The Role of the Endocannabinoid System in Fertility Control

Thesis submitted for the degree of

Doctor of Philosophy (PhD)

at the University of Leicester

by

Tülay Karasu

State Exam, MD, MRCOG

Reproductive Sciences Section

Department of Cancer Studies and Molecular Medicine

University of Leicester

September 2012

Author's Declaration

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

_____

(31/08/2013)

Tülay Karasu

Reproductive Sciences Section

Department of Cancer Studies and Molecular Medicine

University of Leicester Medical School, Leicester,

United Kingdom

ABSTRACT

The Role of the Endocannabinoid System in Fertility Control

Tülay Karasu

Endocannabinoids are endogenous ligands for cannabinoid receptors that play a pivotal role in fertilisation, embryo development, transport, implantation and pregnancy maintenance

The endocannabinoid system (ECS) consists of ligands (e.g. anandamide, AEA), receptors (e.g. CB1, CB2 and TRPV1) and ligand modulating enzymes (e.g. NAPE-PLD and FAAH).

The ECS interacts with sex steroid hormones and cytokines to regulate reproduction. Progesterone (P4), essential for pregnancy maintenance, increases FAAH activity in human lymphocytes, keeping AEA levels low.

An elevated plasma AEA level is detrimental for implantation and pregnancy maintenance and any ECS dysregulation adversely affects pregnancy outcome. The hypothesis therefore was that manipulating the ECS could be an effective way of interrupting implantation.

(a) The effect of RU486, a P4 antagonist used to initiate medical termination of pregnancy (MTOP), on plasma AEA levels and levels of AEA and the ECS in trophoblast were investigated. These were examined using UHPLC-MS/MS, immunohistochemistry, qRT-PCR and Western Blotting.

(b) The effect of exogenously administered AEA to female rats during the implantation window was studied.

The results show that ethnicity and BMI can affect the ECS, increasing AEA levels. RU486 administration causes a rise in plasma ($p=0.005$) and trophoblast ($p=0.0062$) AEA levels. Trophoblast NAPE-PLD ($p=0.0006$), FAAH ($p=0.021$), TRPVR1 ($p=0.042$) and CB1 ($p=0.03$) are significantly elevated at the mRNA level but not at the protein level though protein levels were generally higher.

Exogenous administration of AEA to rats, around the day of implantation causes a reduction in viable implantation sites and an increase in resorbed units. At gestation day 14 there was a significant correlation between number of viable embryos and plasma AEA levels ($p=0.0091$). The complete ECS was detected in implantation, inter-implantation and resorbed units at day 14.

These studies have shown that manipulating the ECS can interrupt implantation showing the importance of this system in pregnancy maintenance.

ACKNOWLEDGEMENTS

My gratitude goes to everyone who helped me in completing this work.

I would like to acknowledge all the women who participated in the studies. They enabled me to complete my work.

I am indebted to my supervisors, Prof. Justin C. Konje, Dr. Timothy H. Marczylo and Mr. Emeka Oloto. They gave me constant support, help, advice and encouragement to pursue my work and achieve my goals.

I would like to thank all the staff at the Reproductive Sciences Section for their help, especially Dr. Jonathon M. Willets for his support with Western Blotting, Dr. Patricia Lam and Shashi Rana for their invaluable technical assistance; Zoe Skidmore, Sally Munton and Jenny McNair for their great administrative support. A special thank you goes to Dr. Katerina N. Bambang for her help, support and for the wonderful time we had together and to Dr. Alpha Gebeh.

I would like to thank all the staff at the Family Planning Clinic at St. Peters Healthcare Centre, the Leicester Royal Infirmary, Leicester General Hospital and the gynaecology theatre at the Leicester Royal Infirmary for their continuous help in the collection of samples. It was such a pleasure to work with them.

I would like to acknowledge Dr. Emma Marczylo and Kate Dudek for their great support during my PCR work at the MRC Toxicology Unit, Leicester.

During this research, I was able to work at the Faculty of Pharmacy, University of Oporto, Porto, Portugal, where I made many new friendships. My gratitude goes to Prof. Natércia A Teixeira, Dr. Georgina Correia-da-Silva and Dr. Bruno Fonseca for all their hard work, their invaluable help, advice and support and for the wonderful and memorable time I had in Porto. Muito obrigada!

Finally, I would like to thank my husband Dr. Adrian Ionescu for his continuous support, encouragement and simply being there for me during all this time. I would also like to thank my parents who always believed in me and supported me.....

LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AA	Arachidonic acid
2-AG	2-arachidonoyl glycerol
Abh4	α/β hydrolase 4
ACTB	ß-actin
AEA	*N*-arachidonylethanolamine
AEA-d8	Deuterated Anandamide
APS	Ammonium persulfate
ART	Assisted reproductive technology
ATP	Adenosine-5'-triphosphate
ATP5B	ATP synthase
B2M	ß-2-microglobulin
BMI	Body mass index
CB1	Cannabinoid receptor type 1
CB2	Cannabinoid receptor type 2
cDNA	Copy DNA
COX2	Cyclooxygenase 2
CYC1	Cytochrome c1
Ct	Cycle threshold
Δ^9 (THC)	Delta-9-tetrahydrocannabinol
DAB	3, 3'- diaminobenzidine
DAG	Diacylglycerol
DAGL	*sn*-1-DAG lipase
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DSU	Day surgery unit
EC	Endocannabinoid
ECS	Endocannabinoid System
EDTA	Ethylene diamine tetra-acetic acid
EIF4A2	Eukaryotic translation initiation factor 4A, isoform-2
ELISA	Enzyme-linked immunosorbent assay
EMT	Endocannabinoid membrane transporter
ERG	Endocannabinoid Research Group

E2	17β-estradiol
ERK	Extracellular regulated kinases
FAAH	Fatty acid amide hydrolase
FSH	Follicle stimulating hormone
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GOI	Gene of interest
GPR119	Gastrin releasing peptide 119
GPR55	Gastrin releasing peptide 55
HPLC	High-performance liquid chromatography
H_2O_2	Hydrogen peroxide
hCG	Human chorionic gonadotrophin
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
i.p.	Intraperitoneal
IMS	Industrial methylated spirits
LIF	Leukaemia inhibitory factor
LOD	Limit of detection
LOQ	Limit of quantification
12-LOX	12-lipoxygenase
LPE	Liquid Phase Extraction
LH	Luteinising Hormone
MAPK	Mitogen-activated protein kinase
MAGL	Monoacylglycerol lipase
MRM	Multiple reaction monitoring
mRNA	Messenger RNA
MTOP	Medical termination of pregnancy
NAAA	*N*-acylethanolamine-hydrolyzing acid amidase
NaCl	Sodium chloride
NAE	*N*-acylethanolamine
NAT	*N*-acyltransacylase
NADA	*N*-arachidonoyl dopamine
NAPE	*N*-arachidonoylphosphatidylethanolamine

NAPE-PLD	*N*-arachidonoylphosphatidylethanolamine hydrolyzing phosopholipase D
NGS	Normal goat serum
OEA	*N*-oleoylethanolamide
OEA-d2	Deuterated OEA
OD	Optical density
PBS	Phosphate-buffered saline
PEA	*N*-palmitoylethanolamide
PEA-d4	Deuterated PEA
PGE2	Prostaglandin
PLC	Phospholipase C
PPAR	Peroxisome proliferative-activated receptor
P4	Progesterone
PKA	Protein kinase A
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
RIN	RNA integrity number
rRNA	Ribosomal RNA
RU486	Antiprogesterone (Mifepristone)
18s	18s rRNA gene
SDS	Sodium dodecyl sulphate
SDHA	Succinate dehydrogenase complex
SEA	*N*-stearoylethanolamide
SEM	Standard error of the mean
SF3A1	Splicing factor 3a, subunit 1
SPE	Solid phase extraction
STAT3	Signal transducer and activator of transcription 3
STOP	Surgical termination of pregnancy
TBE	Tris borate EDTA
TBS	Tris-buffered saline
Th1	type-1 T-helper
Tm	Melting temperature
TOP	Termination of pregnancy
TOP1	Topoisomerase (DNA) 1

TRPV1	Transient receptor potential vanilloid type 1
UBC	Ubiquitin C
UHPLC-MS/MS	Ultra high performance liquid chromatography- tandem mass spectrometry
YWHAZ	Phospholipase A2

Chapter 1

Introduction

1.1 FERTILISATION

Human fertilisation is the fusion of an egg with a spermatozoon. In preparation for fertilisation, the oviduct captures the cumulus-oocyte complex after it is released from the ovarian follicle, maintains its viability and controls the ascent of the spermatozoa to the site of fertilisation. The muscular, ciliated and secretory cells of the oviduct are responsible for this tubal transport of the ovum and spermatozoa. The capacitated sperm and oocyte interact in the ampulla of the fallopian tube (Ikawa et al. 2010). The sperm head binds to the zona pellucida, a glycoprotein coat surrounding the egg. The binding of the spermatozoon to the receptor galactosyltransferase (GalT) initiates the acrosome reaction, whereby the enzyme hyaluronidase is released and digests the matrix of hyaluronic acid surrounding the oocyte and thereby degrading the zona pellucida. This is followed by the fusion of the oocyte and sperm plasma membranes to form a diploid cell called zygote. Once the sperm enters the cytoplasm of the oocyte a cortical reaction occurs which prevents other sperm from fertilizing the same oocyte (Nixon et al. 2001). The zygote then begins to divide during its passage to the uterine cavity and becomes a blastocyst by the time it reaches the uterus and is ready for implantation into the endometrium; usually 6 days post-fertilisation (Guzeloglu-Kayisli, Kayisli & Taylor 2009). The blastocyst consists of trophectoderm and the inner cell mass (ICM). The placenta and extraembryonic membranes are developed from the trophectoderm whereas the embryo is developed from the ICM (Dey et al. 2004).

1.2 IMPLANTATION

Successful implantation is crucial for procreation and requires the interplay of many systems which ensure that the endometrium is receptive to the blastocyst at a defined period known as the "implantation window" (Harper 1992). The synchronized development of the embryo to the blastocyst stage and the differentiation of the uterus to the receptive state are critical to this process. For this purpose, an effective 'cross-talk', which involves several endocrine, paracrine and autocrine factors, exists between maternal and fetal tissues (Guzeloglu-Kayisli, Kayisli & Taylor 2009). Implantation has three stages: apposition, adhesion and invasion. Immune cells, cytokines, growth factors, chemokines and adhesion molecules are among the many components involved in this process (Dimitriadis et al. 2005).

The ovarian steroid hormones have a central role in initiating implantation. Estrogen and progesterone (P4) are necessary for uterine differentiation. P4 is the crucial determinant of the window of implantation in humans, which starts after 6 days of P4 production and lasts for 3-4 days (Nikas et al. 2000) (Dominguez et al. 2003). This window is approximately between days 19-23 of the menstrual cycle. P4 influences endometrial receptivity indirectly by enhancing or suppressing expression of apposition and attachment molecules, cytokines and growth factors, e.g. interleukins (IL), vascular growth factor (VEGF) are up-regulated and ß-3-inegrin and leukaemia inhibiting factor (LIF) are down-regulated (Hoozemans et al. 2004). The endometrium changes from fibroblast-like stromal cells into larger more rounded cells (decidualisation) (Dunn, Kelly & Critchley 2003) and large apical protrusions (pinopodes) and microvilli emerge on the luminal epithelium (Paria et al. 2002). During apposition, the blastocyst attaches to the endometrium using adhesion molecules. This interaction becomes stronger during adhesion and is mediated by adhesion molecules, immune cells and cytokines (Dominguez et al. 2005). The trophectoderm develops into trophoblast tissue. Trophoblast invasion and decidualisation are critical for the establishment of a pregnancy. Many growth factors, cytokines and angiogenic molecules control trophoblast motility suggesting that a complex network of cell types, mediators and signalling pathways are involved in the regulation of trophoblast invasiveness (Pollheimer, Knofler 2005, Fitzgerald et al. 2008, Burrows, King & Loke 1996, Fitzgerald et al. 2005). During invasion, the trophoblast penetrates the endometrial epithelium, destroys the basal membrane and invading the stroma, up to the uterine vessels (Pijnenborg et al. 1981b) and the inner third of the myometrium (Pijnenborg et al. 1981a). Metalloproteinases (MMP) digest the major constituents of the endometrial matrix (Bischof, Meisser & Campana 2002). Their activity is controlled by specific inhibitors including tissue inhibitors of metalloproteinases (TIMPs) or indirectly by cytokines. A balance between trophoblast invasion and maternal control of invasion is paramount for the successful process of implantation (Graham, Lala 1992). While the features of trophoblast invasion have been characterised, the interplay between different cell types and growth factors and the “cross talk” between distinct signalling pathways involved in maternal control of implantation remains to be well characterized.

1.3 FEMALE FERTILITY

Fecundability, the probability of conceiving per menstrual cycle, declines with age with this decline accelerating markedly after the age of 35 (Practice Committee of the American Society for Reproductive Medicine 2006). The overall fertility rate in the UK reached 1.96 children per woman in 2008 and this has been the highest since 1973. There has been a steady increase in the average maternal age at the birth of a first child. In 2008 the mean age at giving birth to a first child was 29.3 years, 3 years older than in 1978 (Office for National Statistics 2009). The major reasons for this age increase are education, employment and the need to be socially and financially ready for a child (Bromer, Patrizio 2008). These lifestyle choices have led to an increase in fertility control.

1.4 FERTILITY CONTROL

There are three main strategies for birth control:

- prevention of fertilization in the form of contraception
- prevention of blastocyst implantation
- termination of a pregnancy (TOP).

There are many different contraceptive options available with different degrees of efficacy. Barrier methods like the male condom have been used for a long time but hormonal methods are more effective. In 1960 the FDA approved the first form of hormonal contraception, the combined oral contraceptive in the form of synthetic estrogen and progesterone.

Where any of the methods fail or are not used, emergency (postcoital) contraception is an option. Unintended pregnancy especially in teenagers is common and remains a major problem in the United Kingdom (Harden et al. 2009). Emergency contraception is currently offered in the form of either the levonorgestrel-only pill (levonelle), Ulipristal acetate or the copper containing intrauterine contraceptive device (Prabakar, Webb 2012). Levonelle is a high dose progestin-only pill which can be taken within 72 hours of unprotected sexual intercourse and reduces the risk of a pregnancy by 57-93% (Hamoda et al. 2004, von Hertzen et al. 2002). Its effectiveness is highest if taken

within 12 hours and declines with time (Anonymous1998). Levonelle has an effect on the developing follicle and delays or prevents ovulation before the rise in luteinizing hormone (LH) has begun (Gemzell-Danielsson 2010). Ullipristal acetate is a synthetic selective progesterone receptor modulator which can be used for up to 120 hours after unprotected sexual intercourse (Richardson, Maltz 2012). It prevents or delays ovulation, even when taken on the day of LH peak (Brache et al. 2010). A recent study comparing Levonelle and Ullipristal acetate within 72 hours after unprotected sexual intercourse showed similar effectiveness (Creinin et al. 2006). The copper intrauterine device (IUCD) can be used for up to 5 days after unprotected intercourse. It works primarily by stopping fertilisation through its toxic effects on the sperm and secondarily by preventing implantation (Stanford, Mikolajczyk 2002). The estimated efficacy is 99% (Trussell 1995). However, emergency contraception has not reduced the number of unintended pregnancies and the need for a termination of pregnancy (Glasier et al 2013).

1.5 TERMINATION OF PREGNANCY

In 1995 it was estimated that there were 46 million terminations of pregnancy (TOP) worldwide. Only 26 million of these were legal terminations. World-wide, there are 26 induced abortions per 100 pregnancies, excluding miscarriages and stillbirths (Henshaw, Singh & Haas 1999). In the United States in 2003 88.2% of the TOP were performed at 12 gestational weeks or earlier, about 10% from 13-20 weeks and just above 1% at or after 21 weeks (Strauss et al. 2006). The total number of abortions in England and Wales in 2010 was nearly 200,000. Ninety percent of terminations were before 13 weeks; 43% of those reported were medical using a progesterone-antagonist RU486 (MTOP) and surgical vacuum extraction was the method of choice in 52% of cases (Department of Health 2011). In 5% of the cases dilatation and evacuation (D&E) was performed, recommended for pregnancies above 15 weeks gestation (Department of Health 2011). The highest termination rate was for women aged 19 years (Department of Health 2011). In the UK, termination of pregnancy is allowed until 24 gestational weeks where there are no abnormalities or threat to the mother's life (Human Fertilisation and Embryology Act 1990). Surgical TOP (STOP) using vacuum aspiration is performed up to 15 weeks gestation. Risk factors with STOP are bleeding,

uterine perforation, cervical trauma, infection and failed procedure which occur in 2.3 out of every 1000 cases (RCOG 2011).

Medical termination undertaken up to 63 days of amenorrhoea was first approved in 1991 in the UK (Smith et al. 1991). It is performed with oral anti-progesterone RU486 (200 mg Mifepristone) followed by prostaglandin (Misoprostol, 800 μg) vaginally 24 to 48 h after the RU486. Over 90% of women will pass products of conception within 4 h of Misoprostol administration (el-Refaey et al. 1995). Misoprostol can be repeated after 4 h if the products have not been expelled (RCOG 2011). Combining RU486 and misoprostol offers a higher efficacy than RU486 alone and lower rates of side effects (Kulier et al. 2011). A large case series showed that 200 mg mifepristone followed by 800 μg misoprostol vaginally was effective in 97.5% of cases (Ashok et al. 1998). This regimen is currently recommended by several guidelines for terminating pregnancies up to 63 days (World Health Organisation 2003, RCOG 20011). The efficacy of RU486 is only 60-80% if used alone (Ulmann et al. 1992).

1.5.1 RU486 (Mifepristone)

RU486 (Mifepristone) is a progesterone receptor antagonist with only slight agonistic action (Baulieu 1991), a glucocorticoid receptor antagonist and a weak androgen receptor antagonist (Cadepond, Ulmann & Baulieu 1997). It is a 19-norsteroid substituted at the 11β position by a *p*-(dimethylamino) phenyl group. RU486 enters target cells and binds to the progesterone receptor, glucocorticoid receptor and to a lesser extent, the androgen receptor. RU486 has dose dependent antiglucocorticoid effects with a significant increase of adrenocorticotropic hormone and cortisol concentrations after administration of ≥ 200 mg of RU486 (Gaillard et al. 1984). Progesterone suppresses uterine contractions due to hyperpolarisation of the cell membrane, which makes the myocytes less sensitive to electrical stimulation and inhibition of gap junction formation which counteracts coordinated uterine contraction (Garfield, Blennerhassett & Miller 1988, Gemzell-Danielsson, Bygdeman & Aronsson 2006) . RU486 blocks the action of P4 and thereby increases uterine contractility and sensitises the myometrium to prostaglandins (PG) (Bygdeman, Swahn 1985) which is known to stimulate uterine contractions (Tang, Gemzell-Danielsson & Ho 2007). Additionally, RU486 increases PG release from the decidual cells and reduces the

activity of the prostaglandin dehydrogenase, which is the main inactivating enzyme of PG (Norman, Thong & Baird 1991, Cheng et al. 1993).

