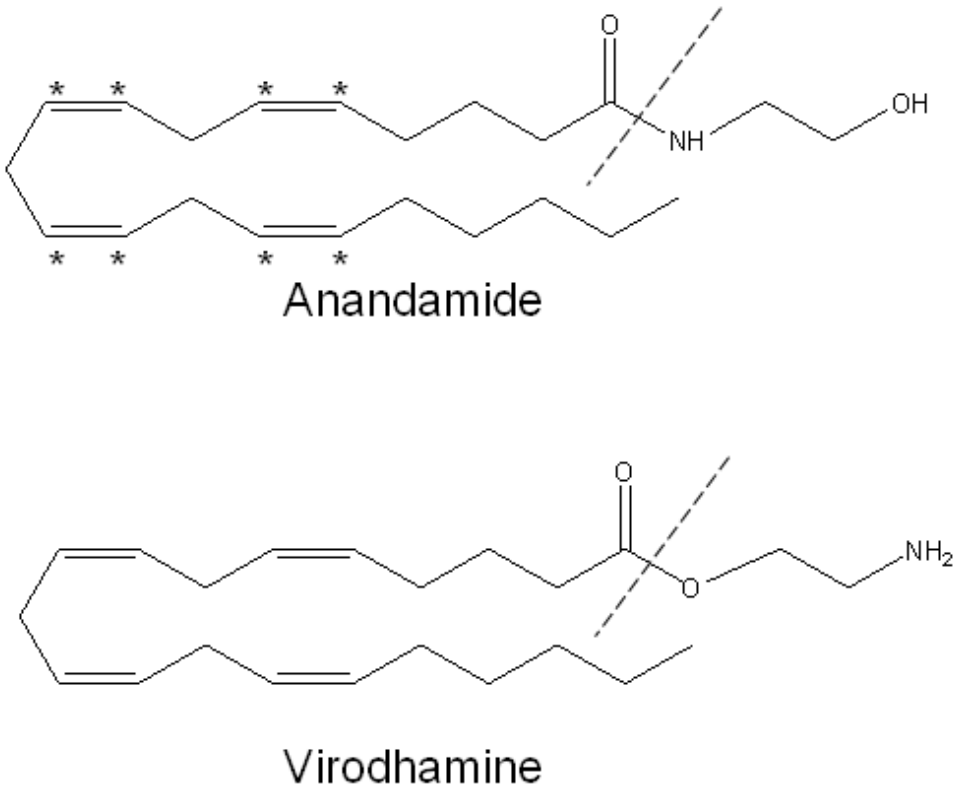

Figure 2.9. Structures of AEA and virodhamine showing the proposed site of fragmentation during UPLC/MS/MS and sites of deuteration in AEA-d8.

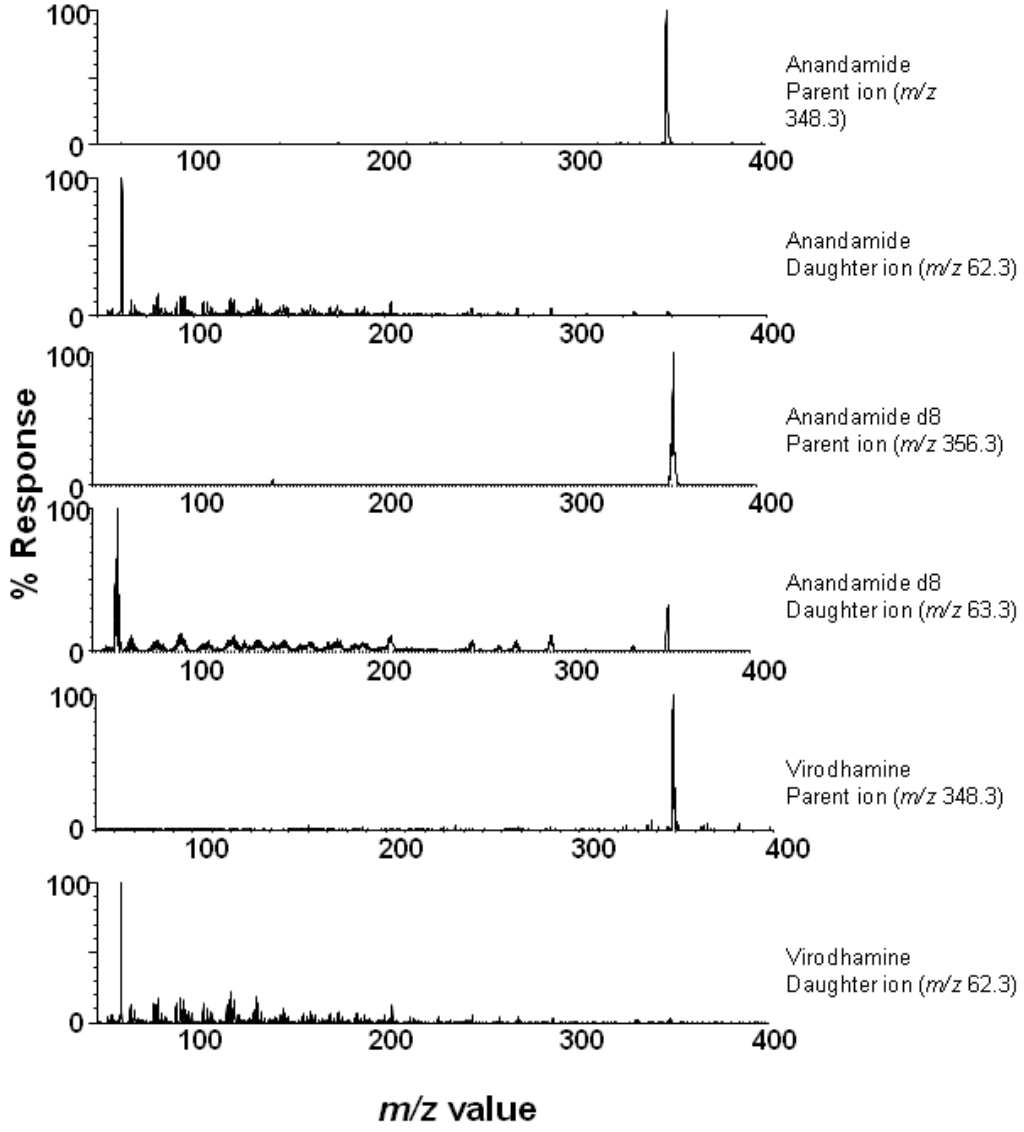

Figure 2.10. Parent and daughter ion mass spectra for AEA, AEA-d8 and virodhamine.

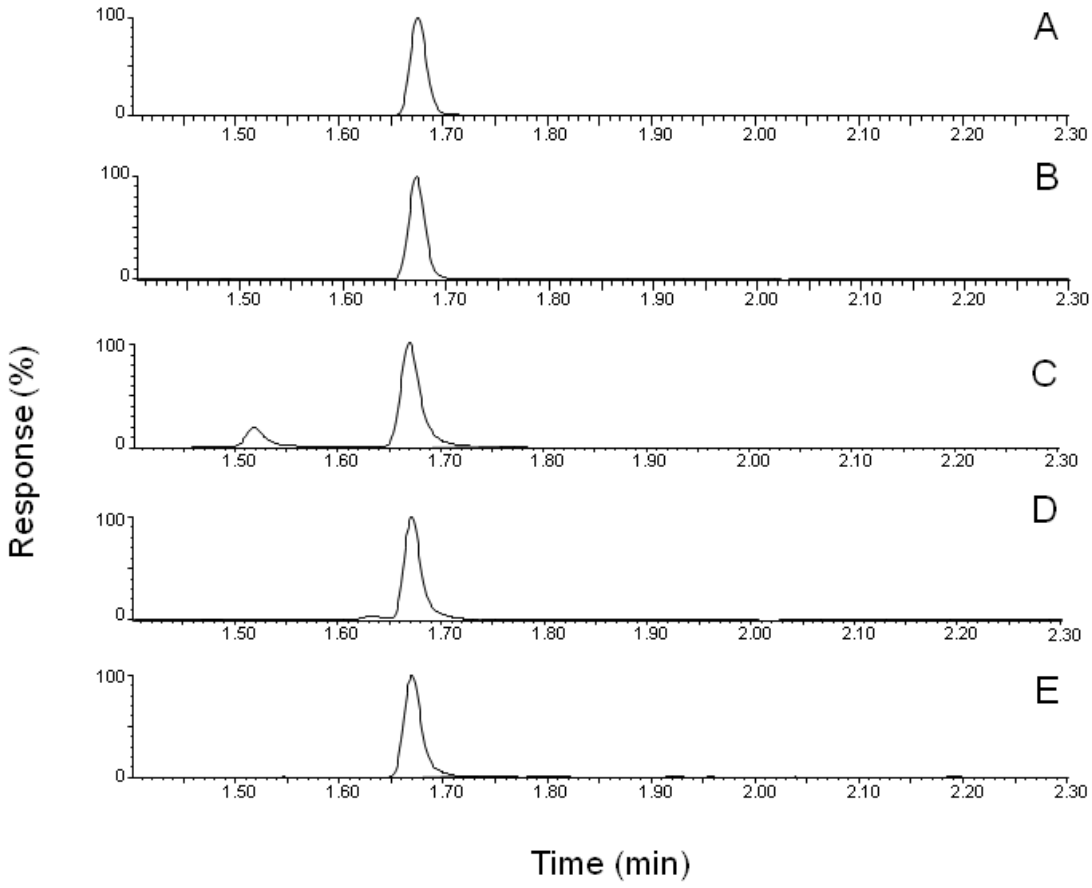

Figure 2.11. (**A**) UPLC-MS/MS spectrograms of an AEA standard in acetonitrile (*m/z* 348.3>62.3); (**B**) an AEAd8 standard in acetonitrile (*m/z* 356.3>63.3); (**C**) AEA standard (19nM) spiked with 9nM virodhamine in acetonitrile (*m/z* 348.3>62.3; (**D**) AEA extracted from 2mL human plasma (*m/z* 348.3>62.3); and (**E**) AEA-d8 internal standard extracted from 2mL human plasma (*m/z* 356.3>63.3).

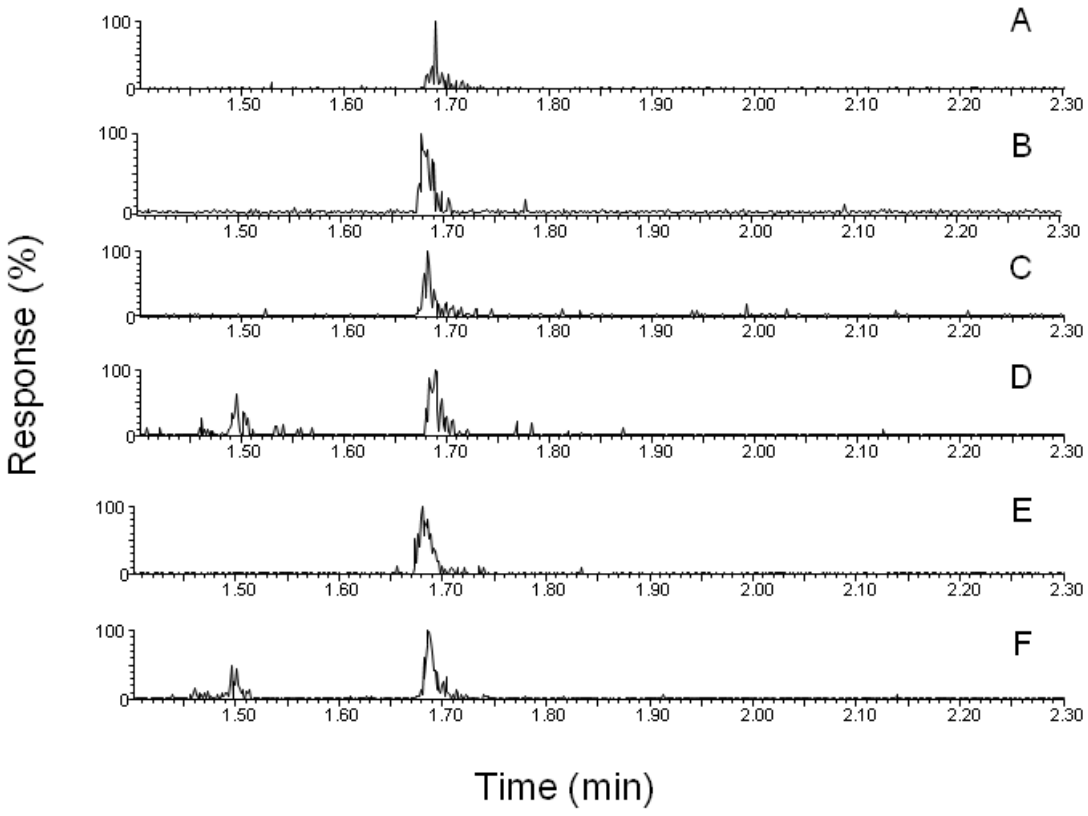

Figure 2.12. Detection by UPLC-MS/MS of low levels of AEA and AEA-d8 extracted from plasma and saline. Spectrograms are representative of five injections of: (**A**) AEA-d8 extracted from plasma at the limit of detection (LOD) (18.75fmol/mL); (**B**) AEA-d8 extracted from plasma at the limit of quantification (LOQ) (25fmol/mL); (**C**) AEA-d8 extracted from saline at the limit of detection (0.78fmol/mL); (**D**) AEA extracted from saline at the limit of detection (0.78fmol/mL); (**E**) AEA-d8 extracted from saline at the limit of quantification (6.25fmol/mL); and (**F**) AEA extracted from saline at the limit of quantification (6.25fmol/mL).

2.5.1 Results of Validation

Linearity as derived from calibration curves (n=15) was described by the equation

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

The response was linear over the non-extracted concentration range of 1.66 to 133fmol on column (equivalent to 0.23-19nM). Precision for the 133fmol injection of AEA (19nM) on column (20 injections) was calculated to have a mean of 19 nM ± 0.70 with a relative standard deviation (RSD) of 3.7% which is the best precision described for the measurement of AEA. Similarly, precision for 20 injections of 0.24nM (1.66fmol) and 0.95nM (6.65fmol) which resemble the lower AEA concentrations observed in plasma yielded mean values of 0.238 ± 0.009nM and 0.945 ± 0.046nM, with RSD of 3.9 and 4.8 %, respectively.

For non-extracted AEA-d0 and AEA-d8, limits of quantification (LOQ) were 0.22fmol on column (a signal to noise ratio > 10) and the limits of detection (LOD) were 0.055fmol on column (signal to noise ratio = 3). These represent improvements of an order of magnitude over the previous best LOD [19]. On column, the accuracy for 3.33fmol of AEA was 97.5 ± 9.5%, 98.5 ± 6.1% for 6.65fmol and was 104.5 ± 3.2% for 133 fmol. In Figure 2.12. spectra demonstrating the LOD and LOQ for the extraction of AEA-d8 from 2mL of plasma are shown to be 18.75fmol/mL (a signal to noise ratio > 3) and 25fmol/mL (a signal to noise ratio > 10), respectively. Likewise, AEA and AEA-d8 extracted from saline yielded LOD and LOQ values of 0.78fmol/mL (signal to noise ratio > 3) and 6.25fmol/mL (signal to noise ratio > 10), respectively.

2.6 Description of the Final Method

The UPLC-MS/MS system comprised of an Acquity UPLC system connected inline with a Quattro Premier tandem mass spectrometer (Waters Ltd., Elstree, UK). The column used was an Acquity UPLC BEH C_{18} (2.1 x 50 mm) maintained at 40°C. Mobile phases were A (2mM ammonium acetate containing 0.1% formic acid) and B (acetonitrile containing 0.1% formic acid). LC gradient conditions were as follows: 0-0.5 min, 80% A; 1.5 min, 0% A; 2.5 min, 80% A then re-equilibrated at 80% A until 3.5 min. Samples were maintained at 4°C throughout.

Analytes were quantified using tandem electrospray mass spectrometry in positive ion mode (ES). Source parameters included capillary voltage of 0.6kV, cone voltage of 18V, source temperature of 120°C, desolvation temperature of 440°C, cone gas flow of 49L/h, desolvation gas flow of 800L/h. Subsequently, MS/MS conditions for monitoring each precursor [M+H] ion comprised entry, collision and exit energies of -2, 17 and -17eV, respectively. Product ions were monitored in single reaction monitoring (SRM) mode.

Injection volumes for samples and standards were identical: 7µl with needle overfill.

Eight-point calibration curves were performed in triplicate and AEA peaks were integrated using Masslynx software version 4.1(Waters Inc., Milford, MA, USA, 2005). Quanlynx software (Waters Inc. Milford, MA. USA, 2005) was used to calculate the concentration of AEA using calibration curves of concentration against relative response calculated as follows:

Relative response (y) = $\frac{Peak\ area}{(IS\ area/[AEA-d8])}$

y = mx + c

m = gradient of calibration curve

c = intercept

x = concentration of AEA

Where IS = the peak area of the AEA-d8 internal standard

[AEA-d8] = the concentration of the internal standard.

Peak area = the peak area of AEA.

2.7 Validation of plasma AEA levels through pregnancy

Once a method for the measurement of AEA in human plasma had been proven to be reliable, sensitive and reproducible, it was decided to validate it against data obtained/published previously by our group for plasma AEA throughout pregnancy. The data was obtained using an 'old' HPLC-MS method, with a run time of 60 minutes per sample.

2.7.1 Introduction

The work done previously by this group had established the levels of plasma AEA throughout pregnancy, and found that AEA fell from the first to second trimester, was then maintained at a relatively low level during the third trimester, and then rose at term, before exhibiting a significant increase during labour (Habayeb et al., 2004).

These findings pointed to a possible role for the endocannabinoid system in reproduction, firstly low levels/activity being needed to maintain uterine quiescence during pregnancy, and then a sudden rise to play a role in the initiation and maintenance of uterine contractions during labour.

2.7.2 Methods

In order to replicate the work done by the group using the new method to measure AEA, the same parameters were used to group the pregnant volunteers:

First trimester – 6 to 11 weeks.

Second trimester – 15-27 weeks.

Third trimester – 28 to 35 weeks.

Term – 37 to 42 weeks.

Labouring - cervical dilatation of at least 4 cm and three to four regular uterine contractions every 10 min.

The grouping of the trimesters does not fit entirely with convention, but were used to replicate those of the original work. The reason for the choosing of these groups originally was because of gaps in the patient numbers, especially in the late first / early second trimester groups, and also to have a separate term non-labouring group to allow comparison with those women in labour. Blood was collected from women attending for routine USS, attending for blood tests during pregnancy, attending for induction of labour, and in labour. Plasma was collected and processed as detailed in 2.1.2.3 and plasma AEA levels calculated.

2.7.3 Results

2.7.3.1 Original Experiments:

Seventy seven pregnant women were recruited; there were 10 each in the first, second, and third trimesters; 22 in term and non-laboring groups; and 25 in term and laboring groups.

The plasma levels of AEA during pregnancy, excluding the laboring women, fell from 0.89 ± 0.14 nM in the first trimester to 0.44 ± 0.12 nM in the second trimester and 0.44 ± 0.11 nM in the third trimester (P ♠0.04) and thereafter rose to 0.68 ± 0.09 nM in the term non-labouring group (P ♠ 0.03). There was, however, a very dramatic change in the levels in the term laboring women, which rose 3.7 times the values in term non-labouring women and six times those in the third trimester to 2.5 ± 0.22 nM. The differences between term laboring and the first, second, and third trimesters and non-labouring levels were all statistically significant (P ♠ 0.0001).

Patient Group	Number of Patients	Plasma AEA (Li et al.)
1st Trimester	10	0.89 ± 0.14
2nd Trimester	10	0.44 ± 0.12
3rd Trimester	10	0.44 ± 0.11
Term	22	0.68 ± 0.09
Labouring	25	2.5 ± 0.22

Table 2.3. Plasma AEA levels for each group. (Mean ± SEM)

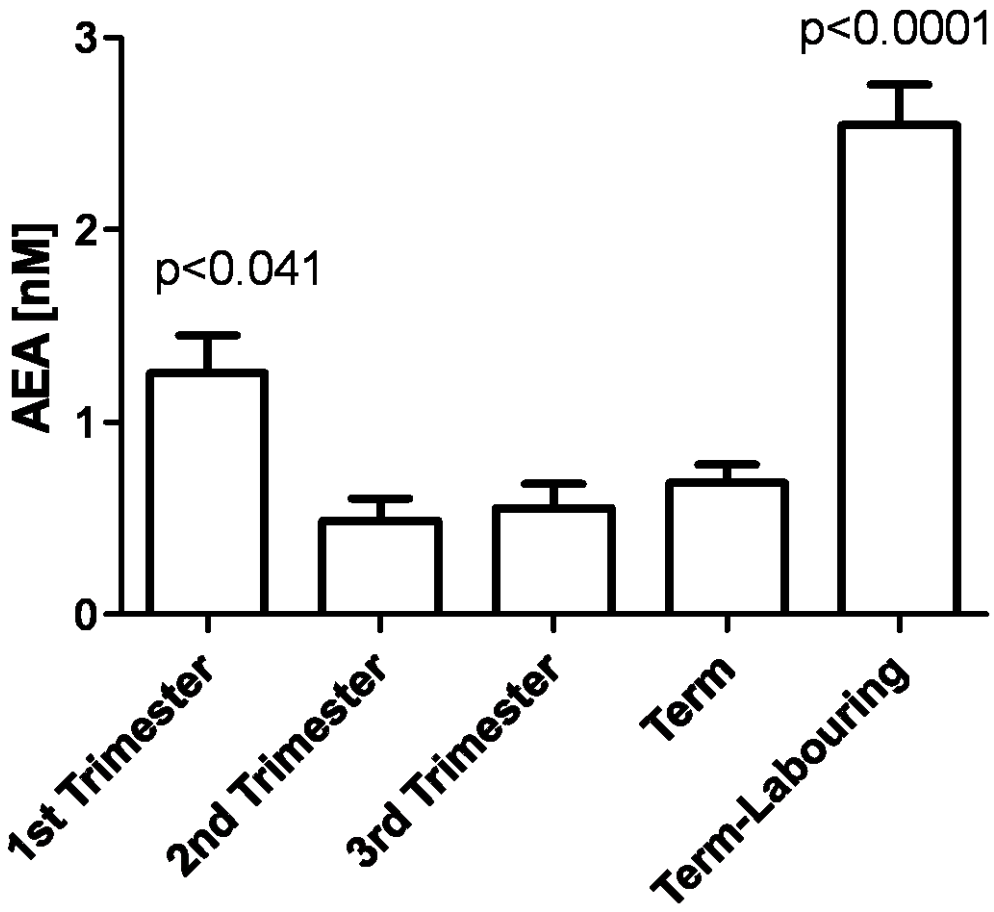

Fig 2.11. AEA levels in pregnancy, and in non-labour and labor women at term. Data are means ± SEM. Comparisons were performed using the Student's unpaired t test with Welch's correction. P .0.05, first trimester vs. second trimester and third trimester vs. term non-labouring; P .0.0001, laboring vs. term non-labouring or third trimester.

2.7.3.2 New Method

Fifty seven pregnant women were recruited for this new method; 15 were in the first trimester, 6 in the second trimester, and 3 in the third trimester; 20 at term and non-laboring groups; and 13 at term and laboring.

The levels of AEA during pregnancy, excluding the laboring women, remained relatively static in the first, second and third trimesters; 0.95 ± 0.07 nM in the first trimester to 1.08 ± 0.07 nM in the second trimester and 0.94 ± 0.14 nM in the third trimester, and thereafter rose to 1.2 ± 0.09 nM in the term non-labouring group (P ♠ 0.03). Again there was a significant change in the levels in the term laboring women, which rose to 1.9 ± 0.16 nM. The differences between term laboring and the first, second, and third trimesters and non-labouring levels were all statistically significant (P ♠ 0.002) Table 2.4.

Patient Group	Number of Patients	Plasma AEA
1st Trimester	15	0.95 ± 0.07
2nd Trimester	6	1.08 ± 0.07
3rd Trimester	3	0.94 ± 0.14
Term	20	1.2 ± 0.09
Labouring	13	1.9 ± 0.16

Table 2.4. Plasma AEA levels for each group. (Mean ± SEM)

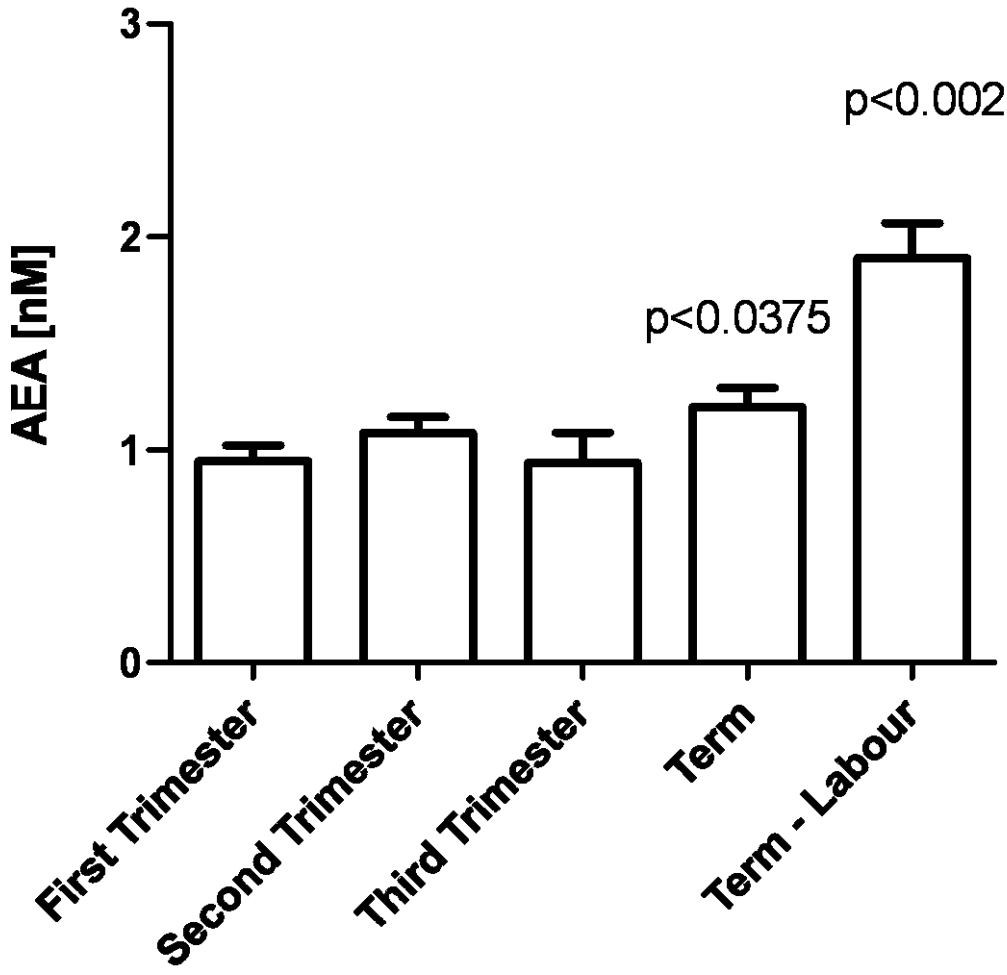

Fig 2.12. AEA levels in pregnancy, and in non-labour and labor women at term. Data are means ± SEM. Comparisons were performed using the Student's unpaired t test with Welch's correction. P .0.0375; P .0.0001, laboring vs. term non-labouring or first to third trimesters.

Patient Group	2nd trimester	3rd trimester	Term	Labouring
1st trimester	0.219	0.9567	0.0375	< 0.0001
2nd trimester		0.436	0.3802	0.0004
3rd trimester			0.191	0.0021
Term				0.0014

Table 2.5. P values of different AEA levels by trimester using Student unpaired *t*-test with Welch's correction for unequal variances.

Patient Group	Original Experiment	New Method	p-value
1st trimester	0.89 ± 0.14 (n=10)	0.95 ± 0.07 (n=15)	p = 0.15
2nd trimester	0.44 ± 0.12 (n=10)	1.08 ± 0.07 (n=6)	p = 0.0008
3rd trimester	0.44 ± 0.11 (n=10)	0.94 ± 0.14 (n=3)	p = 0.08
Term	0.68 ± 0.09 (n=22)	1.2 ± 0.09 (n=20)	p = 0.0003
Labour	2.5 ± 0.22 (n=25)	1.9 ± 0.16 (n=13)	p = 0.02

Table 2.6. Comparison between the two studies AEA levels per trimester. P-values comparing levels per trimester.

2.8 Discussion

The new method developed is an improvement over MS alone, as with MS there is a possibility that a different compound may have the same mass as the one being studied leading to inaccuracies. With MS-MS there is a much smaller chance that a compound different to the one studied will have the same mass for both parent and daughter ions. This means that much greater certainty can be placed upon the fact that what is being measured is the desired compound.

The method developed and described here represents the shortest analysis time for any method published to date for the analysis of AEA in a biological matrix. When it is compared with the next fastest published method, the run time employed represents an improvement of over 4min/per sample previously for measurement of AEA (Schmidt et al., 2006). Furthermore, this method also represents a marked improvement in limit of quantification (LOQ), limit of detection (LOD), precision and accuracy when compared with previously validated methods. The sensitivity is a marked improvement on previous published methods and implies that smaller plasma samples could be used in analysis or that this method could be employed to analyse biomatrices that might have significantly lower AEA concentrations than those found in plasma.

The significantly less sensitive limits of detection and quantification for AEA-d8 measurement following extraction from plasma compared with extraction from saline will need further investigation and implies that greater sensitivity could be achieved by improvements in the extraction method. LOD and LOQ for plasma samples are conservative here because it was decided to employ a relatively large re-suspension volume (80μL) and three injections of each sample (7μL) with needle overfill, to

maintain a high degree of accuracy and reproducibility. Consequently, if desired, the lower limits of detection and quantification for plasma AEA-d8 and AEA could be improved by re-suspending the extracted samples in a smaller final volume of acetonitrile.

In comparing the new method with the method previously employed by the group, the results obtained showed a similar trend in plasma AEA throughout pregnancy, however there were some significant differences.

Similarities were that levels in the first trimester were not significantly different ($p = 0.1549$); term labouring levels were significantly raised in both groups when compared to both first trimester and term pregnancies ($p<0.002$). The trend for a rise during the third trimester of pregnancy was also present in both groups.

There was, however, significant differences in the actual AEA levels found in the 2nd trimester, at term and in labour. Also the trend of a drop in AEA levels form the 1st trimester to the second and third trimesters and term seen using the old method were not replicated.

The differences seen in the second and third trimester groups are likely to be attributable to the low numbers in the new method group [6 and 3 respectively], leading to possible errors, and this may account in some way for the lower levels that were seen in the labouring group using the new method.

Plasma AEA levels measured in humans have varied 10 fold depending upon the method used. This was part of the reason for the development of a new measurement. The fact that the 'new' method is a great improvement in terms of sensitivity, may also partially explain the differences seen versus that previously found.

In general, despite the lack of perfect correlation between the two methods, important trends present in the original work have remained, and because of this it is now possible to explore how plasma AEA levels are changed in different circumstances related to pregnancy.

In terms of a new method for measuring AEA, the combination of UPLC with MS/MS has yielded a method for the analysis of AEA at concentrations found in plasma with a sensitivity, reproducibility and precision that are improvements on previous methods and which make this method suitable for the analysis of clinical samples. The improved sensitivity suggests that this method could be used for the analysis of AEA in reduced sample volumes or in biomatrices with lower levels of AEA. The faster analysis time for this method represents a saving both financially (solvents/salary) and in time, allowing the analysis of larger sample numbers.

CHAPTER 3

AEA levels in viable threatened miscarriage

3.1 Introduction

The term ‘miscarriage’ is used to describe the failure of a pregnancy before viability is reached. In the UK, this usually occurs before 24 weeks of gestation, although the actual upper limit of viability is reducing. Miscarriage is traditionally divided into two groups; those occurring in the first trimester (i.e. up to 12 weeks gestation) and those occurring in the second trimester (i.e. from 13 to 24 weeks gestation). The reason for this distinction is that the causes and treatment of 1st and 2nd trimester miscarriage tend to differ, meaning they are usually treated as two separate entities, with 2nd trimester loss being of much less common occurrence (French and Bierman, 1962), and usually such loss is investigated to find a possible cause, in order to aid future pregnancies. By contrast, 1st trimester loss is a much more common problem, with 15-20% of clinically recognised pregnancies failing during this time (Wilcox et al., 1988, Norwitz et al., 2001a) and that loss is not usually investigated.

Threatened miscarriage is defined as that point when a woman presents with vaginal bleeding (usually light), with or without pain, but has an ongoing viable pregnancy confirmed by an ultrasound scan (USS). Although women with threatened miscarriage have viable pregnancies at the time of presentation, as the name implies there is a chance or threat that the pregnancy will fail, which is significantly greater than that seen in the general pregnant population (Farrell and Owen, 1996, Chung et al., 1999). Unfortunately, at present, it is not possible to predict which of these patients will subsequently go on to suffer a loss of the pregnancy (miscarry) and who will have an ongoing pregnancy that goes to term, and results in a ‘take-home’ baby. The ability to do this would lead to reduced anxiety amongst women and their families, and allow investigations and any provided treatment to be better targeted.

