

Cannabinoids and Reproduction

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I dedicate this thesis to the memory of my late father, Hisham. May his memory be a blessing.

To my wonderful wife, Jumana and our son Hisham Jnr.

To my mother Salwa, sisters May & Maha and brother Hani.

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Cannabinoids and Reproduction

Osama Habayeb

Abstract

The success of implantation depends on the synchronous development of the embryo and the endometrium. This process is now recognised to be regulated by the endocannabinoid system, the most widely studied of which is anandamide. Unfortunately while there is evidence from animals to confirm the role of anandamide in reproduction, there is very little on human pregnancy, despite the fact that as many as 30-50% of pregnancies are lost soon after fertilisation. One of the key constraints on studies in humans has been the technique of assaying anandamide levels in plasma.

The first stage of this study focused on the development of a robust reproducible assay to measure anandamide in human plasma based on a HPLC-MS technique and was used to measure anandamide concentrations during the menstrual cycle, in pregnancy and in labour. The findings suggested that anandamide concentrations are regulated by sex steroid hormones. The most striking finding, however, was a 3.7-fold increase in plasma anandamide concentrations of women in active labour.

The measurement of plasma anandamide concentrations in a group of women with threatened miscarriage with confirmed viable pregnancy before testing showed that plasma anandamide levels were elevated in those women who subsequently miscarried. This very exciting result from a single plasma anandamide measurement provided a sensitivity of 100% and a specificity of 94.4% with a negative predictive value of 100% and a positive predictive value of 81.8% for subsequent miscarriage. These findings suggest that anandamide plays a role in the maintenance of early pregnancy and possibly in labour.

Finally, to try and identify potential targets of anandamide action on first trimester placenta, the expression of the 2 known cannabinoid receptors (CB₁ and CB₂) and the enzyme FAAH was studied both at the RNA and protein levels in first trimester placentas. Both receptors and the enzyme were present in the tissues studied with the expression of the CB₁ diminishing after 9 weeks gestation and FAAH disappearing by 11 weeks with little changes in the expression of CB₂ with gestation. Similarly, anandamide inhibited the growth of BeWo cells (a first trimester trophoblast cell model) suggesting that elevated anandamide levels could adversely affect pregnancies through trophoblast growth inhibition. Taken together, these observations suggest that anandamide is involved in the regulation of early pregnancy maintenance.

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Abbreviations

2-AG	2-Arachidonoyl glycerol
ABC	Avidin biotin complex
AEA	Anandamide (<i>N</i> -Arachidonylethanolamide)
AM404	<i>N</i> -(4-hydroxy-phenyl) arachidonylamine
ATP	Adenosine triphosphate
BT	Biotinyl Tyramide
cAMP	Cyclic adenosine monophosphate
CB ₁	Cannabinoid receptor type 1
CB ₂	Cannabinoid receptor type 2
CB ₃	Cannabinoid receptor type 3
CD	Cluster of differentiation
COX-1	Cyclooxygenase enzyme type 1
COX-2	Cyclooxygenase enzyme type 2
CRL	Crown-rump length
CV	Co-efficient of variation
DAB	3, 3-diaminobenzamidine
Δ^9 -THC	Delta-9-tetrahydrocannabinol
DFP	Di-isopropyl fluorophosphates
DMDCS	Dimethyldichlorosilane
EDTA	Ethylene diamine tetra-acetic acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPCR	G _{iv} o-protein coupled receptor
EGF	Epidermal growth factor
ELISA	Enzyme-linked immuno-sorbent assay
EPAU	Early pregnancy assessment unit
FAAH	Fatty acid amide hydrolase
FAK	Focal adhesion kinase
FCS	Fetal calf serum
FSH	Follicle-stimulating hormone
GC-MS	Gas chromatography mass spectrometry
HCG	Human chorionic gonadotrophin hormone

HLA-G	Human leukocyte antigen-G
HPLC-MS	High performance liquid chromatography mass spectrometry
IF	Interferon
IL	Interleukin
IP ₃	Inositol triphosphate
IVF	In-vitro fertilisation
LIF	Leukaemia inhibitory factor
LH	Luteinising hormone
MAFP	Methyl arachidonoyl fluorophosphates
MAPK	Mitogen-activated protein kinase
MHC	Major- histocompatibility-complex
μM	Micro-molar
mM	Milli-molar
mRNA	Messenger ribonucleic acid
NAPE	<i>N</i> -Arachidonylethanolamide
NAPE-PLD	<i>N</i> -Acyl-phosphatidyl-ethanolamine selective phospholipase D
NAT	<i>N</i> -Acyltransferase
NAE	<i>N</i> -Acyl-ethanolamide
nM	Nano-molar
NMDA	<i>N</i> -methyl-D-aspartate
NO	Nitric acid
PBS	Phosphate buffered saline
PLD	Phospholipase-D
PMNCs	Peripheral mononuclear cells
PMSF	Phenylmethylsulfonyl fluoride
PPAR-δ	Peroxisome-proliferator-activated receptor- δ
PPAR-γ	Peroxisome-proliferator-activated receptor-γ
RT-PCR	Reverse transcriptase-polychain reaction
SA-HRP	Streptavidin-Horseradish Peroxidase
SD	Standard deviation
SEM	Standard error of the mean
SIM	Selected ion monitoring

TBS	Tris-buffered saline
TRPV1	Transient receptor potential vanilloid
TSA	Tyramide signal amplification
VEGF	Vascular endothelial growth factor
VR ₁	Vanilloid receptor type 1

Publications arising from this thesis

Papers

1. Habayeb OMH, Bell SC, Konje J.
Endogenous cannabinoids: Metabolism and their role in reproduction.
Life Sciences 2002; **70**: 1963-77.
2. Habayeb OMH, Taylor AHT, Evans ME, Cooke MS, Bell SC, Taylor DJ, Konje JC.
Changes in the plasma levels of the endogenous cannabis (endocannabinoid),
anandamide, in women of reproductive age, during gestation and in the menopause.
Journal of Clinical Endocrinology and Metabolism 2004; **89**: 5482-7.
3. Habayeb OMH, Taylor AH, Finney M, Evans MD, Konje JC.
Plasma anandamide concentration and pregnancy outcome in women with threatened
miscarriage.
Journal of the American Medical Association (JAMA) 2008; **299**: 1135-6.
4. Habayeb OMH, Taylor AH, Bell SC, Taylor DJ, Konje JC.
Expression of the endocannabinoid system in human first trimester placenta and its
role in trophoblast proliferation.
Endocrinology 2008; **149**: 5052-60.

Abstracts

1. Habayeb OMH, Taylor, AH; Bharkhada, RJ; Taylor, DJ; Bell, SC; Konje, JC.
Immunohistochemical localisation of cannabinoid receptors CB1 and fatty acid amide hydrolase (FAAH) in maternal and fetal tissues.
Book of abstracts, 30th British Congress of Obstetrics and Gynaecology 2004; Page 86.
2. Habayeb OMH, Taylor AHT, Evans ME, Cooke MS, Bell SC, Taylor DJ, Konje, JC.
Plasma concentrations of Anandamide, an endocannabinoid, during gestation.
Abstracts of the Blair Bell's competition meeting in December 2003. British Journal of Obstetrics and Gynaecology 2004; 111:1151-2.
3. Habayeb OMH, Taylor AHT, Evans ME, Cooke MS, Bell SC, Taylor DJ, Konje JC.
The development of a method to assay anandamide levels in stored plasma samples.
Book of abstracts, 2nd Society for Gynecologic Investigation International Summit on Reproductive Medicine 2007; Page 127.
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Expression of the endocannabinoid system in human first trimester placenta.
Reproductive Sciences 2008; 15 (2): 199A.
5. Anthony H Taylor, Osama MH Habayeb, Mark E Evans, Marcus S Cooke, Stephen C Bell, David J Taylor, Justin C Konje.
The outcome of pregnancies complicated by threatened miscarriage can be predicted by plasma anandamide concentrations.
Reproductive Sciences 2008; 15 (2): 165A.

Chapter 1

Introduction

Chapter 1

Introduction

1.1. Early pregnancy survival

Human reproduction entails a fundamental paradox, in that it is critical to the survival of the species, but at the same time the process is relatively inefficient with 40-50% of all conceptions failing to advance beyond 20 weeks of gestation, most of these being lost before 12 weeks (Norwitz *et al.*, 2001). For couples wishing to bear and raise children this can become frustrating and a difficult problem for the gynaecologist, who maybe ill-equipped to deal with such situations. It is thus clear that a better understanding of the factors involved in implantation and early pregnancy maintenance, and in the mechanisms that lead to early loss of a seemingly healthy pregnancy would improve our ability to diagnose and manage early pregnancy complications such as miscarriages.

1.1.1. Normal implantation

Most of the knowledge related to early human development is inferred from studies performed in laboratory animals and non-human primates, but as the cellular and molecular mechanisms involved in implantation vary between different species, the relevance of this information to humans is not always clear (Norwitz *et al.*, 2001). Nevertheless, certain important steps that have been identified in implantation and

placentation in eutherian mammals are probably applicable to human pregnancy (Norwitz *et al.*, 2001).

Pregnancy starts with the process of fertilisation, whereby male and female gametes fuse. In humans this normally occurs in the Fallopian tube (oviduct) 24 to 48 hours after ovulation. The initial development of the pregnancy occurs in the Fallopian tube with the fertilised ovum (zygote) developing into a mass of 12 to 16 cells (morula) by a process of mitosis (Norwitz *et al.*, 2001). Two to three days after fertilisation, the morula enters the uterine cavity. Next, the morula develops into the blastocyst by the establishment of a fluid filled cavity within the mass of cells. This is accompanied by a process of cellular differentiation with the outer cells (towards the surface) becoming the trophoblasts that ultimately develop into the extra-embryonic structures such as the placenta, while the inner cells develop into the embryo (Norwitz *et al.*, 2001). Up to this stage, the developing embryo is surrounded by the zona pellucida, a non-adhesive protective coating layer. Before the process of implantation can occur, the embryo needs to hatch from the zona pellucida. This process occurs some 5-6 days after fertilisation (conception) (Norwitz *et al.*, 2001) and the blastocyst is now able to implant into the wall of the uterus.

The process of implantation in humans commences 6-7 days after conception and is divided into three stages (Norwitz *et al.*, 2001). During the first stage, called “apposition”, the blastocyst aligns itself with microvilli on the apical surface of the syncytiotrophoblast inter-digitating with micro-protrusions from the apical surface of the uterine epithelium (pinopodes). The nature of this process is necessarily unstable to enable the blastocyst to find the most appropriate adhesion point for implantation

(Norwitz *et al.*, 2001). In the second stage, known as “stable adhesion”, there is increased physical interaction between the blastocyst and the uterine epithelium through a series of molecular interactions that are poorly understood (Norwitz *et al.*, 2001). In the third stage, known as “trophoblast invasion”, the syncytiotrophoblasts produce digestive enzymes that allow the trophoblasts to penetrate through the uterine epithelium with the embryonic pole orientated towards the uterine epithelium (Norwitz *et al.*, 2001). During the process of invasion the uterine epithelium is destroyed.

By the 10th day post conception, the blastocyst is completely embedded in the stromal tissue of the uterus, the uterine epithelium has re-grown to cover the site of implantation and the cytotrophoblasts stream out of the trophoblast layer and begin the process of endometrial invasion, which extends into the inner third of the myometrium (interstitial invasion) and the uterine vasculature (endovascular invasion). These processes enable the trophoblasts to come into direct contact with maternal blood (Figures 1.1 & 1.2) (Norwitz *et al.*, 2001). As can be seen in the figures, several chemical mediators are involved in these processes including prostaglandins, leukaemia inhibitory factor (LIF) and epidermal growth factors. Successful implantation is the end result of the complex molecular interactions that occur between the blastocyst and the hormone-primed uterus. Failure to synchronise the component processes involved in these interactions with a receptive uterus results in failed implantation and early pregnancy loss (Norwitz *et al.*, 2001).

1.1.2. Uterine receptivity

Uterine receptivity is defined as the state during the period of endometrial maturation

during which the blastocyst can achieve implantation (Psychoyos, 1986). From assisted reproductive experiments, the optimal time (window of implantation) has been found to be between days 20 and 24 in a regular 28 day menstrual cycle (Bergh & Navot, 1992). The features of uterine receptivity include histological changes with the endometrium becoming more vascular and oedematous and the endometrial glands displaying enhanced secretory activity with the development of pinopodes on the luminal surface of the epithelium (Norwitz *et al.*, 2001).

Multiple signals synchronise the development of the blastocyst and the preparation of the uterus (Norwitz *et al.*, 2001). Among these factors are sex steroid hormones. Implantation requires (a) a pre-ovulatory surge in 17β -estradiol, that stimulate the proliferation and differentiation of uterine epithelial cells and (b) progesterone produced from the corpus luteum after ovulation, which stimulates the proliferation and differentiation of stromal cells (Norwitz *et al.*, 2001). Several factors have been identified as potential markers of endometrial receptivity (Norwitz *et al.*, 2001). For example, the levels of LIF in both the luminal and glandular epithelium of the uterus rise dramatically in the mid-secretory phase of the menstrual cycle (Cullinan *et al.*, 1996) and decreased secretion of LIF has been found to be associated with recurrent pregnancy loss (Chaouat *et al.*, 1995). Other molecules that are involved in the regulation of endometrial receptivity include adhesion molecules (Lessey, 1998) and mucins which are proteins that have high sugar content and cause an increase in the expression of oligosaccharide receptors on the surface of the uterine epithelial cells (Lagow *et al.*, 1999).

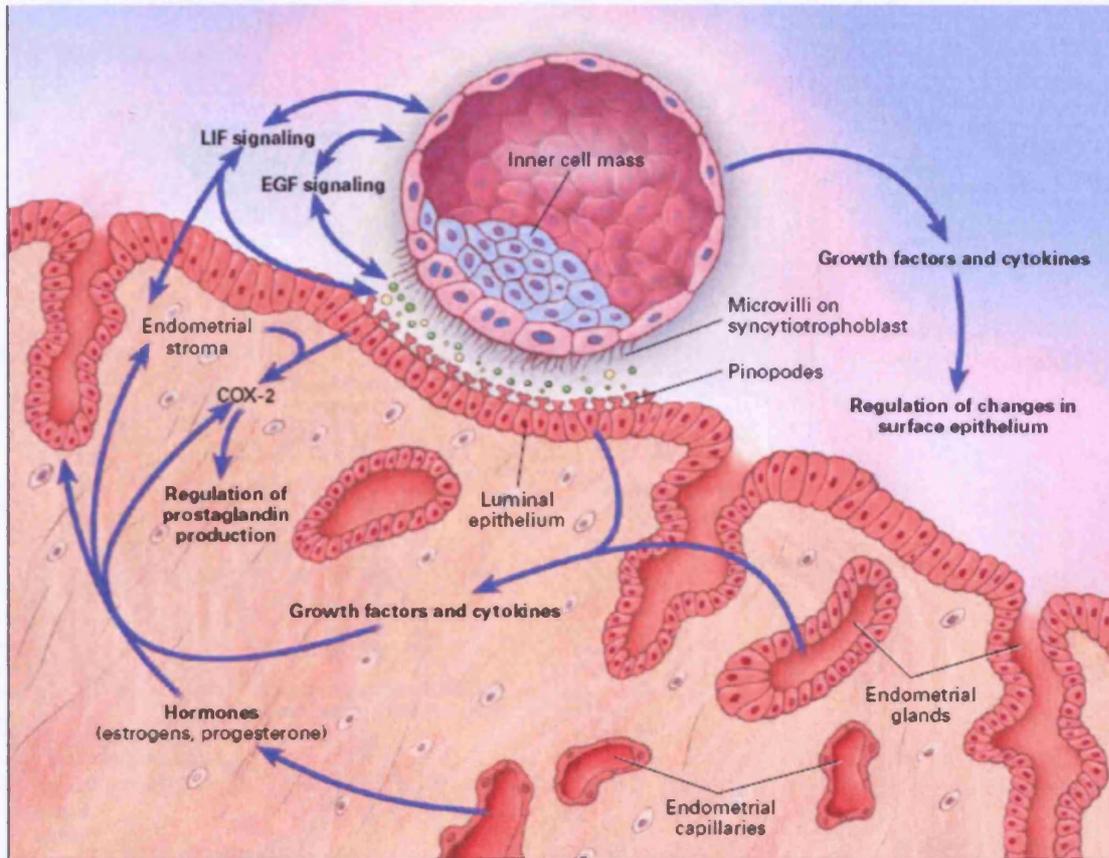


Figure 1.1: Blastocyst apposition and adhesion.

The diagram shows a peri-implantation blastocyst 6-7 days after conception. These processes are thought to be necessary for uterine receptivity and blastocyst apposition and adhesion. COX-2: cyclooxygenase-2; EGF: epidermal growth factor; LIF (leukaemia inhibitory factor) are some of chemical mediators of these processes. From: (Norwitz *et al.*, 2001).

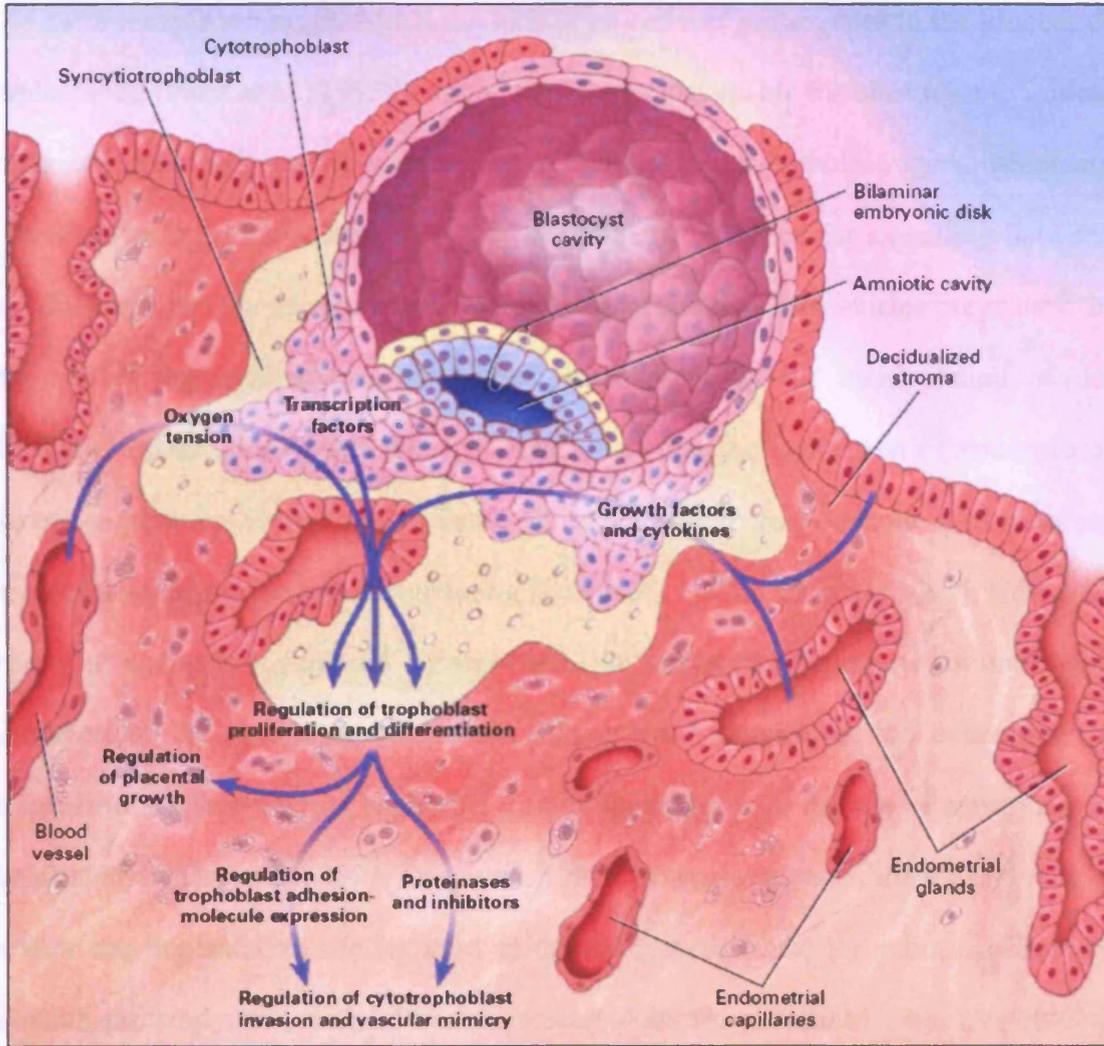


Figure 1.2: The diagram shows an invading blastocyst (9-10 days after conception) and the various processes necessary for trophoblast invasion. From: (Norwitz *et al.*, 2001).

The current evidence suggests that the blastocyst actively participates in the process of implantation (Paria *et al.*, 1993). Among the factors that enable the blastocyst to initiate implantation through a process known as “activation”, are catecholestrogens, which are a class of estrogen metabolites (Paria *et al.*, 1998a). Evidence for signalling between the blastocyst and the uterus is derived from animal studies in the murine pregnancy. In the mouse, implantation can be delayed indefinitely by the manipulation of the hormonal milieu. During this delay, it was found that the expression of endometrial heparin-binding epidermal growth factor genes does not increase despite the blastocyst being positioned next to the uterine lining (Das *et al.*, 1994; Das *et al.*, 1997). However, when the estrogen is replaced by injection, the implantation process resumes with activation of the blastocyst associated with a rapid increase in the expression of endometrial heparin-binding epidermal growth factor genes at the site of apposition of the blastocyst (Das *et al.*, 1994; Das *et al.*, 1997). To complete this process, embryos at or near the implantation site, express epidermal growth factor receptors and heparin sulphate proteoglycans, both of which interact with the endometrial heparin-binding epidermal growth factor-like ligands. Indeed, addition of heparin-binding epidermal growth factors to cultured embryos stimulates their proliferation and maturation (Das *et al.*, 1994; Wang *et al.*, 2000). It is considered that these findings are directly applicable to human pregnancy, as the addition of heparin-binding epidermal growth factor has similar effects on the human embryo *in-vitro* (Martin *et al.*, 1998). Thus, the interaction between an activated blastocyst and a receptive uterus is part of a complex process that leads to successful implantation (Norwitz *et al.*, 2001).

1.1.3. Regulation of implantation

Many of the regulatory mechanisms that have been identified appear to control multiple steps in the process of implantation. For example, LIF appears to be important for both decidualisation and implantation (Stewart *et al.*, 1992; Cullinan *et al.*, 1996). LIF is produced by both uterine glandular epithelial cells before implantation and stromal cells surrounding the active blastocyst at the time of implantation (Song *et al.*, 2000). The synthesis of prostaglandins is also essential for the success of implantation. Cyclooxygenase, the rate limiting enzyme in the synthesis of prostaglandin E₂, has two isoforms: a constitutive (COX-1) and an inducible (COX-2) isoform. Estrogen and progesterone reduce the production of COX-1 in the endometrium; hence COX-1 level is decreased in the mid-luteal phase of the menstrual cycle (coinciding with the period of increased uterine receptivity) in anticipation of implantation (Norwitz *et al.*, 2001). On the other hand, COX-2 production, which is not affected by sex steroid hormones, is restricted to the site of implantation and depends on the presence of a blastocyst that is ready for implantation for its induction (Lim *et al.*, 1997; Marions & Danielsson, 1999). In addition, interleukin-1, detected in the medium in which human embryos have been cultured (Sheth *et al.*, 1991), induces the expression of COX-2 in stromal endometrial cells in culture (Huang *et al.*, 1998). Prostaglandin I₂ (prostacyclin), produced by the actions of COX-2 is a ligand for the nuclear receptor peroxisome proliferator activated receptor δ (PPAR δ) (Lim *et al.*, 1999), which appears to be critical for implantation, as fetal mice lacking a related receptor (PPAR γ) die in the middle of the gestational period because of defective placentation (Barak *et al.*, 1999). The exact molecular mechanisms involved in the regulation of the trophoblast of the endometrium are poorly understood (Norwitz *et al.*, 2001). The temporal and spatial

expression of several growth factors and cytokines within the uterus including LIF (Cullinan *et al.*, 1996), interleukin-1 and its receptors (Simon *et al.*, 1996), insulin-like growth factors I and II and their binding proteins (Giudice & Irwin, 1999), colony stimulating factor 1 (Cohen *et al.*, 1997) and transforming growth factors α and β (Slowey *et al.*, 1994; Godkin & Dore, 1998) suggests that they all may have important functional roles in the regulation of cytotrophoblast invasion. Physiological factors may also be important, for example oxygen tension promotes some aspects of trophoblast differentiation including the production of integrin $\alpha_1\beta_1$ (Genbacev *et al.*, 2001). The exact roles of these various factors are yet to be elucidated.

1.2. Early pregnancy maintenance

The incidence of pregnancy loss after implantation (miscarriage) is higher than most people believe and has been estimated at anything between 25 and 40% (Wilcox *et al.*, 1988). Although many miscarriages involve genetic abnormalities (Simpson, 1980), there is often no known cause (Norwitz *et al.*, 2001).

Many factors play important roles in the maintenance of early pregnancy although considering the complexity of implantation it is likely that many, as yet undetermined, factors will be shown to play important roles in pregnancy maintenance (Norwitz *et al.*, 2001). After more than 50 years of research, it has become clear that ovarian steroid hormones are involved in implantation and early pregnancy success (Norwitz *et al.*, 2001).

1.2.1. Steroid hormones and pregnancy failure

The finding that progesterone-receptor antagonists readily induce miscarriage if given before seven weeks of gestation (Peyron *et al.*, 1993) and that surgical removal of the corpus luteum, the source of progesterone in early pregnancy, may result in miscarriage (Csapo & Pulkkinen, 1978) suggest that adequate progesterone production from the corpus luteum is critical to the maintenance of early pregnancy until the trophoblast takes over this function at seven to nine weeks of gestation (Norwitz *et al.*, 2001). The corpus luteum is maintained during the early pregnancy by the actions of the human chorionic gonadotrophin hormone (HCG), which is produced by the trophoblast (Norwitz *et al.*, 2001). The mode of action of progesterone in early pregnancy is not exactly defined, but it appears to be partially independent of the interaction with either progesterone or glucocorticoid receptors (Schust *et al.*, 1996). Studies in pregnant women with spontaneous mutations in genes that encode steroidogenic enzymes and or hormone receptors indicate that other hormones are important in this process (Miller, 1998). Estrogen, mineralocorticoids and androgens do not appear to play any roles in early pregnancy maintenance although the potential role of glucocorticoids is not known (Norwitz *et al.*, 2001).

1.2.2. Prostaglandins

The concentrations of prostaglandins in the human decidua in early pregnancy are lower than those in the endometrium at any stage of the normal menstrual cycle (Maathuis & Kelly, 1978; Abel *et al.*, 1980), essentially because of reduced prostaglandin synthesis (Norwitz & Wilson, 2000). On the other hand, the

administration of exogenous prostaglandins induces miscarriages in all species studied (Norwitz *et al.*, 2001). These data suggest that pregnancy is maintained by a mechanism that continuously suppresses uterine prostaglandin production (Norwitz *et al.*, 2001). Moreover, a defect in this inhibitory mechanism has been speculated to be associated with miscarriage (Abel *et al.*, 1980; Jaschevatzky *et al.*, 1983). It seems likely that systemic rather than local factors are involved in maintaining a low level of endometrial prostaglandins as evidenced by the observation that ectopic pregnancy is also associated with reduced endometrial prostaglandin production (Abel *et al.*, 1980). What those factors might be is the subject of intense research, but as yet not defined.

1.2.3. Immunological factors

One of the most important functions of the placenta is to regulate the maternal immune response so that the fetal semi-allograft is tolerated during pregnancy (Norwitz *et al.*, 2001). Trophoblasts are essential to this phenomenon as they lie within the maternal-fetal interface; hence they are in direct contact with the maternal immune cells (Norwitz *et al.*, 2001). Two factors are considered to be critical indicators that immune suppression is crucial to early pregnancy success. Firstly, trophoblasts do not express classic major-histocompatibility-complex (MHC) class II molecules (Redman, 1983), but instead increase their expression of human leukocyte antigen-G (HLA-G), an MHC class Ib molecule, as they invade the uterus (Kovats *et al.*, 1990). This observation coupled with the fact that HLA-G exhibits little polymorphism (Bainbridge *et al.*, 1999), suggest that HLA-G plays a functional role in this process (Norwitz *et al.*, 2001). Secondly, maternal lymphocytes, which constitute at least 10-15 percent of all cells in the decidua (Starkey *et al.*, 1988; King *et al.*, 1998) are almost exclusively

CD56⁺ natural killer cells that uniquely have low cytotoxic activity compared to peripheral blood lymphocytes taken from the same woman (Deniz *et al.*, 1994). An interesting observation is that trophoblasts appear to recruit these unusual cells by means of chemokine production (Drake *et al.*, 2001). Therefore, cytotoxicity against semi-allogeneic trophoblasts must be selectively inhibited to maintain the pregnancy (Norwitz *et al.*, 2001). The exact mechanism of this immunosuppression remains unknown but it is thought that cytotrophoblast-derived interleukin-10, a cytokine that suppresses alloimmune responses in mixed lymphocyte preparations (Roth *et al.*, 1996) may play a role.

Progesterone is also implicated in suppressing the alloimmune responses (Pavia *et al.*, 1979) as is the complement system, as deletion of a complement regulator (Crry) in mice leads to fetal loss secondary to placental inflammation (Xu *et al.*, 2000). Figure 1.3 summarises the current understanding of the processes involved in the maintenance of early pregnancy. As can be seen in this figure, progesterone is involved in the regulation of prostaglandin production and the facilitation of immune tolerance. The trophoblasts also actively participate in the regulation of immune tolerance and the process of angiogenesis. Thus the maintenance of early pregnancy is a complex process and an increasing understanding of this process means the continuous inclusion of more molecules and the description of alternate mechanisms. One of this new class of molecules implicated as being key to implantation and pregnancy success are the cannabinoids.

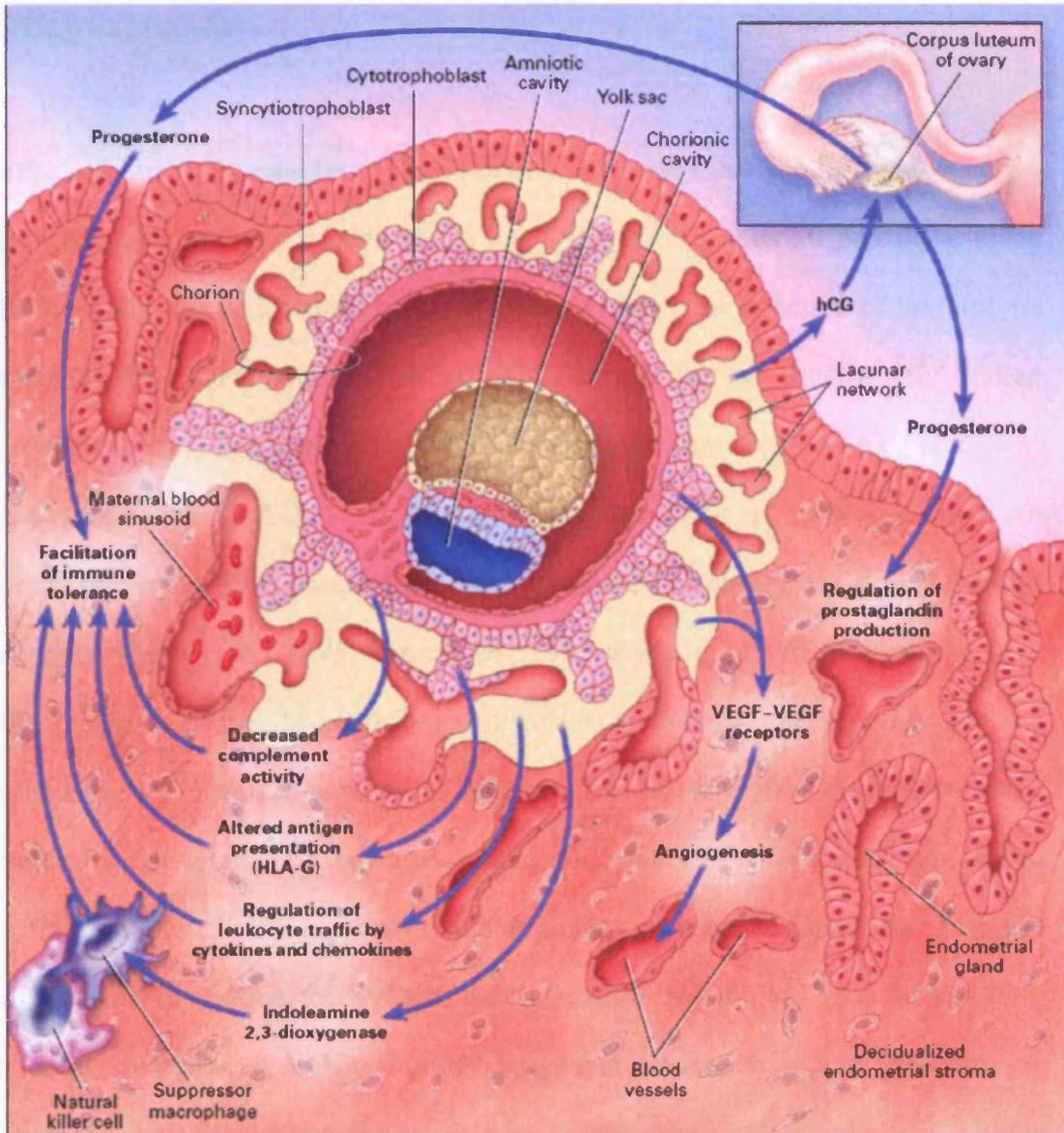
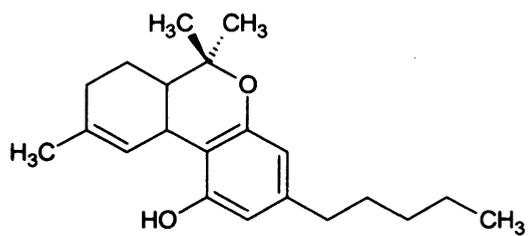


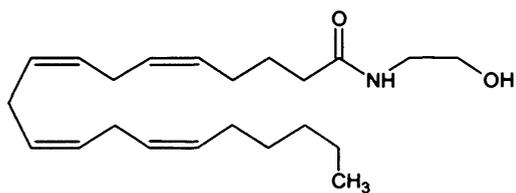
Figure 1.3: The diagram shows an implanted embryo (14 days after conception) and the processes necessary for the maintenance of early pregnancy. VEGF: vascular endothelial growth factor; hCG: human chorionic gonadotrophin hormone; HLA-G: human leukocyte antigen G. From: (Norwitz *et al.*, 2001).

1.3. Cannabinoids

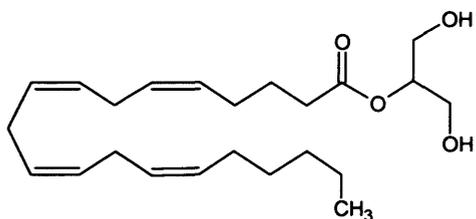
Cannabinoids are a diverse class of lipid soluble molecules that can be divided to two major classes; those made outside of the body, the exocannabinoids and those made within the body, the endocannabinoids. Figure 1.4 shows the structure of the prototype exocannabinoid Δ^9 tetrahydrocannabinol (Δ^9 -THC) and the two most widely studied endocannabinoids *N*-arachidonylethanolamide (anandamide; AEA) and 2-arachidonylglycerol (2-AG). The evidence that this class of molecules play an important role in pregnancy implantation and maintenance comes from several animals experiments (Paria *et al.*, 1995; Paria *et al.*, 1996; Paria *et al.*, 1998b; Paria & Dey, 2000) and human studies (Maccarrone *et al.*, 2000d). In rodents (mice) for example, the endocannabinoid, anandamide plays an important role in early pregnancy maintenance by regulating the window of implantation and the number of embryos in each uterine horn (Paria & Dey, 2000). Although similar studies on anandamide (AEA) have not been performed in humans, the levels of the enzyme that metabolises anandamide, fatty acid amide hydrolase (FAAH), in early pregnancy appear to predict miscarriages (Maccarrone *et al.*, 2000d) suggesting that plasma AEA has a critical role to play in the prevention of ongoing pregnancy in women. These roles will be reviewed in detail in Section 1.5.



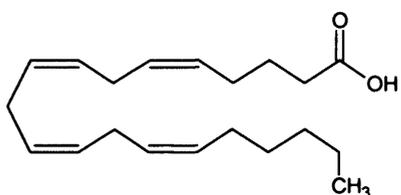
Δ^9 -Tetrahydrocannabinol (THC)



N-arachidonyl ethanolamide
(Anandamide; AEA)



2-arachidonyl glycerol (2-AG)



Arachidonic acid

Figure 1.4: Structures of Δ^9 -THC, AEA and 2-AG. Note that both AEA and 2-AG are derivatives of arachidonic acid (structure shown).

1.3.1. Exocannabinoids

The most studied source of exocannabinoids is the marijuana plant *Cannabis Sativa*. Marijuana has been used as a traditional medicine and a pleasure inducing drug for thousands of years in various parts of the world. The major active component of marijuana is Δ^9 -tetrahydrocannabinol (Δ^9 -THC; Figure 1.4) (Gaoni & Mechoulam, 1964). Exocannabinoids have significant effects on reproduction and pregnancy. For example, marijuana smoking during pregnancy is significantly associated with an increased risk of preterm labour (Gibson *et al.*, 1983) and intrauterine growth restriction (evidenced by reduction of birth weight, height, head circumference, mean arm muscle circumference and non-fat area of the arm (Zuckerman *et al.*, 1989; Frank *et al.*, 1990; Sherwood *et al.*, 1999). All of these 'obstetrical' problems probably indicate that inappropriate action of the endocannabinoids might cause the same problems in susceptible women. Acute marijuana smoking suppresses plasma luteinising hormone (LH) in the luteal phase and in chronic users, it shortens the menstrual cycle, the effect being predominately a short luteal phase leading to menstrual irregularities and anovulation (Mendelson *et al.*, 1986).

The mechanism by which marijuana and Δ^9 -THC exhibited their effects remained obscured until the 1980s when the first cannabinoid receptor (cannabinoid receptor 1) was discovered (Devane *et al.*, 1988). Following this discovery, a search for other receptor isoforms and the endogenous compounds that act as ligands for these receptors was initiated, as it was unlikely that mammalian species developed these receptors purely for the effects of the exocannabinoid marijuana.

1.3.2 Endocannabinoids

In 1992, the first compound that was shown to act as an endogenous ligand for the cannabinoid receptor was discovered as an isolate from porcine brain, and was named anandamide (*N*-arachidonylethanolamide; AEA; Figure 1.4) (Devane *et al.*, 1992) from the Sanskrit word “ananda” meaning bliss (Devane *et al.*, 1992). Since then a number of fatty acid derivatives that act as ligands for the cannabinoid receptors have been discovered, including 2-arachidonylglycerol (2-AG; Figure 4) (Mechoulam *et al.*, 1995), *N*-dihomo- γ -linolenylethanolamide (Hanus *et al.*, 1993), *N*-docosatetraenylethanolamide (Hanus *et al.*, 1993), *N*-arachidonyldopamine (Bisogno *et al.*, 2000) and 2-arachidonoyl glyceryl ether (noladin ether) (Hanus *et al.*, 2001) (Figure 1.5). Collectively, this group of unsaturated fatty acid derivatives has been named endogenous cannabinoids or endocannabinoids and because it was discovered first, anandamide is the most extensively studied member of this group.

1.4. The endocannabinoid, arachidonylethanolamide (anandamide)

Anandamide is similar to Δ^9 -THC in that it exerts several effects on the nervous system, through interactions and modulation at the level of neurotransmitters in different regions of the nervous system (Di Marzo *et al.*, 1998c). In rodents for example, it inhibits memory consolidation (Castellano *et al.*, 1997), exhibits analgesic properties (Mechoulam *et al.*, 1995), and inhibits motor coordination (Di Marzo *et al.*, 1998c) and anxiety like responses (Navarro *et al.*, 1997) and decreases arousal (Santucci *et al.*, 1996). All these effects suggest that it might act as a “stress recovery-

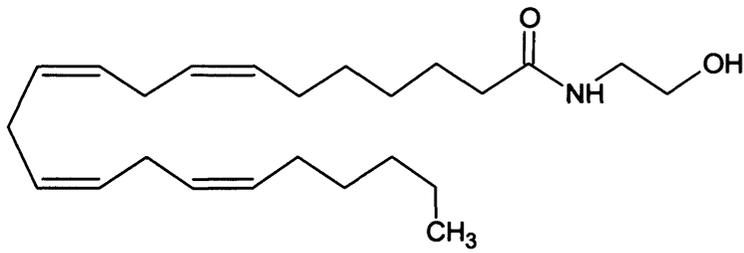
factor” relieving some of the stress-induced responses and sending messages such as “relax, eat, sleep, forget and protect” (Di Marzo *et al.*, 1998c).

Anandamide’s action is not only confined to the brain as it has several effects on many other biological systems. For example, in the vascular system AEA affects the tone of blood vessels leading to alteration in the blood flow (Hillard, 2000). In the immune system, AEA acts as an immunomodulator (Berdyshev, 2000) resulting in regulation of immune responses and anandamide also plays an important role in apoptosis (Maccarrone & Finazzi-Agro, 2003) and has been shown to inhibit the proliferation of cancer cells *in-vitro* (De Petrocellis *et al.*, 1998).

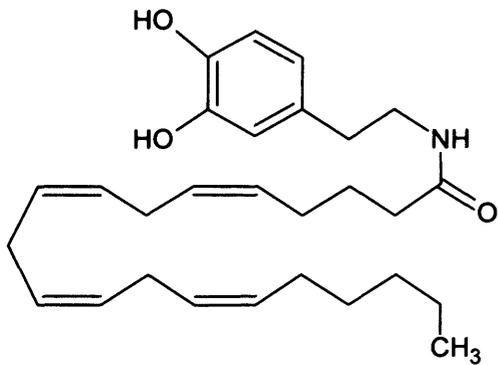
There is thus extensive ongoing research interest into anandamide and its actions because of its widespread effects and there is relatively extensive literature on its exact roles in mammalian systems. There is however, a less than clear evidence of the precise role of the action of endocannabinoids in human reproduction, especially in light of the evidence from animal studies. This thesis, therefore, was designed to focus on the potential role of anandamide, its receptors and the enzyme that apparently regulates its levels, in human reproduction.

1.4.1. Tissue distribution of anandamide

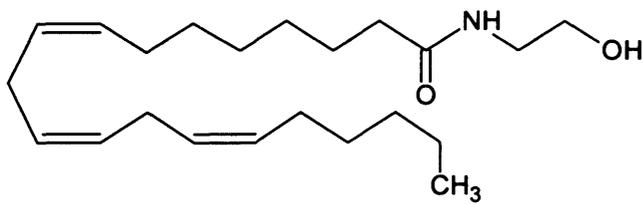
In the human, anandamide has been isolated from the brain, spleen, heart (Felder *et al.*, 1996), plasma (Maccarrone *et al.*, 2002b), seminal fluid, mid-cycle oviductal fluid, follicular fluid, breast secretions (Schuel *et al.*, 2002a) and breast cancer cells (Bisogno *et al.*, 1998a). Its distribution appears to be more ubiquitous in the rat where it has been



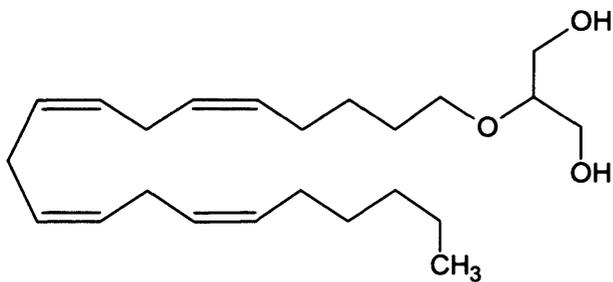
N-docosatetraenylethanolamide



N-arachidonoyl dopamine (NADA)



N-dihomo- γ -linolenylethanolamide



Noladin ether (2-AG ether)

Figure 1.5: Structures of other less studied endocannabinoids.

found in phaeochromocytoma cells (Bisogno *et al.*, 1998a), the kidneys (Deutsch *et al.*, 1997a), brain (Felder *et al.*, 1996; Koga *et al.*, 1997), testicles (Sugiura *et al.*, 1996c), spleen, skin (Felder *et al.*, 1996) and plasma (Giuffrida *et al.*, 2000). It has also been found in the bovine and porcine brains (Schmid *et al.*, 1995) and the murine uterus (Schmid *et al.*, 1997).

The highest concentrations of anandamide in the rat brain are found in the hippocampus and striatum (Felder *et al.*, 1996). The concentration of anandamide in these various tissues ranges from 5 to 150 pmol/g tissue (Di Marzo, 1998). In both the human and the murine brain, the highest concentrations in the brain are in the hippocampus (148 and 29 pmol/g tissue respectively) (Felder *et al.*, 1996). However, in relation to human reproduction, even though there is no direct evidence, the observations that the highest anandamide tissue concentrations ever found were in the murine uterus (20 nmol/g tissue) (Schmid *et al.*, 1997) suggest that AEA is critical to reproduction success. The evidence that fatty acid amide hydrolase may regulate plasma AEA levels in the human suggest that factors that control AEA destruction (Maccarrone *et al.*, 2000d) and production could be the key to reproductive success in the human.

1.4.2. Metabolism of anandamide

The effects of anandamide are dependent on the change of its steady-state levels, which in turn are dependent on the rate of production (i.e. synthesis) and its rate of destruction (i.e. degradation).

1.4.2.1. Synthesis of anandamide

Anandamide is produced on demand and released from the cells when the need arises (i.e. it is not stored in cells) (Piomelli *et al.*, 2000) and it appears to be produced in response to several physiological and pathological stimuli such as neuronal depolarization and bacterial lipopolysaccharides (Di Marzo *et al.*, 1998c). Two mechanisms for the synthesis of *N*-acyl ethanolamides (NAEs) including anandamide have been described (Schmid *et al.*, 1990). The first is direct, energy free condensation between a fatty acid and ethanolamine (*de novo* synthesis) and the second is phospholipase D-catalyzed hydrolysis of *N*-acyl-phosphatidylethanolamines. Both of these mechanisms have been studied in the case of anandamide (Di Marzo, 1998) (Figure 1.6). The first mechanism, *de novo* synthesis, has been described in mammalian brain particulate fractions independently of adenosine triphosphate (ATP) in the presence of micro-molar (μM) concentrations of arachidonic acid and milli-molar (mM) concentrations of ethanolamine (Kruszka & Gross, 1994). The enzyme catalyzing this reaction was named “anandamide synthase” (Kruszka & Gross, 1994). Several observations suggest that this mechanism plays a negligible role in the production of anandamide; firstly, the cellular concentrations of both arachidonic acid and ethanolamine are much lower than those necessary for the enzyme activity (Di Marzo, 1998), secondly, the enzyme has never been fully characterized (Di Marzo *et al.*, 1998c) and thirdly, this mechanism has never been demonstrated in living cells (Di Marzo *et al.*, 1998c). The main mechanism for the synthesis of anandamide is the hydrolysis of the cellular membrane precursor. In this mechanism, the anandamide precursor, *N*-arachidonylphosphatidylethanolamine (NAPE), is hydrolyzed by the enzyme *N*-acylphosphatidylethanolamine-selective phospholipase-D (NAPE-PLD)

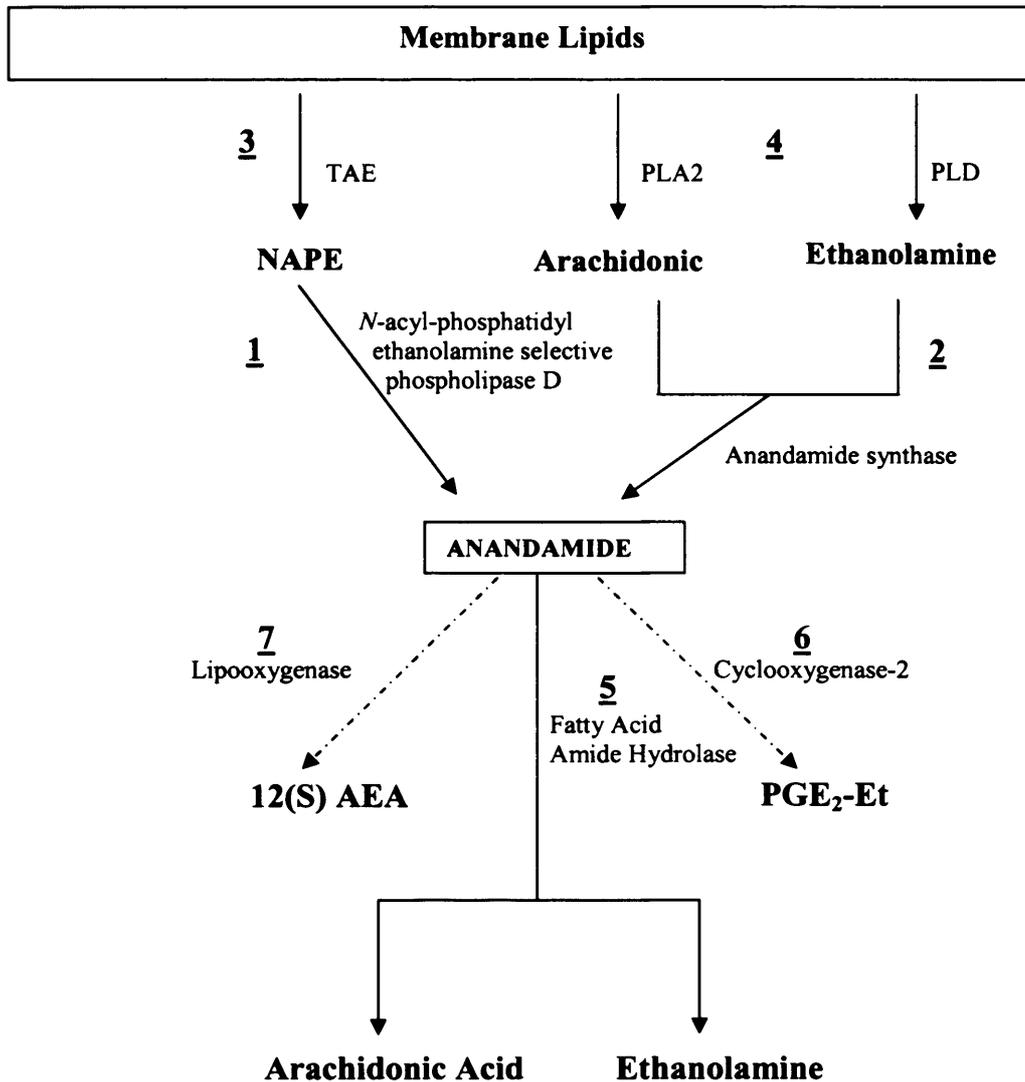


Figure 1.6: Metabolism of Anandamide. 1) Anandamide production by NAPE-PLD from *N*-arachidonylphosphatidylethanolamine. 2) Anandamide production by Anandamide Synthase. 3) *N*-arachidonylphosphatidylethanolamine is produced by the activity of transacylase enzyme. 4) Production of arachidonic acid and ethanolamine from membrane lipid precursors. 5) Degradation of anandamide by fatty acid amide hydrolase. 6) Degradation of anandamide by cyclooxygenase-2 enzyme. 7) Degradation of anandamide by lipooxygenase enzyme. Abbreviations; TAE: Transacylase enzyme; PLA-2: Phospholipase-2 enzyme; PLD: Phospholipase D enzyme; NAPE: (*N*-arachidonylphosphatidylethanolamine); PGE2-Et: Prostaglandin E2-ethanolamine; 12 (S) AEA: (12(S)-hydroxy-arachidonylathanolamide). From: (Habayeb *et al.*, 2002).

(Di Marzo *et al.*, 1994; Sugiura *et al.*, 1996c), a process reported to be initiated by calcium ion influx into the cell and has been shown to occur in several mammalian tissues including intact rat neurons (Di Marzo *et al.*, 1994) and rat testes (Sugiura *et al.*, 1996c). The precursor NAPE is produced in cell membranes by the activity of a transacylase enzyme called *N*-acyltransferase (NAT). This enzyme transfers an arachidonic acid group from the *sn*-1 position of phosphatidylcholine to the head group of phosphatidylethanolamine resulting in the formation of NAPE (Di Marzo *et al.*, 1994; Sugiura *et al.*, 1996b; Cadas *et al.*, 1997). Figure 1.6 shows the different stages of this complex process. Two intracellular second messengers control NAT activity: Ca^{2+} and cyclic adenosine monophosphate (cAMP). NAT is Ca^{2+} -dependent and the enzyme is inactive in the absence of Ca^{2+} , whereas cAMP works through protein kinase A-dependent phosphorylation to enhance NAT activity (Cadas *et al.*, 1996). The precursor/product relationship between NAPE and anandamide has been supported by the findings of a similar distribution of the two compounds in different areas of the brain (Bisogno *et al.*, 1999) and the fact that both NAPE and anandamide levels increase correspondingly in the brain during its development (Berrendero *et al.*, 1999). Recent evidence in the murine model suggests that NAPE-PLD is generated in the endometrium and thus may have a role to play in the generation of AEA in the implanting or pregnant uterus (Guo *et al.*, 2005).

1.4.2.2. Release and uptake of anandamide

Once synthesized, anandamide is released from the cell into the extra-cellular space where it has a short half-life ranging from 2.5 and 7 minutes depending on the system studied (Di Marzo, 1998) and where it acts in either an autocrine or paracrine manner

(Piomelli *et al.*, 2000). The exact mechanisms involved in the release of anandamide are still unknown (Piomelli *et al.*, 2000) but as it is a lipophilic molecule, AEA is thought to diffuse freely across the cell membrane by a process of simple diffusion (Di Marzo, 1998).

The main mechanism for internalization of AEA appears to be via a process of facilitated diffusion, using a selective, saturable, temperature-dependent and Na⁺-independent protein known as the “anandamide membrane transporter” (AMT) (Di Marzo *et al.*, 1994; Beltramo *et al.*, 1997; Bisogno *et al.*, 1997; Hillard *et al.*, 1997; Maccarrone *et al.*, 1998; Piomelli *et al.*, 1999; Maccarrone *et al.*, 2000a). This transporter is yet to be identified and fully characterized, but once the AEA is internalized, it has been suggested that intracellular degradation of anandamide by the enzyme, fatty acid amide hydrolase (FAAH), is sufficient to drive the facilitated diffusion of anandamide across the cell membrane (Deutsch *et al.*, 2001). However, several observations support the presence of a distinctive transport mechanism not related to the enzymatic degradation of anandamide (Beltramo *et al.*, 1997; Maccarrone *et al.*, 2000a; Maccarrone *et al.*, 2001a). For example, several compounds such as *N*-(4-hydroxy-phenyl) arachidonoylamine (AM404) inhibit anandamide uptake without inhibiting FAAH activity (Beltramo *et al.*, 1997). Furthermore, lipopolysaccharides inhibit FAAH expression without affecting anandamide uptake (Maccarrone *et al.*, 2001a) while nitric oxide, peroxynitrite, and superoxide anions stimulate anandamide uptake without affecting FAAH activity (Maccarrone *et al.*, 2000a). However, until the proposed transporter is characterized, the debate about the mechanisms involved in the uptake of anandamide will continue.