1.5.2 RU486 Pharmacokinetics and Metabolism

Peak plasma concentrations of RU486 are reached 1-2 h following oral administration of 100 and 200 mg in women (Shi et al. 1993) and its half-life is 24-48 h analysed by high- performance liquid chromatography (HPLC) (Heikinheimo 1989, He et al. 1989). The bioavailability is about 40% due to the first-pass effect *i.e.*, metabolism and excretion by the liver before entry into the general circulation (Van Look, Bygdeman 1989). In human serum, RU486 binds to α1-acid glycoprotein (AAG) which is an acute phase protein primarily synthesised in hepatocytes (Heikinheimo et al. 1987b). Three metabolites of RU486 have been identified which are excreted in the urine (10%) and in faeces via bile (90%) (Lahteenmaki et al. 1987, Heikinheimo et al. 1987a). These metabolites are mono-demethylated (RU 42633) and di-demethylated (RU 42848) derivatives and the hydroxylated metabolite (RU 42698) (Heikinheimo et al. 1987a, Heikinheimo et al. 1987b). These metabolites are active like RU486. They add 23-33% of the antiprogestagenic and 47-61% of the antiglucocorticoid effect of RU486 (Heikinheimo et al. 1987a).

1.5.3 Misoprostol

Misoprostol (15-deoxy-16-hydroxy-16-methyl prostaglandin E1) is a prostaglandin E1 (PGE1) analogue. It is inexpensive and is effective when given orally, sublingually, vaginally or rectally. The side effects are mainly shivering and diarrhoea (Hamoda, Templeton 2010). Despite misoprostol only being licensed for the treatment and prevention of gastric ulcers it has been used in MTOP since the early 1970s (Weeks, Fiala & Safar 2005, Kulier et al. 2011).

1.5.4 Misoprostol Pharmacokinetics

Misoprostol is rapidly absorbed after oral or sublingual administration with human plasma concentrations peaking at about 12 min and has a short half-life of only 20-40 min. Vaginal administration has the advantage of a delayed time to peak concentration

and a longer half life compared to the oral administration (Zieman et al. 1997, Tang et al. 2002).

1.6 PHYTOCANNABINOIDS AND REPRODUCTION

Cannabinoids are found naturally in *Cannabis sativa* (phytocannabinoids or exocannabinoids) or endogenously in both vertebrates and invertebrates (endocannabinoids). *Cannabis* is the most widely used recreational drug (UNODC. World drug report 2010). Its' medical properties have been known for millennia but only in the second half of the twentieth century was its mechanism of action understood following the identification of Δ^9-tetrahydrocannabinol (THC) the primary psychoactive component and the cannabinoid receptors through which THC exerts its' effect (Devane et al. 1988). These discoveries then led to the identification of the endogenous cannabinoid receptor ligands, the endocannabinoids (Devane et al. 1992). These initial investigations in brain and CNS were soon followed by identification of endocannabinoids and their receptors in peripheral organs and systems and an association with several pathophysiological roles.

Cannabis is the most commonly used illicit drug amongst women of reproductive age. In 2004, a Survey of Drug use and Health showed that 4.6% of American women smoked marijuana during pregnancy (Substance Abuse and Mental Health Services Administration 2005). The detrimental effect of cannabis on female fertility is still controversial. However THC is detectable in reproductive organs (Huizink 2009) and cannabis use during pregnancy is associated with reduced birth weight, impaired neurodevelopment, spontaneous miscarriage, preterm birth and mild developmental abnormalities (Hatch, Bracken 1986, Fergusson et al. 2002, Huizink, Mulder 2006, Campolongo et al. 2009, Jutras-Aswad et al. 2009). Moreover cannabis is associated with both male and female infertility (Maccarrone, Finazzi-Agrò 2004, Klonoff-Cohen, Natarajan & Chen 2006,). The negative effects of exogenous cannabinoids on pregnancy suggest that endocannabinoids and their receptors are involved in the normal reproductive function.

1.7 THE ENDOCANNABINOID SYSTEM AND REPRODUCTION

Endocannabinoids are fatty acid derivates that bind to and activate signalling pathways via the cannabinoid receptors (Di Marzo 1998) and have several roles within both the CNS and the periphery (Fride 2002). The first endocannabinoid, anandamide (*N*-arachidonylethanolamide, AEA) (Devane et al. 1992) was isolated from porcine brain, closely followed by 2-arachidonoylglycerol (2-AG) (Mechoulam et al. 1995, Sugiura et al. 1995). Most studies conducted to date involve either AEA or 2-AG, however, several novel endocannabinoids have been identified including *O*-arachidonoylethanolamine (virodhamine) (Porter et al. 2002), *N*-arachidonoyldopamine (Bisogno et al. 2000) and *N*-arachidonoyl taurine (Saghatelian et al. 2004). In addition, *N*-oleoylethanolamide (OEA), *N*-palmitoylethanolamide (PEA) and *N*-stearoylethanolamide (SEA) are examples of endocannabinoid congeners that are thought to exhibit an 'entourage' effect by inhibiting AEA and 2-AG degradation by acting as sacrificial substrates (Ben-Shabat et al. 1998, De Petrocellis, Cascio & Di Marzo 2004).

The roles of endocannabinoids in reproduction are varied and include regulation of processes like folliculogenesis (El-Talatini et al. 2009), spermatogenesis (Wang, Dey & Maccarrone 2006, Taylor et al. 2007, Battista et al. 2008b), fertilization, oviductal transport (Horne et al. 2008), implantation and embryo development (Wang, Dey & Maccarrone 2006, Taylor et al. 2007, Battista et al. 2007, Battista et al. 2008a, Taylor et al. 2010, Bambang et al. 2010). These events are under the control of steroid hormones and cytokines that have direct effects on various elements of the endocannabinoid system (Maccarrone et al. 2000, Maccarrone et al. 2003, Karasu et al. 2011).

1.8 THE ENDOCANNABINOID SYSTEM

Endocannabinoids including AEA and 2-AG bind to G protein-coupled cannabinoid receptors (CB1 and CB2) (Pertwee, Ross 2002, Sugiura et al. 2002). The cannabinoid receptor-dependent biological effects of AEA and 2-AG are negated by cellular uptake via a putative endocannabinoid membrane transporter (EMT) followed by intracellular

enzymatic degradation (see below). The endocannabinoids, their congeners, the cannabinoid receptors, the EMT, together with the enzymes involved with endocannabinoid synthesis and degradation form the endocannabinoid system (ECS). This system is summarised in Figure 1.1.

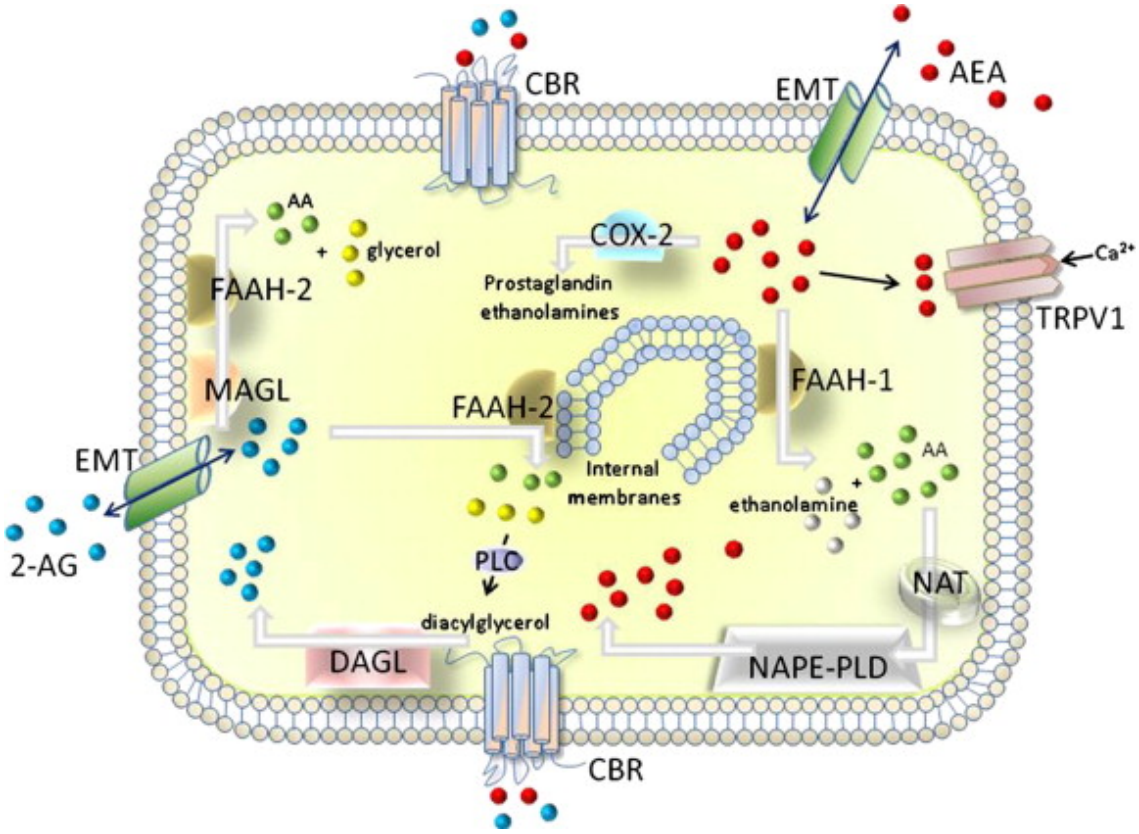

Figure 1.1: The endocannabinoid system (ECS).

The ECS: synthesis and degradation of AEA and 2-AG (Taylor et al. 2010). AEA and 2-AG bind to the putative endocannabinoid membrane transporter (EMT). Intracellular AEA can also bind to the transient vanilloid receptor type 1 (TRPV1). AEA is synthesised from lipid precursors by the enzymes *N*-acyltransacylase (NAT) and *N*-arachidonoylphosphatidyl-ethanolamine-hydrolyzing phosopholipase D (NAPE-PLD) and degraded by fatty acid amide hydrolase isoforms (FAAH1/2) to arachidonic acid (AA) and ethanolamine. Cyclooxygenase-2 (COX-2) converts AEA to prostaglandin-ethanolamide. 2- AG is synthesised by *sn*-1-DAG lipase (DAGL) and degraded by monoacylglycerol lipase (MAGL)/FAAH-2 to AA and glycerol. Abbreviations: PLC, phospholipase C; CBR, cannabinoid receptor.

1.8.1 Metabolism: Biosynthesis, Transport and Degradation of AEA and 2-AG

1.8.1.1 Biosynthesis

The biosynthesis of AEA occurs on demand from its precursor *N*-arachidonoylphosphatidylethanolamine (NAPE) and it is formed by the transfer of arachidonic acid from the *sn*-1 position of 1,2-*sn*-di-arachidonylphosphatidylcholine to phosphatidylethanolamine. NAPE synthesis is catalyzed by a calcium-dependent *N*-acyltransacylase (NAT) (Sugiura et al. 2002). NAPE is hydrolysed to AEA and phosphatidic acid by NAPE phosopholipase D (NAPE-PLD) a calcium sensitive metallo-lactamase (Okamoto et al. 2004b, Wang et al. 2006).

Recently, three alternative pathways for the synthesis of AEA have been proposed: (1.) the double deacylation of NAPE by α/β hydrolase 4 (Abh4) to generate glycerophospho-*N*-arachidonylethanolamine (GP-AEA) which is then cleaved by a phosphodiesterase to AEA (Simon, Cravatt 2008), (2.) cleavage of NAPE by a phospholipase C to phosphoanandamide (pAEA) followed by dephosphorylation to release AEA (Liu et al. 2006) and (3.) secretory phospholipase A_2 ($sPLA_2$) hydrolyzes NAPE to *lyso*-NAPE, which is then further hydrolyzed to AEA by a *lyso*-phospholipase D (*lyso*-PLD) (Sun et al. 2004). Figure 1.2 summarizes these synthetic pathways of AEA. The synthesised AEA is released into the extracellular space where it may act in an autocrine or paracrine way through activation of cannabinoid receptors (see below) (Piomelli et al. 2000).

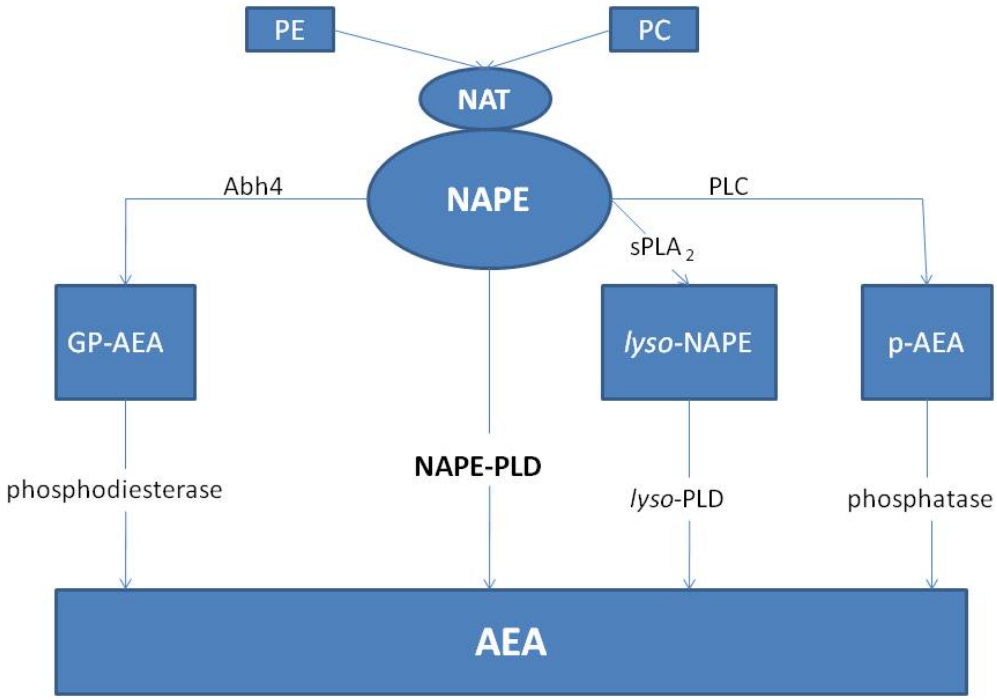

Figure 1.2: Biosynthetic pathways of AEA.

NAPE, produced from membrane phospholipids by NAT is the key intermediate for AEA synthetic pathways as described in the text. Abbreviations: PE, phosphatidylethanolamine; PC, 1,2-*sn*-di-arachidonylphosphatidylcholine; NAT, *N*-Acyltransferase; NAPE, *N*-arachidonoylphosphatidylethanolamine; Abh4, α/β hydrolase 4; GP-AEA, glycerophospho-*N*-Arachidonylethanolamine; $sPLA_2$, secretory phospholipase A_2; *lyso*-PLD, *lyso*-phospholipase D; PLC, phospholipase C; pAEA, phosphoanandamide.

2-AG is released from phospholipid membranes on demand by the hydrolysis of diacylglycerol (DAG) by *sn*-1-DAG lipase (Piomelli 2004). The key intermediate DAG is produced from phosphatidylinositol (PI) by phospholipase C (PLC) activity or from phosphatidic acid (PA) by a phosphatidic acid hydrolase (Bisogno et al. 1999). Another pathway for 2-AG synthesis involves the actions of a phosphatidylinositol-preferring phospholipase A_1 (PLA_1), producing *lyso*-PI, and of lysophosphatidylinositol selective phospholipase C (*lyso*-PLC) (Sugiura et al. 2002) (Figure 1.3).

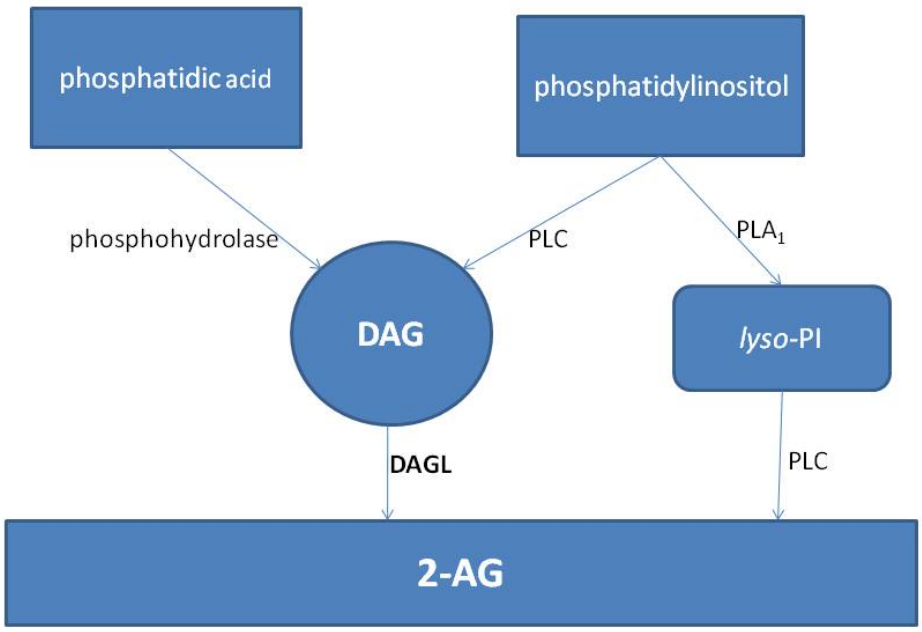

Figure 1.3: Biosynthetic pathways of 2-AG.

2 AG is produced via a DAG or *lyso*-PI intermediate. DAG can also be produced via phosphatic acid. Abbreviations: DAG, Diacylglycerol; DAGL, *sn*-1-DAG lipase; PLC, phospholipase C; PLA_1, phospholipase A_1; lyso-PI, *lyso*-phosphatidylinositol.

1.8.1.2 Transport and Degradation

The activity of AEA is terminated by its removal from the extracellular space via a putative endocannabinoid membrane transporter (EMT) (Ben-Shabat et al. 1998) and then by intracellular degradation by fatty acid amide hydrolases, FAAH1 (McKinney, Cravatt 2005), FAAH2 (Wei et al. 2006) or the lysosomal *N*-acylethanolamine-hydrolyzing acid amidase (NAAA) (Tsuboi et al. 2005) to arachidonic acid (AA) and ethanolamine (De Petrocellis, Cascio & Di Marzo 2004). There are alternative pathways for AEA degradation such as transformation to 12- hydroxyeicosatetraenoyl ethanolamide (12-HETE-EA) by 12-lipoxygenase (12-LOX) (Van der Stelt et al. 2002) or oxidation by COX2 to prostaglandin-ethanolamide (PGE_2-EA) (Rouzer, Marnett 2008) (Figure 1.4a). There is still controversy over the transmembrane movement of AEA. Some cellular models support the hypothesis of a carrier protein for AEA transport in a process of facilitated diffusion (Hillard, Jarrahian 2000, Giuffrida et al. 2000) but this protein has not been identified (Glaser et al. 2003). Other hypotheses include simple diffusion (Kathuria et al. 2003) or intracellular sequestration of AEA (McFarland et al. 2004). In general, AEA cellular uptake is dependent on its concentration gradient and does not require ATP (Hillard et al. 1997).