In this regard, anandamide may hold the key to the problem. The reason for this supposition is that *in-vitro* studies in the mouse have suggested that endocannabinoids, especially AEA, are pivotal for synchronous development of the blastocyst and endometrium in preparation for implantation (Paria et al., 1996, Schmid et al., 1997a, Habayeb et al., 2002), an important event for successful early pregnancy, as discussed in Chapter 1. At the implantation site, levels of FAAH have been shown to increase and AEA levels to decrease prior to successful implantation, and exposure of the blastocyst to high levels of AEA leads to failure of implantation (Paria et al., 1995, Paria and Dey, 2000)

The work of Maccarrone and colleagues in women with IVF-ET pregnancies showed that a 'high' plasma AEA at 6 weeks gestation was associated with subsequent pregnancy failure, whilst lower levels were associated with a successful pregnancy (Maccarrone et al., 2002a) Similarly, the same research group showed that this risk of subsequent pregnancy failure is due to elevated FAAH levels in peripheral blood mononuclear cells (Maccarrone et al., 2000c). These studies, however, have only been performed by this group on a cohort of Italian women, and have not been validated in other clinics and laboratories around the world. If these findings hold true for those women presenting with symptoms of threatened miscarriage in the first trimester, then the likely outcome of the pregnancy will be predictable.

The aims of this part of the thesis were therefore to use the new validated method of measuring plasma AEA in a cohort of pregnant women presenting with threatened miscarriage to determine whether a single plasma AEA levels is predictive of

subsequent miscarriage, what level of AEA could be used as a cut-off and how good such an assay might be in the clinical setting.

3.2 Subjects and Methods

A prospective study was undertaken of 45 women presenting to the Early Pregnancy Assessment Unit [EPAU] at the Leicester Royal Infirmary with symptoms of threatened miscarriage. For the purpose of the study, threatened miscarriage was taken as vaginal bleeding in the absence of any abdominal pain. The women selected were between 6 and 12 weeks of pregnancy and all had USS confirming a single live fetus at presentation.

The inclusion criteria also ensured that all were healthy non-smokers with a BMI <30. Women with pre-existing medical conditions, for example diabetes mellitus, hypertension, connective tissue disease, or who were on any medication when presenting, were excluded. This was to remove possible confounding factors from the study.

In each case, a single blood sample was taken from the ante-cubital fossa and placed into EDTA tubes, mixed and put immediately on ice. Samples were transported to the research laboratory on ice and the plasma separated within 2 hours of collection. The plasma was spiked with 25 pmoles of AEA-d8 and then processed as previously described [Section 2.2.3] to purify the lipid component of plasma and the dried sample from the second to last step, stored at -80^{0}C. After storage of less than 42 days, samples

were reconstituted in acetonitrile, and analysed using the UPLC-MS/MS method outlined previously [Section 2.6.].

3.2.1 Statistical Methods

A power calculation of previously published plasma AEA data (Di Marzo et al., 2001a, Maccarrone et al., 2002a, Habayeb et al., 2004) indicated that the minimum number of subjects in each group that would allow a 3-fold increase in AEA was 5 with α=0.05 and β=0.8.

Receiver Operating Characteristic (ROC) curve analysis were undertaken to assess to ability of AEA to act as a predictive test. Analysis in pregnancy outcome was determined using Mann-Whitney U-test with $p<0.05$ considered as significant.

3.3 Results

Of the 45 pregnancies; 9 subsequently miscarried [miscarriage group] during the first trimester, and 36 went to have live births [live birth group]. The miscarriage group and the group that went on to deliver at term were equally matched in terms of gestation at the time of blood collection, age and BMI [Table 3.1].

The median plasma AEA levels were in the miscarriage group were 3-fold higher than the levels in the live birth group [3.47nM, IQR 2.83-3.86 nM *v.* 1.07nM, IQR 0.81-1.45nM; respectively]. This 3-fold difference (Table 3.1) was a highly significant elevation [$p<0.001$; Mann-Whitney U test] (Figure 3.1).

Table 3.1. Comparison of Live Birth Group and Miscarriage Group in terms of demographics. (NA = not applicable)

Characteristic	Live Birth Group (N=36)	Miscarriage Group (N=9)
Mean ± SD Age (Years)	28 ± 1.5	29 ± 1.6
Mean (range) gestational age at recruitment ($week^{+day}$)	8 (6-11+1)	8+1 (6+5 – 10)
Median (IQR) plasma AEA concentrations [nM]	1.065 (0.81-1.45)	3.47 (2.83-3.86)
Mean (range) interval between recruitment and miscarriage (days)	NA	7.83 (5-14)
Mean ± SD gestation at delivery or miscarriage ($weeks^{+days}$)	$39^{+1} \pm 1^{+5}$	8±1
Mean ± SD birth weight (kg)	3.4 ± 0.52	NA

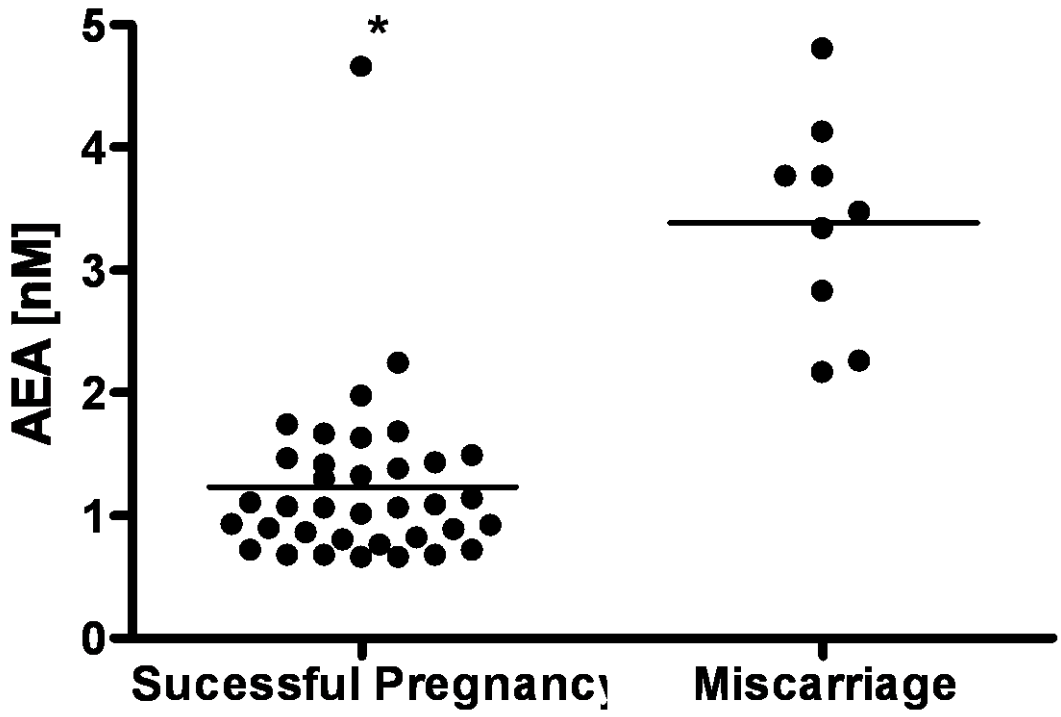

Fig 3.1. Comparison of plasma AEA levels in the successful pregnancy and miscarriage groups. The horizontal bars indicate the medians. The plasma levels in the miscarriage group were significantly higher than the successful pregnancy group [$p<0.0001$; Mann-Whitney U test]. The * denotes the patient who presented preterm with a small for gestational age baby and suffering from very severe pre-eclampsia.

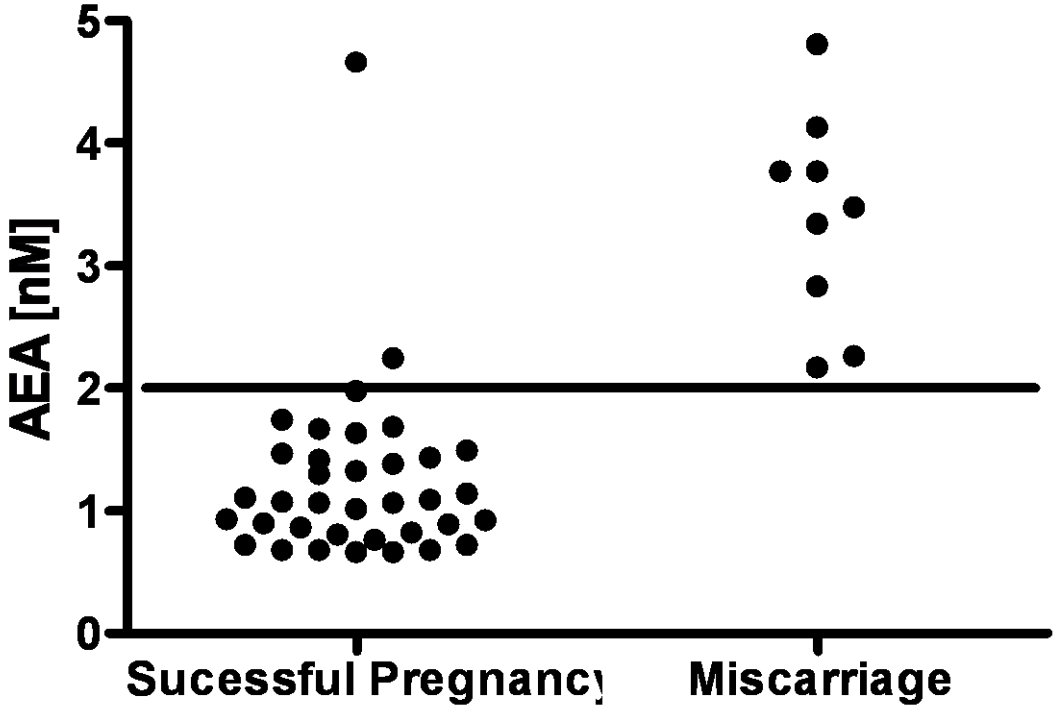

Fig 3.2. Comparison of plasma AEA levels in the successful pregnancy and miscarriage groups demonstrating the number of patients using the threshold level of 2nM as a cut-off point for predicting subsequent miscarriage. This value gave a sensitivity of 100% and specificity of 94%, with a negative predictive value of 100% and positive predictive value of 78% for subsequent miscarriage.

All the women who subsequently when on to miscarry had a plasma AEA of greater than 2nM (Figure 3.2). Of the women with successful pregnancies, 34 of the 36 had plasma AEA < 2nM. The other 2 women within the successful pregnancy group had plasma AEA >2nM. These women, however, did not have 'trouble-free' pregnancies as one patient, (the highest; denoted by the presence of an asterisk in Figure 3.2) went on to develop severe pre-eclampsia and was delivered at 33 weeks gestation with a birth weight of 1.85kg and the other patient (the lower one) delivered at term following an uneventful pregnancy, with a birth weight of 3.7 kg.

Taking a single plasma AEA level of 2.0nM as cut-off for predicting subsequent miscarriage gave a sensitivity of 100% (95% confidence interval [CI] 66.4%-100%) and specificity of 94% (95% CI, 81.3%-99.3%), with a negative predictive value of 100% (95% CI, 66.4%-100%) and positive predictive value of 82% (95% CI, 48.2%-97.7%) for subsequent miscarriage. ROC analysis (see Fig 3.3) showed the area under the curve was 0.972 (95% CI, 0.882-0.999).

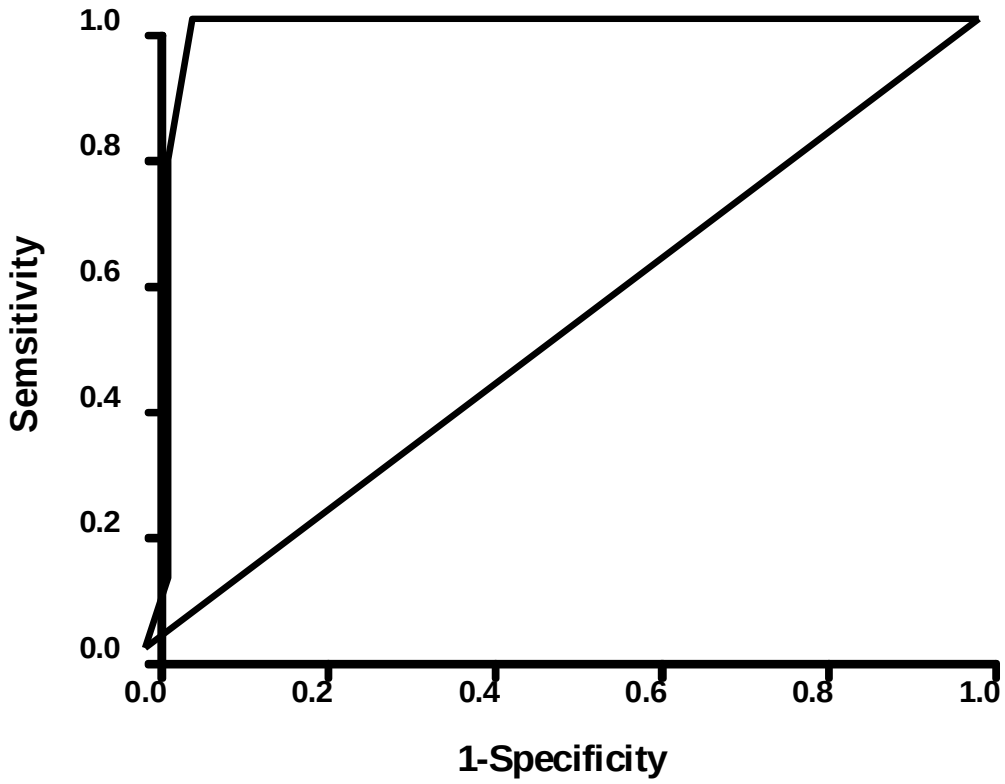

Figure 3.3. ROC analysis of the plasma AEA values for women with threatened miscarriage. Area under the ROC was 0.972 (95% CI, 0.882-0.999). The ROC curve and co-efficients were calculated using an arbitrary cut-off of 2nM.

3.4 Discussion

In the group of women presenting with threatened miscarriage, with a confirmed viable pregnancy, 9 out of 45 (20%) subsequently when on to miscarry in the first trimester, which is in keeping with published data (Sotiriadis et al., 2004, Johns and Jauniaux, 2006). It was found that a single measurement of plasma anandamide in these women was predictive of pregnancy outcome [miscarriage *v.* successful pregnancy], with a sensitivity of 100% and a specificity of 94%. When a threshold plasma level of AEA of 2nM was taken, none of the pregnancies below this critical value went on to miscarry. This suggests that if plasma AEA was measured in all women presenting with threatened miscarriage, a significant number of these women could be reassured that, should they have a plasma AEA concentration of <2nM, that their pregnancies would almost certainly continue, with a successful outcome. This would also allow healthcare resources to be better targeted, as this group should not require further follow up once viability had been confirmed.

From this study it is evident that women with plasma AEA concentration that were >2nM were more likely to subsequently suffer loss of the pregnancy. Such women would therefore require support and close surveillance of the pregnancy on the basis of the AEA result. In this group of patients, unlike the successful pregnancy group, there is a degree of uncertainty, as not all women with plasma AEA >2nM went onto miscarry. Since the majority {9 out of 11: i.e. 82%} of women in this group did miscarry, close monitoring of these pregnancies is essential, not only to confirm if miscarriage occurs, but also if the pregnancy does continue so that other pregnancy problems can be identified early.

Of the 2 women with successful pregnancies and a plasma AEA concentration of >2nM, one had a 'normal' pregnancy whilst the other was complicated by severe pre-eclampsia that required early delivery at 33 weeks gestation. The cohort of women we chose for this study was from the 'normal pregnancy population' and thus are likely to be representative of the type of women one normally sees in EPAUs around the UK. The only caveat is that all the women we studied were spontaneous pregnancies and not from IVF-ET/ICSI treatment groups. That was not by design, simply the way the patients presented. Therefore, it is difficult to directly correlate these data with that of Maccarrone and co-workers (Maccarrone et al., 2002a). Nevertheless, the data tend to suggest that high plasma AEA levels are detrimental to early pregnancy success.

The other possible confounder is that women who were smokers were excluded. That might have meant that women that use recreational drugs such as marijuana were also excluded. It is well recognised that exposure to exogenous cannabis [marijuana] during pregnancy can lead to a host of adverse effects, including increased risk of preterm labour, fetal growth restriction, low birth weight, perinatal death and delay in the commencement of respiration (Gibson et al., 1983, Zuckerman et al., 1989, Sherwood et al., 1999, Fergusson et al., 2002). Since the active part of marijuana [THC] acts *via* the same receptors (CB1 and CB2) as does AEA to exert its effects (Devane et al., 1988, Matsuda et al., 1990, Devane et al., 1992, Munro et al., 1993), it is likely that exposure to high levels of endogenous cannabinoids, such as AEA, during pregnancy will also cause pregnancy problems, such as miscarriage. This means that women with an ongoing pregnancy, and a plasma AEA >2nM would need close surveillance throughout their pregnancy to identify such problems early.

The raised plasma AEA levels found to be predictive of subsequent miscarriage in this group of women is similar to the work of Maccarrone previously in IVF pregnancies (Maccarrone et al., 2002a), and these results may reflect what is happening at a local level, i.e. at the embryo-endometrial interface. From animal models, elevated AEA levels are associated with failure of the blastocyst to implant, and AEA levels are greatly reduced at the site of implantation within the uterus (Das et al., 1995, Paria et al., 1996, Schmid et al., 1997a). Therefore, any divergence from 'normal' AEA levels through failure of the endocannabinoid system in early pregnancy may cause an abnormal embryo-endometrium interaction and subsequent pregnancy failure.

Whether the endocannabinoid system works alone or in combination with other pregnancy related systems, is unclear. However, progesterone, a hormone already known to be vital for normal human reproduction, is likely to be involved as it up-regulates the expression of FAAH in peripheral blood mononuclear cells, which in turn is thought to be the sole regulator reducing plasma AEA levels. It may therefore be that an initial deficiency in progesterone production during the luteal phase of the menstrual cycle or during the early stages of pregnancy causes the following chain of events: reduced FAAH production, subsequent increase in plasma AEA, which in turn acting at the fetal-maternal interface causes damage to the pregnancy, leading to miscarriage. This speculative hypothesis could be easily tested in the human as the levels of hCG, which is used extensively in the management of early pregnancy problems, especially ectopic pregnancies, and which has also been used to predict pregnancy outcomes with varying success, is produced by the developing trophoblast and thus is a critical marker of early placentation events and success. Since it is also a vital component of early

trophoblastic development (Mannavola et al.), its measurement in relation to plasma AEA might reveal a possible interaction with the endocannabinoid system.

In conclusion, the endocannabinoid system appears to play a pivotal role in human reproduction, and measurable parts of the system, in this case a single plasma AEA level, allowed the outcome of a pregnancy presenting with bleeding in the first trimester to be predicted with a level of certainty that left existing predictive markers far behind. However, how the endocannabinoid system 'fits' with other established markers of early pregnancy success (such as hCG) remains unclear, and further investigation of their possible relationships are needed.

CHAPTER 4

The levels of AEA, hCG, Progesterone and PAPP-A in viable and non-viable early pregnancies

4.1. Introduction

The mechanisms underlying early pregnancy development and success or failure are poorly understood. The process in humans is inefficient with up to 60% of conceptuses being lost during the first trimester (Wilcox et al., 1988, Ellish et al., 1996, Zinaman et al., 1996). Most of these are lost at the time of the expected menses, however, approximately 15-20% of clinically recognised pregnancies miscarry in the first trimester (Garcia-Enguidanos et al., 2002, Savitz et al., 2002). This high rate of pregnancy loss leads to significant emotional distress for the women involved, and a significant financial and organisational burden upon healthcare providers. Because of this, a detailed search for biochemical markers which could be used to help predict a failing pregnancy or confirm miscarriage has been undertaken, with the result that hCG and progesterone have been the two most widely studied (Ong et al., 2000a, Homan et al., 2000, Poikkeus et al., 2002, Barnhart et al., 2004b, Ioannidis et al., 2005b). Both play essential roles in early pregnancy development with hCG being produced by the trophoblast and progesterone primarily by the corpus luteum. Studies have shown associations between lower levels of each, either singularly or in combination, with pregnancy failure. However, the sensitivity and specificity of hCG and progesterone as predictors of pregnancy failure is only about 80%, and wide variations have been seen between studies when defining a threshold level for each hormone (Homan et al., 2000, Poikkeus et al., 2002, Al-Azemi et al., 2003, Barnhart et al., 2004b, Wallace et al., 2004a, Elson et al., 2005b, Ioannidis et al., 2005b, Takata et al., 2005). Additionally, since both hCG and progesterone are not produced directly by the embryo, changes in their levels may occur after embryonic demise, and therefore not give a true representation of what is happening *in utero*.

Progesterone and hCG are not the only biomarkers that have been examined. Other biochemical markers studied include:

1. Inhibin A – low levels have been associated with subsequent miscarriage, but as a predictor was found to be no better than hCG or progesterone (Al-Azemi et al., 2003).
2. Leptin – because deficiency of leptin in mice is associated with sterility. In humans, low leptin levels have been found in failed pregnancy, but not prior to this (Laird et al., 2001, Tommaselli et al., 2006).
3. Macrophage Inhibitory Cytokine 1 (MIC 1) – this molecule has Th2 immunity actions and is localised at the materno-fetal interface. In a small study, MIC 1 was shown to be significantly lower in women with subsequent miscarriage (Tong et al., 2004a), although the authors concluded that more work is needed for this molecule to be used as a predictor of miscarriage
4. Pregnancy Associated Plasma Protein-A (PAPP-A) – this protein is a trophoblast derived protease. Low levels in the 2nd trimester are associated with increased pregnancy complications, and have also been found prior to subsequent miscarriage (Tong et al., 2004b).
5. Anandamide – as discussed in Chapter 3, is an endocannabinoid with high levels associated with miscarriage in women with threatened miscarriage.

How all or any of these markers interact at the mechanistic level to help predict miscarriage is obscure, although it is likely that successful implantation and maintenance of pregnancy occur through a complex interaction between fetal and maternal tissues that involve several of these different factors. In addition to those factors described above, a balance between type 1 T-helper (Th1) and type 2 T-helper

(Th2) cytokines (Maccarrone and Finazzi-Agro, 2004) are thought to play a key role in the process of early pregnancy maintenance. Although a unique immunological interplay is essential for the lack of rejection of the fetus by the mother, little is still known about how the factors named, act, singularly or in tandem, to enable synchronous development of the embryo and the endometrium to ensure timely and successful implantation.

Recent data from animal models and older data from studies in humans have indicated that marijuana use has an adverse influence on reproduction, affecting the menstrual cycle, lowering LH levels, and is associated with prematurity, fetal growth restriction and low birth weight (Gibson et al., 1983, Asch and Smith, 1986, Zuckerman et al., 1989, Fergusson et al., 2002). After the discovery of the different elements of the endocannabinoid system, work began looking for a possible role of the endocannabinoids in reproductive success and failure.

To date, various areas of interest have been explored, and evidence of a role for endocannabinoids gained. Initially, elements of the endocannabinoid system were isolated throughout reproductive tissues and fluids (Felder et al., 1996, Paria et al., 1996, Schmid et al., 1997a, Beltramo and Piomelli, 2000, Schuel et al., 2002, Dennedy et al., 2004, Habayeb et al., 2004), with the highest levels of AEA being found at the inter-implantation sites within the mouse uterus, and the levels at implantation sites being significantly lower (Schmid et al., 1997a). Further to this, exposure of the mouse blastocyst *in-vitro* to high concentrations of AEA led to implantation failure, however, when lower levels of AEA were used, the blastocysts were rendered competent for implantation (Paria et al., 1995).

These findings suggested an important role for the endocannabinoid system in both pregnancy success and failure.

These types of studies are difficult, if not impossible, to replicate in humans, however, peripheral measurements for the activity of the endocannabinoid system have been possible, yielding some interesting results. For example, plasma AEA levels have been observed to vary both through the menstrual cycle, and through pregnancy (Habayeb et al., 2004, Lazzarin et al., 2004). The levels in the luteal phase of the menstrual cycle are continuous with those in the first trimester of pregnancy, and significantly lower than the follicular phase of the cycle (Habayeb et al., 2004). These data suggest that plasma AEA levels need to be reduced during the implantation window and in the first trimester for a successful pregnancy outcome and could be reflecting the levels of AEA in the uterus. This is borne out by the plasma AEA and FAAH levels seen in viable first trimester pregnancies in which pregnancies with high AEA and low FAAH were characterised by a high probability of subsequent miscarriage, whilst those with AEA levels similar to those identified in the luteal phase had continuing pregnancies (Habayeb et al., 2004). The causal link between these factors is a hormone known to play a vital role in reproductive success, progesterone, which is upregulated by FAAH *in vivo* (MacCarrone et al., 2000, Maccarrone et al., 2003a). The available evidence therefore points to a significant role played by the endocannabinoid system in early pregnancy success or failure, and as seen in Chapter 3, it may be possible to use the measurement of plasma AEA to predict outcomes in early pregnancy. It is unlikely that AEA acts alone, and it is more likely that this molecule is part of a network of substances involved in early pregnancy success and development. It is therefore of interest to see how AEA correlates with the known markers of early pregnancy success

and failure, i.e. β-hCG, progesterone, and PAPP-A, to see if there is an interplay with the levels of AEA.

Since progesterone, hCG and PAPP-A appear to be involved in the regulatory process responsible for early pregnancy maintenance (Yovich et al., 1986, Ho et al., 1997, Tong et al., 2004b, Potdar and Konje, 2005b, Boldt, 2007, Chetty and Elson, 2007), and progesterone has also been suggested to be involved in the regulation of AEA (Maccarrone et al., 2002b, Gasperi et al., 2005, El-Talatini et al., 2009b), the aim of this part of the project was designed to examine whether:

- there is a difference in AEA levels in pregnancies which are confirmed to be non-viable when compared to those being viable, and
- there is a relationship between plasma AEA, serum progesterone, hCG and PAPP-A in these groups.

Although high levels of plasma AEA are associated with pregnancy failure, it is unclear what happens once the pregnancy has failed, or whether the levels of plasma AEA during spontaneous miscarriage are affected by other reproductive hormones.

4.2. Materials and Methods

4.2.1. Subjects

All subjects gave signed, informed consent to take part in the study, which was undertaken with the approval of and under the guidelines of the institutions ethics committee. Women presenting to the Early Pregnancy Assessment Unit (EPAU), requiring a reassurance ultrasound because of a previous miscarriage, were randomly selected at 6-12 weeks amenorrhoea and had an ultrasound scan to determine viability.

Women with pregnancies of unknown viability undergoing ultrasound scanning in the first trimester [5-12 weeks gestation] were recruited to the study. These were divided into two groups; those that had viable pregnancies, and those that had non-viable pregnancies at the time of the ultrasound scan. A total of 14 ml of blood was collected from each woman and sub-divided for analyses; 4 ml into an EDTA tube for AEA quantification and 10 ml into a plain tube for serum P4, β-hCG and PAPP-A measurement. The tubes containing the blood were placed on ice and transported to the research laboratory where plasma and sera were separated. The serum was then stored at -80°C for later hormonal [hCG, progesterone and PAPP-A] analysis.

4.2.2. Measurement of AEA

The plasma was processed for AEA quantification as described in section 2.2.3 Following the extraction of AEA from plasma, it was quantified for each patient using UPLC-MS/MS as described in section 2.6.

4.2.3. Measurement of Serum hCG, Progesterone and PAPP-A

Serum hCG and progesterone levels were processed by the Biochemistry Department of the University Hospitals of Leicester NHS Trust, using the ADVIA Centaur Immunoassay System (Bayer HealthCare LLC, Diagnostics Division; Tarrytown, NY, USA).

For hCG the ADVIA Centaur Total hCG two-side sandwich immunoassay was used. This had a detection range of 2mIU/ml to 1000mIU/ml. If a level of >1,000mIU/ml was

found a dilutional step was preformed and the assay repeated, allowing up to 100,000mIU/ml to be detected. Intra- and inter-assay coefficients of variability were 2.8% and 2.9%, respectively.

For progesterone the ADVIA Centaur Progesterone competitive immunoassay using direct chemiluminescence was used. The assay range was 0.48 nmol/l to 190.8 nmol/l. Intra-assay coefficient of variability was 5.3%, and inter-assay coefficient of variability was 3.6%.

Serum PAPP-A was measured using a commercially available ELISA kit [Gamma S.A., Liege, Belgium]. Intra-assay coefficient of variability was 5.4%, and inter-assay coefficient of variability was 2.9%.

4.2.4. Statistical Analysis

A power analysis based on previous work of the Endocannabinoid Research Group and that of other published data of viable and non-viable pregnancies (Maccarrone et al., 2002a) with $\alpha = 0.05$ and $\beta = 0.8$ showed that a minimum of 6 subjects was required in both the miscarriage and on-going pregnancy groups to allow a difference of 40% in plasma AEA levels to be observed with 80% power.

Statistical analysis of the data was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Data were non-parametric in nature and thus are expressed as medians and inter-quartile ranges (IQR) where appropriate, and comparisons between groups performed using

Mann-Whitney U-test. Correlations between hormone measurements were made using Spearman's correlation. The level of significance was set at $P<0.05$.

4.3. Results

A total of 45 women attending the EPAU were recruited into the study; 25 with viable pregnancies on USS (viable group) and 20 with non-viable pregnancies on USS (non-viable group). There were no significant differences in the age and BMI distribution in the two groups as shown in Table 4.1.

Plasma AEA levels in the non-viable group were significantly higher than those in the viable group at the time of ultrasound scan (1.48nM; IQR 1.01-2.34nM, versus 1.21nM; IQR 0.92-1.39nM respectively: $P=0.013$) (Fig 4.1.).

Serum P4 levels in the non-viable group were lower than those in the viable group, but this did not achieve statistical significance (41nmol/L; IQR 9.5-65.0ng/mL, versus 51.5ng/mL; IQR 42.0-63.5ng/mL respectively; $P=0.052$) (Fig 4.2.). Although the levels of β-hCG were lower in the non-viable group than in viable group (6560mU/mL; IQR 386-49254mU/mL versus 28650mU/mL; IQR 14172-38707 mU/ml respectively) (Fig 4.3.), this was not statistically significant ($P=0.144$). The median serum PAPP-A levels in the non-viable group were approximately seven times those in the viable group (12.25mg/L; IQR1.99-19.63mg/L, versus 1.82mg/L respectively; IQR1.56-5.73mg/L) (Fig 4.4.), but this was not statistically significant $P=0.071$.

The relationship between plasma AEA levels and the levels of the hormones measured are shown in Table 4.2. There was no statistically significant correlations between plasma AEA levels and serum progesterone (r=0.017; *P*=0.926), AEA and β-hCG levels (r=-0.258, *P*=0.162) and between AEA and PAPP-A levels (r=0.35, *P*=0.086) in the 45 patients (Figs 4.5. to 4.7.). There was, however, a statistically significant negative correlation between plasma AEA levels and the length of gestation (r=-0.327, *P*=0.028). There were also statistically significant correlations between β-hCG and progesterone levels (r=0.575; *P*=0.001) and between PAPP-A and β-hCG levels (r=0.506, *P*=0.027).

A further analysis of the relationship between plasma AEA levels and PAPP-A, β-hCG and P4 in the viable and non-viable groups was undertaken (Table 4.3). In the viable group, there was a negative correlation between β-hCG and plasma AEA levels (r = -0.466, *P*=0.51), although this did not achieve statistical significance. There were also negative but statistically insignificant relationships between P4 and AEA and between PAPP-A and AEA (*P*>0.05). In the non-viable group, there was a statistically significant positive correlation between P4 and β-hCG (r=0.739, *P*=0.004), P4 and PAPP-A (r=1.00, *P*<0.0001) and between AEA and PAPP-A (r=0.697, P=0.025). As expected β-hCG had a statistically significant positive correlation with gestation (r=0.75, *P*=0.003). Plasma AEA also had a negative correlation with gestational age (r=-0.475; *P*=0.035). The other relationships were not statistically significant.

	Viable Pregnancy	Non-Viable Pregnancy	
Age	29 (23-34)	27 (23-29.5)	*P* = 0.34
BMI (kg/m^2)	23 (22-26)	24.5 (21.3-26)	*P* = 0.87
AEA (Li et al.)	1.20 (0.92-1.39)	1.48 (1.01-2.34)	*P* = 0.013
P4 (ng/mL)	51.5 (42-63.5)	41.0 ± (9.5-65.0)	*P* = 0.052
β–hCG (mIU/mL)	28650 (14172-38707)	6560 (386-49254)	*P* = 0.144
PAPP-A (mg/L)	1.82 (1.56-5.73)	12.25 (1.99-19.63)	*P* = 0.071

Table 4.1. Patient Demographics and Hormonal Measurements

Age, BMI, plasma AEA levels, serum progesterone, β-hCG and PAPP-A levels are all shown as median and (IQR).