1.4.2.3. Degradation of Anandamide

Fatty acid amide hydrolase (FAAH) is the main enzyme responsible for degradation of AEA, breaking it down to arachidonic acid and ethanolamine (Cravatt *et al.*, 1996). In addition, two other mechanisms for the degradation of AEA have been described. The first is mediated by the COX-2 enzyme to produce prostaglandin E₂-ethanolamide (Yu *et al.*, 1997) and the second is via lipoxygenase to form 12 (S)-hydroxy-arachidonylethanolamine (Figure 1.6) (Hampson *et al.*, 1995; Ueda *et al.*, 1995b). The physiological significance of these metabolic pathways is unclear but the first pathway does suggest a close link between anandamide and eicosanoid metabolism, which are known to be important in early pregnancy and implantation (see section 1.2.2).

The amidase activity of FAAH that hydrolyzes anandamide was first described in 1993 (Deutsch & Chin, 1993) and in 1998 it was shown that FAAH also has an esterase activity, hydrolyzing the ester bond of 2-AG (Di Marzo *et al.*, 1998b; Goparaju *et al.*, 1998). The enzymatic activity of FAAH had been described long before the discovery of anandamide when it was described as an enzyme that breaks down the NAEs (Schmid *et al.*, 1985).

FAAH is an intracellular membrane-bound serine hydrolase (Schmid *et al.*, 1985; Cravatt *et al.*, 1996), mostly found in the mitochondrial and microsomal fractions of tissue homogenates (Schmid *et al.*, 1985; Hillard *et al.*, 1995; Ueda *et al.*, 1995a). Transgenic mice lacking FAAH, have high concentrations of endogenous cannabinoids which correlate with increased cannabinoid receptor 1 (CB₁) dependent analgesia

(Cravatt *et al.*, 2001) indicating that FAAH is the major enzyme involved in endocannabinoid degradation.

The enzyme belongs to the amidase signature superfamily of serine hydrolases (Patricelli *et al.*, 1999), but differs from the rest of the enzymes of this superfamily by two key features; (1) its integration into the cell membrane and (2) its preference for hydrophobic substrates (Patricelli & Cravatt, 2001). Studies using bacterial amidases defined a highly conserved region “the amidase signature sequence” (Mayaux *et al.*, 1991) within this enzyme. A similar “amidase signature sequence” was described in the mammalian FAAH which suggests a strong evolutionary relationship between the amidases from bacteria and yeast, and the mammalian FAAH enzymes (Cravatt *et al.*, 1996). The amidase signature sequence of FAAH is approximately 50 amino acids in length and is rich with both serine and glycine residues (Kobayashi *et al.*, 1997).

The human, porcine, mouse and rat, FAAH enzymes are 73% identical at the amino acid level and 90% identical in the amidase signature sequence (amino acids 215-257) (Cravatt *et al.*, 1996). Although the mammalian FAAH mRNAs differ in size across species, the mammalian FAAH proteins are all made of 579 amino acids (Ueda *et al.*, 2000) with a molecular mass of ~63 kDa (Giang & Cravatt, 1997). This degree of sequence homology suggests that the enzymatic mechanism for the metabolism of anandamide has been conserved throughout mammalian evolution (Wan *et al.*, 1998).

In the human, the enzyme is encoded by a single gene on the short arm of chromosome one (1p34-35) and is made of 15 exons (Wan *et al.*, 1998) that is transcribed into a fully mature mRNA that is 2.1 kb in length (Wan *et al.*, 1998). The protein form of the

enzyme can be solubilised from membranes with detergents such as sodium taurodeoxycholate (Schmid *et al.*, 1985) and Triton X-100 (Ueda *et al.*, 1995a; Cravatt *et al.*, 1996), but the membrane association make the measurement of this enzyme's activity and expression technically very difficult to achieve (Maccarrone *et al.*, 2002b).

FAAH has been shown to have a wide substrate specificity for long-chain fatty acid derivatives (Schmid *et al.*, 1985; Ueda *et al.*, 1995a), however, when the partially purified enzyme from porcine brain was allowed to react with various NAEs, anandamide was found to be the best substrate, followed in order by ethanolamides of linoleic, oleic and palmitic acids (Ueda *et al.*, 1995a), despite the fact that these compounds are present in much larger quantities *in-vivo* compared to anandamide (Hansen *et al.*, 2000; Schmid, 2000). *N*-oleoylethanolamine, a satiety inducing compound (Fu *et al.*, 2003) and *N*-palmitoylethanolamine, an anti-inflammatory analgesic mediator (Mazzari *et al.*, 1996; Calignano *et al.*, 1998) potentiate the effects of anandamide by competing with anandamide for FAAH, an effect referred to as an 'entourage' effect (Ueda, 2002).

Recombinant rat FAAH has been purified from *Escherichia coli* (Patricelli *et al.*, 1998) and *baculovirus*-Sf9 insect cell systems (Katayama *et al.*, 1999). The k_{cat} value of the recombinant enzyme is 7.1 s^{-1} with oleamide as a substrate in *E. coli* (Patricelli *et al.*, 1998) and 6.5 s^{-1} with anandamide as a substrate in the *baculovirus*-Sf9 system (Katayama *et al.*, 1999). The k_m for anandamide ranges between 2-67 μM depending on the enzyme preparation and assay conditions (Desarnaud *et al.*, 1995; Hillard *et al.*, 1995; Maurelli *et al.*, 1995; Omeir *et al.*, 1995; Ueda *et al.*, 1995a; Bisogno *et al.*, 1997; Katayama *et al.*, 1999; Maccarrone *et al.*, 1999a). The enzyme works best at

alkaline pH with an optimum pH of between 8.5 and 10 (Hillard *et al.*, 1995; Maurelli *et al.*, 1995; Omeir *et al.*, 1995; Ueda *et al.*, 1995a; Bisogno *et al.*, 1997; Goparaju *et al.*, 1998). It has been demonstrated *in-vitro* that FAAH is capable of acting in a reverse manner (i.e. synthesis of anandamide from ethanolamine and arachidonic acid) (Arreaza *et al.*, 1997; Kurahashi *et al.*, 1997; Katayama *et al.*, 1999), however, due to the extremely high K_m value (27-50 mM) for ethanolamine, the physiological significance of this reaction seems to be questionable (Ueda *et al.*, 1995a; Sugiura *et al.*, 1996b; Arreaza *et al.*, 1997; Kurahashi *et al.*, 1997).

FAAH is widely distributed in mammalian organs (Ueda, 2002). Studies in the rat showed a good correlation between the enzymatic activity assays and FAAH mRNA (Cravatt *et al.*, 1996; Katayama *et al.*, 1997) with the enzyme activity being highest in the liver, small intestine, testis, cerebrum and stomach (Katayama *et al.*, 1997). FAAH was reported to be present to a lesser extent in the retina (Yazulla *et al.*, 1999) and uterus (Paria *et al.*, 1996). In addition, both FAAH mRNA and FAAH activity were detected in the murine uterus (Paria *et al.*, 1996), but not in the human uterus. FAAH mRNA has been found in several human tissues including the placenta, liver, brain, pancreas, kidney, skeletal muscles, mononuclear cells and platelets but not in the lungs or the heart (Giang & Cravatt, 1997; Maccarrone *et al.*, 1998; Maccarrone *et al.*, 1999b). In addition, FAAH mRNA has been detected in several human cell lines including H358 bronchioalveolar non-small cell lung carcinoma (Deutsch & Chin, 1993), EFM-19 breast carcinoma cell (Bisogno *et al.*, 1998a) and CHP100 neuroblastoma cell (Maccarrone *et al.*, 1998).

Many inhibitors of FAAH activity have been reported, with inhibition leading to potentiation of anandamide activities (Childers *et al.*, 1994; Adams *et al.*, 1995; Pertwee *et al.*, 1995; Compton & Martin, 1997). Non-specific serine hydrolase inhibitors such as phenylmethylsulfonyl fluoride (PMSF) and diisopropyl fluorophosphates (DFP) and sulfhydryl blockers such as *p*-chloromercuribenzoic acid inhibit FAAH (Deutsch & Chin, 1993; Hillard *et al.*, 1995; Ueda *et al.*, 1995a). Free fatty acids such as arachidonic acid and palmitic acid are weak inhibitors of FAAH (Hillard *et al.*, 1995; Qin *et al.*, 1998; Goparaju *et al.*, 1999; Katayama *et al.*, 1999). Other fatty acid derivatives act as potent inhibitors of FAAH including arachidonoyl trifluoromethyl ketone (Koutek *et al.*, 1994), methyl arachidonoyl fluorophosphate (MAFP) (De Petrocellis *et al.*, 1997; Deutsch *et al.*, 1997b), palmitylsulfonyl fluoride (Deutsch *et al.*, 1997b), arachidonoyl-diazo-methyl-ketone (De Petrocellis *et al.*, 1997) and arachidonoylserotonin (Bisogno *et al.*, 1998b). In addition, many α -keto heterocycle derivatives of fatty acids are potent inhibitors of FAAH (Boger *et al.*, 2000) and high concentrations of non-steroidal anti-inflammatory drugs such as ibuprofen also inhibit FAAH activity (Fowler *et al.*, 1999).

1.4.3. Mechanism of action

Anandamide is thought to exert its various effects by three main mechanisms, (1) by acting on cannabinoid receptors, (2) by binding to vanilloid receptors and (3) by non-receptor mediated mechanisms (Di Marzo, 1998; Maccarrone *et al.*, 2000c). The exact contribution of these various mechanisms depends on the type of system under study and varies from one species to another.

1.4.3.1. Cannabinoid receptors

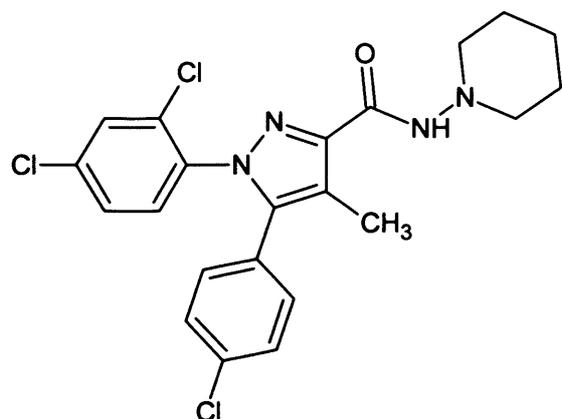
Anandamide acts as an agonist for the cannabinoid receptors. Two subtypes of cannabinoid receptors have been discovered (Munro *et al.*, 1993; Devane *et al.*, 1988). The first, cannabinoid receptor 1 (CB₁), was first described and characterized in the rat brain (Devane *et al.*, 1988) and subsequently cloned from a complementary DNA from a rat brain cDNA library in 1990 (Matsuda *et al.*, 1990). The human CB₁ was cloned in 1991 (Gerard *et al.*, 1991) and was found to share 97.3% sequence homology with the rat CB₁ with 100% homology within the trans-membrane regions (Gerard *et al.*, 1991). A second receptor isotype, cannabinoid receptor 2 (CB₂), was first characterized from macrophages of the marginal zone of the spleen (Munro *et al.*, 1993). The CB₂ receptor appears to be a separate receptor as it shows only 48% homology with CB₁ and only 68% homology within the trans-membrane regions (Munro *et al.*, 1993).

CB₁ was originally called the “central receptor”, because it is the main cannabinoid receptor in brain. It is found in several areas of the brain including the hippocampus (Herkenham *et al.*, 1990), cerebellum (Herkenham *et al.*, 1990), basal ganglia (Herkenham *et al.*, 1990), brainstem (Herkenham *et al.*, 1990) and cerebral cortex (Herkenham *et al.*, 1990). Other tissues that express CB₁ include testes (Gerard *et al.*, 1991), peripheral leukocytes (Bouaboula *et al.*, 1993), macrophages (Sacerdote *et al.*, 2000) sperm (Chang *et al.*, 1993), uterus (Das *et al.*, 1995), vascular endothelial and smooth muscle cells (Sugiura *et al.*, 1998; Liu *et al.*, 2000), human eye (retina, iris, corneal epithelium and ciliary body) (Straiker *et al.*, 1999; Porcella *et al.*, 2000) human placenta (Kenney *et al.*, 1999), ovary (Galiegue *et al.*, 1995), myometrium (Dennedy *et al.*, 2003) and the mouse embryo (Paria *et al.*, 1995).

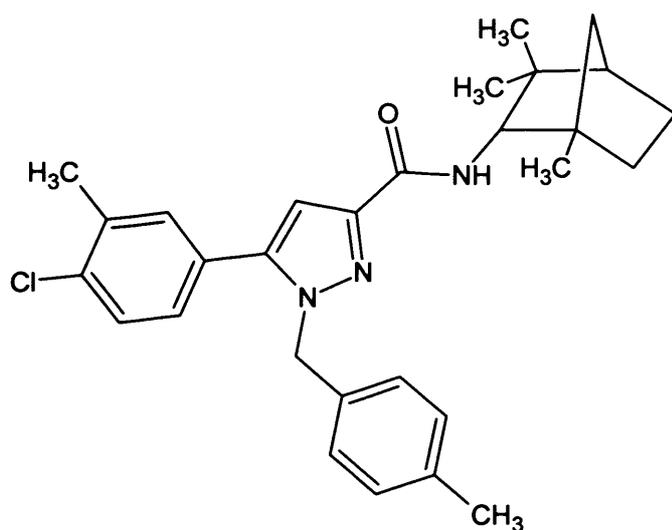
The CB₂ receptor, on the other hand, because it was first isolated from splenic cells has also been called the “spleen-type” cannabinoid receptor (Munro *et al.*, 1993). It is found in a number of immune system-derived cells including some T-cell lines EL4, IL2, Jurkat E6-1 and HPB-ALL (Condie *et al.*, 1996), monocytic cell lines, HL60 (Munro *et al.*, 1993) and RAW264.7 (Jeon *et al.*, 1996) and the mast cell line, RBL-2H3 (Facci *et al.*, 1995). CB₂ has also been isolated in the mouse embryo (Paria *et al.*, 1995).

Both receptors belong to the superfamily of G_{i/o}-protein coupled receptors (GPCR) (Howlett, 2002) and the two receptor sub-types can be selectively antagonized using either SR141716A (Sanofi Research), which when given orally antagonizes the CB₁ receptor or SR144528 which antagonizes the CB₂ receptor (Figure 1.7) (Di Marzo, 1998; Piomelli *et al.*, 2000). Stimulation of cannabinoid receptors activates a number of signal transduction pathways (Howlett *et al.*, 2002). These include:

- 1) Inhibition of adenylyl cyclase activity : Stimulation of CB₁ and CB₂ in brain tissue and human lymphocytes respectively results in inhibition of adenylyl cyclase activity leading to reduction in the production of cyclic AMP (Pertwee, 1999). CB₁ and CB₂ receptor-mediated inhibition of adenylyl cyclase is a pertussis toxin-sensitive cellular event, indicating the requirement for G_{i/o}-proteins (Howlett *et al.*, 2002).
- 2) Regulation of ion channels: Several ion channels are regulated by the stimulation of cannabinoid receptors:
 - i) K⁺ channel activation: CB₁ couples to the inwardly rectifying K_{ir} channels in



SR 141716 A



SR144528

Figure 1.7: Structure of the cannabinoid receptors inhibitors; SR141716A is a CB₁ inhibitor whilst SR144528 is a CB₂ inhibitor.

a pertussis toxin-sensitive manner, indicating that $G_{i/o}$ -proteins serve as transducers of this response (Mackie *et al.*, 1995).

- ii) Inhibition of voltage-gated L, N, P and Q Ca^{2+} channels: Stimulation of CB_1 but not CB_2 leads to inhibition of these various Ca^{2+} channels. These effects are mediated by $G_{i/o}$ -proteins as they are blocked by pertussis toxin (Howlett *et al.*, 2002).
- 3) Regulation of intracellular Ca^{2+} transients: Stimulation of CB_1 by 2-AG and to a lesser extent by AEA in undifferentiated NG108-15 neuroblastoma-glioma hybrid cells causes a transient increase in intracellular Ca^{2+} (Sugiura *et al.*, 1996a). This effect is inhibited by pertussis toxin and phospholipase C inhibitors suggesting a mechanism by which stimulation of CB_1 is coupled to activation of phospholipase C leading to inositol triphosphate (IP_3) release (Sugiura *et al.*, 1996a). However, studies of CHO cells that express recombinant human CB_1 or CB_2 do not show the same findings (Felder *et al.*, 1995). This suggests that this particular transduction pathway varies according to the cellular system studied (Howlett *et al.*, 2002) or that the original data are incorrect.
- 4) Regulation of focal adhesion kinase: CB_1 stimulation in hippocampal slices stimulates tyrosine-phosphorylation of focal adhesion kinase (FAK); a response mediated by $G_{i/o}$ -proteins (Howlett *et al.*, 2002). FAK is important in integrating cytoskeletal changes with signal transduction events (Howlett *et al.*, 2002).
- 5) Signal transduction via mitogen-activated protein kinase (MAPK): Stimulation of CB_1 leads to phosphorylation of MAPK which in turn leads to activation of

the Na⁺/H⁺ exchanger (Howlett *et al.*, 2002).

- 6) Signal transduction via ceramide metabolism: Studies with primary astrocyte cultures demonstrate that anandamide increases glucose metabolism, and phospholipid and glycogen synthesis by a mechanism that is inhibited by SR141716A but not by pertussis toxin (Guzman *et al.*, 2001) suggesting that this mechanism is not mediated by G_{i/o}-proteins. Instead, activation of CB₁ leads to stimulation of sphingomyelinase activation via an adaptor protein called Fan (factor associated with neutral sphingomyelinase) (Sanchez *et al.*, 2001). Stimulation of sphingomyelinase increases the production of ceramide which in turn stimulates ketogenesis and fatty acid oxidation (Blazquez *et al.*, 1999). Prolonged elevation of ceramide is associated with events that lead to apoptosis and decreased proliferation of cells (Blazquez *et al.*, 1999).
- 7) Regulation of nitric acid synthase: Anandamide stimulates the production of nitric oxide (NO) in endothelial cells (Howlett *et al.*, 2002).

1.4.3.2. Vanilloid receptors

The vanilloid receptor type 1 (Transient receptor potential vanilloid- TRPV1) was cloned in 1997 (Caterina *et al.*, 1997) and is a ligand-gated ion channel that plays an important role in nociception (Szallasi & Blumberg, 1999). The receptor belongs to a group of transient receptor channels (TRP) that are mostly found in primary afferent nerve fibres (Benham *et al.*, 2002). The TRPV1 receptor has been implicated in the pathophysiology of many clinical conditions [(Szallasi 2002) for review], for example patients with inflammatory bowel disease have increased expression of the TRPV1

receptor in the colonic nerve fibres (Yiangou *et al*, 2001). Its role in many clinical conditions is subject to ongoing research, but the current evidence suggest that in conditions characterised by chronic pain, the expression and sensitivity of the TRPV1 receptor are altered (Di Marzo *et al*,2002).

Recently, anandamide was found to be an agonist at this receptor (Di Marzo *et al.*, 1998a; Zygmunt *et al.*, 1999; Smart *et al.*, 2000). Interestingly, AEA efficacy as an agonist for the TRPV1 receptor varies from being a partial agonist in tissues with low receptor reserve to a full agonist in tissues with high receptor reserve [(Ross, 2003) for review]. In addition, the efficacy varies between tissues, for example AEA acts as a full agonist in Guinea-pig basilar artery (Zygmunt *et al.*, 1999) while it is a partial agonist in Guinea-pig bronchi (Tucker *et al*, 2001).

The relationship between the TRPV1 and CB₁ receptors has been studied in the dorsal root ganglia (DRG) (Ross, 2003). AEA has a dual effect on the release of neuropeptides. Acting via CB₁ at low AEA concentrations, it inhibits neuropeptide release (Ross *et al*, 2001) whilst acting via TRPV1 at high AEA concentrations, it stimulates neuropeptide release from the DRG neurons (Tognetto *et al*, 2001). Interestingly, in human neuroblastoma CHP100, human lymphoma U937, leukaemia DAUDI cells and Rat C6 glioma cells AEA induces programmed cell death (PCD) (apoptosis) via its action on the TRPV1 receptors as capsazepine, a selective antagonist of TRPV1, inhibited AEA-induced PCD (Maccarrone *et al*, 2000). Both C6 and DAUDI cells contain CB₁ and CB₂ respectively but neither CHP100 nor U937 cells express any of the cannabinoid receptors (CBs) (Maccarrone *et al*, 2000). SR141716A or SR144528 inhibited the AEA-PCD effect in C6 and DAUDI cells respectively but had no effect on

CHP100 or U937 (Maccarrone *et al.*, 2000). These findings suggest that the presence of the CBs on the surface of live cells protects these cells from the apoptotic activity of AEA which is mediated by TRPV1 and when these CBs are lost/ inhibited, the apoptotic activity of AEA takes place (Maccarrone *et al.*, 2000). Therefore, the interaction between the CBs and the TRVP1 appear critical in determining cell fate and it is likely that a better understanding of this relationship will provide insight into many physiological and pathophysiological conditions.

1.4.3.3. Non-receptor mediated actions

Anandamide can also affect cellular functions without interacting with receptors (Di Marzo, 1998). It can liberate arachidonic acid and positively modulate *N*-methyl-D-aspartate (NMDA)-mediated Ca^{2+} currents in cells that do not express the cannabinoid receptors (Hampson *et al.*, 1998). In rabbit skeletal muscles, rat cardiac cell and brain membranes, anandamide inhibits L-type Ca^{2+} -channels by direct interaction with dihydropyridine binding sites of these channels (Shimasue *et al.*, 1996; Jarrhian & Hillard, 1997). This has led to the suggestion that a third CB₃ cannabinoid receptor may exist (Breivogel *et al.*, 2001; Fride *et al.*, 2003).

1.4.4. Effects of anandamide

Anandamide has widespread effects on many systems. Two of the systems that are relevant to reproduction are discussed.

1.4.4.1. The vascular system

Anandamide has significant and complex effects on the vascular system (Hillard, 2000). Some of these effects result from activation of the CB₁ receptors located on axon terminals of the sympathetic neurons which results in decreased noradrenalin release (Hillard, 2000). The physiological consequence of this mechanism is dependent upon the sympathetic tone of the subject, for example, in conscious healthy animals the outcome is vasodilatation leading to an increase in peripheral blood flow accompanied by tachycardia secondary to baroreceptor activation while in anaesthetized animals with high sympathetic tone, the outcome is transient hypertension (Varga *et al.*, 1995; Hillard, 2000). In addition, anandamide causes vasodilatation in various vascular beds in different species either by direct action on the vascular smooth muscles or by altering endothelial factors that regulate vascular smooth muscle (Hillard, 2000). An example of the latter mechanism is demonstrated in rat endothelial cells, in which anandamide increases the synthesis of nitric oxide (NO) that acts as a potent vasodilator (Deutsch *et al.*, 1997a). The same effect of anandamide on NO has been described in human endothelial cell lines (Fimiani *et al.*, 1999).

1.4.4.2. The immune system

The identification of a peripheral cannabinoid receptor (CB₂) in a variety of immune cell types suggests that anandamide plays a role in the modulation of immune responses (Salzet *et al.*, 2000). Several effects of the activation of this receptor type have been described. Activation in macrophages and macrophage-like cells reduces the ability of these cells to process antigens that are necessary for the activation of CD4⁺ T

lymphocytes (McCoy *et al.*, 1999). Stimulation of both CB₁ and CB₂ reduces migration *in-vitro* in the rat (Sacerdote *et al.*, 2000). Activation of both CB₁ and CB₂ receptors inhibits the activity of natural killer cells (Massi *et al.*, 2000) and CB₂ receptor expression is down-regulated at the mRNA and protein levels during B-cell differentiation, with the lowest expression noted in germinal centres and proliferating centroblasts of tonsillar tissues (Carayon *et al.*, 1998). This suggests functional involvement of CB₂ receptors during B-cell differentiation. Activation of both CB₁ and CB₂ receptors augments the immune inhibitory Th-2 cytokines, IL-10 and transforming growth factors and suppresses the Th-1 stimulatory cytokine INF- γ (Howlett *et al.*, 2002), an effect that accounts for some of the immunosuppressive effects of cannabinoids.

1.5. Anandamide and reproduction

1.5.1. Effects of anandamide on gamete function and the hypothalamic-pituitary-ovarian axis

1.5.1.1 The sperm

Anandamide has been shown to have adverse effects on sperm function. It reduces the sperm fertilising capacity of sea urchins by inhibiting the acrosome reaction, an effect mediated via CB₁ (Schuel *et al.*, 1994). Human CB₁ transcripts have been detected in the human testis (Gerard *et al.*, 1991; Galiegue *et al.*, 1995) and in human sperm where a functional response has been observed (Schuel *et al.*, 2002b). AEA has also been detected in human seminal fluid (Schuel *et al.*, 2002b). It has been postulated that

during sperm capacitation, a dual-stage dependent endocannabinoid effect is in operation where AEA in seminal plasma and uterine fluids prevents capacitation in freshly ejaculated sperm, whereas sperm that have travelled through the uterus and into the Fallopian tube are exposed to gradually reduced AEA concentrations that release the molecular 'brake' of CB₁ inhibition so that capacitation occurs (Schuel *et al.*, 2002b).

1.5.1.2 The hypothalamus and pituitary

In the rat, the CB₁ receptor has been identified in the anterior pituitary (Gonzalez *et al.*, 1999; Wenger *et al.*, 1999a) and the expression of the CB₁ receptor gene is regulated by sex steroids (Gonzalez *et al.*, 2000). Anandamide levels in the anterior pituitary inversely correlate with the amounts of CB₁ receptor mRNA transcripts in the anterior pituitary during the ovarian cycle in the rat (Gonzalez *et al.*, 2000). In contrast, anandamide concentration in the hypothalamus inversely correlates with its concentration in the anterior pituitary during the ovarian cycle (Gonzalez *et al.*, 2000). Therefore, anandamide appears to play an important role in the regulation of the hypothalamic-pituitary-ovarian axis possibly affecting the process of ovulation and the regulation of the menstrual cycle (Gonzalez *et al.*, 2000).

1.5.1.3 The Ovary

The cannabinoid receptor CB₁ is expressed in the ovary (Galiegue *et al.*, 1995) and anandamide has been detected in follicular fluid obtained after egg collection in women undergoing in-vitro fertilisation (IVF) (Schuel *et al.*, 2002a). There have been no

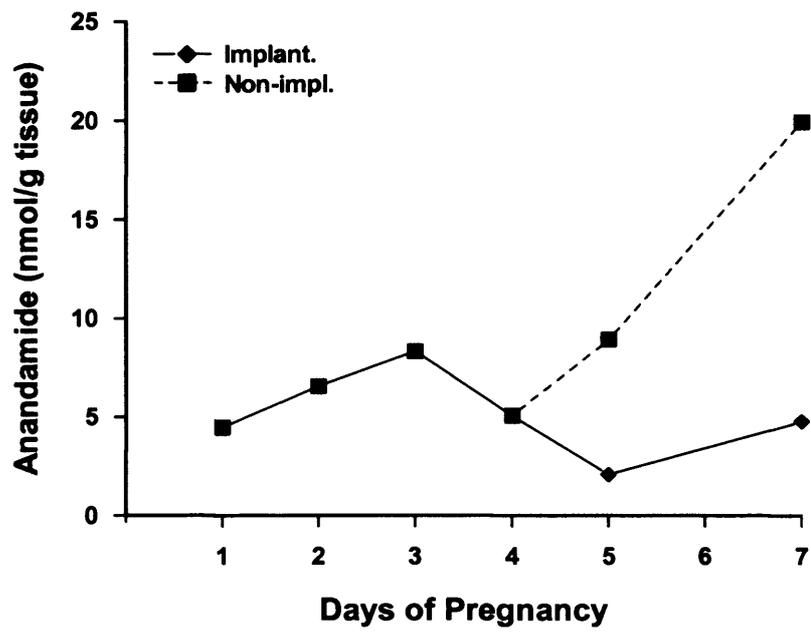
studies on the exact effects of anandamide on the development of and maturation of ovarian follicles, but the fact that exocannabinoids have adverse effects on ovulation (Mendelson *et al.*, 1986), the presence of anandamide in the follicular fluid and the fact that anandamide decreases serum concentrations of LH and prolactin although not follicular stimulating hormone (FSH) in the rat (Wenger *et al.*, 1999b), suggest that endocannabinoid signalling may help regulate follicle maturation and development (Schuel *et al.*, 2002b).

1.5.2. Effects of anandamide on embryo development and pregnancy implantation

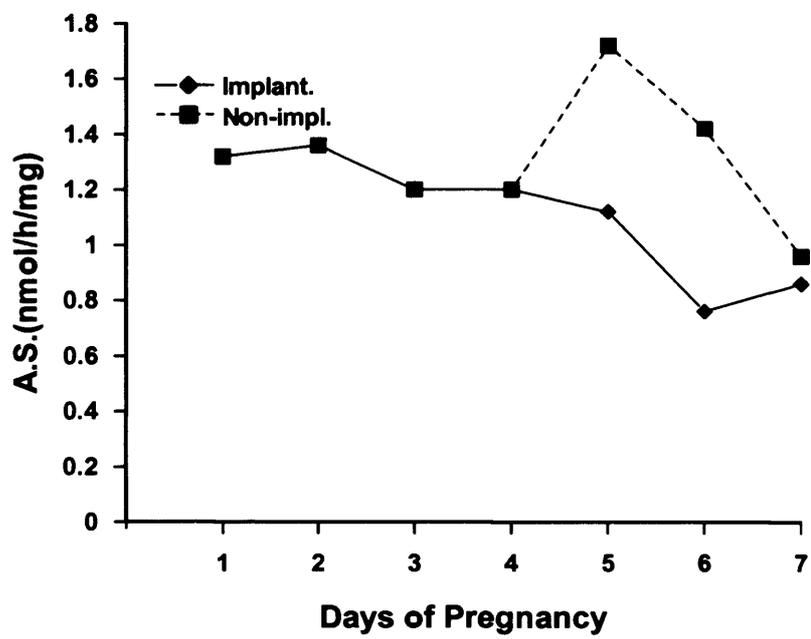
The murine uterus has the capacity to synthesize anandamide (*de novo* synthesis) and indeed contains the highest level of anandamide yet discovered in mammalian tissues (Schmid *et al.*, 1997). The CB₁ receptor mRNA is expressed in the epithelial cells of the mouse uterus (Das *et al.*, 1995). The murine uterus is receptive for implantation on day 4 (implantation day) but by day five it becomes non-receptive (Paria *et al.*, 1993). The concentrations of anandamide and the enzymatic activities involved in the synthesis and degradation of anandamide show little changes in the first four days of pregnancy (before implantation) apart from a significant decrease in FAAH activity between days three and four.

Anandamide concentrations at the implantation and non-implantation sites diverge after implantation with non-implantation site levels increasing dramatically with a concomitant increase in anandamide synthase activity and a decrease in FAAH activity (Figure 1.8). As a result of these changes, the concentrations of anandamide and anandamide synthase activity are significantly higher in the non-implantation sites

A



B



C

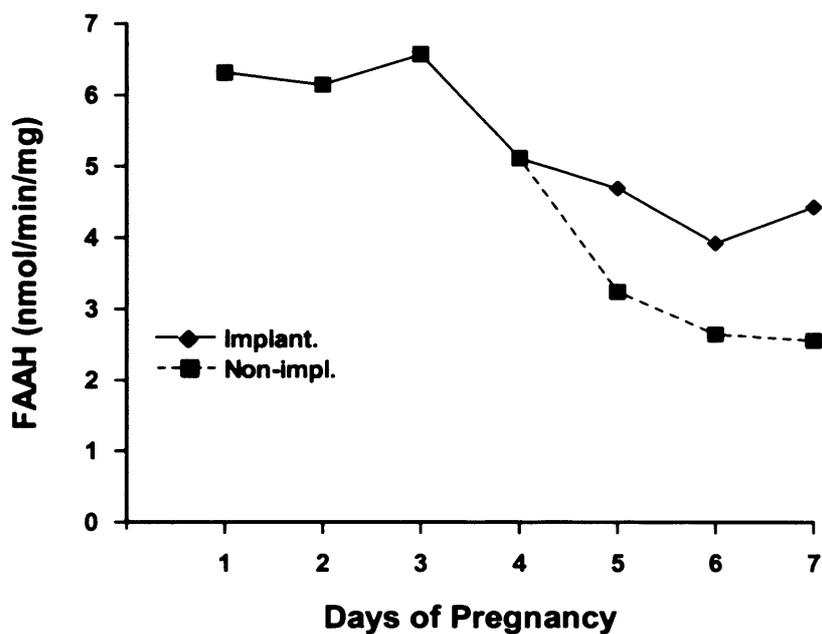


Figure 1.8. Changes in anandamide levels (a), anandamide synthase (b) and FAAH (c) in early mouse pregnancy. Note the increase in both anandamide and anandamide synthase from days four (implantation day) in the non-implantation sites compared to the implantation sites. FAAH activity, however, is much higher in the implantation sites compared to the non-implantation sites.

Abbreviations: non-implant, Non-implantation sites; implant, Implantation sites; FAAH, Fatty Acid Amide Hydrolase; A.S., Anandamide synthase enzyme. From: (Habayeb *et al.*, 2002).

(Data in 1.8 (a) derived from reference (Schmid *et al.*, 1997) with permission from National Academy of Sciences, U. S. A. "Copyright 1997". Data in 1.8 (b) & 1.8 (c) derived from (Paria *et al.*, 1996) with permission of Wiley-Liss, a subsidiary of John Wiley and Sons, 'Copyright 1996').

compared to the implantation sites. FAAH activity is also higher in the implantation sites compared to the non-implantation sites (Paria *et al.*, 1996). Analysis of AEA concentrations and the enzymatic activities in pseudo-pregnant uteri on day five reveals findings similar to those encountered at the non-implantation sites (Paria *et al.*, 1996; Schmid *et al.*, 1997). In keeping with these findings, FAAH expression has been found to be reduced on day five compared to the first four days of pregnancy (Paria *et al.*, 1999) suggesting that the systemic endocrine milieu in early pregnancy is characterized by the increased levels of anandamide and FAAH enzymatic changes described. Indeed, these findings are supported by the recent observation that estrogen and progesterone derived from the maternal ovary suppress the activity of FAAH in the mouse uterus (Maccarrone *et al.*, 2000b). The fact that the implantation and non-implantation sites, and the pseudo-pregnant uterus have different concentrations of anandamide and also show different enzymatic profiles, suggests that local factors such as uterine decidualisation or the presence of the embryo prevent an increase in anandamide concentrations (Habayeb *et al.*, 2002).

Both CB₁ and CB₂ mRNAs are expressed in the pre-implantation mouse embryo (Paria *et al.*, 1995) and anandamide arrests the development of embryos at or after the 2-cell stage *in-vitro*, primarily between the 4-cell and 8-cell stage (Paria *et al.*, 1995). A reduction in trophoectodermal cell numbers is seen in those embryos that escape the developmental arrest in the presence of anandamide and develop into blastocysts (Yang *et al.*, 1996). Anandamide also reduces the rate of zona-hatching of blastocysts *in-vitro* (Schmid *et al.*, 1997). These effects appear to be mediated through the CB₁ receptor as they are abolished when SR141716A is co-administered with anandamide (Yang *et al.*, 1996), but not when SR144528 is co-administered with anandamide (Paria *et al.*,

1998b). The exact role of the CB₂ receptor in the embryo is yet to be defined (Paria & Dey, 2000). Embryos at the 1 and 2- cell stages and at the blastocyst (day 4) stage all express FAAH, but the enzyme is not expressed in embryos at the 8-cell stage (Paria *et al.*, 1999). This finding suggests that FAAH in the embryo plays an important role in controlling the levels of anandamide to curtail the effects of anandamide on the embryo's development. Embryos at the blastocyst stage exposed in culture to low levels of anandamide show accelerated trophoblast differentiation and outgrowth while higher concentrations of anandamide inhibit trophoblast differentiation (Wang *et al.*, 1999). There are no data on the levels of anandamide and the enzymatic activities beyond the seventh day of the murine pregnancy.

Anandamide may be involved in the regulation of the "window" of implantation by synchronizing embryo development with preparation of the uterus up to the receptive state (Schmid *et al.*, 1997). The higher levels of anandamide in the non-implantation sites could be responsible for the inhibition of trophoblast proliferation while the lower levels at the implantation sites might help the proliferation of trophoblast, hence two different effects of anandamide are observed depending on its local concentrations (Paria & Dey, 2000; Wang *et al.*, 2003). On the other hand, excessive anandamide levels might induce the non-receptive phase prematurely leading to early pregnancy failure (Paria & Dey, 2000).

In embryos that are deficient in CB₁ and/or CB₂ receptors, development is asynchronous with uterine development (Paria *et al.*, 2001). On the morning of day four, 98% of wild-type embryos are at the blastocyst stage compared to only 62%, 71% and 61% of embryos that are deficient in CB₁, CB₂ or both receptors, respectively (Paria *et al.*, 2001). This further supports the hypothesis that anandamide may regulate the

window of implantation.

Another possible role for anandamide in early pregnancy is that it might act as a reservoir of arachidonic acid for the generation of prostaglandins which are important for the embryo development and implantation (Schmid *et al.*, 1997). Whether anandamide plays a role in the regulation of implantation in humans remains uncertain, as the mechanism of implantation in the mouse is distinctly different from that in humans. However, a study on the levels and activity of FAAH in maternal peripheral mononuclear cells (PMNCs) suggests that FAAH in these cells might be a useful marker for early pregnancy failure (Maccarrone *et al.*, 2000d). Levels of FAAH were measured in 50 women in early pregnancy (7–11 weeks gestation) and were found to be significantly lower in 7 women who miscarried compared to that in the 43 women who had successful pregnancies (Maccarrone *et al.*, 2000d). From the results of this study, a threshold concentration represented by an optical density (after ELISA) of 0.15 absorbance unit at 450 nm was identified. In a later study by the same group, the enzyme activity was measured in 150 healthy pregnant women at 8 weeks gestation. In 15 of these women FAAH activity was below the threshold and all of them miscarried. In contrast, only one woman out of 135 who had FAAH activity above the threshold miscarried (Maccarrone *et al.*, 2000d). These findings are interesting as they imply that a reduced activity of FAAH is associated with a poor outcome in contrast with the findings in the mouse that FAAH activity is suppressed in the uterus with the advancement of the pregnancy. More recently, in a study by the same group, it was found that progesterone but not β -HCG stimulates the activity of FAAH (Maccarrone *et al.*, 2001b). They also showed that anandamide inhibits the production of LIF (Maccarrone *et al.*, 2001b) and because LIF is reported to be defective in cases of

recurrent miscarriages (Piccinni *et al.*, 1998), a low level of FAAH might lead to spontaneous miscarriage by increasing the levels of anandamide which in turn inhibits the production of LIF (Maccarrone *et al.*, 2001b). What remains unclear at this stage is whether the changes in FAAH in early pregnancy failure are a result of the failing pregnancy or a cause of the failure. It is well recognized that failed pregnancies are associated with decreased levels of progesterone and it is possible that the reduced levels of progesterone leads to a reduction of FAAH in peripheral mononuclear cells (Lockwood, 2000). There are no studies, however, that evaluate the association between anandamide levels in the peripheral mononuclear cells, the uterus and placenta.

1.5.3. Effects of anandamide on myometrial activity

Studies on the effects of marijuana, whose active substance Δ^9 -tetrahydrocannabinol, is the exogenous ligand for the cannabinoid receptors, in pregnancy suggests that cannabinoids and their receptors may have a role in normal birth (Gibson *et al.*, 1983; Marbury *et al.*, 1983; Hingson *et al.*, 1986). A recent study on human myometrial tissues obtained from a pregnant uterus at the time of elective Caesarean section demonstrated that the human myometrial tissue contains both CB₁ and CB₂ mRNA transcripts (Dennedy *et al.*, 2003). Both anandamide and Δ^9 -THC cause relaxation of human myometrial strips *in-vitro*, an effect that was inhibited by SR141716A but not SR144528 suggesting the involvement of CB₁ rather than CB₂ receptors (Dennedy *et al.*, 2003).

1.6. Conclusions

Ever since the isolation of anandamide in 1992, there has been an exponential rise in research not only on its metabolism but also on its pharmacological characteristics and the various effects *in-vitro* and *in-vivo* (in humans and animals). Despite this extensive interest and the plethora of recent published data on the reproductive system of experimental animals especially in implantation and maintenance of pregnancy, there has been very little work on human reproduction. Although the levels of anandamide have been quantified in different body fluids, there have been no reported changes in human pregnancy, despite the recognition that it may regulate not only implantation but the maintenance of pregnancy. Since the levels of the enzyme regulating its activity and concentration have been measured in humans, it is highly relevant to investigate this compound in human reproduction.

1.7. Hypotheses

From the data of both animal and human studies, it appears that anandamide is involved in the processes that lead to the initiation and maintenance of pregnancy. The studies in this thesis were therefore designed to test the parent hypothesis that the endogenous cannabinoid, anandamide, acting *via* receptor-mediated mechanisms, is involved in the maintenance of early pregnancy. The effect is likely to be mediated by alterations in either the systemic or local levels of anandamide and/or of its receptors in the fetal-maternal interface.

The studies were designed to test the following sub-hypotheses:

- 1) That the plasma levels of the endogenous cannabinoid anandamide will change during the menstrual cycle depending upon the relative levels of estrogens and progesterone. (Because plasma anandamide levels appear to be controlled by the ovarian steroids; estradiol which promotes the release of anandamide from endothelial cells and progesterone, which increases the activity of FAAH, the enzyme that destroys anandamide).
- 2) That the levels of plasma anandamide will also vary during normal human pregnancy in response to hormonal changes.
- 3) That due to 'functional progesterone withdrawal', plasma levels of anandamide will increase at the onset of labour.
- 4) The plasma levels of anandamide in women with threatened miscarriages in the first trimester of pregnancy predict the success of ongoing pregnancies.
- 5) That systemic level of FAAH in the peripheral mononuclear cells or the presence of FAAH protein at the fetal-maternal interface will affect the levels of plasma anandamide in early pregnancy.

The aims of this thesis were:

- a. To develop a robust and accurate method to measure plasma anandamide levels.
- b. Determine the most appropriate method for processing clinical samples.

- c. Determine the plasma levels in normal women of reproductive age during uncomplicated pregnancies and labour.
- d. Determine the levels of plasma anandamide in women with threatened miscarriage and determine whether such levels could be used as a diagnostic test.
- e. Study the expression of the endocannabinoid system in tissues from the 1st trimester of human pregnancy to determine if there are changes that are similar to that reported in animal models.

Chapter 2

The development of an assay to measure plasma anandamide concentrations

Chapter 2

The development of an assay to measure plasma anandamide concentrations

2.1. Introduction

The aim of this part of the thesis was to develop a robust and reproducible assay technique for the extraction and measurement of anandamide (AEA) in maternal plasma/serum. Several factors contribute to the difficulties encountered in measuring plasma AEA concentrations. Firstly, AEA is a lipophilic compound, hence a method that involves lipid extraction is needed; secondly, it is rapidly oxidised and exposure of a sample to air can therefore lead to loss of AEA before quantification; thirdly: AEA has a tendency to “stick” to glassware and plastic and fourthly, the presence of peripheral mononuclear cells in the blood that contains high levels of FAAH means that AEA is rapidly degraded by the activity of FAAH if the enzyme is allowed to act on AEA.

At the beginning of this project, two methods described for the measurement of AEA, both described by Giuffrida and co-workers in the USA were available for use. The first was based on gas chromatography mass spectrometry (GC-MS) technology (Giuffrida & Piomelli, 1998) whilst the second was based on high performance liquid chromatography mass spectrometry (HPLC-MS) technology (Giuffrida *et al.*, 2000). Neither of these methods had been used for measurement of plasma AEA in humans

although the HPLC-MS method had been used successfully to measure AEA in rat plasma. GC-MS requires partial purification and chemical derivatisation of AEA, resulting in variable recovery and time-consuming sample preparation. HPLC-MS, on the other hand, requires very clean samples and thus sample preparation is time consuming. Contacts were made with Dr Giuffrida who advised that the HPLC-MS is an easier technique and is more robust as it does not have the same limitations of GC-MS. Therefore, a decision was taken to adapt the HPLC-MS method and try to develop the assay in order to measure AEA concentrations in human plasma.

Developing a robust and reproducible assay took lots of effort and many hours of hard work. All the developmental work and indeed subsequent assays were performed “in-house” at the Genome Instability Research Group’s laboratory at the University of Leicester. The next section describes the original method developed by Giuffrida and the steps undertaken to develop and validate an assay for the measurement of AEA in human plasma.

2.2. Methods

2.2.1. The HPLC-MS method as described by Giuffrida

The following sections (2.2.1.1; 2.2.1.2; 2.2.1.3 and 2.2.1.4) describe the original method as described by Giuffrida for the measurement of anandamide concentrations in the rat plasma (Giuffrida *et al.*, 2000).

2.2.1.1. Collection of blood samples

Blood was collected from rats anesthetized with methoxyflurane either after the animals were decapitated or taken by cardiac puncture. Either way the blood was collected to a glass tube containing Krebs–Tris buffer plus EDTA (4.5 mM) (Appendix 1). The volume of buffer used was half the volume of the blood sample collected.

2.2.1.2. Processing of the blood samples

- 1) Separation of plasma from blood cells: Samples were centrifuged in Accuspin tubes (Sigma) at 800 x *g* for 10 min at 22°C.
- 2) Plasma recovered was spiked with 500 pmol of deuterium-labeled AEA (d_8 -AEA) that acts as an internal standard.
- 3) Protein precipitation was achieved by adding ice-cold acetone (1 volume) followed by centrifugation at 1000 x *g* for 10 minutes at 22°C. Residual acetone in supernatants was evaporated under a stream of nitrogen (N_2) gas.
- 4) Lipid extraction was performed on the supernatants from step 3 by the addition of chloroform: methanol (2:1, volume: volume). The chloroform layer was recovered and evaporated to dryness under a stream of N_2 gas.

- 5) The sample was then reconstituted in a mixture of chloroform/methanol (1:3, 80 μ l), and transferred to 2.0-ml screw top vials with 0.1-ml conical glass inserts and injected into the HPLC/MS.

2.2.1.3. Preparation of the standard curve

- 1) Both AEA standards and deuterium-labeled AEA (d_8 -AEA) were synthesized “in house” by the reaction of arachidonylchloride with ethanolamine or deuterium labelled ethanolamine respectively. Identity and chemical purity (98%) of AEA and d_8 -AEA were determined by thin layer chromatography (TLC) and HPLC-MS.
- 2) A standard calibration curve was constructed by adding constant amounts of d_8 -AEA (500 pmol) to increasing amounts of AEA. The standards were then dried under a stream of N_2 and reconstituted in a mixture of chloroform/ methanol (1:3, 80 μ l), and transferred to 2.0-ml screw top vials with 0.1-ml conical glass inserts and injected into the HPLC/MS.
- 3) Analysis was made in the positive ion mode for the sodium adducts $[M + NA]^+$, $[M + Na^+; m/z=370.3 \text{ AEA and } 378.3 \text{ } d_8\text{-AEA}]$ which were used for quantification in the selected ion recording mode (SIM). SIM peaks were integrated, and the ratios of the area of AEA and d_8 -AEA labelled ions were plotted against the amount of AEA injected.

- 4) The lowest point on the standard curve corresponding to no AEA (0) resulted in a ratio of 0.011 AEA: d₈-AEA. This value was plotted as corresponding to 0 pmol AEA. The MS response was linear over the range 0-500 pmol, and the correlation coefficient value (r^2) of the regression curve was greater than 0.99.
- 5) The limit of detection, that is, the lowest amount that produced a peak height of three times the signal-to-noise ratio, was 0.3 pmol of AEA injected.

2.2.1.4. HPLC-MS conditions

A Hewlett Packard (HP) 1100 Series HPLC/MS system equipped with a Hewlett Packard octadecylsilica (ODS) Hypersil column (100 X 4.6 mm i.d., 5 μ m) was used.

2.2.1.4.1. HPLC conditions

AEA was fractionated by reversed-phase HPLC using an ODS Hypersil column interfaced with the MS. HPLC conditions were optimized for resolution and elution time. The mobile phase consisted of methanol (B) and water (A):

1. 25% A, 75% B for 2 minutes.
2. 15% A, 85% B for 3 minutes.
3. 5% A, 95% B for 20 minutes.
4. 100% B for 5 minutes.

The flow rate was maintained at 0.5 ml/min. Column temperature was kept at 20°C. Under these conditions, AEA standards eluted from the column with a retention time of 15.4 minutes.

2.2.1.4.2. MS conditions

Mass spectrometry (MS) analyses were performed with an electrospray ion source set in the positive ionization mode. Capillary voltage (V_{cap}) was set at 3.5 kV, and fragmentor voltage was varied from 80 to 100 V. Nitrogen was used as the drying gas at a flow rate of 12 litres/minute. The drying gas temperature was set at 350°C and the nebulizer pressure at 50 psi. For quantitative analyses sodium adducts of the molecular ions $[M + \text{Na}]^+$ were detected in the selected ion monitoring (SIM) mode. Complete system control and data evaluation were done using an online system software (HP Chemstation).

2.2.2. Modifications of the HPLC-MS method

The initial experiments in the measurement of AEA were designed to overcome some of the problems associated with AEA measurement, especially the adherence of the molecule to glassware and plastic.

2.2.2.1. Standards and glassware

AEA and d_8 -AEA were obtained from Cayman chemicals (Ann Arbor, MI, USA). In the first stage attempts at infusing variable amounts of both AEA and d_8 -AEA to the HPLC-MS under the same conditions as described by Giuffrida (Giuffrida *et al.*, 2000), produced peaks that were disappointingly small.

Because adherence to glassware is a known problem (Giuffrida *et al.*, 2000), both silanised (Appendix 2) and un-silanised vials were used. It was noted that with the un-silanised vials there was no recovery of the standards injected. At this stage, contacts were again made with Dr Giuffrida and these problems discussed; he advised that AEA has an ability to stick to the glassware and explained that he has had similar difficulties in his laboratory. Dr Giuffrida recommended the use of special vials, (7-ml Kimble scintillation vials) (Fisher Scientific UK, Loughborough, UK) that he had used successfully to overcome this problem. Subsequently it was found that the use of these vials resulted in the recovery of both AEA and d₈-AEA with peaks that were easily detectable by the HPLC-MS. Therefore, all subsequent quantifications of plasma AEA were performed using these vials.

2.2.2.2. The internal standard

The use of the internal standard in HPLC-MS helps to improve the accuracy of the assay and indicates the efficacy of the lipid extraction. Initially, 533.1 pmol of d₈-AEA was used as an internal standard (Giuffrida *et al.*, 2000). The data obtained indicated that the peaks for d₈-AEA exceeded the upper detection limit of our system. The difference was attributed to (a) the fact that d₈-AEA used in the assay was different from that used by Dr Giuffrida and (b) the possibility that sensitivity of the HPLC-MS used was higher. In order to determine the optimal amount of internal standard to be used for the standard curve and for subsequent assays, variable amounts of d₈-AEA were injected and the response of the detector determined by measuring the peaks obtained.

2.2.2.3. Blood collection

Krebs-Tris-EDTA buffer was prepared “in house” (Appendix 1) and used to assess its ability to provide correct anti-coagulation properties that had been achieved with the rat blood samples by Giuffrida (Giuffrida *et al.*, 2000). The results were disappointing in that human blood rapidly clotted. Therefore, venous blood was collected from veins in the cubital fossa after the application of an appropriate tourniquet in an evacuated EDTA containing tube. A total volume of four millilitres (ml) was collected. Blood was transferred immediately into a 120 mm polypropylene conical tube (Becton Dicknson Labware Europe, France) containing 2 mls of the buffer. Samples were collected on ice to reduce the activity of the FAAH enzyme which is abundant in the peripheral blood mononuclear cells (PMNCs) and to reduce the cellular uptake of AEA by PMNCs. To determine the optimal processing time of the blood samples, it was stored on ice for 1, 2, 3, 4 and 6 hours before being processed for the extraction of AEA.

2.2.2.4. Processing of the blood samples

- 1) The polypropylene conical tubes were centrifuged to separate cells from the plasma. Initially, this was at 800 x g for 10 minutes at 22 °C as described in the original method. This resulted in unsatisfactory separation of the blood into plasma and cells layers. Therefore, different centrifugation rates were used to determine the optimal conditions for centrifugation.
- 2) Once separated from cells the plasma was transferred to 7-ml Kimble scintillation vials using either a plastic pipette or a glass Pasteur pipette which

were either straight out of the box or silanised) (Appendix 2) to determine the effects of those different approaches on the recovery of AEA.

- 3) As described in the original method, ice cold acetone was used to precipitate the proteins. As human blood contains more protein than rat blood, the effects of performing two versus one protein precipitation steps were studied.
- 4) Lipid extraction was performed on the supernatants obtained from step 3 by the addition of chloroform:methanol (both from Fisher Scientific UK) (2:1, volume: volume) and by mixing by careful inversion. The original method then required the sample to be allowed to stand for the aqueous and the lipid (chloroform) layers to separate (Giuffrida *et al.*, 2000). This proved to be time consuming as it took between 40 and 60 minutes. A comparison of this time-consuming step and a short centrifugation step (1000 x g for 5 minutes at 22 °C) was made.
- 5) The chloroform layer was recovered and evaporated to dryness under a stream of N₂ and then reconstituted in a mixture of chloroform/methanol (1:3, 80 µl), and transferred to 2.0-ml screw top HPLC vials modified with 0.1-ml conical glass inserts, two or three 20 µl aliquots of samples were inserted to the HPLC injection loop and analysis made on a 10 µl inject.

2.2.2.5. HPLC-MS conditions

The system consisted of a Waters 1525 binary liquid chromatography pump and a Waters 717 plus auto-sampler fitted with a 100 µl injection loop (Waters MS Technology, Manchester, UK) (Figure 2.1). The HPLC was interfaced to a Quattro Ultima triple quadrupole mass spectrometer via a Z-spray ion source and controlled by MassLynx NT software version 3.5 (Waters MS Technology, Manchester, UK).