2-AG is degraded by both FAAH and monoacylglycerol lipase (MAGL) to arachidonic acid (AA) and glycerol (Fergusson et al. 2002). MAGL is primarily found in the cytosol and FAAH in membranes of the microsomal and mitochondrial sub-cellular fractions. In addition, COX2 and lipoxygenase can metabolise 2-AG to prostaglandin-glyceryl esters (PGE_2-GE) (Kozak et al. 2002) and hydroxyeicosatetraenoyl-glycerols, respectively (Van der Stelt et al. 2002) (Figure 1.4b).

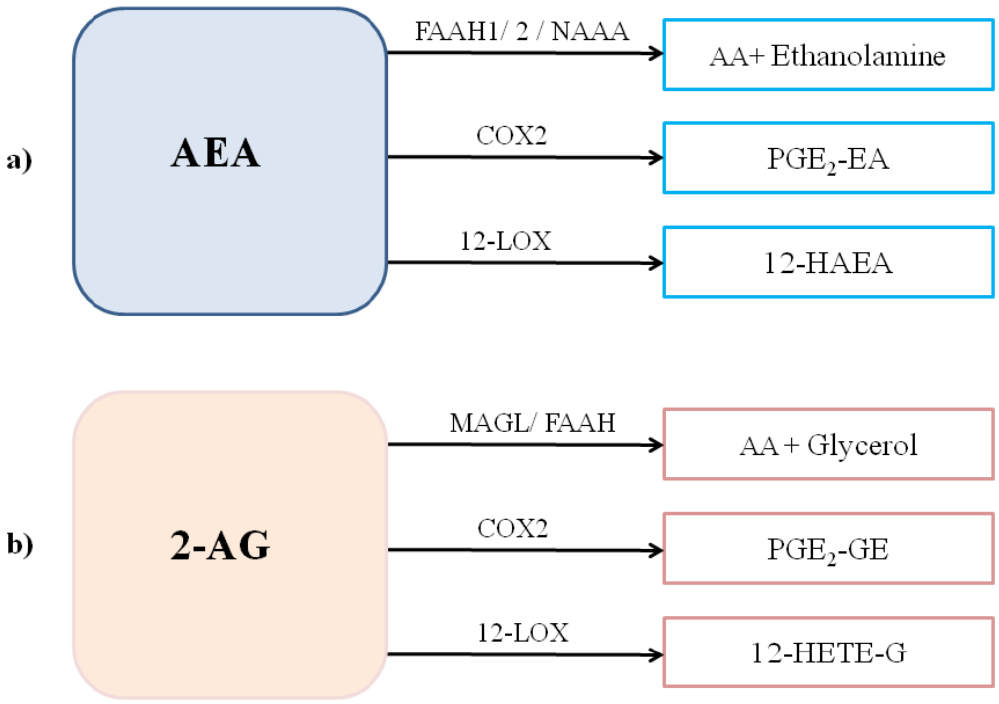

Figure 1.4: Degradation of AEA (a) and 2-AG (b).

Abbreviations: AA, arachidonic acid; FAAH, fatty acid amide hydrolase; COX2, cyclooxygenase2; FAAH , fatty acid amide hydrolase; 12-HETE-EA, 12-hydroxyeicosatetraenoyl -ethanolamide; 12-HETE-G, hydroxyeicosatetraenoyl-glycerols; 12-LOX, 12-lipoxygenase ; MAGL, monoacylglycerol lipase, NAAA, *N*-acylethanolamine-hydrolyzing acid amidase PGE_2-EA, prostaglandin E2 ethanolamide; PGE_2-GE, prostaglandin E2 ethanolamide -glyceryl esters.

1.8.1.3 Endocannabinoid Receptors

1.8.1.3.1 Cannabinoid Receptors – CB1 and CB2

Endocannabinoids are ligands for the cannabinoid receptors type 1 (CB1) and type 2 (CB2) (Howlett et al. 2002). These are G protein-coupled seven transmembrane receptors that share 44% overall identity (Devane et al. 1988, Howlett et al. 2002). CB1 was first described in rat brain (Devane et al. 1988) and thought to be present mainly in the central nervous system, but now is known to be present in peripheral tissues like the ovary, uterine endometrium, testis, liver, heart, small intestine, urinary bladder and lymphocytes (Bouaboula et al. 1993, Pertwee 1997, Pertwee, Ross 2002). The CB2 receptor was first isolated from rat spleen and human myeloid cells (Munro, Thomas & Abu-Shaar 1993) and was initially thought to be mainly expressed in immune cells (Pertwee 1997, Howlett et al. 2002, Pertwee, Ross 2002) but has now been localised in tissues such as embryonic stem cells (Sharov et al. 2003), placenta (Helliwell et al. 2004), myometrium (Dennedy et al. 2004), ovary (El-Talatini et al. 2009), gastrointestinal tract (Fioramonti, Bueno 2008), liver (Mallat, Lotersztajn 2008) and heart (Pacher, Steffens 2009).

Activation of the CB1 receptor stimulates mitogen-activated protein (MAP) kinases (Bouaboula et al. 1995) and inhibits adenylyl cyclase (Paria, Das & Dey 1995) causing reduced levels of cyclic adenosine monophosphate (cAMP). A reduction in cAMP inhibits the opening of voltage gated calcium channels and stimulates potassium channels (Howlett et al. 2004). CB2 activation like CB-1 activation stimulates MAP kinases but also stimulates cytosolic phospholipase A2 (PLA2). CB2 does not regulate ion channels. In addition CB2 activation inhibits nitric oxide synthase whereas CB1 activates it (Howlett et al. 2004, Demuth, Molleman 2006). Nitric oxide has an important role in several critical processes in female reproduction including ovulation, implantation, pregnancy maintenance, labour and delivery (Maul et al. 2003, Vaisanen-Tommiska 2008, Krause, Hanson & Casanello 2011). The regulation of nitric oxide levels by CB2 activation suggests a critical role for CB2.

The relevance of ligand levels for this receptor-related research is complex. Whereas AEA has a high affinity for the CB1 receptor 2-AG has a low affinity for the receptor but a high efficacy (Sugiura et al. 1999). Alternatively, AEA is only a partial agonist of

CB2, but 2-AG has a high affinity for the CB2 receptor. 2-AG is therefore a full agonist for both the CB1 and CB2 receptor (Howlett et al. 2004, Demuth, Molleman 2006).

1.8.1.3.2 Non-CB1/ CB2 G-protein-coupled Receptors

In addition to CB1 and CB2, two putative cannabinoid receptors GPR55 and GPR119, have been identified. These are orphan G protein-coupled receptors (McPartland et al. 2006) which recent data have associated with the ECS (Ross 2009, Godlewski et al. 2009, Moriconi et al. 2010, Pertwee et al. 2010).

Little is known about GPR55 but GPR55 mRNA has been located in various brain regions, testis, ileum, spleen, tonsil and adipose tissue (Brown 2007). Several studies have shown that AEA and 2-AG have no consistent effect on GPR55, but PEA is a potent and selective agonist of GPR55 (Ryberg et al. 2007, Henstridge et al. 2009, Yin et al. 2009). However, lysophosphatidylinositol (LPI) appears to be a more potent ligand for GPR55 than AEA or 2AG (Henstridge et al. 2009, Yin et al. 2009, Nevalainen, Irving 2010) and triggers extracellular signal-regulated kinase (ERK) phosphorylation and a rise in intracellular calcium levels (Oka et al. 2007). GPR55 seems to be involved in pain control (Staton et al. 2008).

Less is known about GPR119, but GPR119 mRNA has been found mainly in pancreatic and gastrointestinal tissues (Chu et al. 2007, Lauffer, Iakoubov & Brubaker 2009) and seems to play a role in obesity and diabetes. OEA has a strong affinity for GPR119 and thereby increases intracellular cAMP and may impact on diabetes and obesity (Overton et al. 2006). GPR119 activation in addition to stimulating adenylyl cyclase and protein kinase A activities also promote glucose-induced insulin secretion and improved glucose control (Chu et al. 2007, Lauffer, Iakoubov & Brubaker 2009). Therefore GPR119 may be a new therapeutic target for treatment of type 2 diabetes (Ohishi, Yoshida 2012).

1.8.1.3.3 Vanilloid Receptor

The transient receptor potential vanilloid type-1 (TRPV1) (Szallasi, Blumberg 1999) is a ligand-gated non-selective cationic channel that belongs to the TRP family of proteins (Pertwee et al. 2010). TRPV1 is activated by capsaicin and stimuli like heat and pH (Szallasi, Blumberg 1999). TRPV1 is synthesised in cells outside the peripheral nervous

system, like keratinocytes, epithelial and endothelial cells (Caterina 2003) and has also been found in various brain areas which are involved in pain transmission or modulation (Mezey et al. 2000).

Endovanilloids are the endogenous ligands for TRPV1 (Di Marzo et al. 2001a, Van Der Stelt, Di Marzo 2004). The first identified endovanilloid was AEA (Zygmunt et al. 1999). AEA, but not 2-AG, binds to TRPV1 at a cytosolic binding site triggering non-selective ion channel activation of protein kinases, calcium influx and release of cytochrome c (Szallasi, Blumberg 1999, Maccarrone, Finazzi-Agro 2003).

TRPV1 receptors are found in the same organs, tissues and cells as cannabinoid receptors, where receptor binding by AEA can have either opposing or similar functions (Ahluwalia et al. 2003, Cristino et al. 2006).

TRPV1 is involved in pain modulation and also plays a pivotal role in thermoregulation (Gavva 2008). Furthermore, TRPV1 has been detected in the gastrointestinal tract and there is some evidence for a role in diabetes, pancreatitis and obesity (White, Urban & Nagy 2011). TRPV1 in urothelial cells of the urinary tract takes part in pain sensation and frequency of bladder contractions (Avelino, Cruz 2006). Less is known about the role of TRPV1 when expressed in the respiratory and auditory systems (White, Urban & Nagy 2011).

1.9 ENDOCANNABINOID SYSTEM AND FEMALE REPRODUCTION

In animal studies the ECS plays a pivotal role in fertilization, oviductal transport, implantation, embryo development and maintenance of early pregnancy (Taylor et al. 2007, Battista et al. 2007, Battista et al. 2008a, Taylor et al. 2010). AEA is thought to be the key link between the developing embryo and the endometrium, ensuring their synchronous development for on-time implantation.

After fertilisation in the oviduct, the fertilised egg undergoes mitotic divisions to form a morula. The morula develops into a blastocyst, which consists of an inner cell mass (ICM) and the trophectoderm. The ICM forms the embryo and the trophectoderm develops to become the placenta and extra-embryonic membranes. A reciprocal

interaction between the blastocyst and receptive uterus is essential for successful implantation (Sun, Dey 2008).

Mice express CB1 and CB2 receptor mRNA and protein in pre-implantation embryos (Paria, Das & Dey 1995, Paria et al. 2001, Wang et al. 2004). CB1 mRNA is detected from the 4 cell stage to the blastocyst stage and CB2 mRNA is detected from the 1 cell stage onwards (Paria, Das & Dey 1995, Battista et al. 2007) (Figure 5). CB2 is expressed in the embryonic stem cells but not in the trophectoderm while CB1 is found in the trophectoderm, but not in the inner cell mass (Paria, Das & Dey 1995). In contrast, only CB1 mRNA has been detected in the oviduct and uterus (Das et al. 1995, Wang et al. 2004).

CB1 plays an important role in the control of oviductal transport and embryo implantation. CB1 knockout mice showed pregnancy loss (Paria et al. 2001, Wang et al. 2004) suggesting that the expression of CB1 is required for implantation. CB1 deficiency in CB1 knockout female mice also caused embryo retention in the oviduct and resultant ectopic pregnancy (Wang et al. 2004). Embryos were also retained in the oviduct when wild type females were exposed to high doses of either the stable AEA analogue methanandamide or to THC (Wang et al. 2004). Recently, a lower CB1 mRNA expression was confirmed in fallopian tubes of women with tubal pregnancies compared to normal fallopian tubes in the luteal phase or menstrual from women undergoing surgery for benign gynaecological conditions (Horne et al. 2008). A lower CB1 mRNA expression was also measured in the endometrium of women with tubal pregnancies compared to endometrium from intra-uterine pregnancies (Horne et al. 2008). Therefore, perturbation of cannabinoid signalling can impair correct embryo implantation.

Normal gestation requires an early immunological adaptation involving peripheral T-lymphocytes (Maccarrone, Finazzi-Agrò 2004). CB2 is involved in releasing cytokines related to fertility (Correa et al. 2005, Borner, Hollt & Kraus 2006). CB2 receptors have been found in first trimester human placenta (Helliwell et al. 2004, Habayeb et al. 2008) suggesting a role in placentation and maternal-fetal signalling (Maccarrone 2008).

The enzymes involved in the synthesis and degradation of endocannabinoids normalize levels of AEA for successful implantation. NAPE-PLD is present in the cytoplasm of cells in the pre-implantation mouse embryo from the 1 cell stage to the blastocyst stage

while FAAH is expressed from the 2 cell stage in the outer cell layers of morulae and trophectoderm. NAPE-PLD is also found in the oviduct with higher levels at the isthmus than the ampullary region, whereas the expression of FAAH is higher in the ampullary region (Wang et al. 2006) (Figure 1.5).

AEA plays an important role in directing uterine implantation (Paria et al. 2001). High levels of NAPE-PLD and low levels of FAAH are present in the inter-implantation sites of the mouse uterus on day 5-7, whereas at the same time high levels of FAAH and low levels of NAPE-PLD are found in the implantation sites (Wang et al. 2007) (Figure 1.5). Consequently, a four-fold reduction in AEA levels is associated with the implantation site (Paria, Deutsch & Dey 1996, Schmid et al. 1997). The implanting blastocyst also regulates uterine AEA levels by an inhibitory effect on uterine NAPE-PLD (Guo et al. 2005) by the release of an as yet unidentified putative lipid "FAAH activator" (Maccarrone et al. 2004).

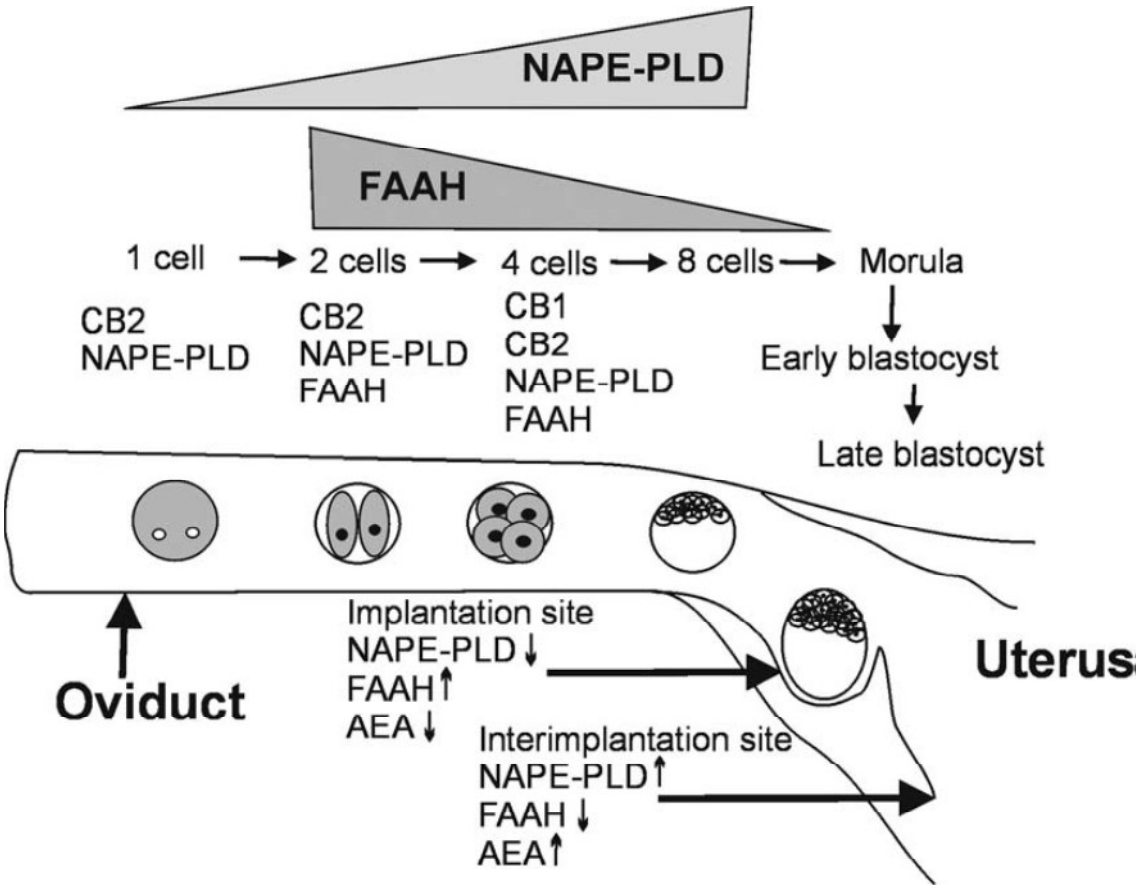

Figure 1.5: The ECS in the pre-implantation embryo and at the blastocyst implantation site in mice (Karasu et al. 2011).

Low NAPE-PLD and high FAAH levels were found in the ampullary region of the oviduct and high NAPE-PLD and low FAAH levels in the isthmus. During embryo development CB2 and NAPE-PLD were present from the 1 cell stage, FAAH from the 2 cell stage and CB1 from the 4 cell stage onwards. In implantation sites low NAPE-PLD and high FAAH levels led to low AEA levels whereas high AEA levels were measured in inter-implantation sites.

The "FAAH activator", produced by both the ICM and trophectoderm, up-regulates FAAH in the uterine cavity and thereby reduces AEA levels.

The exposure of two-cell embryos to high levels of endocannabinoids *in-vitro* results in developmental arrest (Paria, Das & Dey 1995, Paria et al. 1998). This arrest can be prevented by selective CB1 antagonists (SR141716A, AM251), but not by a specific CB2 antagonist (SR144528), suggesting a CB1-dependent effect of the endocannabinoids on the pre-implantation embryo. High AEA levels have a pro-apoptotic potential in mouse blastocysts (Maccarrone et al. 2000).

Endocannabinoid signalling downstream of CB1 in the embryo is concentration-dependent. In mice, low concentrations of AEA (7nM) activate the extracellular regulated kinase (ERK) signalling pathway via CB1 in the trophectoderm cell surface of the blastocyst and thereby make the blastocyst competent for implantation whereas higher levels (28nM) do not activate ERK but inhibit calcium mobilisation (Wang et al. 2003). This is clinically relevant as elevated peripheral AEA levels in women are associated with spontaneous miscarriage (Maccarrone et al. 2000b). A pilot study of women with threatened miscarriage showed that all women who subsequently miscarried had higher peripheral AEA levels (greater than 2.0 nM) than women that didn't go on to miscarry (Habayeb et al. 2008). In IVF pregnancies higher plasma AEA levels were associated with failure to achieve an ongoing pregnancy after embryo transfer (Maccarrone et al. 2002). Similarly, women undergoing IVF/ ICSI required low AEA levels at the time of implantation for a successful pregnancy (El-Talatini, Taylor & Konje 2009).