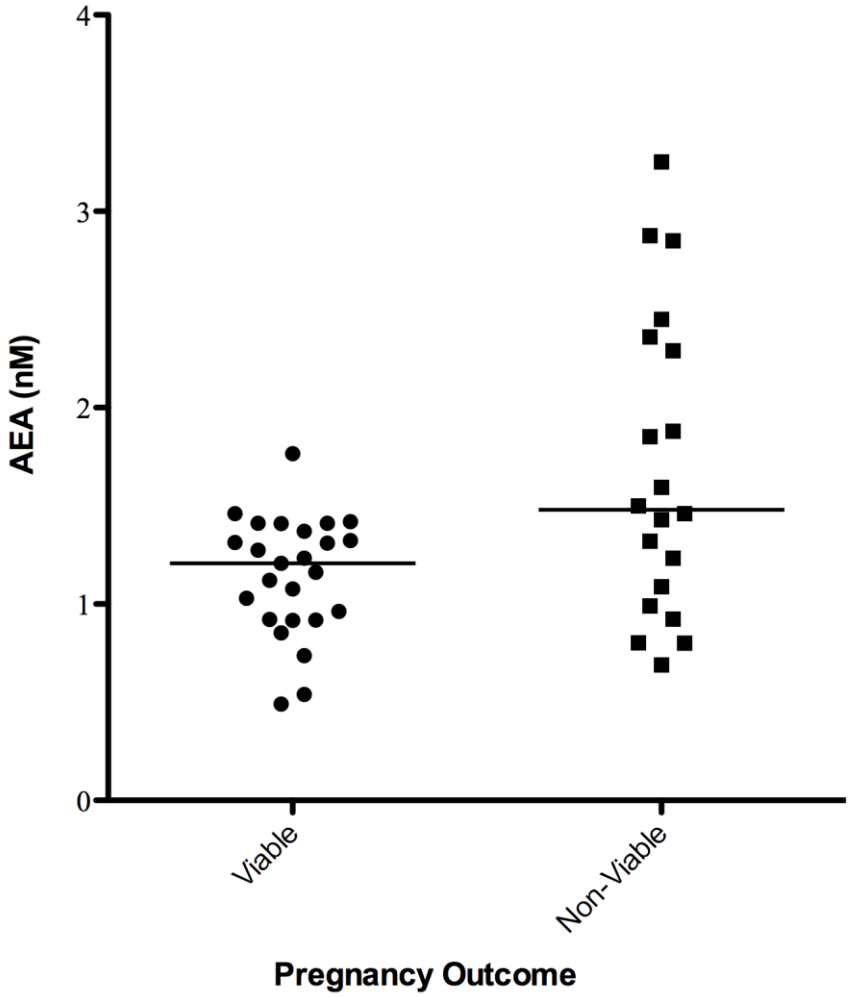

Fig 4.1. – Plasma AEA levels in viable and non-viable pregnancies were significantly higher in non-viable pregnancies compared to viable pregnancies at the time of USS. (P=0.013; Mann-Whitney U-test). Medians are shown in each case.

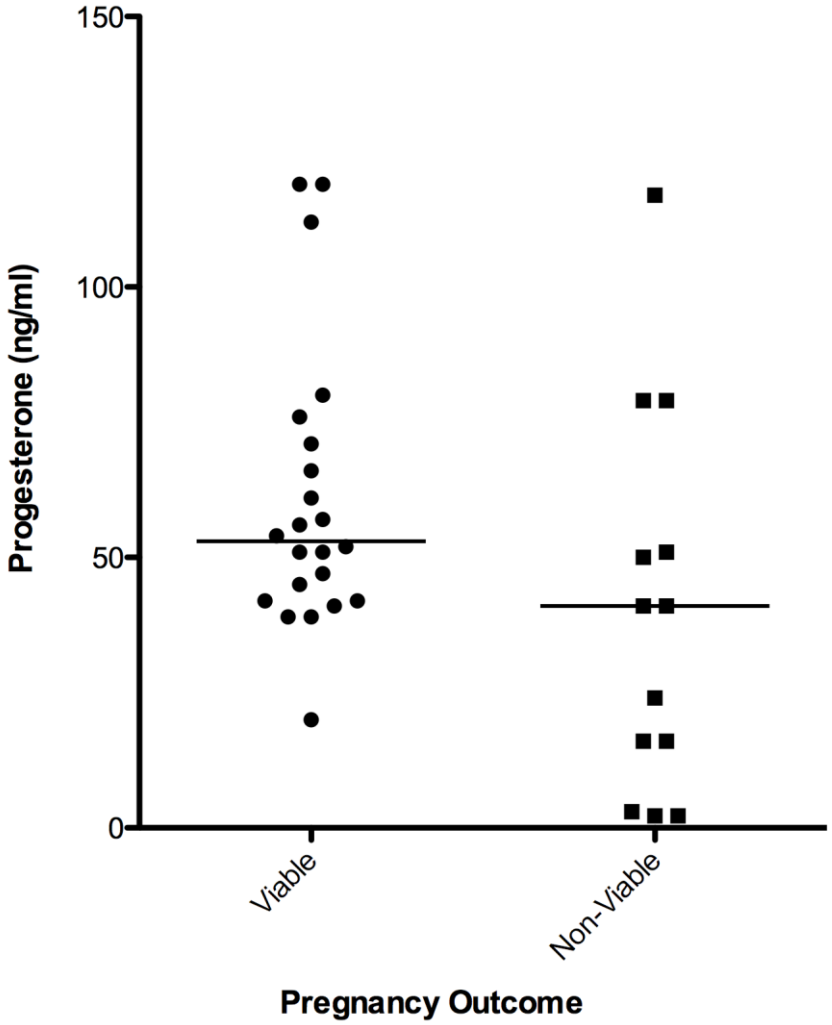

Fig 4.2. - Serum progesterone levels in viable and non-viable pregnancies at the time of USS were higher in viable pregnancies compared to non-viable pregnancies, although this did not reach statistical significance (P=0.052; Mann-Whitney U-test). Data shows medians in each case.

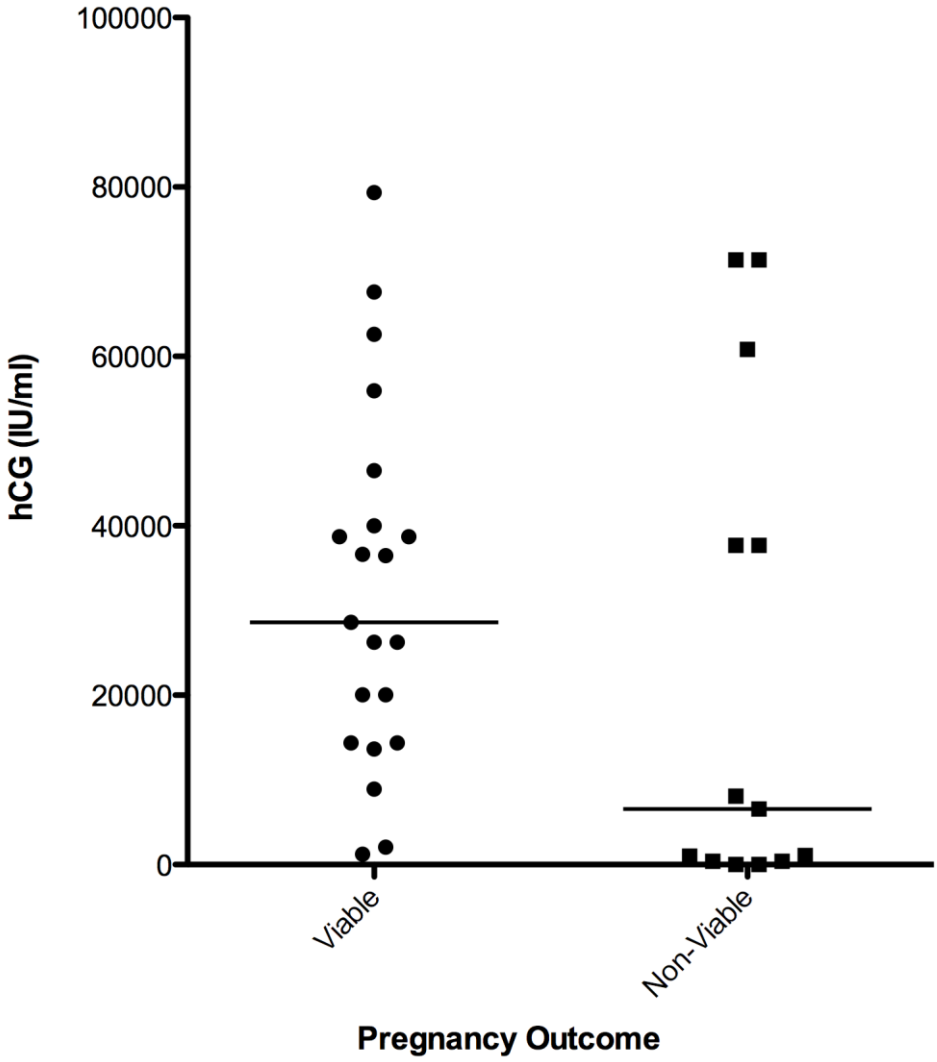

Fig 4.3. Serum hCG levels in viable and non-viable pregnancies were higher in viable pregnancies compared to non-viable pregnancies at the time of USS, although the difference did not reach statistical significance (P= 0.144; Mann-Whitney U-test). Data shows medians in each case.

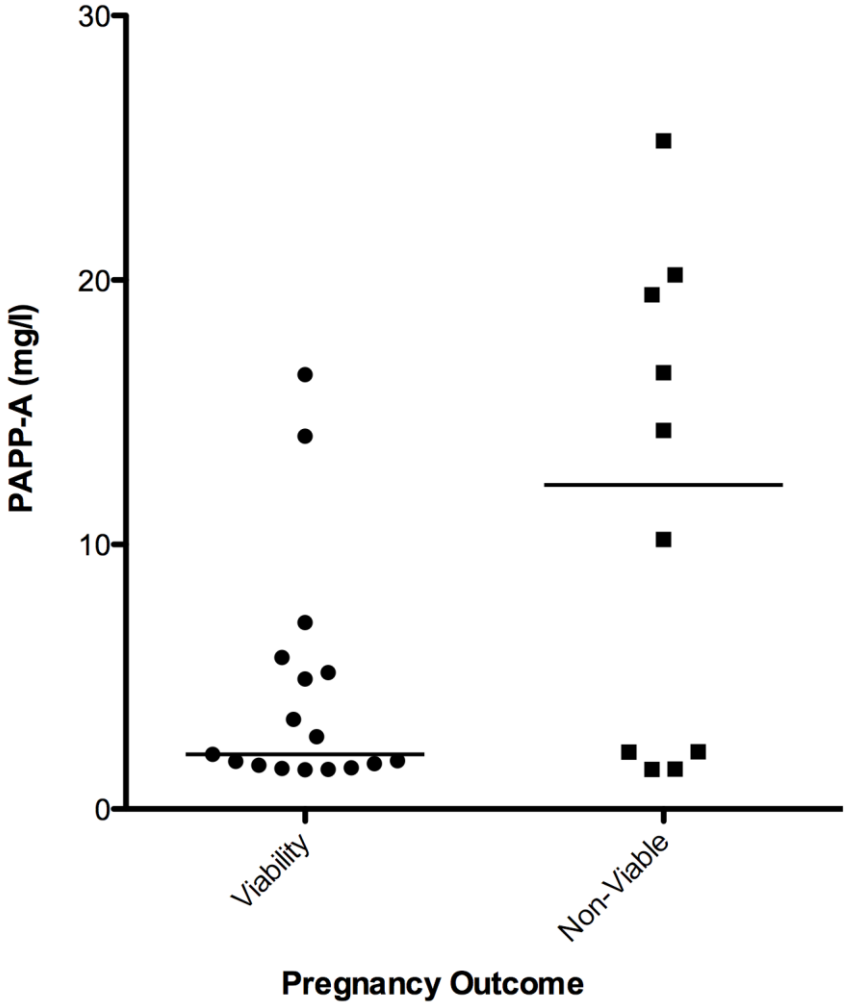

Fig 4.4. Serum PAPP-A levels in viable and non-viable pregnancies were higher in viable pregnancies compared to non-viable pregnancies at the time of USS, although this did not reach statistical significance (P= 0.071; Mann-Whitney U-test). Data shows medians in each case.

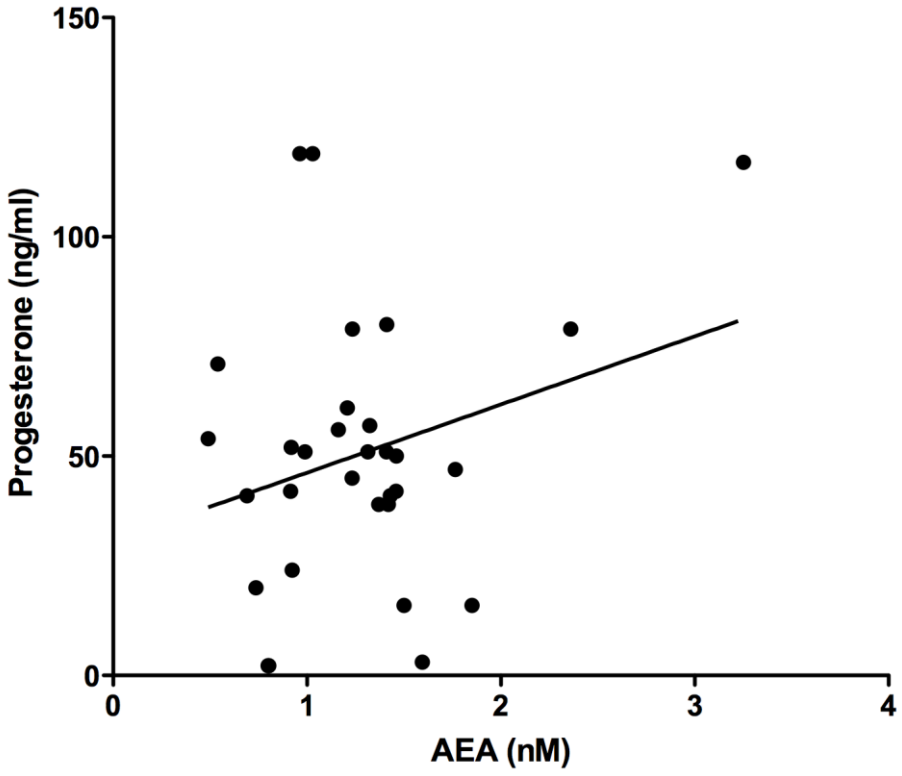

Fig 4.5. Correlation between AEA and progesterone levels. The data indicate that there is a weak positive correlation which is not significant. (r=0.017, P=0.926, n=31)

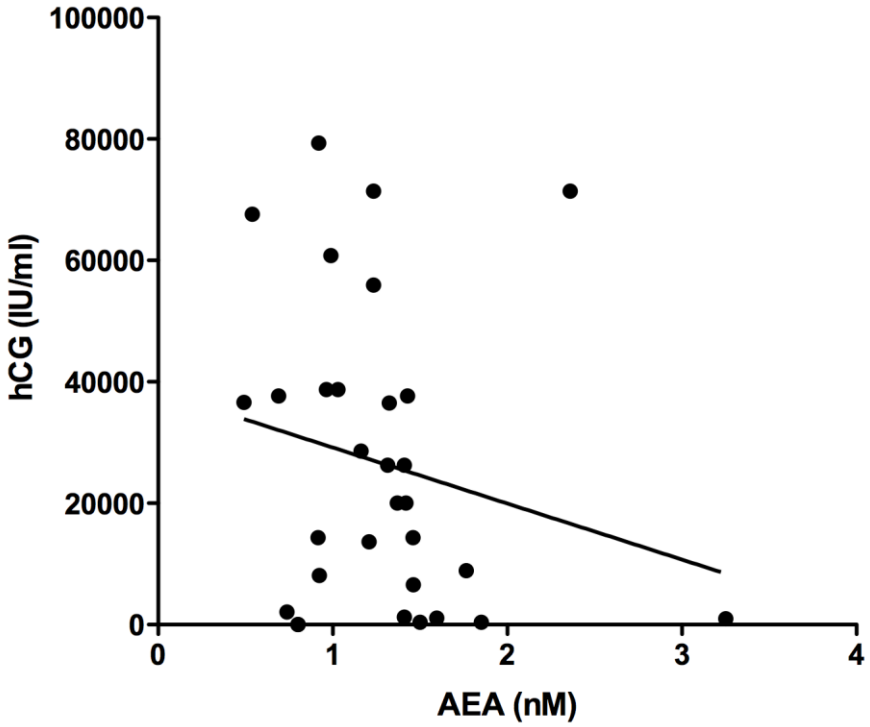

Fig 4.6. Correlation between AEA and hCG levels. The data indicate there a weak negative correlation which is not significant. (r=-0.258, P=0.162, n=31).

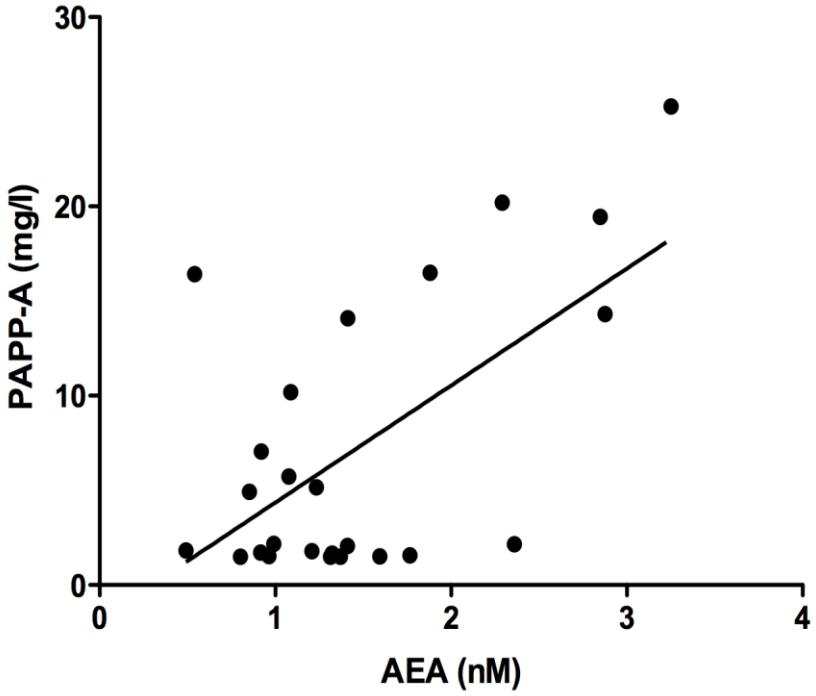

Fig 4.7. Correlation between AEA and PAPP-A levels. The data indicate a positive, statistically significant, correlation which is significant. (r=0.351, P=0.086, n=25).

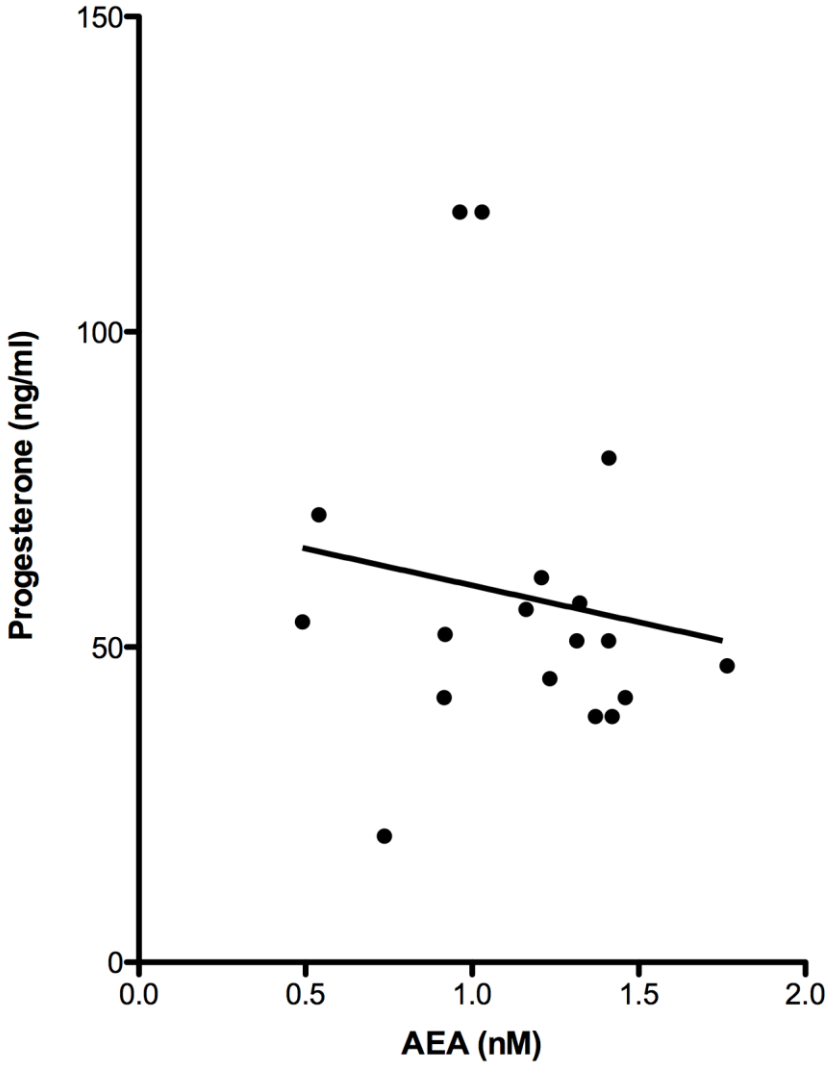

Fig 4.8. Correlation between AEA and progesterone levels in the viable pregnancy group. The data indicate that there is a weak negative correlation, which is not significant. (r=-0.247, P=0.323, n=18)

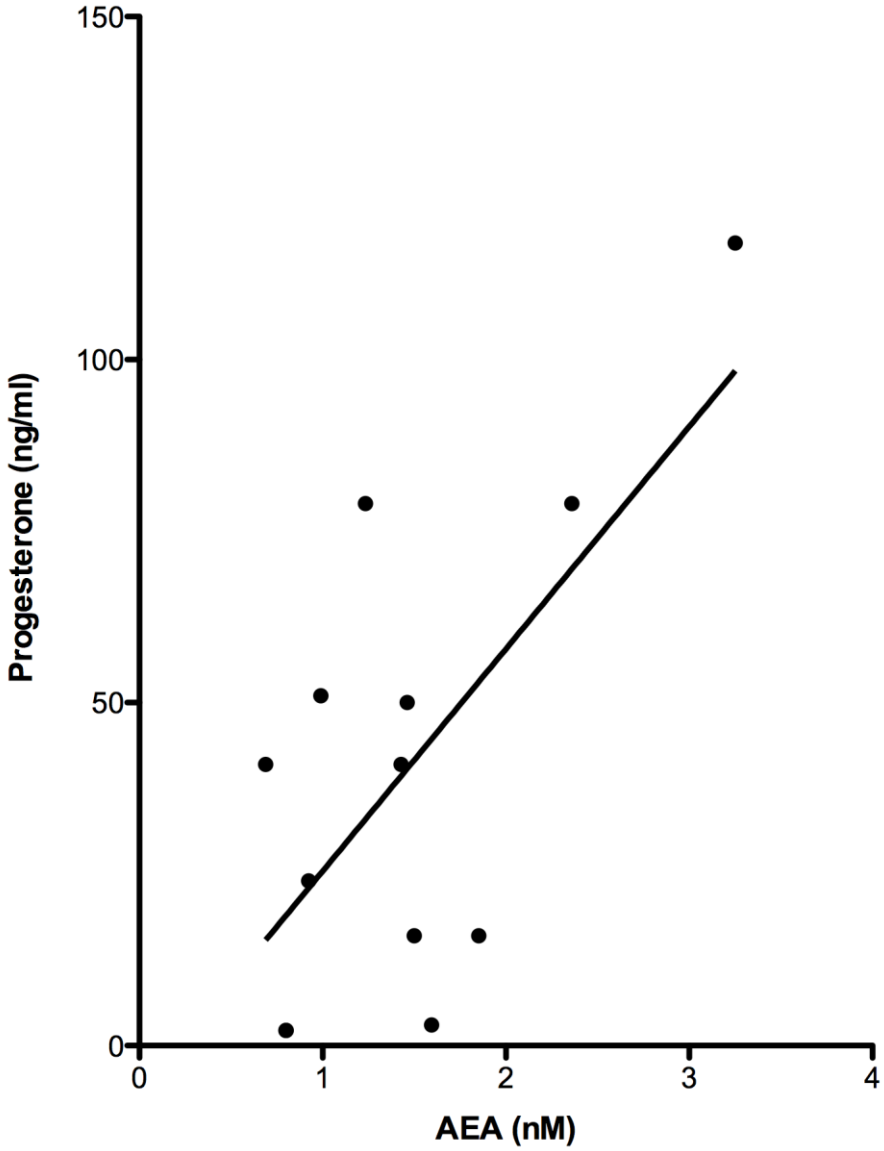

Fig 4.9. Correlation between AEA and progesterone levels in the non-viable group. The data indicate that there is a positive correlation, which is not significant. (r=0.401, P=0.175, n=13)

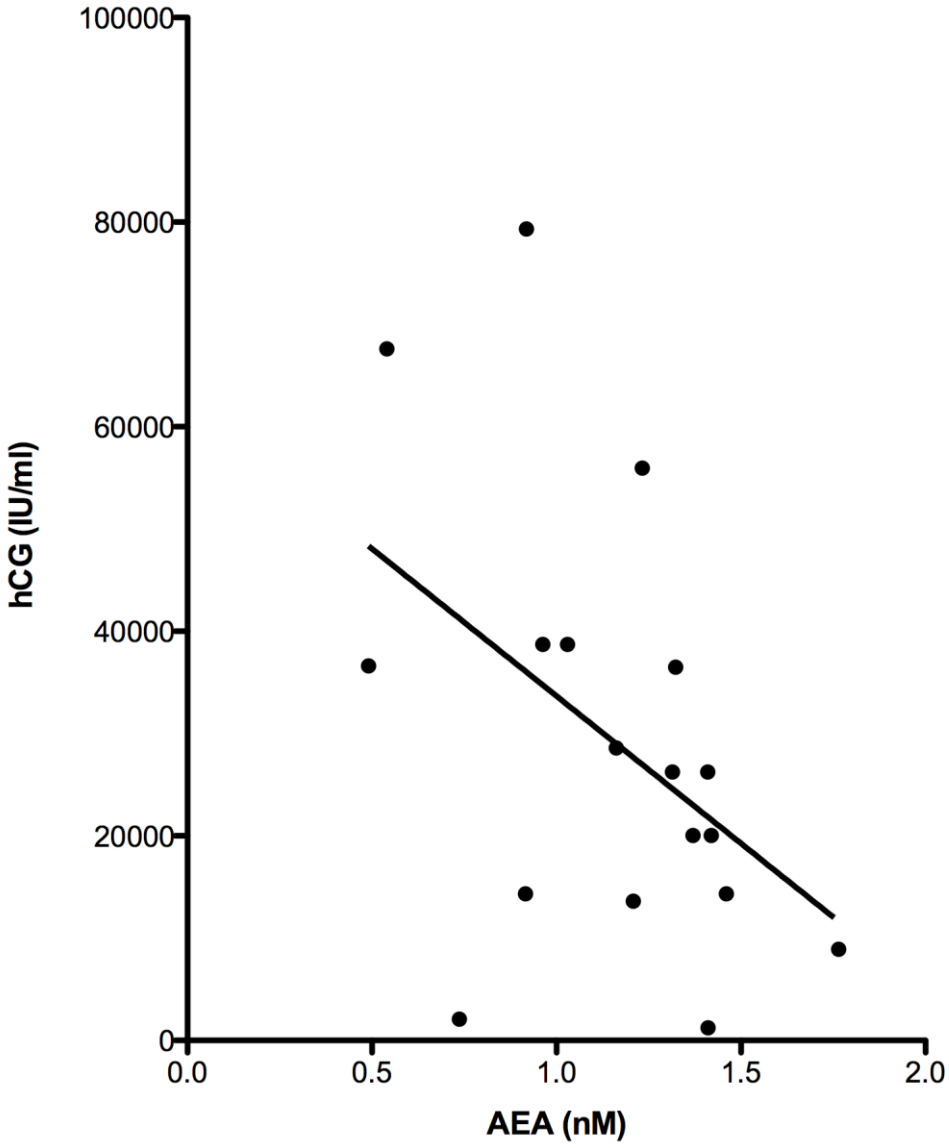

Fig 4.10. Correlation between AEA and hCG levels in the viable group. The data indicate that there is a significant negative correlation. (r=-0.466, P=0.05, n=18).

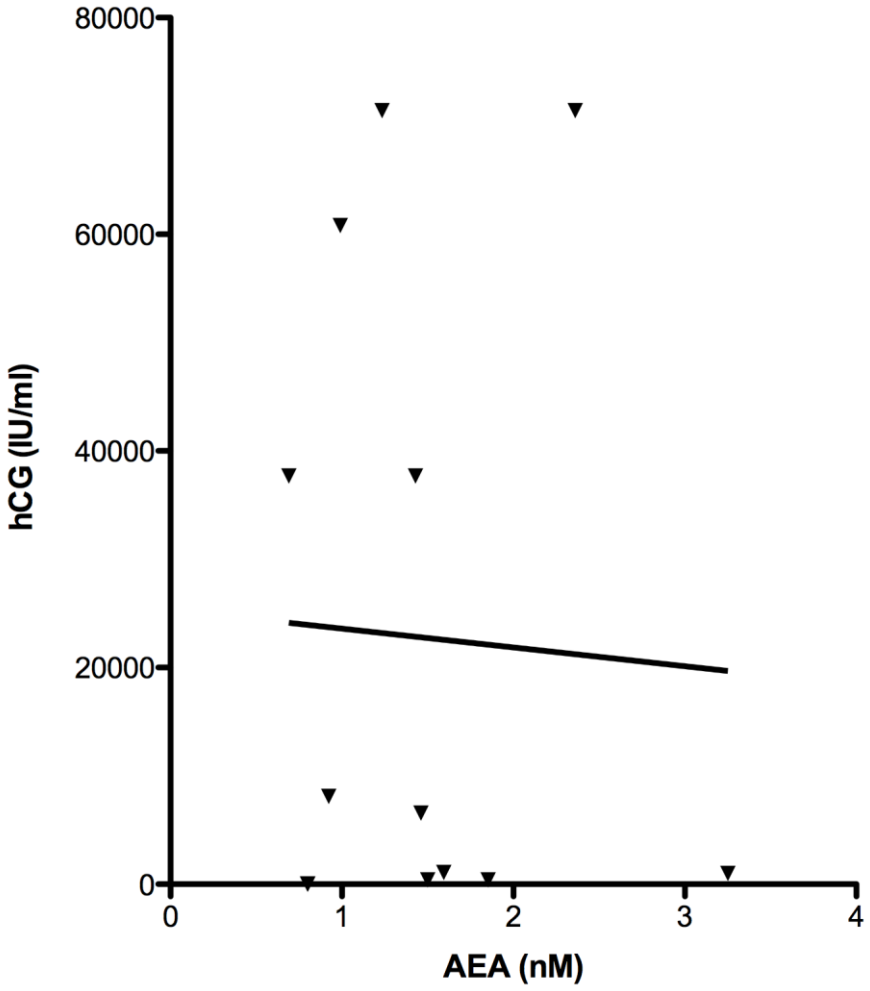

Fig 4.11. Correlation between AEA and hCG levels in the non-viable group. The data indicate that there is no correlation between these factors. (r=0.052, P=0.865, n=13).

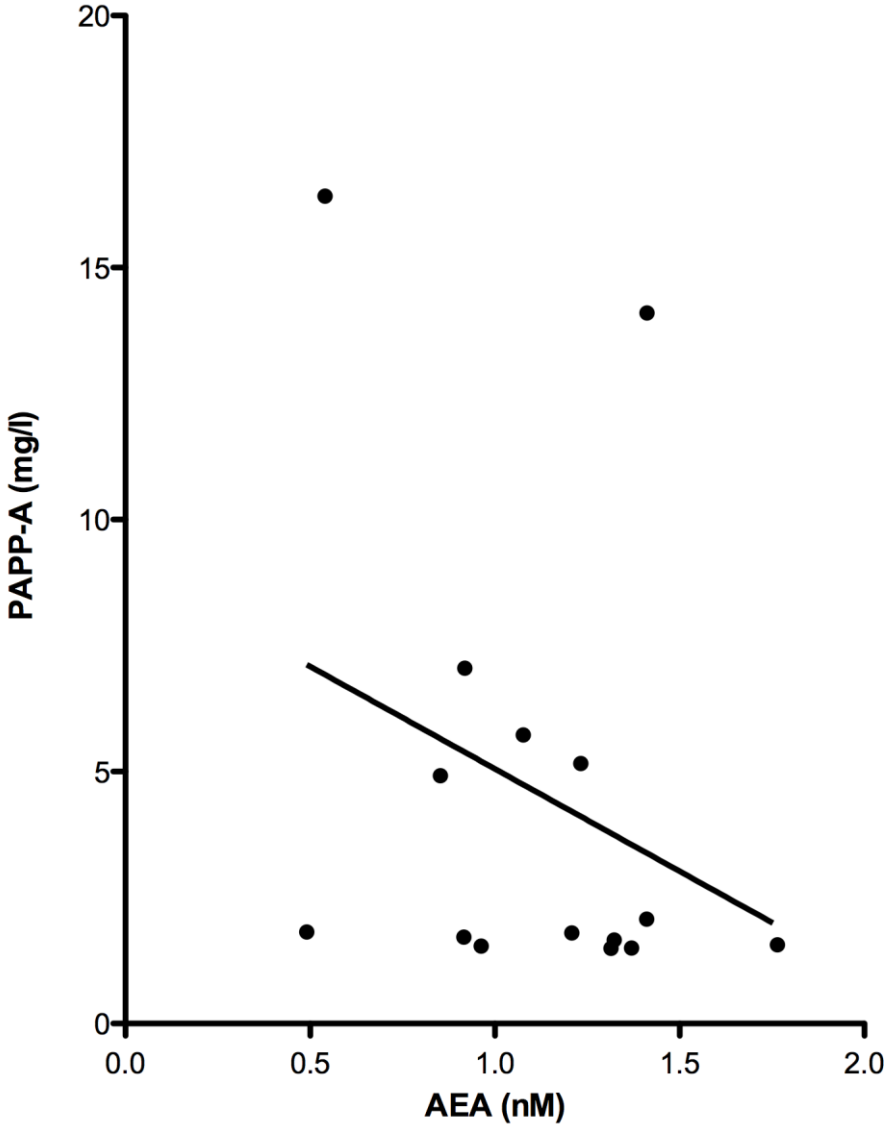

Fig 4.12. Correlation between AEA and PAPP-A levels in the viable group. The data indicate that there is a weak negative correlation, which is not significant. (r=-0.004, P=0.990, n=15).