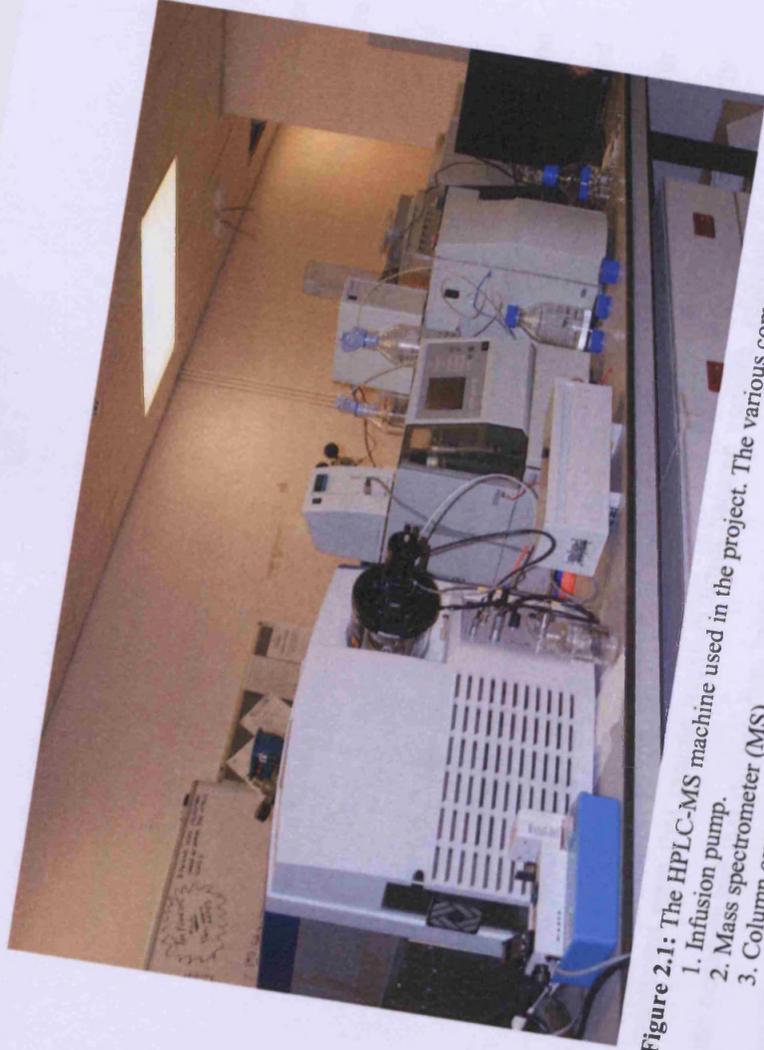
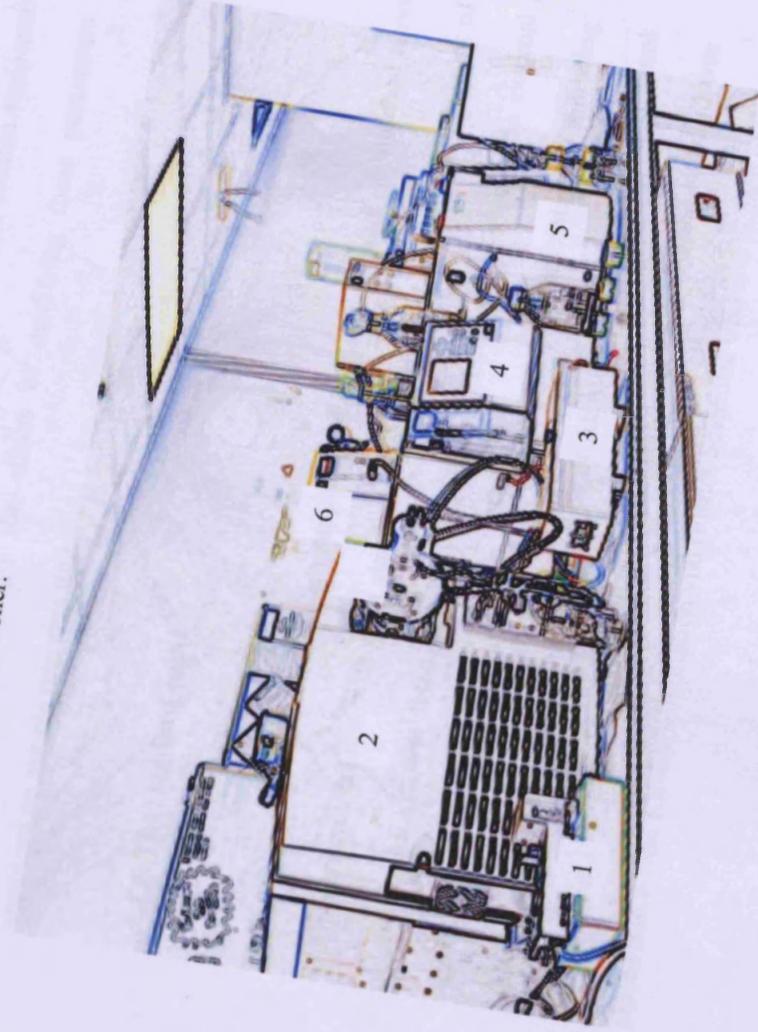


Figure 2.1: The HPLC-MS machine used in the project. The various components are:

1. Infusion pump.
2. Mass spectrometer (MS).
3. Column oven.
4. Autosampler.
5. Solvent delivery system.
6. Temperature controller.



Reversed-phase chromatographic separations were performed on a Hypersil octadecylsilane C18 column (4.6 X 100 mm, 3 µm particle size; Phenomenex, Macclesfield, UK). The column temperature was maintained at 20 °C using a column temperature controller (Jones Chromatography, Hengoed, UK). Samples were maintained at 4 °C in a refrigerated injection system.

Initially, the HPLC-MS conditions were kept as were described in the original method (Giuffrida *et al.*, 2000) and authentic AEA and the d₈-AEA were eluted at 17.51 and 17.67 minutes (Figure 2.2) respectively. As expected, in view of the larger molecular mass of d₈-AEA, its retention time is shorter than that of AEA. However, early indications were that sample retention times became variable, reaching 9 minutes on one occasion and that the variation tended to occur more towards the end of a run of samples or standards. This could have been due to incorrect column temperatures or buffer conditions. Accordingly the effects of modifying these parameters were examined on sample retention times.

2.2.2.6. The standard curve

Both AEA and d₈-AEA stocks were supplied and diluted in ethanol. A standard curve of 5 points was created with each standard point represented by a variable amount of unlabelled AEA and a fixed amount of d₈-AEA. These are referred to as external standards (S₁₋₅). The five external standards were prepared by combining the following amounts of AEA (S₁: 1 pmol AEA, S₂: 2.5 pmol AEA, S₃: 5 pmol AEA, S₄: 10 pmol AEA and S₅: 17.5 pmol AEA) with the internal standard. Each external standard was placed into a 7-ml Kimble scintillation vial (Fisher Scientific UK) and to each of these

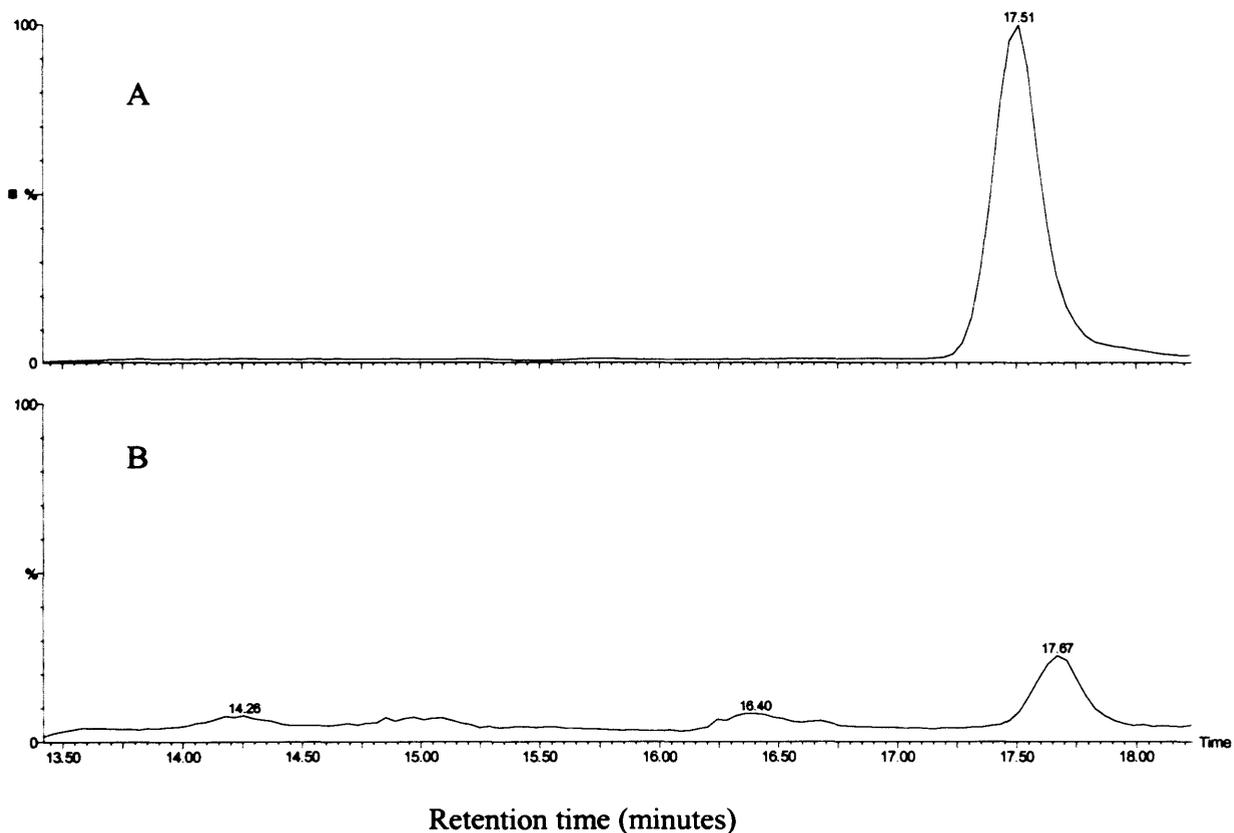


Figure 2.2 Shows anandamide (A) and d_8 -anandamide (B) as eluted from the HPLC-MS. Peaks are for the sodium adducts of AEA (370.3) and d_8 -anandamide (378.3). Note the molecular weight of AEA is 347.3 and d_8 -anandamide is 355.3. The x-axis represents the retention time in minutes while the y-axis is peak size in arbitrary units (AU). Note the small peaks in “B” at 14.25 and 16.40 which probably represent impurities in the d_8 -AEA.

standards, the internal standard was added. After vortex mixing for 20 seconds, the standards were then dried completely under a gentle stream of either nitrogen (N₂) or argon for less than 5 minutes. Each standard was next reconstituted in 80 µl methanol: chloroform (3:1) and vortex mixed for 1 minute. Of the 80µl sample, either two or three 20µl aliquots were injected into the HPLC sample loop with analysis made on a 10µl inject. As the analyzed inject was 10 µl, the AEA quantity on the standard curve representing the amount of AEA on the column is only 1/8 of the actual amount of each standard point, hence S₁₋₅ on the standard curve were 0.125, 0.312, 0.625, 1.25 and 2.18 pmol AEA on the column, respectively. These values constitute the abscissa on the calibration curve, and the AEA: d₈-AEA response ratio the ordinate.

2.2.2.7. Accuracy of the method

To ensure that the observed peaks of AEA in a blood sample were due to the presence of authentic AEA and not some other interfering substance, 1 pmol and 2.5 pmol of AEA were added to plasma samples and the resulting change in the peak size compared to that of samples that had not been supplemented with authentic AEA. Assay precision was assessed by measuring the inter-assay and intra-assay coefficient of variations for the standard curve at the five standard points.

2.3. Results

2.3.1. Modifications of the original method

2.3.1.1. The internal standard

In this experiment, the effects of varying the amounts of d₈-AEA using 533.1, 251, 50.2 and 25.1 pmols were investigated. As can be seen in Figure 2.3, the sizes of the peaks observed varied in proportion to the amounts of d₈-AEA injected. From the sizes of the peaks, it was determined that 25.1 pmol was the ideal amount to be used in this assay.

2.3.1.2. Collection of blood samples

In this experiment, the effects of leaving the samples on ice for 1, 2, 3, 4 and 6 hours compared to immediate processing were investigated. Blood was collected from three volunteers and the AEA concentrations compared at each time point. As can be seen in Table 2.1 and Figure 2.4, delaying the processing by more than 2 hours resulted in an unacceptable loss up to 50% of AEA compared to processing the samples within 2 hours. Therefore, the processing of blood samples for later studies always commenced within 2 hours for all blood samples collected.

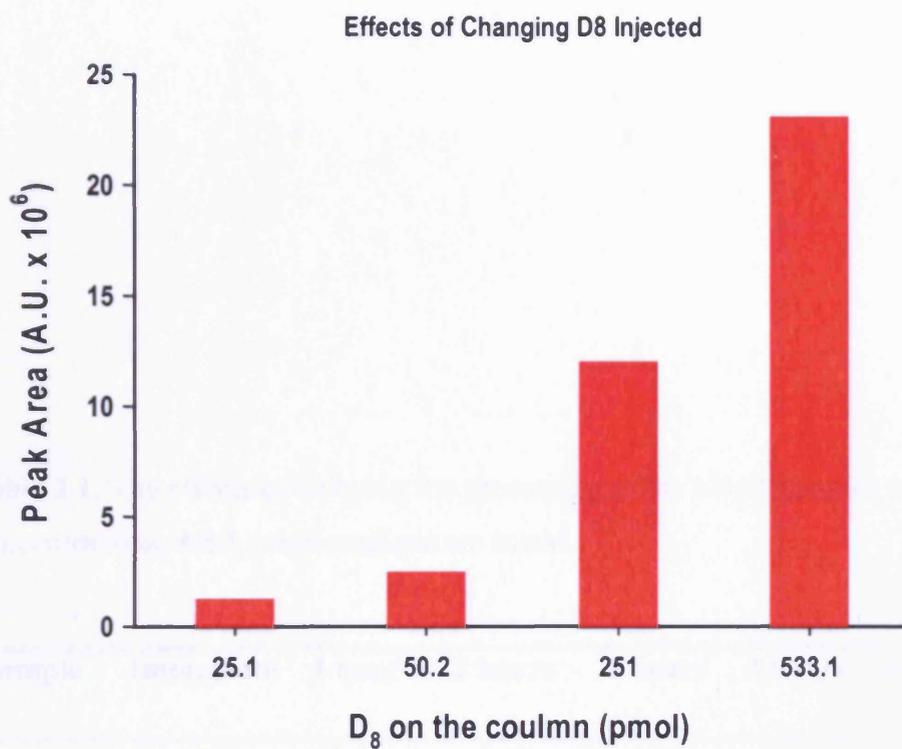


Figure 2.3: The effects of varying the amounts of d8-AEA injected on the column.
AU: arbitrary units.

Table 2.1. The effects of delaying the processing of the blood samples on the AEA concentrations. AEA concentrations are in nM.

Sample	Immediate	1 hour	2 hours	3 hours	4 hours	6 hours
1	1.62	1.57	1.67	0.83	0.92	0.79
2	0.84	0.87	0.79	0.36	0.28	0.41
3	1.14	1.07	1.09	0.72	0.58	0.46

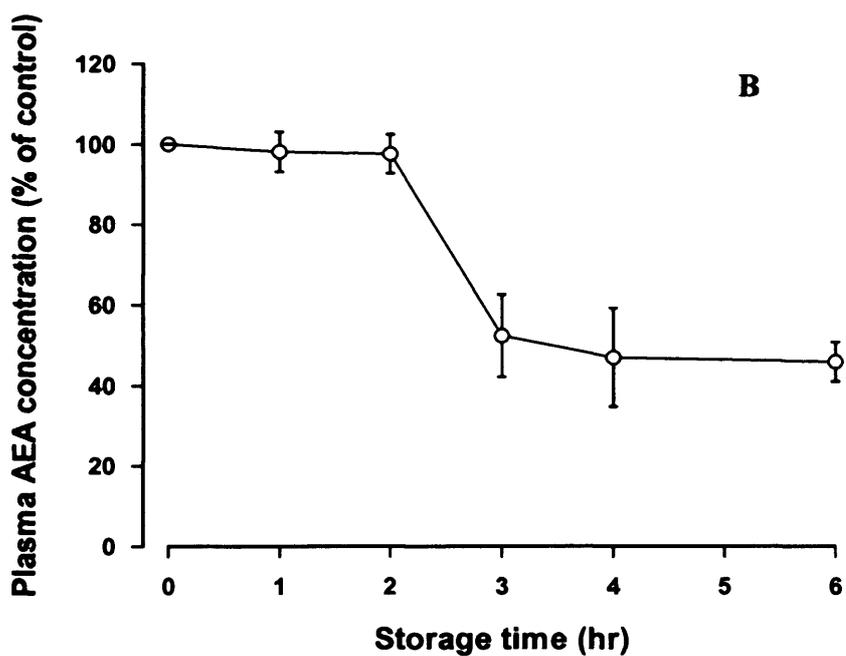
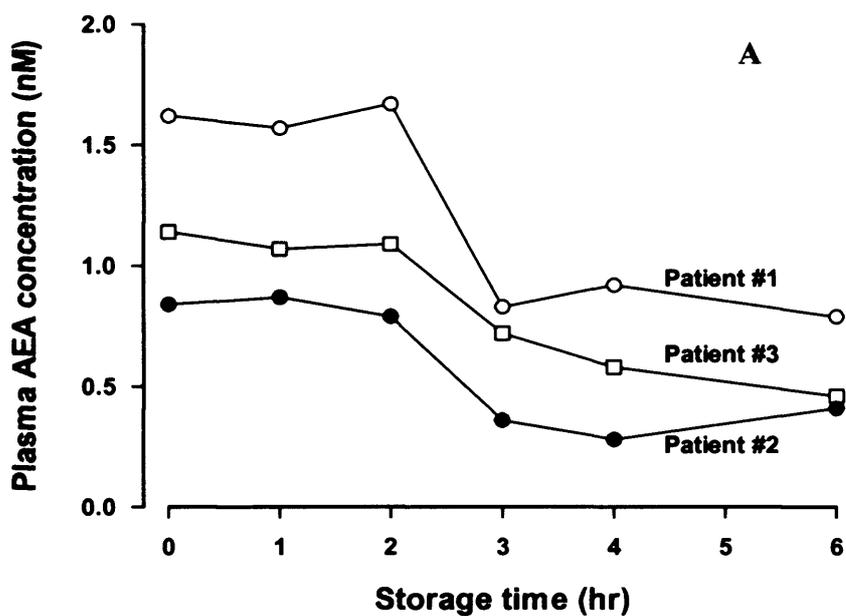


Figure 2.4 The effects of delaying the processing of the blood samples on the AEA concentrations. A: A representation of the actual values measured for the three volunteers. B: Represents the cumulative data for the three volunteers represented as percentage of change in AEA concentrations over time. The data are presented as the mean \pm SD.

2.3.1.3. Processing of blood samples

- 1) Centrifugation of the blood sample to separate cells from the plasma: After a series of experiments, it was found that centrifuging the sample at 1200 x g at 22 °C for 30 minutes resulted in clear separation of the cells from the plasma layer containing the buffer. Two millilitres of plasma mixed with the buffer were recovered.

- 2) The effects of using silanised pipettes: In this experiment, duplicate samples were collected from three volunteers and the AEA concentrations with and without the use of silanised pipettes were compared. The use of silanised pipettes resulted in AEA concentrations that were 28-55 % higher than with non silanised pipettes (Table 2.2). Therefore, all subsequent studies were performed using silanised pipettes.

- 3) Protein precipitation: To determine if performing two protein precipitation results in better recovery of AEA, duplicate blood samples were obtained from three volunteers and the AEA concentrations compared. As can be seen in Table 2.3, performing two precipitation steps offered no advantages as the difference between the two was less than 6%. Therefore, in all subsequent studies, only one precipitation step was performed.

- 4) Lipid extraction: Comparing centrifugating the sample at 1000 x g for 5 minutes at 22 °C with allowing the sample to stand revealed no significant difference in the AEA concentrations (Table 2.4). As this reduces the time of the assay significantly, all subsequent samples were processed using centrifugation.

Table 2.2: The effects of silanisation of the pipettes on the AEA concentrations. AEA concentrations are in nM.

Sample	Silanized (A) nM	Non Silanized (B)	%Change [(A-B/A) x100]
1	1.19	0.76	36%
2	1.38	0.62	55%
3	0.88	0.63	28%

Table 2.3: The effects of performing two acetone steps on AEA concentrations.

Sample	One acetone step	Two acetone steps	%Change
	(A) (nM)	(B) (nM)	[(A-B/A) x100]
1	1.42	1.38	3%
2	0.87	0.92	6%
3	0.64	0.68	6%

Table 2.4: Comparing centrifugation to allowing the sample to stand after the addition of methanol: chloroform.

Sample	Standing	Centrifugation	%Change
	(A) (nM)	(B) (nM)	[(A-B/A) x100]
1	0.97	0.93	4%
2	1.37	1.45	6%
3	1.17	1.24	6%

2.3.1.4. HPLC-MS

In order to solve the problem of variable retention times, the column temperature was increased from 22°C to 30°C. This had a positive impact on the consistency of retention times, however, a variation of the retention times to as low as 12 minutes was still being observed although the variation was less than initially observed. In view of this variation, a decision was made to increase the length of the mobile phase by adding an extra step to allow column re-equilibration (25% A, 75% B for 20 minutes). After these modifications, the retention time remained stable all through subsequent runs. Therefore, the mobile phase for all subsequent studies was:

Water (A) and methanol (B) (both HPLC grade, Fisher Scientific UK) at a flow rate of 0.5 ml/min in the following manner:

- 1) 25% A, 75% B for 2 minutes.
- 2) 15% A, 85% B for 3 minutes.
- 3) 5% A, 95% B for 20 minutes.
- 4) 100% B for 5 minutes.
- 5) 25% A, 75% B for 20 minutes.

Electrospray ionization was carried out at the same settings as described in the original method (Giuffrida *et al.*, 2000). Initially, both protonated ions [$M+H^+$; $m/z=348.3$ AEA and 356.3 d_8 -AEA] sodium adducts of the molecular ions [$M+Na^+$; $m/z=370.3$ AEA and 378.3 d_8 -AEA] (Figure 2.2) were used for quantification in the selected ion recording mode. In subsequent experiments, it was observed that the results of the protonated ions were less reproducible than those with the sodium adducts, hence the

use of protonated ions was abandoned. This is in keeping with the original method described by Giuffrida (Giuffrida *et al.*, 2000).

2.3.2. The standard curve

Peak areas were measured for both AEA and d₈-AEA and the ratios of AEA/AEA-d₈ were plotted for the external standards to obtain a calibration (standard) curve. The curve was linear in the range of 0.03125 pmol AEA to 2.0 pmol AEA on the column ($r^2=0.9998$) (Figure 2.5). The lower limit of detection in the assay was 0.03125 pmol of AEA on the column (equivalent to 0.25 pmol AEA in the sample). The value on the column represented 1/8 of the actual standard prepared since the final reconstitution volume was 80µl and the analysis made on a 10µl injection. The ratios of AEA/d₈-AEA for the plasma samples were then calculated and based on the standard curve, the amount of AEA on the column calculated. This value was then multiplied by 8 to obtain the concentration in the plasma sample. Although 2 ml of plasma were collected, this was diluted by half by the buffer; hence the value obtained in the sample was equal to the amount of AEA in one millilitre of plasma.

2.3.3. Accuracy of the method

Table 2.5 shows the results of the experiment where 1 and 2.5 pmol AEA were added to a plasma sample. There was an increase in the peak areas and the ratios of AEA/d₈-AEA with the addition of AEA. The actual values measured with the addition of AEA were very close to the expected ones. The results of this experiment confirmed that the observed peak represented AEA in the sample. Precision was assessed by measuring

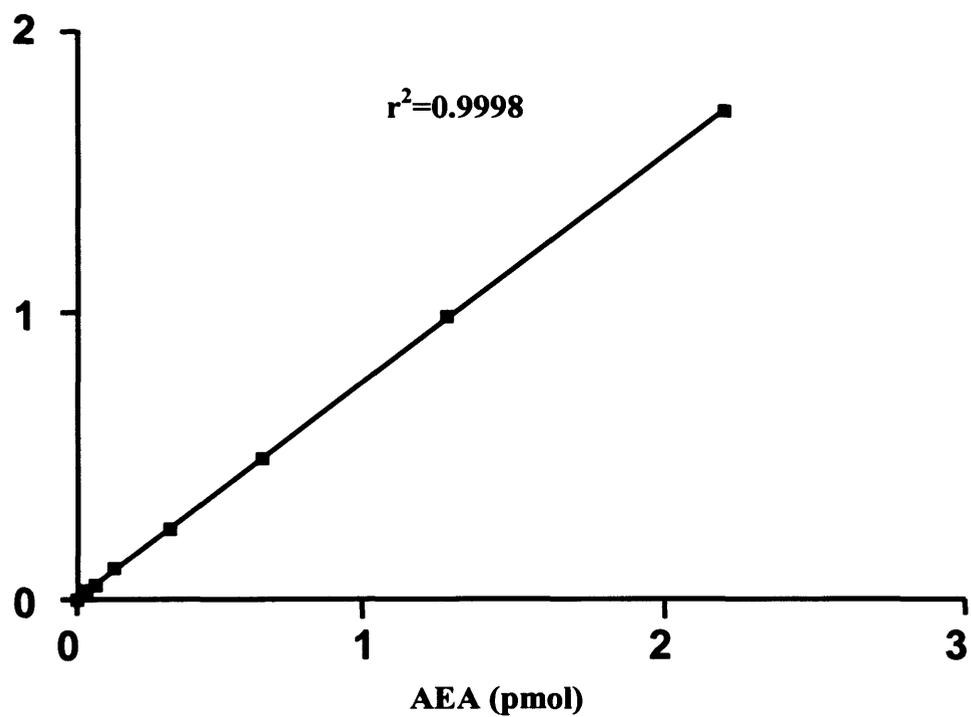


Figure 2.5. A typical standard curve: The x-axis represents amount of AEA injected on the column (pmol). The y-axis is the ratio between the peak areas of AEA/d₈-AEA.

Table 2.5: The results of adding 1 and 2.5 pmol AEA to a plasma sample. The effects of adding authentic AEA to the sample is expected to result in a larger peak size for AEA without affecting the d₈-AEA. Note the small difference between the actual plasma AEA and the expected one, which confirms the accuracy of the method and confirms that the observed peaks are actually AEA. AU: Arbitrary unit.

Sample	AEA peak area (AU)	d₈-AEA peak area (AU)	Ratio of AEA/d₈-AEA peak	AEA on the column (pmol)	Actual Plasma AEA (pmol/	Expected Plasma AEA (pmol/ml)
Neat	647888	2405623	0.27	0.281	2.25	NA
+ 1 pmol	1110538	2849614	0.39	0.406	3.24	3.25
+ 2.5 pmol	1469717	2654213	0.55	0.57	4.58	4.75

the intra-assay and inter-assay coefficient of variations (CV) for the calibration curve at the five standard points. These were calculated as 6% and 9% respectively (Figure 2.6). At this stage of the project, all the blood samples were being analyzed “fresh”, hence it was not possible to calculate inter assay CV for blood samples. The intra-assay CV for blood samples was, however, 7%. Figure 2.6 also demonstrates that all blood samples subsequently measured were well within the range of the standard curve.

2.4. Discussion

In the assay method, a far lower amount of the internal standard compared to the original method described by Giuffrida *et al* (25.1 pmol compared to 500 pmol respectively) was used. Two important differences between the two assays might explain this; firstly, Dr Giuffrida used an internal standard that was manufactured in-house (Giuffrida *et al.*, 2000) while an internal standard that was available commercially was used here. It is possible that the commercially available internal standard is of a higher purity which might explain why a smaller amount was needed for the assay. Secondly, the HPLC/MS machine used in this assay is different to the one used by Giuffrida. It is recognised that different HPLC/MS machines have different sensitivities and therefore it is possible that the observed difference reflects this.

From the various experiments, it was noted that delaying the processing of the samples by more than two hours resulted in an unacceptable loss of anandamide. This might be explained by the fact that such delays encourage uptake of anandamide into the cells either by the proposed anandamide transporter or by the FAAH enzyme. Another explanation could be that the time delay allows the enzyme FAAH to breakdown AEA.

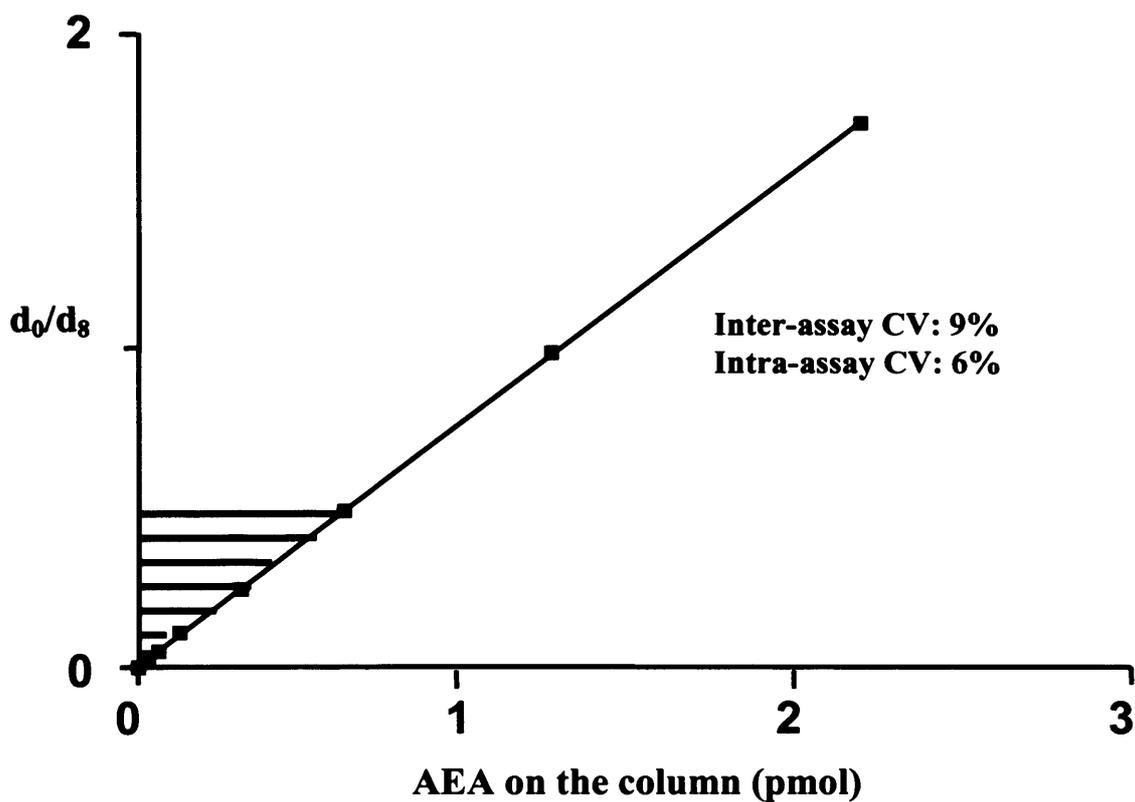


Figure 2.6: The standard curve for the measurement of AEA. Shaded area represents the values of the blood samples tested. The x-axis represents the amount of AEA on the coulumn while the y-axis is the ratio between AEA peak and d₈-AEA peak. All blood samples measured in this project had peak area ratios of < 1.0 (the shaded area).

The fact that anandamide concentrations dropped significantly after two hours have important implications for any future assays for the measurement of anandamide in human plasma.

It is interesting to note that certain vials (Kimble vials) enabled the best recovery of anandamide. Although these vials are not silanized, in the method described here and the original method described by Dr Giuffrida, it was noted that the recovery was significantly better with these vials compared to “standard vials”. This suggests that the actual type of glassware used in these vials potentially does not allow anandamide to “stick” to the vials. The observation that silanised glassware resulted in better recovery is not surprising considering the fact that anandamide is known to adhere to glassware.

Within the procedure of processing blood samples, it was noted that performing 2 acetone steps offered no particular advantage over performing one as originally described by Giuffrida *et al.* A possible explanation is that any protein that remains settles into the aqueous layer after the lipid extraction step. As only the lipid layers were recovered, any protein that might remain in the aqueous layer would not interfere with the assay. It was also demonstrated that a short step of centrifugation after the addition of chloroform: methanol resulted in the same recovery of anandamide compared to allowing the sample to “stand” as was described in the original method. This has a clear advantage in reducing the total length of time needed to process each sample.

The assay developed was both robust and reproducible with intra and inter coefficient variations of 6 and 9% respectively (0.03-2.1 pmol AEA injected), values which are acceptable in any chemical assay. The lower limit of detection in the assay was 0.03125

pmol of AEA injected on the column which is 10 times lower than what was described in the original method. Again this probably reflects the differences in both the HPLC/MS machine used and the differences in the methodology.

2.5. Conclusions

The data presented in this chapter show an improvement on the existing method for the measurement of AEA in plasma and also demonstrates robustness and reproducibility of the assay in the measurement of anandamide concentrations in human plasma. The ability to measure AEA opens many possibilities, not only in the field of reproductive sciences, but also in many other disciplines where AEA is involved. The development of such an assay may suggest that other more difficult matrices, including tissues like the human uterus may eventually become amenable to the extraction and accurate measurement of AEA levels.

Chapter 3

Measurement of plasma anandamide concentrations in women during their reproductive life

Chapter 3

Measurement of plasma anandamide concentrations in women during their reproductive life

3.1. Introduction

The endocannabinoid AEA, as discussed in Chapter 1, has been studied extensively in animals. It has been found to play a key role in blastocyst development and implantation in the murine pregnancy (Schmid *et al.*, 1997; Wang *et al.*, 1999). Despite the evidence from these studies, little has been done in humans partly because of the difficulty in measuring AEA in blood. The findings of low level of FAAH in asymptomatic pregnant women who subsequently miscarry (Maccarrone *et al.*, 2000d) is extremely exciting, as it does suggest that changes in AEA levels might mirror changes in FAAH. In order to establish a potential role for AEA in early pregnancy maintenance, knowledge of the levels in normal pregnancy, of any changes in the levels of AEA that occur through the menstrual cycle, labour and the menopause is essential. The aim of this part of the thesis therefore, was to use the modified method described in Chapter 2 to measure plasma AEA concentrations during the menstrual cycle, pregnancy, labour and the menopause.

Although there was a substantial literature on the measurement of AEA in animal models (Schmid *et al.*, 1995; Felder *et al.*, 1996; Sugiura *et al.*, 1996c; Koga *et al.*, 1997; Schmid *et al.*, 1997; Di Marzo, 1998), there were only few studies on the measurement of anandamide in humans (Felder *et al.*, 1996; Maccarrone *et al.*, 2002b; Schabitz *et al.*, 2002; Schuel *et al.*, 2002a). Additionally, there had been only one study that measured plasma AEA in women

undergoing IVF before conception (Maccarrone *et al.*, 2002b), but no studies on plasma AEA concentrations during the pregnancy. Although there was a suggestion that AEA production could be regulated by gonadal steroids (Maccarrone *et al.*, 2001a; Maccarrone *et al.*, 2001b; Maccarrone *et al.*, 2002a; Maccarrone *et al.*, 2003), there were no studies that had made a connecting relationship between AEA and sex steroid concentrations. To aid in this process, the levels of AEA in the plasma of post-menopausal women was used as a control. There was one study (Dennedy *et al.*, 2003; Dennedy *et al.*, 2004) that implicated AEA in the process of myometrial inactivation during the process of labour using human myometrial smooth muscle cells in culture, but no data to show that AEA was involved in the process. Therefore, plasma levels of AEA were also quantified during labour.

3.2. Subjects and methods

This was a prospective observational study to measure plasma AEA concentrations in a cohort of pregnant and non-pregnant women.

3.2.1. Subjects

All subjects gave a signed informed consent to take part in the study, which was approved by the University Hospitals of Leicester Research Ethics Committee (Appendix 3).

The non-pregnant women were divided into two groups; pre- and post-menopausal. The inclusion criteria for the pre-menopausal women included: aged between 22 and 40, regular menstrual cycle (28-30 days), body mass index (BMI) <27 kg/m², had not

been on any hormonal contraception or therapy for at least three months, not on any drugs (e.g. glucocorticoids, anti-hypertensives, analgesics, recreational drugs) or suffered from any medical disorders (e.g. diabetes mellitus, hypertension, epilepsy, immune disorders, etc). The samples were collected in two sub-groups based on the menstrual cycle (follicular: day 2-7 and luteal: day: 20-25 assessed from patient-reported last menstrual period).

For the purposes of determining the changes in the plasma levels of AEA during pregnancy, 5 groups of women were studied as follows; first, second and third trimesters, term non-labour and term labour. The first trimester was defined as between 6-11⁺⁶ post-menstrual weeks, the second trimester as 13-27⁺⁶ post-menstrual weeks and the third trimester as 28-35 post-menstrual weeks. Term was defined as 37-42 completed post-menstrual weeks.

The inclusion criteria for the pregnant women were uncomplicated singleton pregnancies, accurately dated first trimester ultrasound scans performed between 6 and 10 post-menstrual weeks and no co-existing maternal diseases. Those with multiple pregnancies, intrauterine growth restriction, hypertensive or other vascular disorders of pregnancy, medical disorders (such as diabetes mellitus, epilepsy, thyroid disorders), or were on any medication (including steroids, analgesics over the previous month) or were known to have taken recreational drugs, were excluded. Only women in established labour, defined as cervical dilatation of at least 4 centimetres and 3-4 regular uterine contractions every 10 minutes, were included in the term labouring group.

The inclusion criteria for the post-menopausal women were a minimum of 2 years post-menopause, BMI of $<27 \text{ kg/m}^2$, aged between 55 and 60 years, not on hormone replacement therapy for the preceding three months and not on any drugs (e.g. glucocorticoids, anti-hypertensives, analgesics, recreational drugs) or suffering from any medical disorders (e.g. diabetes mellitus, hypertension, epilepsy, immune disorders, etc).

3.2.2. Methods

The plasma levels of AEA were measured using the developed method described in Chapter 2 and summarised below:

- 1) Blood samples (4 ml) were collected in EDTA tubes on ice and transferred immediately into 120 mm polypropylene conical tubes containing 2 ml of Krebs-Tris-EDTA buffer. All samples were processed within two hours of collection.
- 2) Samples were centrifuged at $1200 \times g$ for 30 min at $22 \text{ }^\circ\text{C}$ to separate plasma from cells. Plasma (2 mls) was recovered with silanised glass pipettes and transferred into 7-ml Kimble scintillation vials. Samples were then spiked with $25.1 \text{ pmol d}_8\text{-AEA}$ as an internal standard.
- 3) Protein was precipitated by the addition of ice-cold acetone (1:1 volume: volume). The resulting samples were mixed by inversion followed by centrifugation at $1000 \times g$ for 10 minutes at $22 \text{ }^\circ\text{C}$. The supernatants were

recovered with silanised glass pipettes and transferred into fresh 7-ml Kimble scintillation vials. Excess acetone was dried under a stream of N₂, a process that took 40 minutes.

- 4) Lipid extraction was performed on the supernatants by the addition of methanol: chloroform (1:2) (1:1 volume: volume). Samples were mixed by inversion and then centrifuged at 1000 x g for 5 minutes at 22 °C. The chloroform (bottom) layer was recovered with silanised glass pipettes and transferred into fresh 7-ml Kimble scintillation vials and evaporated completely under a stream of N₂.
- 5) The dried extracts were reconstituted in 80 µl of methanol: chloroform (3:1) then vortex mixed for one minute. Duplicate 20 µl were injected into the HPLC-MS with the analysis performed on 10 µl.
- 6) HPLC-MS conditions were as described in sections (2.3.1.4) and (2.2.1.4.2).
- 7) The standard curve was as shown in sections (2.2.2.5) and (2.3.2).

3.2.3. Statistical analyses

Power analysis of published AEA data (Maccarrone *et al.*, 2002b) with $\alpha = 0.05$ and $\beta = 0.8$ indicated that the minimum number of subjects required in each study group that

would allow a significant change in plasma AEA concentrations to be observed was six. Data are expressed as mean \pm standard error of the mean (SEM) or standard deviation (SD) where appropriate for each test group and comparison between groups was performed using unpaired Student t-test with Welch's Correction for data with non-equivalent standard deviations and $P < 0.05$ was considered statistically significant. A parametric test (Student t-test) was chosen as the data in this chapter came from a normal distribution (tested by Shapiro-Wilk test for normality).

3.2.4. The accuracy of the assay

Figure 3.1 demonstrates the standard curves obtained from 10 of the "runs" performed in the study. There was good reproducibility of the standard curves between the different runs that were performed over a period of 9 months.

3.3. Results

3.3.1. Subjects

One hundred and two pregnant, menstruating and post-menopausal women were studied. Table 3.1 shows the details of these women. There were 10 subjects in each of the first, second and third trimesters, 22 at term and not in labour and 25 at term and in labour. There were no significant differences in the ages of the women in the reproductive age bracket but as was expected, the age of the post-menopausal women was significantly greater than that of the non-pregnant menstruating group (57 ± 1.98

vs. 32.7 ± 6.1 ; $P < 0.05$). The mean \pm SD gestational age of the term non-labouring and labouring women was similar (39.3 ± 1.28 vs. 39.56 ± 1.26).

The clinical characteristics of the 25 term labouring women are shown in Table 3.2. The mean cervical dilatation at the time of sampling was 6.04 cm (range 4-10 cm), while the mean duration of uterine contractions before the blood sample collection was 5.15 hours (range 1.2-12 hours). Thirteen women had epidural analgesia in labour before sampling and the remaining 12 had a combination of nitrous oxide and oxygen otherwise known as 'gas and air'. None of the women had injectable opioids (Pethidine, Morphine or Dihydromorphine).

3.3.2. Plasma anandamide concentrations

Plasma AEA levels in normal menstruating women, differed between the early follicular phase and the late luteal phases (Figure 3.2). The values in the early follicular phase of the menstrual cycle (1.68 ± 0.16 nM) were significantly higher than those in the late luteal phase of the menstrual cycle (0.87 ± 0.19 nM) ($P < 0.005$). By contrast, plasma AEA levels in the post-menopausal women (0.67 ± 0.005 nM) were significantly lower than those found in women in the follicular phase ($P=0.0002$), but not significantly lower than those in the luteal phase of the menstrual cycle ($P=0.3$) (Figure 3.2), suggesting the involvement of sex steroids in the regulation of plasma AEA levels. The levels of plasma AEA during the first, second and third trimesters of pregnancy were 0.89 ± 0.14 nM, 0.44 ± 0.12 nM and 0.44 ± 0.11 nM respectively. The reduction in levels from the first to second/third trimesters was significantly different ($P=0.04$) (Figure 3.2). There was a significant increase in the plasma AEA levels of

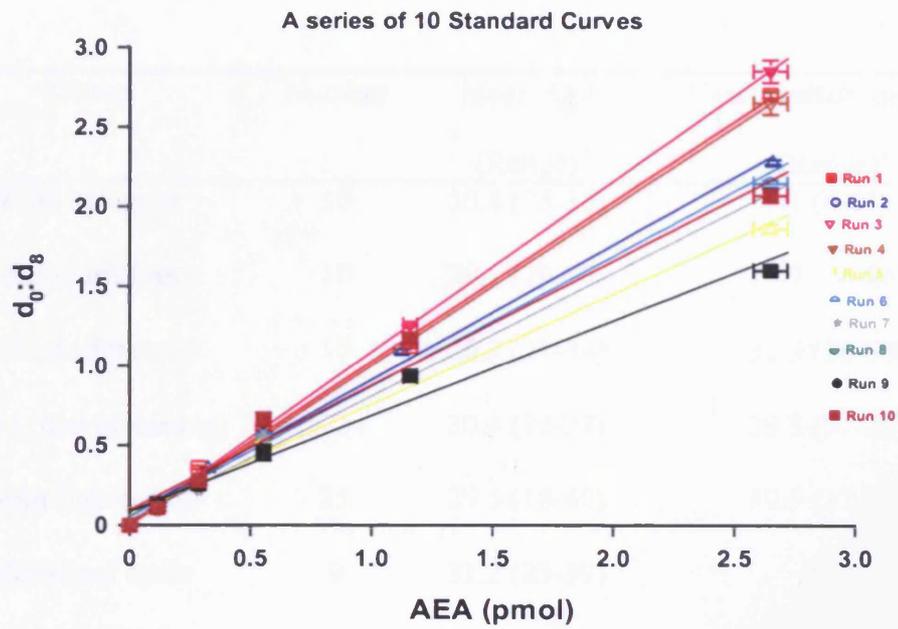


Figure 3.1: A series of 10 standard curves performed over a 9 month period. Note that the variation between the standard curves is at it lowest for at the first three standard points.

Table 3.1. The distribution of 102 women recruited into this study. The number of women in each experimental group, mean age and mean gestational age of the pregnant women are shown. From: (Habayeb *et al.*, 2004).

Group	Number	Mean Age (Range)	Mean Gestational Age (Range)
First trimester	10	30.4 (25-36)	9.1 (6-11)
Second trimester	10	28.1 (20-37)	18.7 (14-26)
Third trimester	10	26.3 (21-34)	31.9 (28-35)
Term (non labouring)	22	30.4 (22-37)	39.3 (37-42)
Term (labouring)	25	29.5 (18-40)	39.5 (37-42)
Menstrual cycle (follicular)	9	31.2 (23-39)	NA
Menstrual cycle (luteal)	8	34.2 (22-40)	NA
Post-menopausal	8	57.7 (55-60)	NA

(NA: Not applicable).

Table 3.2. Clinical characteristics of the 25 women in the labouring group; data are means with ranges in parentheses.

Characteristic	Value: Mean (range)/ Number of women
Mean cervical dilatation (cm)	6.04 (4-10)
Mean length of labour before collection (hours)	5.15 (1.2-12)
Mean interval between sample collection and delivery (hours)	5.26 (0.5-11.25)
Epidural analgesia	13
Entonox (Nitrous Oxide and Oxygen)	12
Mode of delivery:	
1) Spontaneous vaginal delivery	17
2) Forceps	3
3) Vacuum extraction	2
4) Caesarean section	3

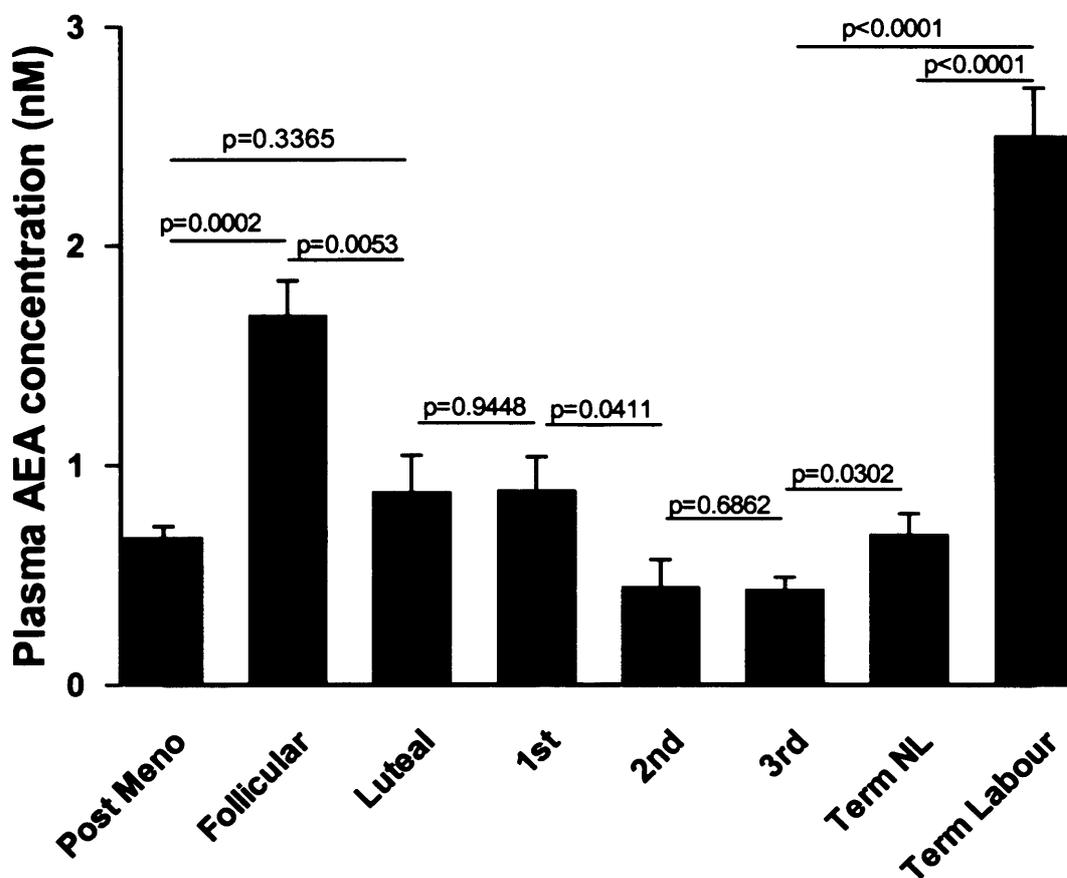


Figure 3.2: Changes in plasma AEA concentrations in postmenopausal, menstruating, pregnant, term non-labouring and term labouring women. Note that the levels in the follicular phase are significantly higher than those in the luteal phase. The levels in the first trimester are similar to those in the luteal phase, but are significantly higher than those in the second and third trimesters. AEA levels increase significantly in the term non-labour group compared to those in the third trimester with the highest increase seen in the labour group. From: (Habayeb *et al.*, 2004).

women who were at term but were not in labour (0.68 ± 0.09 nM) compared to the second and third trimesters ($P=0.03$), but this was over-shadowed by the very dramatic change in the plasma AEA levels of the women in the labour group (Figure 3.2) whose average increase to 2.5 nM was 3.7 times the values in term non-labouring women and 6 times those of women in the third trimester of pregnancy. The plasma AEA levels for women at term and in labour were significantly higher than those of women in the first, second and third trimesters of pregnancy ($P<0.0001$).

When the levels of AEA in the menstruating and post-menopausal women were compared to those during pregnancy, significant patterns also emerged (Figure 3.2). The plasma AEA levels in the luteal phase were similar to those in the first trimester, while the levels in postmenopausal women were lowest amongst all groups tested. The luteal phase and first trimester values were higher than those of second, third trimester and non-labouring groups, but were significantly lower than the levels in the term labouring group. The follicular phase levels were significantly higher ($P<0.001$) than the levels during pregnancy but significantly lower ($P<0.005$) than those in the labouring group.

Further analysis of the characteristics of the group of women in labour revealed that the plasma anandamide concentrations were related to the length of labour (as estimated from sampling to delivery). As can be seen in Figure 3.3 there was a direct linear relationship between AEA levels and the length of time from sampling to the onset of labour ($r^2= 0.2193$, $P<0.0182$), suggesting that plasma AEA and the duration of labour are linked in some way.

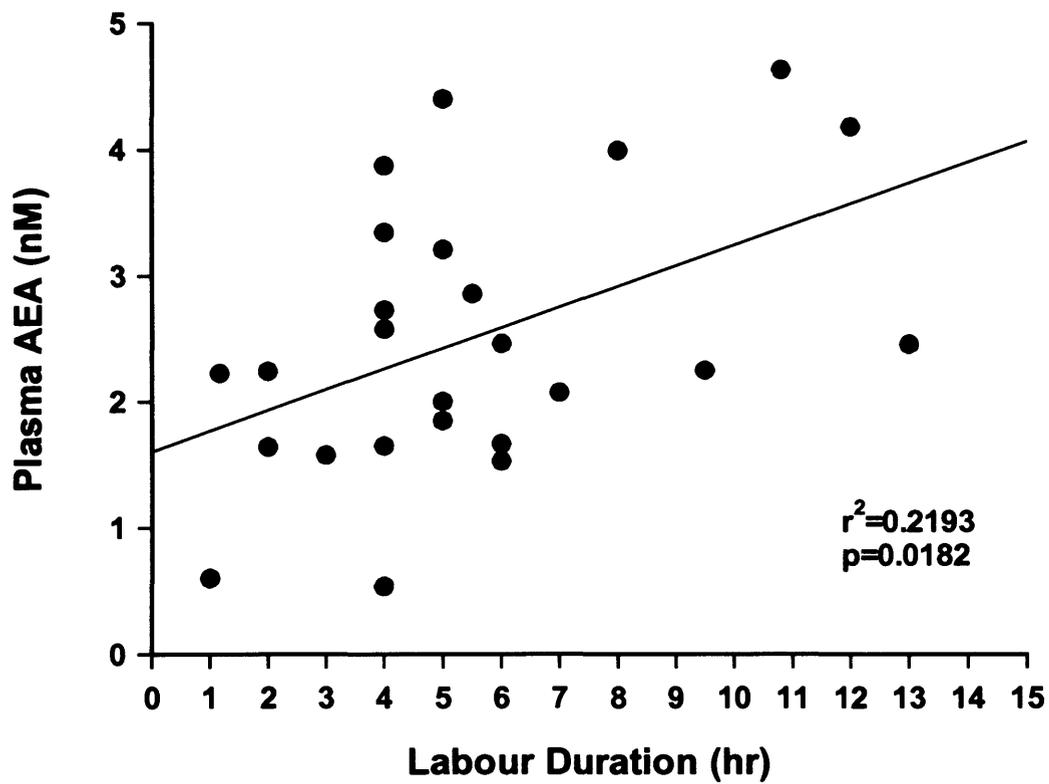


Figure 3.3: Relationship between plasma AEA concentrations and the duration of labour. Note the trend of increase in AEA levels with the increase in the duration of labour. From: (Habayeb *et al.*, 2004).

When the labouring women were divided into three groups based on the total duration of labour (group I =0-5 hours, Group II=6-10 hours and group III=11-15 hours). The mean \pm SEM levels of AEA in the three groups were respectively; 2.1 ± 0.31 nM, 2.58 ± 0.29 and 3.8 ± 0.66 nM. Another characteristic analysed was the relationship between anandamide concentrations and the cervical dilatation at the time of sampling. As can be seen in Figure 3.4 there was no correlation between the cervical dilatation at the time of sampling and plasma AEA levels; ($r^2 =0.092$, $P=0.104$). Although there was a trend for AEA levels to increase with decreasing interval between sampling and delivery, this did not achieve statistical significance ($P>0.05$).

The mean levels in the 13 women who had an epidural analgesia before sampling were similar to those in the group that had only entonox (gas and air) prior to sampling (2.24 v 2.78 nM, respectively) and for the rest of their labour (Figure 3.5). AEA concentrations were similar in smokers and non-smokers irrespective of the physiological state at testing.

3.3. Discussion

These results demonstrate remarkable differences in the concentrations of plasma anandamide during a women's reproductive life. There was a significant reduction in the levels from early follicular to late luteal phase suggesting that hormonal regulation may be involved. The early follicular phase of the menstrual cycle is dominated mainly by estradiol, whereas the late luteal phase is dominated mainly by progesterone. These data suggest that plasma AEA levels could be regulated by these two hormones.