With regards to FAAH, decreased activity in peripheral blood lymphocytes may be a marker of early spontaneous miscarriage (Maccarrone et al. 2000b). Taken together these results suggest that FAAH activity and plasma AEA may be used for the monitoring of early pregnancies.

Although the ECS has not been studied extensively during pregnancy, two cross-sectional studies of plasma AEA levels show that these are high in the first trimester comparable to the luteal phase, fall between first and second trimesters consistent with successful pregnancy maintenance and then rise significantly at term and still further during labour (Habayeb et al. 2004, Lam et al. 2008). Low AEA levels are thus thought to maintain pregnancy while high levels are associated with labour onset (Habayeb et

al. 2004). Recently, a crucial role for CB1 was demonstrated for the onset of labour in mice (Wang, Xie & Dey 2008). CB1 deficiency altered corticotrophin-releasing hormone activity leading to abnormal ovarian E2 (17β-estradiol) and P4 secretion and induction of preterm birth (Wang, Xie & Dey 2008). The effects of ECS on female fertility are summarised in Table 1.1.

The precise mechanism by which endocannabinoids influence reproduction is uncertain, but cyclooxygenase 2 (COX2) is thought to have a major role. For example, one route of AEA degradation is by COX2-dependent biotransformation to prostamides (prostaglandin ethanolamides) (Yu, Ives & Ramesha 1997, Kozak et al. 2002). COX2 is an inducible enzyme which is elevated during inflammation, carcinogenesis and pyrexia. COX2 is essential in female reproduction where it is involved in several critical processes including ovulation, fertilization, implantation and decidualisation (Lim et al. 1997). In mice, COX2 is expressed at the implantation site, but is hardly detectable at the inter-implantation sites (Wang et al. 2007) and therefore contributes with FAAH to the differential concentrations of AEA at these sites (Wang et al. 2007).

Table 1.1: Main effects of the endocannabinoid system on female fertility (Karasu et al. 2011)

Effects of low levels	ECS Component	Effects of high levels
Embryo implantation Embryo development	**AEA**	Miscarriage Pro-apoptotic mouse blastocyst Induction of labour Preterm birth
Miscarriage	**FAAH**	Embryo implantation Embryo development
Ectopic pregnancy	**CB1**	Oviductal transport
	CB2	Embryo development

1.10 ENDOCANNABINOIDS AND SEX STEROID HORMONES

In addition to the direct effects proposed for endocannabinoids on reproduction the ECS also interacts with sex steroid hormones and cytokines to regulate reproduction indirectly. These interactions are discussed in the following sections.

1.10.1 The Role of Progesterone

Progesterone (P4) is a steroid hormone produced predominantly after ovulation by the corpus luteum and in pregnancy by the placenta. Its' major physiological functions are in reproduction like ovulation, implantation and maintenance of a pregnancy (Graham, Clarke 1997). Furthermore, P4 is involved in the lobular-alveolar development in the mammary gland for milk secretion and mediates signals in the brain. Its primary action is through the nuclear progesterone-receptor which is induced by estrogen (Graham, Clarke 1997). This amplification by estrogen may be mediated through the estrogen receptors which have been shown to up regulate the expression of progesterone receptors.

It is well known that reproduction is dependent upon tight immunoregulation. Type-2 T-helper (Th2) cytokines promote fertility and type-1 T-helper (Th1) cytokines inhibit fertility. P4 creates a suitable endometrial environment for implantation and maintains the pregnancy by contributing to a protective immune milieu. Consequently, P4 induces the production of the pro-fertility Th2 cytokines and inhibits the anti-fertility Th1 cytokines (Piccinni, Romagnani 1996). P4 stimulates leukaemia inhibitory factor (LIF) release through IL-4, which has also been demonstrated to promote implantation and pregnancy continuation (Maccarrone et al. 2001). Furthermore P4 is involved in the maintenance of endocannabinoid levels. P4 up-regulates lymphocyte FAAH activity through the transcription factor Ikaros (Maccarrone et al. 2001, Maccarrone et al. 2003) and thereby decreases AEA levels in lymphocytes (Table 1.2) (Figure 1.6). P4 has a minimal effect on EMT, NAPE-PLD and CB1 expression in lymphocytes (Maccarrone et al. 2001, Maccarrone et al. 2003) but has been shown to down-regulate uterine NAPE-PLD expression in mice, possibly leading to a decrease in uterine AEA levels consistent with a decline in AEA levels in first trimester pregnancy (see earlier) (Guo et al. 2005). Unexpectedly, the activity of uterine FAAH, localised in murine glandular

and luminal epithelium, is also decreased below basal levels by progesterone which is expected to lead to an increase in AEA levels (Maccarrone et al. 2000).

Changes in P4 levels and lymphocyte FAAH expression are well correlated during the menstrual cycle (Lazzarin et al. 2004). This is in agreement with the previously observed up-regulation of the FAAH gene by P4 (Maccarrone et al. 2003). However there does not seem to be a correlation between plasma AEA levels and those of P4 in normally cycling women (El-Talatini, Taylor & Konje 2010) and of pregnant women during implantation and early pregnancy (El-Talatini, Taylor & Konje 2009). It has therefore been concluded that plasma AEA in women is most likely not regulated by P4 (El-Talatini, Taylor & Konje 2009). However, the source of plasma AEA has not been identified and we cannot rule out possible regulation of AEA by P4 in placenta or other target tissues.

Table 1.2: Effects of progesterone and estrogen on the ECS in female fertility

Hormone	Reproductive Process	Effect on ECS
Progesterone	Implantation Pregnancy maintenance	▪ Increases FAAH through transcription factor Ikaros and reduces AEA ▪ Increases LIF via IL4 and IL4 enhances FAAH activity ▪ Promotes pro-fertility Th2 cytokines and thereby enhances FAAH activity
Estrogen	Folliculogenesis Implantation	▪ Stimulates NAPE-PLD activity and inhibits FAAH activity and leads to an increased AEA content in endothelial cells ▪ Down regulates NAPE-PLD and inhibits FAAH in uterine epithelium

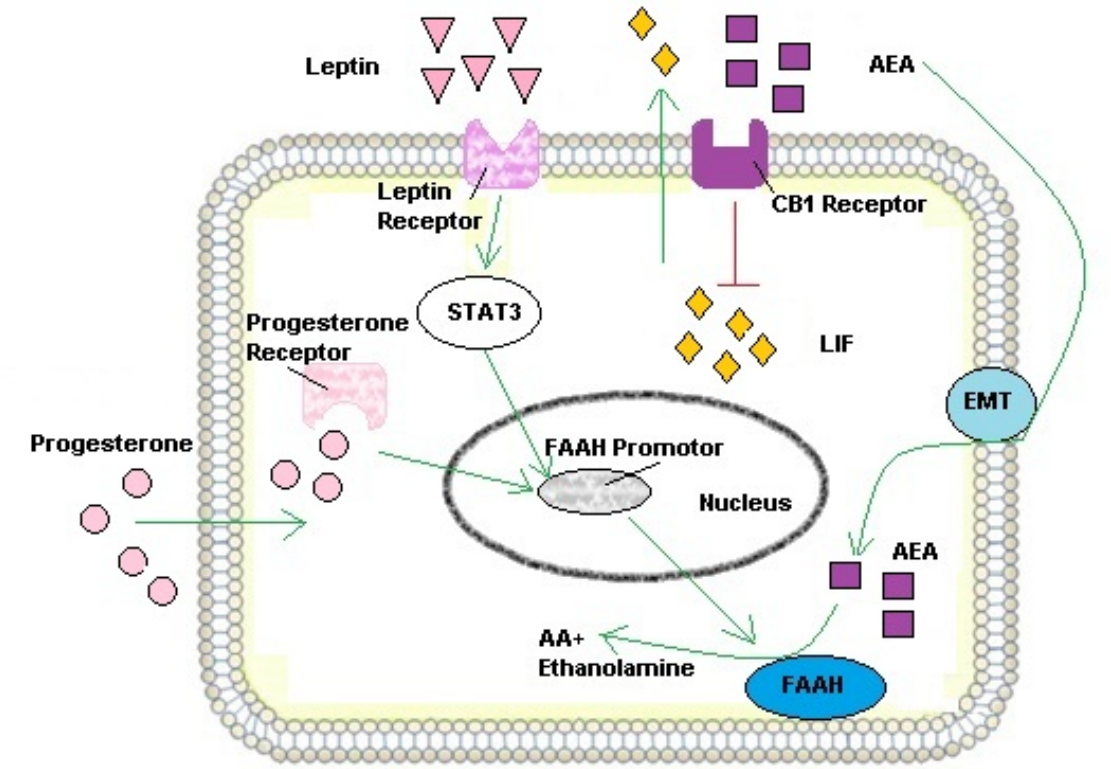

Figure 1.6: AEA-hormone-cytokine network.

AEA is removed from the extracellular space via the putative, not yet identified endocannabinoid membrane transporter (EMT) and degraded by FAAH to arachidonic acid (AA) and ethanolamine. Leptin promotes up regulation of the FAAH promoter via signal transducer and activator of transcription 3 (STAT3) and thereby decreases AEA levels. P4 up regulates the FAAH promoter via the transcription factor Ikaros. AEA reduces LIF release via the CB1 Receptor.

1.10.2 The Role of Estrogen

Estrogens are steroid hormones which up-regulate the expression of many genes via activation of estrogen receptors (ERα, ERβ).

Estrogens are involved in reproduction, cardiovascular system, bone integrity and cognitive function (Deroo, Korach 2006). Estrogens are produced primarily by developing follicles and also by the corpus luteum in ovaries, and by the placenta. Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) stimulate the production of estradiol (E2) by the ovaries. Some estrogens are produced in smaller quantities by other tissues such as the liver, adrenal glands, and the breasts. E2 modifies

many responses and is known to increase prolactin secretion (Fink 1988) and to be involved in the regulation of the ECS (Gorzalka, Dang 2012).

E2 stimulates NAPE-PLD activity and inhibits FAAH activity, causing the accumulation of AEA in endothelial cells which then modulates the cardiovascular and immune systems (Maccarrone et al. 2002). In contrast, uterine NAPE-PLD expression (mRNA) is down-regulated by E2 suggesting a role in decreasing AEA levels (Guo et al. 2005). NAPE-PLD activity correlates with uterine AEA levels (Guo et al. 2005). In a separate study a decrease in mouse uterine FAAH activity was demonstrated by E2 (Maccarrone et al. 2000) (Table 1.2). The contradictory nature of these studies demonstrate that it is difficult to speculate on the effects of E2 on AEA levels based upon the expression of the ECS and that studies measuring the actual levels of AEA may be more useful.

Circulating plasma AEA levels during the menstrual cycle of healthy women demonstrated a positive correlation with E2 suggesting that E2 may be involved in the regulation of AEA (El-Talatini, Taylor & Konje 2010) or that both AEA and E2 are co-regulated by another factor. A positive correlation between E2 and AEA was also demonstrated in non pregnant women after *in-vitro* fertilization and embryo transfer (El-Talatini, Taylor & Konje 2009).

1.11 ENDOCANNABINOIDS AND CYTOKINES

1.11.1 The Role of Leukaemia Inhibitory Factor

Leukaemia inhibitory Factor (LIF), a member of the interleukin (IL) 6 family, plays important roles in the immune, haematopoetic and reproductive systems (Smith, Charnock-Jones & Sharkey 1998). Amongst its biological roles are cell proliferation, differentiation and survival (Hilton 1992). Downstream signalling is triggered after binding of the LIF receptor-β (LIF-Rβ) to glycoprotein gp130 (Heinrich et al. 2003). Signal transduction involves activation of several different pathways, but the main ones are JAK/Stat, Src homology 2-domain-containing tyrosine phosphatase (SHP-2)/Ras/ extracellular signal regulated kinase (ERK) and phosphatidyl-inositol-3-kinase (PI3K)/Akt pathways.

LIF knockout mice are infertile due to failed implantation, however, $LIF^{-/-}$ embryos can implant in wild type female mice or after supplementation with LIF to female $LIF^{-/-}$ mice (Stewart et al. 1992, Chen et al. 2000). Endometrial LIF and not embryonic LIF is therefore critical for implantation. Interestingly, gp130 knockout mice also present with failed embryo implantation (Ernst et al. 2001). *In-vitro* studies in mice have demonstrated a role for LIF in blastocyst hatching, trophoblast outgrowth and implantation of cultured mouse embryos (Lavranos, Rathjen & Seamark 1995, Cai, Cao & Duan 2000). LIF expression has been detected in the human endometrium with a peak LIF mRNA concentration in the luteal phase at the time of implantation (Charnock-Jones et al. 1994, Kojima et al. 1994). Furthermore, LIF is expressed in human fallopian tubes and may therefore be involved in blastocyst development (Keltz et al. 1996). LIF-Rβ mRNA, but not LIF, has been demonstrated in human blastocysts (Charnock-Jones et al. 1994).

IL1β and leptin up-regulate LIF-Rβ in human endometrium (Gonzalez et al. 2004). Studies in cultured endometrial cells show that TNF-α, and IL6 also stimulate LIF production (Laird et al. 1997). Addition of seminal fluid increases LIF expression in human endometrial cells in-vitro (Gutsche et al. 2003). Furthermore, it is suggested, that the human blastocyst is involved in the regulation of endometrial LIF expression (Perrier d'Hauterive et al. 2004), whereby human chorionic gonadotrophin (hCG) has a stimulating effect on LIF expression. LIF can stimulate hCG production by the trophoblast (Nachtigall et al. 1996). LIF also enhances blastocyst development and differentiation *in-vitro* (Dunglison, Barlow & Sargent 1996, Cai, Cao & Duan 2000).
LIF is involved in decidualisation as high LIF expression is detected in trophoblast and placenta (Hilton 1992, Nachtigall et al. 1996, Laird et al. 1997, Sharkey et al. 1999). Furthermore, LIF production by cultured endometrial tissue from women affected by idiopathic infertility is lower than that of fertile women (Delage et al. 1995) and lower levels of LIF are found in some women with recurrent miscarriage (Piccinni et al. 1998). Secretion of gp130 is reduced in infertile women (Sherwin et al. 2002). LIF levels in uterine flushings have been investigated as a predictor of successful implantation. LIF levels decrease in the late luteal phase of the menstrual cycle (Laird et al. 1997, Sharkey et al. 1999) and increased LIF levels are measured in women who fail to conceive (Ledee-Bataille et al. 2002). A suggested explanation for a failed

conception is a delayed LIF expression following a delayed development of the endometrium.

T-lymphocytes play a significant role in implantation and successful pregnancy (Piccinni et al. 1998); Type 2 T-helper (Th2) cytokines inhibit type 1 T- helper (Th1) cytokine responses allowing the survival of the fetus (Piccinni, Romagnani 1996). Th2 cytokines such as interleukins IL-3, IL-4 and IL-10 stimulate trophoblast growth through inhibition of natural killer cells. Th1 cytokines like IL-2, IL-12 and interferon (INF)-γ damage the trophoblast through stimulation of natural killer cells and secretion of tumour necrosis factor (TNF)-α. The ECS is thought to be involved in the modulation of this immune system in human fertility. For example FAAH expression is regulated by the Th1 and Th2 cytokines (Maccarrone et al. 2001): IL-4 and IL-10 enhance FAAH activity whereas IL-2 and INF-γ reduce FAAH expression.

IL-2 inhibits the release of LIF and IL-4 stimulates its release (Maccarrone et al. 2001, Maccarrone et al. 2002). AEA reduces the release of LIF from T cells via a CB1 receptor-dependent mechanism (Maccarrone et al. 2001, Brown 2007) and thereby carries out its anti-fertility action (Figure 1.6).

P4 induces pro-fertility Th2 cytokines and stimulates LIF release through IL-4 (Piccinni et al. 1998, Maccarrone et al. 2001). Treatment of women with the anti-progesterone RU486 after ovulation reduces the LIF expression of the glandular epithelium but not in the luminal epithelium or stromal cells (Danielsson, Swahn & Bygdeman 1997). Furthermore RU486 was shown to have no effect on LIF expression in the fallopian tube (Li et al. 2004). These results suggest that there are different regulatory mechanisms in the different tissues.

LIF is essential for implantation and the ECS is involved in the modulation of the immunological changes occurring during early pregnancy.

1.11.2 The Role of Leptin

Leptin, a 16kDa helical cytokine, is a product of the obese gene (*ob*) (Zhang et al. 1994). It is produced by adipose tissue, ovary and the placenta (Henson, Castracane 2000, Reitman et al. 2001, Margetic et al. 2002) and was first described in relation to food uptake and energy homeostasis (Friedman, Halaas 1998). Mutations in *ob* are associated with obesity and pituitary dysfunction in humans (Clement et al. 1998). Homozygous, knockout ($ob/ob^{-/-}$) female mice are infertile though exogenous leptin can restore their fertility (Chehab, Lim & Lu 1996). Therefore leptin has a critical role in reproduction (Clarke, Henry 1999).

Leptin regulates the growth and development of the conceptus (Kiess et al. 1998) and angiogenesis (Park et al. 2001, Bouloumie et al. 1998). The human leptin receptor exists in long and short isoforms which couple to different signal transduction pathways. The long isoform (OB–Rl) couples to the Janus kinase 2 and signal transducer and activator of transcription 3 (JAK2/ Stat3) signalling pathway (Tartaglia 1997) and the short isoform (OB–Rs) signals through the mitogen-activated protein kinase (MAPK) pathway (Bjorbaek et al. 1997). Leptin and its receptors have been located in human placental syncytiotrophoblast (Ashworth et al. 2000, Maymo et al. 2011) and the human endometrium (Gonzalez et al. 2000). Low leptin levels have been found in women with spontaneous miscarriage in the first trimester (Lage et al. 1999). Enhanced leptin secretion from the endometrium occurs in the presence of a blastocyst in human. Therefore it seems that leptin is important for implantation (Gonzalez et al. 2000). Leptin stimulates the development of mouse embryos *in-vitro* (Kawamura et al. 2002). However, high levels of leptin also interfere with mouse embryo development and zona pellucida hatching and cause apoptosis in blastocysts (Fedorcsak, Storeng 2003).