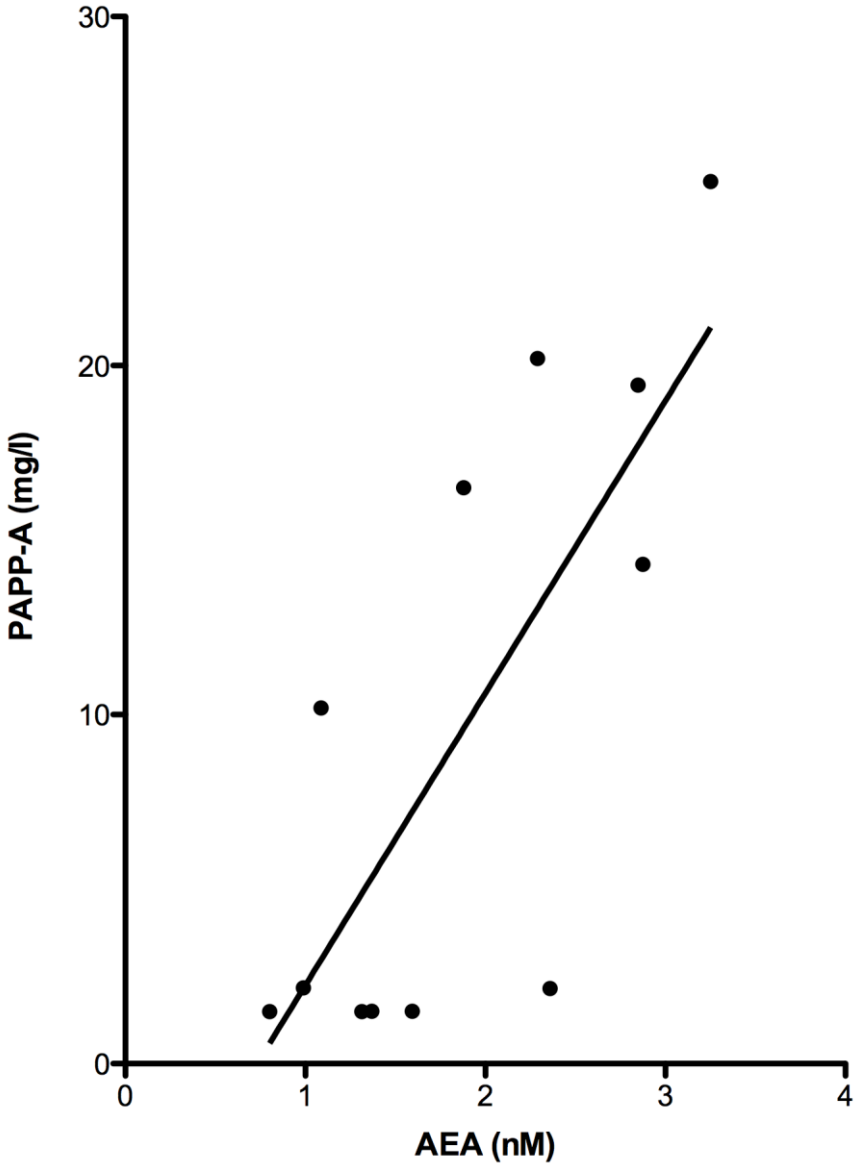

Fig 4.13. Correlation between AEA and PAPP-A levels in the non-viable group. The data indicate that there is a significant positive correlation (r=0.697, P=0.025, n=10).

Table 4.2. Spearman rank correlations between serum hormone and plasma AEA levels in women within the 1st trimester of pregnancy with viable and non-viable pregnancies.

Viable Pregnancies	**r-values**	***P*-values**	**n**	**r-values**	***P*-values**	**n**	**r-values**	***P*-values**	**n**	**r-values**	***P*-values**	**n**
	Prog			**β-hCG**			**PAPP-A**			**AEA**		
β-hCG	0.391	0.109	**18**									
PAPP-A	0.266	0.404	**12**	0.427	0.167	**12**						
AEA	-0.247	0.323	**18**	-0.466	***0.051***	**18**	-0.004	0.990	**15**			
Gestation	-0.086	0.735	**18**	0.386	0.114	**18**	0.156	0.579	**15**	-0.246	0.236	**25**

Non-viable Pregnancies	**r-values**	***P*-values**	**n**	**r-values**	***P*-values**	**n**	**r-values**	***P*-values**	**n**	**r-values**	***P*-values**	**n**
	Prog			**β-hCG**			**PAPP-A**			**AEA**		
β-hCG	0.739	***0.004***	**13**									
PAPP-A	1.000	***<0.0001***	**4**	0.300	0.624	**5**						
AEA	0.401	0.175	**13**	0.052	0.865	**13**	0.697	***0.025***	**10**			
Gestation	0.490	0.089	**13**	0.750	***0.003***	**13**	-0.532	0.114	**10**	-0.475	***0.035***	**20**

Progesterone (Prog) was measured in ng/mL, β-hCG was measured in mIU/mL, PAPP-A was measured in mg/L, AEA was measured in nM and gestation was measured in weeks. The Spearman co-efficients (r) were calculated using GraphPad Prism software and significant correlations are in ***bold***. The number of samples (n) is given for each correlation.

Table 2. Spearman rank correlations between serum hormone and plasma AEA levels in women within the 1st trimester of pregnancy.

All samples	**r-values**	***P*-values**	**n**	**r-values**	***P*-values**	**n**	**r-values**	***P*-values**	**n**	**r-values**	***P*-values**	**n**
	Prog			**β–hCG**			**PAPP-A**			**AEA**		
β-hCG	0.576	***0.001***	**31**									
PAPP-A	0.506	***0.027***	**19**	0.512	***0.025***	**19**						
AEA	0.017	0.926	**31**	-0.258	0.162	**31**	0.351	0.086	**25**			
Gestation	0.045	0.809	**31**	0.338	0.063	**31**	-0.192	0.337	**27**	-0.327	***0.028***	**45**

Progesterone (Prog) was measured in ng/mL, β-hCG was measured in mIU/mL, PAPP-A was measured in mg/L, AEA was measured in nM and gestation was measured in weeks. The Spearman co-efficients (r) were calculated using GraphPad Prism software and significant correlations are in ***bold***. The number of samples (n) is given for each correlation.

4.4. Discussion

This study demonstrates that plasma AEA levels are elevated in women with non-viable first trimester pregnancies when compared to the levels in confirmed viable pregnancies. The levels of plasma AEA in viable pregnancies were similar to those previously reported by Maccarrone *et al*. 2002 and our group (Habayeb et al., 2004) and also similar to those reported in the luteal phase of the menstrual cycle (Habayeb et al., 2004), affirming the suggestion that for successful implantation, plasma AEA levels need to be maintained at a low level during both the implantation window (El-Talatini et al., 2009b) and early pregnancy development (Habayeb et al., 2008b).

In the mouse, a significant fall in local AEA and a rise in FAAH are essential at the implantation site compared to the inter-implantation site for successful implantation and early pregnancy maintenance (Schmid et al., 1997b, Paria et al., 2002c). As seen in Chapter 3, and in the observations of Maccarrone *et al*. 2000, peripheral mononuclear cell FAAH levels and the levels of AEA in humans behave in a similar way as the local uterine endocannabinoid changes reported in the mouse uterus (Schmid et al., 1997a, Liu et al., 2002). The demonstration of raised plasma AEA concentrations in non-viable pregnancies at the time of presentation suggests that an aberration in the endocannabinoid system may have occurred resulting in a disruption of the normal implantation and developmental process, and may thus be the cause of the miscarriage. Precisely, when this might have occurred, is uncertain but since the levels of AEA in non-viable pregnancies are much lower than those we found in women presenting with a threatened miscarriage and a viable pregnancy who subsequently miscarried (as shown in Chapter 3), then this observation may represent the AEA levels returning to a 'baseline' following a peak that occurred at the time of fetal demise. This conclusion is

supported by the finding that the plasma AEA levels observed in this cohort of women were close to those observed during the follicular phase of the menstrual cycle, suggesting that the plasma AEA levels may be measure of the woman's reproductive tract preparing for the recommencement of the new menstrual cycle.

The serum progesterone and β-hCG levels found in these women were similar to those reported in the literature (Yovich et al., 1986, Nulsen and Peluso, 1992, Elson et al., 2003b, Barnhart et al., 2004b, Condous et al., 2004, Ioannidis et al., 2005a, Elson et al., 2005a) and as expected, serum progesterone levels were significantly lower in the non-viable group. This observation was important in the context of this study because progesterone levels have been used to help in the diagnosis and management of failed pregnancies and more recently, those of unknown location (Norwitz et al., 2001b, Elson et al., 2003b, Condous et al., 2004, Ioannidis et al., 2005a, Potdar and Konje, 2005a, Gevaert et al., 2006). Therefore, it is reasonable to assume that the women recruited 'fit' into the categories assigned and that no 'rogue' cases that could skew the data had appeared. Although median β-hCG levels were 4.4 times higher in the viable group, this difference was not statistically significant (P=0.144). Various studies have reported similar observations (Ong et al., 2000b, Barnhart et al., 2004b, Wallace et al., 2004b, Elson et al., 2005a) and it is thought that the absence of significant differences is due largely to differences in the timing of the sample collection. For example, it was likely that some samples would have been taken immediately after the pregnancy failed, and in others it would have been taken days later, when β-hCG levels would have already fallen significantly. Also, it is known that as a predictor of pregnancy failure, β-hCG is poor, with a sensitivity and a specificity of only around 80%. In addition, wide variations have been reported between studies when defining a threshold level for

viability at a specific gestational time point (Al-Azemi et al., 2003, Barnhart et al., 2004b).

The lack of a correlation between AEA and either serum β-hCG or progesterone in the cohort as a whole could easily be interpreted to mean that plasma AEA levels are linked to early pregnancy failure through a mechanism independent of these factors. The lack of a correlation between AEA and progesterone differs from the studies of Maccarrone *et al*. who observed that low P4 levels were associated with high plasma AEA levels, through a mechanism that involved the direct stimulation of FAAH (the main enzyme that degrades AEA) in peripheral mononuclear cells, because their plasma AEA levels were directly inversely related to FAAH expression levels (Maccarrone et al., 2002a, Maccarrone et al., 2003b). The lack of a correlation between P4 and AEA levels was therefore surprising. Two possible explanations could be offered for this disparity.

Firstly, the timing of the sampling in our series: samples were obtained after fetal demise and in some cases, the miscarriages had already occurred (i.e. the uterus had already been spontaneously evacuated). At present, it is not exactly clear how rapidly the levels of these biomarkers return to normal. If, as may be the case, one biomarker is cleared faster than the other, a previously existing correlation may be absent.

Secondly, by combining the viable and non-viable groups to perform the correlations, any correlations in the viable data and *vice versa* may have been hidden. Re-examining the data under the discrete groups of viable and non-viable pregnancies (Table 4.3, Figs 4.8. to 4.13.) then revealed a negative, though non-statistically significant correlation between AEA and β-hCG ($r=-0.466$; $P=0.051$) in the viable group and a significant

correlation between β-hCG and P4 only in the non-viable group (r=0.739; *P*=0.004), as would be expected based on published literature (Yovich et al., 1986, Schindler, 2005, Chetty and Elson, 2007). The serum levels of progesterone and PAPP-A were perfectly correlated (r=1.000; *P*<0.0001), despite the number of samples assayed being small. There was also a correlation (r=0.697, *P*=0.025) between plasma AEA levels and PAPP-A levels, but only in the non-viable pregnancies and a negative correlation between the length of gestation and plasma AEA levels in the non-viable group (r=-0.475, *P*=0.035), but not in the viable group (r=-0.246, *P*=0.236).

A third possibility is that genetic abnormalities in some of the fetuses might have altered the expression of the enzymes controlling the levels of these biomarkers. These data suggest that a complex interplay, which needs further investigation, is involved between these factors and early pregnancy success. Future work with a larger sample may also show the expected correlations.

Although PAPP-A levels were increased in the non-viable group, the increase was not statistically significant. This was a rather unexpected result as a decrease in PAPP-A level is now considered to be a marker of problems in pregnancy (Ong et al., 2000b, Tong et al., 2004b). This discrepancy may, however be due either to the low numbers involved in this study, or because the pregnancies had already failed (Westergaard et al., 1983, Ruge et al., 1990, Cuckle et al., 1999). There was, however, a strong correlation between P4, β-hCG and PAPP-A levels in both viable and non-viable pregnancies, suggesting a causal link between pregnancy and the production of each of these factors. At present, the factors involved in the production of PAPP-A are unknown and although there are a number of factors that have been implicated in its generation, P4 is not one

of them (Boldt and Conover, 2007, Ong et al., 2000b, Tong et al., 2004b). Therefore, the correlation of these 3 factors presumably represents a marker of trophoblast/placental function and wellbeing.

The finding of an association between raised plasma AEA levels and non-viable pregnancies could mean either, (a) that a disruption in the endocannabinoid system led to a disruption of the normal implantation and developmental process, causing miscarriage or, (b) that a failed pregnancy, for whatever reason, is associated with a disruption in the endocannabinoid system in favour of a higher AEA level. Which of these possibilities is the more likely is currently uncertain.

Although the trophoblast, the endometrium, and the embryo all contain CB1 and CB2 receptors and FAAH, the lack of correlation between plasma AEA levels, and trophoblast dependent markers of pregnancy success suggest that the trophoblast may not be the target for the action of AEA in early pregnancy failure. This would suggest that AEA is likely acting at the level of the local endometrial-embryo interface. Further studies of these interactions with larger patient numbers may eventually lead to the identification of potential areas for interventions to reduce miscarriages.

It remains to be seen if these changes in plasma AEA levels do actually correlate with those at the endometrial-embryo interface, and what the exact role AEA plays in the whole process might be.

CHAPTER 5

Expression of the endocannabinoid system in human first trimester placenta in viable and non-viable pregnancies

5.1. Introduction

In Chapters 3 and 4, it was clearly demonstrated that the levels of anandamide measured in plasma differed in women with:

a. Viable ongoing pregnancies and those with viable pregnancies which subsequently miscarry.
b. Viable ongoing pregnancies and those with a confirmed miscarriage / non-viable pregnancy.

Those observations clearly point towards an important role for the endocannabinoid system in the establishment and maintenance of early pregnancy and that problems within the endocannabinoid system may play a role in miscarriage. These findings are similar to those previously reported by Maccarrone *et al* (Maccarrone et al., 2002a) in which elevated plasma anandamide levels were associated with failure to achieve an ongoing pregnancy after IVF/ET. The exact mechanism by which these effects are exerted is unclear, but it is highly likely that the levels of anandamide measured peripherally in plasma may reflect changes occurring naturally within the tissues at the feto-maternal interface and that differences in peripheral AEA levels reported in the different groups of women that miscarry are merely a reflection of changes in the endocannabinoid system at the feto-maternal interface.

Previously, CB1, CB2 and FAAH have been demonstrated to be present in the first trimester trophoblast, term placenta and fetal membranes (Habayeb et al., 2008a) . It has also been demonstrated that the intensity of CB2 immunoreactivity in the syncytiotrophoblast remains constant throughout the 1st trimester, which differs from the staining patterns for FAAH and CB1 (Habayeb et al., 2008a).

In those studies, FAAH immunoreactivity in the syncytiotrophoblast layer increased gradually between the 7th and 10th gestational week and then diminished to the point where it was barely detectable within large parts of the trophoblast by the 11th week. By contrast, CB1 immunoreactivity in the syncytiotrophoblast layer diminished in intensity, but did not disappear in weeks 10 to 12 of gestation.

Although this pattern of staining appeared to be of great interest, it is not known if differences exist in CB1, CB2 and FAAH immunoreactivity in viable versus non-viable early pregnancy tissue, and also how their presence on the maternal side of the feto-maternal interface in the decidua differs from that of the trophoblast. Additionally, there are no data relating to the expression of NAPE-PLD (the enzyme responsible for the formation of AEA) in either the first trimester trophoblast or decidua, and whether this enzyme's expression is affected by spontaneous miscarriage.

The aims of this part of the project were therefore to; (1) examine CB1, CB2, FAAH and NAPE-PLD expression in trophoblast and decidua from viable 1st trimester pregnancies, and (2) to compare that pattern of expression with that in similar tissues from non-viable 1st trimester pregnancies.

5.2. Methods

5.2.1. Subjects

The study was approved by the University Hospitals of Leicester Research Ethics Committee, and women participating in the study gave informed written consent (Appendix 3). Tissues from viable pregnancies were obtained from women undergoing surgical termination of the pregnancy between seven and twelve weeks gestation. Tissues from women undergoing medical termination of the pregnancy between seven and twelve weeks gestation were also obtained and all women had an ultrasound scan prior to the procedure, which confirmed viability and gestational age. Medical termination was with the oral administration of mifepristone (a progesterone antagonist) followed by vaginal misoprostol (a prostaglandin), 48 hours later. Furthermore, tissues from non-viable pregnancies were obtained from women undergoing surgical evacuation of retained products of conception (ERPOC) between seven and twelve weeks gestation, following the diagnosis of a failed pregnancy on ultrasound scan. Women known to suffer with any medical illnesses or who were on any chronic medications were excluded.

5.2.2. Tissue collection

Tissue samples were collected immediately after surgical or medical evacuation and placed on ice before transportation to the laboratory. The time from collection to arrival in the laboratory was always within 10 minutes. Excess blood was then removed by washing the tissues in copious quantities of ice-cold sterile phosphate-buffered saline. The trophoblast and decidua were dissected free with the aid of a dissecting microscope (Carl Zeiss Ltd., Welwyn Garden City, Hertfordshire, UK) and the tissue transferred to

tissue cassettes containing foam pads to prevent them floating in the fixative. Tissues were fixed in 10% neutral-buffered formalin for 4 days before embedding in paraffin wax.

5.2.3. Immunocytochemistry

Immunocytochemistry for CB1, CB2, FAAH and NAPE-PLD were based upon those previously developed by the group (Habayeb et al., 2008a, El-Talatini et al., 2009a).

5.2.3.1 Antibodies

Anti-CB1 rabbit polyclonal antibody was obtained from Sigma-Aldrich Limited (Poole, Dorset, UK) (catalogue number: C1108; Lot number: 045K1103) and used at a dilution of 1:4000. Anti-CB2 rabbit polyclonal antibodies were from Sigma-Aldrich Limited (Catalogue number: C1358; Lot number: 026K1234) and used at a dilution of 1:500. Anti-FAAH rabbit polyclonal antibody was obtained from Alpha Diagnostics International (San Antonio, TX) (Catalogue number: FAAH11-S; Lot number: 549798S) and used at a dilution of 1:2000. Anti-NAPE-PLD rabbit polyclonal antibodies were obtained from Cayman Chemicals (catalogue number: ABIN110270; Cayman Chemicals, Ann Arbor, MI) and used at a dilution of 1:200.

Negative controls for anti-CB1 and anti-CB2 were rabbit IgG from Vector Laboratories (Peterborough, UK; Catalogue number X0903. Lot number: 000255509). The negative controls for the immunohistochemistry were used at the same concentrations as for the primary antibodies. Anti-FAAH antibody was made from normal rabbit serum; hence

normal rabbit serum from DAKO (Catalogue number: X0902**;** Lot number: 013) was used at the same dilution that was used for the primary antibody as a negative control.

Avidin-Biotin blocking kit was obtained from Vector Laboratories (Catalogue number: SP 2001. Lot number: S0201). ABC detection system (ABC Elite; Vector Laboratories) (catalogue number: PK-6100 series. Lot number: S0818) was used in conjunction with 3,3'-diaminobenzamidine (DAB; Vector laboratories; Catalogue number: Sk4100. Lot Number: 040108) to detect the presence of immunoreactive complexes for the anti-CB2 and anti-FAAH antibodies. The tyramide signal amplification system from Perkin-Elmer LAS (Beaconsfield, Bucks., UK; Catalogue Number: NEL 700A00; Lot number: 432521) was substituted for ABC Elite for the detection of anti-CB1 complexes. Biotinylated goat anti-rabbit antibody from DAKO (Catalogue number: E 0432. Lot number: 00024051) was used at a dilution of 1:400 as the secondary antibody. DPX mounting medium was from BDH (Poole, Dorset, UK).

5.2.3.2. Detection of immunoreactive FAAH

Tissues sections (4 μm thick) were mounted onto silanized glass microscope slides and dried for 7 days at 37 ^{o}C prior to use.

1) Slides were de-waxed in xylene three times for 3 minutes and re-hydrated in graded alcohol for 9 minutes (3 minutes in 99% IMS twice and 3 minutes in 95%) followed by incubation in distilled water for 3 min.
2) Endogenous peroxidase activity was then blocked by incubation in 6% hydrogen peroxide (H_2O_2) in water for 10 minutes. Slides were then washed in running tap water for 5 minutes then in TBS [Tris-buffered saline (0.5M Trizma, 1.5M

NaCl, 2mM $MgCl_2$, pH 7.6) containing 0.1% bovine serum albumin (Fraction V; Sigma-Aldrich Ltd.)] for 5 minutes.

3) 100 µl of 10% normal goat serum (NGS) (prepared in TBS) was added to each slide to block non-specific protein binding sites. Slides were incubated in a humid chamber for 20 minutes at room temperature.
4) The slides were drained and the sections wiped around.
5) 100 µl of Avidin blocking solution [prepared by adding 1 drop of avidin blocking solution to 250 µl of (NGS in TBS 1 in 10)] was added and the slides were incubated in a humid chamber for 20 minutes at room temperature. The slides were then washed in TBS for 5 minutes.
6) 100 µl of Biotin blocking solution [prepared by adding 1 drop of avidin blocking solution to 250 µl of (NGS in TBS 1 in 10)] was then applied and the slides incubated in a humid chamber for 20 minutes at room temperature.
7) 100 µl of primary anti FAAH antibody or normal rabbit serum (NRS) both diluted at 1 in 2000 in a solution of (NGS in TBS 1 in 10) was added and the slides incubated overnight (18 hours) at 4 ^{o}C in a humid chamber.
8) On the following day, the slides were washed in TBS for 30 minutes. The slides that had anti-FAAH were washed separately to those with NRS.
9) 100 µl of the secondary antibody diluted at 1 in 400 in TBS was added to each slide and the slides incubated in a humid chamber at room temperature for 30 minutes. This was followed by a 20 minute wash in TBS.
10) 100 µl of the ABC solution [prepared by adding 1 drop of A and 1 drop of B to 2.5 ml of tris-buffered saline (TBS) and vortex mixed was prepared at least 30 minutes before use] was added and the slides incubated in a humid chamber at room temperature for 30 minutes.

11) The slides were then washed in TBS for 20 minutes.
12) 100 µl of DAB (prepared adding 1 drop of buffer, then 1 drop of H_2O_2 followed by vortex mixing, to 2.5 ml of distilled water. This was followed by the addition of two drops of DAB. which was then mixed by gentle inversion. The solution was prepared immediately before use as recommended by the manufacturer) was added to each slide. The slides were then incubated in a humid chamber for 5 minutes at room temperature.
13) The slides were then washed in distilled water for 5 minutes before being counterstaining in Mayer's haematoxylin for 15 seconds. This was followed by washing in running tap water for 5 minutes.
14) Finally the slides were dehydrated in graded alcohols (3 minutes in 95% IMS once then 3 minutes in 99% IMS twice), cleared in xylene (for 3 minutes twice) and then mounted in DPX mounting medium.

Once the slides had dried completely, representative images were taken on an Axioplan transmission microscope equipped with a Sony analogue camera connected to a computer running Axiovision image capture and processing software (Axiovison version 4.4, Carl Zeiss Ltd., Welwyn Garden City, Hertfordshire, UK). Images were captured at either 5x, 10x, 20x or 40x magnification in the presence of daylight and medium value neutral density filters with the lamp set at 6400K. Image backgrounds were colour corrected to neutral grey with the use of ColorPilot software (version 4.62; www.colorpilot.com).

5.2.3.3. Detection of immunoreactive CB1

The method used for the detection of anti-CB1 was similar to the one described in **5.2.3.1** for anti-FAAH except:

1) Microwave antigen retrieval was performed. This step was performed immediately after dewaxing and rehydration of the slides. Slides were placed in citric acid buffer (10mM; pH=6.0) and microwaved at 700 watts for 10 minutes. They were then allowed to cool for 20 minutes before being washed in running tap water for 5 minutes.
2) Anti-CB1 antibody was used at a dilution of 1 in 4000 (concentration 0.020 µg/ml) with rabbit IgG (0.25 µg/ml) acting as a negative control. Both were diluted in TNT buffer (100 ml of 1M Tris-HCl, 30 ml of 5M NaCl, 1 ml of Tween 20 and 869 ml of distilled water, pH 7.5).
3) The tyramide signal amplification system replaced the ABC system for the detection of anti-CB1 complexes. The following steps replaced steps 8-10 in **5.2.3.1.**:
    I. 100 µl of the secondary antibody diluted at 1 in 400 in TNT buffer was added to each slide. The slides were incubated in a humid chamber at room temperature for 20 minutes. The slides were then washed in TNT buffer for 5 minutes three times.
    II. 100 µl of blocking solution (0.5% blocking reagent in TNT buffer) was added to each slide. The slides were incubated in a humid chamber at room temperature for 20 minutes.
    III. 100 µl of Streptavidin-Horseradish peroxidase (SA-HRP) (diluted 1 in 100 in TNT buffer) was added to each slide. The slides were incubated in a

humid chamber at room temperature for 30 minutes and then washed in TNT buffer for 5 minutes three times.

IV. 100 µl of Biotinyl Tyramide (BT) (prepared by diluting BT 1 in 50 in amplification diluent) was added to each slide, which was then incubated in a humid chamber at room temperature for 5 minutes. The slides were then washed in TNT buffer for 5 minutes three times.

V. Repeat step III

The remaining parts of the method are similar to steps 12-14 in **5.2.3.1.**

5.2.3.4. Detection of immunoreactive CB2

The method for Anti-CB2 was similar to the one described for anti-FAAH in **5.2.3.1:**

1) For anti-CB2, antigen retrieval by microwave was performed as described above.
2) Anti-CB2 was used at a dilution of 1 in 500 (concentration of 0.43 µg/ml). Rabbit IgG was used as negative control (concentration of 0.50 µg/ml).

5.2.3.5. Detection of immunoreactive NAPE-PLD

The method for the detection of NAPE-PLD followed the manufacturer's recommendations and required slightly thicker tissues sections (5 µm compared to the 4mm sections used for the CB1, CB2 and FAAH immunohistochemistry). The tissues were mounted onto silanized glass microscope slides and dried at 37°C prior to use.

1. Slides were de-waxed in xylene twice times for 5 min and re-hydrated in graded ethanol from 100 to 50% in 5% steps for 5 min followed by incubation in distilled water for 5 min.
2. Endogenous peroxidase activity was then blocked by incubation in 3% H_2O_2 in ice-cold water for 15 min. Blocking of non-specific protein binding sites was performed by incubation with 5% normal goat serum in Tris-buffered saline (TBS; 0.5M Trizma, 1.5M NaCl, pH 7.4; 100 µl/slide) for 30 min at room temperature.
3. Primary NAPE-PLD rabbit antibodies (ABIN110270; Cayman Chemicals, Ann Arbor, MI) diluted to a dilution of 1:200 in TBS were added and the slides incubated in a humid chamber overnight at room temperature. Slides were then washed in Tris-buffered saline containing 0.1% Tween 20 (TBS-T; Sigma-Aldrich Ltd.) three times for 5 min with buffer changes.
4. After washing, biotinylated goat anti-rabbit antibody (Dako; Glostrup, Denmark) diluted to 1:400 in TBS was applied for 30 min at room temperature.
5. This was then followed by an additional wash in TBS-T, and ABC Elite reagent (Vector Laboratories) was applied according to the manufacturer's instructions, followed by an additional washing in TBS-T, 3,3'-diaminobenzidine (Vector Laboratories) was added to each slide (100µl/slide) for 5 min.
6. The slides were then washed in distilled water for 5 min before counterstaining in Mayer's haematoxylin (Sigma-Aldrich) for 15 seconds. After washing in running tap water for 5 min, slides were dehydrated in graded alcohols to 100% ethanol, cleared in xylene twice for 6 min before mounting with DPX mounting medium (BDH Poole, Dorset).

5.3. Results

Firstly, controls were undertaken using term fetal membranes and ovarian tissue, both of which are known to contain CB1, CB2, FAAH and NAPLE-PLD (Helliwell et al., 2004, El-Talatini et al., 2009a). This demonstrated positive, antibody specific, staining for all, with negative controls using serum IgG. (Figure 5.1).

As shown previously (Habayeb et al., 2008a) immunoreactive staining for CB1, CB2 and FAAH were all demonstrated in the trophoblast of viable 1st trimester pregnancies. In addition to this, immunoreactive CB1, CB2 and FAAH were also detected within the decidual tissue of the viable pregnancy samples. NAPE-PLD immunoreactivity was found in both the trophoblast and the decidua of 1st trimester tissue.

When looking at non-viable pregnancies immunoreactive CB1, CB2, FAAH and NAPE-PLD were detected in both the trophoblast and the decidua.

The samples were all run at the same time to ensure that the staining patterns observed are related to the states of the tissue rather than to the vagaries of methodology

5.3.1. CB1 Immunoreactivity

CB1 showed consistent staining in the extra-villous trophoblast, immature and mature villi of a 7 week placenta. CB1 immunoreactivity was demonstrated in circulating blood and syncytial knots, but there was a lack of CB1 immunoreactivity in the fetal blood cells and infiltrating maternal plasma cells. In the syncytiotrophoblast the

immunoreactivity was confined to the cytoplasm, but in the cytotrophoblast there was immunoreactivity in both the cytoplasm and nuclei. (Figure 5.2)

CB1 immunostaining was present throughout the first trimester in both the trophoblast and decidua (Figure 5.6). The majority of the staining was confined to the cytotrophoblast and syncytiotrophoblast layers, whereas intense immunoreactivity was observed in the decaying endometrial glands and decidualised stromal fibroblasts. By contrast blood vessels had much lower staining intensities.

In the trophoblast, medical termination of pregnancy and spontaneous abortion were characterised by a marked decrease in the expression of CB1 when compared to that found from surgical termination (Figure 5.10). In the decidua, medical termination showed no change in CB1 staining. By contrast, spontaneous miscarriage was characterised by a decrease in CB1 expression (Figure 5.11).

5.3.2. CB2 Immunoreactivity

CB2 also showed consistent staining in extra-villous trophoblast, immature and mature villi of a 7 week placenta. CB2 immunoreactivity was seen in some but not all fetal blood cells and infiltrating maternal plasma cells, and also showed strong immunoreactivity in circulating maternal blood cells. The immunoreactivity for CB2 was observed in the cytoplasm and nuclei of both the syncytiotrophoblast and the cytotrophoblast layers (Figure 5.3).

CB2 immunostaining was present throughout the first trimester in both the trophoblast and decidua (Figure 5.7) with the majority of the staining confined to the cytotrophoblast and syncytiotrophoblast layers and infiltrating maternal blood cells, whereas intense immunoreactivity was observed in the decaying endometrial glands and decidualised stromal fibroblasts. The intensity of decidual cell staining increased and reach a peak around week 10 of gestation. By contrast blood vessels had much lower staining intensities.

In the trophoblast, medical termination of pregnancy and spontaneous abortion were characterised by a marked increased CB2 expression (Figure 5.10). Similarly, in the decidua, medical termination of pregnancy and spontaneous abortion were characterised by an increase in the expression of CB2 staining (Figure 5.11).

5.3.3 NAPE –PLD Immunoreactivity

NAPE-PLD immunostaining was present throughout the first trimester in both the trophoblast and decidua (Figure 5.8). The majority of the staining was confined to the cytotrophoblast and syncytiotrophoblast layers, whereas intense immunoreactivity was observed in the decaying endometrial glands and decidualised stromal fibroblasts. The intensity of decidual cell staining increased and reached a peak around week 10, with the intensity essentially gone by the end of week 12 (Figure 5.8), although staining remained present. By contrast blood vessels had much lower and sporadic staining intensities, but consistent staining for NAPE-PLD was seen in extra-villous trophoblast, immature and mature villi of a 7 week placenta. NAPE-PLD immunoreactivity was present in the basal membrane in some thinner syncytiotrophoblast, but only nuclear

staining in the cytotrophoblast within the same villous (Figure 5.4.). Intense nuclear staining was found in syncytial knots and some but not all extravillous trophoblasts. The endothelial cells of blood vessels also showed nuclear NAPE-PLD immunoreactivity.

In the trophoblast, medical termination of pregnancy and spontaneous abortion showed no differences in NAPE-PLD expression, compared with surgical termination (Figure 5.10). However, in the decidua, medical termination of pregnancy was characterised by an increase in the expression NAPE-PLD protein, but no change in the spontaneous abortion tissue, when compared to the surgical termination group (Figure 5.11).

5.3.4. FAAH Immunoreactivity

FAAH immunostaining was present throughout the first trimester in both the trophoblast and decidua (Figure 5.9). The majority of the staining was confined to the cytotrophoblast and syncytiotrophoblast layers, whereas intense immunoreactivity was observed in the decidualised stromal fibroblasts but not the decaying endometrial glands and by week 12 gland staining was absent. The intensity of decidual cell staining increased and reached a peak around week 10 and diminished by week 12 (Figure 5.9). By contrast, blood vessels had much lower staining intensities except in mesenchymal cells surrounding the vessels. Nuclear staining was found in extra-villous trophoblast, immature and mature villi of a 7 week placenta.