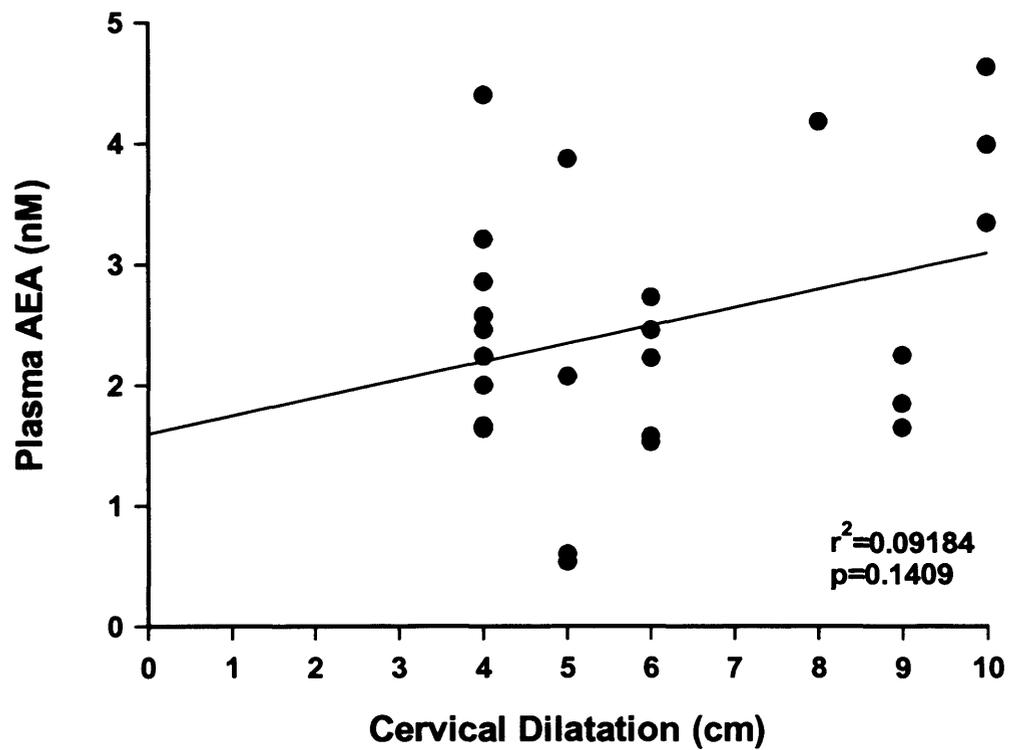


Figure 3.4: Relationship between plasma AEA concentrations and cervical dilatation at the time of sampling. Note that there is no correlation between the cervical dilatation and AEA levels. From: (Habayeb *et al.*, 2004).

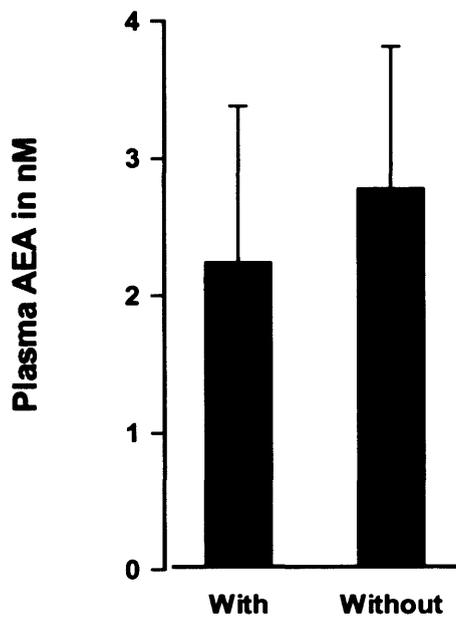


Figure 3.5: The effect of epidural analgesia on plasma anandamide levels. There was no significant difference between the two groups in AEA concentrations with the means of 2.24 nM in the epidural group compared to 2.78 in the group that did not have epidural. Values are means \pm SD. 13 women had epidurals and 12 did not.

The plasma levels did not appear to change in the transition from late luteal to early first trimester of pregnancy, but this is not surprising as these two phases of reproduction are often considered to be similar as far as hormonal regulation is concerned (see Chapter 1).

Plasma AEA concentrations progressively reduced as the pregnancy advanced although there was no change between levels when the levels in the second and third trimesters were compared. There were no obvious reasons for this but it could be argued that the numbers in the study were small and its cross-sectional nature may have masked other patterns. Plasma AEA increased in term non-labouring compared to those of women in the second and third trimesters of the pregnancy, suggesting that the mechanism causing the reduction of plasma AEA levels in the 1st and 2nd trimester of pregnancy was failing. Plasma AEA concentrations in labour were 6-fold higher than those in women in the third trimester and 3.7-fold higher than those in women at term but non labouring group, suggesting that either the mechanism that keeps plasma AEA levels during the 2nd and 3rd trimester of pregnancy have failed altogether, or that a new mechanism has come into play that actively increases plasma AEA levels during labour.

A high plasma AEA level in the follicular phase of the menstrual cycle and very high level in labour both occur in states that are characterized by low and high levels of plasma progesterone, respectively (Borth, 1967; Pomini *et al.*, 1967; Runnebaum & Zander, 1967), suggesting an association between progesterone and AEA levels. Evidence from the literature, suggests that progesterone up-regulates the expression and activity of the enzyme fatty acid amide hydrolase (FAAH), which is responsible for the

breakdown of AEA in peripheral blood mononuclear cells, without affecting the CB1 receptor or the anandamide transporter (Maccarrone *et al.*, 2001b; Maccarrone *et al.*, 2003). The increase in plasma AEA concentrations in the follicular phase compared to the luteal phase could therefore be explained by the very low levels of progesterone in the follicular phase of the cycle and the increased expression of FAAH during the luteal phase of the cycle. The marked increase in plasma AEA levels during labour is more difficult to explain as the plasma levels of progesterone reach their peak during this phase of gestation. However, labour has been considered to be a hyper-estrogenic state (akin to that of the follicular phase of the menstrual cycle) because of the notion of 'functional progesterone withdrawal', where the plasma levels of progesterone are exceedingly high, but the tissues become refractory to its action (Mesiano *et al.*, 2002; Astle *et al.*, 2003). It is therefore easy to speculate that the PMNC would also have to exhibit 'functional progesterone withdrawal', or that another mechanism was active during labour.

If progesterone was the only regulator for plasma AEA levels, then we would expect the levels of AEA to be high in the post-menopausal women, as those women have characteristically extremely low levels of progesterone. The fact that the AEA concentrations are low in that group compared to the follicular phase suggests that estrogen plays a role in the regulation of AEA. The low levels in the post-menopausal women suggest that the presence of estrogen is associated with an increase in the concentrations of AEA. Indeed, it has been shown that estrogen increases AEA concentrations in endothelial cells (Maccarrone *et al.*, 2002a), and of course, although the luteal phase of the menstrual cycle is dominated by progesterone, there is also a

peak of estrogen production during this phase too, albeit smaller than the follicular phase (Pomini *et al.*, 1967).

Messenger RNA (mRNA) for the cannabinoid receptors (CB₁ and CB₂) have been demonstrated in the uterus (Dennedy *et al.*, 2003; Dennedy *et al.*, 2004), although the exact distribution of the receptors within the uterus is unknown. Stimulation of these receptors *in-vitro* results in muscle relaxation (Dennedy *et al.*, 2003; Dennedy *et al.*, 2004). This action and the dramatic rise in AEA levels in labour pose a paradox. The paradox is that the contractions of labour and the relaxing effects of AEA would appear to neutralize each other. How, therefore, could the rise in AEA be explained? It is recognized that in the early phases of labour, the upper segment of the uterus exerts the greatest contractile effect while the cervix has to dilate (Naeslund & Snellman, 1953; Dobson, 1988; Cabrol *et al.*, 1990). Unfortunately, without studies that have quantitatively examined the differential expression/distribution of the receptors in the pregnant myometrium, only speculation can be used to explain the paradox. It may therefore be speculated that there may be a differential distribution of CB₁ and CB₂ with a preponderance of CB₁ to the lower uterine segment. In this scenario, during early labour the high levels of AEA cause relaxation of the lower segment, which then enables the cervix to dilate. An alternative scenario would be that the high levels of AEA allow the muscle fibres to relax between the labour contractions, in preparation for the next series of uterotonic stimuli.

In early pregnancy, low FAAH levels in the PMNCs have been associated with miscarriage (Maccarrone *et al.*, 2000d). As FAAH in PMNCs appears to be the main regulator of plasma AEA concentrations, it would be expected that low FAAH

expression would be associated with high plasma AEA concentrations. Additionally, endocannabinoids are known to have adverse effects on successful pregnancy in mice (Paria *et al.*, 1995; Yang *et al.*, 1996; Paria *et al.*, 1998b; Paria & Dey, 2000), where increased levels have been demonstrated to block the development of two-cell mouse embryos to the blastocyst stage and blastocyst hatching (Paria *et al.*, 1995; Paria *et al.*, 1998b), a mechanism, which may be associated with pregnancy failure or growth restriction of early onset. The lower concentrations of AEA in the luteal phase and the first trimester of the pregnancy compared to concentrations in the follicular phase and late pregnancy and labour will support the importance of adequate regulation of the production of this compound. Since endocannabinoids share a similar mechanism of action to exogenous cannabinoids and the latter are associated with miscarriages, fetal growth restriction and preterm labour (Gibson *et al.*, 1983; Zuckerman *et al.*, 1989; Frank *et al.*, 1990; Sherwood *et al.*, 1999; Lockwood, 2000; Nygren & Andersen, 2001), it may be expected that where there is dysregulation of AEA metabolism, these complications may occur. The low AEA concentrations in the luteal phase and the first trimester are potentially a protective mechanism for the fetus.

Another possible mechanism through which exogenous and endogenous cannabinoids cause adverse pregnancy outcome is via their action on vascular CB₁ receptors where they cause vasodilatation (Hillard, 2000). Such vasodilatation, which has also been described in advanced liver cirrhosis (Batkai *et al.*, 2001), results in hypotension and a decrease in circulating blood volume. In pregnancy, such hypotension will be associated with reduced uterine perfusion with resultant hypoxia; the consequences of which could be intrauterine fetal death or growth restriction. AEA has also been demonstrated to inhibit the hypothalamic-pituitary-axis, specifically inhibiting the

production of growth hormone in the pregnant rat (Wenger *et al.*, 1999b). These combined effects may result in fetal growth restriction. Obviously other mechanisms through which endogenous and exogenous cannabinoids can affect pregnancy may also exist.

It is not clear from the results of this study whether the increase in AEA during labour is a consequence of labour or a cause of labour. It is unlikely that the increases in AEA concentrations are due to pain because there were no correlation between AEA concentrations and the use of epidural analgesia during labour. Labour is associated with increased production of prostaglandins, which are generated from arachidonic acid (Challis *et al.*, 2002), and since AEA may act as a reservoir for arachidonic acid, the increase in plasma AEA levels during labour could potentially be to provide a high reservoir for this precursor for prostaglandin synthesis.

The higher concentrations of plasma AEA in the term non-labouring group compared to the third trimester group suggest that plasma AEA levels might start to increase well before the onset of clinically recognised labour. If this is indeed the case, then measuring plasma AEA concentrations at term before the onset of labour might predict the success of labour induction. If the same increase in plasma AEA concentrations occurs in preterm labour, and if such an increase is preceded by an increase in AEA concentrations before the onset of clinically recognised preterm labour, then measuring AEA in cases of threatened preterm labour might predict those women who are destined to deliver prematurely from those who are not.

3.4. Conclusions

In conclusion, this study demonstrated very distinct patterns of plasma AEA concentrations in pregnancy and two non-pregnant states. The patterns of AEA concentrations in these groups suggest an interplay with gonadal steroids, but the exact relationship remains unclear. It is likely that estradiol increases plasma AEA concentrations whilst progesterone decreases them. The exponential rise in plasma AEA levels during labour compared to those in the third trimester and non-labouring women levels leads to the inevitable question of the value of AEA in labour and how its levels are regulated?

The lower plasma AEA levels in the luteal phase and the first trimester compared to the follicular phase, suggest that a reduction in AEA concentration from values in the follicular phase is essential for implantation and early pregnancy maintenance. It is, therefore, reasonable to postulate that high plasma AEA levels are detrimental to early pregnancy and might be associated with miscarriage.

One technical difficulty in applying this assay to physiological and pathological processes was highlighted during the execution of the study in that samples needed to be processed and analysed on the day of collection. This limited the number of samples that could be collected and analysed. Therefore, ways of storing the samples whilst being able to obtain the same reliable plasma AEA concentrations to improve the performance of this assay became the next step in this project.

Chapter 4

The development of a method to assay anandamide concentrations in stored plasma samples

Chapter 4

The development of a method to assay anandamide concentrations in stored plasma samples

4.1. Introduction

Despite the increasing interest in endocannabinoid research, a major ‘constraint’ in its progress has been the difficulty in measuring AEA. This is primarily because AEA is a highly lipophilic agent that is relatively unstable and rapidly oxidised when exposed to air. The measurements of AEA described in Chapter 3 were performed on freshly obtained blood samples that were processed and measured on the day of collection. This process took ~2-3 hours for a small number of samples (1-6), and more than 6-7 hours for a larger number of samples (7-12). Such a laborious process meant that several man-hours were required to complete the analysis of a relatively small number of samples thereby limiting the number of studies that could be undertaken. It was clear, therefore, that if a method to measure AEA in stored plasma samples was possible, faster progress could be made in this research area. Previous attempts at storage were unsuccessful, as they resulted in loss of AEA that was approximately 50%. To overcome this difficulty, a series of experiments were undertaken in order to develop a reliable method of processing and storing AEA from plasma samples.

4.2. Subjects and methods

4.2.1. Subjects

Twenty four pregnant women who were in the first trimester of the pregnancy, aged between 22 and 42 years were recruited into the study. All participants gave written informed consent to take part in the study. The inclusion criteria were singleton pregnancies with confirmed gestational age by ultrasound scan examination with crown-rump length measurement (CRL) at 6-12 weeks gestation, a body mass index (BMI) of $<27 \text{ Kg/m}^2$, not on any drugs (e.g. glucocorticoids, anti-hypertensives, analgesics, recreational drugs) and not suffering from any medical disorders (e.g. diabetes mellitus, hypertension, epilepsy, immune disorders etc). Every effort was made to ensure that the characteristics in this cohort were similar to those in cohort in chapter 3 to enable comparison.

4.2.2. Methods

Two blood samples (4 ml each) were collected from the antecubital fossa of each woman into EDTA tubes and stored on ice until plasma separation. The samples were analysed using an isotopic dilution HPLC/MS method as described in Chapters 2 and 3. Briefly, proteins were precipitated with acetone, the acetone evaporated under a stream of nitrogen gas and lipids extracted into chloroform/methanol. Samples were either injected into the HPLC/MS and measured against a standard (calibration) curve of known quantities of anandamide “fresh” or stored, once the optimum storage

conditions were defined, for variable length of time “stored” at -80°C until the day of analysis. On the day of analysis, the “stored” samples were thawed on ice, reconstituted in methanol: chloroform (3:1; $80\mu\text{l}$) and subsequently analysed in the HPLC-MS in the same way as the “fresh” samples.

4.2.3. Experiments

In order to determine the optimal way of storing samples before analysis, blood samples were collected from three healthy volunteers and divided into four categories:

1. Fresh plasma (‘Fresh’).
2. Separated plasma spiked with the internal standard ($\text{d}_8\text{-AEA}$; 25.1pmol) and stored at -80°C (‘Plasma’) for seven days.
3. The same as step 2, but storage after protein precipitation (‘Protein Precipitated’).
4. Lipids extraction into chloroform: methanol evaporated to dryness and then stored as dry powders at -80°C (‘Dried Extract’).

4.2.4. Analysis

Quantified plasma AEA from each of the three options of storage was compared to the levels from the established method (i.e. fresh). From the results of these initial experiments, the most appropriate method of storage was determined. Plasma AEA levels measured in both freshly prepared and stored samples from the 24 first trimester

patients were compared and an analysis for variability and robustness was undertaken using the equation:

$$\% \text{ Change} = 100\% \times \frac{[AEA]_{\text{stored}} - [AEA]_{\text{fresh}}}{[AEA]_{\text{stored}}}$$

4.3. Results

4.3.1. Experiments

The results of the storage experiment are shown in Table 4.1 and graphically in Figure 4.1. Storage of plasma, either with proteins ('plasma') or without ('protein precipitated') led to a significant (~50%) reduction in the levels of measurable AEA. If the samples were processed through to a 'dry extract' and then reconstituted in chloroform/methanol before being injected into the HPLC/MS the levels were the same as the levels in immediately processed ('fresh') samples with the mean percentage change of $-0.83 \pm 3.5 \%$ (SD). These data suggest that the only accurate way of storing plasma samples is as dry extracts after lipid extraction. To ensure the robustness of this approach and to study the effects of storage for a longer period of time, i.e. up to 6 weeks, the 24 blood samples from pregnant women were processed to "dry extracts" powders and stored between 7 and 42 days before measurement.

4.3.2. Study group

Table 4.2 shows the patient demographics, the gestational age at which the samples were collected, length of storage and the plasma AEA concentrations obtained from "fresh" and "stored" samples. The results in this cohort revealed little difference in plasma AEA concentrations between the stored and fresh samples (Figure 4.2) with the mean percentage change in plasma AEA levels of $-1.26 \pm 3.7 \%$ (SD), which compared favourably with the results from the experimental study value of $-0.83 \pm 3.5 \%$ (SD).

Table 4.1. Results of the storage experiments. Note that AEA concentrations showed little change when the samples were processed as dry extracts but varied significantly when stored as plasma with without protein precipitation.

Volunteer Number	AEA nM (Fresh)	AEA nM (Stored as plasma) (% change)	AEA nM (Stored after protein precipitation) (% change)	AEA nM (stored as dry sample) (% change)
1	1.32	0.74 (-44)	0.80 (-40)	1.28 (-3.3)
2	1.18	0.68 (-42)	0.64 (-46)	1.23 (+4.2)
3	0.89	0.32 (-64)	0.45 (-49)	0.68 (-3.4)

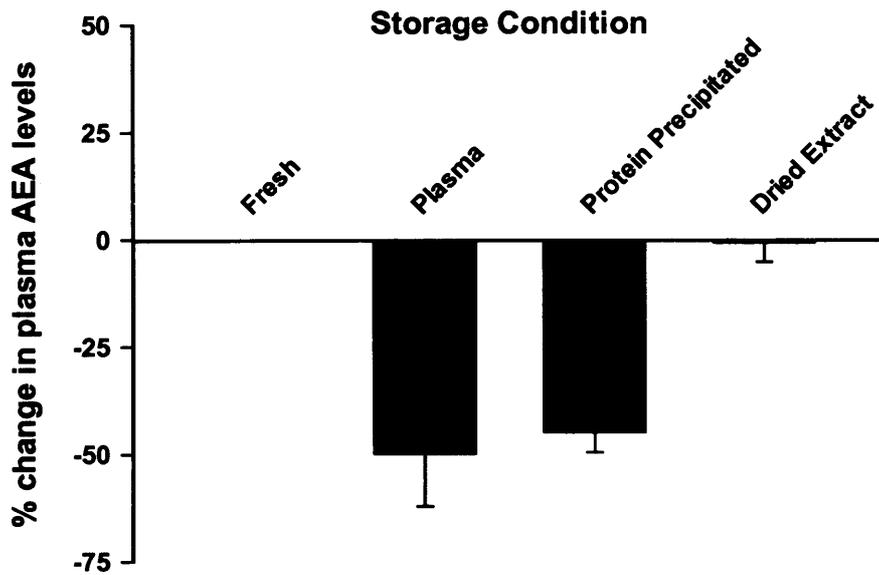


Figure 4.1: The effect of storage at different extraction stages on AEA levels. The % change in measured AEA levels is shown for plasma samples stored for 7 days either without ('plasma') or with protein precipitation ('protein precipitation') compared to the level in samples processed and analysed immediately ('fresh'). This shows that plasma cannot be stored fresh, but needs to be either extracted and assayed immediately or processed to a dry powder for storage. Data are mean \pm SD; n=3.

Table 4.2. Patient demographics and plasma AEA concentrations in freshly analysed and stored samples. A decrease in AEA plasma measurement is indicate with a “-“and an increase indicated with a “+”.

Patient Number	Gestational Age (Weeks ⁺ days)	Maternal Age (Years ⁺ month)	Plasma Anandamide Concentration (nM) Fresh	Plasma Anandamide Concentration (nM) Stored	Storage Interval (Days)	Change in AEA concentration (%)
1	7 ⁺⁴	29 ⁺⁷	1.408	1.459	7	+3.6
2	6 ⁺⁴	42 ⁺⁸	1.664	1.743	7	+4.7
3	6	30 ⁺⁸	1.384	1.424	18	+2.9
4	9	35 ⁺⁸	2.240	2.151	16	-4.0
5	6	29 ⁺⁴	3.340	3.263	15	-2.3
6	6 ⁺⁶	31 ⁺¹	1.320	1.259	15	-4.6
7	6 ⁺⁵	29 ⁺⁹	1.648	1.554	12	-5.7
8	6 ⁺⁶	25 ⁺⁵	1.800	1.730	26	-3.9
9	11 ⁺⁵	25 ⁺⁶	1.440	1.373	26	-4.6
10	8 ⁺⁵	32 ⁺⁹	1.490	1.427	26	-4.2
11	6	38 ⁺⁸	2.160	2.100	26	-2.8
12	7	25 ⁺⁶	1.456	1.376	26	-5.5
13	10	38 ⁺⁸	2.160	2.093	26	-3.1
14	7 ⁺³	27 ⁺⁶	1.144	1.094	25	-4.4
15	7 ⁺³	22 ⁺⁹	1.056	1.004	25	-4.9
16	7	29 ⁺⁹	0.920	0.875	21	-4.9
17	7 ⁺¹	27 ⁺⁹	0.890	0.916	28	+2.9
18	8 ⁺¹	24 ⁺⁶	0.930	0.901	28	+3.1
19	9 ⁺¹	33 ⁺¹	3.760	3.865	28	+2.8
20	11 ⁺¹	27 ⁺⁹	0.68	0.701	28	+3.1
21	7 ⁺⁶	26 ⁺¹¹	0.660	0.637	24	+3.5
22	10 ⁺¹	22 ⁺⁹	1.060	1.040	24	-1.9
23	8	30 ⁺⁹	0.760	0.781	42	+2.7
24	8 ⁺⁴	26 ⁺²	3.470	3.37	42	-2.9

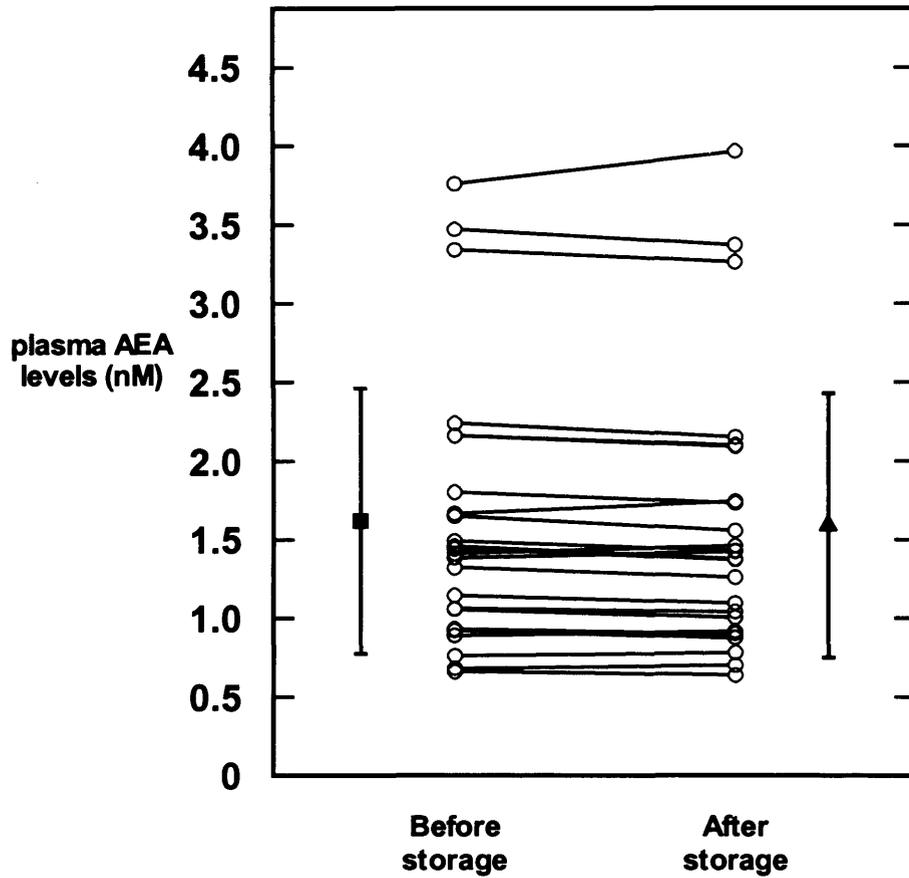


Figure 4.2: The effect of storage on plasma anandamide levels from pregnant women. Samples were analysed immediately or stored as dried extracts at -80°C for the intervals shown in Table 1 before analysis and are joined by a line for each individual patient. The data are also presented as mean \pm SD for the fresh samples (■) and for the stored samples (▲); n=24.

An analysis of the amount of error in the measurement of the sample as a function of storage duration (Figure 4.3) indicates that all the data were within a 5% band of error and that storage duration had no effect on this error or the final measurement. There was a strong correlation between the AEA levels before and after storage with $r^2=0.995$ (Figure 4.4) indicating that there was a good spread of samples to analyse and a Bland-Altman plot of the variation indicated that the variation on measurement was within the acceptable constraints of 5% from the norm (Figure 4.5).

4.4. Discussion

Due to the lipophilic nature of AEA and the fact that AEA is relatively unstable and rapidly oxidised when exposed to air, plasma measurements of AEA are often difficult (Giuffrida & Piomelli, 1998; Giuffrida *et al.*, 2000). Up to this point analysis of plasma AEA had been performed on freshly collected and processed samples. Whilst the method is reproducible and robust, the fact that the samples had to be analysed fresh meant that there were limitations on the number of samples that could be tested. Initial experiments of storing whole blood samples, plasma samples and partially processed samples after precipitation of proteins were all disappointing as they showed variation between the “fresh” samples and “stored” samples of almost 50%. This study demonstrated that the one reliable way of storing samples within this lipid extraction method of analysis was by storing “dried extracts”.

The ability to store samples offers many advantages as it allows a higher throughput of samples, sharing of samples through different laboratories to obtain a consensus on plasma AEA concentrations, allows multi-centre sample collection and contributes to

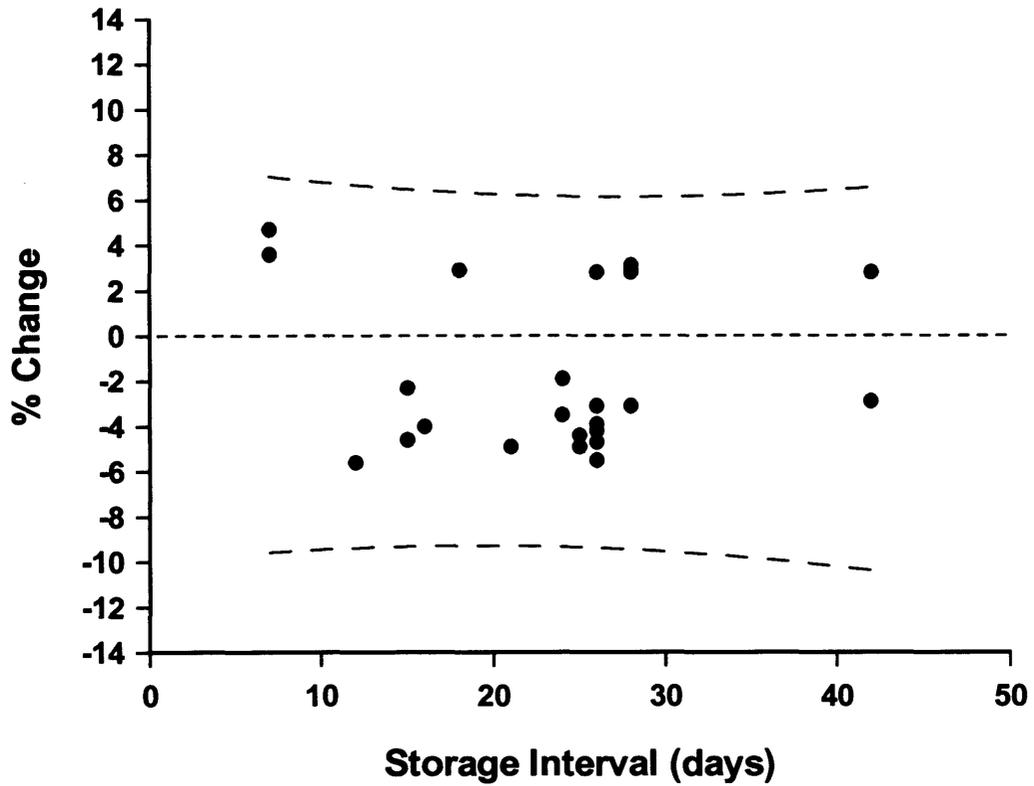


Figure 4.3: Lack of effect of storage duration on plasma anandamide measurement values. The data are the % change in AEA measurement values for each of the 24 samples and are bounded by the 95% confidence intervals for the data (dashed line). Patient numbers 18 and 20 produced similar variances in measurement and so appear as a single point.

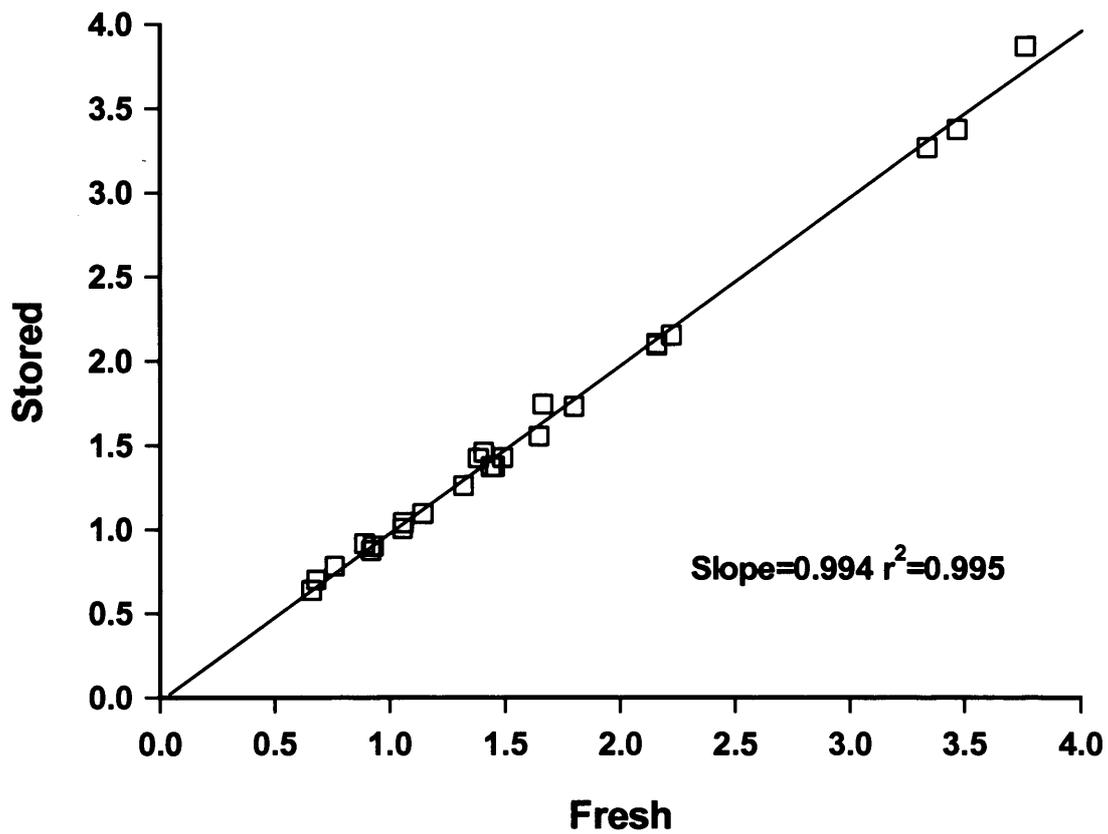


Figure 4.4: Evaluation of the degree of agreement for the effect of sample storage. The figure shows the correlation between 'stored' anandamide levels and 'fresh' anandamide levels in first trimester plasma. Linear regression analysis was used to calculate the 'line of best fit' and the regression co-efficient (r^2) is shown. The data are the % change in AEA measurement values for each of the 24 samples and are bounded by the 95% confidence intervals for the data (dashed lines). Patients 18 and 20 produced similar variances in measurement and so appear as a single point.

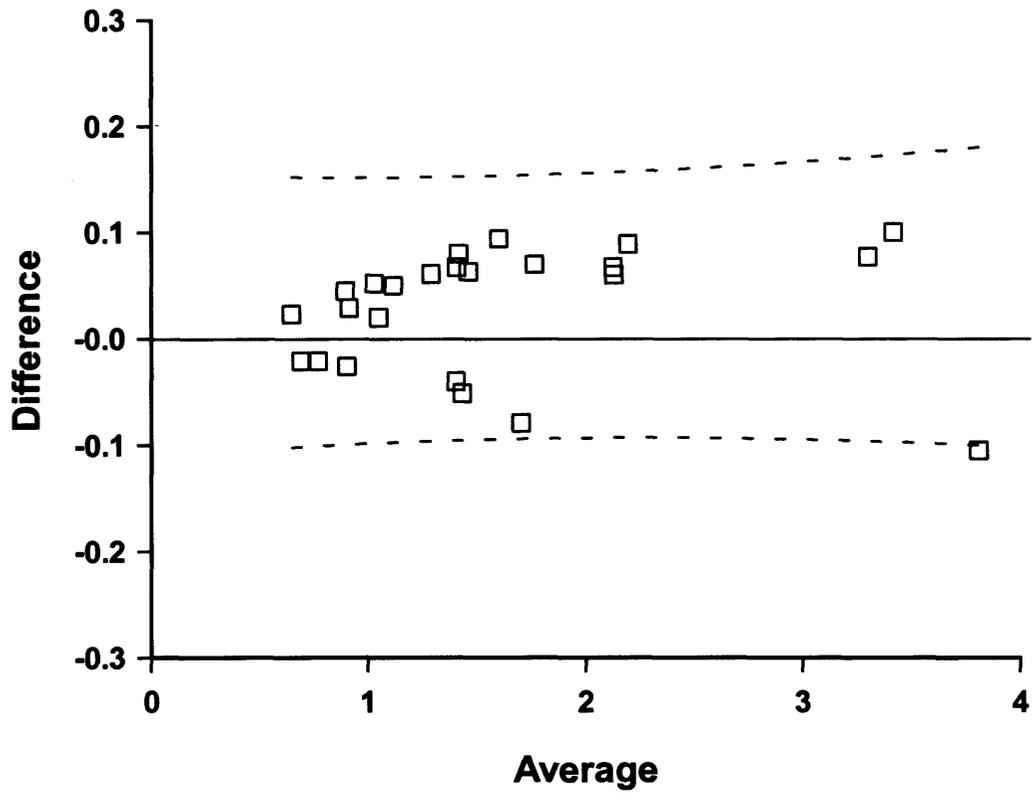


Figure 4.5: A Bland-Altman plot showing the lack of effect of storage duration on plasma anandamide measurement values.. The data are the % change in AEA measurement values for each of the 24 samples and are bounded by the 95% confidence intervals for the data (dashed lines).

the development of a more standardised test in analytical laboratories. The ability to store samples also allows the recruitment of a larger number of women and thus larger studies to investigate other potential roles for AEA in reproduction.

4.5. Conclusions

The data presented in this chapter indicate that extraction to the 'dry extract' stage provides a reliable method to store plasma samples for later "batch" quantification of AEA since the recovery of AEA remained relatively unchanged over the study period which varied between 7 and 42 days. Whilst samples have not been stored for longer than 42 days, it is anticipated that longer storage times would yield similar data.

Chapter 5

The measurement of plasma anandamide concentrations in women with threatened miscarriages

Chapter 5

The measurement of plasma anandamide concentrations in women with threatened miscarriages

5.1. Introduction

The results from Chapter 3 revealed that AEA plasma concentrations in the luteal phase of the menstrual cycle were significantly lower than those in the follicular phase and that the levels in the first trimester of the pregnancy were similar to those in the luteal phase. These data suggested that low plasma levels may be associated with early pregnancy success. Previous studies by Maccarrone and co-workers showed that the levels of FAAH in peripheral blood mononuclear cells (PMNCs) in early pregnancy predicted spontaneous miscarriage (Maccarrone *et al.*, 2000d). In addition, the levels and activity of FAAH in PMNCs in women who failed to achieve ongoing pregnancy following embryo transfer in IVF pregnancies were lower than in those who achieved an ongoing pregnancy (Maccarrone *et al.*, 2002b). Taken together, these findings suggest that the concentrations of plasma AEA in women who miscarry may be higher than levels in those in whom the pregnancy continues. Since failed pregnancies are known to be associated with lower progesterone levels and progesterone has been found to be the main regulator of FAAH in PMNCs (Maccarrone *et al.*, 2001b)

(Maccarrone *et al.*, 2003), it could be adduced that low plasma progesterone levels would lead to lower FAAH activity and therefore high plasma AEA concentrations.

Although there have been no previous reports about the possible relationship between early pregnancy failure and plasma AEA concentrations, the fact that low levels of FAAH in PMNCs predicted spontaneous miscarriage suggests that elevated plasma AEA levels might predict the outcome of pregnancies when measured in the first trimester.

The aim of this study was therefore to measure plasma AEA levels in a group of women with threatened miscarriages, to establish:

- A. Firstly, that plasma AEA levels are indeed higher in threatened miscarriages destined to fail compared to those that end in viable pregnancy.
- B. Secondly, if there is definitive level of plasma AEA above which a woman is likely to miscarry.
- C. Thirdly, to establish a possible predictive value for such a test.

5.2. Subjects and methods

5.2.1. Subjects

This was a prospective comparative study. All women participating in the study gave informed written consent. For this study, only women with a threatened miscarriage were included. Threatened miscarriage was defined as any form of bleeding from the genital tract before 24 weeks of gestation that was associated with minimal abdominal

pain and confirmed fetal viability on ultrasound. Women were recruited from the Early Pregnancy Assessment Unit (EPAU) at the Leicester Royal Infirmary.

Inclusion criteria were gestational age between 6 and 12 weeks, healthy women not on any drugs (e.g. glucocorticoids, anti-hypertensives, analgesics, recreational drugs) or suffering from any medical disorders (e.g. diabetes mellitus, hypertension, epilepsy, immune disorders, etc), a body mass index (BMI) $<30 \text{ Kg/m}^2$, and singleton pregnancy. Power analysis of plasma AEA (see Chapter 3) and FAAH levels with $\alpha=0.05$ and $\beta=0.8$ indicated that the minimum number of subjects in each group that would allow a two-fold difference in plasma AEA levels to be observed was six. Recruitment therefore continued until at least 6 women of those presenting with threatened miscarriage miscarried.

5.2.2. Methods

Blood samples were collected and analysed fresh. The blood samples were collected immediately after the scan. A subgroup of the women in this study had two blood samples collected in order to evaluate the ability to store blood samples (see Chapter 4). All blood samples were processed within two hours of collection. Blood samples were processed and then analysed by HPLC-MS as described in Chapters 2 and 3.

5.2.2.1. Statistical analyses

Data are expressed as median (interquartile range-IQR) and the two groups were compared using the (2-tailed Mann-Whitney *U* test). A non-parametric test was used as

the data did not come from a normal distribution (i.e. failed Shapiro-Wilk test for normality).

5.3. Results

A total of 45 women were recruited into this study. Of these, 36 had live births and 9 had spontaneous miscarriages. The demographic characteristics of these women are summarised in Table 5.1. There were no differences between the women who miscarried and those who had live births in terms of age and gestational age at recruitment.

The median plasma anandamide concentration in the miscarriage group (3.47 nM; interquartile range, 2.83-3.86) was approximately 3-fold that in the live birth group (1.07 nM; interquartile range, 0.81-1.45) (2-tailed Mann-Whitney *U* test $P < 0.001$) (Figure 5.1). For the 36 women who had live births, the mean plasma AEA concentrations were 1.24 ± 0.12 nM (mean \pm SEM). Of the 36 women who had live births, 34 had plasma AEA concentrations that were < 2.0 nM. The other two women had plasma AEA concentrations of 4.65 and 2.25 nM, respectively. The woman who had plasma AEA concentration of 2.25 nM at the time of threatened miscarriage (the solid square in Figure 5.1), went on to have a difficult pregnancy with severe pre-eclampsia and was delivered of a growth restricted baby who weighed 1.85 kg at 33 weeks gestation. By contrast, the woman who had a plasma AEA concentration of 4.2 nM at the time of threatened miscarriage had a pregnancy that was uncomplicated and a healthy 3.35 kg baby was delivered following spontaneous labour 39⁺¹ weeks of gestation.

Table 5.1: Characteristics of the study group.

	Live Birth	Spontaneous Miscarriage
Number	36	9
Body mass index*, mean	24.6 (2.4)	25.1 (1.9)
Gestation at recruitment: Mean (Range)	8 (6-11 ⁺¹)	8 ⁺¹ (6 ⁺⁵ -10)
Anandamide concentrations, median (IQR), nM	1.07 (0.81-1.45)	3.47 (2.83-3.86)
Age: Mean \pm SD	28 \pm 1.5	29 \pm 1.6
Interval between recruitment and miscarriage in days: Mean (Range)	NA	7.83 (5-14)
Gestation at delivery or miscarriage (weeks): Mean \pm SD	39 ⁺¹ \pm 1 ⁺⁵	8 ⁺⁰ \pm 1 ⁺¹
Birth weight (kilograms): Mean \pm SD	3.40 \pm 0.52	NA

Abbreviations: IQR, interquartile range; NA, not applicable.

*Calculated as weight in kilograms divided by height in meters squared.

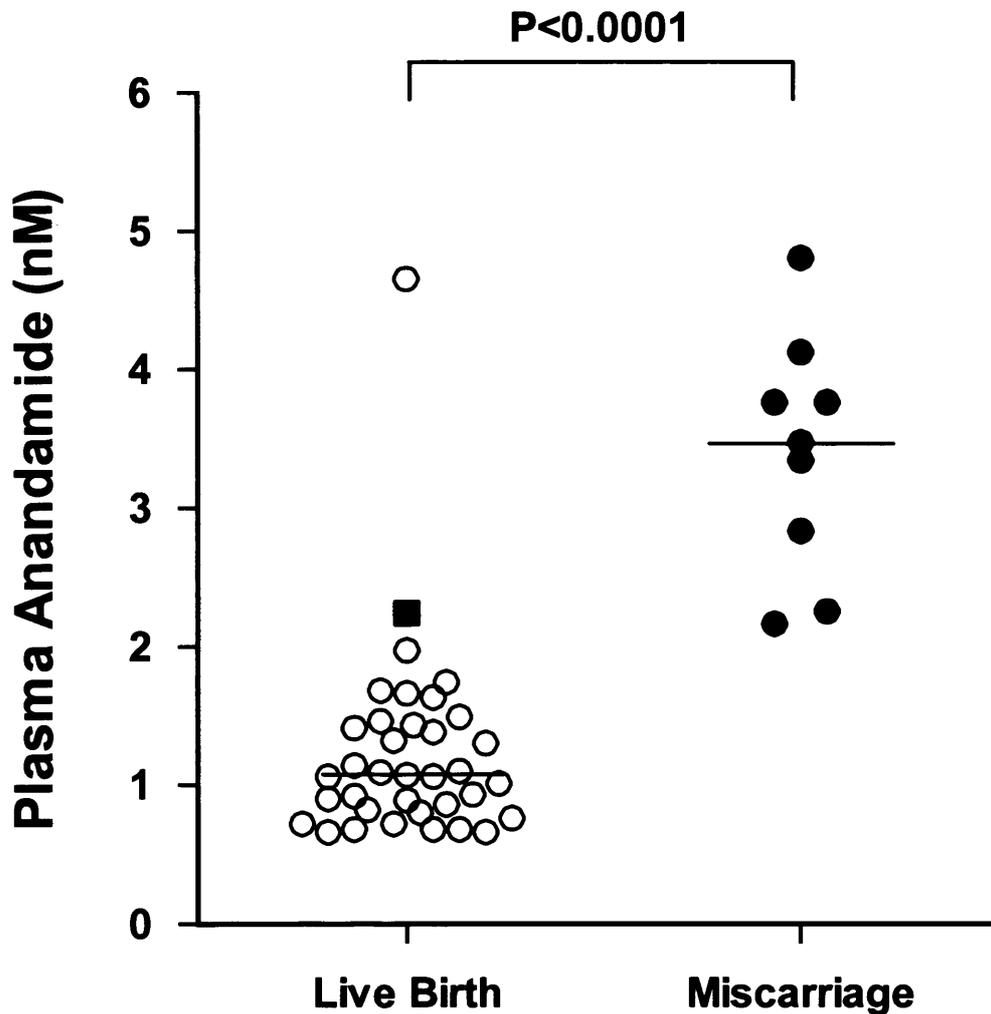


Figure 5.1: The data are grouped according to those who proceeded to have a live birth and those who miscarried and are shown as individual cases. The median for each group is indicated by a horizontal solid line. The plasma anandamide levels were significantly different ($P<0.001$; 2-tailed Mann-Whitney U test). Square indicates patient who developed severe preeclampsia and delivered at 33 weeks' gestation of baby that weighed 1.85 kg. From (Habayeb *et al.*, 2008b).

All of the nine women who subsequently miscarried had AEA values > 2.0 nM and six out of these nine women had AEA values > 2.2 nM. An ROC curve analysis (Figure 5.2) of the threatened miscarriage data revealed that by using a plasma AEA level of 2.0 nM as the optimal cut-off point, a single plasma AEA measurement in women with threatened miscarriages in the first trimester, provided a sensitivity of 100% (95% CI 66.37-100) and a specificity of 94% (95% CI 81.34-99.32) with a negative predictive value of 100% (95% CI 89.6-100) and a positive predictive value of 81.8% (95% CI 48.2-97.2) for subsequent miscarriage. In other words, if the plasma AEA concentrations were < 2.0 nM, there was a 100% chance of a live birth for this group of women.

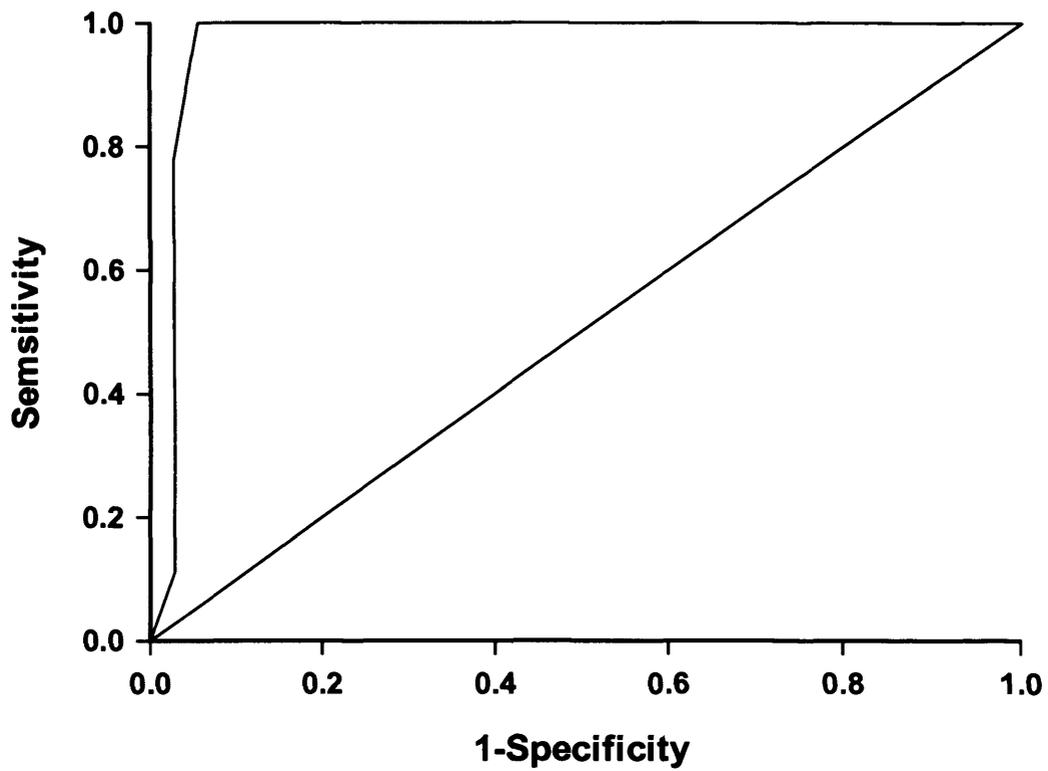


Figure 5.2: ROC analysis of the plasma AEA values for women with threatened miscarriage. The ROC curve and co-efficients were calculated using an arbitrary cut-off of 2 nM.

5.4. Discussion

The results of this study revealed that the plasma concentrations of AEA > 2 nM in cases of threatened miscarriages may help predict which of these pregnancies will ultimately end up in a spontaneous miscarriage and which will end in a live birth. This is very important clinically as most patients with a threatened miscarriage are very anxious and an appropriate and accurate AEA plasma measurement might help to categorise patients into those who are at risk of miscarriage and those who are likely to have an ongoing pregnancy.

Previous studies have revealed that levels of FAAH might have a similar predictive value; FAAH levels being lower in cases of miscarriages compared to those in pregnancies that end up with ongoing pregnancies (Maccarrone *et al.*, 2000d). In the FAAH study, levels of FAAH were measured in 50 women in early pregnancy (7–11 weeks gestation) and were found to be significantly lower in 7 women who miscarried compared to that in the 43 women who had successful pregnancies (Maccarrone *et al.*, 2000d).

From the results of this study, a threshold value for FAAH activity was identified. In a later study by the same group, the enzyme activity was measured in 150 asymptomatic healthy pregnant women at 8 weeks gestation. In 15 of these women the activity of the enzyme was below the threshold and all of them miscarried. In contrast, only one out of the 135 who had FAAH activity above the threshold miscarried (Maccarrone *et al.*, 2000d).

The AEA findings are in keeping with these in Chapter 3 and the levels of plasma AEA appear to be inversely related to the levels of progesterone. Therefore, low plasma progesterone levels leads to lower levels and activity of FAAH leading to higher AEA concentrations. What is particularly interesting about this study is that plasma AEA concentrations were raised 5-13 (mean 7.83) days before the miscarriage. This suggests that a single measurement of plasma AEA in the first trimester may act as a useful predictor for the risk of miscarriage. With the improvement of the assay and the ability to measure AEA in stored plasma samples (see Chapter 4), there is the potential for the development of an bed-side assay, with the potential to significantly improve the prediction of the outcome and the counselling of women who present with threatened miscarriages.

As discussed earlier (see Chapter 1), animal studies in the murine pregnancy have shown that the local concentrations of AEA in the uterus regulate its receptivity towards the developing blastocyst with the implantation sites containing significantly lower AEA concentrations compared to the non-implantation sites (Schmid *et al.*, 1997). AEA in the mouse has been shown to be embryotoxic (Yang *et al.*, 1996) (Paria *et al.*, 1998b). Studies on the levels of FAAH enzyme in the murine uterus show an opposite pattern to that of AEA with FAAH higher in implantation sites compared to the non-implantation sites (Paria *et al.*, 1996). These findings suggest that AEA has to be kept below a critical level in order to achieve successful implantation and protect the fetus. Whether the same changes occur in the human pregnancy is not known, as the implantation mechanisms are distinctively different. There are no data on the expression of the endocannabinoid system in early human pregnancy or on the effects

of AEA on the development and growth of the trophoblasts. This will be covered in the next chapter.

The high AEA concentrations in the woman, who had an ongoing pregnancy, though with severe pre-eclampsia (PET) and fetal growth restriction (FGR), are very interesting and need further investigation to explore whether it was a one-off observation or an observation related to complicated pregnancies. It is well established that PET and FGR are two conditions that have a common pathophysiological mechanism, namely failure of the trophoblasts to invade the walls of blood vessels and alter the placental circulation into a high capacity low resistance system (Pijnenborg *et al.*, 1980; Olofsson *et al.*, 1993; McMaster *et al.*, 1994). Whilst it is not possible to draw any meaningful conclusions from one result, if this observation is confirmed, it suggests that plasma AEA levels might act as an early predictor of implantation problems that might result in a spectrum of conditions, including miscarriage, PET and FGR. In this context, a single measurement of AEA in the first trimester has the potential to identify these pregnancies that are destined to result in adverse obstetric outcomes.

What is not very clear is exactly what the nature of the interaction between the developing placenta and plasma AEA might be; it is possible that early human placental tissue expresses FAAH, which protects the developing fetus from the potential harmful effects of high maternal plasma levels of AEA. It may therefore be postulated that pregnancies that are destined to miscarry may have defective FAAH either locally in the placenta or peripherally in the PMNCs or in both, which results in a higher plasma AEA concentrations in these women. Therefore, as a first step in trying

to understand if plasma AEA levels are an indicator of impending miscarriage, or its expression is the cause of irrevocable trophoblast damage during the early stages of pregnancy, it would be important to study the expression of the endocannabinoid system in first trimester placenta and determine whether the receptors or FAAH enzyme is modulated.

5.4. Conclusions

Plasma AEA levels measured in the first trimester in women with threatened miscarriages revealed a threshold above which subsequent miscarriages could be predicted. An AEA concentration below 2.0 nM had a 100% predictive value of an ongoing pregnancy, whilst a value >2.0 nM had an 81.8% predictive value of miscarriage. Since the AEA concentrations were raised 5-13 days before the actual miscarriage, there is the potential to investigate strategies that could modify AEA levels to prevent the occurrence of miscarriages.

Chapter 6

Expression of the endocannabinoid system in human first trimester placenta and its role in trophoblast proliferation

Chapter 6

Expression of the endocannabinoid system in human first trimester placenta and its role in trophoblast proliferation

6.1. Introduction

From the studies in Chapters 3 and 5 that demonstrated that plasma anandamide levels change during pregnancy and labour and that the levels in women with threatened miscarriages that subsequently miscarried were significantly higher than in those who went on to have live births, it can be extrapolated that anandamide most likely plays a role in the success of pregnancy. What remains unclear is what the potential target tissues might be for anandamide especially in women with threatened miscarriage. The demonstration that both FAAH and the cannabinoid receptor CB₁ are expressed in term human placenta and fetal membranes (Park *et al.*, 2003) suggest that the endocannabinoid system may be present throughout gestation, however, this has not been extensively investigated especially in early pregnancy and there are no data on the expression of CB₂ in early gestational tissues. Additionally, the effect of AEA on trophoblast growth and survival has not been examined.

The observed changes in AEA levels during early pregnancy raise the question of what effects AEA has, if any, on the growth and development of the trophoblast. Studies in the murine pregnancy have shown that AEA affects the development of the mouse embryo (Yang *et al.*, 1996), but whether AEA has similar effects on the human embryo remains unknown.

The aims of this part of the project were to; (a) determine whether FAAH and the cannabinoid receptors, CB₁ and CB₂, are expressed in the trophoblast during the first trimester of pregnancy, (b) describe any changes that might occur during gestation and (c) determine if AEA has any effect on the growth of a human cell line model of the first trimester trophoblast.

6.2. Subjects, materials and methods

6.2.1. Subjects

The study was approved by the University Hospitals of Leicester Research Ethics Committee. All women participating in the study gave signed written informed consent. The study population consisted of women undergoing surgical termination of the pregnancy between seven and twelve weeks. All women had an ultrasound scan for confirmation of gestational age prior to the procedure. Women known to suffer from any medical illnesses or who were on any medications were excluded.

6.2.2. Tissue collection

Products of conception were collected immediately after the surgical procedure. Collected samples were transported on ice to the laboratory, where blood was washed away with sterile phosphate-buffered saline and two pieces of trophoblast dissected free with the aid of a dissecting microscope. One piece was fixed in 10% neutral-buffered formalin for 4 days before embedding in paraffin wax. A second portion of tissue (~100mg) was placed into a sterile polypropylene tube and snap-frozen in liquid

nitrogen for 5 minutes. The latter samples were stored at -80 °C for RNA analysis. Archival term placental tissues from women undergoing elective Caesarean section acted as positive controls.