Leptin levels vary in relation to gender and body composition. For example, women of reproductive age have higher serum levels than men (Hickey et al. 1996) and postmenopausal women (Shimizu et al. 1997). Serum leptin levels also change during the menstrual cycle with lower levels during the follicular phase compared with the secretory phase (Hardie et al. 1997). Maternal serum leptin concentrations are greater than in non pregnant women indicating that leptin may play a role in pregnancy

maintenance. During early pregnancy leptin concentrations rise in conjunction with E2 levels (Hardie et al. 1997). E2 regulates leptin levels via the leptin promoter (Machinal et al. 1999). Leptin concentrations correlate well with P4 levels during the luteal phase of the menstrual cycle and with hCG concentrations during pregnancy (Hardie et al. 1997). Consequently, this suggests a relationship between obesity, leptin levels and reproduction (Linne 2004, Henson, Castracane 2006, Metwally, Ledger & Li 2008). Leptin concentrations in plasma are related to the amount of body fat (Considine et al. 1996, Hardie et al. 1997). Obese women undergoing IVF treatment have lower conception rates (Wang, Davies & Norman 2000, Fedorcsak et al. 2004) and there is an increased risk of early pregnancy loss with obesity (Fedorcsak et al. 2000, Wang, Davies & Norman 2002, Maheshwari, Stofberg & Bhattacharya 2007). Weight reduction before IVF treatment increases the chances for a successful pregnancy (Fedorcsak et al. 2004). Successful appetite control and therefore reduction of obesity has been demonstrated with the CB1 antagonist rimonabant (Leite et al. 2009) but there is insufficient data available on the effects of this drug with regard to the reproductive system. As a centrally acting CB1 antagonist rimonabant showed unacceptable side effects preventing further development and application as a weight loss drug. However, CB1 receptor blockers selective for peripheral receptors may achieve weight loss without the adverse psychiatric effects (Christopoulou, Kiortsis 2011).

Leptin is also integrated into the regulation of the endocannabinoid-hormone-cytokine network. It reduces the levels of AEA and 2-AG in the hypothalamus of *ob/ob*$^{-/-}$ mice (Di Marzo et al. 2001b, Kirkham et al. 2002). Uterine AEA and 2-AG are up regulated in *ob/ob*$^{-/-}$ mice due to reduced activity of EMT, FAAH and MAGL and increased activity of DAGL. Normal endocannabinoid levels were rescued by treatment with leptin (Maccarrone et al. 2005). These results suggest that leptin down-regulates the endocannabinoid signalling.

In humans, leptin up-regulates the promoter region of the FAAH gene expression through STAT3 signalling (Maccarrone et al. 2003) and concomitantly reduces AEA levels in T cells (Figure 1.6). Consequently inhibition of LIF release by AEA is reduced (Maccarrone et al. 2002).

1.12 HYPOTHESIS

Endocannabinoids play a pivotal role in gametogenesis, early embryo development including blastocyst maturation, oviductal transport, implantation and maintenance of early pregnancy. Manipulating this system may be an effective way of interrupting implantation leading to the development of new post coital contraceptives which would be effective within five to six days of unprotected sexual intercourse.

This leads to the following hypotheses:

1. The anti-progesterone RU486 will elevate AEA levels during MTOP because progesterone normally up-regulates FAAH leading to low levels of AEA pivotal for successful implantation.
2. Exogenously administered AEA will prevent implantation.

These hypotheses were investigated independently:

a) The effect of RU486 on AEA levels were investigated in a cohort of women undergoing a termination of pregnancy. If high AEA levels were indeed detrimental to successful implantation and maintenance of pregnancy, then this anti-progesterone should cause a rise in the AEA levels.
b) Secondly the effects of exogenously administered AEA to female rats following mating were investigated on the basis that these will increase AEA levels in the uterus and thus prevent implantation.

These hypotheses were tested by a series of experiments designed to answer these specific questions in humans and rats:

1. Do plasma levels of AEA increase following the administration of RU486 during MTOP?
2. What are the effects of RU486 on the ECS in human trophoblast?
3. How do the changes (if any) in AEA levels relate to those of leptin which is essential for pregnancy maintenance?
4. Does exogenously administered AEA prevent embryo implantation in rats?
5. What is the dosing window for successful post-coital contraception with AEA in rats?

Chapter 2

The Effect of the Anti-progesterone Mifepristone (RU486) on Plasma Ethanolamides and Leptin in First Trimester Pregnancy

2.1 INTRODUCTION

Blastocyst implantation is a complicated process involving regulatory molecules like sex steroids, proteases, growth factors and cytokines (Fitzgerald et al. 2005). The ECS is also a key element involved in the synchronous development of the blastocyst and endometrial receptivity required for a successful early pregnancy (Taylor et al. 2007). It has been suggested that the correct "AEA tone" is important throughout the reproductive process for successful implantation (Schuel 2006). P4 is an essential steroid hormone for pregnancy maintenance and it participates in sustaining the correct "AEA tone" by up-regulating the AEA degrading enzyme FAAH and thereby lowering plasma AEA levels (Maccarrone et al. 2003). Leptin has been shown to participate in implantation, placental development and fetal growth and development (Castellucci et al. 2000, Henson, Castracane 2000). Leptin supports trophoblast cell proliferation through the JAK-2 and STAT-3 pathways (Magarinos et al. 2007) and prevents apoptosis by the mitogen-activating protein kinase (MAPK) pathway (Perez-Perez et al. 2008). Leptin together with progesterone interact with the ECS and reduces AEA levels by up-regulation of the FAAH promoter (Maccarrone et al. 2003). This advocates a cross-talk between cytokines, steroid hormones and the ECS. The studies in this part of the thesis were to determine in women undergoing termination of pregnancy the effect of the anti-progesterone RU486 on the plasma levels of the ethanolamides *N*-arachidonoylethanolamine (AEA), *N*-oleoylethanolamine (OEA) and palmitoylethanolamine (PEA) and serum leptin levels and to investigate the effect of factors like ethnicity, gestational age and BMI on *N*-acylethanolamides (NAE) and leptin levels.

2.2 MATERIAL AND METHODS

2.2.1 Subjects

These were women undergoing medical termination (study group) and those undergoing surgical termination (control group) of pregnancy at the University Hospitals of Leicester NHS Trust, at the Leicester Royal Infirmary. All women had a pelvic ultrasound scan prior to the procedure to confirm gestational age and viability.

All volunteers gave written informed consent to take part in the study. The study was approved and conducted according to the guidelines of the Leicestershire and Rutland Local Research Ethics Committee (Appendix 4).

2.2.2 Power Calculation

Data from previous publications by the Endocannabinoid Research Group (ERG) of the University of Leicester and of other groups suggested that a minimum of 50 volunteers in the study and 50 women in the control group were required to detect a minimum difference of 20% in AEA levels for an alpha of 0.05 and a power of 80%.

2.2.3 Inclusion Criteria

These were as follows:

1. BMI<30 kg/m^2
2. Accurately dated singleton pregnancy using ultrasound
3. Written informed consent

2.2.4 Exclusion Criteria

These were as follows:

1. Previous or current use of recreational drugs
2. Presence of any systemic disease like diabetes, hypertension
3. Smoker of more than 20 cigarettes a day

2.2.5 Chemicals

All chemicals and solvents were of high-performance liquid chromatography (HPLC) grade. AEA, OEA, PEA, deuterated AEA (AEA-d8), deuterated OEA (OEA-d2) and deuterated PEA (PEA-d4) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Each had a purity of >98%. HPLC grade water was obtained using a water

purification system (Maxima ELGA, ELGA, High Wycome, UK). Acetonitrile, chloroform, formic acid, ammonium acetate and methanol were obtained from Fisher Scientific (Loughborough, UK). Polytetrafluoroethylene (PTFE) filters of 0.2 µm, 47 mm diameter and Oasis HLB solid phase extraction (SPE) cartridges (1cc, 30mg) were purchased from Waters Ltd (Elstree, UK).

2.2.6 Preparation of Standards

Supplied stock solutions of AEA, OEA, PEA, AEA-d8, OEA-d2 and PEA-d4 were dried under a gentle stream of nitrogen gas and reconstituted in acetonitrile to a concentration of 5 mg/ml for AEA, OEA and PEA and 100 µg/ml for their deuterated equivalents. All stock solutions were stored at -20°C. Further dilutions were made in acetonitrile on ice on the day of analysis.

The preparation of AEA-d8, OEA-d2 and PEA-d4 (internal standard) is as follows:

A. Deuterated internal standards for calibration curves

1:25 dilution: 20µl 6.272 pmol/µl AEA-d8 + 20µl 6.272 pmol/µl OEA-d2 + 20µl 6.272 pmol/µl PEA-d4 + 440µl acetonitrile = 500µl 0.25 pmol/µl

B. Deuterated internal standards for sample extraction

1:100 dilution: 10µl 6.272 pmol/µl AEA-d8 + 10µl 6.272 pmol/µl OEA-d2 + 20µl 6.272 pmol/µl PEA-d4 + 960µl acetonitrile = 1000µl 0.06272 pmol/µl

2.2.7 Recruitment and Blood Sample Collection

Volunteers were recruited at the time of their first visit to the Fertility Control Clinic when requesting a termination of pregnancy. An ultrasound scan was performed to date the pregnancy. Those women opting for a medical termination of pregnancy were recruited as the study group while those opting for a surgical termination were recruited into the control group. Women were matched for age and their demographic characteristics. Blood samples were collected from the clinic from both groups at the time of their routine blood investigations.

A second blood sample was collected from those on the study group when they attended the hospital for their RU486 medication. The blood sample was collected immediately

prior to RU486 administration (200mg). A third blood sample was taken about 24 hours later, just prior to the administration of Misoprostol, a prostaglandin (800µg).
For the control group, a second blood sample was taken on the day of their surgery, just before the routine vaginal administration of prostaglandin (400µg). A third blood sample was taken in the anaesthetic room before induction of general anaesthesia. A final blood sample was collected in the recovery area approximately 10 min following the STOP procedure.

On each occasion above approximately 8 ml was collected into EDTA monovettes for plasma and into serum gel monovettes for serum. The blood samples were kept on ice and transported to the analytical laboratory for processing within 60 min of collection.

2.2.8 Sample Processing

In the laboratory, the blood samples were centrifuged at 1200 x g for 30 min at 4°C to separate plasma/ serum from blood cells. Plasma and serum were transferred into clean 7 ml Kimble scintillation vials (Kinesis, St. Neots, Cambs, UK). EC extraction was either performed immediately or the samples were stored at -80°C for later analysis.

2.2.9 Extraction of AEA, OEA and PEA from Human Plasma

Solid phase extraction (SPE) was used to extract the EC from human plasma (Marczylo et al. 2009). Plasma (0.5 ml) was spiked with 2.5 pmol/ml AEA-d8, 2.5 pmol/ml OEA-d2 and 5 pmol/ml PEA-d4 internal standards and diluted to 1 ml with deionized water. All samples were vortexed thoroughly for 10s. The samples were loaded onto Oasis HLB 1 cc cartridges (Waters UK Ltd) which had been preconditioned with 1 ml of methanol followed by 1 ml of distilled water. The samples were drawn through the cartridges under gentle vacuum at a flow rate of approximately 1 ml/min using a Vacmaster vacuum manifold (Biotage, Uppsala, Sweden). Cartridges were washed with 1 ml of 40% aqueous methanol and the EC were eluted in 1 ml of acetonitrile. The eluant was evaporated under a gentle stream of nitrogen. The dried extract was reconstituted in 80µl of acetonitrile and transferred to an HPLC vial. Quantification of AEA, OEA and PEA was achieved using Ultra Performance Liquid Chromatography-

Tandem Mass Spectrometry (UPLC-MS/ MS, Waters Ltd) (Lam et al. 2008, Marczylo et al. 2009, Marczylo et al. 2010).

2.2.10 Construction of Calibration Curves

Seven point calibration curves were prepared in triplicate from stock solutions in acetonitrile (ACN) on ice. The concentration ranges for AEA and OEA were 0.2375-19 pmol/ml and 0.9-76 pmol/ml for PEA. Standards were spiked with deuterated internal standards (IS, 2.5 pmol of AEA-d8, OEA-d2 and PEA-d4). The serial dilutions of AEA, OEA and PEA were conducted as shown in Table 2.1.

Table 2.1: Serial dilutions of stock solutions of AEA, OEA and PEA

Standards	Serial Dilution	NAE Concentration	
		AEA, OEA	PEA
A	200 µl stock[1] + 200 µl ACN	1 µM	4 µM
B	100 µl A + 900 µl ACN	100 nM	400 nM
C	200 µl B + 300 µl ACN	20 nM	80 nM
D	500 µl C + 500 µl ACN	10 nM	40 nM
E	500 µl D + 500 µl ACN	5 nM	20 nM
F	400 µl E + 600 µl ACN	2 nM	8 nM
G	500 µl F + 500 µl ACN	1 nM	4 nM
H	500 µl G + 500 µl ACN	0.5 nM	2 nM
I	500 µl H + 500 µl ACN	0.25 nM	1 nM

[1] 1 ml stock solution of 2 µM AEA, 2 µM OEA and 8 µM PEA (MM) was prepared from 20µl 100 µM AEA + 20µl 100 µM OEA + 80µl 100 µM PEA + 880µl acetonitrile (ACN).

Standard curves were conducted in triplicate. Each replicate was from a separate serial dilution. 190µl of standard (C-I) was transferred to an HPLC vial and 10µl of 0.25 pmol/µl AEA-d8, OEA-d2 and 0.5 pmol/µl PEA-d4 added. Following UHPLC-MS/MS analysis, linear regression analysis was used to establish the slope, intercept and coefficient of correlation of the calibration lines of concentration versus relative response ([peak area endocannabinoid] / [internal standard (IS) area/ IS concentration]).

2.2.11 Stability of Endocannabinoids in Human Blood

One concern with respect to EC measurement is that EC concentrations may change in proportion to processing time. To examine the effect of processing time on the concentrations of the EC, whole blood samples were divided into 4 identical aliquots. The aliquots were centrifuged and EC extracted at different time points: immediately, 30, 60 and 120 min after collection.

The stability of AEA, OEA and PEA in human plasma during storage at -80°C was also examined in samples after a single freeze-thaw cycle. One aliquot was processed on the day of sample collection and 3 other aliquots were stored at -80°C. After 1 week, 2 weeks and 4 weeks of storage the aliquots were thawed on ice, and EC extracted as described above and analysed. Values from the day of sample collection were compared with the values obtained after one freeze-thaw cycle and the stability expressed as the percentage change in concentration.

2.2.12 Quantification of AEA, OEA and PEA

Quantitative analysis of AEA, OEA and PEA was performed using an ultra high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/ MS) system comprising an Acquity UHPLC connected to a Quattro Premier tandem mass spectrometer (Waters Ltd.) The column, an Acquity UPLC BEH C_{18} (92.1 x 50 mm, 1.7 µm) was maintained at 40°C. The mobile phases comprised A (2mM ammonium acetate containing 0.1% formic acid) and B (acetonitrile containing 0.1 % formic acid) and were filtered through 0.2 µm PTFE filters prior to use. LC gradient conditions were: 0-0.5 min, 20% B; 2.5 min, 100% B; 3.5 min, 20% B and then re-equilibrated at

20% B until 4 min. Samples were maintained at 4°C. Tandem electrospray mass spectrometry in positive ion mode (ES^+) was used to quantify the analytes of interest in multiple reaction monitoring (MRM) mode. Source parameters were as follows: capillary voltage of 1 kV, cone voltage 21 V, source temperature 120°C, desolvation temperature 440°C, cone gas flow 49 L/ h and desolvation gas flow 800 L/ h. Subsequently, MS/MS conditions for monitoring each precursor $[M+H]^+$ ion included entry, collision and exit energies of 6, 16 and 2eV, respectively. MRM mode was used to monitor product ions with mass transitions employed as follows: AEA (*m/z* 348.25>61.9), AEA-d8 (*m/z* 356.25>62.9), OEA (*m/z* 326.5>61.9), OEA-d2 (*m/z* 328.2>61.9), PEA (*m/z* 300.5>61.9) and PEA-d4 (*m/z* 304.2>61.9). Samples and standards (7µl) were injected with the needle overfill preference. 7-point calibration curves in triplicate were performed. Peaks from standards and analytes were integrated using Masslynx software version 4.1 (Waters Inc., Milford, MA). Quanlynx software (Waters Inc.) and the concentrations of AEA, OEA and PEA were determined using calibration curves of concentration against relative response using the formula:

$$relative\ response\ (y) = \frac{Peak\ area}{(IS\ \ area/[IS])}$$

Peak area= the peak area of AEA
IS area= the peak area of the AEA-d8 internal standard
[IS]= the concentration of the internal standard

2.2.13 Quantification of Serum Leptin Concentration

Blood samples were taken from women attending for MTOP and serum extracted by centrifugation as described above. Serum leptin concentrations were measured using a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) for total leptin (Invitrogen, California, USA Cat. No KAC2281). All samples were measured in duplicate and the content of leptin was calculated from a standard curve generated for each plate with recombinant leptin. The minimal detectable level of leptin for this assay was 3.5 pg/ml. The intra-assay and inter-assay precision were 3% and 3.9%,

respectively. The serial dilutions employed to generate the standard is shown in Table 2.2.

Serum samples were diluted to 1:100 with Standard Diluent Buffer and mixed well. 100µl of the standards and samples were pipetted into the microtiter wells, which were coated with a monoclonal antibody specific to human leptin. This was followed by 100µl of the secondary biotinylated monoclonal anti-human leptin antibody. During the 2 hour incubation the leptin binds to the immobilised human leptin antibody and is also bound by secondary antibody. Following washing to remove any excess secondary antibody, 100µl of Streptavidin-Horse Radish Peroxidase (HRP enzyme) was added to each well except the blank well which was followed by an incubation of 30 min to allow Streptavidin-HRP to bind to the biotinylated antibody. Unbound streptavidin was removed with washes and 100µl of stabilized chromogen was added to each well and left for 30 min. The stabilized chromogen is biotransformed by the bound enzyme and produces colour. The intensity of the colour in each well is proportional to the concentration of leptin in the sample. Finally 100µl of the STOP solution was added to each well. The absorbance was read by a plate reader at 450 nm. The values obtained were multiplied by 10 to correct the 1:100 dilution.

Table 2.2: Human leptin standard curve

Human Leptin	Reconstituted Standard	Standard Diluent Buffer
1000 pg/ml	100µl resuspended standard	900µl
500 pg/ml	300µl of 1000 pg/ml	300µl
250 pg/ml	300µl of 500 pg/ml	300µl
125 pg/ml	300µl of 250 pg/ml	300µl
62.5 pg/ml	300µl of 125 pg/ml	300µl
31.2 pg/ml	300µl of 62.5 pg/ml	300µl
15.6 pg/ml	300µl of 31.2 pg/ml	300µl
0 pg/ml	0	300µl

2.2.14 Statistical Analysis

Statistical analyses were performed using GraphPad Instat version3, Graphpad Software, San Diego, California, USA. The data is presented as mean ± SEM. A p-value <0.05 was considered statistically significant.

2.3 RESULTS

2.3.1 Stability of NAEs in Human Blood and the Consequences of Sample Handling

AEA, OEA and PEA concentrations were measured in plasma and serum from the same volunteers and no significant difference was found between them (Table 2.3). Consequently, all further studies measured the AEA, OEA and PEA concentrations in plasma alone.

There were no differences in AEA, OEA and PEA plasma values, irrespective of whether the blood samples were centrifuged at 4°C or 20°C (Table 2.4). It was therefore decided to centrifuge all samples at 4°C, as done in previous studies by the Endocannabinoid Research Group.