FAAH immunoreactivity was in the mesenchymal core, syncytiotrophoblast and cytotrophoblast of the same villus. There was also intense staining in syncytial knots

and extravillous trophoblast. The presence of FAAH immunoreactivity was also observed in infiltrating maternal blood cells, but not in fetal blood cells (Figure 5.5).

Medical termination of pregnancy was characterised by a marked increase in cytotrophoblastic FAAH expression in the trophoblast, that did not occur in spontaneous miscarriage, but was characterised by a marked decrease in mesenchymal FAAH expression (Figure 5.10).

In the decidua, medical termination of pregnancy FAAH expression was unaffected. By contrast, spontaneous miscarriage was characterised by a marked increase FAAH expression compared with decidua obtained from surgical terminations (Figure 5.11).

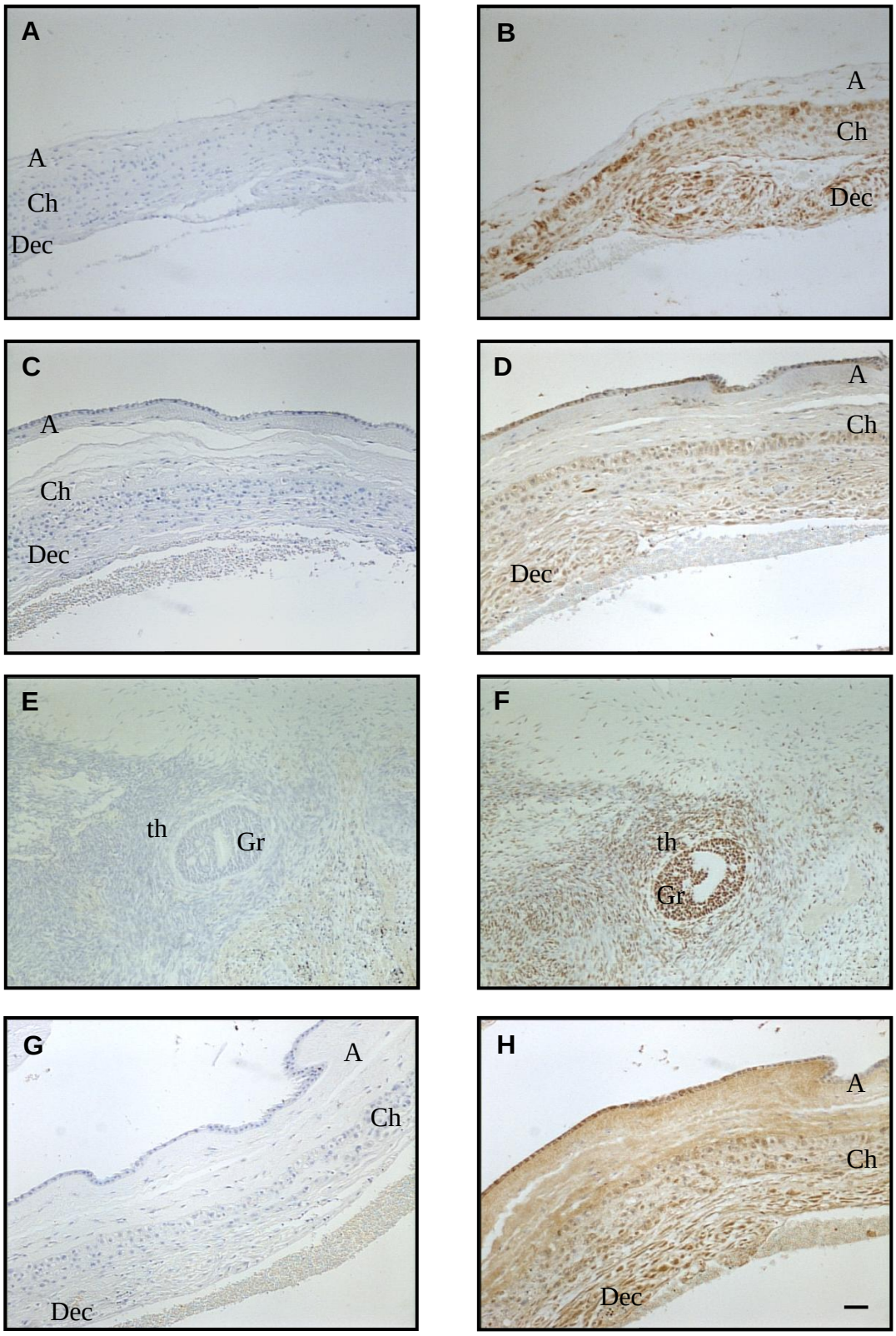

Figure 5.1 Demonstration of antibody specificity for CB1 (panels A and B), CB2 (panels C and D), NAPE-PLD (panels E and F) and FAAH (panels G and H) antibodies on term fetal membranes (panels A, B, C, D, G and H) and ovary (panels E and F). The IgG or serum controls are shown in the left hand panels and the antibody samples in the right hand panels. All images were taken at 100x magnification. Bar = 50 μm. Am = amnion, Ch = chorion, Dec = deciduas, Gr = granulose cells, th =thecal cells.

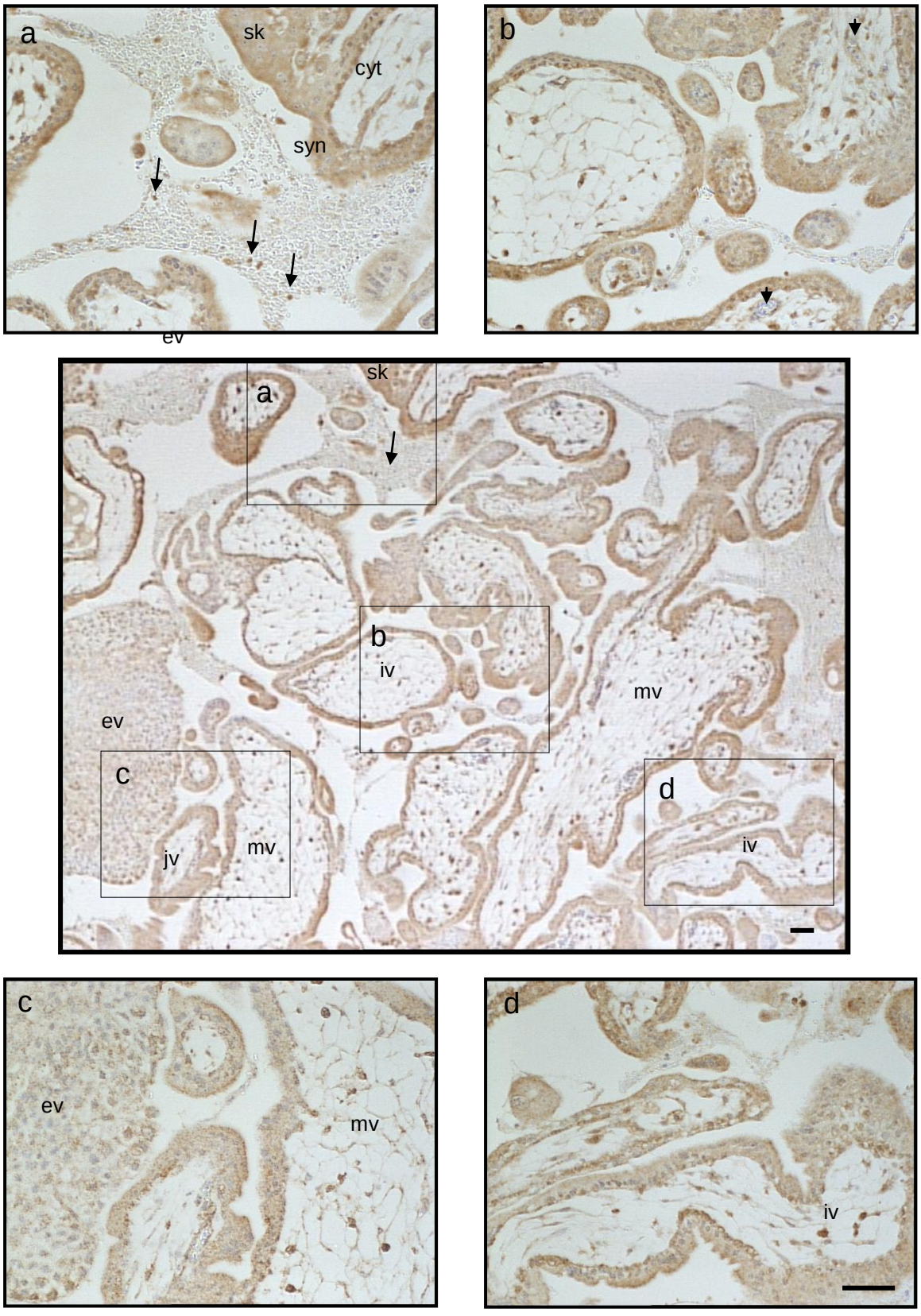

Figure 5.2 Showing CB1 immunoreactivity and anatomical features of the first trimester placenta and consistent staining in extra (ev), immature (iv) and mature villi (mv) of a 7 week placenta. Note the lack of CB1 immunoreactivity in the fetal blood cells (short arrows) and infiltrating maternal plasma cells, but the presence of CB1 immunoreactivity in circulating plasma cells (long arrow) and syncytial knots (sk). The immunoreactivity is confined to the cytoplasm of the syncytiotrophoblast (syn) but also found in both the cytoplasm and nuclei of the cytotrophoblast (cyt). Images were captured at 50x [centre] and 100x magnification [a/b/c/d]. Bar = 50μm.

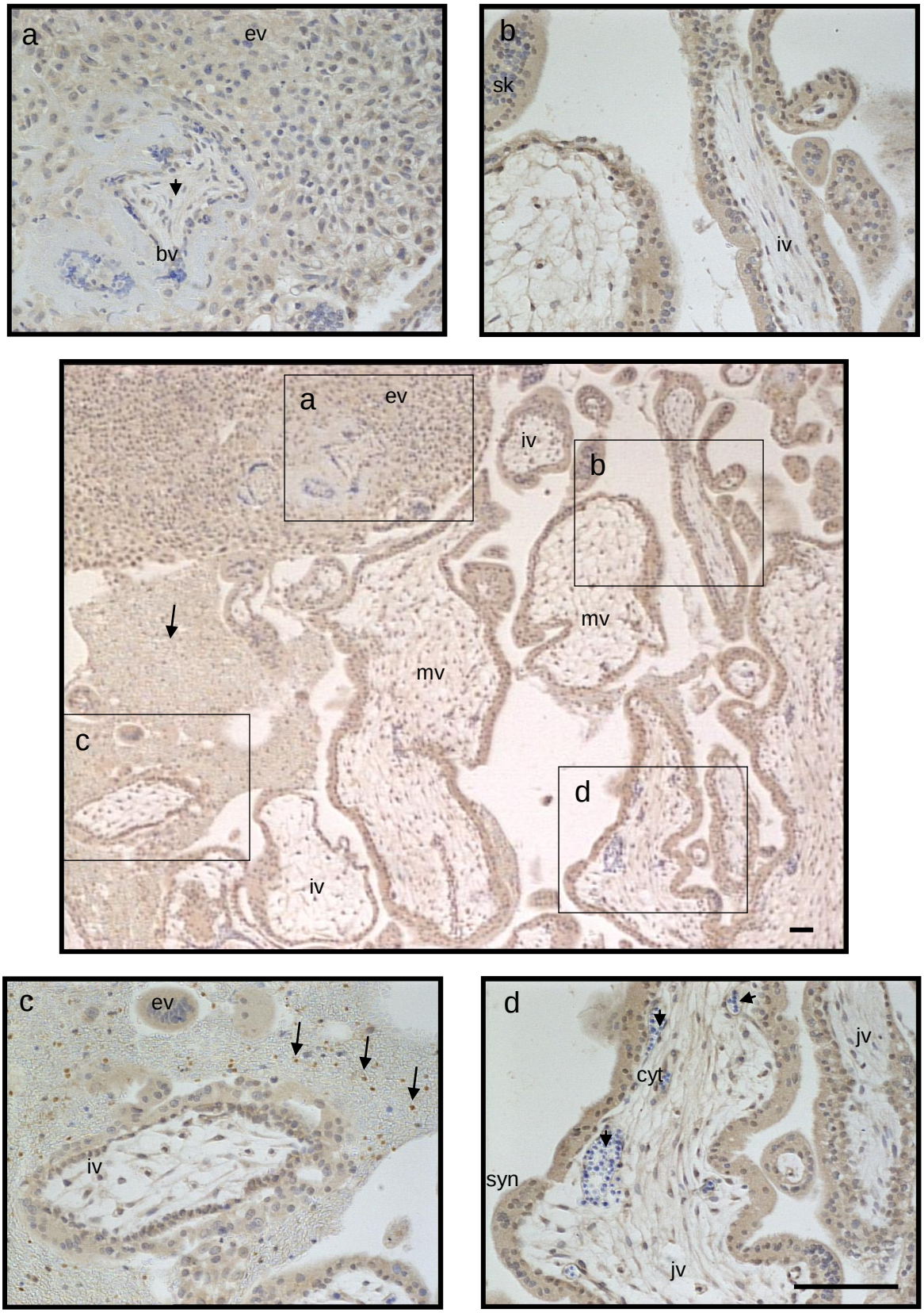

Figure 5.3 Showing CB2 immunoreactivity and anatomical features of the first trimester placenta and consistent staining in extra (ev), immature (iv) and mature villi (mv) of a 7 week placenta. Note the presence of CB2 immunoreactivity in some but not all fetal blood cells (short arrows) and infiltrating maternal plasma cells, and the presence of strong CB2 immunoreactivity in circulating maternal plasma cells (long arrow). The immunoreactivity is present in the cytoplasm and nuclei of both the syncytiotrophoblast (syn) and the cytotrophoblast (cyt). Images were captured at 50x [centre slide] and 200x magnification [a,b,c,d]. Bar = 50μm.

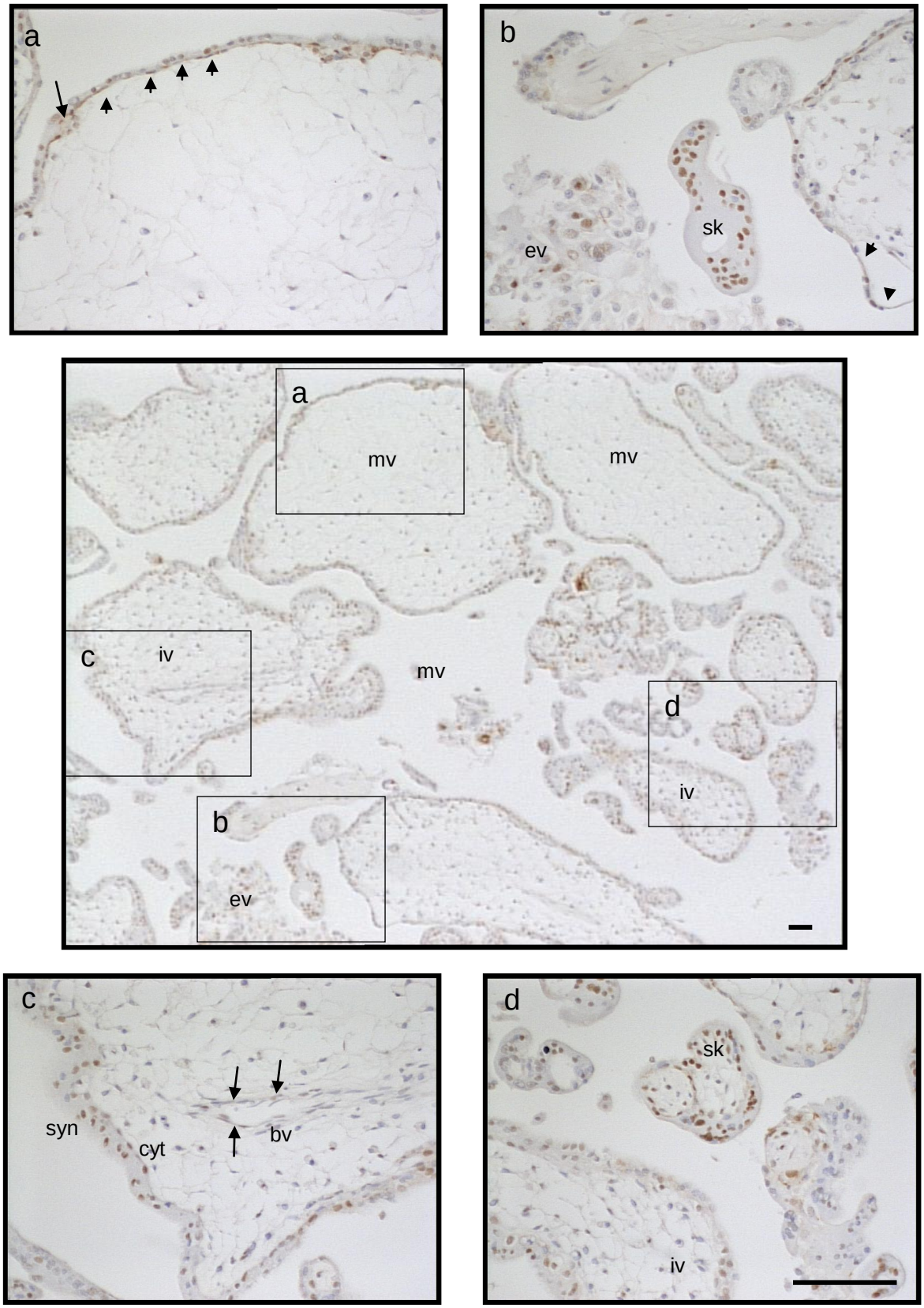

Figure 5.4 Showing NAPE-PLD immunoreactivity and anatomical features of the first trimester placenta and consistent staining in extra (ev), immature (iv) and mature villi (mv) of a 7 week placenta. Note the presence of basal membrane NAPE-PLD immunoreactivity in some thinning syncytiotrophoblast (syn) (short arrows, panels a and b) but only nuclear staining in the cytotrophoblast (cyt) within the same villous (long arrow, panel a). Intense nuclear staining was found in syncytial knots (sk) and some but not all extravillous trophoblasts (panel b). The endothelial cells of blood vessels (panel c) also showed nuclear NAPE-PLD immunoreactivity. Images were captured at 50x [centre] and 200x magnification [a,b,c,d]. Bar = 50μm.

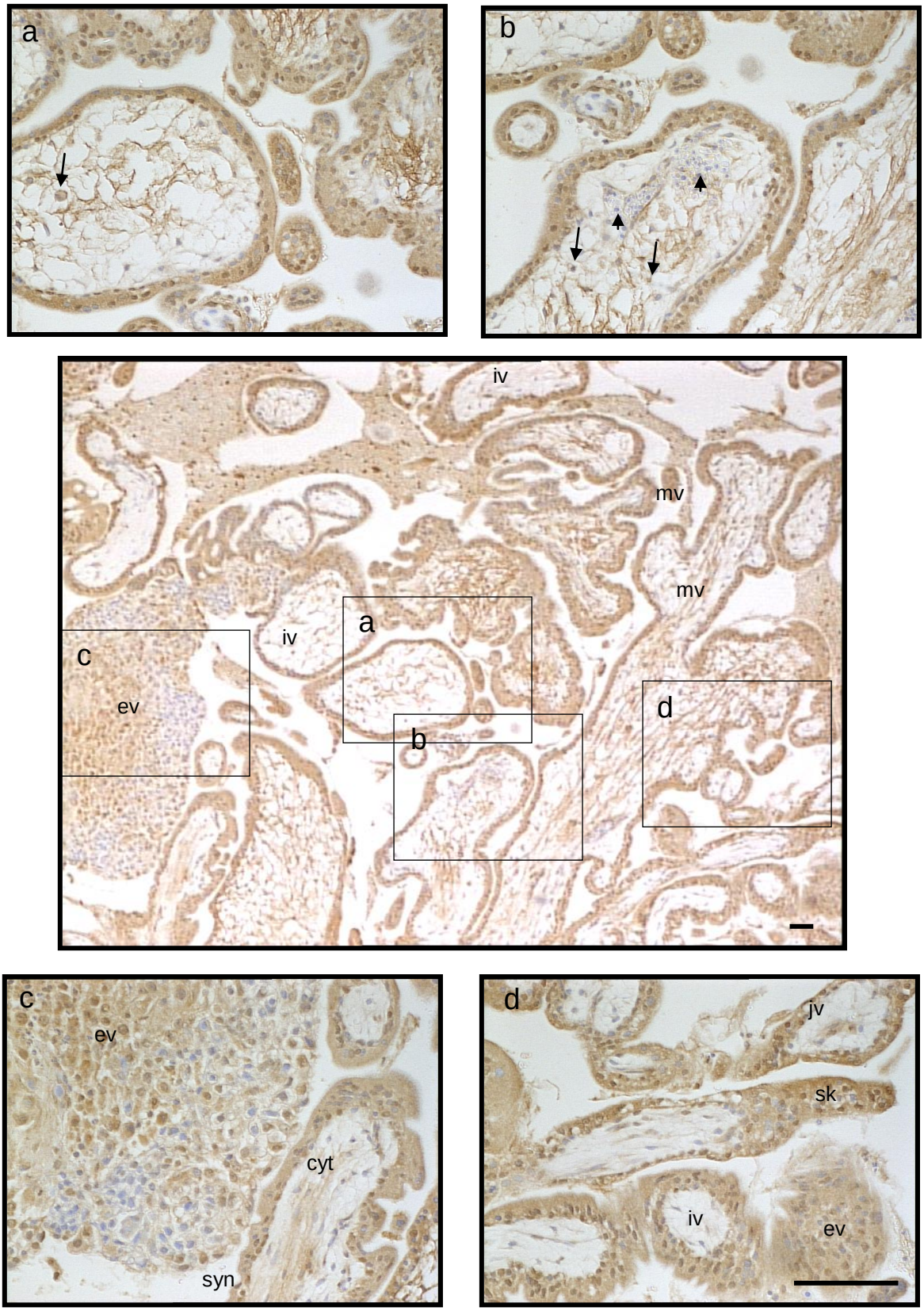

Figure 5.5 Showing FAAH immunoreactivity and anatomical features of the first trimester placenta and consistent nuclear staining in extra (ev), immature (iv) and mature villi (mv) of a 7 week placenta. Note the presence of FAAH immunoreactivity in the mesenchymal core, syncytotrophoblast (syn) and cytotrophoblast (cyt) of the same villus (panel a). Note also the intense stain in syncytial knots (sk) and extravillous trophoblasts (ev). The presence of FAAH immunoreactivity was also observed in infiltrating maternal plasma cells (long arrows, panels a and b), but in fetal plasma cells (short arrows, panel b). Images were captured at 50x [centre slide] and 200x magnification [slides a,b,c,d]. Bar = 50μm.

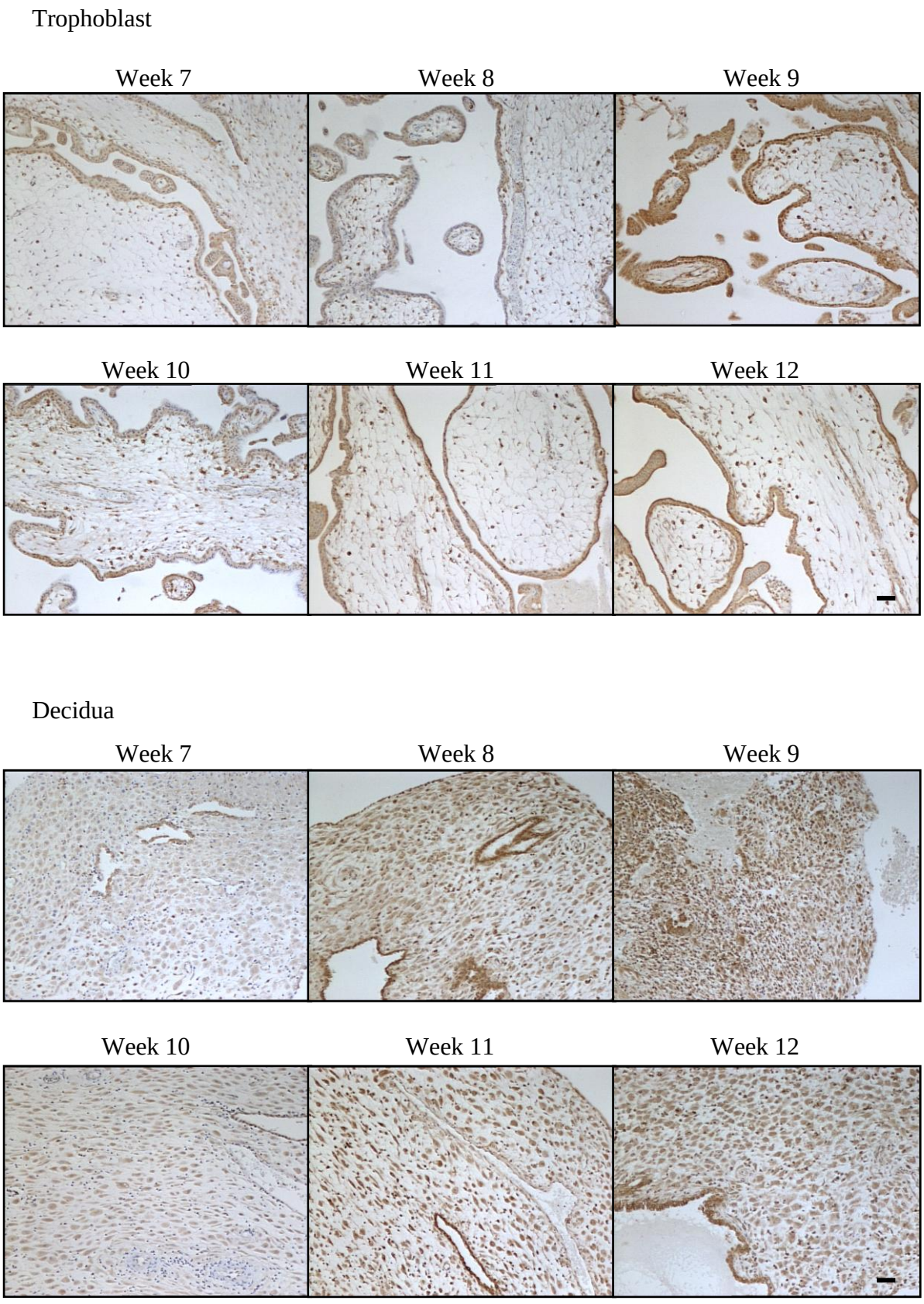

Figure 5.6 The effect of gestation on the expression of CB_1 immunoreactivity. CB_1 immunostaining is present throughout the first trimester in both the trophoblast (upper panels) and decidua (lower panels). The majority of the staining was confined to the cytotrophoblast and syncytiotr0phoblast layers, whereas intense immunoreactivity was observed in the decaying endometrial glands and decidualised stromal fibrobalsts. By contrast blood vessels had much lower staining intensities. Images were taken at 100x magnification. Bar = 50μm.

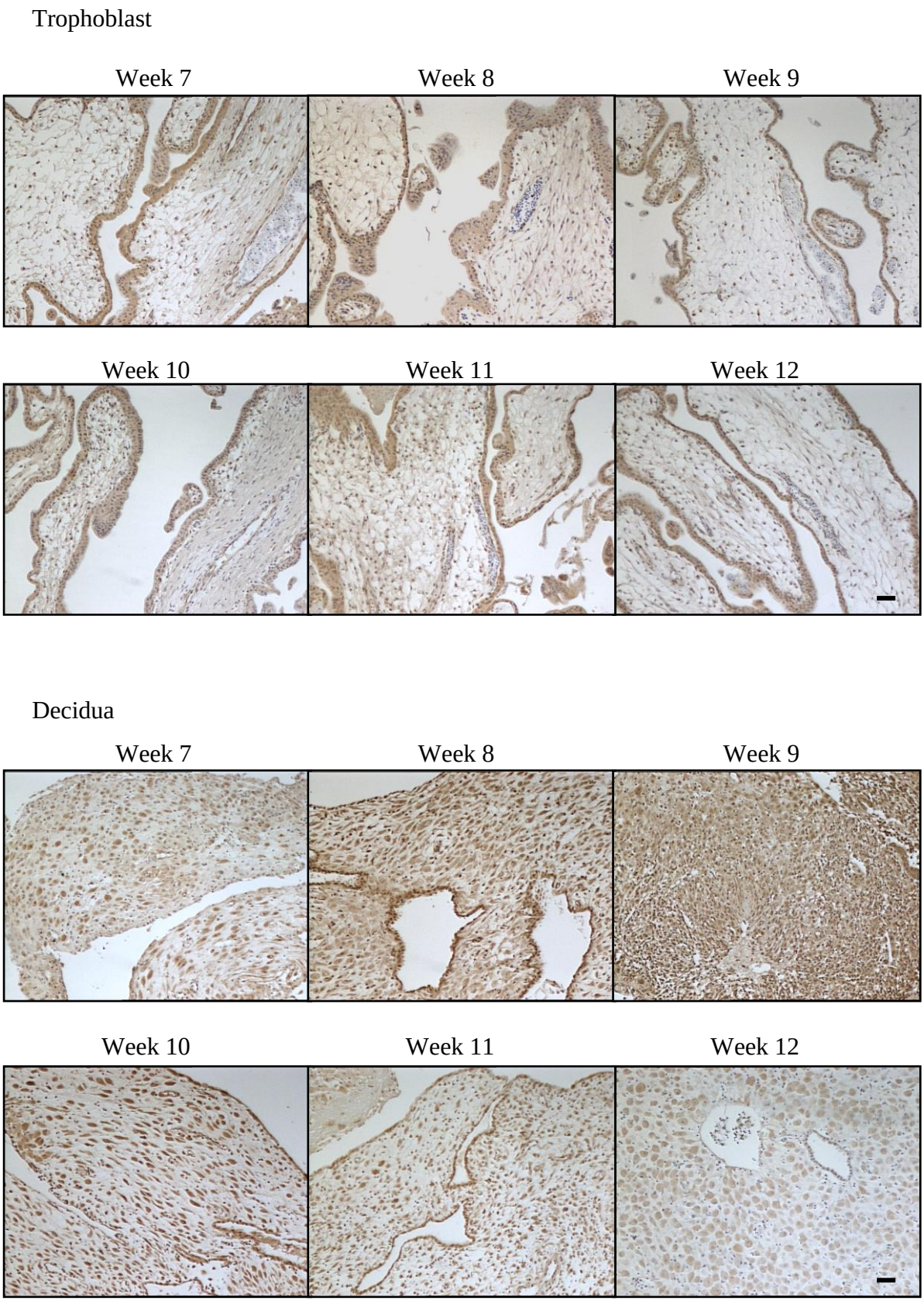

Figure 5.7 The effect of gestation on the expression of CB_2 immunoreactivity. CB_2 immunostaining is present throughout the first trimester in both the trophoblast (upper panels) and decidua (lower panels). The majority of the staining was confined to the cytotrophoblast and syncytiotrophoblast layers and infiltrating maternal plasma cells, whereas intense immunoreactivity was observed in the decaying endometrial glands and decidualised stromal fibroblasts. The intensity of decidual cell staining increased and reach a peak around week 10. By contrast blood vessels had much lower staining intensities. Images were taken at 100x magnification. Bar = 50μm.

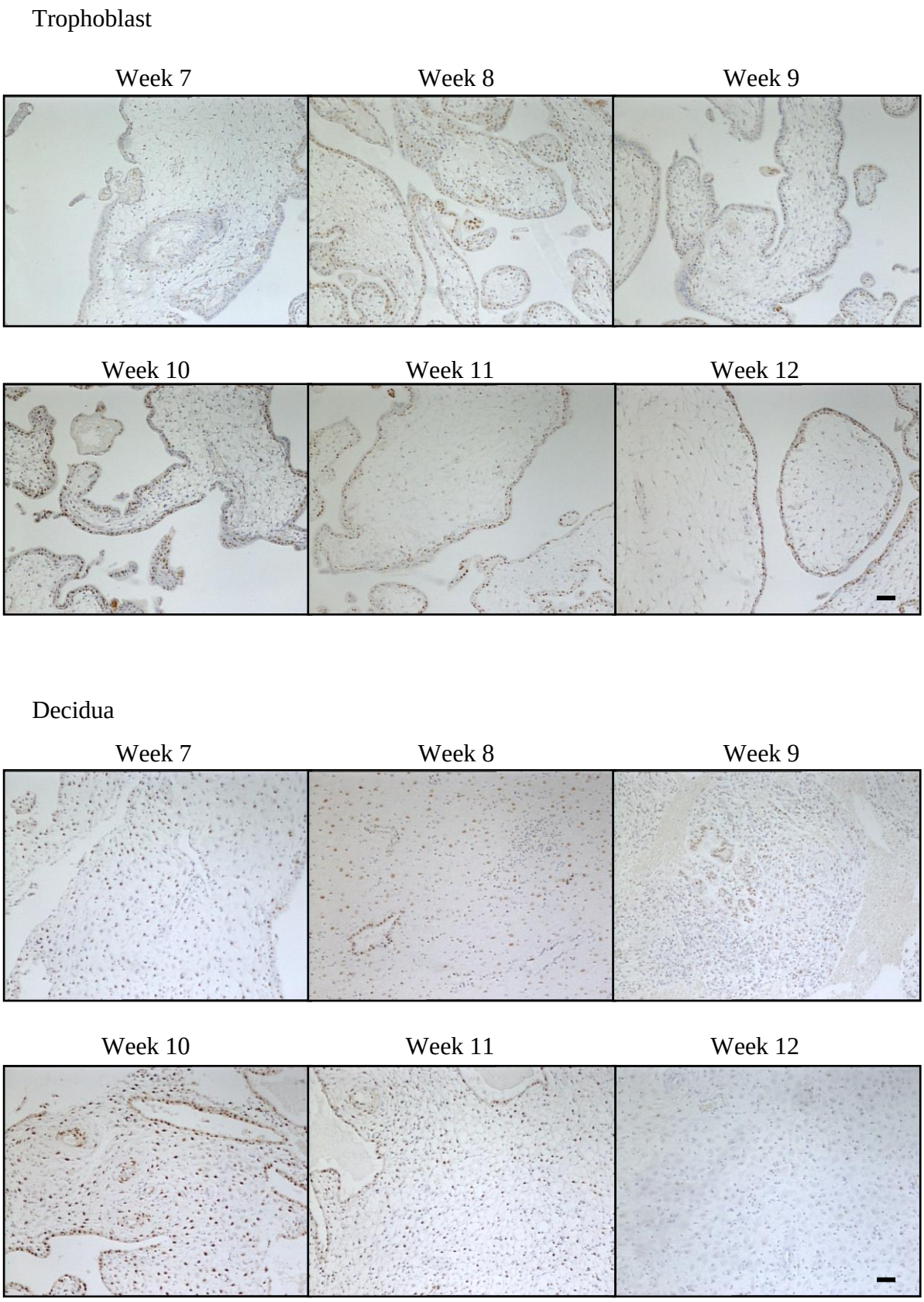

Figure 5.8 The effect of gestation on the expression of NAPE-PLD immunoreactivity. NAPE-PLD immunostaining is present throughout the first trimester in both the trophoblast (upper panels) and decidua (lower panels). The majority of the staining was confined to the cytotrophoblast and syncytiotrophoblast layers, whereas intense immunoreactivity was observed in the decaying endometrial glands and decidualised stromal fibroblasts. The intensity of decidual cell staining increased and reach a peak around week 10 and was essentially gone by the end of week 12. By contrast blood vessels had much lowerand sporadic staining intensities. Images were taken at 100x magnification. Bar = 50μm.