6.2.3. RNA studies

6.2.3.1. RNA preparation

Total cellular RNA was extracted from frozen placenta samples using TRIZOL reagent (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Briefly, approximately 100 mg of defrosted trophoblast sample was homogenised in 1 ml of TRIZOL reagent using an UltraTurrex® blade homogeniser followed by 20 strokes in a tight fitting glass-glass Dounce® homogeniser. The homogenate was transferred to a microfuge tube and 200 µl chloroform added. The mixture was shaken vigorously for 15 seconds and then allowed to stand at room temperature for 3 minutes. After centrifugation for 15 minutes at 7,500 rpm at 4°C in a Sanyo Hawk 15/05 microfuge (Fisher Scientific, Loughborough, UK), the supernatant was transferred to a fresh microfuge tube and 500 µl of isopropanol added. The mixture was thoroughly vortexed for 10 seconds, and then allowed to stand at room temperature for 10 minutes. The RNA was pelleted at 13,000 rpm at 4°C for 10 minutes and re-suspended in 500µl of 75% ethanol in DEPC-treated de-ionised water by careful vortexing. After collecting the RNA pellet, the ethanol was removed and the RNA allowed to air-dry inverted over clean towels for 7 minutes. Excess liquid was carefully removed with paper tissues and the clean, extracted RNA then dissolved in 100 µl of DEPC-treated distilled water and

immediately treated with 1 Unit of RNase-free DNase I (Promega Corp., Southampton, UK) for 1 hour in the presence of 5 Units of RNase inhibitor (RNasin, Promega Corp). The reaction was stopped by the addition of 150 µl of Phenol: Chloroform: Iso-Amyl Alcohol (25:24:1) and vortexing for 1 minute. The phases were separated by centrifugation at 13,000 rpm for 2 minutes at 4°C and the top aqueous layer transferred to a fresh microfuge tube. Any residual phenol was removed by the addition of 160 µl of Chloroform: Iso-Amyl Alcohol (24:1) and by vortexing for 1 minute. The phases were separated again and the top aqueous layer transferred to a new microfuge tube. The RNA was precipitated by the addition of 100µl of isopropanol, the mixing of the contents and by being placed at -20°C for 1 hour. The RNA was collected by centrifugation at 13,000 rpm at 4°C for 5 minutes, and the pellet was washed first with 500 µl of 75% ethanol in DEPC-treated de-ionised water and then with 1 ml of 75% ethanol in DEPC-treated de-ionised water. The RNA pellet was allowed to air-dry inverted over clean paper towels for 7 minutes before being dissolved in PCR-grade distilled water at 56°C for 5 minutes. The concentration, purity and integrity of the resulting RNA were determined spectrophotometrically before the presence of mRNA for CB₁, CB₂, FAAH and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was determined using end-point reverse transcriptase polymerase chain reaction (RT-PCR).

6.2.3.2. End point RT-PCR

One microgram of total RNA was reversed transcribed with avian myeloblastosis virus-reverse transcriptase (AMV-RT; Promega, Southampton, UK) at 42 °C for 1 hour in the presence of 5 Units of RNase inhibitor (RNasin; Promega). A minus RT reaction was

obtained by omitting the AMV-RT enzyme. At the end of the reaction, the enzymes were denatured by heating at 95 °C for 5 minutes and the cDNA stored at -20 °C. One microlitre of cDNA was subjected to PCR using 10 pmol/μl of specific primers for CB₁, CB₂, FAAH and GAPDH using the annealing temperatures given in Table 6.1. PCR products were resolved on 3% agarose gels and stained with ethidium bromide (2 μg/ml; ICN Biomedicals, Basingstoke, UK) for 15 minutes, before being de-stained with distilled water for 30 minutes. Gel images were captured using a Syngene Gene Genius system (Syngene, Cambridge, UK) equipped with Gene Snap version 6 gel documentation software. For quantification of relative transcript levels cDNA was subjected to quantitative real-time RT-PCR.

6.2.3.3. Quantitative Real-Time RT-PCR

Amplification of CB₁, CB₂, FAAH and GAPDH cDNA was performed in a thermal Roche Lightcycler (Roche Diagnostics Ltd., Lewes, UK) using 1 μl of cDNA for CB₂ and GAPDH, and 2 μl of cDNA for CB₁ and FAAH, with the Lightcycler FastStart DNA master SYBR Green I kit (Roche Diagnostics). Mastermix reactions were prepared as per the manufacturer's instructions on ice with 10 pmol of each primer pair (Table 6.1) and the cDNA added last. Controls included a water blank, minus RT control for each trophoblast sample, a series of diluted cloned human CB₁, CB₂, (CNR01LTN00 and CNR020TN00, respectively; UMR cDNA Resource Centre, Rolla, MO, USA), FAAH and GAPDH (BioChain Institute Inc. Hayward, CA, USA) cDNA targets adjusted so that a standard curve from 1 to 10,000,000 pmol of cDNA could be constructed. Additionally, a series of diluted pooled trophoblast cDNA (1/10 to 1/10,000 dilutions) were used to calculate amplification efficiencies for each gene that

Table 6.1: Primer design and expected amplification size. CB1 and CB2 primers from (Kenney *et al.*, 1999). GAPDH primers from (Hall *et al.*, 1998). S: Sense; A: Antisense.

mRNA target	Annealing Temperature (°C)	Primer Sequences	Expected size (bp)
CB ₁	62.4	5'-CTTCCCACAGAAATTCCC-3' (S) 5'-TACCTTCCCATCCTCAGA-3' (A)	853
CB ₂	62.4	5'-CGTGGCTGTGCTCTATCTGA-3' (S) 5'-ATCTCGGGGCTTCTTCTTTT-3' (A)	851
FAAH	59	5'-GGCCGTCAGCTACACTATGC-3' (S) 5'-ATCAGTCGCTCCACCTCCC-3' (A)	249
GAPDH	60	5'-AGAACATCATCCCTGCCTC-3' (S) 5'-GCCAAATTCGTTGTCATACC-3' (A)	347

was amplified. The cycle conditions were 95 °C for 10 minutes, followed by 45 cycles of 95 °C for 10 seconds, 60 °C for 5 seconds, 72 °C for 15 seconds for GAPDH, 95 °C for 12 seconds, 62 °C for 7 seconds, 72 °C for 15 seconds for CB1 and CB2; and 95 °C for 12 seconds, 59 °C for 9 seconds, 72 °C for 12 seconds for FAAH. The F1 derivative crossing points were then used to measure the transcript concentrations from the standard curves and the predicted melting temperatures of the amplified products used to confirm that only the target DNA was amplified.

6.2.4. Immunocytochemistry

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 antibody was 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, Texas, USA) (Catalogue number: FAAH11-S; Lot number: 549798S) and used at a dilution of 1:2000.

Negative controls for anti-CB₁ and anti-CB₂ were rabbit IgG from Vector Laboratories (Peterborough, UK; Catalogue number X0903. Lot number: 000255509). In order to determine the protein content of the primary antibodies, as this information was not available by the manufacturer, a Bradford assay was performed on an aliquot of the antibody using IgG as the standard (Appendix 4). 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'-diaminobenzamide (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). UK).

6.2.4.1. Anti-FAAH Immunocytochemistry

- 1) Tissue sections (4 μm thick) were mounted onto silanized glass microscope slides and dried for 7 days at 37 °C prior to use.
- 2) De-waxing and re-hydration: Slides were de-waxed in xylene three times for 3 minutes and re-hydrated in graded alcohol for 9 minutes [6 minutes in 99% industrial methylated spirit (IMS) twice and 3 minutes in 95%] followed by incubation in distilled water for 3 minutes.

- 3) **Blocking of endogenous peroxidase activity:** This was achieved by incubation in 6% hydrogen peroxide (H₂O₂) in water for 10 minutes. Slides were then washed in running tap water for 5 minutes then in TBA [Tris-buffered saline (0.5 M Trizma, 1.5 M NaCl, 2 mM MgCl₂, pH 7.6) containing 0.1% bovine serum albumin (Fraction V; Sigma-Aldrich Ltd.)] for another 5 minutes.

- 4) **Blocking of non-specific protein binding:** Achieved by adding 10% normal goat serum (NGS) (100 µl/slide) and incubation in a humid chamber for 20 minutes at room temperature.

- 5) **Blocking of avidin and biotin:**
 - A. **Avidin blocking:** to each slide, 100 µl of Avidin blocking solution [prepared by adding 1 drop of avidin blocking solution to 250 µl of (NGS in TBA 1 in 10)]. Slides were incubated in a humid chamber for 20 minutes at room temperature and then washed in TBA for 5 minutes.
 - B. **Biotin blocking:** to each slide, 100 µl of Biotin blocking solution [prepared by adding 1 drop of biotin blocking solution to 250 µl of (NGS in TBA 1 in 10)]. Slides were incubated for 20 minutes in a humid chamber at room temperature.

- 6) **Primary antibody:** To each slide, 100 µl of primary anti FAAH antibody or normal rabbit serum (NRS) both diluted at 1 in 2000 in a solution of (NGS in TBA 1 in 10) was added. Slides were incubated overnight (18 hours) at 4 °C in a humid chamber.

- 7) On the following day, slides were washed in TBA for 30 minutes. The slides that had anti-FAAH were washed separately to those with NRS.
- 8) Secondary antibody: To each slide, 100 μ l of the secondary antibody diluted at 1 in 400 in TBA was added. The slides were incubated in a humid chamber at room temperature for 30 minutes, and then washed for 20 minutes in TBA.
- 9) The detection system: To each slide, 100 μ l of the Avidin Biotin Complex (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 added. Slides were incubated in a humid chamber at room temperature for 30 minutes followed by a 20 minute wash in TBS.
- 10) Detection system: 100 μ l of DAB (prepared adding 1 drop of buffer, then 1 drop of H₂O₂ followed by vortex mixing, and then adding two drops of DAB to 2.5 ml of distilled water. The solution was then mixed by inversion. The solution was prepared immediately before use as recommended by the manufacturer) was added to each slide. Slides were then incubated in a humid chamber for 5 minutes at room temperature. This was followed by a five minute wash in distilled water.
- 11) Counterstaining: This was achieved by incubating the slides in Mayer's haematoxylin for 15 seconds. This was followed by a 5 minute wash in running tap water.

12) Dehydration and clearing: Slides were dehydrated in graded alcohols (3 minutes in 95% IMS once then 6 minutes in 99% IMS) and cleared in xylene (for 9 minutes). Slides were then mounted in DPX mounting medium.

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 (Axiovision version 4.4, Carl Zeiss Ltd., Welwyn Garden City, Hertfordshire, UK). Images were captured at either 10x 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).

6.2.4.2. Anti-CB₁ Immunocytochemistry

The method used for anti-CB₁ was similar to the one described in section 6.2.4.1 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 to detect anti-CB₁ complexes. The following steps replaced steps 8 and 9 in section 6.2.4.1:

- 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 and then washed in TNT buffer for 5 minutes three times.
- II. 100 µl of blocking solution (0.5% blocking reagent in TNT buffer) was then added to each slide. The slides were then 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 next 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 then added to each slide. This was followed by

incubation in a humid at room temperature for 5 minutes. They were then washed in TNT buffer for 5 minutes three times.

The remaining parts of the method are similar to steps 10-12 in section 6.2.4.1.

6.2.4.3. Anti-CB₂ Immunocytochemistry

The method for Anti-CB₂ is similar to the one described for anti-FAAH in section 6.2.4.1 except:

- 1) For anti-CB₂, antigen retrieval by microwave was performed as described in section 6.2.4.2.
- 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).

6.2.5. Effect of anandamide on BeWo cell morphology and growth

The human choriocarcinoma cell line BeWo (ECACC 86082803, European Collection of Cell Cultures, Salisbury, Wiltshire, UK), chosen because it is a good model for the human first trimester trophoblast (Al-Nasiry *et al.*, 2006) and because the cells contain the cannabinoid receptors (Kenney *et al.*, 1999) and respond to cannabinoid stimulation (Khare *et al.*, 2006), was maintained in Ham's F12 medium (Invitrogen) supplemented with 10% fetal calf serum (FCS) (Invitrogen) and cultured at 37 °C in humidified atmosphere of 5% CO₂ in air. For cell morphology studies, BeWo cells were plated onto Nunc 6-well multi-well plates in triplicate for each data point (Fisher Scientific, Loughborough, Leicestershire, UK) at a density of 8 x 10⁵ cells per well (Nomura *et*

al., 2004). After 48 hours in culture the medium was exchanged with one that contained 5% FCS and up to 30 μ M anandamide [final concentration of 0.1% (v/v) ethanol]. The control consisted of a culture medium containing 0.1% (v/v) ethanol. Cells were cultured in the media plus additives for 1 hour to allow non-specific binding of the anandamide to plastic to reach equilibrium. The medium was then replaced and culture continued for an additional 48 hours, with fresh medium replaced at 24 hours. Photomicrographs were obtained using a Nikon Eclipse TE2000-U inverted microscope equipped with a DN- 100 digital camera image capture system (Nikon UK Limited, Kingston upon Thames, Surrey, UK).

To examine the effect of anandamide on cell survival and cell proliferation indices, BeWo cells were plated onto Nunc 96-well plates (Fisher Scientific UK, Loughborough, UK) at 1×10^4 cells per well, in 200 μ l of normal growth medium and allowed to proliferate for 48 hours. The medium was then changed to one that contained anandamide for 1 hour to allow for non-specific binding of the endocannabinoid to the plastic-ware. The medium was then replaced with the same anandamide-containing media and cultured for an additional 23 hours, after which the media was changed again and the cell re-incubated for an additional 24 hours.

In order to determine which receptor type mediated might be mediating AEA effects, a separate set of experiments were performed in which BeWo cells were treated with 10 μ M AEA in the presence of either SR141716A (CB₁-antagonist) or SR144528 (CB₂-antagonist) both used at a receptor blocking concentration of 300 nM).

After 48 hours of culture in the presence and absence of anandamide and with or without the receptor antagonists, cell numbers were assessed using the Cell Proliferation and Apoptosis Kit II (Roche Diagnostics Ltd., Lewes, East Sussex, UK) as per the manufacturer's instructions with measurements taken on a Multiskan Ascent ELISA plate reader (Labsystems Oy, Helsinki, Finland) with the detection filter set at 492 nm and the reference set at 620 nm. Cell numbers were obtained by calibration against a standard curve of untreated BeWo cell numbers grown in parallel (Taylor *et al.*, 2001; Khare *et al.*, 2006). To make direct comparisons between cultures and to account for small variability between cultures, cell numbers were converted to a percentage of the untreated control.

6.2.6. Data analysis

The absolute levels of CB₁, CB₂, FAAH and GAPDH transcript were calculated using Pfaffl's method (Pfaffl, 2001). In order to compare the results of the various weeks, the data was normalised to the week 7 samples [i.e. week 7 data converted into 1 arbitrary unit (AU) for CB₁, CB₂ and FAAH]. All data were analysed for differences using one-way ANOVA with Tukey's honestly significant difference (HSD) test within the InStat version 3.0 software package (GraphPad Software, San Diego, CA, USA, 1998; <http://www.graphpad.com>). Statistical significance was accepted when $P < 0.05$.

6.3. Results

6.3.1. Expression of the endocannabinoid system in the first trimester placenta/trophoblast

RT-PCR of RNA extracts taken from trophoblast placentas of between seven and twelve weeks of gestation indicated the presence of amplified DNA consistent with the expected amplicon sizes of 853 bp for CB₁, 851 bp for CB₂, 249 bp for FAAH and 347 bp for GAPDH (Figure 6.1). Additionally, there was an absence of products in the lanes obtained for the -RT controls indicating that the amplified products were obtained from mRNA and not contaminating genomic DNA. Detailed analysis of the expression patterns for CB₁ transcript indicated that the amount of product diminished from a normalized value of 1.0 to ~0.1 after week ten of gestation, whereas the CB₂ and FAAH levels of products remained constant throughout (Figure 6.1). Such semi-quantitative methods of determining relative transcript levels are often criticised as being inaccurate, therefore quantitative real-time RT-PCR analyses on the extracts was also performed (Figure 6.2). The data indicated that there was a significant decrease in the expression of CB₁ transcript from 4.77 ± 1.28 at week 10 of gestation to 0.40 ± 0.15 arbitrary units at week 12: a significant decrease of 91% in expression ($P < 0.01$; Student's t-test), which was similar to that of the end-point analysis (Figure 6.1 and Figure 6.3 A). Although there appeared to be a differential expression of both CB₂ and FAAH transcripts in the trophoblast during the first trimester, these data did not reach statistical significance (Figure 6.3).

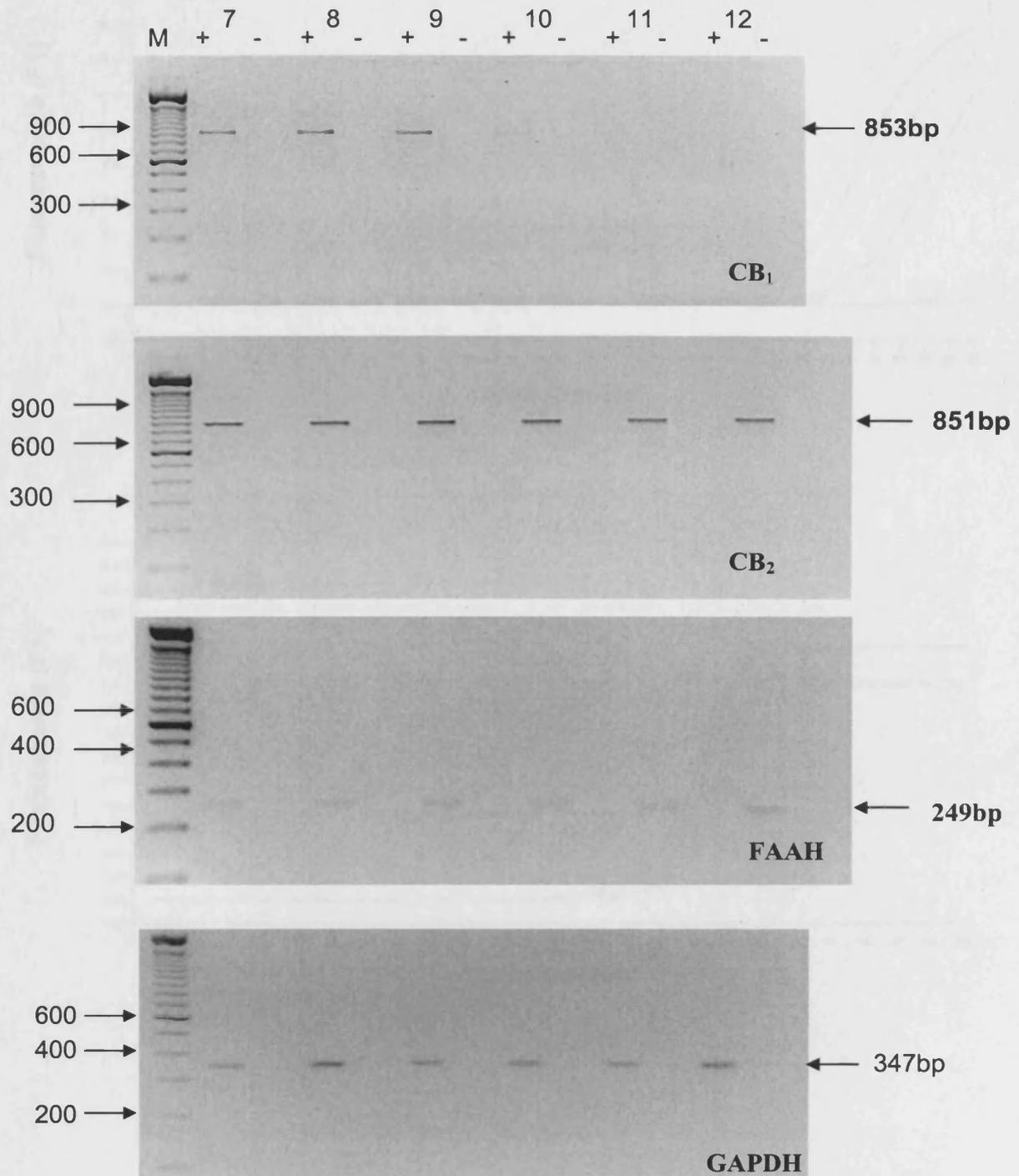


Figure 6.1: Ethidium bromide-stained agarose gels of RT-PCR products for CB₁, CB₂, FAAH and GAPDH for placental samples that were taken from weeks 7 and 12 of the first trimester. The (+) indicates the presence of AMV-RT in the reverse transcriptase reaction. The (-) indicates the absence of enzyme. The relative size markers from the 100 bp DNA ladder are shown on the left.

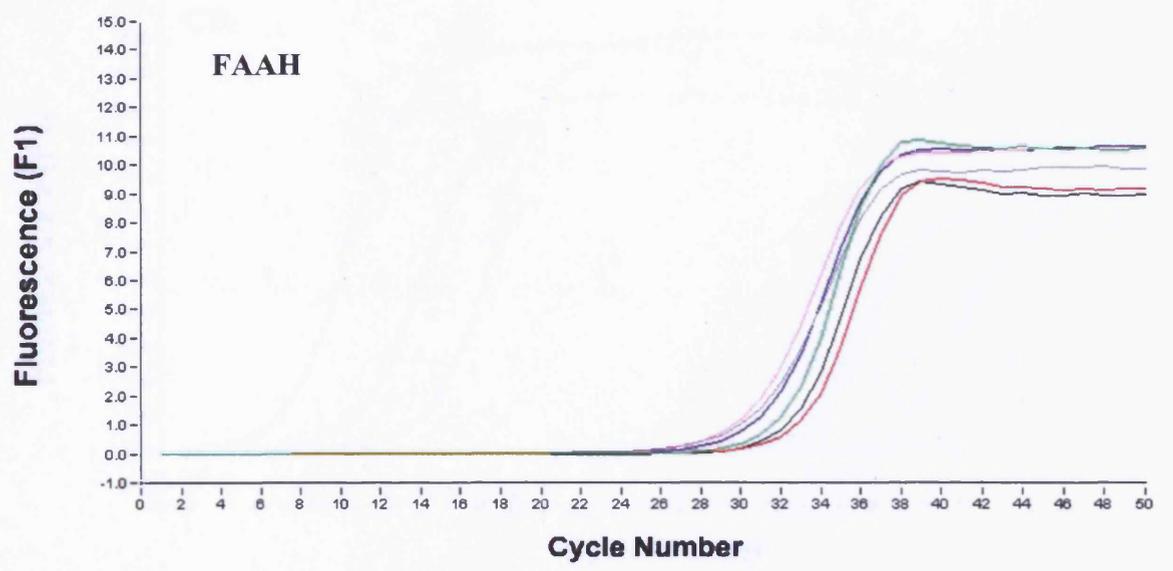
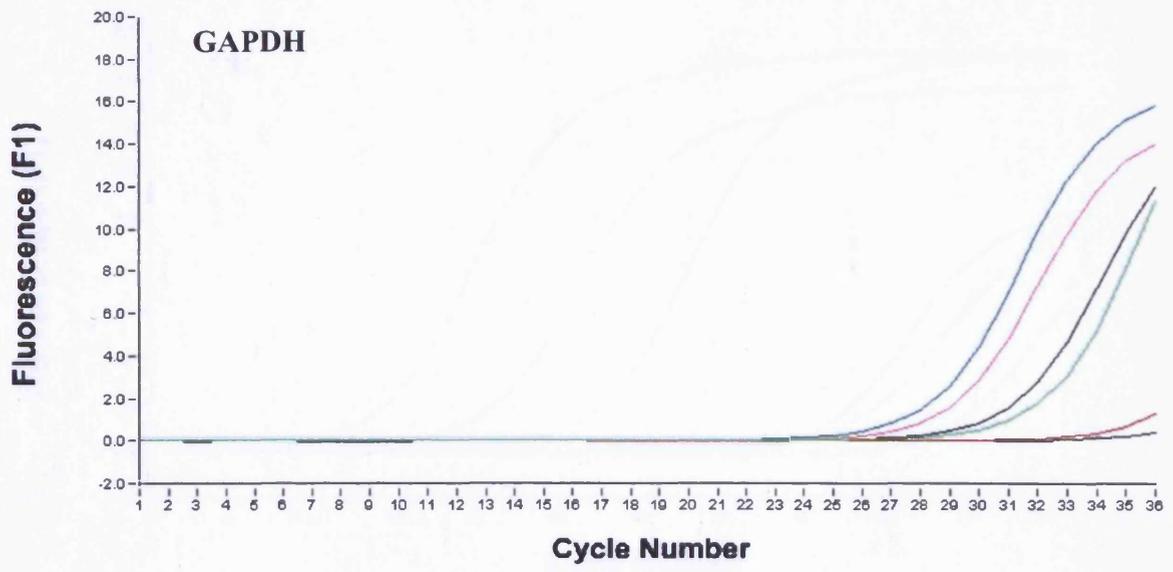


Figure 6.2: Continued on the following page.

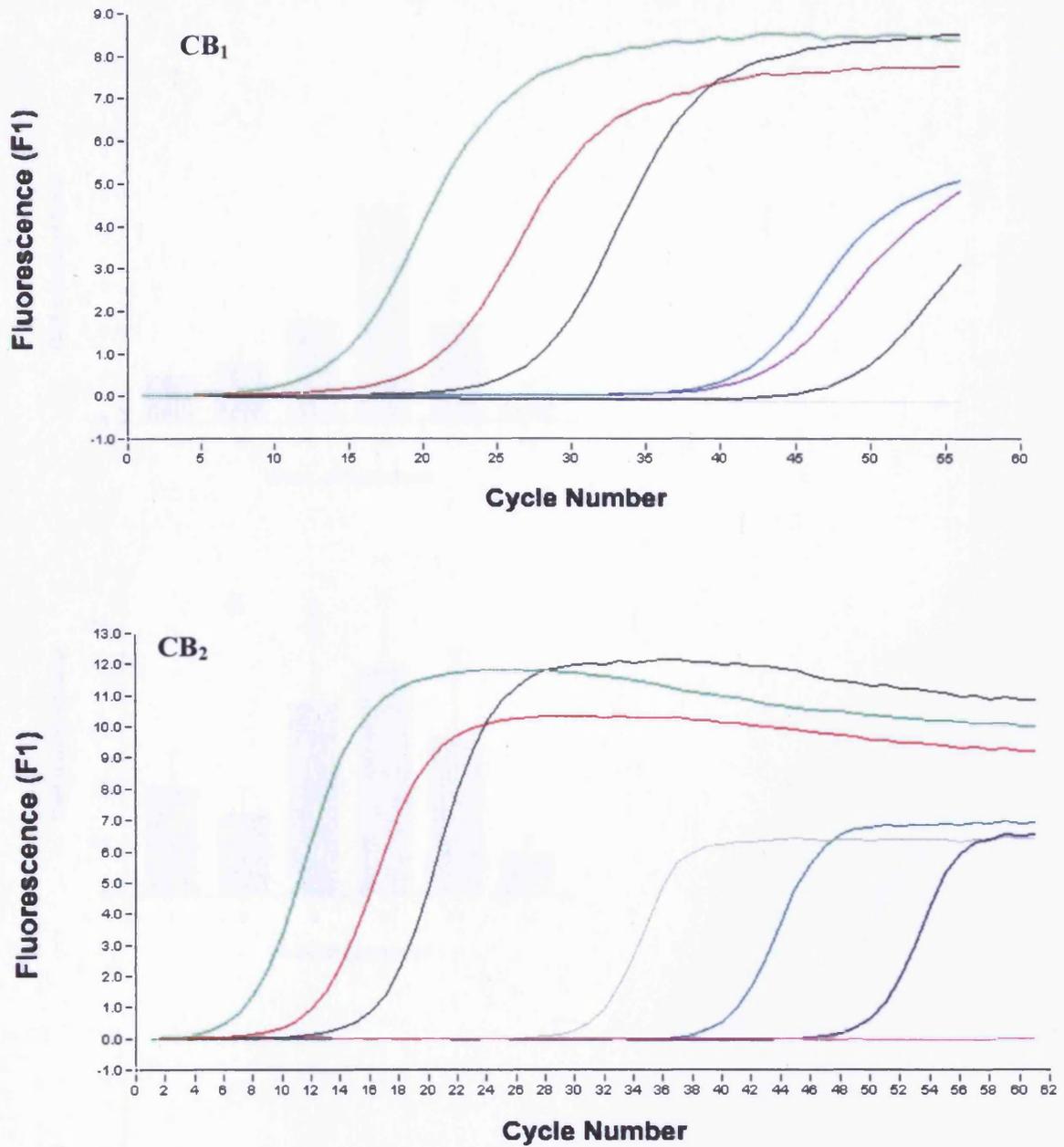


Figure 6.2: Sample of the growth charts obtained from the real time PCR experiments for GAPDH (housekeeping gene), FAAH, CB₁ and CB₂. The green, brown and blue traces are 1 in 100, 1 in 1000 & 1 in 10000 dilutions of the standard respectively. Other curves represent individual samples. Note the relative high cycle numbers with CB₁ and CB₂. These findings might indicate low transcript levels or low amplification efficiency.

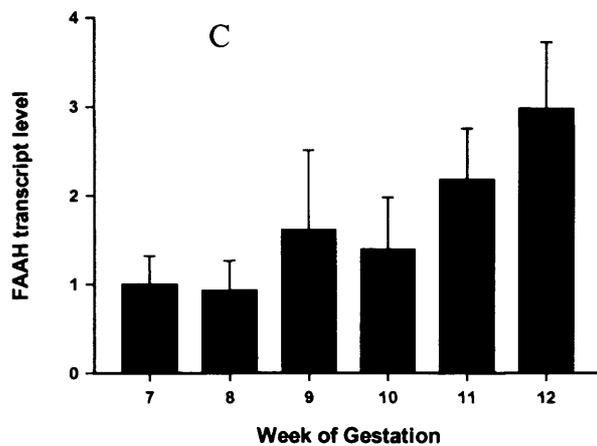
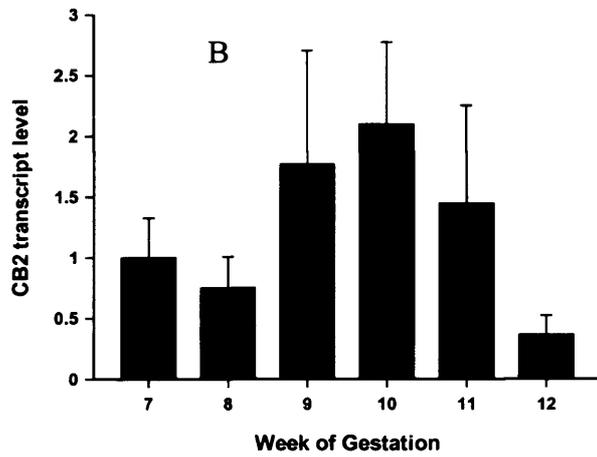
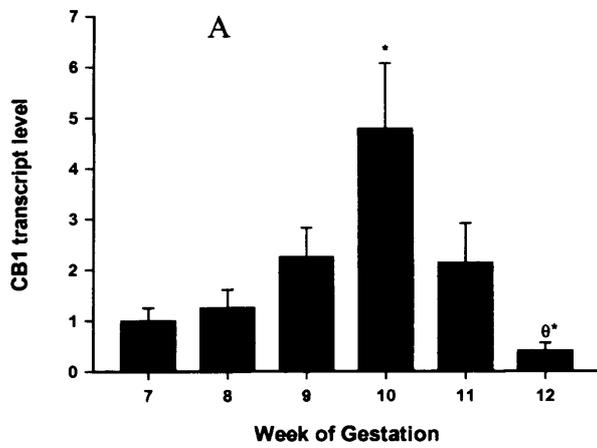


Figure 6.3: Relative levels of CB₁, CB₂ and FAAH transcripts compared to week 7 of gestation using quantitative real-time RT-PCR. Data are the mean values \pm SD after normalization to the relative levels of GAPDH according to the method of Pfaffl and are from 3 experiments performed in triplicate for each sample. Each week of gestation is represented by 4 independent samples from 4 patients. *: $P < 0.05$; One-way ANOVA with Tukey's HSD test compared to week 7; θ : $P < 0.001$ compared to week 10.

6.3.2. Detection of immunoreactive CB₁, CB₂ and FAAH

Fetal membranes obtained at term were used as a positive control tissue (Park *et al.*, 2003), to optimize the conditions for the immunohistochemistry studies. The results of these optimisation experiments indicated that the primary antibody dilutions for FAAH, CB₁ and CB₂ were 1:2000, 1:4000 and 1:500, respectively. Rabbit serum or isotype IgG diluted to the same concentrations were non-reactive (Figure 6.4). Using these dilutions of primary antibodies and a common secondary-detection system, there was also clear specific immunostaining for FAAH, CB₁ and CB₂ on first trimester trophoblast samples (Figures 6.4, 6.5 and 6.6).

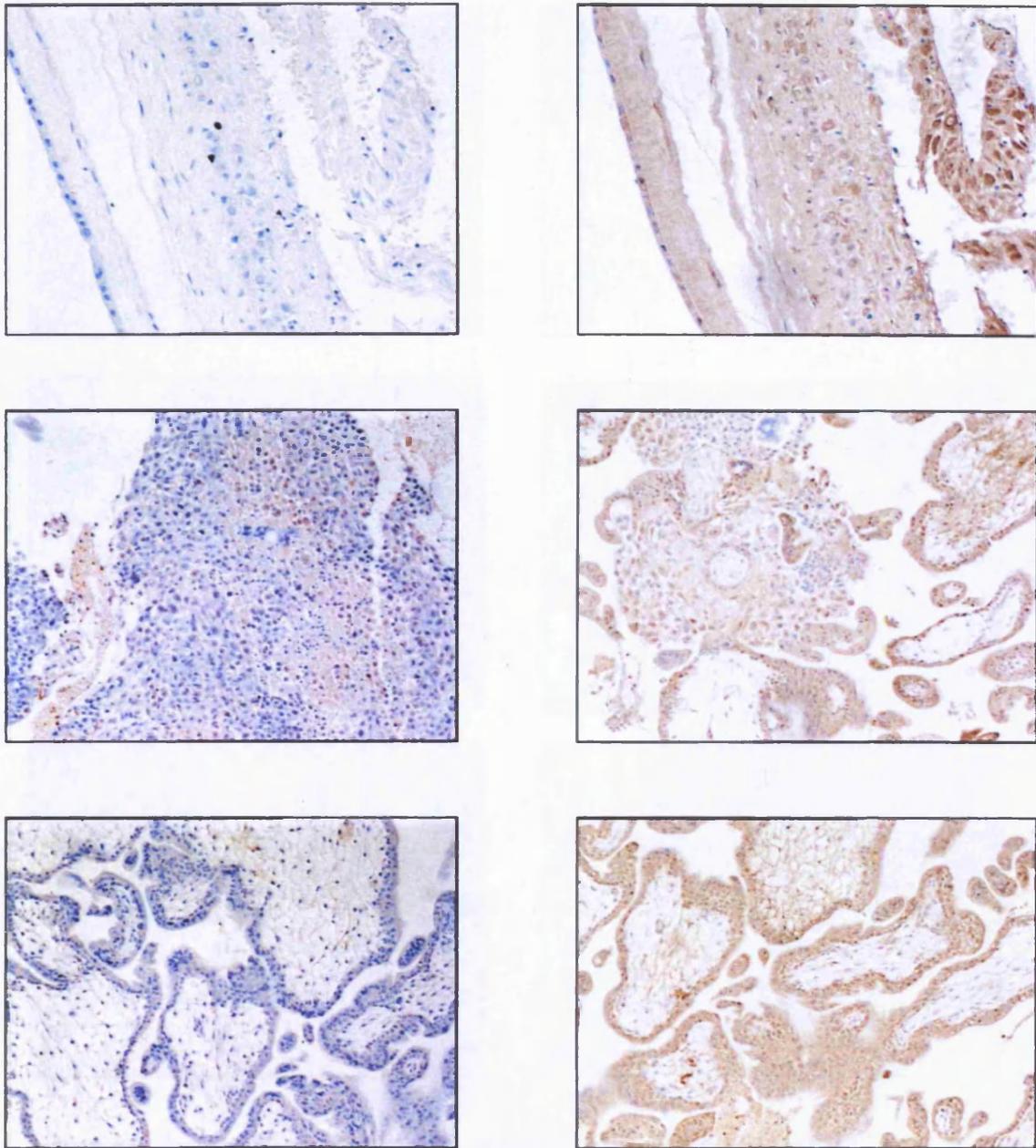


Figure 6.4: Demonstration that the FAAH polyclonal antibody is specific to particular cell types using term fetal membranes (upper panels), 1st trimester deciduas (middle panels) and 1st trimester placenta (lower panels) as control tissues. Note the apparent increase in decidual staining from 1st trimester to term. Note negative staining in the panels on the left (negative control) compared to the panels on the right (Anti-FAAH antibody).

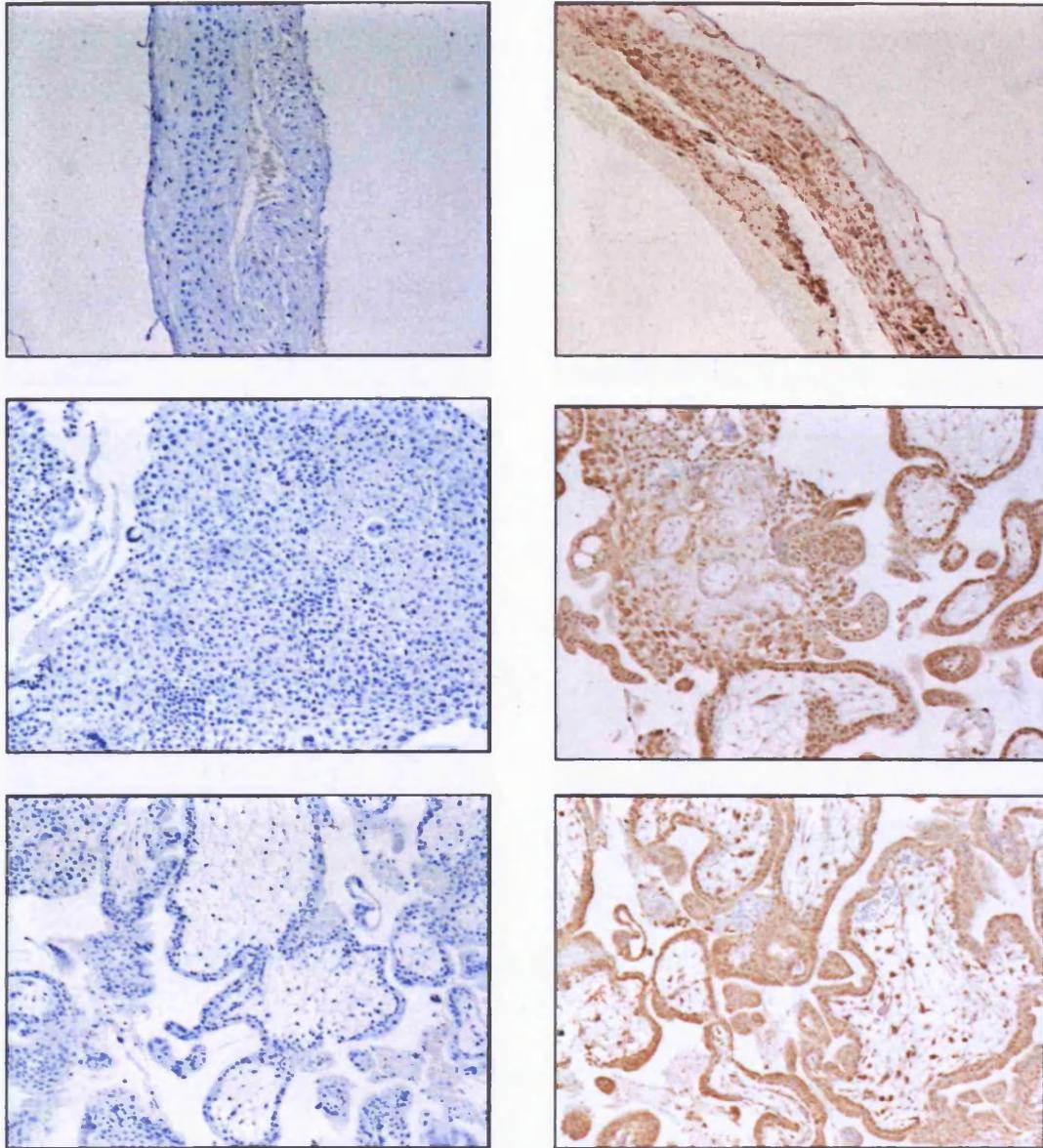


Figure 6.5: Demonstration that the CB₁ antibody is specific to particular cell types using term fetal membranes (upper panels), 1st trimester deciduas (middle panels) and 1st trimester placenta (lower panels) as control tissues. Note the amnion is missing from the term fetal membrane sample. Note the negative staining in the panels on the left (negative control) compared to the panels on the right (Anti- CB₁ antibody).

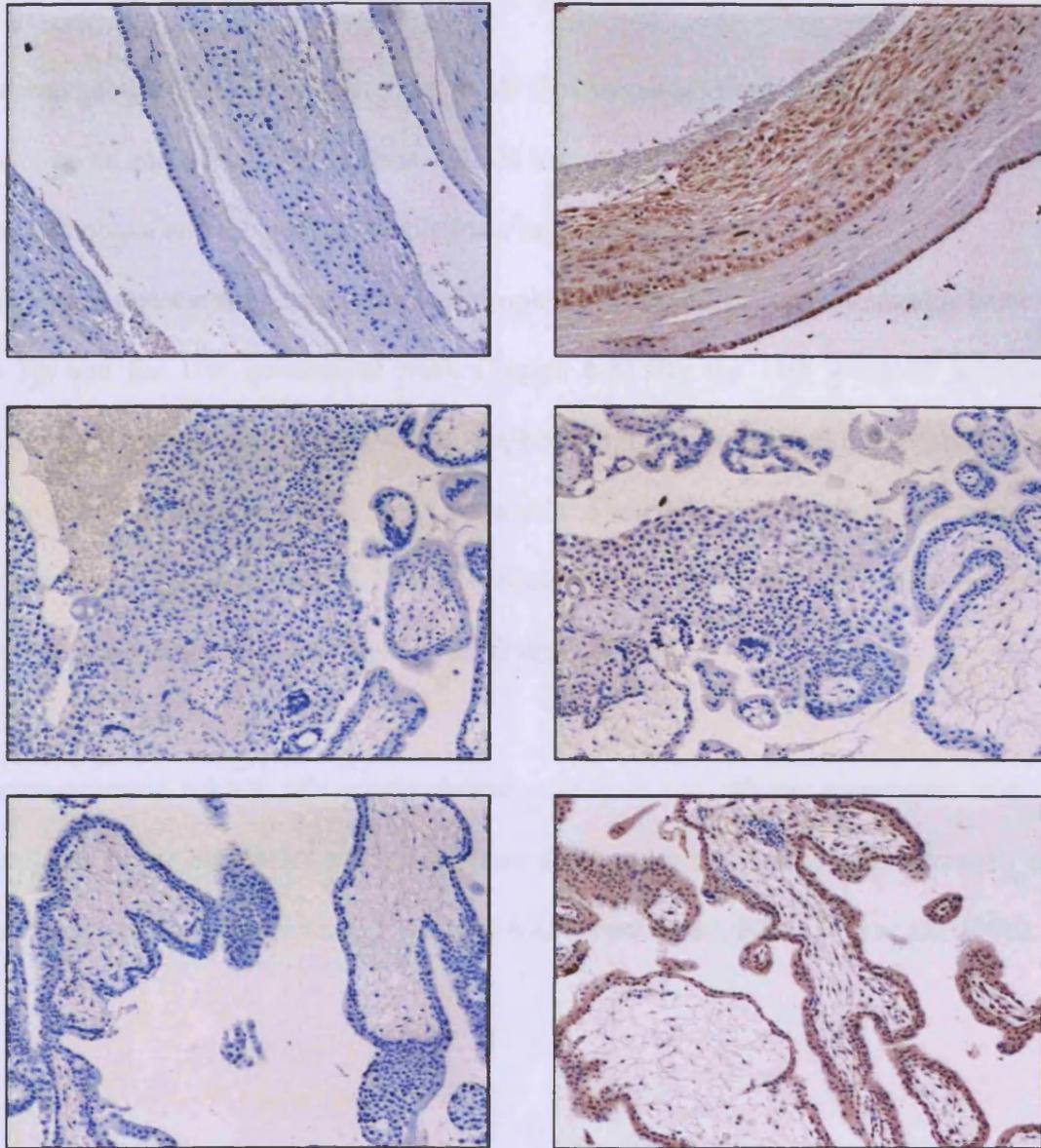


Figure 6.6: Demonstration that the CB₂ antibody is specific to particular cell types using term fetal membranes (upper panels), 1st trimester deciduas (middle panels) and 1st trimester placenta (lower panels) as control tissues. Note the decidua lack CB₂ immunoreactivity where the syncytium and cytotrophoblasts are immunoreactive. The left panels are the negative controls while the right ones are the anti-CB₂ antibody.

6.3.2.1. Immunoreactive FAAH staining patterns

Immunoreactive FAAH was detected in all first trimester trophoblast tissues between weeks seven and twelve of gestation. FAAH immunoreactivity was detected in both the cytotrophoblast and the syncytiotrophoblast layers (Figure 6.7).

FAAH immunoreactivity in the syncytiotrophoblast layer increased gradually between the 7th and the 10th gestational week (Figure 6.8). By the 11th week of gestation, FAAH immunoreactivity at the syncytiotrophoblast layer diminished to the point where it was barely detectable within large parts of the trophoblast, an effect that persisted into the 12th week (Figure 6.8). FAAH immunoreactivity was also demonstrated in the mesenchymal core of the developing villi (Figure 6.7).

Immunoreactive FAAH was predominantly noted at the cellular membrane and the cytoplasm of the cells with no nuclear staining, in keeping with the membranous and intracytoplasmic vesicular localisation of FAAH (Park *et al.*, 2003; Wei *et al.*, 2006).

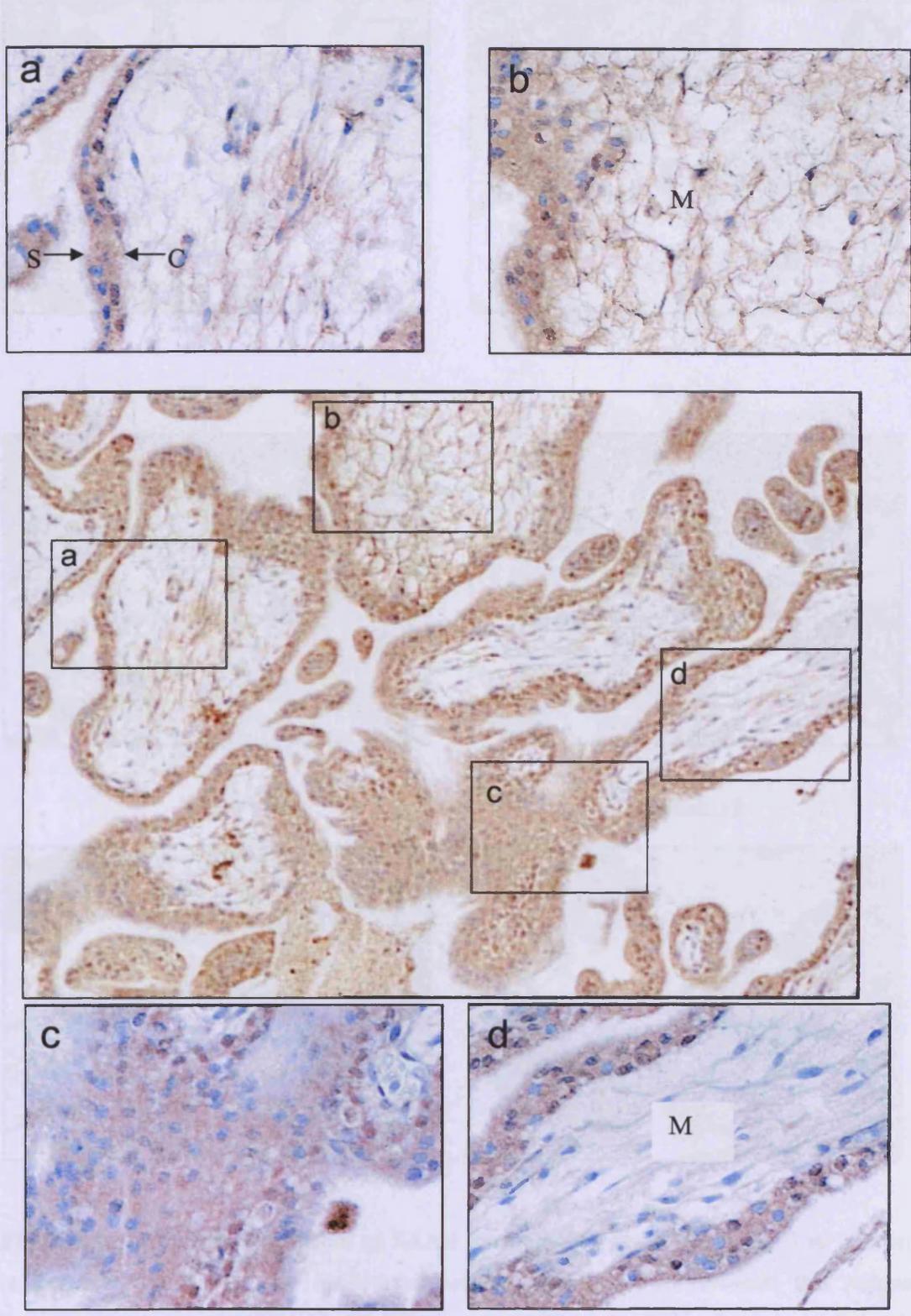


Figure 6.7: Showing placental anatomical features and consistent staining in (a) immature, (b) mature, (c) intermediate and (d) juvenile villi of a 7 week placenta. Note the increased presence of FAAH immunoreactivity in the mesenchyme as the villi matures. S: Syncytiotrophoblast; C: Cytotrophoblast; M: Mesenchymal core.

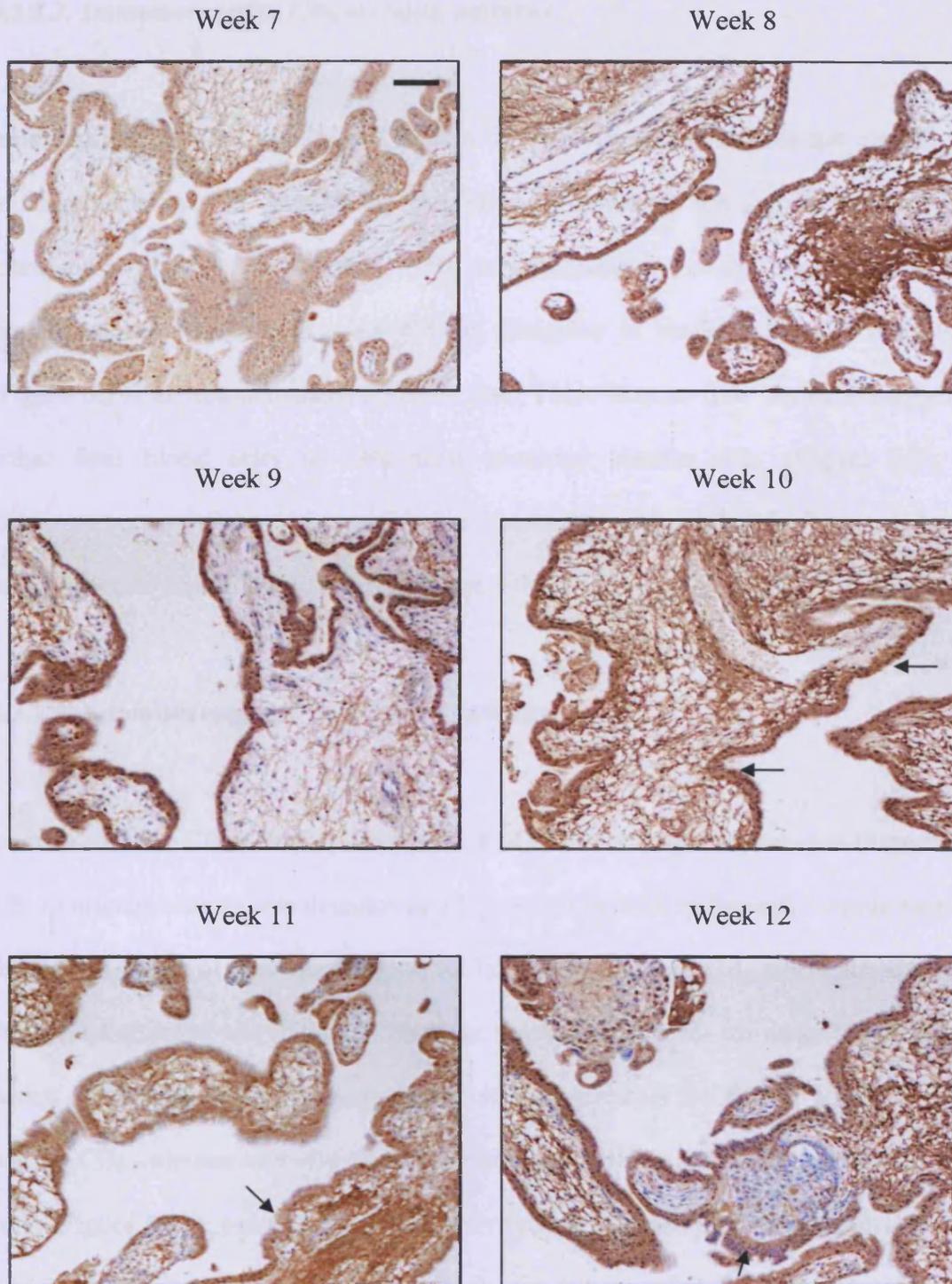


Figure 6.8: The effect of gestation on FAAH immunoreactivity. From weeks 7 to 10, there is increased staining in the syncytiotrophoblast layer (arrows), however this reduced dramatically in weeks 11 and 12. Note also that staining was demonstrated in the mesenchymal core of the villi. All representative images of placenta were taken at 10x magnification. (Bar = 50 μ M).

6.3.2.2. Immunoreactive CB₁ staining patterns

Immunoreactive CB₁ was also detected in first trimester trophoblast tissue. CB₁ immunoreactivity was detected in all types of trophoblast through various stages of maturation (Figures 6.5 and 6.9). CB₁ immunoreactivity in the syncytiotrophoblast layer diminished in intensity, but did not disappear in weeks 10 to 12 of gestation (Figure 6.10) as immunoreactive FAAH had. There was no CB₁ immunoreactivity in either fetal blood cells or infiltrating maternal plasma cells (Figure 6.9), but CB₁ immunoreactivity was detected in the endothelial cells of the blood vessels but not in the vascular smooth muscle cells (Figure 6.9).