Table 2.3: Plasma NAE levels in plasma and serum

Analyte	Plasma	Serum	p-value
AEA (nM)	0.8±0.05	0.8±0.07	0.982
OEA (nM)	4.38±0.27	4.42±0.26	0.7249
PEA (nM)	4.72±1.39	4.03±1.26	0.09

Data are presented as mean ±SEM, p-values were calculated using paired Student's t-test (n=10)

Table 2.4: Plasma NAE levels and the effects of centrifugation temperature

Analyte	4° C	20° C	p-value
AEA (nM)	1.1±0.23	0.83±0.12	0.089
OEA (nM)	4.52±0.40	3.47±0.33	0.116
PEA (nM)	4.51±0.99	3.79±1.34	0.265

Data are presented as mean ±SEM, p-values were calculated using paired Student's t-test (n=6).

When whole blood samples were kept on ice prior to centrifugation, there was a non-significant, time-dependent increase in the plasma levels of AEA, OEA and PEA after 30 and 60 min compared to samples processed immediately. AEA, OEA and PEA levels after keeping on ice for 120 min were significantly higher than samples processed immediately (p=0.0002, p=0.018, p=0.041, respectively) (Table 2.5). Consequently, all blood samples in the following studies were processed within 1 h of sample collection.

Concentrations of AEA, OEA and PEA did not change significantly after a single freeze-thaw cycle over 2 weeks storage at -80° C. However, there was a significant variation in the concentrations of AEA, OEA and PEA after 4 weeks storage at -80° C. AEA levels rose significantly (p=0.0059) while OEA and PEA levels declined after 4 weeks storage (p=0.0013 and p=0.0185). It was therefore recommended that storage of plasma samples should not exceed 2 weeks before processing.

Table 2.5: The effect of blood processing time on plasma NAE concentrations

Analyte	0 min	30 min	60 min	120 min	p-value
AEA (nM)	0.47±0.11	0.67±0.08	0.61±0.09	0.86 ±0.07***	***0.0002
OEA (nM)	2.91±0.64	3.55±0.54	3.78±0.49	3.90±0.50*	*0.018
PEA (nM)	3.86±0.68	5.1±0.99	5.31±1.27	6.06±1.53*	*0.041

Data are presented as mean ±SEM, p-values were calculated using the paired one way analysis of variance (ANOVA) test with Bonferroni ad hoc post test (n=7, 5 and 5, respectively for AEA, OEA and PEA).

2.3.2 Concentrations of AEA, OEA and PEA in healthy First Trimester Pregnancies

113 patients attending the LRI for a termination of pregnancy were recruited for this study at their first clinic visit. The demographic characteristics of the women are summarised in Table 2.6. Of these, there were 68 Whites, 31 Asians, 9 Black Africans and 5 of mixed ethnicity as shown in Figure 2.1.

Table 2.6: Characteristics of women recruited prior to termination of pregnancy

	Mean (range)
Number (n)	113
Age (years)	26.9 (15- 43)
BMI (kg/m^2)	24.3 (18.0- 29.6)
Parity	1.1 (0-6)
Gestation at recruitment (days)	54.9 (35-73)
Gestation at procedure (days)	61.1 (39-84)
Interval between clinic and procedure (days)	5.8 (1-16)

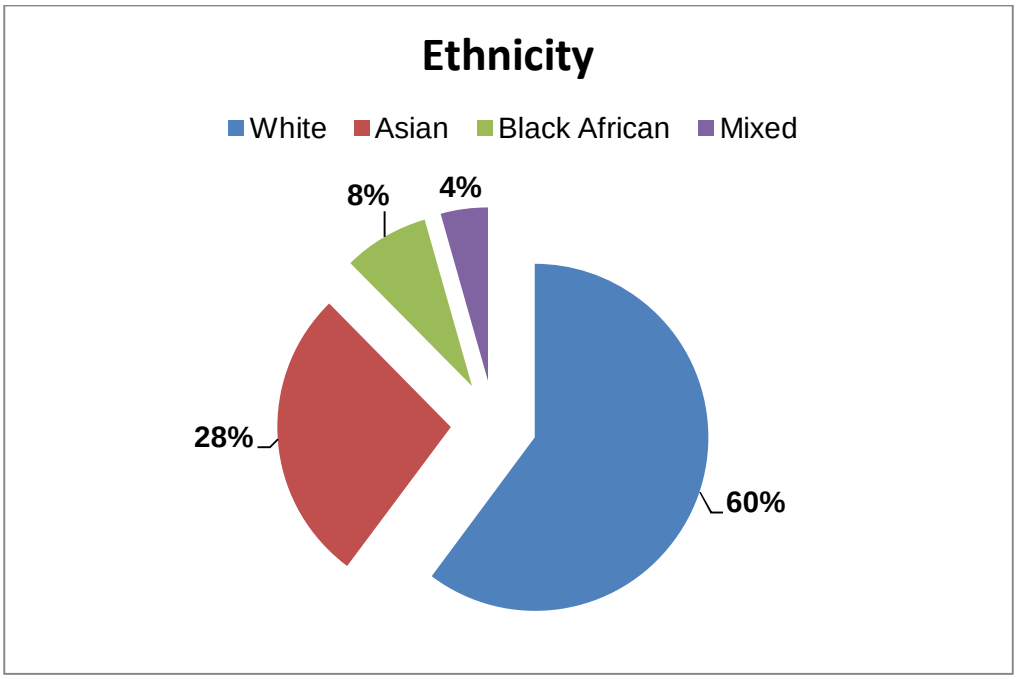

Figure 2.1: The percentage of different ethnicities recruited in clinic.

Additionally eight women with a BMI>30 kg/m^2 were recruited to allow for the examination of the effect of weight on NAE levels. Their mean age was 30 years (range 23-42) with a mean BMI of 33.0 kg/m^2 (range 30-35) and a mean gestation at the first clinic visit of 56.4 days (range 35-81).

The mean plasma concentrations, SEM and 95 % Confidence Interval (95% CI) of AEA, OEA and PEA of women with a BMI<30 kg/m^2 are shown in Table 2.7.

There was no significant correlation between NAE plasma levels and gestational age in women attending clinic for TOP independent of ethnicity (AEA: R^2=0.0018; p=0.65, OEA: R^2= 0.0018; p=0.1572, PEA: R^2= 0.0086; p=0.4412) (Figure 2.2).

Likewise, when all non Caucasian women were excluded from the data, there was no significant increase in AEA, OEA and PEA concentrations with increasing gestational age (AEA: R^2=0.011; p=0.4, OEA: R^2=0.0267; p=0.2 and PEA: R^2=0.011; p=0.5).

There was a significant positive correlation between plasma concentrations of AEA and OEA (R^2=0.4983; p<0.0001) and OEA and PEA (R^2=0.076; p=0.0189) (Figure 2.3).

Table 2.7: Concentrations of AEA, OEA and PEA in human plasma during first trimester pregnancy

Analyte	n	Mean	SEM	95% CI
AEA (nM)	113	0.53	0.02	0.48-0.57
OEA (nM)	111	3.27	0.12	3.02- 3.51
PEA (nM)	74	7.75	0.41	6.94- 8.57

All samples were measured in duplicate.

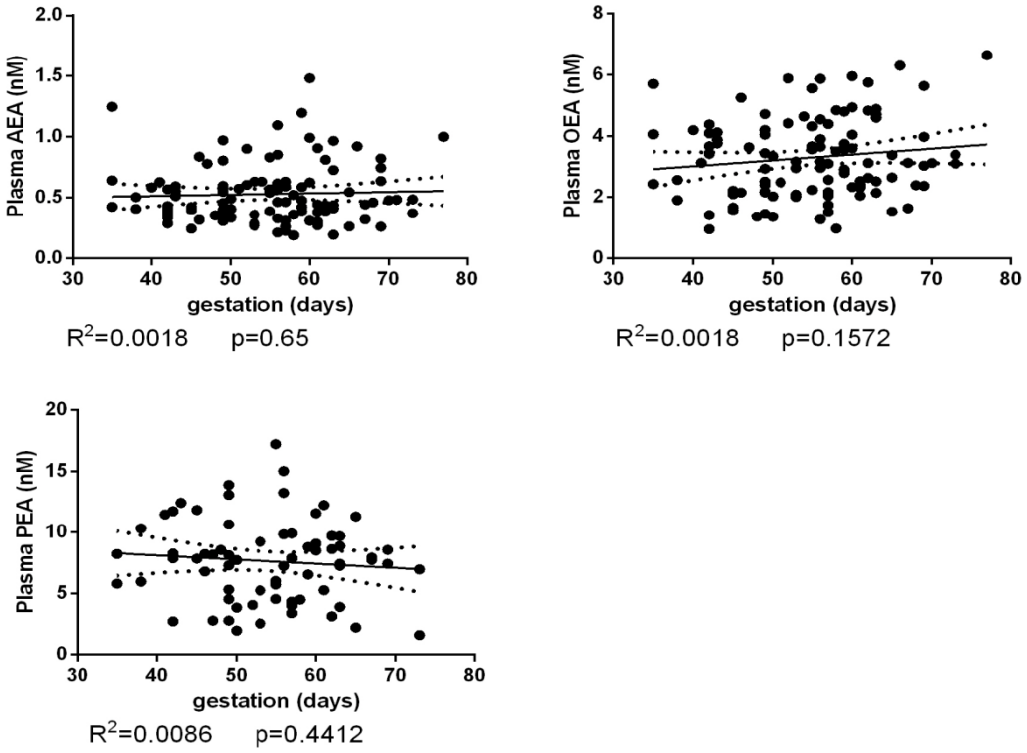

Figure 2.2: Correlation between plasma AEA, OEA and PEA levels and gestation. There was no correlation between NAE levels and gestational age (linear regression analysis).

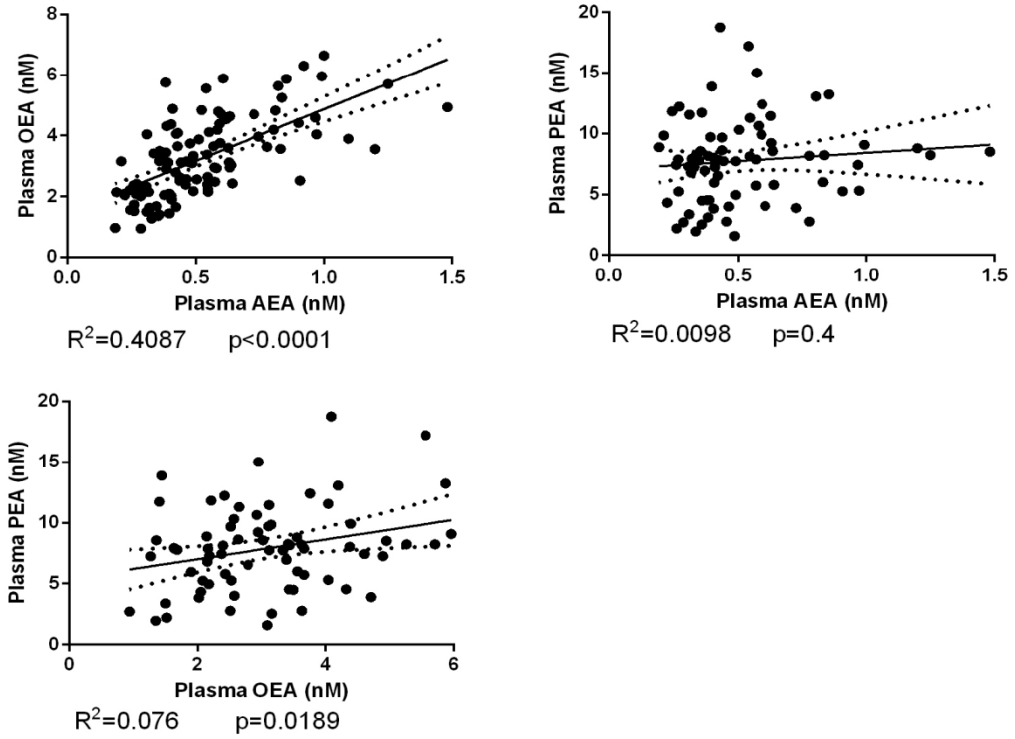

Figure 2.3: Correlation between plasma concentrations of AEA, OEA and PEA. There was a significant positive correlation between plasma concentrations of AEA and OEA as well as OEA and PEA (linear regression analysis).

2.3.3 Ethnicity and Plasma Concentrations of NAEs

Gestational age was not significantly different in the different ethnic groups (p=0.1881) (Table 2.8). Plasma AEA levels were significantly higher in women of Asian and Black African decent compared to White women (p=0.01 and p=0.0003, respectively). Plasma OEA levels were higher in Black African women compared to White women, but this was not statistically significant (p=0.2). Plasma PEA levels did not show any significant differences between the three different ethnic groups (p=0.8) (Figure 2.4).

Table 2.8: Ethnicity and gestational age at recruitment

Ethnicity (n)	Gestation in days (range)
White (68)	55.2 (35-78)
Asian (31)	53.4 (40-69)
Black African (9)	59.1 (42-67)

Data is presented as mean (range).

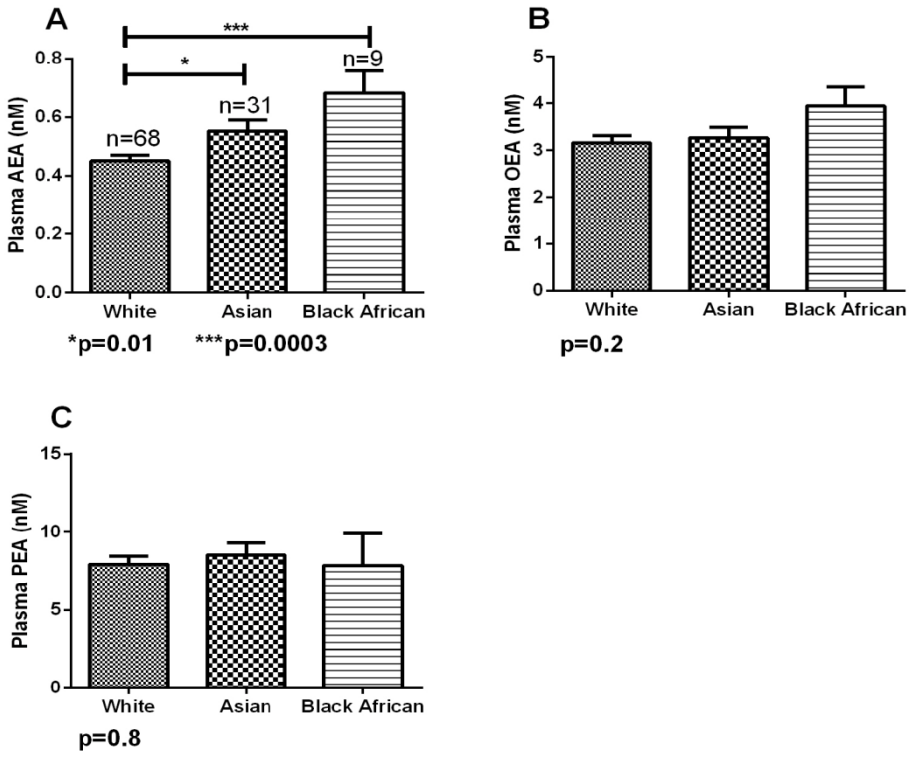

Figure 2.4: Ethnicity and plasma NAE levels.
Plasma AEA levels were significantly lower in white women compared to Asian and Black African women (p=0.01 and p=0.0003). Plasma OEA and PEA were not significantly different in different ethnic groups (One Way ANOVA with Bonferroni post hoc test for AEA, OEA and PEA).

2.3.4 BMI and Plasma Concentrations of NAEs

Plasma AEA levels were statistically higher in women with a BMI>30 kg/m^2 compared with women with a BMI<25 and a BMI of 25-30 kg/m^2 (p=0.007). Furthermore, plasma OEA levels of obese women were higher than in those women with a BMI<25 and a BMI of 25-30 kg/m^2 (p=0.029 and p=0.016). Plasma PEA levels did not significantly change with BMI (Figure 2.5).

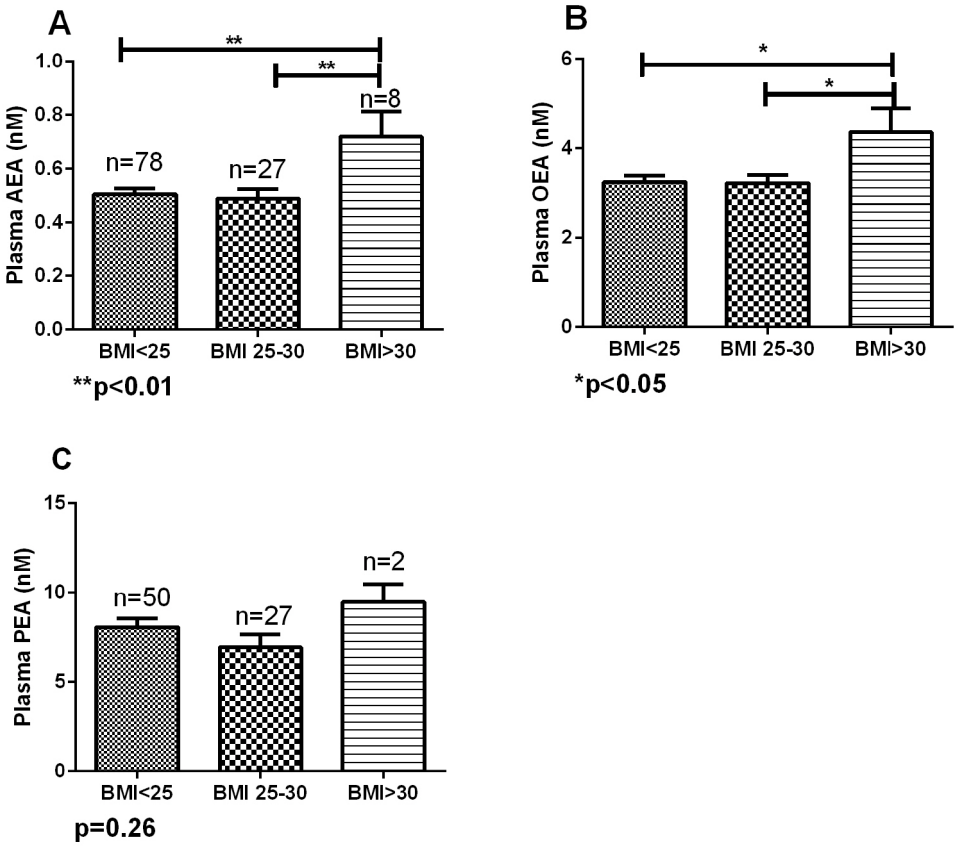

Figure 2.5: BMI and Plasma NAE levels.
Plasma AEA and OEA levels were statistically higher in women with a BMI>30 kg/m^2 compared to women with a BMI<25 and BMI 25-30 kg/m^2. Plasma PEA levels were not significantly different (One way ANOVA with Bonferroni post hoc test).

2.3.5 Plasma NAE Concentrations in Women attending for STOP

Fifteen White women attending the day surgical unit (DSU) for surgical termination provided serial blood samples**.** The first blood sample was taken when they arrived at the DSU and before receiving misoprostol. The second blood sample was taken in the anaesthetic room before the administration of an anaesthetic agent and 1-4 h after

receiving misoprostol and the last in the recovery room following removal of products of conception. The demographic data of this cohort of women are presented in Table 2.9.