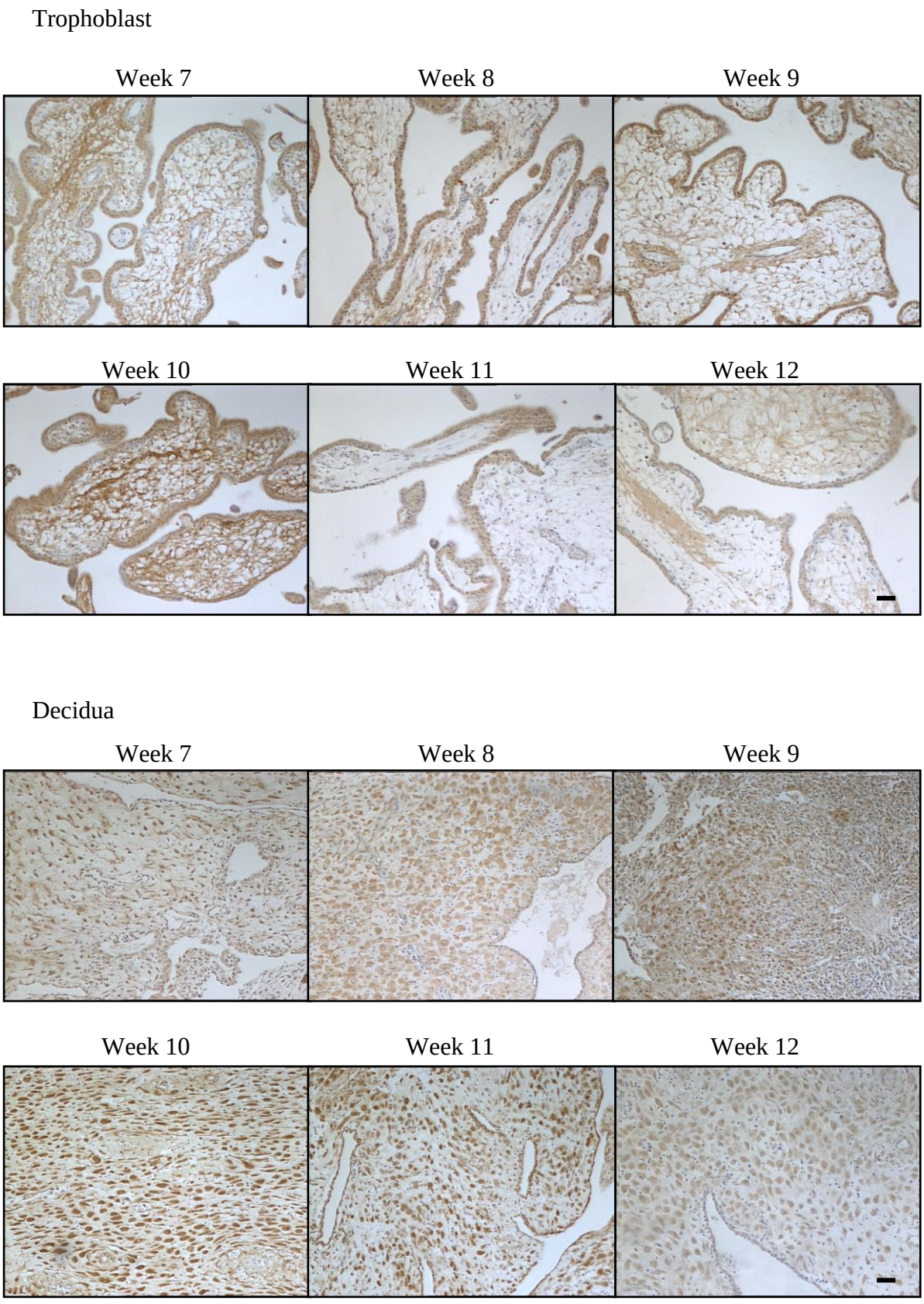

Figure 5.9 The effect of gestation on the expression of FAAH immunoreactivity. FAAH immunostaining is present throughout the first trimester in both the trophoblast (upper panels) and decidua (lower panels). The majority of the staining was confined to the cytotrophoblast and syncytiotrophoblast layers, whereas intense immunoreactivity was observed in the decidualised stromal fibroblasts but not the decaying endometrial glands and by week 12 gland staining was absent. The intensity of decidual cell staining increased and reach a peak around week 10 and diminished by 12. By contrast blood vessels had much lower staining intensities except in mesenchymal cells surrounding the vessels. Images were taken at 100x magnification. Bar = 50μm.

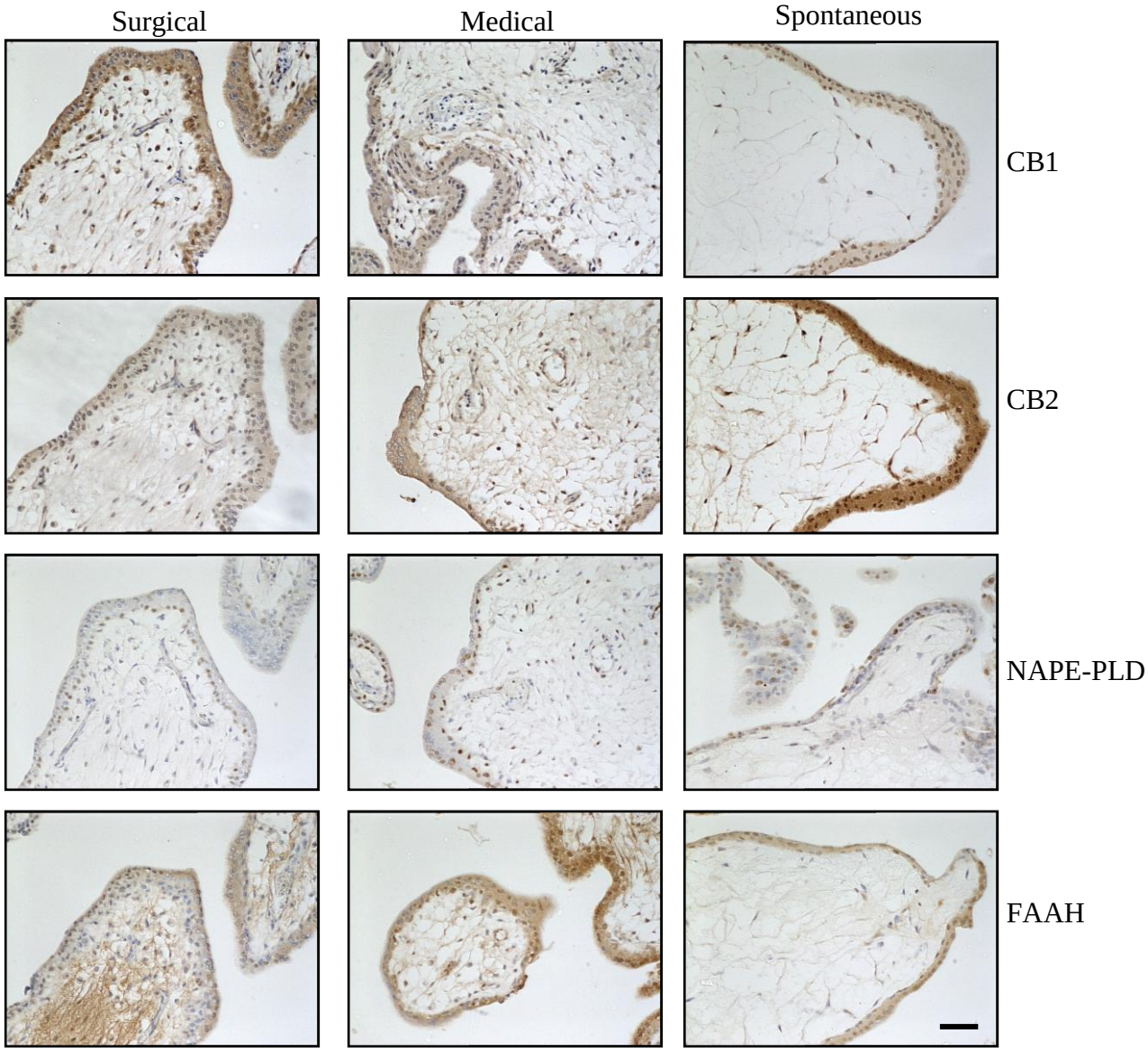

Figure 5.10 The effect of medical and spontaneous abortion on the expression of endocannabinoid system in the first trimester trophoblast. Medical termination of pregnancy and spontaneous abortion is characterised by a marked decrease in the expression of CB1 and increased CB2 expression, whilst NAPE-PLD expression is unaffected. By contrast, medical termination of pregnancy is characterised by a marked increase in cytotrophoblastic FAAH expression that does not occur in spontaneous miscarriage but is characterised by a marked decrease in mesenchymal FAAH expression. Images were taken at 200x magnification. Bar = 50μm. Surgical termination of pregnancy was used as the control condition.

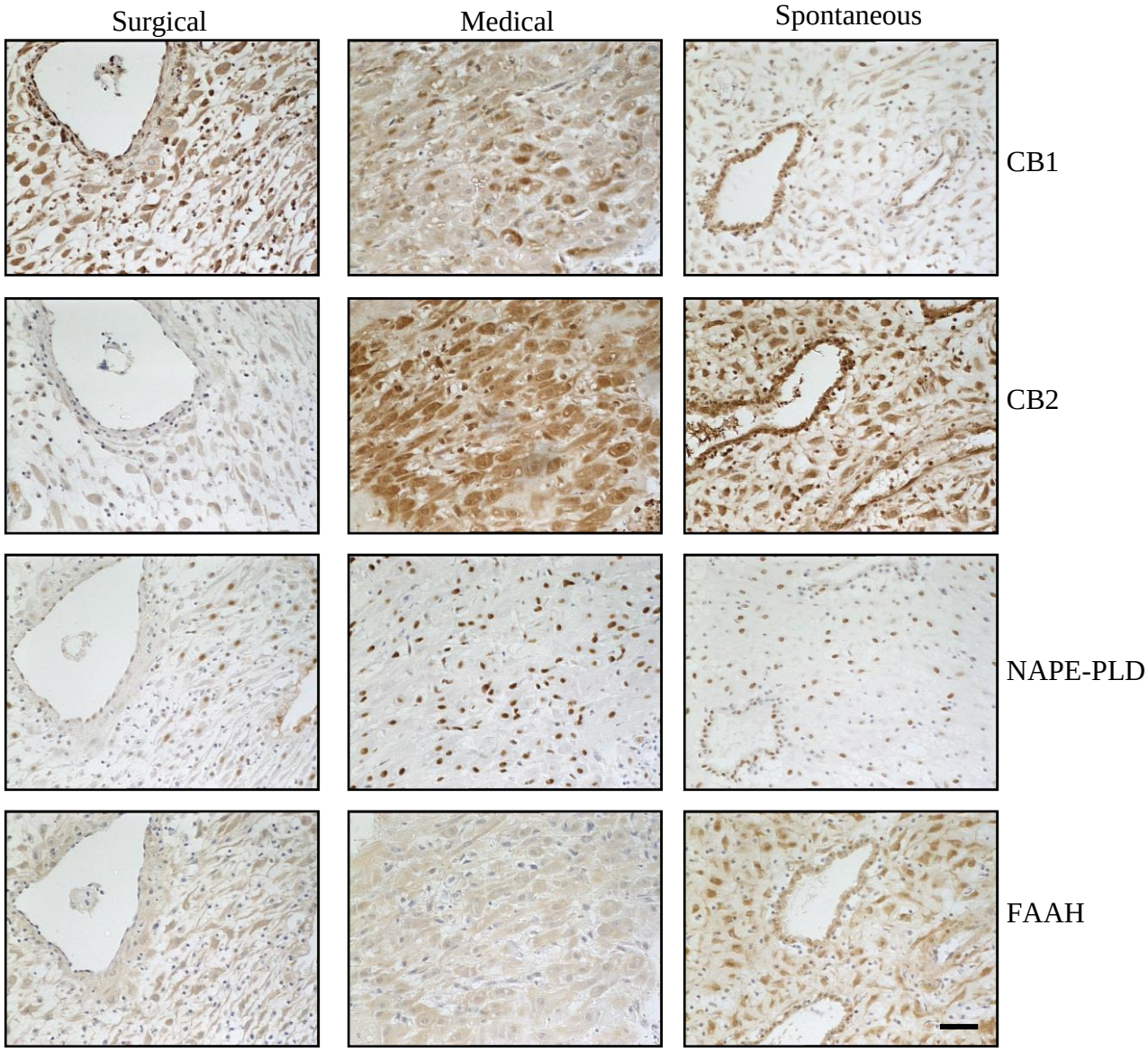

Figure 5.11 The effect of medical and spontaneous abortion on the expression of the endocannabinoid system in first trimester decidua. Medical termination of pregnancy is characterised by an increase in the expression of CB2 and NAPE-PLD protein whilst CB1 and FAAH expression are unaffected. By contrast, spontaneous miscarriage is also characterised by a marked increase in CB2 and FAAH expression and a decrease in CB1 expression that exceeds that of medical termination. Images were taken at 200x magnification. Bar = 50μm. Surgical termination of pregnancy was used as the control condition.

5.4. Discussion

CB1, CB2, FAAH and NAPE-PLD immunoreactivities were all localised to the trophoblast and the decidua of both viable and non-viable first trimester pregnancy tissues. CB1, CB2 and FAAH have all been previously described in 'normal' viable first trimester pregnancy tissue (Helliwell et al., 2004, Habayeb et al., 2008a), but this is the first time they have been described in pregnancies that have either:

1. Miscarried, or
2. Been aborted using medical methods (mifepristone and misoprostol)

It is also the first time that NAPE-PLD has been localised in first trimester human pregnancy tissue, in either viable or non-viable pregnancies. The presence of another piece of the endocannabinoid system in different locations throughout in the feto-maternal interface helps to further strengthen the evidence for a crucial role being played by the endocannabinoid system in the establishment and maintenance of early pregnancy.

In this study, the patterns of CB1, CB2 and FAAH localisation found in the viable (surgical termination) group were very similar to those previously reported (Habayeb et al., 2008a) in normal first trimester human trophoblast, although a few differences were noted:

- CB1 remained constant from 7 to 12 weeks gestation, whereas the previous studies had demonstrated a fall after 10 weeks gestation.
- CB2 was found in some (but not all) fetal blood cells and infiltrating blood cells, which had not been previously reported. Also CB2 immunoreactivity appeared

to increase from 7 weeks and plateau at 10 weeks gestation, whereas previously it was reported as remaining static throughout the first trimester.

- There was no difference in FAAH distribution, with the same peak at 10 weeks followed by rapid fall and disappearance by 12 weeks gestation.

FAAH was localised to mainly the syncytiotrophoblast and cytotrophoblast layers, with no activity in fetal blood cells. As mentioned above, levels peaked at 10 weeks and disappeared by 12 weeks. The intense staining in the syncytiotrophoblast and cytotrophoblast layers point to FAAH potentially acting as a barrier to AEA at the feto-maternal interface to ensure early pregnancy success. The fact that no FAAH is present in fetal blood cells mean that the FAAH in the trophoblast is likely to be the only line of defence against the potentially harmful effects of AEA upon the trophoblast (Helliwell et al., 2004, Habayeb et al., 2008a) and could be the only defence against anandamide-induced embryotoxicity (Wang et al., 1999) .

It has been shown that in blood taken from first trimester pregnant women, FAAH in peripheral lymphocytes had a reciprocal relationship with plasma AEA levels, exemplified by low levels of FAAH being associated with high plasma AEA levels and subsequent pregnancy failure and *vice versa* (Maccarrone et al., 2000b, Maccarrone et al., 2002a). It was hypothesised that this reciprocal expression of FAAH and plasma AEA levels may be a reflection of what is happening at the feto-maternal interface. In the miscarriage/non-viable group, FAAH staining was significantly lower than in the viable group, in keeping with what has been found peripherally.

It has also been suggested that miscarriage may be linked to a deficiency of progesterone in early pregnancy (Norwitz et al., 2001a), and the fact that progesterone is known to cause an up regulation of FAAH expression in peripheral lymphocytes, may also point to this as a mechanism for why FAAH levels are low in feto-maternal tissues of those women that miscarry. However, in those samples obtained from women undergoing a medical termination of pregnancy, using an anti-progestin (mifepristone), the staining intensity for FAAH was actually increased compared with the surgical termination group. This therefore suggests that in the miscarriage group the failure of the pregnancies is independent of the influence of progesterone, and to do with a change within other components of the endocannabinoid system. It may however be a reflection that by the action of mifepristone binding to progesterone at its receptor, the body increases progesterone production, with a parallel increase in FAAH.

The cannabinoid receptors, CB1 and CB2, appear to be differentially expressed, with CB2 having increased staining and CB1 having reduced staining in the miscarriage and the medical termination groups when compared with the surgical termination group, in both the trophoblast and decidual compartments. These data it suggest that the CB2 receptor may play a major role the development and maintenance of early pregnancy. The increase in CB2 may merely either be a reaction to a raised exposure to AEA by the embryo, or speculatively, it may be that pregnancies that fail have a genetic predisposition to do so in terms of over expression of CB2 and under expression of FAAH within the pregnancy tissue. Indeed, recent evidence from the CB1 knockout mouse model indicates that CB1 is not required for implantation and early placentation, because these animals proceed through pregnancy without difficulty, but deliver early due to a lack of CB1 (Wang et al., 2008). From other studies conducted by our research

group, it is clear that some AEA is required for human implantation (El-Talatini et al., 2009b) but that elevated levels are associated with miscarriage (Chapters 3 and 4). The data thus suggest that a pregnant woman requires some anandamide signalling for implantation and early pregnancy success, but too much signalling, perhaps through CB2 leads to placentation failure and pregnancy loss.

There was no significant difference in NAPE-PLD staining between all three groups. As this enzyme is responsible for the formation of AEA, it would appear that the elevated AEA levels associated with subsequent miscarriage in threatened miscarriage, and with non–viable pregnancies, as was demonstrated in Chapters 3 and 4, are produced peripherally rather at a local tissue level, and thus peripheral AEA is not merely a reflection of what is happening at the local level. Nevertheless, this peripherally-derived AEA may act upon the feto-maternal interface to cause pregnancy failure, since AEA acts on trophoblast cell lines to prevent cell proliferation through the CB2 receptor (Habayeb et al., 2008a).

This Chapter has provided new perspective on how miscarriage associated with elevated AEA, and low peripheral FAAH, may act at the feto-maternal interface through changes in the endocannabinoid system in the trophoblast and decidua. These changes all appear to allow the elevated AEA to act upon the trophoblast, and it is known that this causes inhibition of trophoblast growth and proliferation (Khare et al., 2006, Habayeb et al., 2008a). Such actions may ultimately lead to miscarriage.

CHAPTER 6

General Discussion

The aim of this thesis was to investigate the role played by the endocannabinoid system in early pregnancy and the changes in the system associated with early pregnancy loss/miscarriage. Previous work in both animals and humans suggested that changes within the endocannabinoid system may be directly related to implantation failure and early pregnancy maintenance (Maccarrone et al., 2000b, Maccarrone et al., 2002b, Maccarrone et al., 2002a, Paria et al., 2002b, Park et al., 2004, Habayeb et al., 2004, Habayeb et al., 2008a), and the investigations undertaken here aimed to explore these concepts further.

The original hypotheses to be explored were that:

3. anandamide fulfils a critical role in the establishment and maintenance of early human pregnancy and that this role is mediated locally at the endometrial and embryo levels, by changes in the endocannabinoid system (including a decrease in local levels of AEA), and that changes in systemic AEA levels reflect the changes at the local level.
4. abnormalities in the local and systemic endocannabinoid system are associated with early pregnancy failure and measurement of AEA levels in plasma may provide an early indication of impending pregnancy failure and that expression or measurement of the endocannabinoid system at the fetal-maternal interface will provide important clues as to the potential mechanism.

The first hurdle to allow these investigations was the need to develop a robust method for measuring anandamide in human plasma. Although AEA had previously been measured in plasma, the methods used had several disadvantages including:

a. long run times
b. comparatively poor limits of detection, and

c. wide variations in the levels detected

The method developed, as detailed in Chapter 2, allowed for a more rapid throughput of clinical samples than had been previously available, and also was a great improvment with regards to the sensitivity of the assay in terms of the limits of detection and quantification.

Using the newly developed method, changes in plasma AEA levels during pregnancy, were compared with the levels found using the method previously used by our research group (Habayeb et al., 2004). The levels of plasma AEA remained low during pregnancy until term and then increased significantly in labour, corresponding with previously reported data. The levels in the first trimester were very similar to those reported in the luteal phase of the menstrual cycle, suggesting that in 'normal' pregnancy plasma AEA levels need to be maintained throughout the implantation window and into the first trimester for a successful pregnancy outcome. Indeed, in parallel studies performed by a clinical colleague on a cohort of IVF-ET women, plasma anandamide levels were shown to increase significantly during the 4th and 5th week of gestation and then return to luteal levels, if the pregnancy was to progress. If, however, plasma AEA levels remained elevated, then the pregnancy failed (El-Talatini et al., 2009b). These data were qualitatitively similar to previous studies (Maccarrone et al., 2002a) and used the same assay method used within this thesis to measure plasma AEA levels indicating how good the assay truly is.

The findings of elevated plasma AEA levels at term, and especially in women in active labour correlate with that found previously, pointing to the endocannabinoid system

being potentially important for the preparation for, initiation of and maintenance of labour; the nature of this, however, has remained elusive. Indeed, recent evidence in the CB1 knockout mouse has shown that the loss of CB1 receptor signalling leads to preterm labour (Wang et al., 2008), although a similar dysfunctional CB1 signalling pathway in preterm women is yet to be established.

If inappropriate AEA signalling during labour is responsible for preterm birth, then it is possible that elevated AEA levels in failing/failed early pregnancy causes some degree of uterine activity leading to expulsion of the embryo.

Because endocannabinoids and the events that occur early in pregnancy was the focus of this thesis, it seemed sensible to build upon previously published data and examine changes in the endocannabinoid system at the systemic and at the local level, in regards to pregnancy outcomes and to correlate those findings with other pregnancy-related markers (i.e. progesterone, hCG, PAPP-A). The most important finding (described in Chapter 3) was that plasma levels of AEA in women who presented with a viable pregnancy complicated by a threatened miscarriage were significantly elevated in women who subsequently went on to miscarry ($p<0.001$). Taking a single plasma AEA level of 2.0nM as a cut-off for predicting subsequent miscarriage, ROC analysis indicated that at this level, a sensitivity and specificity for predicting spontaneous miscarriage was 100% and 94% respectively. The negative predictive value was 100% and positive predictive value was 82%, for subsequent miscarriage. This was the first really good indicator/biochemical marker (i.e. a single plasma AEA measurement) of pregnancy outcomes in an at risk population and if confirmed in larger studies may provide the first effective predictor of pregnancy outcomes in women with a threatened miscarriage. This association of high plasma AEA levels with subsequent miscarriage is

similar to that previously reported (Maccarrone et al., 2002a), however, direct comparison with the Italian data is not possible as that study involved pregnancies conceived by IVF-ET/ICSI, whilst all pregnancies in this thesis were natural conceptions. However, similarities can be drawn, and the finding of elevated plasma AEA levels in association with subsequent miscarriage is likely to point to a significant role for anandamide and the endocannabinoid system in early pregnancy success or failure. What remained unclear was whether AEA was acting in isolation, or it interacted with other substances that are known to be important in early pregnancy. For the purposes of further investigation progesterone, hCG and PAPP-A were chosen, because they are often used to clinically define successful pregnancy.

In Chapter 4 the levels of these biochemical markers, in association with plasma AEA in viable first trimester pregnancies and in those pregnancies which had failed, as confirmed by USS at the time of blood collection, were compared. The data indicated that plasma AEA levels were significantly elevated in women with non-viable first trimester pregnancies when compared to the levels in confirmed viable pregnancies ($p=0.013$) and that the plasma AEA levels in viable pregnancies were similar to those previously reported by Maccarrone *et al*. 2002 and our group (Habayeb et al., 2004) and also similar to those reported in the luteal phase of the menstrual cycle (Habayeb et al., 2004), confirming the suggestion that for successful implantation, plasma AEA levels need to be maintained at a low level during both the implantation window (El-Talatini et al., 2009b) and during early pregnancy development (Habayeb et al., 2008b).

Raised plasma AEA concentrations in non-viable pregnancies at the time of presentation suggests that an aberration in the endocannabinoid system may have

already occurred resulting in a disruption of the normal implantation and developmental process, and may thus be the cause of the miscarriage. Because the levels of AEA in non-viable pregnancies were much lower than those found in women presenting with a threatened miscarriage and a viable pregnancy who subsequently miscarried (as shown in Chapter 3), this observation may represent the AEA levels returning to 'baseline' following a peak that occurred at the time of fetal demise. This conclusion is supported by the finding that the plasma AEA levels observed in this cohort of women were close to those observed during the follicular phase of the menstrual cycle, suggesting that the plasma AEA levels may be a measure of the woman's reproductive tract preparing for the recommencement of the new menstrual cycle.

Serum progesterone and β-hCG levels found in these women were similar to those reported in the literature (Yovich et al., 1986, Nulsen and Peluso, 1992, Elson et al., 2003b, Barnhart et al., 2004a, Condous et al., 2004, Ioannidis et al., 2005a, Elson et al., 2005a) and as expected, serum progesterone levels were significantly lower in the non-viable group (Elson et al., 2003a). Although median β-hCG levels were 4.4 times higher in the viable group, this difference was not statistically significant (P=0.144). Previous studies have reported similar observations (Ong et al., 2000b, Barnhart et al., 2004a, Wallace et al., 2004b, Elson et al., 2005a) and it is thought that the absence of a significant differences was due largely to differences in the timing of the sample collection (Barnhart et al., 2004b). Also, it is known that β-hCG is a poor predictor of pregnancy failure, with a sensitivity and a specificity of only around 80% (Yovich et al., 1986, Ellish et al., 1996). In addition, wide variations have been reported between studies when defining a threshold level for viability at a specific gestational time point (Al-Azemi et al., 2003, Barnhart et al., 2004b).

The lack of a correlation between plasma AEA and either serum β-hCG or progesterone, in the cohort as a whole, could easily be interpreted to mean that plasma AEA levels are linked to early pregnancy failure through a mechanism independent of these factors. The lack of a correlation between AEA and progesterone differs from the studies of Maccarrone *et al*. who observed that low P4 levels were associated with high plasma AEA levels, through a mechanism that involved the direct stimulation of FAAH in peripheral mononuclear cells, because their plasma AEA levels were directly and inversely related to FAAH expression levels (Maccarrone et al., 2002a, Maccarrone et al., 2003a). The lack of a correlation between P4 and AEA levels was therefore surprising. Recent data from El-Talatini relating P4 and AEA levels also demonstrated a lack of correlation during the normal menstrual cycle, although there was a good correlation between plasma AEA and 17β-estradiol levels, suggesting that P4 is not the only hormone that regulates AEA levels (El-Talatini et al., 2009b).

Although PAPP-A levels were increased in the non-viable group, the increase was not statistically significant. This was a rather unexpected result as a decrease in PAPP-A level is now considered to be a marker of problems in pregnancy (Ong et al., 2000a, Tong et al., 2004b). This discrepancy may however be due either to the relatively low numbers involved in this study, or because the pregnancies had already failed (Westergaard et al., 1983, Ruge et al., 1990, Cuckle et al., 1999). There was, however, a strong correlation between P4, β-hCG and PAPP-A levels in both the viable and non-viable pregnancies, suggesting a causal link between pregnancy and the production of each of these factors. At present, the factors involved in the production of PAPP-A are unknown and although a number of studies have been implicated in its generation, P4 is not one of them (Ong et al., 2000b, Tong et al., 2004b, Boldt and Conover, 2007).

Therefore, the correlation of these 3 factors with each other presumably represents a marker of trophoblast/placental function and well-being.

In common with the data presented in Chapter 3, the finding of an association between raised plasma AEA levels and non-viable pregnancies could mean either, (a) that a disruption in the endocannabinoid system led to a disruption of the normal implantation and developmental process, thereby causing miscarriage or, (b) that a failed pregnancy, for whatever reason, is associated with a disruption in the endocannabinoid system in favour of a higher AEA level. Which of these possibilities is the more likely is currently uncertain.

Although the trophoblast, the endometrium, and the embryo all contain CB1 and CB2 receptors and FAAH, the lack of correlation between plasma AEA levels, and trophoblast dependent markers of pregnancy success suggest that the trophoblast may not to be the target for the action of AEA in early pregnancy failure. This would suggest that AEA is likely acting at the level of the local endometrial-embryo interface. Further studies of these interactions with larger patient numbers or with the use of animal models may eventually lead to the identification of potential areas for interventions to reduce miscarriages. Indeed, very recent evidence suggests that anandamide has a direct role in the development of and regression of the developing decidua in the rat, through a process that initially involves proliferation and then apoptosis (Fonseca et al., 2009).

In the mouse, a significant fall in local AEA and a rise in FAAH are essential at the implantation site compared to the inter-implantation site for successful implantation and early pregnancy maintenance (Schmid et al., 1997b, Paria et al., 2002c). As seen in

Chapter 3, and in the observations of Maccarrone *et al*. 2000, peripheral mononuclear cell FAAH levels and the levels of AEA in humans behave in a similar way as the local uterine endocannabinoid changes reported in the mouse uterus (Schmid et al., 1997a, Liu et al., 2002). To examine the influence of the endocannabinoid system at the feto-maternal interface in humans, immunohistochemistry was used to localise elements of the endocannabinoid system in first trimester pregnancy tissue. The localisation of CB1, CB2, FAAH and NAPE-PLD was investigated, in both surgical termination of pregnancy group and non-viable pregnancies (Chapter 5). The surgical termination of pregnancy group took the form of viable pregnancies undergoing surgical termination of pregnancy, and the non-viable were surgical evacuation for missed miscarriage. A third group consisting of medical terminations of pregnancy, using the progesterone antagonist mifepristone and prostaglandin, was also included because this group should indicate whether progesterone signalling pathways are truly involved in the regulation of the endocannabinoid system at the feto-maternal interface. The data indicated that as was previously reported (Helliwell et al., 2004, Habayeb et al., 2008a) CB1, CB2 and FAAH were all localised in first trimester pregnancy tissue. However, in this study this was the first time they had been described in pregnancies that had either:

1. Miscarried, or
2. Been aborted using medical methods (mifepristone and misoprostol)

It was also the first time that NAPE-PLD had been localised in first trimester human pregnancy tissue, in either viable or non-viable pregnancies. The presence of another member of the endocannabinoid system in different locations throughout the feto-maternal interface helps to further strengthen the evidence for a crucial role being

played by the endocannabinoid system in the establishment and maintenance of early pregnancy.

The patterns of CB1, CB2 and FAAH localisation found in the viable (surgical termination) group were very similar to that previously reported (Habayeb et al., 2008a) in normal first trimester human trophoblast. FAAH was localised in the syncytiotrophoblast and cytotrophoblast layers, with no activity in fetal blood cells, and with levels peaking at 10 weeks and disappearing by 12 weeks. The intense staining in the syncytiotrophoblast and cytotrophoblast layers point to FAAH potentially acting as a barrier to AEA at the feto-maternal interface to ensure early pregnancy success. The fact that no FAAH was present in fetal blood cells means that the FAAH in the trophoblast was likely to be the only line of defence against the potentially harmful effects of AEA upon the trophoblast (Helliwell et al., 2004, Habayeb et al., 2008a) and could be the only defence against anandamide-induced embryotoxicity (Wang et al., 1999).

As stated earlier, FAAH in peripheral lymphocytes of pregnant women in the first trimester had been shown to have a reciprocal relationship with plasma AEA levels, exemplified by low levels of FAAH [associated with high plasma AEA levels] and subsequent pregnancy failure and *vice versa* (Maccarrone et al., 2000b, Maccarrone et al., 2002a). It was hypothesised that this reciprocal expression of FAAH and plasma AEA levels may be a reflection of what is happening at the feto-maternal interface. In the miscarriage/non-viable group, FAAH staining was significantly lower than in the viable group, in keeping with what has been found peripherally. These data have

recently been confirmed in a study of 15 women undergoing spontaneous miscarriage (Trabucco et al., 2009).