6.3.2.3. Immunoreactive CB₂ staining patterns

Immunoreactive CB₂ was also detected in first trimester trophoblast tissue (Figure 6.6). CB₂ immunoreactivity was detected in all types of trophoblast through various stages of the first trimester of gestation (Figure 6.11). The intensity of CB₂ immunoreactivity in the syncytiotrophoblast remained constant throughout the 1st trimester (Figure 6.12), which differed from the immunoreactive staining patterns for FAAH and CB₁. There was no CB₂ immunoreactivity in either fetal blood cells or infiltrating maternal plasma cells (Figure 6.11), but CB₂ immunoreactivity was detected in the endothelial cells of the blood vessels although not in the vascular smooth muscle cells (Figure 6.11).

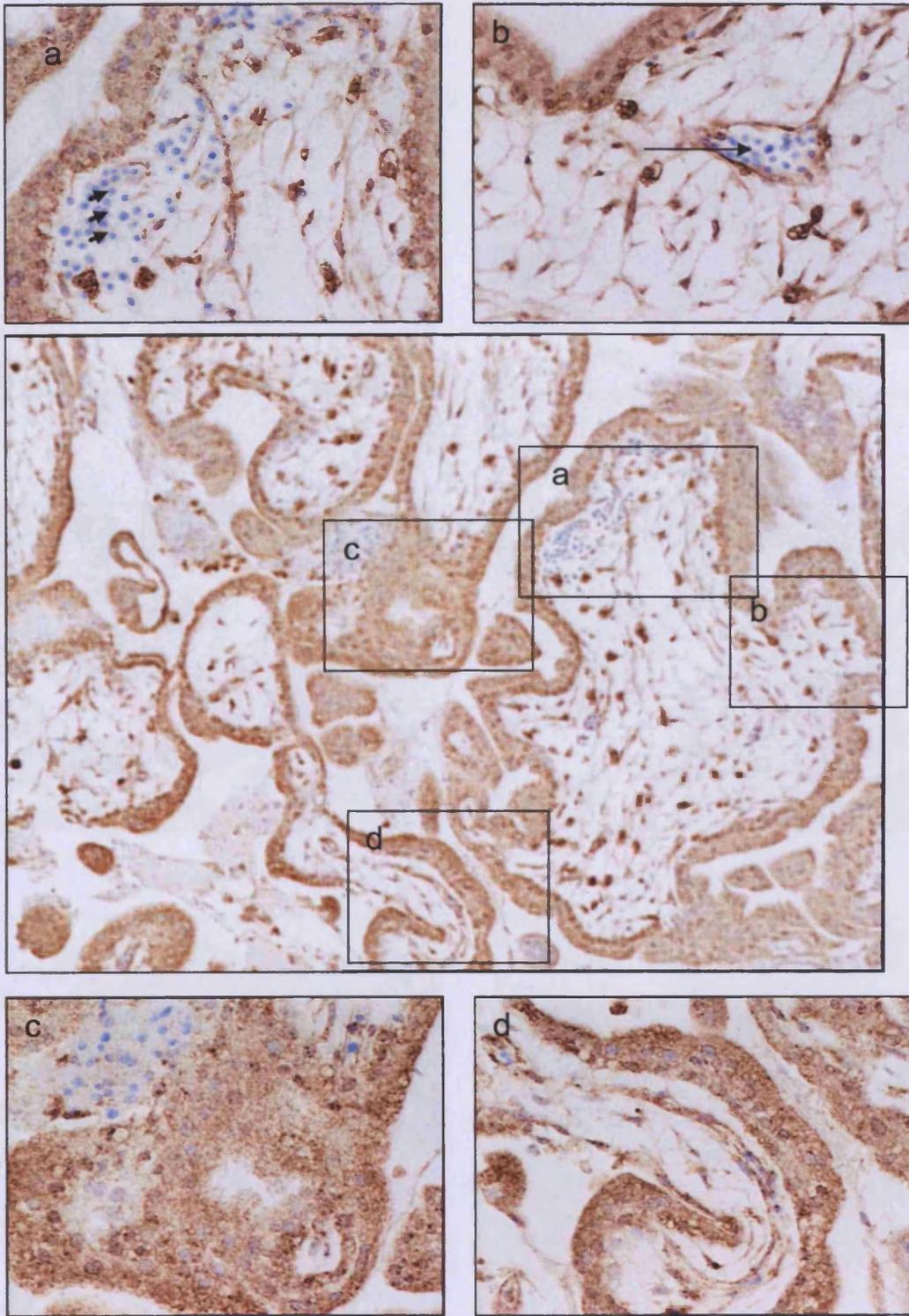


Figure 6.9: Showing placental anatomical features and consistent staining in immature (a), mature (b), intermediate (c) and juvenile villi (d) of a 7 week placenta. Note the lack of CB₁ immunoreactivity in the fetal blood cells (short arrows) and infiltrating maternal plasma cells (long arrow).

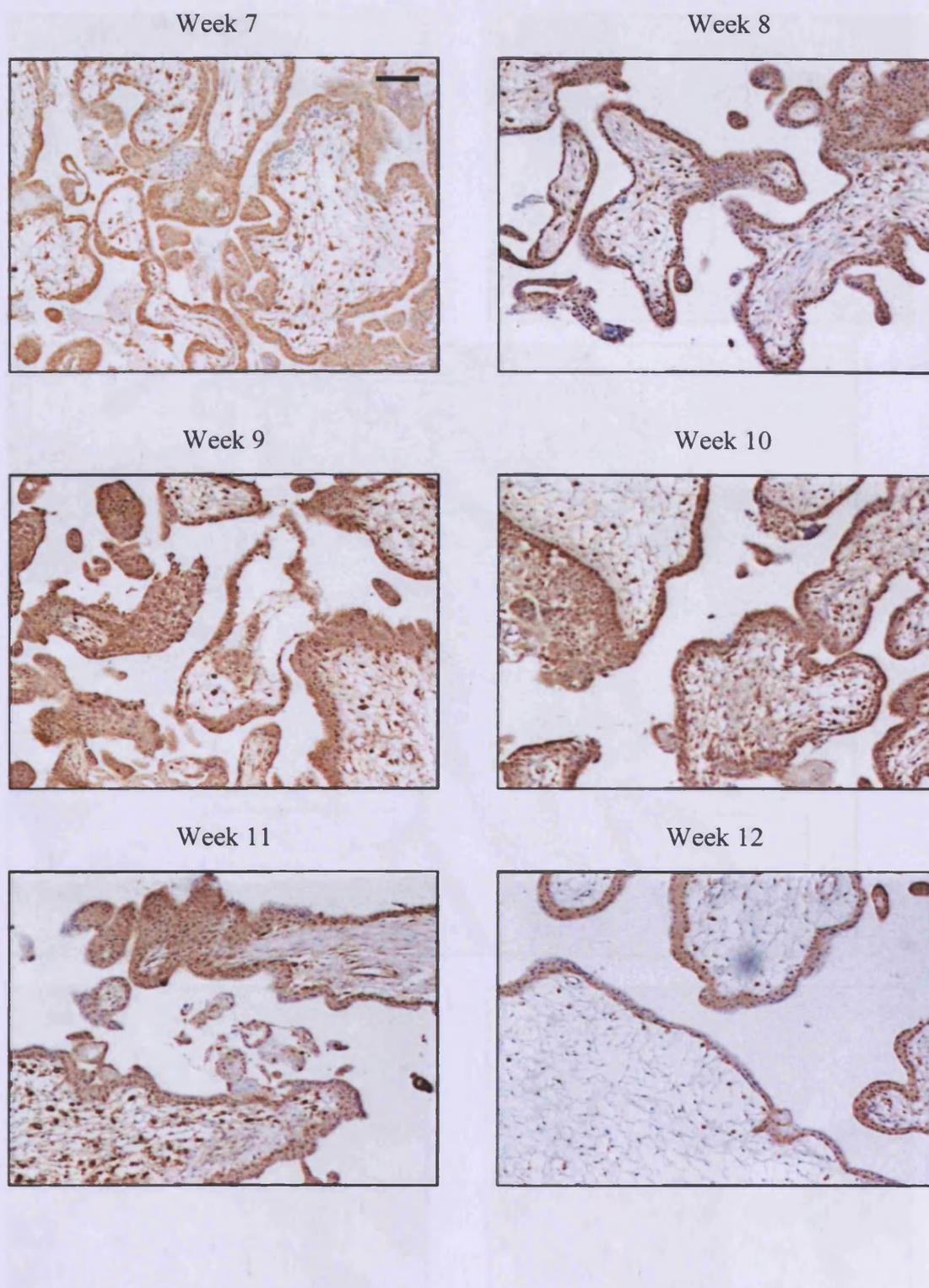


Figure 6.10: The effect of gestation on CB₁ immunoreactivity. Note the general increase in staining intensity in weeks 8 through to 11 that dissipates in week 12. All representative images of placenta were taken at 10x magnification. (Bar = 50 μM).

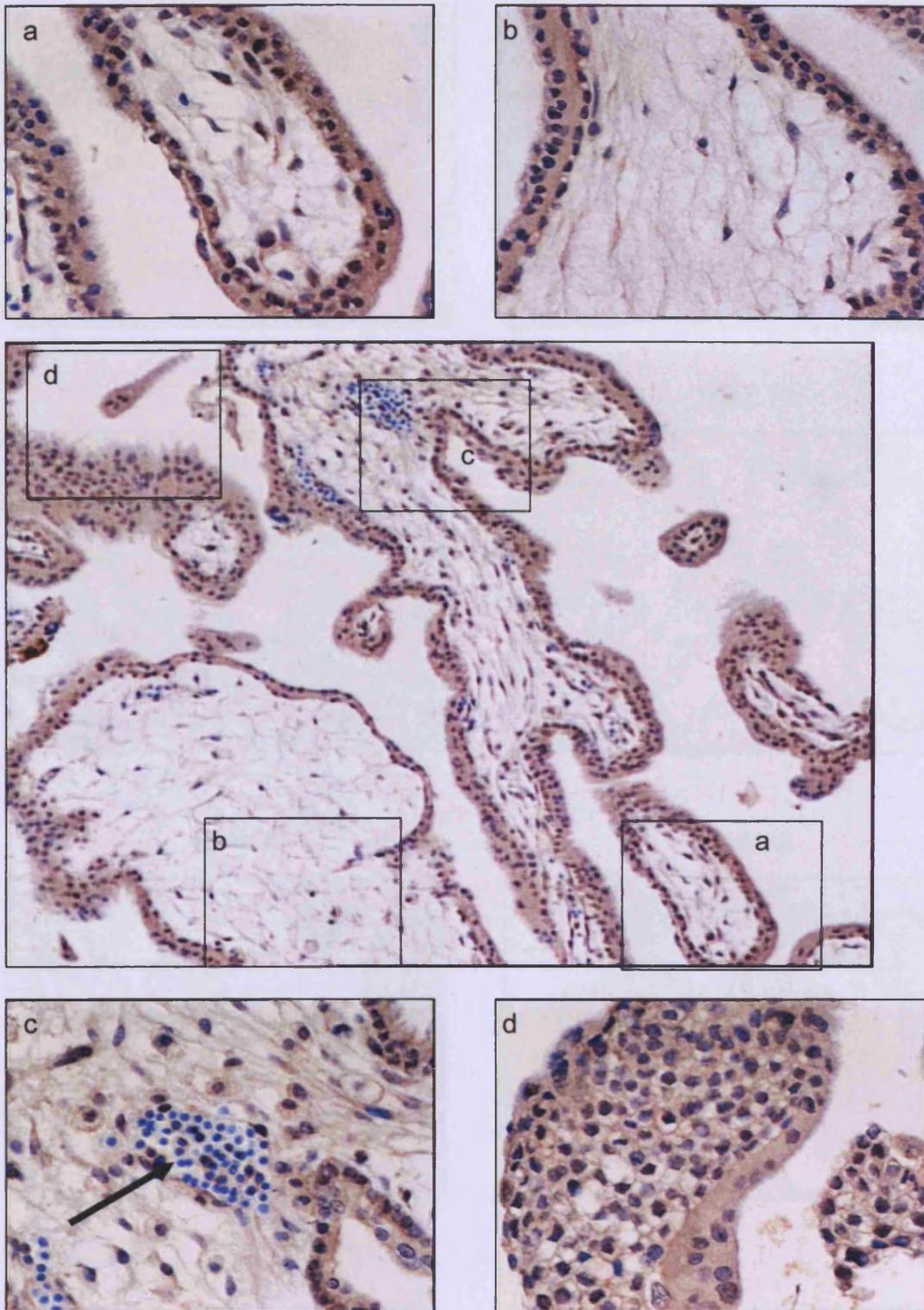


Figure 6.11: CB₂ immunoreactivity: Showing placental anatomical features and consistent staining in (a) immature, (b) mature, (c) intermediate and (d) juvenile villi of a 7 week placenta. Note the lack of CB₂ immunoreactivity in the fetal blood cells.

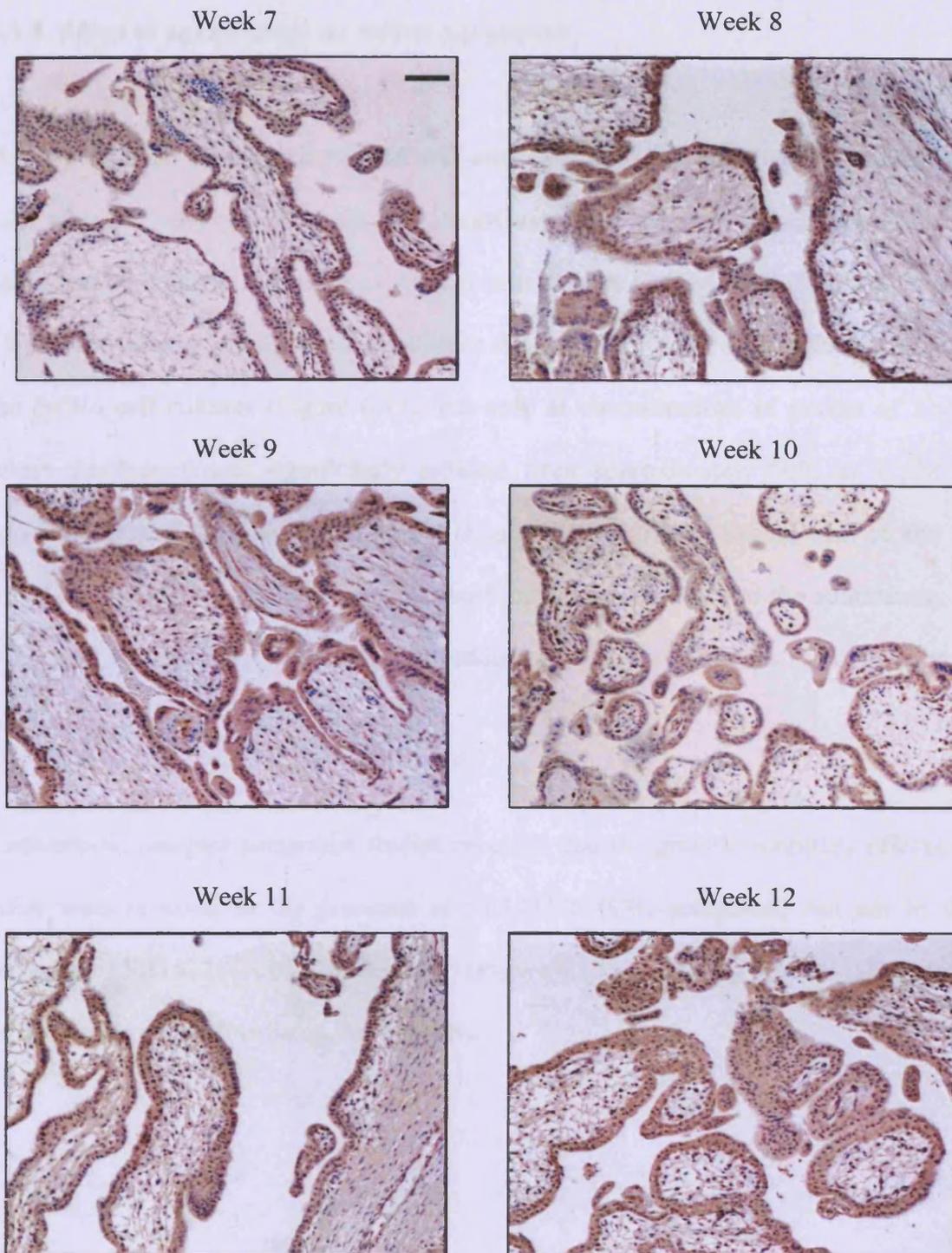


Figure 6.12: The effect of gestation on CB₂ immunoreactivity. Note that there is no real change in the levels of CB₂ immunoreactivity throughout the 1st trimester of pregnancy. All representative images of placenta were taken at 10x magnification. (Bar = 50 μM).

6.3.3. Effect of anandamide on BeWo cell growth

BeWo cells were plated such that cultures achieved approximately 70–80% confluency after 48 hours, the point at which AEA treatments were initiated, and continued for an additional 48 hours with a range of AEA concentrations (10 nM, 100 nM, 1 μ M, 3 μ M, 15 μ M and 30 μ M). Under these conditions AEA demonstrated an inhibitory effect on the BeWo cell cultures (Figure 6.13), but only at concentrations in excess of 3 μ M where confluency was significantly reduced from approximately 80% at 3 μ M to approximately 65-70 % at 15 and 30 μ M (Figure 6.14). Cultures treated with 15 and 30 μ M AEA did not exhibit increased cell death or failure to attach to the substratum, as evidenced by the lack of increase of shedding of cells in the spent medium (Figure 6.13).

Cannabinoid receptor antagonist studies revealed that the growth inhibitory effects of AEA were reversed in the presence of SR144528 (CB₂-antagonist) but not in the presence of SR141716A (CB₁-antagonist) (Figure 6.15), indicating that the AEA effect on the BeWo cells is mediated through CB₂.

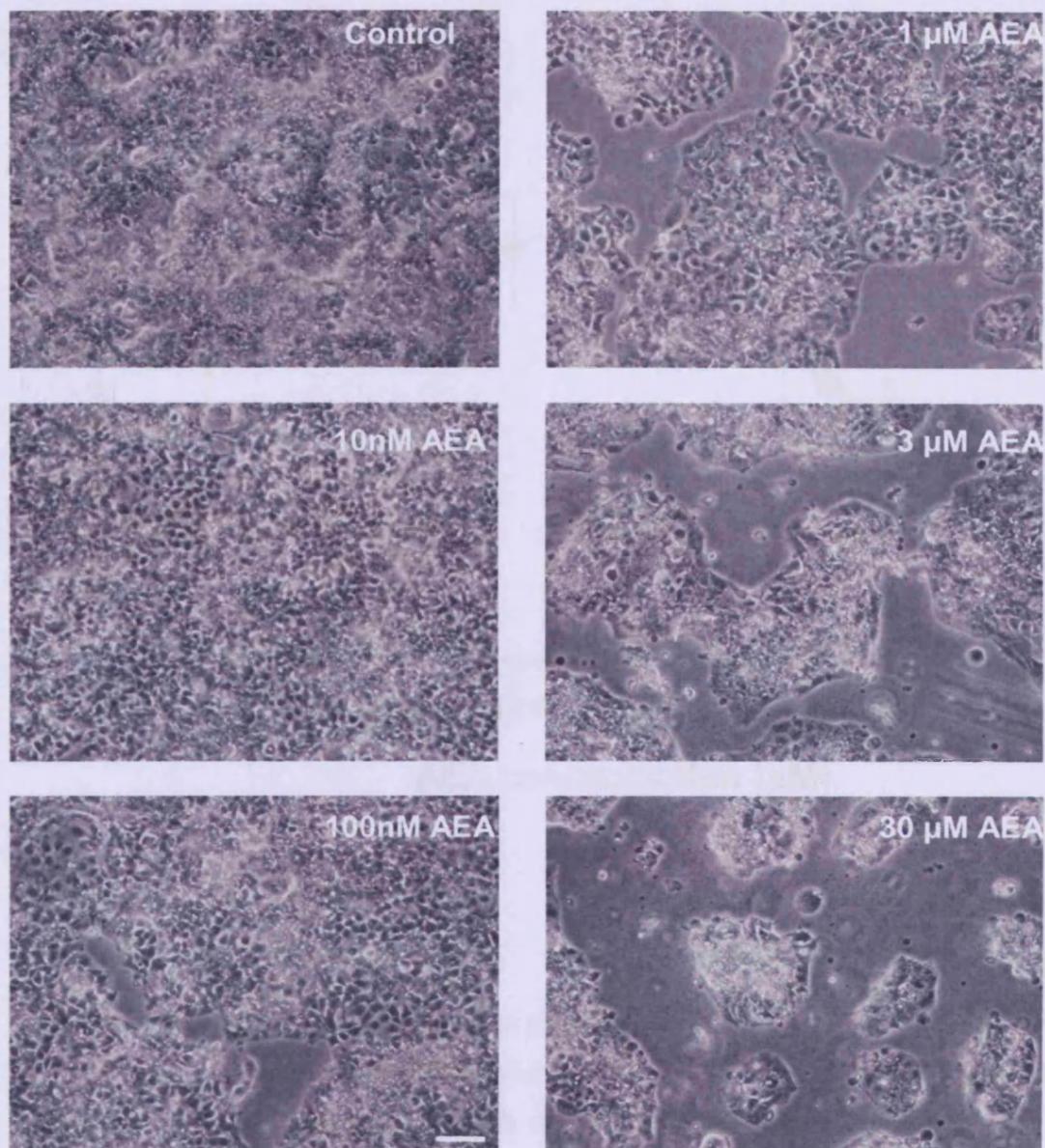


Figure 6.13: Effect of anandamide on BeWo cell growth. BeWo cells plated at 4×10^5 cells/well onto 6-well plates were allowed to proliferate for 48 hours before treatment with the indicated concentrations of anandamide (AEA) (10 nM, 100 nM, 1 μ M, 3 μ M & 30 μ M) for an additional 48 hours with the medium exchanged after 24 hours. Note the decreased cell growth in the cultures treated with AEA (Bar = 10 μ M). From (Habayeb *et al.*, 2008a).

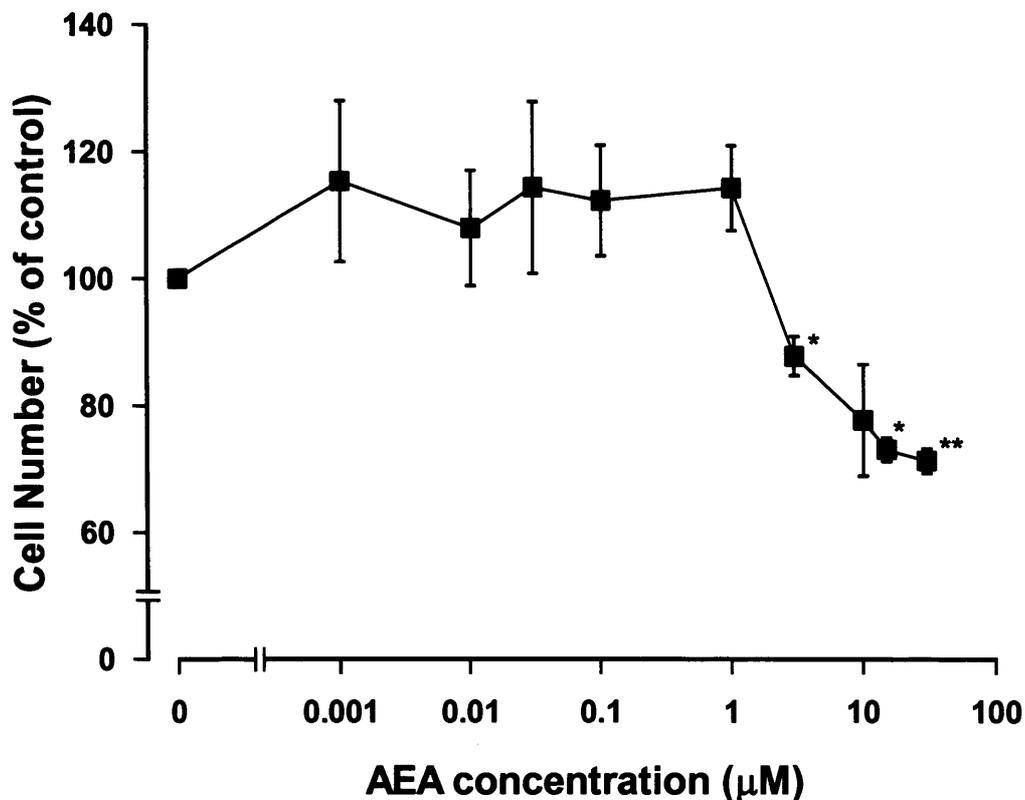


Figure 6.14: Effect of anandamide on BeWo cell growth.

BeWo cells plated at 1×10^4 cells/ well in 96-well plates were allowed to proliferate for 48 hours before treatment with the indicated concentrations of AEA for an additional 48 hours with the medium exchanged after 24 hours. The cells were incubated with XTT proliferation agent for 4 hours and the levels of colour developed measured with absorbance at 492 nm and corrected for microplate imperfections at 620 nm and then converted to the cell numbers relative to the untreated controls for each plate. The data are presented as mean \pm SEM for 4 independent experiments performed in quadruplicate.

(* $p < 0.05$; *** $p < 0.001$ One-way ANOVA with Tukey's honestly significant difference test; $n=4$). From (Habayeb *et al.*, 2008a).

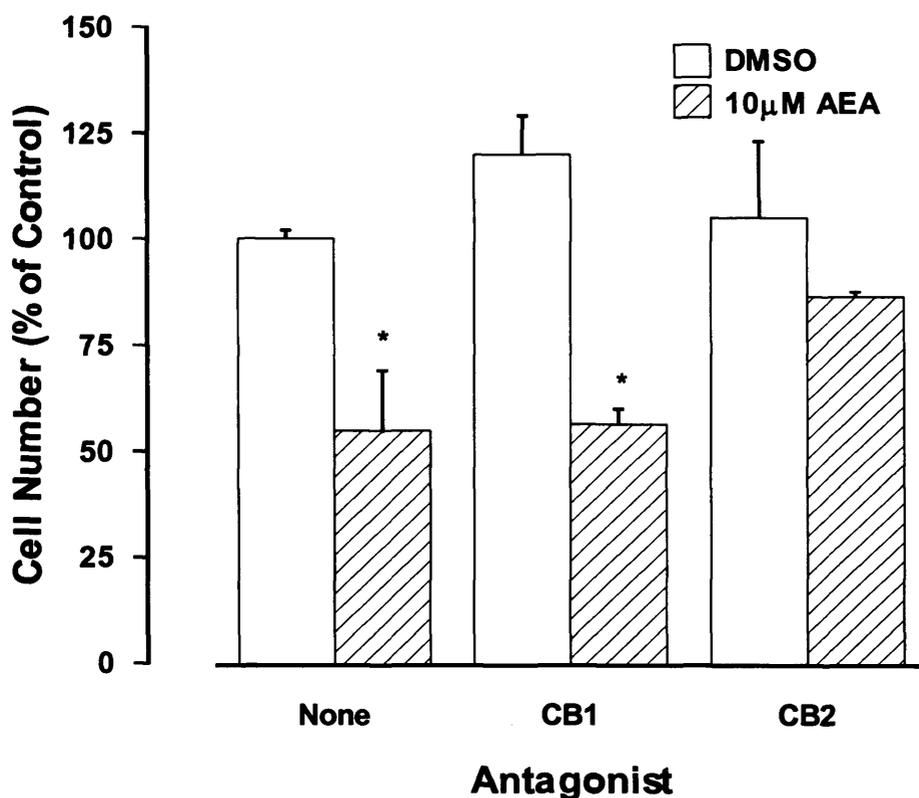


Figure 6.15: The effects of AEA on BeWo cell growth in the presence of cannabinoid receptors antagonists. BeWo cells plated at 1×10^4 cells per well onto 96-well plates were allowed to proliferate for 48 hours before treatment with $10 \mu\text{M}$ AEA for an additional 48 hours with the medium exchanged after 24 hours in the presence or absence of 300 nM of SR141716A (CB_1 -antagonist), SR144528 (CB_2 -antagonist) or the antagonist diluent DMSO (0.1% dimethylsulfoxide) acting as a control. Note that the inhibitory effect of AEA on BeWo cell growth is abolished in the presence of the CB_2 -antagonist but not in the presence of the CB_1 -antagonist or the control. Data presented as the mean \pm SEM for three independent experiments performed in quadruplicate ($n=3$). *, $P < 0.05$ Student's paired t test. From (Habayeb *et al.*, 2008a).

6.4. Discussion

The results of these studies confirm that the various components of the endocannabinoid system (CB₁, CB₂ and FAAH) are expressed in first trimester tissues. Additionally, CB₁ receptor transcript levels diminished after 9 weeks gestation, and immunoreactive FAAH disappeared from the syncytiotrophoblast by the 11th week of gestation. CB₂ expression, however, remained constant, at both the transcript and protein levels, throughout the first trimester.

Since the syncytiotrophoblast is in direct contact with maternal blood, the presence of FAAH in this layer suggests that it could be acting to protect the growing embryo from the detrimental effects of AEA. The disappearance of FAAH at 11 weeks when circulation in the placental unit is fully established is interesting as it suggests that the protective role of the syncytiotrophoblast may be only necessary prior to the establishment of the placental unit. The concurrent increase in FAAH expression in the mesenchymal core of villi in the fully established placental unit suggests that this area of the developing placenta then assumes the protective role of the developing syncytiotrophoblast. This is supported by the results described in Chapter 3, which suggest that there is little change in the plasma concentrations of AEA in the first trimester of the pregnancy compared to those in the luteal phase of the menstrual cycle. This may also explain why there is no concomitant decrease in levels of FAAH mRNA from homogenized tissues (Figure 6.3).

Two studies in the literature have investigated the presence of CB₁ immunoreactive protein and transcripts in the human placenta. In the first, CB₁ transcripts were found in

the term placenta (Kenney *et al.*, 1999), but in the second study performed on first trimester placental tissue neither CB₁ transcripts nor CB₁ immunoreactive proteins were detected (Helliwell *et al.*, 2004). The reasons for the discrepancy in these observations is unclear, but may be related to methodological differences, because the CB₁ immunoreactivity found in this study was only demonstrable after a process of antigen retrieval by microwave energy and the use of the tyramide amplification system to increase the immunoreactive signal (Figure 6.5). The immunostaining pattern in term fetal membranes (Park *et al.*, 2003) were consistent with these findings, suggesting that the methodology that was used here is appropriate for immunohistochemical detection of CB₁.

The immunohistochemical data presented in this chapter were supported by the presence of transcripts for FAAH, CB₁ and CB₂ (Figures 6.1, 6.2 & 6.3). These findings are different from those described by Helliwell *et al* who did not detect CB₁ transcripts and reported a different pattern of FAAH transcripts (Helliwell *et al.*, 2004). A careful BLAST search of the published primer sequences (Helliwell *et al.*, 2004) indicated that the sequences used by Helliwell *et al* are unlikely to detect human CB₁. When the previously used protocols (Helliwell *et al.*, 2004) were applied to the term fetal membrane, CB₁ transcripts were not detectable (data not shown). However, using primer sets of Kenney *et al.*, (Kenney *et al.*, 1999), which were previously validated for first trimester trophoblast samples and BeWo cells, where cannabinoids prevented serotonin transport (Kenney *et al.*, 1999), it was clear that human first trimester trophoblast contain transcripts of both CB₁ and CB₂. Since human FAAH transcripts could not be detected with the published FAAH primer sets under the experimental conditions set out in the same paper (Helliwell *et al.*, 2004), we designed our own set of

primers that were directed against exons 14 and 15 of the human FAAH gene (GeneBank accession number: AH007340). As can be seen (Figure 6.1), the predicted product from RT-PCR was generated in all trophoblast samples and that the levels were not modulated through the first trimester, suggesting that the use of a more sensitive amplification technique in the immunohistochemistry studies and the use of better gene-specific primers could explain the discrepancy between the studies presented in this chapter and those of Helliwell *et al.* (Helliwell *et al.*, 2004).

The findings in this chapter suggest that absolute trophoblastic FAAH levels and CB₂ levels do not alter significantly during the first trimester, but their cellular distribution is altered from the syncytiotrophoblast to the mesenchymal core of the villous. The most significant change was the diminution of CB₁ expression after the 9th week of gestation, a point of critical alteration in the developing placenta, where the maternal and fetal blood meet in a meaningful way for the first time (Norwitz *et al.*, 2001; Moffett & Loke, 2006). The placenta forms an efficient barrier between fetal and maternal blood and separates the fetus from maternal supplies of natural endocannabinoids, such as anandamide and 2-arachidonyl glycerol and natural exocannabinoids, such as Δ^9 -THC; therefore, it was hypothesised that if high levels of maternal anandamide were detrimental to early placental and fetal development, FAAH expression would be high in the trophoblast during normal early pregnancy as has been suggested before (Helliwell *et al.*, 2004).

The fact that the protein levels of FAAH remained constant in early gestation and appeared to diminish after the 9th week of gestation may reflect the protection afforded the fetus during this critical time. Although larger subject numbers would be required

to confirm these findings, the results of this chapter suggest that FAAH levels in the human trophoblast decline between the 10th and 12th week of gestation. These data appear to reflect the results of Maccarrone *et al.*, where maternal FAAH levels and activity in PMNCs peaked at 9 to 10 weeks of gestation (Maccarrone *et al.*, 2000d). An investigation into the factors regulating FAAH levels at the trophoblast level may provide an explanation for the observed changes in plasma AEA at this point in gestation.

There is growing evidence that a major transition in placental physiology occurs at approximately 10th week of gestation with the dissipation of trophoblast plugs from the spiral arteries consequently allowing maternal blood to perfuse the placenta for the first time (Jauniaux *et al.*, 2000; Jauniaux *et al.*, 2001). Thus, at about the 10th week of gestation, the human fetus must begin to protect itself against circulating maternal anandamide, but because the CB₁ and CB₂ receptors are expressed in the syncytiotrophoblast, endocannabinoids are likely to have a regulatory role in the placenta. One suggestion is that endocannabinoids may be involved in the removal of damaged cells by a process of apoptosis. Indeed, there is emerging evidence that endocannabinoids have a significant apoptotic potential in other organs (Maccarrone & Finazzi-Agro, 2003). The studies on the effect of anandamide on BeWo cell growth (Figures 6.13 & 6.14) tend to support this suggestion, as high concentrations of AEA correlated with lower cell numbers and reduced cell growth, an effect that appear to be mediated by CB₂ (Figure 6.15).

6.4. Conclusions

The findings in this chapter revealed that transcripts for CB₁, CB₂ and FAAH are present in human first trimester trophoblast extracts with only the CB₁ transcript being significantly regulated. The significant 4.7-fold increase in expression at 10th week gestation was reduced to 8.9% of the peak value by week 12. Transcripts for CB₂ showed a similar pattern of expression but were not significantly reduced. By contrast, FAAH transcript levels appeared to increase towards the end of the first trimester, but again did not reach significance. These observations were supported by immunohistochemical studies that demonstrated a similar pattern of expression at the protein level, with cellular localisation for all three proteins concentrated within the syncytiotrophoblast layer. Anandamide also prevented BeWo trophoblast cell proliferation in a dose-dependent manner with a 65-70% significant inhibition of cell proliferation with concentrations in excess of 3 μ M.

Taken together, the data in this chapter may explain, in part, how elevated plasma anandamide levels may lead to increased risk of first trimester pregnancy loss.

Chapter 7

Discussion and future directions

Chapter 7

Discussion and future directions

This thesis was designed to test the hypothesis that the endogenous cannabinoid, anandamide, acting *via* receptor-mediated mechanisms, is involved in the maintenance of early pregnancy. It was hypothesised that any effect would be mediated by alterations either through changes in the systemic or local levels of anandamide and/or of its receptors in the fetal-maternal interface.

At the outset, there were no published data to indicate whether it was possible or not to measure AEA in human plasma. Therefore, the first part of this thesis involved the development of a robust and reproducible assay to measure anandamide concentrations in human plasma. This was achieved by modifying an HPLC/MS technique based assay that had been used to measure anandamide concentrations in rat plasma (Giuffrida *et al.*, 2000). Although all studies in this thesis were subsequently performed on freshly processed plasma samples, this was laborious and time consuming making the measurement in large samples collected over a period of time difficult. The next part of the method development was therefore an improvement in the assay technique to enable reliable quantification on stored plasma. The success of this would eventually allow for the storage of samples and the transport of samples between different laboratories; ultimately enabling collaborative studies between laboratories.

The ability to accurately measure AEA in plasma samples potentially opens up many other avenues for further investigation of the roles of AEA in reproduction. Potentially, the method could be used to test other bio-matrices such as follicular or seminal fluids (Schuel *et al.*, 2002a), helping to explore other potential roles for AEA in those fluids.

Once a robust, reproducible assay for plasma AEA was established, the assay was applied to measure the plasma AEA levels in a cohort of healthy women volunteers during the menstrual cycle, through uncomplicated pregnancy, including labour and in a group of postmenopausal women. Anandamide concentrations varied during these physiological states in a pattern suggestive of hormonal regulation with the concentrations in the luteal phase of the menstrual cycle (a state characterised by high progesterone levels) being lower than in the follicular phase (a state characterised by high estrogen and low progesterone levels) suggesting that estrogen and progesterone may modulate plasma anandamide levels. Indeed, in a group of postmenopausal women (a state characterised by low estrogen and low progesterone levels), anandamide concentrations were demonstrated to be low (Habayeb *et al.*, 2004). These findings are consistent with reported literature as estrogen was shown to stimulate the synthesis and release of anandamide (Maccarrone *et al.*, 2002a), while progesterone was found to stimulate FAAH, the main enzyme that metabolises anandamide, leading to a reduction in anandamide concentrations (Maccarrone *et al.*, 2003).

The most striking change in AEA concentrations, however, was an almost 4-fold increase in women who were in active labour compared to the concentrations in women in the third trimester who were not labouring (Habayeb *et al.*, 2004). Human labour,

however, is not associated with a reduction in progesterone concentrations, unlike many animals (Fuchs & Fuchs, 1984; Steer, 1990; Casey & MacDonald, 1997), hence the changes in anandamide concentrations in labour were unexpected. It is possible that the rise in AEA concentrations maybe a manifestation of the “functional withdrawal” of progesterone in labour (Mesiano *et al.*, 2002; Astle *et al.*, 2003). Since plasma AEA levels are considered to be controlled almost exclusively by the actions of FAAH in the peripheral mononuclear cells (PMNC), then these data suggest that a similar “functional progesterone withdrawal” probably occurs in the PMNCs as plasma AEA concentrations are reported to be inversely correlated with the levels of FAAH in these cells (Lazzarin *et al.*, 2004). Potentially, this means that PMNCs could be studied as a model that reflects changes occurring in the myometrium during this important event. The use of these cells offers a clear advantage over studying human myometrial tissue, as PMNCs can be obtained easily compared to obtaining myometrial tissue from labouring uteri.

It is unclear what the role of an increased AEA level at the time of labour might be, but it has been suggested that AEA might act as a reservoir of arachidonic acid that is needed for the production of prostaglandins (Schmid *et al.*, 1997). Alternatively, the relaxing effects of anandamide on the myometrium (Dennedy *et al.*, 2004) might allow the myometrium to relax between contractions. Further work to delineate the actual role of AEA in human labour is required, but the observations on plasma AEA reported in this thesis suggest that AEA may have many putative roles in human pregnancy. For example, the reduction in plasma levels during the latter stages of the menstrual cycle and the fact that it remains low through to the early stages of pregnancy suggests that a

low plasma AEA level could be important for early pregnancy success. By contrast, a high plasma AEA level might be indicative of early pregnancy failure.

Several studies in both animals and humans suggested that AEA is involved in the early stages of reproduction. In the murine pregnancy for example, uterine AEA concentrations differed significantly in the sites where implantation occurred compared to those where it did not (Schmid *et al.*, 1997; Paria *et al.*, 1996). More recently, NAPE-PLD was found to be an important regulator of AEA levels in the murine uterus (Guo *et al.*, 2005). The mouse embryo was found to express both CB₁ and CB₂, and AEA was shown to arrest the development of such embryos (Paria *et al.*, 1995). In humans, Maccarrone *et al* found that the levels of FAAH in PMNCs were significantly lower in women who miscarried compared to the levels in those who did not and that AEA concentrations in the plasma of women undergoing IVF treatment was lower in those who conceived compared to that in those who did not (Maccarrone *et al.*, 2002b). However, there were no studies that had measured the plasma AEA concentrations in relation to miscarriage, whether spontaneous, threatened or recurrent. This is a particularly difficult group of women to treat as there are no proven therapies. Therefore, a cohort of 45 women with confirmed viable intrauterine pregnancy who presented with a threatened miscarriage between 6 and 11 weeks gestation was studied in this thesis. Of these women, 36 had live births and 9 had spontaneous miscarriages. The mean \pm SEM plasma concentration of AEA in the group that miscarried was approximately 2.58- fold higher than that in the group with live births (3.39 ± 0.29 v 1.24 ± 0.12 ; $p < 0.0001$; Mann-Whitney U-test; Figure 5.1). Out of the 36 women who had live births, 34 had plasma AEA levels < 2.0 nM. All the nine women who subsequently miscarried had AEA values > 2.0 nM and six out of these nine had AEA

values > 2.2 nM. Using 2.0 nM as the optimal cut-off point, a single plasma AEA measurement in women with a threatened miscarriage in the first trimester, provided a sensitivity of 100% and a specificity of 94.4% with a negative predictive value of 100% and a positive predictive value of 81.8% (Habayeb *et al.*, 2008b) for subsequent miscarriage. In other words, if plasma AEA concentrations are <2.0 nM, there is a 100% chance of a live birth. This is clinically important, because women in this group are routinely treated with 'expectant management'; essentially 'wait and see'. However, this first biochemical test that has a 100% negative predictive value is good as it allows the clinician to assure those women who are worried that they will not miscarry. However, the main criticism of this study is that the sample size is small (n=45) and so the publication of the data are unlikely to change clinical practice overnight. What is required is a much larger blinded study of not only women with threatened miscarriage, but also those with spontaneous and recurrent miscarriages. However, the raised plasma AEA level in the study described in Chapter 5 is similar to the observations of Maccarrone *et al* in which elevated AEA levels were noted in women who failed to conceive following embryos transfer during IVF treatment (Maccarrone *et al.*, 2002b).

These findings raised the question of precisely how AEA levels adversely affect such pregnancies. One interesting observation comes from studies that have shown that the active ingredient of marijuana, Δ^9 -THC, also affects pregnancy by increasing a women's risk of miscarriage, developing pre-eclampsia, having a baby that is small for gestation age or being born prematurely (Gibson *et al.*, 1983; Zuckerman *et al.*, 1989; Frank *et al.*, 1990; Sherwood *et al.*, 1999). All of these conditions are known to have an aetiology that involves a detrimental effect on the developing placenta and so it is reasonable to hypothesise that both Δ^9 -THC and AEA have detrimental effects at the

fetal-maternal interface. The suggestion is that in those women with elevated AEA levels in early pregnancy, the AEA interferes with the growth and/or function of the trophoblast (i.e. the developing placenta).

In order to study this hypothesis further, the expression of the endocannabinoid system (CB₁, CB₂ and FAAH) in the first trimester pregnancy tissue both at the RNA and the protein level was examined. CB₁, CB₂ and FAAH were all expressed in the first trimester trophoblast tissue, with cellular localisation for all three proteins concentrated within the syncytiotrophoblast layer. Additionally, CB₁ receptor transcript levels diminished after 9 weeks gestation, and immunoreactive FAAH diminished from the syncytiotrophoblast by the 11th week of gestation. CB₂ expression, however, remained constant, at both the transcript and protein levels, throughout the first trimester (Habayeb *et al.*, 2008a). The presence and modulation of the endocannabinoid system in the first trimester trophoblast/placenta suggests that AEA could have a direct effect on its function. To test this, the effects of AEA on BeWo cells were studied, as these cells are a good model for first trimester trophoblast. It was found that higher concentrations of AEA correlated with lower cell numbers and reduced cell growth (Habayeb *et al.*, 2008a). These results suggest that anandamide has a direct detrimental effect on trophoblast development.

It is interesting to note that one of the 2 women in the threatened miscarriage study who had AEA concentrations greater than 2.0 nM and did not miscarry, went on to have a difficult pregnancy with severe pre-eclampsia and was delivered of a growth restricted baby who weighed 1.85 kg at 33 weeks gestation. It is now recognised that conditions such as pre-eclampsia and fetal growth restriction are manifestations of defective

trophoblast function with failure of the trophoblasts to invade the walls of blood vessels and convert the utero-placental circulation into a high capacity low resistance system (Pijnenborg *et al.*, 1980; Olofsson *et al.*, 1993; McMaster *et al.*, 1994). It is therefore possible to suggest that raised levels of AEA in early pregnancy either lead to complete destruction of the trophoblasts resulting in miscarriage or partial destruction leading to PET or FGR. Whilst these findings are exciting and might open the door for the development of a test that can be used to predict the outcome of human pregnancies, one has to emphasise the need for confirmation of these findings in a larger study, before the application of such a test can become a reality.

There have been recent studies on the role of AEA in reproduction both in animals and humans. For example, AEA concentrations have been found to regulate the transport of the mouse embryo in the murine pregnancy (Wang *et al.*, 2004). These findings appear to be mirrored in human ectopic pregnancies where the expression of CB₁ in the fallopian tubes of women affected with ectopic pregnancies was found to be different compared to those in women who did not have ectopic (Schuel, 2006). In addition, anandamide was recently found to stimulate the production of prostaglandins by human gestational tissue (Mitchell *et al.*, 2008), possibly providing an explanation for the potential role of AEA in labour and in early pregnancy.

The main conclusion from all the above observations is that anandamide is involved in the process of implantation, and hence the finding of high anandamide in the miscarriage group. If the mechanisms controlling AEA levels can be elucidated, then a potential therapy might be found.

Future directions

The results of the studies within this thesis have highlighted the potential role of endocannabinoids, and anandamide in particular, in human reproduction. This is a new field with exciting potential and the following are some suggestions for future research projects:

- 1) A study to further investigate more detailed changes in AEA concentrations in the menstrual cycle is warranted. The study presented in this thesis focused on the follicular phase (days 2-7) and the luteal phase (day 20-25) in a cross-sectional manner. There might be more changes in plasma AEA concentration especially around ovulation or at the very end of the cycle that could be related to endometrial breakdown.
- 2) A longitudinal study to determine the changes in plasma anandamide concentrations during human pregnancy. In this thesis a cross-sectional study of 5 groups of women at various gestational ages was undertaken. There might be further changes that can only be elicited by measuring anandamide at weekly intervals during pregnancy.
- 3) The finding of high anandamide concentrations in labour (3.7-fold higher than the group of women at term who were not in labour) was an exciting observation. It also interesting to note that the concentrations of plasma AEA in the term group who were not in labour was higher than the levels in the third trimester.

This raised an interesting question; do the levels of AEA rise before the onset of labour? If so, there are potentially many applications for performing such measurements:

- A) Could plasma AEA concentrations be a predictor for success of induction of labour? It could be hypothesized that AEA concentrations are higher in those women who respond to the process of induction of labour compared to those who do not and that there is a critical threshold of plasma AEA levels above which the induction is likely to succeed.

 - B) Does plasma anandamide rise before the onset of preterm labour? If so, could plasma AEA be used as a predictor of preterm labour? To address this question, a study to measure anandamide concentrations in women who are at high risk of preterm labour is suggested.
- 4) A large study in women with threatened miscarriage to determine if the pilot findings in this thesis apply to larger and more heterogeneous population.
- 5) A study comparing the expression of the endocannabinoid system in first trimester trophoblast tissue comparing samples obtained from surgical termination of the pregnancy (normal pregnancy), medical termination of the pregnancy (normal pregnancy affected by progesterone inhibition) and miscarriage to determine if the expression of the endocannabinoid system changes in pregnancies complicated by miscarriages.

- 6) Further development in the assay of anandamide with the aim of producing a bedside test that is both quick and reliable and cheap.

Appendices

Appendix 1: Krebs-Tris Buffer

Sodium chloride (NaCl)	136 mM
Potassium chloride (KCl)	5 mM
Magnesium chloride (MgCl ₂)	1.2 mM
Calcium chloride (CaCl ₂)	2.5 mM
Glucose	10 mM
Trizma Base	20 mM
Ethylene diamine tetraacetic acid (EDTA)	4.5 mM

Prepared in de-ionised water at pH of 7.4

Appendix 2: Silanisation procedure

Materials

Silanising fluid: 5% of dimethyldichlorosilane (DMDCS) in toluene, both from Supelco (Poole, Dorset, UK).

Procedure

A) Glass pipettes:

- 1) Silanising fluid added to a 2.0 litter glass beaker to a depth of approximately 5 centimetres.
- 2) Pipettes were placed in the beaker, tips down and left for 2 hours to silanised in the silanising vapour.
- 3) Each pipette was then washed with methanol to remove any remnants of silanising fluid.
- 4) The pipettes were then dried in a drying oven for 30 minutes.

B) Conical glass inserts:

- 1) Each insert was filled completely with silanising fluid and left for 30 minutes in an upright position.
- 2) The silanising fluid was removed and the inserts were washed with methanol.
- 3) Inserts were dried in a drying oven for 30 minutes.

Appendix 3a: A copy of the letter of approval for the project

28 NOV 2001

University Hospitals of Leicester **NHS**

NHS Trust

DIRECTORATE OF RESEARCH AND DEVELOPMENT

Leicester General Hospital
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23 November 2001

Dr J.C. Konje
Senior Lecturer/Honorary Consultant
Obstetrics/Gynaecology
Leicester Royal Infirmary

Dear Dr Konje

RE: Project Number: 7516 *(Please quote this number in all correspondence)*
A pilot study to determine the levels of anandamide in the maternal serum during pregnancy and labour

Since all aspects of your LRI R&D notification are complete, I now have pleasure in confirming full approval of the project on behalf of the University Hospitals of Leicester NHS Trust.

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

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

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

In the meantime, in order to keep our records up to date, could you please notify the Research Office if there are any significant changes to the start or end dates, protocol, funding or costs of the project.

I look forward to the opportunity of reading the published results of your study in due course.

Yours sincerely



Dr Nichola Seare
Research and Development Business Manager

Trust Headquarters, Glenfield Hospital, Groby Road, Leicester, LE3 9QP
Website: www.uhl-tr.nhs.uk
Chairman Mr Philip Hammersley CBE Chief Executive Dr Peter Reading

Appendix 3b: A copy of the consent form.

University Hospitals of Leicester 	
NHS Trust	
Principle Investigator Dr. Justin Konje MD, MRCOG Department of Obstetrics & Gynaecology Clinical Sciences Building Leicester Royal Infirmary Leicester LE2 7LX	Leicester Royal Infirmary Leicester LE1 5WW Tel: 0116 2541414 Fax: 0116 2585631 Minicom: 0116 2586878
PATIENT CONSENT FORM	
<u>Title of study</u>	
A pilot study to determine the levels of anandamide in the maternal serum during pregnancy and labour	
I agree to take part in the above study as described in the Patient Information Sheet	
I understand that I may withdraw from the study at any time without justifying my decision and without affecting my normal care and medical management.	
I understand that members of the research team may wish to view relevant sections of my medical records, but that all the information will be treated as confidential.	
I understand medical research is covered for mishaps in the same way as for patients undergoing treatment in the NHS i.e. compensation is only available if negligence occurs.	
I have read the patient information leaflet on the above study and have had the opportunity to discuss the details with and ask any questions. The nature and the purpose of the tests to be undertaken have been explained to me and I understand what will be required if I take part in the study.	
Signature of patient Date.....	
(Name in BLOCK LETTERS)	
.....	
I confirm I have explained the nature of the Trial, as detailed in the Patient Information Sheet, in terms which in my judgement are suited to the understanding of the patient.	
Signature of Investigator Date.....	
(Name in BLOCK LETTERS)	
.....	
<small>Trust Headquarters, Glenfield Hospital, Groby Road, Leicester, LE4 9QP Website: www.uhll.nhs.uk Chairman Mr Philip Hammersley CBE Chief Executive Dr Peter Reading</small>	

Appendix 4: Bradford's Assay to determine the protein content of Anti-cannabinoid antibodies

Materials: 1) Bovine serum albumin (BSA) (Fraction V; Sigma-Aldrich Ltd).
2) Bradford reagent (Biorad,

Methods: 1) Stock of BSA in de-ionised water made at a concentration of 100 µg/ml.
2) Standard curve set with the following standards: 0 µg/ml, 1 µg/ml, 2.5 µg/ml, 5 µg/ml, 7.5 µg/ml, 10 µg/ml and 15 µg/ml.
3) Duplicate of each standard (800 µl) measured into a cuvette.
4) To each cuvette, 200 µl of Bradford reagent was added to make the final volume 1 ml. After a thorough mix, standard were allowed to stand for 20 minutes at room temperature.
5) Optical density of standards read on a spectrophotometer at 595 nm (see Figure A1) and the regression line calculated.
6) From each antibody (anti CB₁ and anti-CB₂), 2 µl taken then diluted in de -ionised water (1 in 2 and 1 in 4 dilution) equivalent to 1 µl and 0.5 µl of IgG).
7) Each antibody sample was diluted further to 800 µl with de-ionised water and 200 µl of Bradford reagent added and thoroughly mixed.
8) The absorbance at 595 nm was measured.

Each antibody contains 1 mg/ml (i.e. 1 µg/µl) of BSA (added by the manufacturer as a stabiliser). Any protein above that value is attributed to IgG.

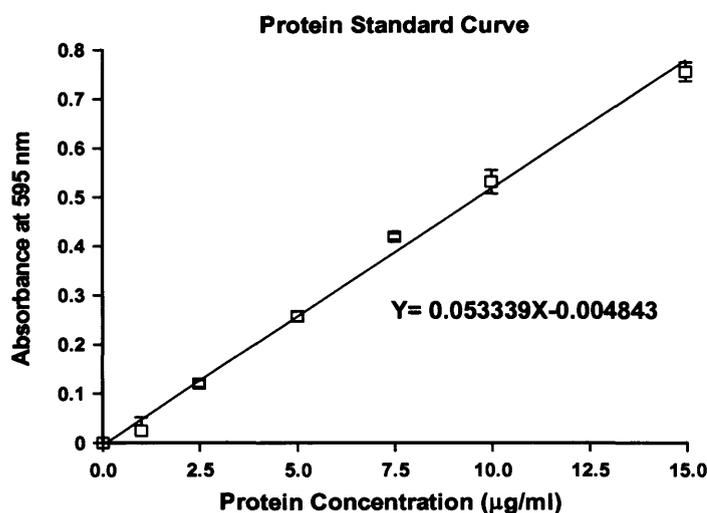


Figure A1: The relationship between the protein concentration and the absorbance at 595 nm.

Results:

- 1) From the standard curve, the following equation was derived:

$$Y = 0.053339X - 0.004843$$

Where Y is the absorbance at 595 nm, X is the concentration of the protein (µg/ml).