There was a significant increase in plasma AEA levels between the first blood sample and that taken in the anaesthetic room ($p=0.0284$). AEA levels fell thereafter, but this was not statistically significant. Likewise OEA levels significantly increased in the anaesthetic room and declined after the procedure. Plasma OEA levels were significantly higher in the recovery room when compared to those on the DSU ($p<0.0001$). Higher plasma PEA levels were also measured in the anaesthetic room, however, this was not statistically significant ($p=0.68$) (Figure 2.6).

In order to evaluate the relevance of these observations to pregnancy, 6 healthy non-pregnant women in the reproductive age group who attended DSU for a diagnostic laparoscopy due to pelvic pain were recruited and serial bloods were collected for AEA, OEA and PEA quantification. These were: on arrival to DSU, in the anaesthetic room and 5-10 min after the procedure in the recovery room. Plasma AEA, OEA and PEA levels increased in the anaesthetic room and decreased in the recovery room, but this was not statistically significant ($p=0.454$, $p=0.052$ and $p=0.06$) (Figure 2.6).

Table 2.9: Characteristics of women attending DSU for serial blood tests

	Mean (range)
Number (n)	15
Age (years)	23 (15-43)
BMI	22.9 (18.1-29.4)
Gestation at recruitment (days)	59 (41-77)
Gestation at procedure (days)	68 (57-85)
Interval between clinic and procedure (days)	7.2 (1-20)

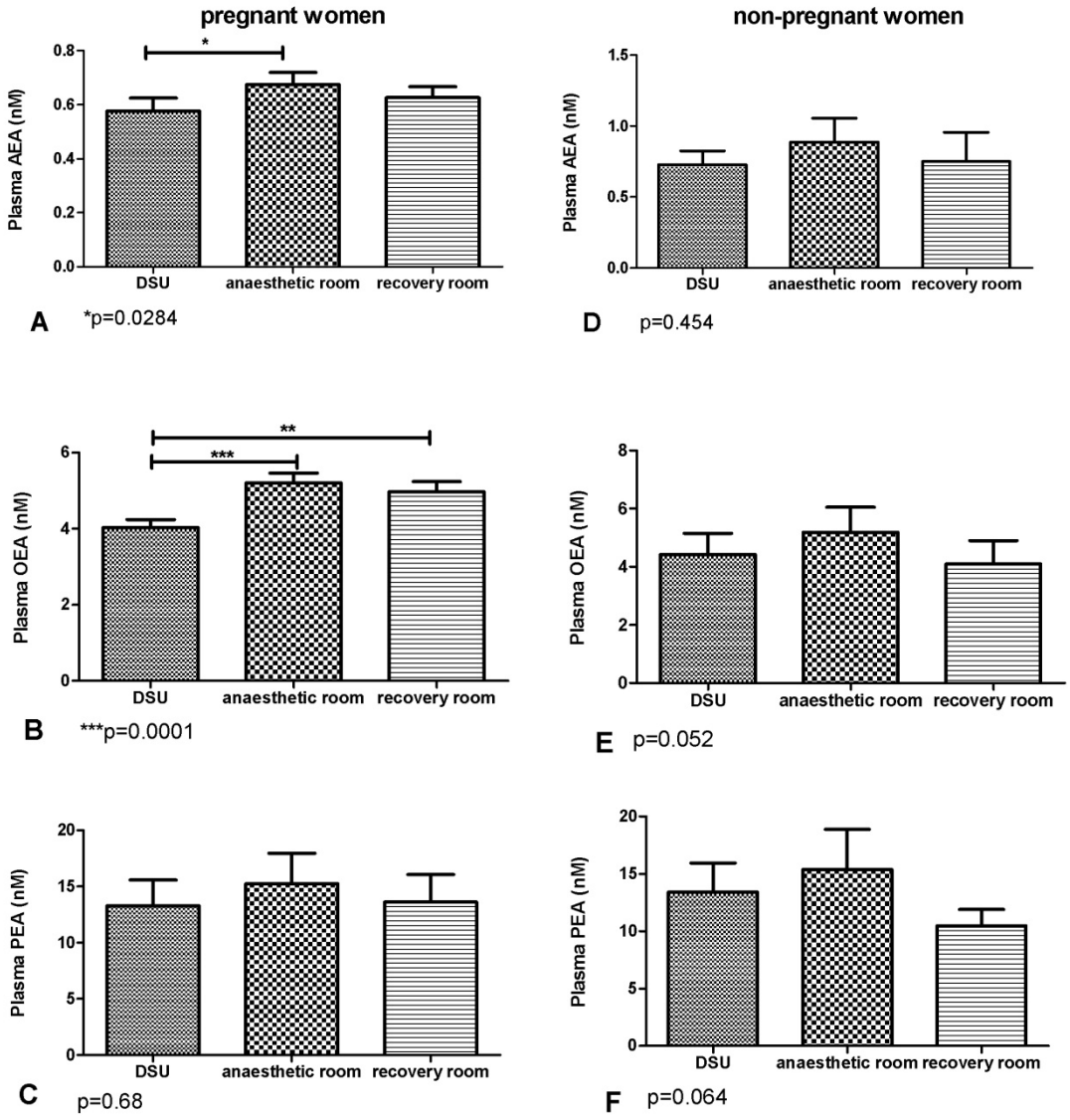

Figure 2.6: Serial plasma concentrations of AEA, OEA and PEA in pregnant (n=15) and non pregnant women (n=6) with White ethnicity.

A significant increase in AEA (A) and OEA (B) levels were observed in pregnant women between arrival at DSU and in the anaesthetic room ($p=0.0037$ and $p<0.0001$). The OEA plasma levels (B) were significantly higher in the recovery room than on DSU ($p<0.0001$). PEA levels (C) showed no statistically significant changes ($p=0.06$). In the non pregnant women AEA (D), OEA (E) and PEA levels (F) did not show any statistical significance ($p=0.454$, $p=0.052$ and $p=0.064$) (n=6) (repeated measures one way ANOVA with Bonferroni post hoc test) and were not statistically different to the pregnant women.

In order to evaluate the relevance of intravaginal misoprostol used for cervical ripening, we recruited 6 White women attending DSU for STOP where misoprostol was omitted and serial blood samples were taken as described above. The mean age was 31 (21-38) years and the mean BMI was 23.6 (19.8-30) kg/m^2. The mean gestational age at procedure was 66 (54-77) days.

In the women not receiving misoprostol, plasma AEA, OEA and PEA levels increased between arrival at DSU and in the anaesthetic room, however this was only statistically significant for OEA levels (p=0.0014). The AEA levels dropped significantly in the recovery room after surgery (p=0.02). The PEA levels did not show any significant changes (Figure 2.7).

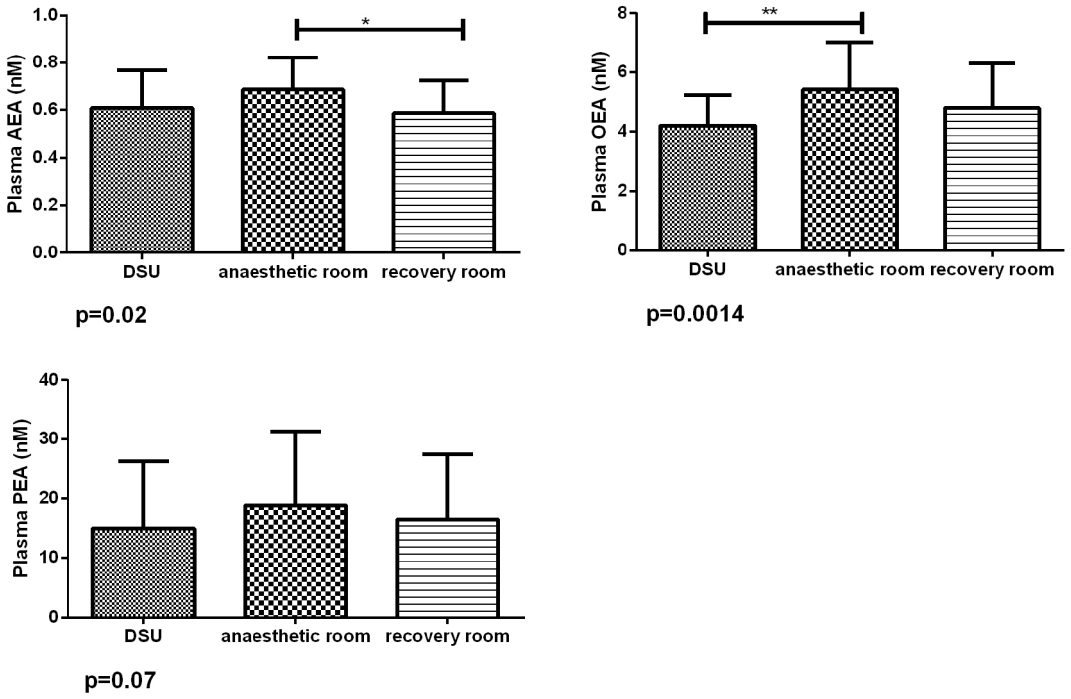

Figure 2.7: Serial NAE plasma concentrations.
Plasma AEA, OEA and PEA levels in 6 pregnant women without the use of intravaginal misoprostol prior to surgery. Plasma AEA levels in the anaesthetic room were significantly higher than in the recovery room (p=0.02), whereas OEA levels taken on DSU were significantly higher than in the anaesthetic room (p=0.0014) (repeated measures One way ANOVA with Bonferroni post hoc test).

With regards to ethnicity, plasma AEA and PEA concentrations were consistently higher in Asians (n=5) compared to White women (n=15) but these were not statistically significant ($p>0.05$) (Figure 2.8). No Black African women were recruited into this study.

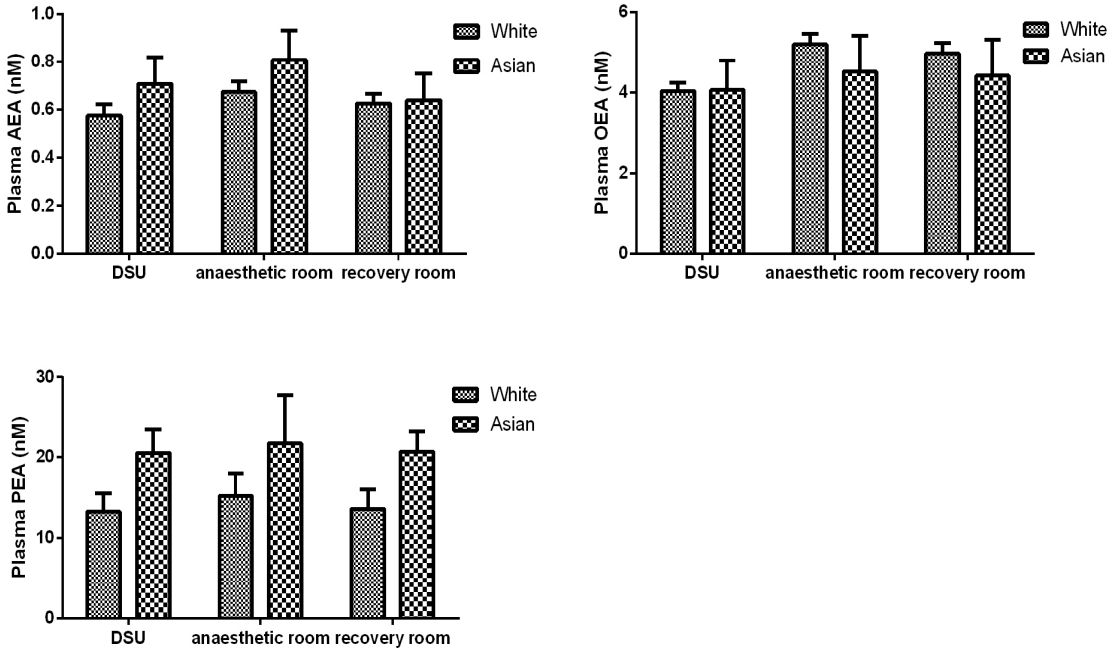

Figure 2.8: Plasma AEA levels in White and Asian women attending for STOP.
Plasma AEA and PEA levels were higher in Asian compared to White women, but not reaching statistically significance ($p>0.05$) (Two way ANOVA with Bonferroni post hoc test).

Women with a BMI greater than 30 kg/m^2 (n=4) had significantly higher AEA concentrations on DSU compared to women with a BMI lower than 30 kg/m^2 (n=22) ($p<0.05$). The difference was even more significant when AEA levels were taken in the anaesthetic room and in the recovery room ($p<0.01$). OEA levels did not show any significant difference and PEA levels are not determined in a large enough sample set for analysis (Figure 2.9).

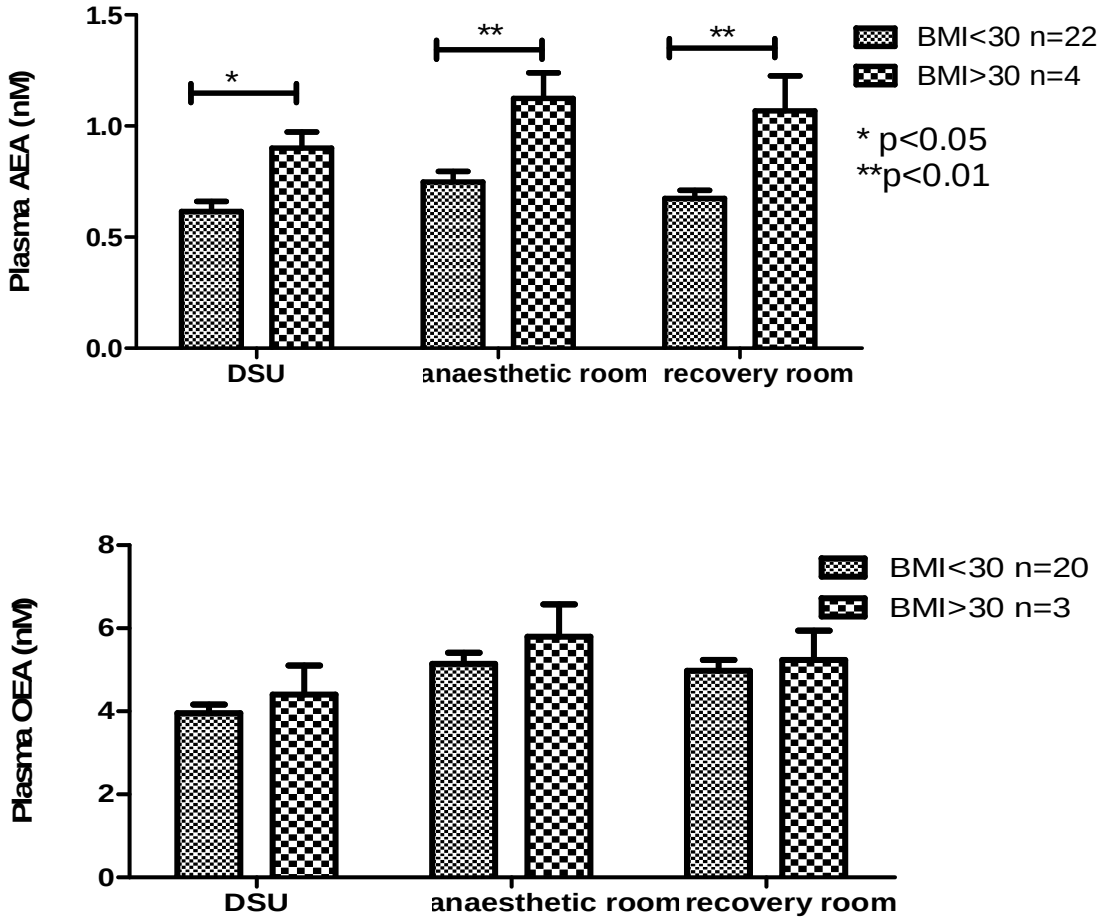

Figure 2.9: Serial blood tests in women with STOP with a BMI<30 and BMI>30 kg/m^2.
AEA levels in women with a BMI>30 kg/m^2 were significantly higher than in women with a BMI<30 kg/m^2 when taken in DSU ($p<0.05$), anaesthetic room ($p<0.01$) and recovery room ($p<0.01$). OEA levels were not significantly different in women with BMI>30 kg/m^2 compared to women with a BMI<30 kg/m^2. The time difference was significant for AEA ($p=0.0003$) and OEA ($p=0.0004$) (Two-way ANOVA with Bonferroni post hoc test).

2.3.6 Plasma NAE Concentrations of Women attending for MTOP

Sixty eight women undergoing MTOP were recruited. Their demographic characteristics are shown in Table 2.10. Plasma AEA, OEA and PEA levels increased one day after the administration of RU486, but this was only significant for AEA and PEA values ($p=0.005$ and $p=0.025$) (Figure 2.10).

Table 2.10: Demographics of women attending for MTOP

Ethnicity	White	Asian	Black African
Number (n)	48	15	5
Age (years)	26 (16-41)	26 (18-40)	28 (22-39)
BMI (kg/m^2)	23.2 (18-30)	22.8 (18-28)	26.1 (23-28)
Gestation at recruitment (days)	49 (35-63)	47 (31-57)	51 (42-61)
Gestation at procedure (days)	54 (44-64)	52 (44-59)	56 (49-62)

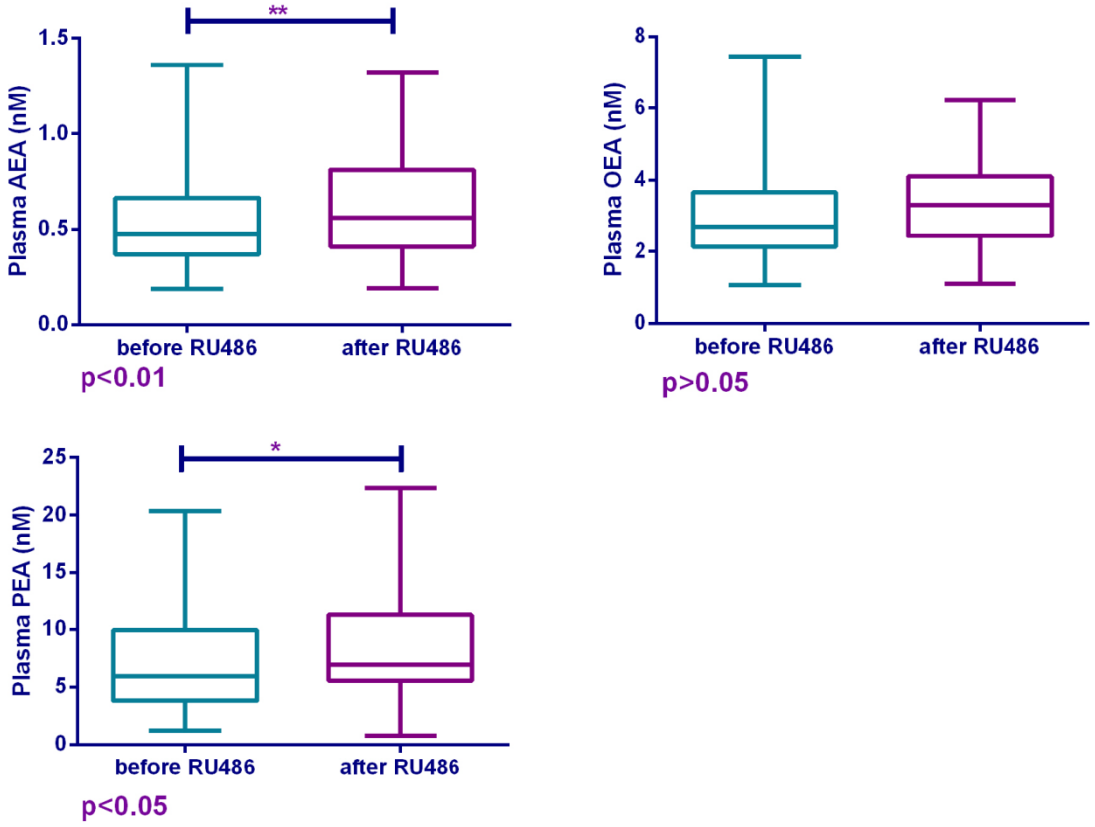

Figure 2.10: Plasma NAE concentrations in all women with BMI≤30 kg/m^2 (n=68). Plasma AEA and PEA levels increased significantly 1 day after RU486 (p=0.005 and p=0.025 respectively), (Wilcoxon matched-pairs signed rank test).