Deficiency of progesterone in early pregnancy has been linked to miscarriage (Norwitz et al., 2001a), and progesterone is known to cause an up regulation of FAAH expression in peripheral lymphocytes (Maccarrone et al., 2001b). It may therefore be that because of reduced progesterone levels one would expect FAAH levels to be low in feto-maternal tissues of those women that miscarry. However, in those samples obtained from women undergoing a medical termination of pregnancy, with an anti-progesterone (mifepristone), the staining intensity for FAAH was actually increased compared with viable pregnancies. This may suggest that in the miscarriage group the failure of the pregnancies is independent of the influence of progesterone, and to do with a change within other components of the endocannabinoid system, drawing a parallel with the finding of a lack of correlation between serum progesterone and plasma AEA levels observed in Chapter 4. Alternatively it may be that by blocking progesterone receptors, mifepristone leads to an increase in plasma progesterone and hence an increase in FAAH.

CB1 and CB2, on the other hand were differentially expressed, with CB2 having increased staining and CB1 having reduced staining in the miscarriage and the medical termination groups when compared with the surgical termination of pregnancy group, in both the trophoblast and decidual compartments. This suggests that it is the CB2 receptor that plays the major role in terms of AEA affecting the development and maintenance of early pregnancy. The increase in CB2 may merely be a reaction to a raised exposure to AEA by the embryo, or speculatively, it may be that pregnancies that

fail have a genetic predisposition to do so in terms of over expression of CB2 and under expression of FAAH within the pregnancy tissue.

No significant difference in NAPE-PLD staining was seen between all three groups, suggesting that the elevated plasma AEA levels associated with subsequent miscarriage in threatened miscarriage, and with non–viable pregnancies, as was demonstrated in Chapters 3 and 4, are produced peripherally rather than being produced at a local tissue level, and thus peripheral AEA is not merely a reflection of what is happening at the local level, but the peripheral AEA may play a direct role in pregnancy failure. Nevertheless, this peripherally-derived AEA may act upon the feto-maternal interface to cause pregnancy failure, since AEA acts on trophoblast cell lines to prevent cell proliferation through the CB2 receptor (Habayeb et al., 2008a). Contrary to the data presented here, and contrary to their own published work, in a single study indicating that there is high CB1 expression and low FAAH expression associated with spontaneous miscarriage (Trabucco et al., 2009) and this is related to decreased transcript levels for NAPE-PLD. The data are however counter-intuitive, as decreased NAPE-PLD and increased CB1 expression should result in decreased AEA levels in the tissue, whilst decreased FAAH expression should result in increased AEA levels. Thus the authors indicated that they considered the local production of AEA as being critical. Indeed, the immunohistochemical studies in Chapter 5 also demonstrated the existence of NAPE-PLD protein at the feto-maternal interface, supporting the data of Trabucco *et al*., and a fall in the levels of FAAH suggesting that a very fine balance in the expression and activities of these two enzymes is critical in early pregnancy. Thus, the exact role and function of the endocannabinoid system at the tissue level is likely to be even more complex than was originally envisioned (Maccarrone et al., 2002a).

This Chapter has provided new ideas upon how miscarriage associated with elevated AEA, and low peripheral FAAH, may act at the feto-maternal interface by changes in the endocannabinoid system in the trophoblast and decidua. These changes all appear to allow the elevated AEA to act upon the trophoblast, and it is known that this causes inhibition of trophoblast growth and proliferation (Khare et al., 2006, Habayeb et al., 2008a) and decidual apoptosis (Moghadam et al., 2005, Fonseca et al., 2009). Such actions may ultimately lead to miscarriage.

Conclusion

Prior to starting the studies in this thesis, it had become clear from other studies that the endocannabinoid system was an important part of reproduction. During the course of these studies it became apparent that some AEA is required for human implantation to occur (El-Talatini et al., 2009b), but that elevated levels are also associated with adverse pregnancy outcome (Habayeb et al., 2008b). Elevated plasma AEA in humans has been linked with miscarriage in IVF pregnancies (Maccarrone et al., 2002a), and in animals elevated AEA at the site of implantation has a negative effect upon implantation (Paria et al., 2002b, Wang et al., 2004), and high levels of AEA affect the blastocyst's ability to implant (Wang et al., 1999). The data presented in this thesis add to this existing knowledge by providing:

a. A much improved method of measuring plasma anandamide than previously described.
b. The finding that elevated plasma AEA levels in naturally conceived pregnancies presenting with a threatened miscarriage is a very good predictive marker of subsequent pregnancy failure.

c. Data showing that plasma AEA levels are also elevated in pregnancies which have already miscarried at presentation.

d. But that there appears to be no correlation between plasma AEA and other pregnancy related hormones such as progesterone, hCG and PAPP-A, leading to the conclusion that AEA exerts its negative effects upon the pregnancy through mechanisms that are likely to be independent of these other factors.

e. Evidence that CB1, CB2 and FAAH are all localised in pregnancy tissue from miscarriages and for the first time that NAPE-PLD protein is present in both surgical termination of pregnancy and non-viable pregnancy tissues.

f. Confirmatory evidence that the cannabinoid receptors, CB1 and CB2, are differentially expressed through the first trimester, with CB2 having increased staining and CB1 having reduced staining in the miscarriage and the medical termination groups when compared with the surgical termination of pregnancy group, in both the trophoblast and decidual compartments, whilst in the miscarriage/non-viable group, FAAH staining was significantly lower than that observed in the surgical termination of pregnancy group, in keeping with what has been found peripherally.

g. That women undergoing a medical termination of pregnancy, using the anti-progesterone (mifepristone), demonstrated increased staining for FAAH compared to that of viable pregnancies, suggesting that in the miscarriage group the failure of the pregnancies may be independent of the influence of progesterone.

In summary, there are obviously still many unanswered questions, but given further time others areas of investigation could include:

h. Increasing to numbers in the work of Chapter 3 to strengthen the case for AEA being used as a predictive test for miscarriage.
i. Examining FAAH expression and activities in peripheral lymphocytes in the same group of patients [threatened miscarriage with a viable pregnancy at presentation], to see if the same reciprocal pattern is seen as reported in IVF pregnancies (Maccarrone et al., 2000b, Maccarrone et al., 2002a).
j. Examining the absolute AEA levels in viable and non-viable pregnancy tissues, to determine if a difference comparable to that seen systemically is also manifest at the feto-maternal interface.
k. Manipulating the endocannabinoid system, for example with anandamide antagonists/blockers to see if pregnancy outcome can be improved/altered. Obviously it would not be possible to do this in human subjects, but this could be done in experimental animals, such as laboratory rats, in which elevated AEA levels is known to adversely affect pregnancy outcomes.

From all of the above, a much clearer picture is emerging about the role of endocannabinoids in early pregnancy and it is anticipated that future studies will elucidate just how important anandamide, its receptors, and the factors that regulate the endocannabinoid system really are in human reproduction.

Appendix 1: Publications arising from this thesis

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

Analytical Biochemistry, *Volume 380, Issue 2, 15 September 2008, Pages 195-201*

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

2. **Plasma Anandamide Concentration and Pregnancy Outcome in Women With Threatened Miscarriage**

Osama M. H. Habayeb; Anthony H. Taylor; Mark Finney; et al.

JAMA. 2008;299(10):1135-1136

Appendix 2: Presentations arising from this thesis

Poster Presentations :

1. **Early Pregnancy Failure is Characterised by Elevated Plasma Levels of Anandamide and Inhibition of Trophoblast Cell Growth**

Blair Bell Competition Meeting, Royal College of Obstetricians and Gynaecologists, London, December 2007.

2. **Plasma levels of Anandamide are Elevated in Early Pregnancy Failure**

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

Appendix 3: Ethics Documentation, patient information leaflet and consent forms.

Bibliography

AL-AZEMI, M., LEDGER, W. L., DIEJOMAOH, M., MOUSA, M., MAKHSEED, M. & OMU, A. (2003) Measurement of inhibin A and inhibin pro-alphaC in early human pregnancy and their role in the prediction of pregnancy outcome in patients with recurrent pregnancy loss. *Fertility & Sterility,* 80**,** 1473-9.

ASCH, R. H. & SMITH, C. G. (1986) Effects of delta 9-THC, the principal psychoactive component of marijuana, during pregnancy in the rhesus monkey. *J Reprod Med,* 31**,** 1071-81.

BARNHART, K., SAMMEL, M. D., CHUNG, K., ZHOU, L., HUMMEL, A. C. & GUO, W. (2004a) Decline of serum human chorionic gonadotropin and spontaneous complete abortion: defining the normal curve. *Obstetrics & Gynecology,* 104**,** 975-81.

BARNHART, K., SAMMEL, M. D., CHUNG, K., ZHOU, L., HUMMEL, A. C. & GUO, W. (2004b) Decline of serum human chorionic gonadotropin and spontaneous complete abortion: defining the normal curve. *Obstet Gynecol,* 104**,** 975-81.

BEGG, M., PACHER, P., BATKAI, S., OSEI-HYIAMAN, D., OFFERTALER, L., MO, F. M., LIU, J. & KUNOS, G. (2005) Evidence for novel cannabinoid receptors. *Pharmacol Ther,* 106**,** 133-45.

BELTRAMO, M. & PIOMELLI, D. (2000) Carrier-mediated transport and enzymatic hydrolysis of the endogenous cannabinoid 2-arachidonylglycerol. *Neuroreport,* 11**,** 1231-5.

BELTRAMO, M., STELLA, N., CALIGNANO, A., LIN, S. Y., MAKRIYANNIS, A. & PIOMELLI, D. (1997) Functional role of high-affinity anandamide transport, as revealed by selective inhibition. *Science,* 277**,** 1094-7.

BIFULCO M & DI MARZO V (2002) Targeting the endocannabinoid system in cancer therapy : a call for further research. *Nature Medicine,* 8**,** 547-550.

BOLDT, H., B. CONOVER, CHERYL, A. (2007) Pregnancy-associated plasma protein-A (PAPP-A): A local regulator
of IGF bioavailability through cleavage of IGFBPs
. *Growth Hormone & IGF Research***,** 10-18.

BOLDT, H. B. & CONOVER, C. A. (2007) Pregnancy-associated plasma protein-A (PAPP-A): a local regulator of IGF bioavailability through cleavage of IGFBPs. *Growth Hormone & Igf Research,* 17**,** 10-8.

BOUABOULA, M., POINOT-CHAZEL, C., BOURRIE, B., CANAT, X., CALANDRA, B., RINALDI-CARMONA, M., LE FUR, G. & CASELLAS, P. (1995) Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB1. *Biochem J,* 312 (Pt 2)**,** 637-41.

BOUABOULA, M., RINALDI, M., CARAYON, P., CARILLON, C., DELPECH, B., SHIRE, D., LE FUR, G. & CASELLAS, P. (1993) Cannabinoid-receptor expression in human leukocytes. *Eur J Biochem,* 214**,** 173-80.

BREIVOGEL, C. S. & CHILDERS, S. R. (2000) Cannabinoid agonist signal transduction in rat brain: comparison of cannabinoid agonists in receptor binding, G-protein activation, and adenylyl cyclase inhibition. *J Pharmacol Exp Ther,* 295**,** 328-36.

BROWN, A. (2001) Identification of modulators of GPR55 activity. Patent WO0186305. *Glaxosmithline*.

BUCKLEY, N. E., HANSSON, S., HARTA, G. & MEZEY, E. (1998) Expression of the CB1 and CB2 receptor messenger RNAs during embryonic development in the rat. *Neuroscience,* 82**,** 1131-49.
CABRAL, G. A., TONEY, D. M., FISCHER-STENGER, K., HARRISON, M. P. & MARCIANO-CABRAL, F. (1995) Anandamide inhibits macrophage-mediated killing of tumor necrosis factor-sensitive cells. *Life Sci,* 56**,** 2065-72.
CADAS, H., DI TOMASO, E. & PIOMELLI, D. (1997) Occurrence and biosynthesis of endogenous cannabinoid precursor, N-arachidonoyl phosphatidylethanolamine, in rat brain. *J Neurosci,* 17**,** 1226-42.
CARSON, D. D., BAGCHI, I., DEY, S. K., ENDERS, A. C., FAZLEABAS, A. T., LESSEY, B. A. & YOSHINAGA, K. (2000) Embryo implantation. *Dev Biol,* 223**,** 217-37.
CASTELLANO, C., CABIB, S., PALMISANO, A., DI MARZO, V. & PUGLISI-ALLEGRA, S. (1997) The effects of anandamide on memory consolidation in mice involve both D1 and D2 dopamine receptors. *Behav Pharmacol,* 8**,** 707-12.
CHANG, M. C., BERKERY, D., SCHUEL, R., LAYCHOCK, S. G., ZIMMERMAN, A. M., ZIMMERMAN, S. & SCHUEL, H. (1993) Evidence for a cannabinoid receptor in sea urchin sperm and its role in blockade of the acrosome reaction. *Mol Reprod Dev,* 36**,** 507-16.
CHEN, B. A. & CREININ, M. D. (2007) Contemporary management of early pregnancy failure. *Clin Obstet Gynecol,* 50**,** 67-88.
CHETTY, M. & ELSON, J. (2007) Biochemistry in the diagnosis and management of abnormal early pregnancy. *Clinical Obstetrics & Gynecology,* 50**,** 55-66.
CHOONG, S., ROMBAUTS, L., UGONI, A. & MEAGHER, S. (2003) Ultrasound prediction of risk of spontaneous miscarriage in live embryos from assisted conceptions. *Ultrasound Obstet Gynecol,* 22**,** 571-7.
CHUNG, T. K., SAHOTA, D. S., LAU, T. K., MONGELLI, J. M., SPENCER, J. A. & HAINES, C. J. (1999) Threatened abortion: prediction of viability based on signs and symptoms. *Aust N Z J Obstet Gynaecol,* 39**,** 443-7.
CONDOUS, G., LU, C., VAN HUFFEL, S. V., TIMMERMAN, D. & BOURNE, T. (2004) Human chorionic gonadotrophin and progesterone levels in pregnancies of unknown location. *International Journal of Gynaecology & Obstetrics,* 86**,** 351-7.
CRAVATT, B. F., DEMAREST, K., PATRICELLI, M. P., BRACEY, M. H., GIANG, D. K., MARTIN, B. R. & LICHTMAN, A. H. (2001) Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. *Proc Natl Acad Sci U S A,* 98**,** 9371-6.
CRAVATT, B. F., GIANG, D. K., MAYFIELD, S. P., BOGER, D. L., LERNER, R. A. & GILULA, N. B. (1996) Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature,* 384**,** 83-7.
CRAVATT, B. F. & LICHTMAN, A. H. (2003) Fatty acid amide hydrolase: an emerging therapeutic target in the endocannabinoid system. *Curr Opin Chem Biol,* 7**,** 469-75.
CUCKLE, H. S., SEHMI, I. K., JONES, R. G. & MASON, G. (1999) Low maternal serum PAPP-A and fetal viability.[comment]. *Prenatal Diagnosis,* 19**,** 788-90.
DAS, S. K., PARIA, B. C., CHAKRABORTY, I. & DEY, S. K. (1995) Cannabinoid ligand-receptor signaling in the mouse uterus. *Proc Natl Acad Sci U S A,* 92**,** 4332-6.
DE BRAEKELEER, M. & DAO, T. N. (1990) Cytogenetic studies in couples experiencing repeated pregnancy losses. *Hum Reprod,* 5**,** 519-28.

DE PETROCELLIS, L., CASCIO, M. G. & DI MARZO, V. (2004) The endocannabinoid system: a general view and latest additions. *Br J Pharmacol,* 141**,** 765-74.

DEAN, J. (1983) Preimplantation development: biology, genetics, and mutagenesis. *Am J Ind Med,* 4**,** 31-49.

DENNEDY, M. C., FRIEL, A. M., HOULIHAN, D. D., BRODERICK, V. M., SMITH, T. & MORRISON, J. J. (2004) Cannabinoids and the human uterus during pregnancy. *Am J Obstet Gynecol,* 190**,** 2-9; discussion 3A.

DERKINDEREN, P., LEDENT, C., PARMENTIER, M. & GIRAULT, J. A. (2001) Cannabinoids activate p38 mitogen-activated protein kinases through CB1 receptors in hippocampus. *J Neurochem,* 77**,** 957-60.

DERKINDEREN, P., TOUTANT, M., BURGAYA, F., LE BERT, M., SICILIANO, J. C., DE FRANCISCIS, V., GELMAN, M. & GIRAULT, J. A. (1996) Regulation of a neuronal form of focal adhesion kinase by anandamide. *Science,* 273**,** 1719-22.

DERKINDEREN, P., VALJENT, E., TOUTANT, M., CORVOL, J. C., ENSLEN, H., LEDENT, C., TRZASKOS, J., CABOCHE, J. & GIRAULT, J. A. (2003) Regulation of extracellular signal-regulated kinase by cannabinoids in hippocampus. *J Neurosci,* 23**,** 2371-82.

DEUTSCH, D. G., GOLIGORSKY, M. S., SCHMID, P. C., KREBSBACH, R. J., SCHMID, H. H., DAS, S. K., DEY, S. K., ARREAZA, G., THORUP, C., STEFANO, G. & MOORE, L. C. (1997) Production and physiological actions of anandamide in the vasculature of the rat kidney. *J Clin Invest,* 100**,** 1538-46.

DEVANE, W. A., DYSARZ, F. A., 3RD, JOHNSON, M. R., MELVIN, L. S. & HOWLETT, A. C. (1988) Determination and characterization of a cannabinoid receptor in rat brain. *Mol Pharmacol,* 34**,** 605-13.

DEVANE, W. A., HANUS, L., BREUER, A., PERTWEE, R. G., STEVENSON, L. A., GRIFFIN, G., GIBSON, D., MANDELBAUM, A., ETINGER, A. & MECHOULAM, R. (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor.[see comment]. *Science,* 258**,** 1946-9.

DI MARZO, V. (1998) 'Endocannabinoids' and other fatty acid derivatives with cannabimimetic properties: biochemistry and possible physiopathological relevance. *Biochim Biophys Acta,* 1392**,** 153-75.

DI MARZO V (1994) Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature,* 372**,** 686-691.

DI MARZO V & DE PETROCELLIS L (2006) Plant, Synthetic, and Endogenous Cannabinoids in Medicine. *Annual Review of Medicine,* 57**,** 553-574.

DI MARZO, V., BISOGNO, T., DE PETROCELLIS, L., BRANDI, I., JEFFERSON, R. G., WINCKLER, R. L., DAVIS, J. B., DASSE, O., MAHADEVAN, A., RAZDAN, R. K. & MARTIN, B. R. (2001a) Highly selective CB(1) cannabinoid receptor ligands and novel CB(1)/VR(1) vanilloid receptor "hybrid" ligands. *Biochem Biophys Res Commun,* 281**,** 444-51.

DI MARZO, V., GOPARAJU, S. K., WANG, L., LIU, J., BATKAI, S., JARAI, Z., FEZZA, F., MIURA, G. I., PALMITER, R. D., SUGIURA, T. & KUNOS, G. (2001b) Leptin-regulated endocannabinoids are involved in maintaining food intake. *Nature,* 410**,** 822-5.

DI MARZO, V., MELCK, D., BISOGNO, T. & DE PETROCELLIS, L. (1998) Endocannabinoids: endogenous cannabinoid receptor ligands with neuromodulatory action. *Trends Neurosci,* 21**,** 521-8.

DRMOTA, T. (2004) Screening assays for cannabinoid-ligand type modulators of GPR55. Patent WO2004074844. *Astrazenica*.
EDDY, C. A. & PAUERSTEIN, C. J. (1980) Anatomy and physiology of the fallopian tube. *Clin Obstet Gynecol,* 23**,** 1177-93.
EL-TALATINI, M. R., TAYLOR, A. H., ELSON, J. C., BROWN, L., DAVIDSON, A. C. & KONJE, J. C. (2009a) Localisation and function of the endocannabinoid system in the human ovary. *PLoS ONE,* 4**,** e4579.
EL-TALATINI, M. R., TAYLOR, A. H. & KONJE, J. C. (2009b) The relationship between plasma levels of the endocannabinoid, anandamide, sex steroids, and gonadotrophins during the menstrual cycle. *Fertil Steril*.
ELLISH, N. J., SABODA, K., O'CONNOR, J., NASCA, P. C., STANEK, E. J. & BOYLE, C. (1996) A prospective study of early pregnancy loss. *Hum Reprod,* 11**,** 406-12.
ELSON, J., SALIM, R., TAILOR, A., BANERJEE, S., ZOSMER, N. & JURKOVIC, D. (2003a) Prediction of early pregnancy viability in the absence of an ultrasonically detectable embryo. *Ultrasound Obstet Gynecol,* 21**,** 57-61.
ELSON, J., SALIM, R., TAILOR, A., BANERJEE, S., ZOSMER, N. & JURKOVIC, D. (2003b) Prediction of early pregnancy viability in the absence of an ultrasonically detectable embryo.[see comment]. *Ultrasound in Obstetrics & Gynecology,* 21**,** 57-61.
ELSON, J., TAILOR, A., SALIM, R., HILLABY, K., DEW, T. & JURKOVIC, D. (2005a) Expectant management of miscarriage--prediction of outcome using ultrasound and novel biochemical markers. *Human Reproduction,* 20**,** 2330-3.
ELSON, J., TAILOR, A., SALIM, R., HILLABY, K., DEW, T. & JURKOVIC, D. (2005b) Expectant management of miscarriage--prediction of outcome using ultrasound and novel biochemical markers. *Hum Reprod,* 20**,** 2330-3.
FACCI, L., DAL TOSO, R., ROMANELLO, S., BURIANI, A., SKAPER, S. D. & LEON, A. (1995) Mast cells express a peripheral cannabinoid receptor with differential sensitivity to anandamide and palmitoylethanolamide. *Proc Natl Acad Sci U S A,* 92**,** 3376-80.
FARRELL, T. & OWEN, P. (1996) The significance of extrachorionic membrane separation in threatened miscarriage. *Br J Obstet Gynaecol,* 103**,** 926-8.
FEGLEY, D., KATHURIA, S., MERCIER, R., LI, C., GOUTOPOULOS, A., MAKRIYANNIS, A. & PIOMELLI, D. (2004) Anandamide transport is independent of fatty-acid amide hydrolase activity and is blocked by the hydrolysis-resistant inhibitor AM1172. *Proc Natl Acad Sci U S A,* 101**,** 8756-61.
FELDER, C. C., NIELSEN, A., BRILEY, E. M., PALKOVITS, M., PRILLER, J., AXELROD, J., NGUYEN, D. N., RICHARDSON, J. M., RIGGIN, R. M., KOPPEL, G. A., PAUL, S. M. & BECKER, G. W. (1996) Isolation and measurement of the endogenous cannabinoid receptor agonist, anandamide, in brain and peripheral tissues of human and rat. *FEBS Lett,* 393**,** 231-5.
FELDER, C. C., VELUZ, J. S., WILLIAMS, H. L., BRILEY, E. M. & MATSUDA, L. A. (1992) Cannabinoid agonists stimulate both receptor- and non-receptor-mediated signal transduction pathways in cells transfected with and expressing cannabinoid receptor clones. *Mol Pharmacol,* 42**,** 838-45.
FERGUSSON, D. M., HORWOOD, L. J. & NORTHSTONE, K. (2002) Maternal use of cannabis and pregnancy outcome. *Bjog,* 109**,** 21-7.
FERNANDEZ-RODRIGUEZ, C. M., ROMERO, J., PETROS, T. J., BRADSHAW, H., GASALLA, J. M., GUTIERREZ, M. L., LLEDO, J. L., SANTANDER, C., FERNANDEZ, T. P., TOMAS, E., CACHO, G. & WALKER, J. M. (2004)

Circulating endogenous cannabinoid anandamide and portal, systemic and renal hemodynamics in cirrhosis. *Liver Int,* 24**,** 477-83.
FONSECA, B. M., CORREIA-DA-SILVA, G. & TEIXEIRA, N. A. (2009) Anandamide-Induced Cell Death: Dual Effects in Primary Rat Decidual Cell Cultures. *Placenta*.
FONTANA, A., DI MARZO, V., CADAS, H. & PIOMELLI, D. (1995) Analysis of anandamide, an endogenous cannabinoid substance, and of other natural N-acylethanolamines. *Prostaglandins Leukot Essent Fatty Acids,* 53**,** 301-8.
FRENCH, F. E. & BIERMAN, J. M. (1962) Probabilities of fetal mortality. *Public Health Rep,* 77**,** 835-47.
FRIED, P. A., WATKINSON, B. & GRAY, R. (2003) Differential effects on cognitive functioning in 13- to 16-year-olds prenatally exposed to cigarettes and marihuana. *Neurotoxicol Teratol,* 25**,** 427-36.
GARCIA-ENGUIDANOS, A., CALLE, M. E., VALERO, J., LUNA, S. & DOMINGUEZ-ROJAS, V. (2002) Risk factors in miscarriage: a review. *Eur J Obstet Gynecol Reprod Biol,* 102**,** 111-9.
GASPERI, V., FEZZA, F., SPAGNUOLO, P., PASQUARIELLO, N. & MACCARRONE, M. (2005) Further insights into the regulation of human FAAH by progesterone and leptin implications for endogenous levels of anandamide and apoptosis of immune and neuronal cells. *Neurotoxicology,* 26**,** 811-7.
GEBER, W. F. & SCHRAMM, L. C. (1969) Effect of marihuana extract on fetal hamsters and rabbits. *Toxicol Appl Pharmacol,* 14**,** 276-82.
GERDEMAN, G. L., RONESI, J. & LOVINGER, D. M. (2002) Postsynaptic endocannabinoid release is critical to long-term depression in the striatum. *Nat Neurosci,* 5**,** 446-51.
GEVAERT, O., DE SMET, F., KIRK, E., VAN CALSTER, B., BOURNE, T., VAN HUFFEL, S., MOREAU, Y., TIMMERMAN, D., DE MOOR, B. & CONDOUS, G. (2006) Predicting the outcome of pregnancies of unknown location: Bayesian networks with expert prior information compared to logistic regression. *Human Reproduction,* 21**,** 1824-31.
GIBSON, G. T., BAGHURST, P. A. & COLLEY, D. P. (1983) Maternal alcohol, tobacco and cannabis consumption and the outcome of pregnancy. *Aust N Z J Obstet Gynaecol,* 23**,** 15-9.
GIUDICE, L. C. (1999) Potential biochemical markers of uterine receptivity. *Hum Reprod,* 14 Suppl 2**,** 3-16.
GIUFFRIDA A & AL., E. (2000) Quantification of bioactive Acylethanolamides in rat plasma by electrospray mass spectrometry. *Analytical Biochemistry,* 280**,** 87-93.
GIUFFRIDA, A., RODRIGUEZ DE FONSECA, F. & PIOMELLI, D. (2000) Quantification of bioactive acylethanolamides in rat plasma by electrospray mass spectrometry. *Anal Biochem,* 280**,** 87-93.
GLASER, S. T., ABUMRAD, N. A., FATADE, F., KACZOCHA, M., STUDHOLME, K. M. & DEUTSCH, D. G. (2003) Evidence against the presence of an anandamide transporter. *Proc Natl Acad Sci U S A,* 100**,** 4269-74.
GOMEZ DEL PULGAR, T., VELASCO, G. & GUZMAN, M. (2000) The CB1 cannabinoid receptor is coupled to the activation of protein kinase B/Akt. *Biochem J,* 347**,** 369-73.
GONSIOREK, W., LUNN, C., FAN, X., NARULA, S., LUNDELL, D. & HIPKIN, R. W. (2000) Endocannabinoid 2-arachidonyl glycerol is a full agonist through

human type 2 cannabinoid receptor: antagonism by anandamide. *Mol Pharmacol,* 57**,** 1045-50.
GONZALEZ, S., BISOGNO, T., WENGER, T., MANZANARES, J., MILONE, A., BERRENDERO, F., DI MARZO, V., RAMOS, J. A. & FERNANDEZ-RUIZ, J. J. (2000) Sex steroid influence on cannabinoid CB(1) receptor mRNA and endocannabinoid levels in the anterior pituitary gland. *Biochem Biophys Res Commun,* 270**,** 260-6.
HABAYEB, O. M., BELL, S. C. & KONJE, J. C. (2002) Endogenous cannabinoids: metabolism and their role in reproduction. *Life Sci,* 70**,** 1963-77.
HABAYEB, O. M. & KONJE, J. C. (2004) The one-stop recurrent miscarriage clinic: an evaluation of its effectiveness and outcome. *Hum Reprod,* 19**,** 2952-8.
HABAYEB, O. M., TAYLOR, A. H., BELL, S. C., TAYLOR, D. J. & KONJE, J. C. (2008a) Expression of the endocannabinoid system in human first trimester placenta and its role in trophoblast proliferation. *Endocrinology,* 149**,** 5052-60.
HABAYEB, O. M., TAYLOR, A. H., EVANS, M. D., COOKE, M. S., TAYLOR, D. J., BELL, S. C. & KONJE, J. C. (2004) Plasma levels of the endocannabinoid anandamide in women--a potential role in pregnancy maintenance and labor? *Journal of Clinical Endocrinology & Metabolism,* 89**,** 5482-7.
HABAYEB, O. M., TAYLOR, A. H., FINNEY, M., EVANS, M. D. & KONJE, J. C. (2008b) Plasma anandamide concentration and pregnancy outcome in women with threatened miscarriage. *Jama,* 299**,** 1135-6.
HELLIWELL, R. J., CHAMLEY, L. W., BLAKE-PALMER, K., MITCHELL, M. D., WU, J., KEARN, C. S. & GLASS, M. (2004) Characterization of the endocannabinoid system in early human pregnancy. *J Clin Endocrinol Metab,* 89**,** 5168-74.
HERKENHAM, M., LYNN, A. B., LITTLE, M. D., JOHNSON, M. R., MELVIN, L. S., DE COSTA, B. R. & RICE, K. C. (1990) Cannabinoid receptor localization in brain. *Proc Natl Acad Sci U S A,* 87**,** 1932-6.
HILLARD, C. J., EDGEMOND, W. S., JARRAHIAN, A. & CAMPBELL, W. B. (1997) Accumulation of N-arachidonoylethanolamine (anandamide) into cerebellar granule cells occurs via facilitated diffusion. *J Neurochem,* 69**,** 631-8.
HILLARD, C. J. & JARRAHIAN, A. (2003) Cellular accumulation of anandamide: consensus and controversy. *Br J Pharmacol,* 140**,** 802-8.
HO, H. H., O'CONNOR, J. F., NAKAJIMA, S. T., TIEU, J., OVERSTREET, J. W. & LASLEY, B. L. (1997) Characterization of human chorionic gonadotropin in normal and abnormal pregnancies. *Early Pregnancy,* 3**,** 213-24.
HOMAN, G., BROWN, S., MORAN, J., HOMAN, S. & KERIN, J. (2000) Human chorionic gonadotropin as a predictor of outcome in assisted reproductive technology pregnancies. *Fertility & Sterility,* 73**,** 270-4.
HUANG, S. M., BISOGNO, T., TREVISANI, M., AL-HAYANI, A., DE PETROCELLIS, L., FEZZA, F., TOGNETTO, M., PETROS, T. J., KREY, J. F., CHU, C. J., MILLER, J. D., DAVIES, S. N., GEPPETTI, P., WALKER, J. M. & DI MARZO, V. (2002) An endogenous capsaicin-like substance with high potency at recombinant and native vanilloid VR1 receptors. *Proc Natl Acad Sci U S A,* 99**,** 8400-5.
IOANNIDIS, G., SACKS, G., REDDY, N., SEYANI, L., MARGARA, R., LAVERY, S. & TREW, G. (2005a) Day 14 maternal serum progesterone levels predict pregnancy outcome in IVF/ICSI treatment cycles: a prospective study. *Human Reproduction,* 20**,** 741-6.

IOANNIDIS, G., SACKS, G., REDDY, N., SEYANI, L., MARGARA, R., LAVERY, S. & TREW, G. (2005b) Day 14 maternal serum progesterone levels predict pregnancy outcome in IVF/ICSI treatment cycles: a prospective study. *Hum Reprod,* 20**,** 741-6.

ISHAC, E. J., JIANG, L., LAKE, K. D., VARGA, K., ABOOD, M. E. & KUNOS, G. (1996) Inhibition of exocytotic noradrenaline release by presynaptic cannabinoid CB1 receptors on peripheral sympathetic nerves. *Br J Pharmacol,* 118**,** 2023-8.

JARRAHIAN, A., MANNA, S., EDGEMOND, W. S., CAMPBELL, W. B. & HILLARD, C. J. (2000) Structure-activity relationships among N-arachidonylethanolamine (Anandamide) head group analogues for the anandamide transporter. *J Neurochem,* 74**,** 2597-606.

JAUNIAUX, E., FARQUHARSON, R. G., CHRISTIANSEN, O. B. & EXALTO, N. (2006) Evidence-based guidelines for the investigation and medical treatment of recurrent miscarriage. *Hum Reprod,* 21**,** 2216-22.

JOHNS, J. & JAUNIAUX, E. (2006) Threatened miscarriage as a predictor of obstetric outcome. *Obstet Gynecol,* 107**,** 845-50.

JOHNSON, D. E., HEALD, S. L., DALLY, R. D. & JANIS, R. A. (1993) Isolation, identification and synthesis of an endogenous arachidonic amide that inhibits calcium channel antagonist 1,4-dihydropyridine binding. *Prostaglandins Leukot Essent Fatty Acids,* 48**,** 429-37.