- 2) The absorbance for 1 µg of BSA is 0.047386 AU (0.053339-0.004843).
- 3) The measured absorbance for 1 µl of CB₁ and CB₂ was 0.048 and 0.0565 AU respectively.
- 4) The difference between the values in "3" and "2" are attributed to IgG in the antibodies. For CB₁ this was 6.14×10^{-4} and for CB₂ was 9.114×10^{-3} AU.
- 5) Applying the equation to the values obtained in "4" results in "X" value for CB₁ and CB₂ of 66.88 and 170.96 µg in the aliquots tested.
- 6) Since only an equivalent of 0.8 of the aliquot was tested (due to the addition of 200 µl of Bradford reagent to the 800 µl diluted sample), the figures obtained

in “5” need to be divided by 0.8 to give the concentration in $\mu\text{g}/\mu\text{l}$. These values were 83.6×10^{-3} and $213.7 \times 10^{-3} \mu\text{g}/\mu\text{l}$ for CB_1 and CB_2 respectively or 83.6 and 213.7 $\mu\text{g}/\text{ml}$ for CB_1 and CB_2 respectively.

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Publications



Minireview

Endogenous cannabinoids: Metabolism and their role in reproduction

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Abstract

Over the past two decades a number of endogenous compounds that act as ligands for the cannabinoid receptors has been discovered. In analogy with the “endorphins” these compounds have been called “endocannabinoids”. Endocannabinoids have been demonstrated in many mammalian tissues including humans and are widely distributed in the CNS, peripheral nerves, uterus, leukocytes, spleen and testicles. The uterus contains the highest levels of anandamide, the first discovered endocannabinoid, suggesting an important role for this substance in reproduction. Several studies have shown anandamide to be involved in the regulation of implantation and reduced activity of the enzyme that degrades anandamide has been associated with early pregnancy loss in humans. The bulk of the literature concerning endocannabinoids is based upon anandamide related studies; therefore, in this review we focus on the metabolism of anandamide and its role in reproduction. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Anandamide; Fatty acid amide hydrolase; Lymphocytes/Uterus

Introduction

Endogenous cannabinoids are unsaturated fatty acid derivatives which act as endogenous ligands for cannabinoid receptors. The first product of this group, Anandamide (arachido-nylethanolamide), was isolated from the porcine brain in 1992 [1]. The name was coined

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from the Sanskrit word “Ananda” meaning bliss. Since then a number of fatty acid derivatives that belong to the group, endocannabinoids has been isolated. These include sn-2 arachidonyl-glycerol [2,3,4], homo- γ -linoleoylethanolamide and docosatetraenoyl-ethanolamide [5].

Anandamide has several recognised effects on the nervous system. Through interactions and modulation at the level of neurotransmitters in different regions of the nervous system [6] it produces several effects on various brain activities. In rodents for example, anandamide inhibits memory consolidation [7], impairs working memory [8], exhibits analgesic properties [2], inhibits motor coordination (See Ref. [6] for review) and anxiety like responses [9] and decreases arousal [10]. All these effects suggest that it might act as a “stress recovery-factor” relieving some of the stress- induced responses and sending messages such as “relax, eat, sleep, forget and protect” (Cited from [6]).

The uterus contains the highest concentrations of anandamide yet discovered in mammalian tissues [11] and this suggests that it might play a role in reproduction. Although there is a dearth of knowledge on the role of endocannabinoids in reproduction, the fact that exogenous cannabinoids such as marijuana and hashish have significant effects on reproduction and pregnancy suggests a potential role for endocannabinoids in reproduction. In human marijuana smoking during pregnancy is significantly associated with an increased risk of preterm labour [12] and intrauterine growth restriction (evidenced by reduction of birth weight, height, head circumference, mean arm muscle circumference and non-fat area of the arm) [13,14,15]. Acute marijuana smoking in humans suppresses plasma LH in the luteal phase. In chronic users, it shortens the menstrual cycle, the effect being predominately a short luteal phase leading to menstrual irregularities and anovulation [16].

Metabolism of anandamide

Analytical studies and tissue distribution

In the human, anandamide has been isolated in the brain, spleen, heart [17] and breast cancer cells [18]. Its distribution is more widespread in the rat where it has been found in pheochromocytoma cells [18], the kidneys [19], brain [17,20], testicles [21], spleen [17], skin [17] and blood plasma [22]. It has also been found in the murine uterus [11], leukocytes and bovine and porcine brain [23]. The highest concentrations of anandamide in the rat brain are found in the hippocampus and striatum [17].

Synthesis of anandamide

Anandamide is an ethanolamine amide of arachidonic acid, the precursor of prostaglandins and leukotrienes. It is produced on demand and released from cells when the need arises (i.e. not stored in cells) [24]. The exact stimulus for its synthesis is poorly understood. Two mechanisms for the production of anandamide have been described. The first involves the hydrolysis of a phospholipid precursor in the cell membrane called N-arachidonyl-phospha-

tidyl ethanolamine (NAPE) to anandamide (Fig. 1). This process is mediated by the enzyme N-acyl-phosphatidyl ethanolamine selective phospholipase D (Na PE-PLD) [6,21,25–27]. This mechanism is initiated by calcium ion influx into the cell and has been shown to occur in several mammalian tissues including cultured intact rat neurons [25] and in rat testes [21].

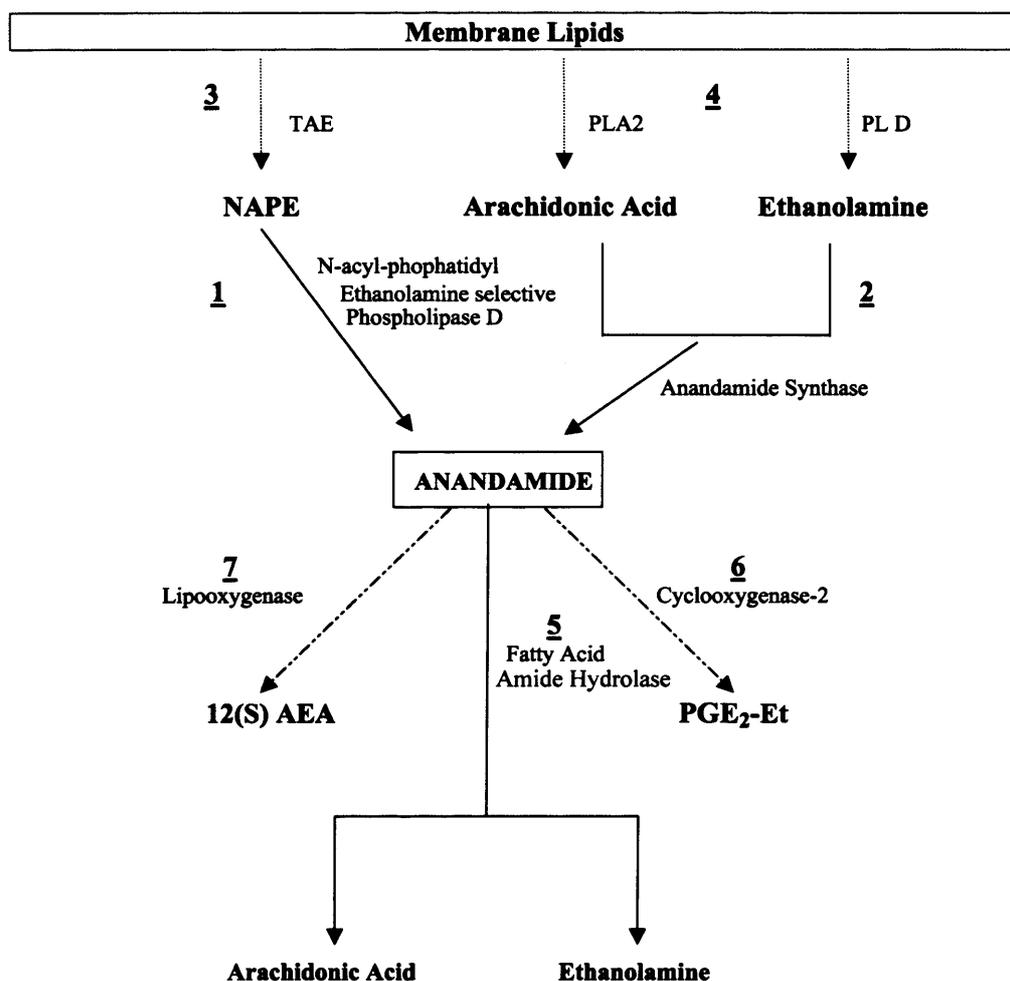


Fig. 1. Metabolism of Anandamide. 1) Anandamide production by Na-PE-PLD from N-arachidonyl-phosphatidyl ethanolamine. 2) Anandamide production by Anandamide Synthase. 3) N-arachidonyl-phosphatidyl ethanolamine is produced by the activity of transacylase enzyme. 4) Production of arachidonic acid and ethanolamine from membrane lipid precursors. 5) Degradation of anandamide by fatty acid amide hydrolase. 6) Degradation of anandamide by cyclooxygenase-2 enzyme. 7) Degradation of anandamide by lipooxygenase enzyme. Abbreviations: TAE, (Transacylase enzyme); PLA-2, (Phospholipase-2 enzyme); PLD, (Phospholipase D enzyme); NAPE, (N-arachidonyl-phosphatidyl ethanolamine); PGE₂-Et, (Prostaglandin E₂-ethanolamine); 12(S) AEA, (12(S)-hydroxy-arachidonyl ethanolamine).

The precursor NAPE is produced in the cell membrane by a Ca^{2+} -dependent transacylase enzyme [6,26,27]. The second mechanism which is de-novo synthesis of anandamide from arachidonic acid and ethanolamine by condensation (Fig. 1) has however not been demonstrated in living cells [6].

Release and uptake of anandamide

Once synthesized, anandamide is released from the cell into the extra cellular space where it can either act in an autocrine or paracrine manner [24]. Due to its lipophilic nature, anandamide may diffuse passively through the cell membrane into the cytosol [28]. It can also be internalised by the process of facilitated diffusion mediated by a selective anandamide transporter - this indeed is the main mechanism for internalisation of anandamide [29,30]. Several compounds, for example phenylmethylsulfonyl fluoride, oleamide [30,31] and *N*-(4-hydroxyphenyl) arachidonoylamine (AM404) [29]) can inhibit anandamide uptake and therefore potentiate its effects.

Degradation of anandamide

Once internalised anandamide is metabolised to arachidonic acid and ethanolamine (Fig. 1) by fatty acid amide hydrolase (FAAH) [32,33]. This enzyme, originally known as “anandamide hydrolase” and “anandamide amidohydrolase”, is encoded by a single gene localised on the short arm of human chromosome one [34]. It is 579 amino acids long and has a molecular mass of 63.0 kDa [33]. It is most abundant in brain and liver tissues and is present to a lesser extent in the spleen, kidney, testis and lungs [28,34].

Human FAAH shares 82% and 84% sequence identity with FAAH in the rat and mouse respectively. This degree of sequence homology suggests that the enzymatic mechanism for anandamide metabolism has been well conserved through mammalian evolution [28,34]. FAAH is inhibited by phenylmethylsulfonyl fluoride (PMSF), arachidonic acid, arachidonyl trifluoromethyl ketone, 2-octyl γ -bromoacetoacetate, and palmitoylsulfonyl fluoride (AM374) [35–40].

Anandamide is also metabolised by cyclo-oxygenase - 2 to prostaglandin E_2 -ethanolamide [41]. Although the physiological significance of this metabolic pathway is unclear, it does, however, further suggest a close link between anandamide and eicosanoids. Anandamide is also metabolised by lipo-oxygenase to form 12 (S)-hydroxy-arachidonylethanolamide, but the physiological significance of this is unclear [42–44].

Mechanism of action

Cannabinoid receptor-mediated actions

Anandamide acts as an agonist for cannabinoid receptors. Two subtypes of receptors have been described to date. The first, CB_1 , was first described and characterised in the rat brain

[45]. The cloning and expression of a complementary DNA from a rat brain cDNA library that encoded CB₁ was reported in 1990 [46]. The human CB₁ shares 97.3% sequence identity with the rat CB₁ with 100% identity within the trans-membrane regions [47]. The second receptor subtype, CB₂, was first characterised from macrophages of the marginal zone of the spleen [48]. The CB₂ receptor shows 48% identity with CB₁ with 68% identity within the trans-membrane regions.

CB₁, originally called central receptor, is the main receptor in the brain. It is found in several areas of the brain including the hippocampus (which probably explains the effects of cannabinoids on memory functions), cerebellum, basal ganglia and cerebral cortex. Other tissues that express CB₁ include the cervical ganglion, peripheral autonomic nerves, testes, spleen, peripheral leukocytes, sperm, uterus, vascular endothelial and smooth muscle cells, human eye (retina, iris, corneal epithelium and ciliary body) and human placenta [47,49–58]. The CB₂ receptor, on the other hand, was first isolated from splenic cells; hence it is called “spleen-type” cannabinoid receptor. It is found in a number of immune system-derived cells including some T-cell lines (EL4.IL2, Jurkat E6-1 and HPB-ALL) [59], monocyte lines HL60 [48] and RAW264.7 [60] and the mast cell line RBL-2H3 [61]. Both receptors belong to the super family of G-protein coupled receptors (GPCR) and their functional responses are mediated by pertussis toxin sensitive GTP-binding (G_{i/o}) proteins. Two effects of stimulation of the CB receptors are recognized. Firstly, inhibition of adenylate cyclase leading to decreased levels of c-AMP [62–66], and secondly, inhibition of N-type and P/Q type calcium channels leading to a reduction of calcium ion influx to the cells [63,65–68]. These two receptor sub-types can be selectively inhibited/antagonised. SR141716A for example, given orally antagonises the CB₁ receptor while SR 144528 antagonises the CB₂ receptor [24,28]. Certain effects of anandamide, for example, activation of arachidonic acid release and inhibition of gap-junction mediated Ca²⁺ signalling in astrocytes are not mediated by either of the two known receptors but are mediated by interactions with G-proteins [69].

Non-cannabinoid receptor mediated actions

Anandamide can also affect cellular functions without interacting with cannabinoid receptors [28]. It can liberate arachidonic acid and positively modulate N-methyl-D-aspartate (NMDA)-mediated Ca²⁺ currents in cells that do not express the cannabinoid receptors [70]. In rabbit skeletal muscles, rat cardiac and brain membranes, anandamide inhibits L-type Ca²⁺-channels by direct interaction with dihydropyridine binding sites of these channels [71,72].

Effects of endogenous cannabinoids on reproduction

Effects on the hypothalamic-pituitary-ovarian axis

Exocannabinoids have several adverse effects on the hypothalamic-pituitary-ovarian axis in both animals and humans. As mentioned earlier, marijuana smoking is associated with the

suppression of LH and a shortened luteal phase in humans [16,73]. Delta-9-Tetrahydrocannabinol (Δ^9 -THC) causes delayed puberty in the female rat [74]. Exposure to Δ^9 -THC suppresses the preovulatory surge of LH in the female rat leading to blocked ovulation [75]. Chronic exposure to Δ^9 -THC in the female rat causes irregular oestrous cycles with reduced serum LH and FSH [76,77] and affects the ultra structure of preovulatory follicles, with a reduction in the mitochondrial and lipid droplet volumes in peripheral granulosa cells [78]. Similar findings of suppression of serum LH and delayed ovulation have been described in the female Rhesus monkey [79]. The inhibitory effect on ovulation is reversed by the administration of gonadotrophin releasing hormones [76]. This suggests that the inhibitory effect is mediated via the inhibition of hypothalamic factors, which naturally stimulate the release of pituitary LH.

The effects of endogenous cannabinoids on the hypothalamic-pituitary-ovarian axis are quite similar to those of Δ^9 -THC. Anandamide decreases serum LH and prolactin levels in rats of both sexes, although it does not affect the level of serum FSH [80]. Initially, the site of action of anandamide was thought to be the hypothalamic regulatory centres with no direct effect on the anterior pituitary. More recently, the CB₁ receptor was identified in the anterior pituitary itself [81,82] and suggests that anandamide may affect the function of the anterior pituitary by direct action as well as by interfering with the hypothalamic regulatory factors; the latter mechanism is supported by the fact that cannabinoid receptors are present in the arcuate nucleus and the preoptic area [83]. The expression of the CB₁ receptor gene in the anterior pituitary is regulated by sex steroids [84]. The amounts of CB₁ receptor-mRNA transcripts in the anterior pituitary fluctuate through the ovarian cycle in rats with the highest levels in the diestrus and the lowest in the oestrus phase. In contrast, anandamide levels in the anterior pituitary peak in the estrus phase and are lowest in the diestrus phase. The concentration of anandamide in the hypothalamus shows the reversed pattern, suggesting that anandamide concentration in both the anterior pituitary and the hypothalamus are influenced by sex steroids [84]. Anandamide might, therefore, play an important role in the regulation of the hypothalamic-pituitary-ovarian axis, possibly affecting the process of ovulation and the regulation of the menstrual cycle.

Anandamide affects the neuroendocrine system of pregnant rats. When administered chronically it prolongs the duration of pregnancy and increases the rate of stillbirth [85]. It also reduces the levels of serum LH, pituitary prolactin, serum prolactin, serum progesterone and prostaglandins PGF₁ and PGF₂ [85]. The reduction in the levels of prostaglandins might partly explain the prolongation of pregnancy. The prolongation of the duration of the pregnancy is different from the effect of exogenous cannabinoids which, as mentioned earlier, are associated with preterm labour. Administration of anandamide has no effect on the levels of FSH or gonadotrophin releasing hormone [85]. The postnatal development of the hypothalamic pituitary axis in the offspring of animals who receive anandamide during the pregnancy is temporarily inhibited [86]. The inhibitory effect is more prominent in males compared to females [86]. This inhibitory effect on the developing hypothalamic pituitary axis disappears by the 20th postnatal day [86].

Effects of endogenous cannabinoids on spermatogenesis, fertilisation, embryo development and pregnancy implantation

In animal studies, both anandamide and Δ^9 -THC have been demonstrated to have adverse effects on sperm function. They reduce sperm fertilizing capacity of sea urchins by inhibiting the acrosome reaction, an effect mediated by the CB₁ receptor [51,52]. Chronic exposure to exogenous cannabinoids in mice leads to impaired spermatogenesis at both the mitotic and meiotic stages [87]. Chronic marijuana use in humans is associated with reduced serum testosterone levels, reduced sperm counts and impotence [88]. Marijuana use in early pregnancy in humans is associated with increased incidence of fetal loss [16]. Several animal studies have demonstrated adverse effects of marijuana exposure in early pregnancy. Marijuana exposure in the rabbit for example, is associated with an increased rate of embryo resorption [89]. The murine uterus has the capacity to synthesise anandamide (*de novo* synthesis) and indeed contains the highest level of anandamide yet discovered in mammalian tissues [11]. The CB₁ receptor-mRNA is expressed in the epithelial cells of the mouse uterus [53]. The uterus becomes receptive for implantation on day 4 (implantation day) and by day five it becomes non-receptive [90]. The concentrations of anandamide and the enzymatic activities involved in the synthesis and degradation of anandamide show little changes in the first four days of pregnancy (before implantation) apart from a significant decrease in FAAH activity between days three and four (Fig. 2a, b and c).

After implantation on day four, anandamide concentrations in the implantation and non-implantation sites diverge with non-implantation site levels increasing dramatically with a concomitant increase in anandamide synthase activity and a decrease in FAAH activity (Fig. 2a, b and c). As a result of these changes, the concentrations of anandamide and the anandamide synthase activity are significantly higher in the non-implantation sites compared to the implantation sites (Fig. 2a and b). FAAH activity is also higher in the implantation sites compared to the non-implantation sites (Fig. 2c). Analysis of anandamide concentrations and the enzymatic activities in pseudopregnant uteri on day five reveals findings similar to those encountered at the non-implantation sites [11,91]. In keeping with these findings, FAAH expression has been found to be reduced on day five compared to the first four days of pregnancy [92]. These findings suggest that the systemic endocrine milieu in early pregnancy is involved in the increased levels of anandamide and the enzymatic changes described. Indeed, these findings are supported by the recent observation that oestrogen and progesterone derived from the maternal ovary suppress the activity of FAAH in the mouse uterus [93]. The fact that the implantation and non-implantation sites, and the pseudopregnant uterus have different concentrations of anandamide and also show different enzymatic profiles suggests that local factors such as uterine decidualisation or the presence of the embryo prevents the increase of anandamide concentrations.

Both CB₁ and CB₂ mRNAs are expressed in the preimplantation mouse embryo [94]. Anandamide arrests the development of embryos at or after the 2-cell stage *in-vitro*, primarily between the 4-cell and 8-cell stages. The effect is dose dependent [94]. A reduction in trophoectodermal cell numbers is seen in those embryos that escape the developmental arrest in the presence of anandamide and develop into blastocysts [95]. Anandamide also reduces

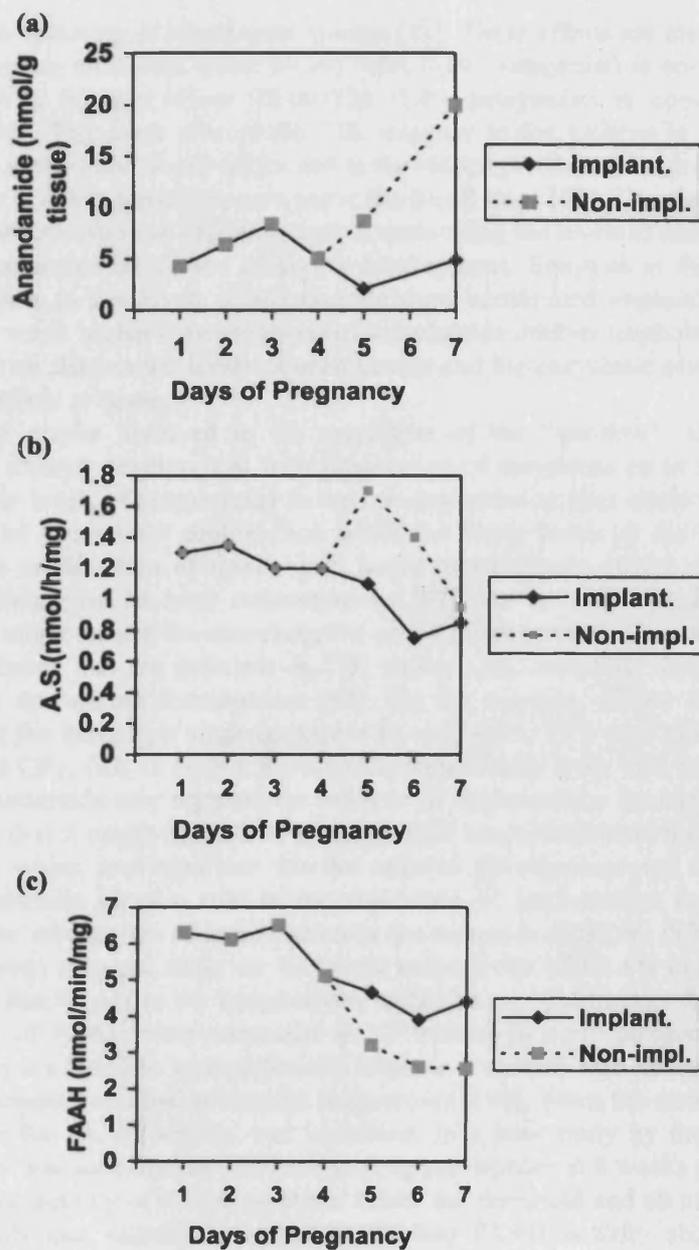


Fig. 2. Changes in Anandamide levels (a), Anandamide synthase (b) and FAAH (c) in Mouse pregnancy. Abbreviations: non-impl, non-implantation sites; implant, Implantation sites; FAAH, Fatty Acid Amide Hydrolase enzyme; A.S., anandamide synthase enzyme. Anandamide concentrations are in nmol/g tissue. (Data in 2 (a) derived from reference [11] with permission from National Academy of Sciences, U. S. A. "Copyright 1997". Data in 2 (b) 2 (c) derived from reference [91] with permission of Wiley-Liss, a subsidiary of John Wiley and sons, "Copyright 1996".

the rate of zona-hatching of blastocysts in-vitro [11]. These effects are mediated by the CB₁ receptor as they are abolished when SR141716A (CB₁ antagonist) is co-administered with anandamide [95], but not when SR144528 (CB₂ antagonist) is co-administered with anandamide [96]. The exact role of the CB₂ receptor in the embryo is yet to be defined [97]. Embryos at the 1 and 2-cell stages and at the blastocyst (day 4) stage all express FAAH, but the enzyme is not expressed in embryos at the 8-cell stage [92]. This finding suggests that FAAH in the embryo plays an important role in controlling the levels of anandamide to curtail the effects of anandamide on the embryo's development. Embryos at the blastocyst stage exposed in culture to low levels of anandamide show accelerated trophoblast differentiation and outgrowth while higher concentrations of anandamide inhibits trophoblast differentiation [98]. There are no data on the levels of anandamide and the enzymatic activities beyond day seven of the mouse pregnancy.

Anandamide maybe involved in the regulation of the “window” of implantation by synchronising embryo development with preparation of the uterus up to the receptive state [11]. The higher levels of anandamide in the non-implantation sites could be responsible for the inhibition of trophoblast proliferation while the lower levels at the implantation sites might help the proliferation of trophoblast, hence two different effects of anandamide are observed depending on its local concentrations [97]. On the other hand, excessive anandamide levels might induce the non-receptive phase prematurely leading to early pregnancy failure. In embryos that are deficient in CB₁ and/or CB₂ receptors, development is asynchronous with the uterine development [99]. On the morning of day four, 98% of wild embryos are at the blastocyst stage compared to only 62%, 71% and 61% of embryos that are deficient in CB₁, CB₂ or both CB₁ and CB₂ respectively [99]. This further supports the theory that anandamide may regulate the window of implantation. Another possible role for anandamide is that it might act as a reservoir of arachidonic acid for the generation of prostaglandins which are important for the embryo development and implantation [11]. Whether anandamide plays a role in the regulation of implantation in humans remains uncertain, as the mechanism of implantation in the mouse is distinctly different from that in humans. However, a recent study on the levels and activity of FAAH in maternal lymphocytes suggests that FAAH in the lymphocytes might be a useful marker for early pregnancy failure. Levels of FAAH were measured in 50 women in early pregnancy (7–11 weeks gestation) and were found to be significantly lower in 7 women who miscarried compared to that in the 43 women who had successful pregnancies [100]. From the results of this study, a threshold value for FAAH activity was identified. In a later study by the same group, the enzyme activity was measured in 150 healthy pregnant women at 8 weeks gestation. In 15 of these women the activity of the enzyme was below the threshold and all of them miscarried. In contrast, only one woman out of 135 who had FAAH activity above the threshold miscarried [100]. These findings are interesting particularly as they imply that a reduced activity of FAAH is associated with a poor outcome in contrast to the findings in the mouse that FAAH activity is suppressed in the uterus with the advancement of the pregnancy. More recently, in a study by the same group, it was found that progesterone but not β -HCG stimulates the activity of FAAH [101]. In the same study, it was shown that anandamide inhibits the production of Leukaemia-inhibitory factor (LIF) [101]. Since LIF is reported

to be defective in cases of recurrent miscarriages [102], a low level of FAAH might lead to spontaneous miscarriage by increasing the levels of anandamide which in turn inhibits the production of LIF [101]. What remains unclear at this stage is whether the changes in FAAH in early pregnancy failure are a result of the failing pregnancy or a cause of the failure. It is well recognized that failed pregnancies are associated with decreased levels of progesterone and it is possible that the reduced levels of progesterone leads to a reduction of FAAH in peripheral lymphocytes [103]. There are no studies, however, that evaluate the association between anandamide levels in the peripheral lymphocytes, the uterus and placenta.

Future Directions

Endocannabinoids are potentially an exciting group of endogenous mediators that are involved in the regulation of mammalian reproduction. The involvement of anandamide in the regulation of the anterior pituitary hormones, the close link between sex steroids and anandamide levels in both the anterior pituitary and the hypothalamus, and the fluctuation of anandamide levels throughout the ovarian cycle all suggest an important role for anandamide in the control of the menstrual cycle. If similar findings are confirmed in humans, then this might lead to a better understanding of menstrual disorders and certain cases of anovulation and infertility.

Anandamide plays a critical role in the regulation of early pregnancy acceptance and maintenance in the animal model. The exact role in humans is yet to be elucidated but the recent findings of a correlation between FAAH activity in peripheral lymphocytes and the outcome of pregnancy suggest that endocannabinoids might play an important role in the regulation/maintenance of early pregnancy. There is need for further work to explore the relation between the levels of anandamide and FAAH in lymphocytes from the uterus. Anandamide levels are influenced by sex steroid hormones. Levels of anandamide may, therefore, differ in and outside pregnancy. If this is the case, then it might explain some of the mood changes encountered during pregnancy and after childbirth. There is a need to investigate all these unanswered questions on the role of anandamide in reproduction.

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Plasma Levels of the Endocannabinoid Anandamide in Women—A Potential Role in Pregnancy Maintenance and Labor?

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Although exposure to exocannabinoids (e.g. marijuana) is associated with adverse pregnancy outcome, little is known about the biochemistry, physiology, and consequences of endocannabinoids in human pregnancy. In these studies, we measured the levels of the endocannabinoid anandamide (N-arachidonylethanolamine, AEA) by HPLC-mass spectrometry in 77 pregnant and 25 nonpregnant women. The mean \pm SEM plasma AEA levels in the first, second, and third trimesters were 0.89 ± 0.14 , 0.44 ± 0.12 , and 0.42 ± 0.11 nM, respectively. The levels in the first trimester were significantly higher than those in either the second or third trimester. During labor,

AEA levels were 3.7 times nonlaboring term levels (2.5 ± 0.22 vs. 0.68 ± 0.09 nM, $P < 0.0001$). During the menstrual cycle, levels in the follicular phase were significantly higher than those in the luteal phase (1.68 ± 0.16 vs. 0.87 ± 0.09 nM, $P < 0.005$). Postmenopausal and luteal-phase levels were similar to those in the first trimester. These findings suggest that successful pregnancy implantation and progression requires low levels of AEA. At term, AEA levels dramatically increase during labor and are affected by the duration of labor, suggesting a role for AEA in normal labor. (*J Clin Endocrinol Metab* 89: 5482–5487, 2004)

ENDOGENOUS CANNABINOIDS, endocannabinoids, are unsaturated fatty acid derivatives that act as ligands for the cannabinoid receptors (1). Arachidonylethanolamide or anandamide (AEA) was the first endocannabinoid isolated from brain tissue (2). It is enzymatically released from cell membrane phospholipid precursors in response to depolarizing agents, hormones, and neurotransmitters (3, 4). The levels of AEA are considered to be controlled by its cellular uptake through an AEA transporter (5, 6) and its subsequent enzymatic degradation by a membrane-bound fatty acid amide hydrolase (FAAH) (7–10) and other enzymes (11–13). AEA exerts its effects through interaction with plasma membrane cannabinoid receptors. Two subtypes of cannabinoid receptors, CB1 and CB2, belonging to the superfamily of G protein-coupled receptors (14) have been reported (15–18). They have been localized in different tissue types including the reproductive tract. The CB1 receptor is referred to as the central receptor because it was first localized in the central nervous system (19), whereas the CB2 receptor is referred to as the peripheral receptor because it was first localized in the spleen and other sites (18, 20, 21).

Several epidemiological and observational studies have been published on the adverse effects of exogenous cannabinoids present in marijuana on pregnancy in the human and

animal models. These effects vary from early fetal loss, fetal growth restriction, and premature birth (22–26). By contrast, there are relatively few reports on the role of endocannabinoids in pregnancy. In the mouse, it has been proposed that AEA plays an important role in the local regulation of uterine implantation (27), because AEA was shown to be embryotoxic and arrests embryo development (28). In the implantation site, tissue AEA levels are only 25% of those in the nonimplantation site (29). This is considered to be due to local tissue levels of FAAH, which is expressed at higher levels in the implantation site compared with the nonimplantation site (30). More recent studies have suggested a further potential role for endocannabinoids (e.g. AEA) in myometrium because CB1 receptor is expressed in the human myometrium and *in vitro* stimulation of this receptor by AEA results in relaxation of myometrial strips (31). CB1 receptor and FAAH immunoreactivity have also recently been identified in the human placenta and fetal membranes (32), suggesting that the endocannabinoid system may also be important in these tissues during gestation.

Little information is available on the systemic levels of AEA during human pregnancy. However, during early pregnancy, blood AEA levels are inversely correlated with FAAH levels in peripheral blood mononuclear cells (33), and, since FAAH levels at 8 wk gestation were lower in women who subsequently miscarried compared with those who progressed beyond the first trimester, this suggested that low AEA levels are required for successful pregnancy progression (34). Plasma AEA levels have also been shown to be higher in patients who fail to achieve an ongoing pregnancy after *in vitro* fertilization (IVF) treatment and embryo transfer

Abbreviations: AEA, N-Arachidonylethanolamine (anandamide); AEA-d₀, unlabeled AEA; AEA-d₈, deuterium-labeled AEA; BMI, body mass index; FAAH, fatty acid amide hydrolase; IVF, *in vitro* fertilization; MS, mass spectrometry.

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compared with those who had successful ongoing pregnancies and were mirrored by lower levels of FAAH in peripheral blood mononuclear cells (33). Whether these differences are influenced by or depend upon the various changes in the sex steroids that modulate implantation and maintain early pregnancy is uncertain. However, the relationship between AEA levels and pregnancy establishment and in any other role is unclear because AEA levels have not been reported beyond the first trimester or indeed during the menstrual cycle. Such studies have been hampered by methodological difficulties in the past, but the tools and techniques used for AEA assays have been and continue to be refined (35).

Our objectives were, firstly, to refine and validate an established method to assay AEA in human plasma and then to use this method to determine, for the first time, plasma levels of AEA in the menstrual cycle and normal pregnancy.

Subjects and Methods

Subjects

All subjects gave signed informed consent to take part in the study, which was approved and conducted according to the guidelines of the institute's research ethics committee. For the purposes of determining the changes in the levels of AEA during pregnancy, we studied cross-sectionally five groups of women in the first, second, and third trimesters; term nonlaboring; and term laboring. To minimize the potential influence of a wide gestational window on AEA levels, we restricted our sampling to 6–11 postmenstrual weeks in the first trimester, 15–27 postmenstrual weeks in the second trimester, and 28–35 postmenstrual weeks in the third trimester. Term was defined as 37–42 completed postmenstrual weeks.

The inclusion criteria for the pregnant women were body mass index (BMI) less than 27 kg/m², accurately dated pregnancies from first-trimester ultrasound scans performed between 6 and 8 postmenstrual weeks, uncomplicated singleton pregnancies, and no coexisting maternal or gestational diseases. None were on any medication or known to have taken recreational drugs. Only women in established labor (defined as cervical dilatation of at least 4 cm and three to four regular uterine contractions every 10 min) were included in the term laboring group.

The nonpregnant women were divided into two groups: pre- and postmenopausal. The inclusion criteria for the premenopausal women included: having had regular menstrual cycles for the last 6 months, having a BMI less than 27 kg/m², not having been on any hormonal contraception or therapy for at least 3 months, and not being on any drugs or suffering from any medical disorders. The samples were collected in two subgroups based on the menstrual cycle (follicular, d 2–7; luteal, d 20–25) assessed from patient-reported last menstrual period date. The inclusion criteria for the postmenopausal women included: a minimum of 2 yr postmenopause, BMI of less than 27 kg/m², not having been on hormone replacement therapy for the preceding 3 months, and not being on any drugs (e.g. glucocorticoids, antihypertensives, analgesics, recreational drugs) or suffering from any medical disorders. We studied only nonobese women, defined by a BMI less than 27 kg/m² (in our environment), as we were uncertain about the effect of adiposity on the homeostasis of AEA.

AEA measurements

Plasma AEA was measured by HPLC-mass spectrometry (HPLC-MS) based on an isotope dilution method as reported previously (36). Blood samples (4 ml) were collected in EDTA tubes on ice and added to polypropylene tubes containing 2 ml of Krebs-Tris-EDTA buffer. Samples were centrifuged at 1200 × g for 30 min at 22 C to separate plasma from cells. After recovery, the plasma was spiked with 25.1 pmol of deuterium-labeled AEA (AEA-d₈; Cayman Chemicals, Ann Arbor, MI) to estimate the efficiency of the lipid extraction procedure. Protein was precipitated by addition of ice-cold acetone followed by centrifugation at 1000 × g for 10 min at 22 C. Lipid extraction was then performed on the supernatants by the addition of methanol:chloroform (1:2) (1:1 vol:

vol). The chloroform layer was recovered, and the samples were dried under a stream of nitrogen before reconstitution in methanol:chloroform (3:1). Duplicate injections of reconstituted samples (10 μl) were used for HPLC-MS analysis.

The HPLC-MS system consisted of a Waters 1525 binary liquid chromatography pump and a Waters 717 plus autosampler fitted with a 100-μl injection loop (Waters MS Technology, Manchester, UK). The HPLC was interfaced to a Quattro Ultima triple quadrupole mass spectrometer via a Z-spray ion source and controlled by MassLynx NT software version 3.5 (Waters MS Technology). Reversed-phase chromatographic separations were performed on a Hypersil octadecylsilane C₁₈ column (4.6 × 100 mm, 3 μm particle size; Phenomenex, Macclesfield, UK). The column temperature was maintained at 30 C using a column temperature controller (Jones Chromatography, Hengoed, UK) and the samples at 4 C in a refrigerated injection system. The mobile phase consisted of water (A) and methanol (B) delivered at 0.5 ml/min using a stepwise increase in methanol, thus: 25% A, 75% B for 2 min; 15% A, 85% B for 3 min; 5% A, 95% B for 20 min; 100% B for 5 min; a column re-equilibration step was then performed by changing the buffer conditions to 25% A, 75% B for 20 min. Under these conditions, the retention times for AEA and AEA-d₈ were 17.12 and 16.98 min, respectively.

Electrospray ionization was carried out in the positive mode using nitrogen as the nebulizing gas. Capillary voltage was set at 3.5 kV, cone voltage 50 V, source temperature 120 C, and desolvation temperature 350 C. MS parameters for unlabeled AEA (AEA-d₀) and AEA-d₈ analysis were established by infusion standards at 1 ng/μl using an infusion pump. Sodium adducts of the molecular ions [M + Na⁺; m/z = 370.3 (d₀) and 378.3 (d₈)] were used for quantification in the selected ion recording mode. The ratio of the area under the curve for AEA-d₀ to AEA-d₈ was determined, and quantification of the amount of AEA in plasma samples spiked with the same amount of AEA-d₈ was made directly from a plot of area ratios vs. known AEA-d₀ amounts (Cayman Chemicals) injected into the HPLC. Standard curves were derived from the quantification of known and increasing quantities of the labeled AEA. The recovery of the AEA was constant over these ranges of concentrations. This enabled validation of the method of quantification. In addition, before quantification of AEA on the study subjects, we had undertaken repeated (three per volunteer from the same blood sample) measurements from nonpregnant adult volunteers to determine the reliability of recovery. The values obtained from each subject after repeated measurements were very similar and reassured us of the recovery. The standard curves were linear up to 2 pmol AEA-d₀, with a lower limit of detection of 0.03125 pmol AEA-d₀ injected on the column. The intra- and interassay coefficients of variation for the range of detection (0.03–2.1 pmol/column) were 6 and 9%, respectively.

All the blood samples were processed within 2 h of collection as recommended by Giuffrida *et al.* (36). Failure to process the sample within this time period led to degradation of AEA, resulting in a recovery of less than 50% (data not shown).

Statistical analyses

Power analysis of published AEA data (33) with $\alpha = 0.05$ and $\beta = 0.8$ indicated that the minimum number of subjects required in each study group that would allow a significant change in plasma AEA concentrations to be observed was six. Therefore, we ensured that at least six subjects were included in each sample group (Table 1). Data are expressed as mean ± SEM or SD where appropriate for each test group, and comparison between groups was performed using the unpaired Stu-

TABLE 1. Distribution, age, and gestational age of the study groups (data are presented as means with ranges in parentheses)

Study group	No.	Age (yr)	Gestational age (wk)
First trimester	10	30.4 (25–36)	9.1 (6–11)
Second trimester	10	28.1 (20–37)	18.7 (14–26)
Third trimester	10	26.3 (21–34)	31.9 (28–35)
Term (not laboring)	22	30.4 (22–37)	39.3 (37–42)
Term (laboring)	25	29.5 (18–40)	39.5 (37–42)
Menstrual cycle (follicular)	9	31.2 (23–39)	
Menstrual cycle (luteal)	8	34.2 (22–40)	
Postmenopausal	8	57.7 (55–60)	

dent's *t* test with Welch's correction for data with nonequivalent sds; a $P < 0.05$ was considered statistically significant.

Results

Subjects

One hundred two pregnant, menstruating, and postmenopausal women were studied. There were 10 each in the first, second, and third trimesters; 22 in term and nonlaboring groups; and 25 in term and laboring groups. Table 1 shows the details of these women. There were no significant differences in the mean \pm SD ages of the women of reproductive age, but the mean \pm SD of the postmenopausal women was significantly greater than that of the nonpregnant menstruating group (57 ± 2.0 yr, cf. 32.7 ± 6.1 ; $P < 0.05$). The mean \pm SD gestational ages of the term nonlaboring and laboring women were similar (39.3 ± 1.3 yr, cf. 39.6 ± 1.3 ; $P > 0.05$).

Seventeen of the 25 women in the term laboring group had a spontaneous vaginal delivery, three had a forceps delivery, two had a vacuum extraction, and three had an emergency cesarean section. The mean \pm SD cervical dilatation at the time of sampling was 6.04 ± 2.2 cm (range 4–10 cm), whereas the mean duration of uterine contractions before the blood sample collection was 5.1 ± 2.2 h (range 1.2–12 h). The mean \pm SD interval between sampling and delivery was 5.3 ± 4.3 h (range 0.5–11.8 h). Thirteen women had epidural analgesia in labor before sampling, and the others (12) had only a combination of nitrous oxide and oxygen, otherwise known as "gas and air," in labor.

AEA levels

The 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 nonlaboring 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 nonlaboring 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 nonlaboring levels were all statistically significant ($P < 0.0001$).

In the menstruating women, the levels of AEA in the follicular phase (1.68 ± 0.16 nM) were significantly higher than those in the luteal phase (0.87 ± 0.19 nM) ($P < 0.0053$). The levels in postmenopausal women (0.67 ± 0.01 nM) were significantly lower than those in the follicular phase ($P = 0.0002$) but not significantly lower than those in the luteal phase.

When the levels of AEA in the menstruating and postmenopausal women were compared with those during pregnancy, significant patterns emerged. These are shown in Fig. 1. The postmenopausal (0.67 ± 0.01 nM) and luteal-phase levels (0.77 ± 0.19 nM) were similar to those in the first trimester, higher (but not statistically, $P > 0.05$) than the second and third trimesters and nonlaboring term levels but significantly lower than the levels in the term laboring group ($P < 0.0002$). The follicular-phase levels were significantly higher ($P < 0.001$) than the levels during pregnancy but significantly lower ($P < 0.005$) than those in the laboring group.

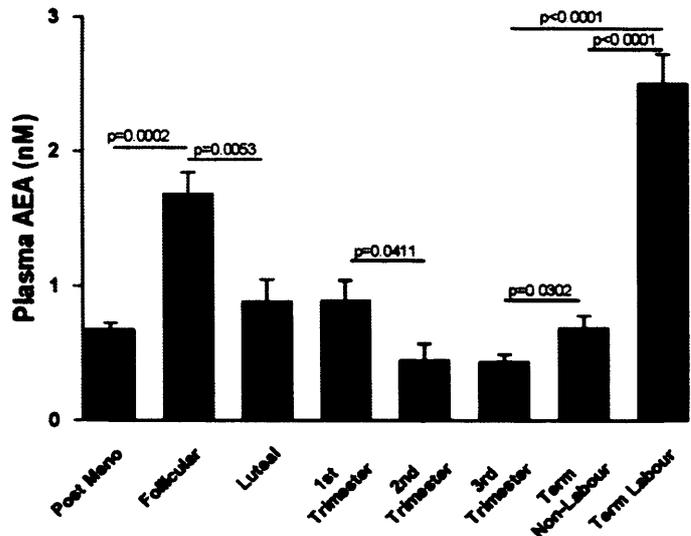


FIG. 1. Plasma AEA levels in postmenopausal women, during the menstrual cycle, pregnancy, and in nonlabor and labor women at term. Data are means \pm SEM; n values are shown in Table 1. Comparisons were performed using the Student's unpaired *t* test with Welch's correction. $P < 0.05$, first trimester vs. second trimester and third trimester vs. term nonlaboring; $P < 0.0001$, laboring vs. term nonlaboring or third trimester; $P < 0.001$ postmenopausal vs. follicular phase of the menstrual cycle; $P < 0.001$ follicular vs. luteal phase of the menstrual cycle.

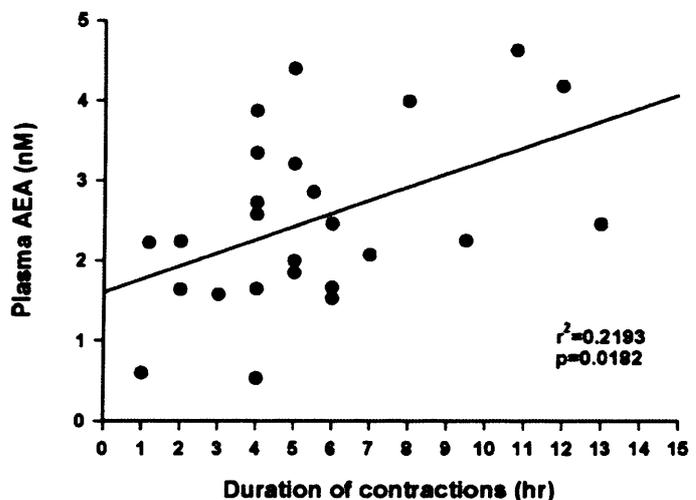


FIG. 2. Relationship between plasma AEA levels and the duration of contractions at the time of sampling.

Figure 2 shows the relationship between plasma AEA levels and the duration of contractions up to the time of sampling. There was a direct linear relationship between AEA levels and this duration ($r^2 = 0.2193$, $P < 0.0182$). The laboring women were further divided into three groups based on the total duration of labor (group I, 0–5 h; group II, 5.1–10 h; and group III, 10.1–15 h). The mean \pm SEM levels of AEA in the three groups were, respectively, 2.1 ± 1.04 , 2.58 ± 0.95 , and 3.8 ± 1.15 nM. Although there was a trend for AEA values to increase with increasing duration of labor, this did not achieve statistical significance ($P > 0.05$). Figure 3 shows an inverse relationship between the levels of AEA at the time of

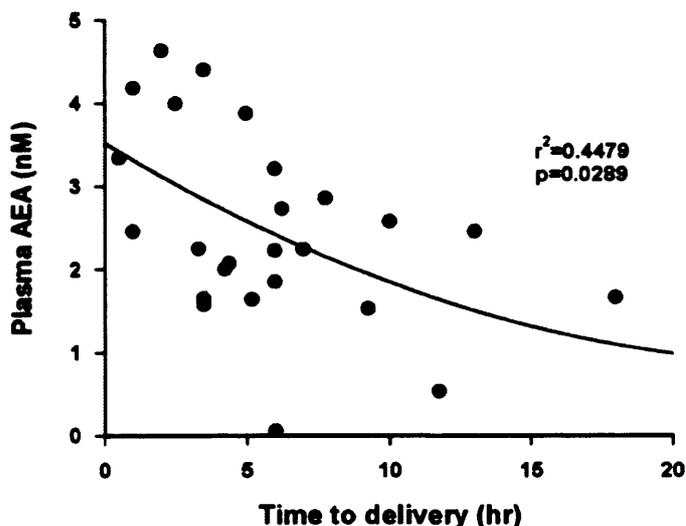


FIG. 3. Relationship between plasma AEA levels and the interval from sampling to delivery.

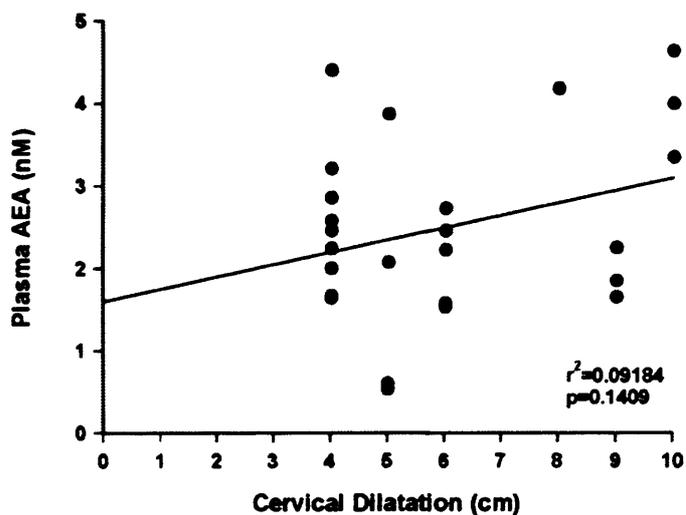


FIG. 4. Relationship between plasma AEA levels and cervical dilatation at the time of sampling.

sampling and the time from sampling to delivery. Figure 4 shows the relationship between the cervical dilatation at the time of sampling and plasma AEA levels ($r^2 = 0.092$, $P = 0.1$). The relationship between plasma AEA and the interval between sampling and delivery is shown in Fig. 3 ($r^2 = 0.4479$, $P = 0.0289$).

The levels in the 13 women who had an epidural analgesia before sampling were similar (2.24 ± 0.32 nM) to those in the 12 that had entonox gas only (2.78 ± 0.30 nM) before sampling and for the rest of their labor. None of the women received opiates.

Discussion

Our results demonstrate remarkable changes in the levels of AEA during pregnancy and labor. Plasma AEA levels fell from the first to the second and third trimesters with no change between the second and third trimesters. The levels

rose at term before the onset of clinically apparent labor and rose further during labor to represent a 6-fold increase from third-trimester levels.

The studies of Maccarrone *et al.* (33) demonstrated that, in IVF pregnancies, low plasma AEA levels were associated with pregnancies that progressed beyond the first trimester of pregnancy, indicating that a successfully developing conceptus suppresses systemic levels of plasma AEA. That this suppression may be required for normal development of the fetus and pregnancy maintenance is supported by the association of high AEA levels with miscarriages (34). Endocannabinoids are also known to have adverse effects on successful pregnancy in mice (29, 37–39). High levels have been demonstrated to block the development of two-cell mouse embryos at the blastocyst stage and at blastocyst hatching (37), a mechanism that may be associated with pregnancy failure or growth restriction of early onset. The requirement for an environment with low levels of AEA is supported by the effects of exogenous cannabinoids, which act upon the endocannabinoid receptors, and which are associated with miscarriages, fetal growth restriction, and preterm labor (22–26).

Surprisingly, in our study the levels detected in the first trimester, which were similar to those reported in successful IVF pregnancies (33), were similar to those in the luteal phase of the menstrual cycle. This implies that the low levels of AEA proposed to be required to support early pregnancy are already established in the luteal phase, enabling successful implantation, and that successful pregnancy represents a successful maintenance of these suppressed levels. The low levels in postmenopausal women and the high levels in the follicular phase suggest that steroid hormones primarily regulate AEA levels, with estradiol increasing the levels and progesterone suppressing them. The effect of progesterone could result from regulation of the degradation of peripheral AEA by peripheral blood mononuclear cells given that the levels of FAAH, the principal enzyme involved in AEA degradation, in these cells are regulated by progesterone (40, 41). The induction of high AEA levels by estradiol could be mediated by its effect upon endothelial cells given that it has been reported that estradiol increases the release of AEA from these cells into the circulation (42).

However, these considerations do not account for the dramatic increase in the levels of AEA at term before the onset of clinically apparent labor and subsequently during labor when there are no changes in systemic steroid hormone levels. It is unlikely that the increase in AEA concentrations is due to pain because there was no correlation between AEA concentrations and the use of epidural analgesia during labor. Unless AEA production is uniquely stimulated by labor, the previous considerations of steroid hormone control of its production and degradation could be explicable if, as has been proposed for the uterus (43), a “functional progesterone withdrawal” occurs in peripheral blood mononuclear cells such that the induction of FAAH by progesterone fails, allowing AEA levels to rise. Although this seems a logical explanation, there are potentially many (yet unidentified) factors that may be involved in this process.

These observations beg the question as to the possible function of AEA in parturition and labor. Labor is associated

with an increased local production of prostaglandins (44) that act on all reproductive tissues and, given that AEA acts as a reservoir for arachidonic acid, the rise in AEA levels could be to provide a large reservoir of the precursor for prostaglandin production. Whether equivalent rises in AEA occur during preterm labor awaits further study. The cannabinoid receptors, CB1 and CB2, are present in the uterus (31), and *in vitro* stimulation of these receptors results in relaxation of myometrial strips (31), which appears paradoxical given the rise in AEA associated with labor in this study. However, it is possible that if the cannabinoid receptors are differentially expressed within the uterus, then AEA action could be associated with relaxation of myometrial cells within the lower uterine segment during labor. This interpretation may be supported by our observation that AEA levels rose at term before the onset of clinically apparent uterine contractions and labor, a period when development of the lower uterine segment and cervical dilation occur.

In conclusion, these observations underline the potential role of endocannabinoids in human pregnancy maintenance and labor. However, little is known about the mechanisms by which these cannabinoids affect these processes, and it is only through an understanding of these mechanisms that the importance of dysregulation of production and metabolism regulation of AEA can be understood. Until this is completely understood, we recommend that the current exploration of the use of exocannabinoids for pain relief in labor be delayed. Previous observations that the highest level of AEA ever measured in any tissue in the mouse was found in the uterus and its local effect on the contracting human pregnant myometrial strips (31) suggest a uterine origin, although there could potentially be other sources.

Acknowledgments

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We do not recommend routine pretransplantation chemotherapy for patients who have received treatment in the past. Our recommendation to treat patients with impending immunosuppression (from human immunodeficiency virus infection, induction for organ transplant, or other etiologies) who have not previously received antitrypanosomal therapy should be interpreted in the context of our overall BII recommendation for *T. cruzi*-infected adults up to age 50 years without advanced heart disease. The major objective of etiologic treatment in this setting is to prevent development or progression of cardiomyopathy.

Our intention in explicitly listing anticipated immunosuppression as a patient category for treatment was to lend additional weight to the recommendation since we assume that antitrypanosomal therapy would be better tolerated, and theoretically more effective, before immunosuppression develops or is induced. Although data are lacking on this point, a secondary theoretical benefit is a possible reduction in the risk of reactivation. Advanced renal, hepatic, or cardiac dysfunction would certainly complicate therapy and constitutes a contraindication.

We agree with Altclas et al that posttransplantation monitoring is indicated whether or not a course of antitrypanosomal treatment was completed prior to transplant. As stated in the Clinical Review, we recommend that treatment decisions be individualized for all adult patients with Chagas disease, balancing the potential benefit vs the risk of drug toxicity.