JONSSON, K. O., HOLT, S. & FOWLER, C. J. (2006) The endocannabinoid system: current pharmacological research and therapeutic possibilities. *Basic Clin Pharmacol Toxicol,* 98**,** 124-34.

KEARN, C. S., GREENBERG, M. J., DICAMELLI, R., KURZAWA, K. & HILLARD, C. J. (1999) Relationships between ligand affinities for the cerebellar cannabinoid receptor CB1 and the induction of GDP/GTP exchange. *J Neurochem,* 72**,** 2379-87.

KEMPE, K., HSU, F. F., BOHRER, A. & TURK, J. (1996) Isotope dilution mass spectrometric measurements indicate that arachidonylethanolamide, the proposed endogenous ligand of the cannabinoid receptor, accumulates in rat brain tissue post mortem but is contained at low levels in or is absent from fresh tissue. *J Biol Chem,* 271**,** 17287-95.

KHARE, M., TAYLOR, A. H., KONJE, J. C. & BELL, S. C. (2006) {Delta}9-Tetrahydrocannabinol inhibits cytotrophoblast cell proliferation and modulates gene transcription. *Mol Hum Reprod,* 12**,** 321-333.

KLINE, J., STEIN, Z. & SUSSER, M. (1989) *Conception to birth : epidemiology of prenatal development,* New York, Oxford University Press.

KOGA, D., SANTA, T., FUKUSHIMA, T., HOMMA, H. & IMAI, K. (1997) Liquid chromatographic-atmospheric pressure chemical ionization mass spectrometric determination of anandamide and its analogs in rat brain and peripheral tissues. *J Chromatogr B Biomed Sci Appl,* 690**,** 7-13.

KOGA, D., SANTA, T., HAGIWARA, K., IMAI, K., TAKIZAWA, H., NAGANO, T., HIROBE, M., OGAWA, M., SATO, T., INOUE, K. & ET AL. (1995) High-performance liquid chromatography and fluorometric detection of arachidonylethanolamide (anandamide) and its analogues, derivatized with 4-(N-chloroformylmethyl-N-methyl)amino-7-N,N-dimethylaminosulp honyl-2,1 ,3- benzoxadiazole (DBD-COCl). *Biomed Chromatogr,* 9**,** 56-7.

KOLODNY, R. C., MASTERS, W. H., KOLODNER, R. M. & TORO, G. (1974) Depression of plasma testosterone levels after chronic intensive marihuana use. *N Engl J Med,* 290**,** 872-4.

LAIRD, S. M., QUINTON, N. D., ANSTIE, B., LI, T. C. & BLAKEMORE, A. I. (2001) Leptin and leptin-binding activity in women with recurrent miscarriage: correlation with pregnancy outcome. *Hum Reprod,* 16**,** 2008-13.

LAZZARIN, N., VALENSISE, H., BARI, M., UBALDI, F., BATTISTA, N., FINAZZI-AGRO, A. & MACCARRONE, M. (2004) Fluctuations of fatty acid amide hydrolase and anandamide levels during the human ovulatory cycle. *Gynecol Endocrinol,* 18**,** 212-8.

LEDENT, C., VALVERDE, O., COSSU, G., PETITET, F., AUBERT, J. F., BESLOT, F., BOHME, G. A., IMPERATO, A., PEDRAZZINI, T., ROQUES, B. P., VASSART, G., FRATTA, W. & PARMENTIER, M. (1999) Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB1 receptor knockout mice. *Science,* 283**,** 401-4.

LEUNG, D., SAGHATELIAN, A., SIMON, G. M. & CRAVATT, B. F. (2006) Inactivation of N-acyl phosphatidylethanolamine phospholipase D reveals multiple mechanisms for the biosynthesis of endocannabinoids. *Biochemistry,* 45**,** 4720-6.

LI, T. C., SPRING, P. G., BYGRAVE, C., LAIRD, S. M., SERLE, E., SPUIJBROEK, M. & ADEKANMI, O. (1998) The value of biochemical and ultrasound measurements in predicting pregnancy outcome in women with a history of recurrent miscarriage. *Hum. Reprod.,* 13**,** 3525-3529.

LIU, J., WANG, L., HARVEY-WHITE, J., OSEI-HYIAMAN, D., RAZDAN, R., GONG, Q., CHAN, A. C., ZHOU, Z., HUANG, B. X., KIM, H. Y. & KUNOS, G. (2006) A biosynthetic pathway for anandamide. *Proc Natl Acad Sci U S A,* 103**,** 13345-50.

LIU, W. M., DUAN, E. K. & CAO, Y. J. (2002) Effects of anandamide on embryo implantation in the mouse. *Life Sci,* 71**,** 1623-32.

LOPEZ-RODRIGUEZ, M. L., VISO, A., ORTEGA-GUTIERREZ, S., LASTRES-BECKER, I., GONZALEZ, S., FERNANDEZ-RUIZ, J. & RAMOS, J. A. (2001) Design, synthesis and biological evaluation of novel arachidonic acid derivatives as highly potent and selective endocannabinoid transporter inhibitors. *J Med Chem,* 44**,** 4505-8.

MACCARRONE M & AL., E. (2001) Progesterone up-regulates anandamide hydrolase in human lymphocytes. Role of cytokines and implications for fertility. *Journal of Immunology,* 166**,** 7183-7189.

MACCARRONE, M., ATTINA, M., CARTONI, A., BARI, M. & FINAZZI-AGRO, A. (2001a) Gas chromatography-mass spectrometry analysis of endogenous cannabinoids in healthy and tumoral human brain and human cells in culture. *J Neurochem,* 76**,** 594-601.

MACCARRONE, M., BARI, M., DI RIENZO, M., FINAZZI-AGRO, A. & ROSSI, A. (2003a) Progesterone activates fatty acid amide hydrolase (FAAH) promoter in human T lymphocytes through the transcription factor Ikaros. Evidence for a synergistic effect of leptin. *J Biol Chem,* 278**,** 32726-32.

MACCARRONE, M., BARI, M., DI RIENZO, M., FINAZZI-AGRO, A. & ROSSI, A. (2003b) Progesterone activates fatty acid amide hydrolase (FAAH) promoter in human T lymphocytes through the transcription factor Ikaros. Evidence for a synergistic effect of leptin. *Journal of Biological Chemistry,* 278**,** 32726-32.

MACCARRONE, M., BARI, M., LORENZON, T., BISOGNO, T., DI MARZO, V. & FINAZZI-AGRO, A. (2000a) Anandamide uptake by human endothelial cells and its regulation by nitric oxide. *J Biol Chem,* 275**,** 13484-92.

MACCARRONE, M., BISOGNO, T., VALENSISE, H., LAZZARIN, N., FEZZA, F., MANNA, C., DI MARZO, V. & FINAZZI-AGRO, A. (2002a) Low fatty acid amide hydrolase and high anandamide levels are associated with failure to achieve an ongoing pregnancy after IVF and embryo transfer. *Molecular Human Reproduction,* 8**,** 188-95.

MACCARRONE, M., DE FELICI, M., BARI, M., KLINGER, F., SIRACUSA, G. & FINAZZI-AGRO, A. (2000) Down-regulation of anandamide hydrolase in mouse uterus by sex hormones. *Eur J Biochem,* 267**,** 2991-7.

MACCARRONE, M., DI RIENZO, M., FINAZZI-AGRO, A. & ROSSI, A. (2003c) Leptin activates the anandamide hydrolase promoter in human T lymphocytes through STAT3. *J Biol Chem,* 278**,** 13318-24.

MACCARRONE, M., FALCIGLIA, K., DI RIENZO, M. & FINAZZI-AGRO, A. (2002b) Endocannabinoids, hormone-cytokine networks and human fertility. *Prostaglandins Leukot Essent Fatty Acids,* 66**,** 309-17.

MACCARRONE, M. & FINAZZI-AGRO, A. (2004) Anandamide hydrolase: a guardian angel of human reproduction? *Trends Pharmacol Sci,* 25**,** 353-7.

MACCARRONE, M., VALENSISE, H., BARI, M., LAZZARIN, N., ROMANINI, C. & FINAZZI-AGRO, A. (2000b) Relation between decreased anandamide hydrolase concentrations in human lymphocytes and miscarriage. *Lancet,* 355**,** 1326-9.

MACCARRONE, M., VALENSISE, H., BARI, M., LAZZARIN, N., ROMANINI, C. & FINAZZI-AGRO, A. (2000c) Relation between decreased anandamide hydrolase concentrations in human lymphocytes and miscarriage. *Lancet,* 355**,** 1326-1329.

MACCARRONE, M., VALENSISE, H., BARI, M., LAZZARIN, N., ROMANINI, C. & FINAZZI-AGRO, A. (2001b) Progesterone up-regulates anandamide hydrolase in human lymphocytes: role of cytokines and implications for fertility. *J Immunol,* 166**,** 7183-9.

MACCARRONE, M., VAN DER STELT, M., ROSSI, A., VELDINK, G. A., VLIEGENTHART, J. F. & AGRO, A. F. (1998) Anandamide hydrolysis by human cells in culture and brain. *J Biol Chem,* 273**,** 32332-9.

MAKRYDIMAS, G., SEBIRE, N. J., LOLIS, D., VLASSIS, N. & NICOLAIDES, K. H. (2003) Fetal loss following ultrasound diagnosis of a live fetus at 6-10 weeks of gestation. *Ultrasound Obstet Gynecol,* 22**,** 368-72.

MANNAVOLA, D., MOELLER, L. C., BECK-PECCOZ, P., PERSANI, L., WEISS, R. E. & REFETOFF, S. (2004) A novel slice variant involving the 5' untranslated region of thyroid hormone receptor β1 (TRβ1). *Journal of Endocrinological Investigations,* 27**,** 318 - 322.

MATARESE, G., LA CAVA, A., SANNA, V., LORD, G. M., LECHLER, R. I., FONTANA, S. & ZAPPACOSTA, S. (2002) Balancing susceptibility to infection and autoimmunity: a role for leptin? *Trends Immunol,* 23**,** 182-7.

MATSUDA, L. A., LOLAIT, S. J., BROWNSTEIN, M. J., YOUNG, A. C. & BONNER, T. I. (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature,* 346**,** 561-4.

MCFARLAND MJ & BARKER EL (2004) Anandamide Transport. *Pharmacology and Therapeutics,* 104**,** 117-135.

MECCARIELLO, R., CHIANESE, R., CACCIOLA, G., COBELLIS, G., PIERANTONI, R. & FASANO, S. (2006) Type-1 cannabinoid receptor expression in the frog, Rana esculenta, tissues: a possible involvement in the regulation of testicular activity. *Mol Reprod Dev,* 73**,** 551-8.

MECHOULAM, R., BEN-SHABAT, S., HANUS, L., LIGUMSKY, M., KAMINSKI, N. E., SCHATZ, A. R., GOPHER, A., ALMOG, S., MARTIN, B. R., COMPTON, D. R. & ET AL. (1995) Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem Pharmacol,* 50**,** 83-90.

MECHOULAM, R., FRIDE, E. & DI MARZO, V. (1998) Endocannabinoids. *Eur J Pharmacol,* 359**,** 1-18.

MECHOULAM, R. & GAONI, Y. (1965) Hashish. IV. The isolation and structure of cannabinolic cannabidiolic and cannabigerolic acids. *Tetrahedron,* 21**,** 1223-9.

MENDELSON, J. H., MELLO, N. K., ELLINGBOE, J., SKUPNY, A. S., LEX, B. W. & GRIFFIN, M. (1986) Marihuana smoking suppresses luteinizing hormone in women. *J Pharmacol Exp Ther,* 237**,** 862-6.

MEREU, G., FA, M., FERRARO, L., CAGIANO, R., ANTONELLI, T., TATTOLI, M., GHIGLIERI, V., TANGANELLI, S., GESSA, G. L. & CUOMO, V. (2003) Prenatal exposure to a cannabinoid agonist produces memory deficits linked to dysfunction in hippocampal long-term potentiation and glutamate release. *Proc Natl Acad Sci U S A,* 100**,** 4915-20.

METWALLY, M., LEDGER, W. L. & LI, T. C. (2008) Reproductive endocrinology and clinical aspects of obesity in women. *Ann N Y Acad Sci,* 1127**,** 140-6.

METWALLY, M., LI, T. C. & LEDGER, W. L. (2007) The impact of obesity on female reproductive function. *Obes Rev,* 8**,** 515-23.

MOGHADAM, K. K., KESSLER, C. A., SCHROEDER, J. K., BUCKLEY, A. R., BRAR, A. K. & HANDWERGER, S. (2005) Cannabinoid receptor I activation markedly inhibits human decidualization. *Mol Cell Endocrinol,* 229**,** 65-74.

MUNRO, S., THOMAS, K. L. & ABU-SHAAR, M. (1993) Molecular characterization of a peripheral receptor for cannabinoids. *Nature,* 365**,** 61-5.

NORWITZ, E. R., SCHUST, D. J. & FISHER, S. J. (2001a) Implantation and the survival of early pregnancy. *N Engl J Med,* 345**,** 1400-8.

NORWITZ, E. R., SCHUST, D. J. & FISHER, S. J. (2001b) Implantation and the survival of early pregnancy. *New England Journal of Medicine,* 345**,** 1400-8.

NULSEN, J. C. & PELUSO, J. J. (1992) Regulation of ovarian steroid production. *Infertility and Reproductive Medicine Clinics of North America,* 3**,** 44-55.

ONG, C. Y., LIAO, A. W., SPENCER, K., MUNIM, S. & NICOLAIDES, K. H. (2000a) First trimester maternal serum free beta human chorionic gonadotrophin and pregnancy associated plasma protein A as predictors of pregnancy complications. *Bjog,* 107**,** 1265-70.

ONG, C. Y., LIAO, A. W., SPENCER, K., MUNIM, S. & NICOLAIDES, K. H. (2000b) First trimester maternal serum free beta human chorionic gonadotrophin and pregnancy associated plasma protein A as predictors of pregnancy complications. *BJOG: An International Journal of Obstetrics & Gynaecology,* 107**,** 1265-70.

ORTEGA-GUTIERREZ, S., HAWKINS, E. G., VISO, A., LOPEZ-RODRIGUEZ, M. L. & CRAVATT, B. F. (2004) Comparison of anandamide transport in FAAH wild-type and knockout neurons: evidence for contributions by both FAAH and the CB1 receptor to anandamide uptake. *Biochemistry,* 43**,** 8184-90.

PAGOTTO, U., MARSICANO, G., FEZZA, F., THEODOROPOULOU, M., GRUBLER, Y., STALLA, J., ARZBERGER, T., MILONE, A., LOSA, M., DI MARZO, V., LUTZ, B. & STALLA, G. K. (2001) Normal human pituitary gland and pituitary adenomas express cannabinoid receptor type 1 and synthesize endogenous cannabinoids: first evidence for a direct role of

cannabinoids on hormone modulation at the human pituitary level. *J Clin Endocrinol Metab,* 86**,** 2687-96.
PARIA, B. C., DAS, S. K. & DEY, S. K. (1995) The preimplantation mouse embryo is a target for cannabinoid ligand-receptor signaling. *Proceedings of the National Academy of Sciences of the United States of America,* 92**,** 9460-4.
PARIA, B. C., DEUTSCH, D. D. & DEY, S. K. (1996) The uterus is a potential site for anandamide synthesis and hydrolysis: differential profiles of anandamide synthase and hydrolase activities in the mouse uterus during the periimplantation period. *Mol Reprod Dev,* 45**,** 183-92.
PARIA, B. C. & DEY, S. K. (2000) Ligand-receptor signaling with endocannabinoids in preimplantation embryo development and implantation. *Chem Phys Lipids,* 108**,** 211-20.
PARIA, B. C., REESE, J., DAS, S. K. & DEY, S. K. (2002a) Deciphering the cross-talk of implantation: advances and challenges. *Science,* 296**,** 2185-8.
PARIA, B. C., SONG, H., WANG, X., SCHMID, P. C., KREBSBACH, R. J., SCHMID, H. H., BONNER, T. I., ZIMMER, A. & DEY, S. K. (2001) Dysregulated cannabinoid signaling disrupts uterine receptivity for embryo implantation. *J Biol Chem,* 276**,** 20523-8.
PARIA, B. C., WANG, H. & DEY, S. K. (2002b) Endocannabinoid signaling in synchronizing embryo development and uterine receptivity for implantation. *Chem Phys Lipids,* 121**,** 201-10.
PARIA, B. C., WANG, H. & DEY, S. K. (2002c) Endocannabinoid signaling in synchronizing embryo development and uterine receptivity for implantation. *Chemistry & Physics of Lipids,* 121**,** 201-10.
PARIA, B. C., ZHAO, X., WANG, J., DAS, S. K. & DEY, S. K. (1999) Fatty-acid amide hydrolase is expressed in the mouse uterus and embryo during the periimplantation period. *Biol Reprod,* 60**,** 1151-7.
PARK, B., GIBBONS, H. M., MITCHELL, M. D. & GLASSA, M. (2003) Identification of the CB1 cannabinoid receptor and fatty acid amide hydrolase (FAAH) in the human placenta. *Placenta,* 24**,** 473-8.
PARK, B., MCPARTLAND, J. M. & GLASS, M. (2004) Cannabis, cannabinoids and reproduction. *Prostaglandins Leukot Essent Fatty Acids,* 70**,** 189-97.
PATTISON, N. S., CHAMLEY, L. W., MCKAY, E. J., LIGGINS, G. C. & BUTLER, W. S. (1993) Antiphospholipid antibodies in pregnancy: prevalence and clinical associations. *Br J Obstet Gynaecol,* 100**,** 909-13.
PERSAUD, T. V. & ELLINGTON, A. C. (1967) Cannabis in early pregnancy. *Lancet,* 2**,** 1306.
PERTWEE, R. G. (1997) Pharmacology of cannabinoid CB1 and CB2 receptors. *Pharmacol Ther,* 74**,** 129-80.
PERTWEE, R. G. (2005) Pharmacological actions of cannabinoids. *Handb Exp Pharmacol***,** 1-51.
PICCINNI, M. P., BELONI, L., LIVI, C., MAGGI, E., SCARSELLI, G. & ROMAGNANI, S. (1998) Defective production of both leukemia inhibitory factor and type 2 T-helper cytokines by decidual T cells in unexplained recurrent abortions. *Nat Med,* 4**,** 1020-4.
PICCINNI, M. P., GIUDIZI, M. G., BIAGIOTTI, R., BELONI, L., GIANNARINI, L., SAMPOGNARO, S., PARRONCHI, P., MANETTI, R., ANNUNZIATO, F., LIVI, C. & ET AL. (1995) Progesterone favors the development of human T helper cells producing Th2-type cytokines and promotes both IL-4 production

and membrane CD30 expression in established Th1 cell clones. *J Immunol,* 155**,** 128-33.
PICCINNI, M. P. & ROMAGNANI, S. (1996) Regulation of fetal allograft survival by a hormone-controlled Th1- and Th2-type cytokines. *Immunol Res,* 15**,** 141-50.
PIOMELLI, D., BELTRAMO, M., GLASNAPP, S., LIN, S. Y., GOUTOPOULOS, A., XIE, X. Q. & MAKRIYANNIS, A. (1999) Structural determinants for recognition and translocation by the anandamide transporter. *Proc Natl Acad Sci U S A,* 96**,** 5802-7.
PIOMELLI, D., GIUFFRIDA, A., CALIGNANO, A. & RODRIGUEZ DE FONSECA, F. (2000) The endocannabinoid system as a target for therapeutic drugs. *Trends Pharmacol Sci,* 21**,** 218-24.
POIKKEUS, P., HIILESMAA, V. & TIITINEN, A. (2002) Serum HCG 12 days after embryo transfer in predicting pregnancy outcome. *Hum Reprod,* 17**,** 1901-5.
POTDAR, N. & KONJE, J. C. (2005a) The endocrinological basis of recurrent miscarriages. *Current Opinion in Obstetrics & Gynecology,* 17**,** 424-8.
POTDAR, N. & KONJE, J. C. (2005b) The endocrinological basis of recurrent miscarriages. *Curr Opin Obstet Gynecol,* 17**,** 424-8.
PUFFENBARGER, R. A., KAPULINA, O., HOWELL, J. M. & DEUTSCH, D. G. (2001) Characterization of the 5'-sequence of the mouse fatty acid amide hydrolase. *Neurosci Lett,* 314**,** 21-4.
QUENBY, S. & FARQUHARSON, R. (2006) Uterine natural killer cells, implantation failure and recurrent miscarriage. *Reprod Biomed Online,* 13**,** 24-8.
RAKHSHAN, F., DAY, T. A., BLAKELY, R. D. & BARKER, E. L. (2000) Carrier-mediated uptake of the endogenous cannabinoid anandamide in RBL-2H3 cells. *J Pharmacol Exp Ther,* 292**,** 960-7.
RICHARDSON, D., ORTORI, C. A., CHAPMAN, V., KENDALL, D. A. & BARRETT, D. A. (2007) Quantitative profiling of endocannabinoids and related compounds in rat brain using liquid chromatography-tandem electrospray ionization mass spectrometry. *Anal Biochem,* 360**,** 216-26.
RINALDI-CARMONA, M., BARTH, F., HEAULME, M., SHIRE, D., CALANDRA, B., CONGY, C., MARTINEZ, S., MARUANI, J., NELIAT, G., CAPUT, D. & ET AL. (1994) SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Lett,* 350**,** 240-4.
ROMERO, J., DE MIGUEL, R., RAMOS, J. A. & FERNANDEZ-RUIZ, J. J. (1998) The activation of cannabinoid receptors in striatonigral GABAergic neurons inhibited GABA uptake. *Life Sci,* 62**,** 351-63.
ROSENKRANTZ, H., GRANT, R. J., FLEISCHMAN, R. W. & BAKER, J. R. (1986) Marihuana-induced embryotoxicity in the rabbit. *Fundam Appl Toxicol,* 7**,** 236-43.
ROTH SH (1979) The non-specific binding properties of delta 9-tetrahydrocannabinol and the effects of various solubilizers. *Journal of Pharmacology,* 31**,** 224-230.
RUEDA, D., GALVE-ROPERH, I., HARO, A. & GUZMAN, M. (2000) The CB(1) cannabinoid receptor is coupled to the activation of c-Jun N-terminal kinase. *Mol Pharmacol,* 58**,** 814-20.
RUGE, S., PEDERSEN, J. F., SORENSEN, S. & LANGE, A. P. (1990) Can pregnancy-associated plasma protein A (PAPP-A) predict the outcome of pregnancy in women with threatened abortion and confirmed fetal viability? *Acta Obstetricia et Gynecologica Scandinavica,* 69**,** 589-95.
RUSSO E (2005) *History of Cannabis as a Medicine*.

SASSENRATH, E. N., CHAPMAN, L. F. & GOO, G. P. (1978) Reproduction in rhesus monkeys chronically exposed to delta-9-tetrahydrocannabinol. *Adv Biosci,* 22-23**,** 501-12.
SAVITZ, D. A., HERTZ-PICCIOTTO, I., POOLE, C. & OLSHAN, A. F. (2002) Epidemiologic measures of the course and outcome of pregnancy. *Epidemiol Rev,* 24**,** 91-101.
SCHINDLER, A. E. (2005) Endocrinology of pregnancy: consequences for the diagnosis and treatment of pregnancy disorders. *Journal of Steroid Biochemistry & Molecular Biology,* 97**,** 386-8.
SCHMID, P. C., PARIA, B. C., KREBSBACH, R. J., SCHMID, H. H. & DEY, S. K. (1997a) Changes in anandamide levels in mouse uterus are associated with uterine receptivity for embryo implantation. *Proc Natl Acad Sci U S A,* 94**,** 4188-92.
SCHMID, P. C., PARIA, B. C., KREBSBACH, R. J., SCHMID, H. H. & DEY, S. K. (1997b) Changes in anandamide levels in mouse uterus are associated with uterine receptivity for embryo implantation. *Proceedings of the National Academy of Sciences of the United States of America,* 94**,** 4188-92.
SCHMIDT, A., BRUNE, K. & HINZ, B. (2006) Determination of the endocannabinoid anandamide in human plasma by high-performance liquid chromatography. *Biomed Chromatogr,* 20**,** 336-42.
SCHUEL, H., BURKMAN, L. J., LIPPES, J., CRICKARD, K., FORESTER, E., PIOMELLI, D. & GIUFFRIDA, A. (2002) N-Acylethanolamines in human reproductive fluids. *Chem Phys Lipids,* 121**,** 211-27.
SCHUEL, H., GOLDSTEIN, E., MECHOULAM, R., ZIMMERMAN, A. M. & ZIMMERMAN, S. (1994) Anandamide (arachidonylethanolamide), a brain cannabinoid receptor agonist, reduces sperm fertilizing capacity in sea urchins by inhibiting the acrosome reaction. *Proc Natl Acad Sci U S A,* 91**,** 7678-82.
SHARKEY, A. (1998) Cytokines and implantation. *Rev Reprod,* 3**,** 52-61.
SHERWOOD, R. A., KEATING, J., KAVVADIA, V., GREENOUGH, A. & PETERS, T. J. (1999) Substance misuse in early pregnancy and relationship to fetal outcome. *Eur J Pediatr,* 158**,** 488-92.
SMITH, P. B., COMPTON, D. R., WELCH, S. P., RAZDAN, R. K., MECHOULAM, R. & MARTIN, B. R. (1994) The pharmacological activity of anandamide, a putative endogenous cannabinoid, in mice. *J Pharmacol Exp Ther,* 270**,** 219-27.
SOTIRIADIS, A., PAPATHEODOROU, S. & MAKRYDIMAS, G. (2004) Threatened miscarriage: evaluation and management. *Bmj,* 329**,** 152-5.
STEFANO, G. B., SALZET, B., RIALAS, C. M., POPE, M., KUSTKA, A., NEENAN, K., PRYOR, S. & SALZET, M. (1997) Morphine- and anandamide-stimulated nitric oxide production inhibits presynaptic dopamine release. *Brain Res,* 763**,** 63-8.
STELLA, N., SCHWEITZER, P. & PIOMELLI, D. (1997) A second endogenous cannabinoid that modulates long-term potentiation. *Nature,* 388**,** 773-8.
SUGIURA-OGASAWARA, M., FURUKAWA, T. A., NAKANO, Y., HORI, S., AOKI, K. & KITAMURA, T. (2002) Depression as a potential causal factor in subsequent miscarriage in recurrent spontaneous aborters. *Hum Reprod,* 17**,** 2580-4.
SZEKERES-BARTHO, J., FAUST, Z., VARGA, P., SZEREDAY, L. & KELEMEN, K. (1996) The immunological pregnancy protective effect of progesterone is manifested via controlling cytokine production. *Am J Reprod Immunol,* 35**,** 348-51.

TAKATA, K., SUGANUMA, N., WADA, H., TAKEUCHI, K., KITAMURA, K. & KANAYAMA, N. (2005) Human chorionic gonadotropin in cervical fluid as a predictor of miscarriage. *Fertility & Sterility,* 84**,** 687-91.

TOMMASELLI, G. A., DI SPIEZIO SARDO, A., DI CARLO, C., BIFULCO, G., CERROTA, G., CIRILLO, D., GRECO, E. & NAPPI, C. (2006) Do serum leptin levels have a role in the prediction of pregnancy outcome in case of threatened miscarriage? *Clin Endocrinol (Oxf),* 65**,** 772-5.

TONG, S., MARJONO, B., BROWN, D. A., MULVEY, S., BREIT, S. N., MANUELPILLAI, U. & WALLACE, E. M. (2004a) Serum concentrations of macrophage inhibitory cytokine 1 (MIC 1) as a predictor of miscarriage. *Lancet,* 363**,** 129-30.

TONG, S., MARJONO, B., MULVEY, S. & WALLACE, E. M. (2004b) Low levels of pregnancy-associated plasma protein-A in asymptomatic women destined for miscarriage. *Fertility & Sterility,* 82**,** 1468-70.

TRABUCCO, E., ACONE, G., MARENNA, A., PIERANTONI, R., CACCIOLA, G., CHIOCCARELLI, T., MACKIE, K., FASANO, S., COLACURCI, N., MECCARIELLO, R., COBELLIS, G. & COBELLIS, L. (2009) Endocannabinoid system in first trimester placenta: low FAAH and high CB1 expression characterize spontaneous miscarriage. *Placenta,* 30**,** 516-22.

TSOU, K., BROWN, S., SANUDO-PENA, M. C., MACKIE, K. & WALKER, J. M. (1998) Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. *Neuroscience,* 83**,** 393-411.

VOGESER, M., HAUER, D., CHRISTINA AZAD, S., HUBER, E., STORR, M. & SCHELLING, G. (2006) Release of anandamide from blood cells. *Clin Chem Lab Med,* 44**,** 488-91.

VOGESER, M., HAUER, D., SCHMOELZ, M. & SCHELLING, G. (2004) Quantification of whole blood anandamide by isotope dilution liquid chromotography - tandem mass spectrometry. *Absract : Society for Neuroscience,* 695.

WALEH, N. S., CRAVATT, B. F., APTE-DESHPANDE, A., TERAO, A. & KILDUFF, T. S. (2002) Transcriptional regulation of the mouse fatty acid amide hydrolase gene. *Gene,* 291**,** 203-10.

WALLACE, E. M., MARJONO, B., TYZACK, K. & TONG, S. (2004a) First trimester levels of inhibins and activin A in normal and failing pregnancies. *Clin Endocrinol (Oxf),* 60**,** 484-90.

WALLACE, E. M., MARJONO, B., TYZACK, K. & TONG, S. (2004b) First trimester levels of inhibins and activin A in normal and failing pregnancies. *Clinical Endocrinology,* 60**,** 484-90.

WANG, H. & DEY, S. K. (2005) Lipid signaling in embryo implantation. *Prostaglandins Other Lipid Mediat,* 77**,** 84-102.

WANG, H., GUO, Y., WANG, D., KINGSLEY, P. J., MARNETT, L. J., DAS, S. K., DUBOIS, R. N. & DEY, S. K. (2004) Aberrant cannabinoid signaling impairs oviductal transport of embryos. *Nat Med,* 10**,** 1074-80.

WANG, H., MATSUMOTO, H., GUO, Y., PARIA, B. C., ROBERTS, R. L. & DEY, S. K. (2003) Differential G protein-coupled cannabinoid receptor signaling by anandamide directs blastocyst activation for implantation. *Proc Natl Acad Sci U S A,* 100**,** 14914-9.

WANG, H., XIE, H. & DEY, S. K. (2008) Loss of cannabinoid receptor CB1 induces preterm birth. *PLoS One,* 3**,** e3320.

WANG, J., PARIA, B. C., DEY, S. K. & ARMANT, D. R. (1999) Stage-specific excitation of cannabinoid receptor exhibits differential effects on mouse embryonic development. *Biol Reprod,* 60**,** 839-44.

WENGER, T., FRAGKAKIS, G., GIANNIKOU, P., PROBONAS, K. & YIANNIKAKIS, N. (1997) Effects of anandamide on gestation in pregnant rats. *Life Sci,* 60**,** 2361-71.

WENGER, T., TOTH, B. E., JUANEDA, C., LEONARDELLI, J. & TRAMU, G. (1999) The effects of cannabinoids on the regulation of reproduction. *Life Sci,* 65**,** 695-701.

WENGER, T., TOTH, B. E. & MARTIN, B. R. (1995) Effects of anandamide (endogen cannabinoid) on anterior pituitary hormone secretion in adult ovariectomized rats. *Life Sci,* 56**,** 2057-63.

WESTERGAARD, J. G., SINOSICH, M. J., BUGGE, M., MADSEN, L. T., TEISNER, B. & GRUDZINSKAS, J. G. (1983) Pregnancy-associated plasma protein A in the prediction of early pregnancy failure. *American Journal of Obstetrics & Gynecology,* 145**,** 67-9.

WILCOX, A. J., WEINBERG, C. R., O'CONNOR, J. F., BAIRD, D. D., SCHLATTERER, J. P., CANFIELD, R. E., ARMSTRONG, E. G. & NISULA, B. C. (1988) Incidence of early loss of pregnancy. *N Engl J Med,* 319**,** 189-94.

WILSON, R. I. & NICOLL, R. A. (2001) Endogenous cannabinoids mediate retrograde signalling at hippocampal synapses. *Nature,* 410**,** 588-92.

XIAO, A. Z., ZHAO, Y. G. & DUAN, E. K. (2002) Expression and regulation of the fatty acid amide hydrolase gene in the rat uterus during the estrous cycle and peri-implantation period. *Mol Hum Reprod,* 8**,** 651-8.

YOVICH, J. L., WILLCOX, D. L., GRUDZINSKAS, J. G. & BOLTON, A. E. (1986) The prognostic value of HCG, PAPP-A, oestradiol-17 beta and progesterone in early human pregnancy. *Australian & New Zealand Journal of Obstetrics & Gynaecology,* 26**,** 59-64.

ZIMMER, A., ZIMMER, A. M., HOHMANN, A. G., HERKENHAM, M. & BONNER, T. I. (1999) Increased mortality, hypoactivity, and hypoalgesia in cannabinoid CB1 receptor knockout mice. *Proc Natl Acad Sci U S A,* 96**,** 5780-5.

ZINAMAN, M. J., CLEGG, E. D., BROWN, C. C., O'CONNOR, J. & SELEVAN, S. G. (1996) Estimates of human fertility and pregnancy loss. *Fertil Steril,* 65**,** 503-9.

ZUCKERMAN, B., FRANK, D. A., HINGSON, R., AMARO, H., LEVENSON, S. M., KAYNE, H., PARKER, S., VINCI, R., ABOAGYE, K., FRIED, L. E. & ET AL. (1989) Effects of maternal marijuana and cocaine use on fetal growth. *N Engl J Med,* 320**,** 762-8.