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RESEARCH LETTER

Plasma Anandamide Concentration and Pregnancy Outcome in Women With Threatened Miscarriage

To the Editor: Approximately 40% to 50% of all human conceptions are lost before 20 weeks of gestation.¹ Recent animal studies suggest that the endocannabinoid anandamide (N-arachidonoyl-ethanolamine) is critical for both the syn-

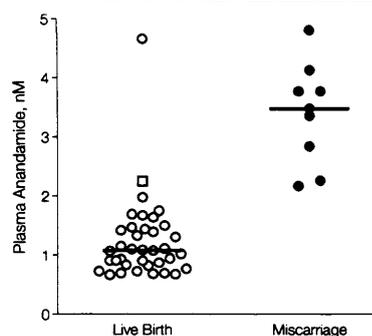
chronous development of the blastocyst and the endometrium in preparation for implantation, with low anandamide levels essential for successful implantation.² Plasma anandamide levels are regulated by fatty acid amide hydrolase (FAAH), the enzyme (up-regulated by progesterone) that metabolizes anandamide into arachidonic acid and ethanolamine.³ Increased FAAH expression and lower anandamide levels have been demonstrated at the implantation site and low FAAH expression and high anandamide levels at the interimplantation site prior to successful implantation.² Levels of FAAH in peripheral blood mononuclear cells from uncomplicated early pregnancies were significantly lower in women who subsequently miscarried.⁴ In women undergoing in vitro fertilization and embryo transfer, high plasma anandamide level at 6 weeks after embryo transfer was associated with failure to achieve an ongoing pregnancy.⁵ We therefore investigated whether plasma anandamide levels could predict outcome in women presenting with threatened miscarriage.

Table. Characteristics of the Study Groups

Characteristic	Live-Birth Group (n = 36)	Miscarriage Group (n = 9)
Age, mean (SD), y	28 (1.5)	29 (1.6)
Body mass index, mean (SD) ^a	24.6 (2.4)	25.1 (1.9)
Gestational age at recruitment, mean (range)	8 wk (6 wk to 11 wk 1 d)	8 wk 1 d (6 wk 5 d to 10 wk)
Anandamide level, median (IQR), nM	1.065 (0.81-1.45)	3.47 (2.83-3.86)
Interval between recruitment and miscarriage, mean (range), d	NA	7.83 (5-14)
Gestation at delivery or miscarriage, mean (SD)	39 wk 1 d (1 wk 5 d)	8 wk (1 wk 1 d)
Birth weight, mean (SD), kg	3.40 (0.52)	NA

Abbreviations: IQR, interquartile range; NA, not applicable.
^aCalculated as weight in kilograms divided by height in meters squared.

Figure. Plasma Anandamide Levels in Women With Threatened Miscarriage



The data are grouped according to those who proceeded to have a live birth and those who miscarried and are shown as individual cases. The median for each group is indicated by a horizontal solid line. The plasma anandamide levels were significantly different ($P < .001$; 2-tailed Mann-Whitney U test). Square indicates patient who developed severe preeclampsia and delivered at 33 weeks' gestation (birth weight of newborn, 1.85 kg).

Methods. Anandamide levels were measured in plasma from 45 healthy pregnant nonsmokers (TABLE) presenting to the early pregnancy assessment unit at 6 to 12 weeks' gestation with a threatened miscarriage (painless vaginal bleeding associated with a viable pregnancy). The assay was conducted with a high-performance liquid chromatography-mass spectrometry isotope dilution method (Waters Micro-mass Quattro Premier Mass Spectrometer; Waters Corporation, Milford, Massachusetts).⁶ The extraction and quantification were undertaken within 2 hours of blood collection. Patients, clinicians, and researchers were blinded to the results during the follow-up period.

Based on published anandamide data,^{5,6} a minimum of 6 spontaneous miscarriages and 6 live births would allow a clinically significant difference of 40% in anandamide concentration to be observed with 80% power, assuming 2-sided $\alpha = .05$. Ethics committee approval for the study and written informed consent from each volunteer were obtained; participants did not receive financial compensation. Groups were compared using the Mann-Whitney *U* test; significance was set at 2-sided $P = .05$. Data were analyzed using InStat Version 3.01 (GraphPad Software Inc, San Diego, California), and the area under the receiver operating characteristic curve was calculated using Stata 9 (StataCorp, College Station, Texas).

Results. Of the 45 women, 9 subsequently miscarried and 36 had live births. Both groups had similar characteristics (Table) and underwent similar treatment. The median plasma anandamide concentration in the miscarriage group (3.47 nM; interquartile range, 2.83-3.86) was approximately 3-fold that in the live birth group (1.07 nM; interquartile range, 0.81-1.45; $P < .001$) (FIGURE). All women who miscarried had anandamide values greater than 2.0 nM; 34 of the 36 in the live birth group (94.4%) had anandamide values less than 2.0 nM. One of the 2 live-birth patients with values greater than 2.0 nM developed severe preeclampsia and delivered a 1.85-kg growth-restricted infant at 33 weeks; the other had an uncomplicated delivery of a 3.7-kg full-term infant. There were no distinguishing characteristics of outliers.

Using an anandamide level of 2.0 nM as an arbitrary cut-off point, a single plasma anandamide measurement provided a sensitivity of 100% (95% confidence interval [CI], 66.4%-100%) and a specificity of 94.4% (95% CI, 81.3%-99.3%) with a negative predictive value of 100% (95% CI, 66.4%-100%) and a positive predictive value of 82% (95% CI, 48.2%-97.7%) for subsequent miscarriage. The area under the receiver operating characteristic curve was 0.972 (95% CI, 0.882-0.999).

Comment. In this pilot study of women with threatened miscarriage, plasma anandamide level was associated with presence or absence of subsequent miscarriage. The study is limited by a small number of participants (with resultant wide confidence intervals) and requires replication in larger and more diverse populations.

Compared with tests based on peripheral blood mononuclear cells, anandamide-level measurement has an advantage of being based on whole blood and not requiring separation. If established as valid and clinically practical, anandamide measurement has the potential for improving the prediction and counseling of women presenting with threatened miscarriages.

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Author Contributions: Dr Konje had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Habayeb, Konje.

Acquisition of data: Habayeb, Finney, Evans.

Analysis and interpretation of data: Habayeb, Taylor, Evans, Konje.

Drafting of the manuscript: Habayeb, Taylor, Finney, Konje.

Critical revision of the manuscript for important intellectual content: Taylor, Evans, Konje.

Statistical analysis: Habayeb, Taylor.

Obtained funding: Konje.

Administrative, technical, or material support: Habayeb, Finney, Konje.

Study supervision: Taylor, Evans, Konje.

Financial Disclosures: Dr Konje reported having a patent (No. PCT/GB2005/050028) for the use of anandamide as a predictive test for preterm labor. PerkinElmer markets radiolabelled anandamide and produces a biochemical diagnostics test for chromosomal abnormalities in the first and second trimesters of pregnancy. PerkinElmer has first right of refusal for any patentable property emanating from Dr Konje's laboratory. No other disclosures were reported.

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Role of the Sponsor: The funding organizations had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation, review, or approval of the manuscript.

Additional Contributions: David J. Taylor, MD, University of Leicester, provided intellectual input into the study and comments on the manuscript. Stephen Bell, PhD, University of Leicester, provided assistance with study concept and design. Marcus Cooke, PhD, University of Leicester, provided advice on the laboratory measurements and his comments on the manuscript. None of these persons received compensation for his role in this study.

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Expression of the Endocannabinoid System in Human First Trimester Placenta and Its Role in Trophoblast Proliferation

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The endocannabinoid, anandamide, which binds to two major receptor proteins, the cannabinoid receptors (CBs) 1 and 2 (CB₁ and CB₂), has been shown to play a role in first trimester miscarriage possibly through impairment of the developing trophoblast. Although the precise molecular mechanisms underlying this are unknown, plasma anandamide levels are known to be regulated by the progesterone-induced enzyme, fatty acid amide hydrolase (FAAH). Here, we tested the hypothesis that temporal-spatial expression of FAAH, CB₁, and CB₂ is regulated during early pregnancy and that anandamide detrimentally alters trophoblast proliferation. Transcripts for CB₁, CB₂, and FAAH were demonstrated in first trimester trophoblast extracts with only the CB₁ transcript being significantly regulated. The significant 4.7-fold increase in expression at wk 10 gestation was reduced to 8.9% of the peak

value by wk 12. Transcripts for CB₂ showed a similar pattern of expression but were not significantly induced. By contrast, FAAH transcript levels appeared to increase toward the end of the first trimester, but again did not reach significance. These observations were supported by immunohistochemical studies that demonstrated a similar pattern of expression at the protein level, with cellular localization for all three proteins concentrated within the syncytiotrophoblast layer. Anandamide also prevented BeWo trophoblast cell proliferation in a dose-dependent manner, with a 50–60% significant inhibition of cell proliferation with concentrations in excess of 3 μ M. This effect was mediated through CB₂. Together, these data provide insights into how elevated plasma anandamide levels increase the risk of first trimester miscarriage. (*Endocrinology* 149: 5052–5060, 2008)

ANANDAMIDE [*N*-arachidonylethanolamine (AEA)] is an endogenous cannabinoid that is involved in human reproduction (1). AEA is released from phospholipid precursors by cells in response to stimulation by neurotransmitters and hormones via the action of *N*-arachidonylphosphatidyl ethanolamine selective phospholipase-D (NAPE-PLD) (1–4), and possibly through the actions of phospholipase C and phosphatases (5).

AEA exerts its effects mainly by acting on two cannabinoid receptors (CBs), CB₁ and CB₂. Although AEA is an endogenous agonist for CB₁ and CB₂, it also is an endogenous agonist for type-1 vanilloid receptors, and can directly affect a variety of other signal systems (6, 7) AEA is rapidly removed by a putative transporter and metabolized by the enzyme, fatty acid amide hydrolase (FAAH) into arachidonic acid and ethanolamine (8). AEA, CB₁, CB₂, and FAAH constitute the major components of the endocannabinoid system (1, 4) and have been subjected to extensive study with regard to reproduction. Other less well-studied members of the cannabinoid system such as 2-arachidonylethanolamine, *N*-oleylethanolamine, *N*-palmitoylethanolamine, and the en-

zyme NAPE-PLD (9, 10) are gaining more prominence as part of this system. A critical balance between AEA release and degradation creates an endogenous AEA tone that regulates oviductal transport of preimplantation mouse embryos (11, 12). A similar process may regulate implantation in humans. Studies in the mouse have demonstrated that the local concentrations of AEA in the uterus regulate its receptivity toward the developing blastocyst with nonimplantation sites containing higher concentrations of AEA than the implantation sites (13). This temporal-spatial distribution of AEA in the uterus is complemented by an inverse correlation with FAAH expression, with high levels at the implantation site and low levels at non-implantation sites (14). In human studies it has been shown that low levels of FAAH in peripheral blood mononuclear cells in early pregnancy predict spontaneous miscarriage (15). In addition, the levels and activity of FAAH in peripheral blood mononuclear cells in women who failed to achieve pregnancy after embryo transfer in *in vitro* fertilization pregnancies are lower than in those who conceived (16). We have previously measured plasma AEA levels in normal human pregnancy and demonstrated that these vary in a pattern, suggestive of the involvement of gonadal steroids in its regulation (17). Combined, these studies suggest that AEA plays an important role in the establishment and maintenance of early human pregnancy.

Previous studies demonstrating that both FAAH and CB₁ are expressed in term human placenta and fetal membranes (18) suggested that the endocannabinoid system is likely to be present from early in gestation, however, this has not been extensively investigated, and there are no data on the ex-

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Abbreviations: AEA, *N*-arachidonylethanolamine; AMV, avian myeloblastosis virus; CB, cannabinoid receptor; FAAH, fatty acid amide hydrolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NAPE-PLD, *N*-arachidonylphosphatidyl-ethanolamine selective phospholipase-D; RT, reverse transcriptase.

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pression of CB₂ in early gestational tissues. In addition, the effect of AEA on human trophoblast growth and survival has not been examined. However, AEA is known to have a biphasic growth/survival effect on the mouse blastocyst with low (7–14 nM) levels stimulating trophoblast outgrowth and high (28–56 nM) levels inhibiting growth (6, 19, 20). These data suggest that a similar effect may be observed in the human trophoblast. Therefore, the aims of this study were to: 1) determine the expression of FAAH and the CBs, CB₁ and CB₂, in the trophoblast during the first trimester of pregnancy; 2) describe any changes that might occur during gestation; and 3) determine whether AEA would have any effect on the growth of a human cellular model of the first trimester trophoblast.

Materials and Methods

Tissue collection

The local Research Ethics Committee approved this study, and all volunteers gave signed informed consent. Products of conception were collected between 7 and 12 wk gestation from women undergoing surgical termination of pregnancy. All had an ultrasound scan examination to date accurately the pregnancies before pregnancy termination; standard practice in the gynecology unit. Collected samples were transported on ice to the laboratory, where blood was washed away with sterile PBS and two pieces of trophoblast dissected free with the aid of a dissecting microscope. One piece was fixed in 10% neutral-buffered formalin for 4 d before embedding in paraffin wax. A second portion of tissue (~100 mg) was placed into a sterile polypropylene tube and snap frozen in liquid nitrogen for 5 min. The latter samples were stored at –80 C for later RNA analysis. Archival term placental tissues from women undergoing elective cesarean section acted as positive controls (21).

Materials

Anti-CB₁ and anti-CB₂ rabbit polyclonal antibodies were obtained from Sigma-Aldrich Ltd. (Poole, Dorset, UK), whereas anti-FAAH rabbit polyclonal antibody was obtained from (Alpha Diagnostics International, Inc., San Antonio, TX). Microwave antigen retrieval (22) was performed for both CB₁ and CB₂ studies but was not required for the FAAH studies. The secondary antirabbit horseradish peroxidase conjugates were from Dako (Glostrup, Denmark), and the tyramide amplification and detection system was from PerkinElmer LAS (Beaconsfield, Buckinghamshire, UK). An ABC detection system (ABC Elite; Vector Laboratories, Peterborough, UK) was used in conjunction with 3,3'-diaminobenzidine (Vector laboratories) to detect the presence of immunoreactive complexes for the anti-CB₂ and anti-FAAH antibodies, whereas the tyramide amplification system was substituted for ABC Elite for the detection of anti-CB₁ complexes. The anti-FAAH antibodies were used at an optimal dilution of 1:2000 in PBS [137 mM NaCl, 9.2 mM Na₂HPO₄, and 1.46 mM KH₂PO₄ (pH 7.6)], CB₁ at 1:4000, and CB₂ at 1:500 dilutions. For each antibody used, negative controls were performed using the same concentrations of rabbit IgG (for CB₁ and CB₂; Vector Laboratories) and normal rabbit serum (for FAAH; Dako).

Expression of the endocannabinoid system in the first trimester placenta

Total cellular RNA was extracted from frozen placenta samples using TRIzol reagent (Invitrogen, Paisley, UK) according to the manufacturer's instructions, and the concentration, purity, and integrity of the resulting RNA were determined spectrophotometrically before the presence of mRNA for CB₁, CB₂, and FAAH, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was determined using end-point RT-PCR.

End-point RT-PCR

One microgram of total RNA was reversed transcribed with avian myeloblastosis virus (AMV)-reverse transcriptase (RT) (Promega, Southampton, UK) at 42 C for 1 h in the presence of 5 U ribonuclease inhibitor (RNasin; Promega). A minus RT reaction was obtained by omitting the AMV-RT enzyme. At the end of the reaction, the enzymes were denatured by heating at 95 C for 5 min and the cDNA stored at –20 C. One microliter of cDNA was subject to PCR using 10 pmol/μl specific primers for CB₁, CB₂, FAAH, and GAPDH using the annealing temperatures given in Table 1. PCR products were resolved on 3% agarose gels and stained with ethidium bromide (2 μg/ml; ICN Biomedicals, Basingstoke, UK) for 15 min, before being destained with distilled water for 30 min. Gel images were captured using a Syngene GeneGenius system (Syngene, Cambridge, UK) equipped with GeneSnap version 6 gel documentation software. For quantification of relative transcript levels, samples were subjected to quantitative real-time RT-PCR.

Quantitative real-time RT-PCR

Amplification of CB₁, CB₂, FAAH, and GAPDH cDNA was performed in a Roche LightCycler (Roche Diagnostics Ltd., Lewes, UK) using 1 μl cDNA for CB₂ and GAPDH, and 2 μl cDNA for CB₁ and FAAH, with the LightCycler FastStart DNA master SYBR Green I kit (Roche Diagnostics). Mastermix reactions were prepared as per the manufacturer's instructions on ice with 10 pmol each primer pair (Table 1), and the cDNA was added last. Controls included a water blank, minus RT control for each trophoblast sample, a series of diluted cloned human CB₁, CB₂ (CNR01LTN00 and CNR020TN00, respectively; University of Missouri-Rolla cDNA Resource Center, Rolla, MO), FAAH (12), and GAPDH (BioChain Institute Inc., Hayward, CA) cDNA targets adjusted so that a standard curve from 1–10,000,000 pmol cDNA could be constructed. In addition, a series of diluted pooled trophoblast cDNA (1:10 to 1:10,000 dilutions) were used to calculate amplification efficiencies for each gene amplified. The cycle conditions were 95 C for 10 min, followed by 45 cycles of 95 C for 10 sec, 60 C for 5 sec, 72 C for 15 sec for GAPDH, 95 C for 12 sec, 62 C for 7 sec, 72 C for 15 sec for CB₁ and CB₂; and 95 C for 12 sec, 59 C for 9 sec, and 72 C for 12 sec for FAAH. The crossing points were then used to measure the transcript concentrations from the standard curves and the predicted melting temperatures of the amplified products used to confirm that only the target DNA was amplified.

Detection of immunoreactive CB₁, CB₂, and FAAH

Tissues sections (4 μm) were mounted onto silanized glass microscope slides and dried for 7 d at 37 C before use. Slides were dewaxed

TABLE 1. Primer design and expected amplicons sizes

mRNA target	Annealing temperature (C)	Primer sequences (5'–3')	Expected size (bp)	Reference
CB ₁	62.4	CTTCCCACAGAAATTCCC sense	853	(21)
		TACCTTCCCATCCTCAGA antisense		
CB ₂	62.4	CGTGGCTGTGCTCTATCTGA sense	851	(21)
		ATCTCGGGGCTTCTTCTTTT antisense		
FAAH	59	GGCCGTCAGCTACACTATGC sense ATCAGTCGCTCCACCTCCC antisense	249	
GAPDH	60	AGAACATCATCCCTGCCTC sense	347	(40)
		GCCAAATTCGTTGTGCATACC antisense		

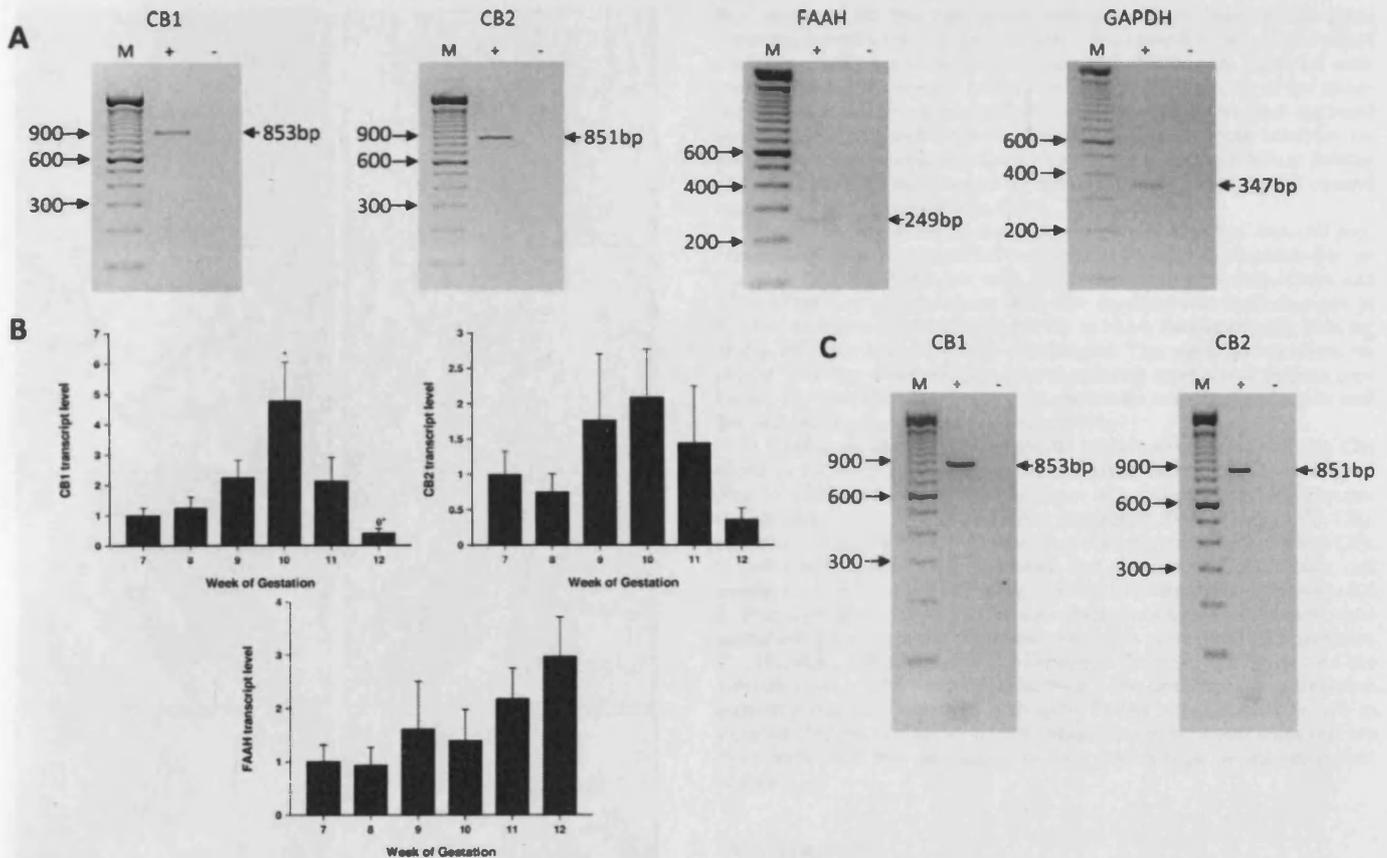


FIG. 1. Expression of the endocannabinoid system in first trimester trophoblast biopsies. **A**, Ethidium bromide-stained agarose gels of RT-PCR products for CB₁, CB₂, FAAH, and GAPDH for placenta samples taken from wk 7 of the first trimester. +, Indicates the presence of AMV-RT in the RT reaction; –, indicates the absence of enzyme. The relative size markers from the 100-bp DNA ladder are shown on the left. **B**, Relative levels of CB₁, CB₂, and FAAH transcripts compared with wk 7 gestation using quantitative real-time RT-PCR. Data are the mean values \pm SD after normalization to the relative levels of GAPDH according to the method of Pfaffl (30) and are from three experiments performed in triplicate for each sample. Each week of gestation is represented by four independent samples ($n = 4$). *, $P < 0.05$; one-way ANOVA with Tukey's honestly significant difference test compared with wk 7; ^o, $P < 0.001$ compared with wk 10. **C**, Ethidium bromide-stained agarose gels of RT-PCR products for CB₁ (left panel) and CB₂ (right panel) from the batch of BeWo cells used in this study. +, Indicates the presence of AMV-RT in the RT reaction; –, indicates the absence of enzyme. The relative size markers (M) from the 100-bp DNA ladder are shown on the left.

in xylene three times for 3 min and rehydrated in graded alcohol for 3 min, followed by incubation in distilled water for 3 min. Microwave antigen retrieval was performed for CB₁ and CB₂ only by incubating the slides in 10 mM citric acid buffer (pH 6.0) heated at 700 W for 10 min (23). Endogenous peroxidase activity was then blocked by incubation in 6% H₂O₂ in water for 10 min. Blocking of nonspecific protein binding sites was performed by incubation in 10% normal goat serum for 10 min at room temperature. Endogenous avidin and biotin sites were blocked using the Avidin-Biotin Blocking Kit (Vector Laboratories) as recommended by the manufacturer. Primary antibodies diluted in Tris-buffered saline [0.5 M Trizma, 1.5 M NaCl, and 2 mM MgCl₂ (pH 7.6); 100 μ l/slide] were added, and the slides were incubated in a humid chamber overnight at 4 C. Slides were then washed in [Tris-buffered saline containing 0.1% BSA (Fraction V; Sigma-Aldrich)] for 30 min. After washing the slides for 30 min in Tris-buffered saline, biotinylated goat antirabbit antibody (Vector Laboratories) diluted to 1:400 in TBA was applied for 30 min at room temperature. After an additional wash in Tris-buffered saline, ABC Elite reagent (for CB₂ and FAAH) and tyramide amplification reagent (for CB₁) were applied according to the manufacturer's detailed instructions. After additional washing in Tris-buffered saline for 20 min, 3,3'-diaminobenzidine was added to each slide (100 μ l/slide) for 5 min. Slides were then washed in distilled water for 5 min before counterstaining in Mayer's hematoxylin for 15 sec. After washing in running tap water for 5 min, slides were dehydrated in graded alcohols,

cleared in xylene twice for 6 min before mounting with di-n-butylphthalate xylene (DPX) mounting medium (BDH, Poole, Dorset, UK).

Images were taken on an Axioplan transmission microscope equipped with a Sony DXC-151P analog camera (Sony Corp., Kanagawa, Japan) (24) connected to a computer running Axiovision image capture and processing software (Axiovision version 4.4; Carl Zeiss Ltd., Welwyn Garden City, Hertfordshire, UK). Images were captured at either $\times 10$ or $\times 40$ magnification in the presence of daylight and medium value neutral density filters with the lamp set at 6400 K. Image backgrounds were color corrected to neutral gray with the use of ColorPilot software (version 4.62; www.colorpilot.com).

Anandamide on BeWo cell growth

The human choriocarcinoma cell line BeWo (86082803; European Collection of Cell Cultures, Salisbury, Wiltshire, UK), chosen because it is a good model for the human first trimester trophoblast (25) and because the cells contain the CBs (21) and respond to cannabinoid stimulation (26), was maintained in Ham's F12 medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) and cultured at 37 C in a humidified atmosphere of 5% CO₂ in air. BeWo cells were plated onto Nunc six-well plates in triplicate for each data point (Fisher Scientific, Loughborough, Leicestershire, UK) at a density of 4×10^5 cells per well (27). After 48 h culture, the medium was exchanged with one

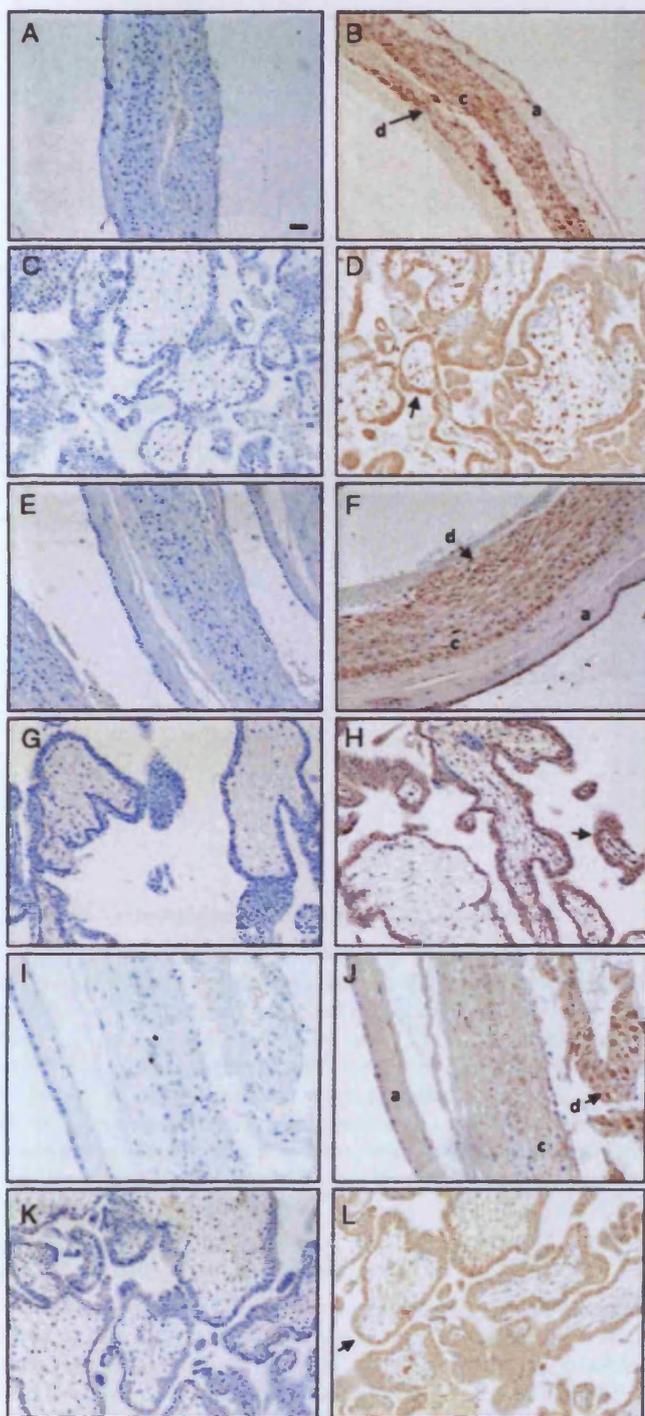


FIG. 2. CB₁, CB₂, and FAAH immunohistochemistry. Demonstration of specific immunostaining for CB₁ (B and D), CB₂ (F and H), and FAAH (J and L), and IgG controls (A and C, E and G, and I and K) in term fetal membranes (A, B, E, F, I, and J; control tissues) and first trimester trophoblast (C, D, G, H, K, and L). Immunoreactive CB₁, CB₂, and FAAH are found in the amnion (a), chorion (c), and decidua (d) of the term fetal membranes, as expected. In first trimester trophoblast samples, specific CB₁, CB₂, and FAAH immunostaining was mainly confined to the cytotrophoblast and syncytiotrophoblast layers (arrows) and the mesenchymal cores of villi at all stages of development. The images are taken from a representative tissue obtained at 8 wk gestation at $\times 10$ magnification. Bar, 50 μ m.

that contained 5% fetal calf serum and up to 30 μ M anandamide [final concentration of 0.1% (vol/vol) ethanol]. The control consisted of culture medium containing 0.1% (vol/vol) ethanol. Cells were cultured with media plus additives for 1 h to allow nonspecific binding of the anandamide to plastic to reach equilibrium. The medium was then replaced and culture continued for an additional 48 h, with fresh medium replaced at 24 h. Photomicrographs were obtained using a Nikon Eclipse TE2000-U inverted microscope equipped with a DN-100 digital camera image capture system (Nikon Corp., Tokyo, Japan).

To examine the effect of anandamide on cell survival and cell proliferation indices, we plated BeWo cells onto Nunc 96-well plates (Fisher Scientific) at 1×10^4 cells per well, in 200 μ l normal growth medium and allowed them to proliferate for 48 h. The medium was then changed to one that contained anandamide for 1 h to allow for nonspecific binding of the endocannabinoid to the plasticware. The medium was then replaced with the same anandamide-containing media and culture continued for an additional 23 h, when the media was changed again and the cell reincubated for an additional 24 h.

In a separate series of experiments to determine which of the CBs could be mediating the effect of anandamide, BeWo cells were treated with 10 μ M anandamide in the presence of a receptor blocking concentration (300 nM) of the CB₁-selective antagonist SR141716A or the CB₂-selective antagonist SR144528 that does not activate other receptors (28).

After 48 h culture in the presence and absence of anandamide, cell numbers were assessed using the Cell Proliferation and Apoptosis Kit II (Roche Diagnostics) as per the manufacturer's instructions with measurements taken on a Multiskan Ascent ELISA plate reader (Labsystems Oy, Helsinki, Finland), with the detection filter set at 420 nm and the reference set at 620 nm. Cell numbers were obtained by calibration against a standard curve of untreated BeWo cell numbers grown in parallel (26, 29). To make direct comparisons between cultures, we then converted the cell numbers to a percentage of the untreated control.

Data analysis

The absolute levels of CB₁, CB₂, FAAH, and GAPDH transcript were calculated using Pfaffl's method (30), data normalized to the wk 7 samples, and expressed as the mean \pm SD relative to those of controls. All data were then analyzed for differences using one-way ANOVA with Tukey's honestly significant difference test within the InStat version 3.0 software package (GraphPad Software Inc., San Diego, CA; <http://www.graphpad.com>). Statistical significance was accepted when $P < 0.05$.

Results

Expression of the endocannabinoid system in the first trimester placenta

RT-PCR of RNA extracts taken from placentas between 7 and 12 wk gestation indicated the presence of amplified DNA, consistent with the expected amplicon sizes of 853 bp for CB₁, 851 bp for CB₂, 249 bp for FAAH, and 347 bp for GAPDH (Fig. 1). In addition, there was an absence of products in the lanes obtained for the $-RT$ controls, indicating that the amplified products were obtained from mRNA and not contaminating genomic DNA. A detailed analysis of the expression patterns for the CB₁ transcript indicated that there was a significant decrease in the expression of CB₁ transcript from 4.77 ± 1.28 at wk 10 gestation to 0.40 ± 0.15 arbitrary units at wk 12: a significant 91% decrease in expression ($P < 0.01$; Student's *t* test). Although there appeared to be a differential expression of both CB₂ and FAAH transcripts in the trophoblast during the first trimester, these data did not reach statistical significance (Fig. 1B).

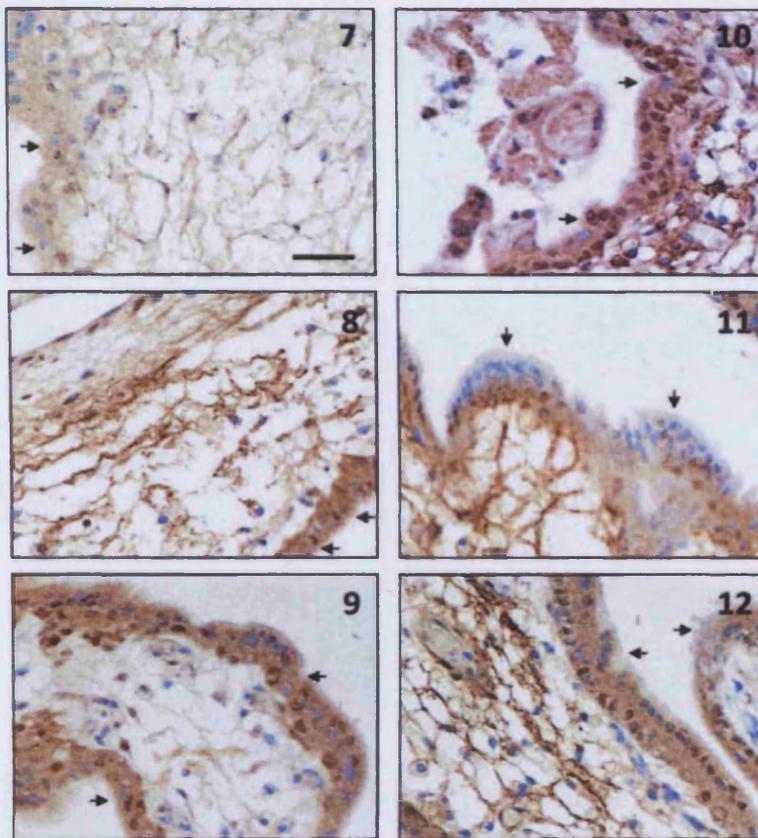


FIG. 3. Effect of gestation on the expression of immunoreactive FAAH in the first trimester trophoblast. Immunohistochemical staining for FAAH was found in the syncytiotrophoblast, cytotrophoblast, and mesenchymal fibroblastic core in all trophoblastic samples from wk 7–12. FAAH immunoreactivity in the syncytiotrophoblast (arrows), diminished by the wk 11 in the brush-border epithelium (predominantly blue cells), an effect that persists at wk 12. Representative images were taken at $\times 40$ magnification.

Detection of immunoreactive CB₁, CB₂, and FAAH

Using term fetal membranes as a positive control tissue (18), the optimal conditions for the immunohistochemistry studies were obtained and indicated that the primary antibody dilutions for FAAH, CB₁, and CB₂ were 1:2000, 1:4000, and 1:500, respectively. Rabbit serum or isotype IgG diluted to the same concentrations were nonreactive (Fig. 2). Using these dilutions of primary reagents and a common secondary detection system, there was also clear specific immunostaining for FAAH, CB₁, and CB₂ on first trimester trophoblast samples.

Immunoreactive FAAH staining patterns

Immunoreactive FAAH was detected in all 1st trimester trophoblast tissues between wk 7 and 12 gestation. FAAH immunoreactivity was detected in both the cytotrophoblast and the syncytiotrophoblast layers (Fig. 3). FAAH immunoreactivity in the syncytiotrophoblast layer increased gradually between the 7th and the 10th gestational week. By the 11th week of gestation, FAAH immunoreactivity at the syncytiotrophoblast layer diminished to the point where it was barely detectable within large parts of the trophoblast, an effect that persisted into the 12th week (Fig. 3). FAAH immunoreactivity was also demonstrated in the mesenchymal core of the developing villi. Immunoreactive FAAH was predominately noted throughout the cytoplasm of the cells with little or no nuclear staining (Fig. 3), in keeping with the

membranous and intracytoplasmic vesicular localization of FAAH (8).

Immunoreactive CB₁ staining patterns

Immunoreactive CB₁ was also detected in first trimester trophoblast tissue. CB₁ immunoreactivity was detected in all types of trophoblast through various stages of maturation (Fig. 2). CB₁ immunoreactivity in the syncytiotrophoblast layer diminished in intensity but did not disappear in gestation (wk 10–12; Fig. 4) as immunoreactive FAAH had. There was no CB₁ immunoreactivity in either fetal blood cells or infiltrating maternal plasma cells, but CB₁ immunoreactivity was detected in the endothelial cells of the blood vessels but not in the vascular smooth muscle cells (Fig. 4).

Immunoreactive CB₂ staining patterns

Immunoreactive CB₂ was also detected in first trimester trophoblast tissue. CB₂ immunoreactivity was detected in all types of trophoblasts through various stages of the first trimester of gestation (Fig. 2). The intensity of CB₂ immunoreactivity in the syncytiotrophoblast remained constant throughout the first trimester (Fig. 5), which differed from the immunoreactive staining patterns for FAAH and CB₁. There was no CB₂ immunoreactivity in either fetal blood cells or infiltrating maternal plasma cells, but CB₂ immunoreac-

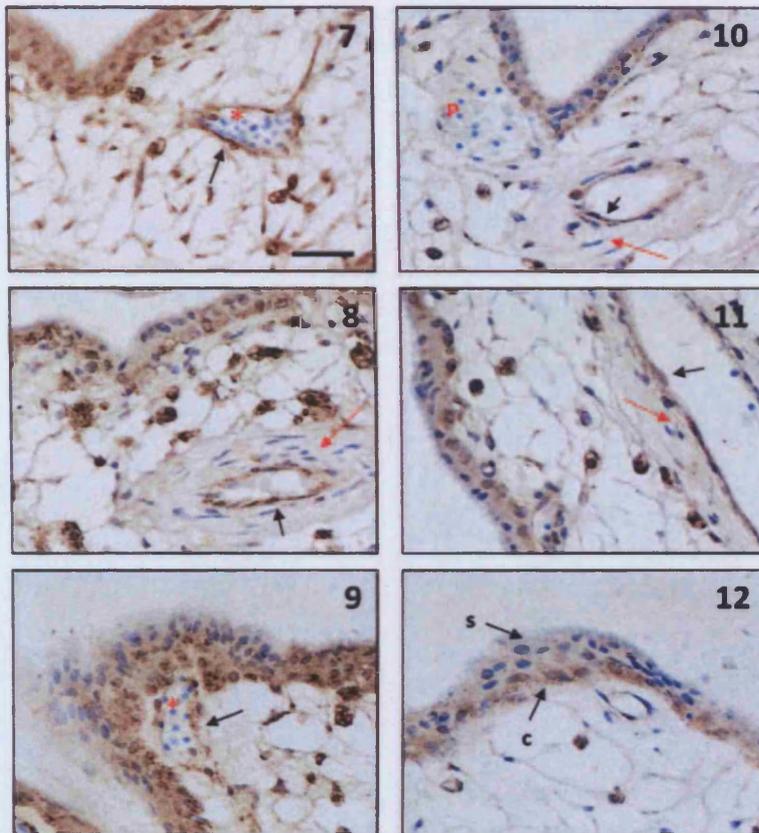


FIG. 4. Effect of gestation on CB₁ expression in the 1st trimester trophoblast. Immunohistochemical staining for CB₁ was found in the syncytiotrophoblast, cytotrophoblast, and mesenchymal fibroblastic core of all placental samples from wk 7–12. CB₁ immunoreactivity in the syncytiotrophoblast layer decreased from wk 10 onward. Positive staining in the endothelial cells of blood vessels (black arrow) was countered by negative staining in the smooth muscle cells of the blood vessels (red arrow) and fetal erythrocytes (red asterisk) and infiltrating maternal plasma cells (P). By the 12th week of gestation, there was a small decrease in the expression of the syncytiotrophoblast (s) compared with the cytotrophoblast (c). Representative images were taken at $\times 40$ magnification. Bar, 50 μ m.

tivity was detected in the endothelial cells of the blood vessels but not in the vascular smooth muscle cells (Fig. 5).

Effect of anandamide on BeWo cell growth

BeWo cells were plated such that cultures achieved approximately 70–80% confluency after 48 h, the point at which AEA treatments were initiated, and continued for an additional 48 h with a range of AEA concentrations (26). Under these conditions AEA demonstrated an inhibitory effect on the BeWo cell cultures (Fig. 6), but only at concentrations in excess of 3 μ M, where confluency was significantly reduced from approximately 80% at 3 μ M, to approximately 65–70% at 15 and 30 μ M. Cultures treated with 15 and 30 μ M AEA did not exhibit increased cell death or failure to attach to the substratum, as evidenced by the lack of increase of shedding of cells in the spent medium (Fig. 6A). The antiproliferative effects of high AEA concentrations were mediated via the CB₂ receptor (Fig. 6C). BeWo cells treated with 10 μ M AEA demonstrated a significant 40–50% reduction in cell density after 48 h treatment. In the presence of 300 nM of the CB₁-selective antagonist SR141716A, the data were very similar to the control, whereas in the presence of 300 nM of the CB₂-selective antagonist SR144528, the growth-inhibitory effect of AEA was abrogated (Fig. 6C).

Discussion

Our observations confirm that various components of the endocannabinoid system (CB₁, CB₂, and FAAH) are ex-

pressed in 1st trimester tissues. In addition, we have demonstrated that CB₁ receptor transcript levels diminish after 9 wk gestation, immunoreactive FAAH disappears from the syncytiotrophoblast by the 11th week of gestation, and that CB₂ expression, at the transcript and protein level, remains constant throughout the 1st trimester.

Because the syncytiotrophoblast is in direct contact with maternal blood, the presence of FAAH in this layer suggests that it could be acting to protect the growing embryo from the detrimental effects of AEA. The disappearance of FAAH at 11 wk when circulation in the placental unit is fully established is interesting because it suggests that the protective role of the syncytiotrophoblast is only necessary before the establishment of the placental unit. Whether this is due to the fact that AEA levels in maternal plasma decrease at this point of gestation (17) is unclear. The concurrent increase of FAAH expression in the mesenchymal core of villi in the fully established placental unit suggests that this area of the developing placenta then assumes the protective role of the developing syncytiotrophoblast. It may also explain why there is no concomitant decrease in the levels of FAAH mRNA from homogenized tissues (Fig. 1).

Our demonstration of CB₁ immunoreactive protein and transcripts in first trimester human trophoblast is in accordance with the findings of Kenney et al. (21) but differs from those of Helliwell et al. (31). The reason for the discrepancy in these observations is unclear but may be related to methodological differences because we found that

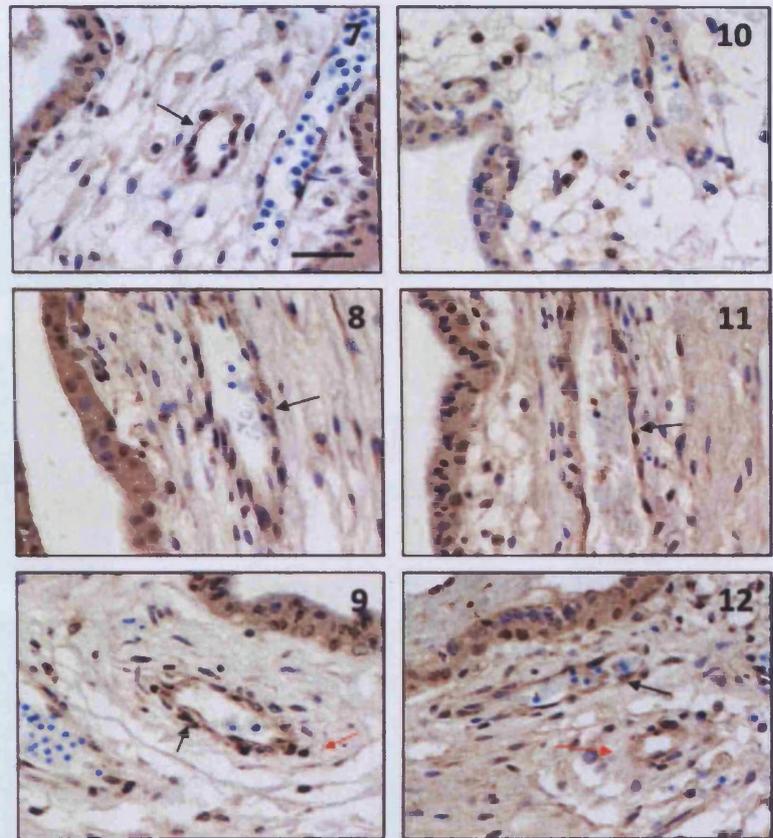


FIG. 5. Effect of gestation on CB₂ expression in the first trimester trophoblast. Immunohistochemical staining for CB₂ was found in the syncytiotrophoblast, cytotrophoblast, and mesenchymal fibroblastic core of all placental samples from wk 7–12. CB₂ immunoreactivity in the syncytiotrophoblast layer remains constant throughout the first trimester. Positive staining in the endothelial cells of blood vessels (black arrow) and the negative staining in the smooth muscle cells of the blood vessels (red arrow). Representative images were taken at $\times 40$ magnification. Bar, 50 μm .

CB₁ immunoreactivity was demonstrable after a process of antigen retrieval by microwave energy and the use of the tyramide amplification system to increase the immunoreactive signal (Fig. 4). The immunostaining pattern in term fetal membranes was consistent with that presented (18), suggesting that the methodology used here is appropriate for immunohistochemical detection of CB₁. Methodological differences in these studies could be one explanation because we used an improved method that allowed the detection of very low levels of CB₁ protein, when compared with that of FAAH and CB₂ in the first trimester trophoblast.

Our immunohistochemical data, which were supported by the presence of transcripts for FAAH, CB₁, and CB₂ (Fig. 1) were different from that previously reported (31). A careful Basic Local Alignment Search Tool (BLAST) search of the published primer sequences (31) indicated that the sequences used are unlikely to detect human CB₁. Indeed, when we applied the previously used protocols, we were unable to detect CB₁ (data not shown). However, using primer sets of Kenney *et al.* (21), which were previously validated for term placenta samples and BeWo cells, where cannabinoids prevented serotonin transport, it was clear that human first trimester trophoblast contain transcripts of both CB₁ and CB₂. Because we failed to detect human FAAH (data not shown) with the published FAAH primer sets under the experimental conditions set out in the paper of Helliwell *et al.* (31), we designed our own set of primers that are directed

against exons 14 and 15 of the human FAAH gene (GenBank accession no. AH007340). As can be seen in Fig. 1, the predicted product from RT-PCR was generated in all trophoblast samples, and the levels were not modulated through the first trimester, suggesting that the use of a more sensitive amplification technique in the immunohistochemistry studies and of better gene-specific primers may explain the discrepancy between our study and that of Helliwell *et al.* (31).

Our observations suggest that trophoblastic FAAH levels and CB₂ levels do not alter significantly during the first trimester but alter their cellular distribution from the syncytiotrophoblast to the mesenchymal core of the villus. The most significant change was the diminution of CB₁ expression after the ninth week of gestation, a point of critical alteration in the developing placenta, where the maternal and fetal blood meet in a meaningful way for the first time (32, 33).

The placenta forms an efficient barrier between fetal and maternal blood. However, it is unlikely to effectively separate maternal endocannabinoids from the fetal compartment. This is because the exocannabinoid, Δ^9 -tetrahydrocannabinol, is known to cross freely the placenta and enter the fetal circulation, whereby it causes detrimental effects on fetal growth and development (34, 35). Therefore, because cannabinoids have a direct effect on the developing placenta (21, 36), it was our hypothesis that if high levels of maternal anandamide were detrimental to early placental and fetal development, FAAH expression would be high in the tro-

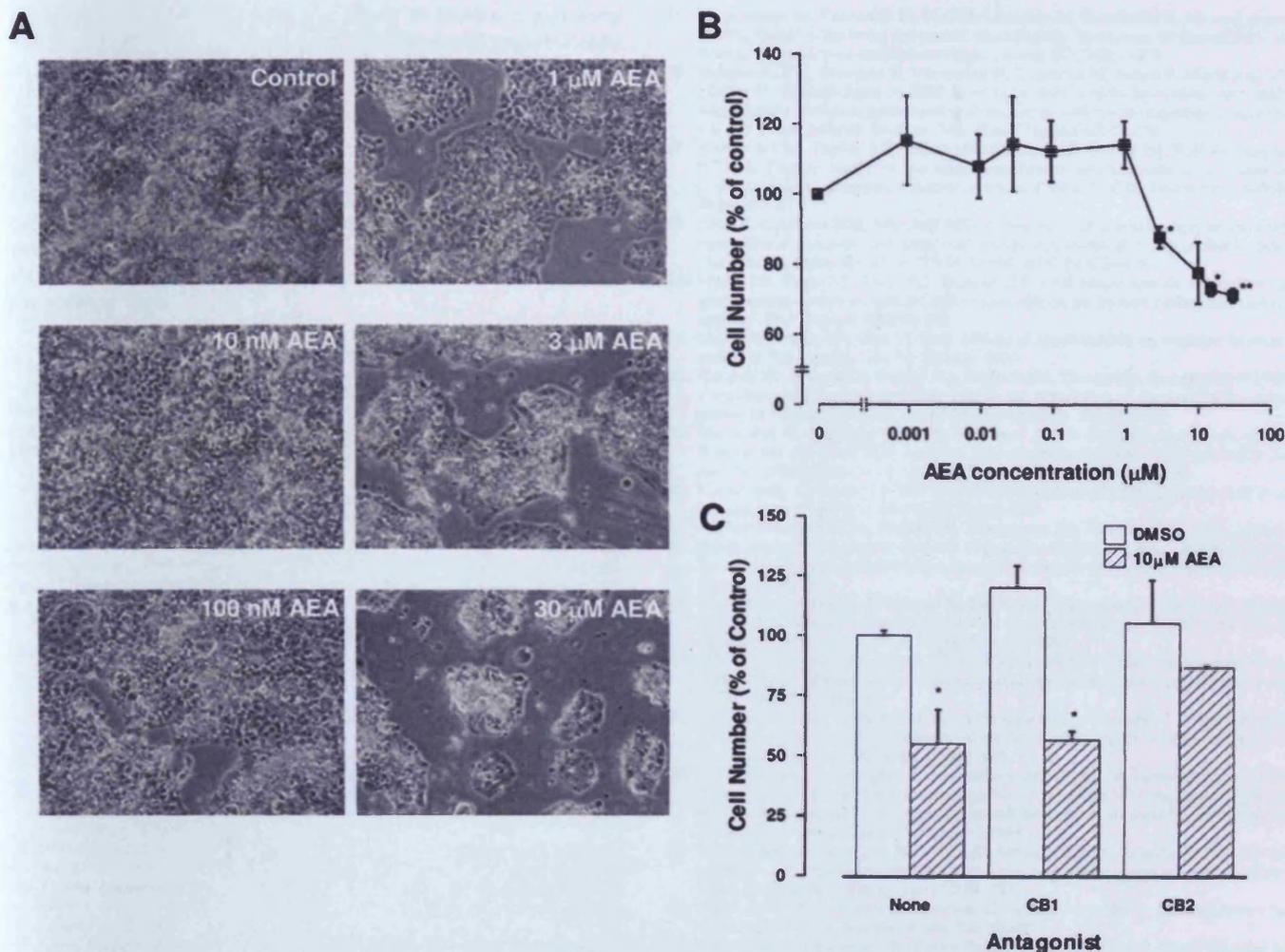


FIG. 6. Effect of anandamide on BeWo cell growth. A, BeWo cells plated at 4×10^5 cells per well onto six-well plates were allowed to proliferate for 48 h before treatment with the indicated concentrations of anandamide (AEA) for an additional 48 h with the medium exchanged after 24 h. Note the decreased cell growth in the cultures treated with AEA (bar, $10 \mu\text{m}$). B, BeWo cells plated at 1×10^4 cells per well in 96-well plates were allowed to proliferate for 48 h before treatment with the indicated concentrations of anandamide (AEA) for an additional 48 h with the medium exchanged after 24 h. The cells were incubated with XTT proliferation agent for 4 h, and the levels of color developed measured with absorbance at 492 nm and corrected for microplate imperfections at 620 nm, and then converted to the cell numbers relative to the untreated controls for each plate. The data are presented as mean \pm SEM for four independent experiments performed in quadruplicate ($n = 4$). *, $P < 0.05$; ***, $P < 0.001$ Kruskal-Wallis one-way ANOVA with Dunn's post test. C, BeWo cells plated at 1×10^4 cells per well onto 96-well plates were allowed to proliferate for 48 h before treatment with $10 \mu\text{M}$ anandamide (AEA) or 0.1% ethanol for an additional 48 h with the medium exchanged after 24 h in the presence or absence of 300 nM of the CB₁-selective antagonist SR141716A or the CB₂-selective antagonist SR144528. The antagonist diluent [0.1% dimethylsulfoxide (DMSO)] acted as the control. The data are presented as the mean \pm SEM for three independent experiments performed in quadruplicate ($n = 3$). *, $P < 0.05$ Student's paired *t* test.

phoblast during normal early pregnancy, as has been hypothesized before (31).

The fact that the protein levels of FAAH remained constant in early gestation and appeared to diminish after 9 wk gestation may reflect the protection afforded the fetus during this critical time. Although larger subject numbers would be required to confirm our findings, this study suggests that FAAH levels in the human trophoblast decline between the 10th and 12th week of gestation. These data are similar to a previous study that examined maternal plasma FAAH levels in 50 women who had normal pregnancies between gestational ages 7–12 wk (15). That study found that the levels of

FAAH activity peaked at 9–10 wk gestation. An investigation into the factors regulating FAAH and AEA levels at the trophoblast level may provide an explanation for the observed changes.

There is growing evidence that a major transition in placental physiology occurs at approximately 10 wk gestation with the dissipation of trophoblast plugs from the spiral arteries, consequently allowing maternal blood to perfuse the placenta for the 1st time (37, 38). Thus, at about the 10th week of gestation, the human fetus must begin to protect itself against circulating maternal anandamide, but because the CB₁ and CB₂ receptors are expressed in the syncytiotro-

phoblast, endocannabinoids are likely to have a regulatory role in the placenta. One suggestion is that endocannabinoids may be involved in the removal of damaged cells by a process of apoptosis. Indeed, there is emerging evidence that endocannabinoids have a significant apoptotic potential in other organs (39). Our studies on the effect of anandamide on BeWo cell growth (Fig. 6) tend to support this suggestion because high concentrations of AEA correlated with lower cell numbers and reduced cell growth. Together, the data presented may explain, in part, how elevated plasma anandamide levels may lead to an increased risk of 1st trimester pregnancy loss.

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