2.3.6.1 Ethnicity and Plasma Concentrations of AEA, OEA and PEA in Women undergoing MTOP

Figure 2.11 shows differences in the NAE levels in women attending for MTOP based on their ethnicity. Black African women had significantly increased plasma AEA and OEA levels than White or Asian women ($p<0.01$ and $p<0.05$). Plasma AEA levels in White women increased significantly after RU486 administration ($p=0.038$); in Asian women plasma AEA and PEA levels increased significantly ($p=0.015$ and $p=0.014$) whereas in Black African women there were no significant changes.

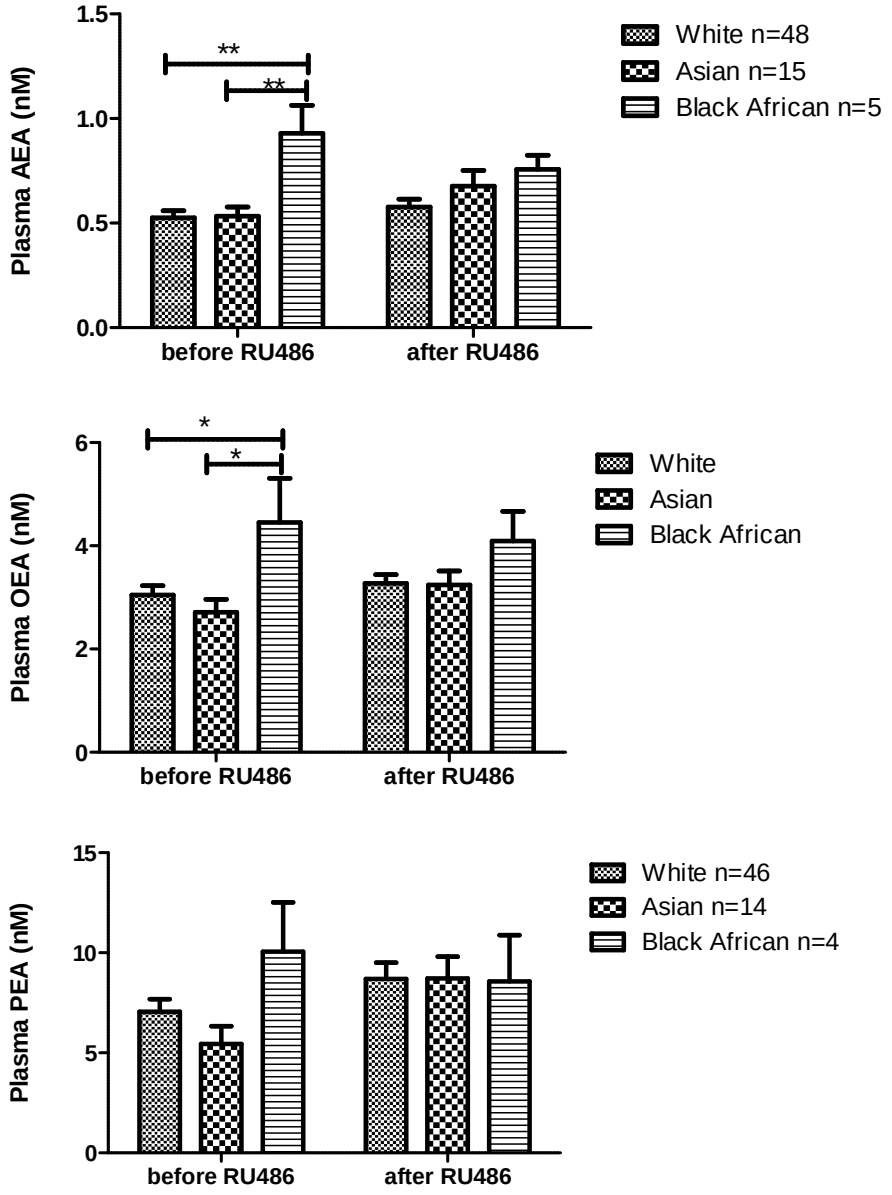

Figure 2.11: Plasma NAE levels before and one day after RU486 in women of different ethnicities.
(Two-way ANOVA test with Bonferroni post hoc test) (*$p<0.05$, **$p<0.01$)

2.3.6.2 Serial Plasma NAE Concentrations after RU486

Twenty three women agreed to have an additional blood test one hour after the administration of RU486. Their demographic data are shown in Table 2.11. Plasma AEA levels dropped slightly one hour after RU486 but increased significantly one day later (p=0.0006) whereas OEA levels did not change significantly and PEA levels increased after both one hour and one day (p=0.044) (Figure 2.12). However, when only women of White and Asian ethnicity with a BMI<30 kg/m^2 were included (n=18) AEA, OEA and PEA levels increased significantly after RU486 (p=0.0005, p=0.02 and p=0.0073) (Figure 2.13).

Table 2.11: Demographics of women with serial blood tests after RU486

	Mean (range)
Number (n)	23
Ethnicity	White n=16 Asian n=6 Black African n=1
Age (years)	29 (19-41)
BMI (kg/m^2)	24.9 (19.2-33.9)
Gestation at procedure (days)	53 (44-63)

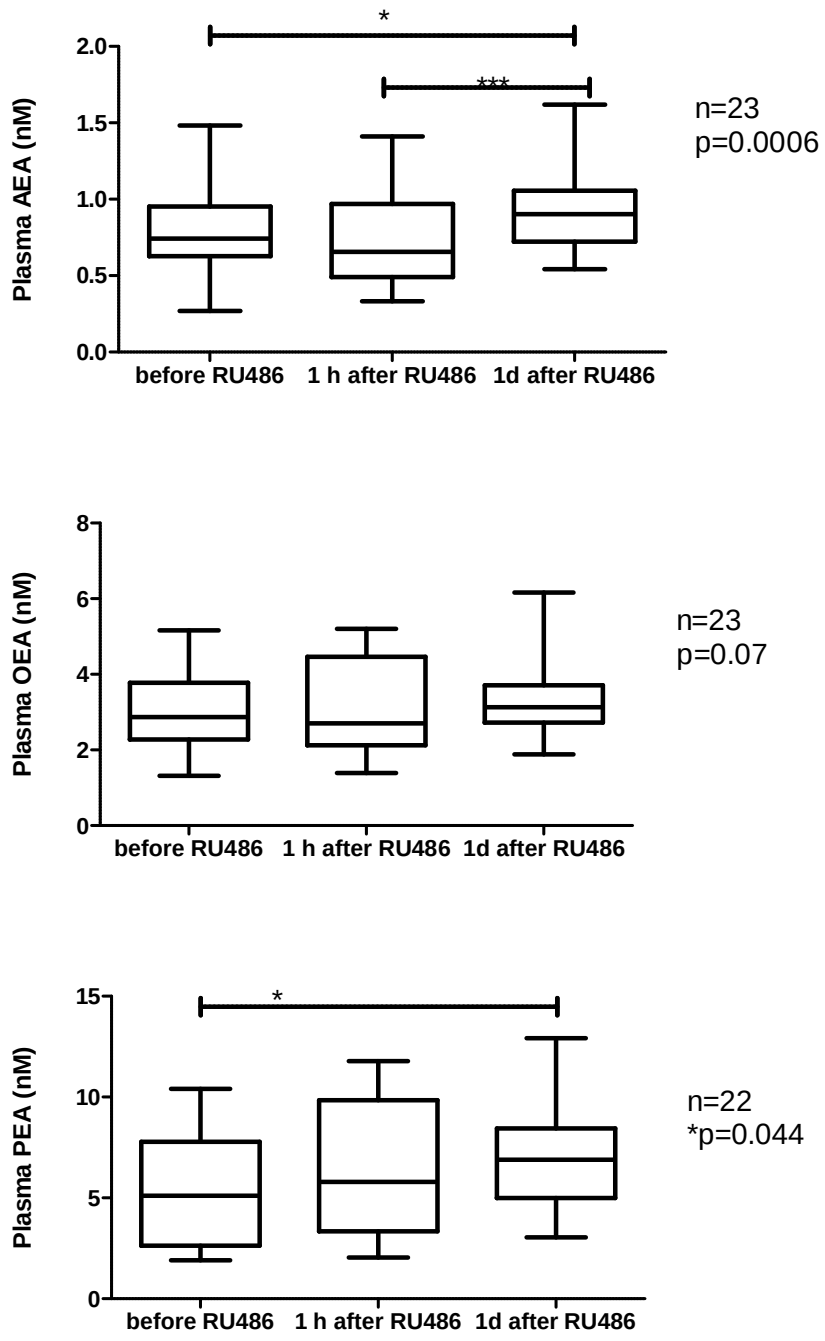

Figure 2.12: Serial plasma NAE concentrations after RU486.
Plasma AEA levels before and 1 hour after the administration of RU486 were significantly lower than 1 day after RU486 (p=0.0006). Plasma PEA levels were also lower before the administration of RU486 than 1 day later (p=0.044) (paired one way ANOVA analysis with Bonferroni post hoc test).

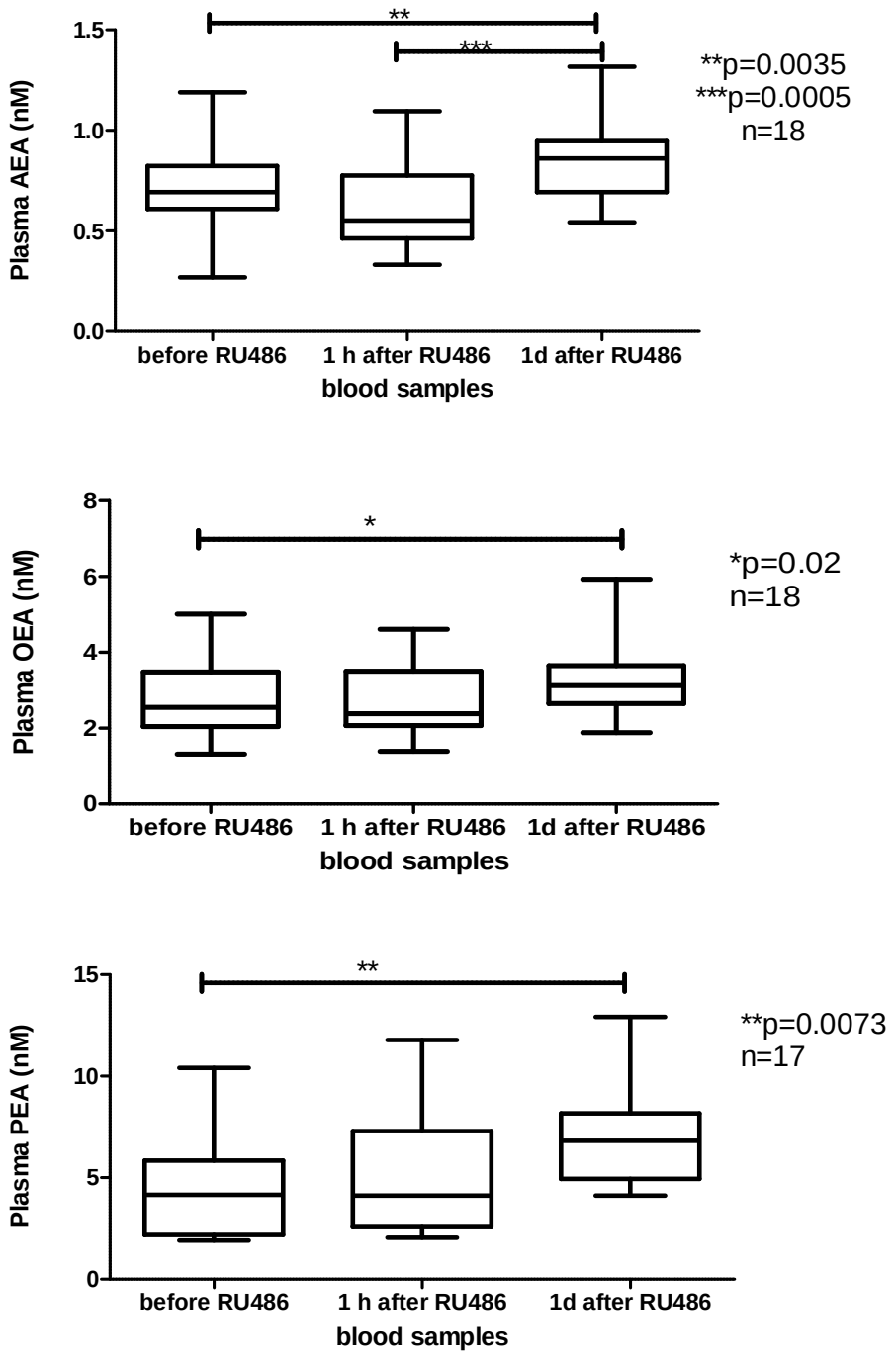

Figure 2.13: Serial plasma NAE concentrations after RU486 of White and Asian women with a BMI<30 kg/m^2.

Plasma AEA, OEA and PEA levels were significantly lower before than 1 day after the administration of RU486 (p=0.0035, p=0.02 and p=0.0073, respectively). Plasma AEA levels 1 hour after the administration of RU486 were also significantly lower than 1 day later (p=0.0005) (paired one way ANOVA analysis with Bonferroni post hoc test).

There were seven White women with a BMI<30 kg/m^2 who had an additional blood sample after expulsion of the products of conception. Plasma AEA, OEA and PEA levels did not change significantly in this cohort of women (p=0.57, p=0.47 and p=0.2) (Figure 2.14).

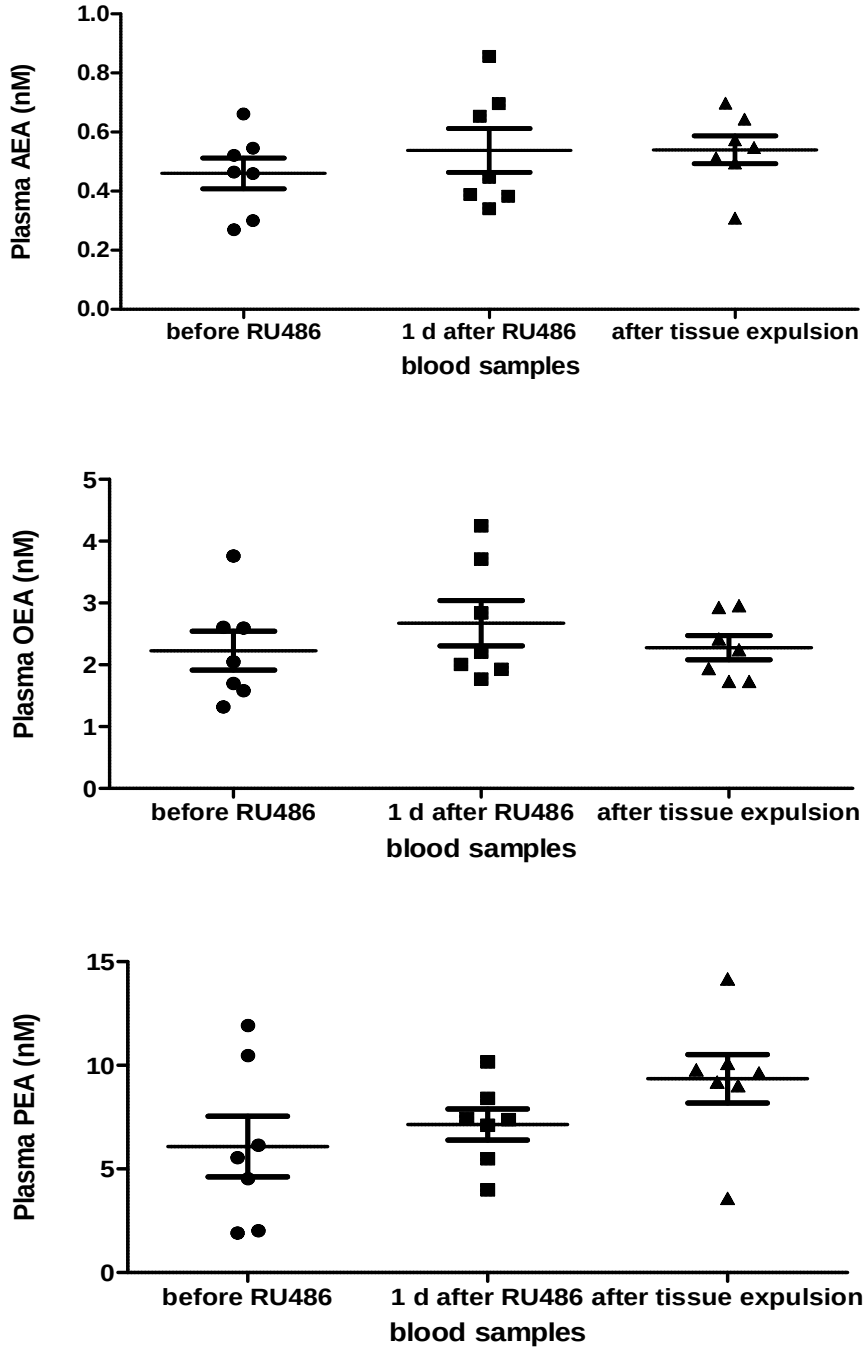

Figure 2.14: Plasma NAE concentrations after RU486 (n=7).
There were no significant differences in plasma AEA, OEA and PEA levels before and 1 day after RU486 and after tissue expulsion (paired one way ANOVA analysis).

2.3.6.3 The Effect of Gestational Age on RU486-induced NAE Concentrations in Women undergoing MTOP

Sixty eight women provided blood samples before and 1 day after the administration of RU486. 12 women were 6 weeks gestation, 28 at 7 weeks and 28 at 8 weeks pregnant at the time of procedure. Plasma AEA, OEA and PEA levels varied with gestation, but this was not statistically significant (Figure 2.15).

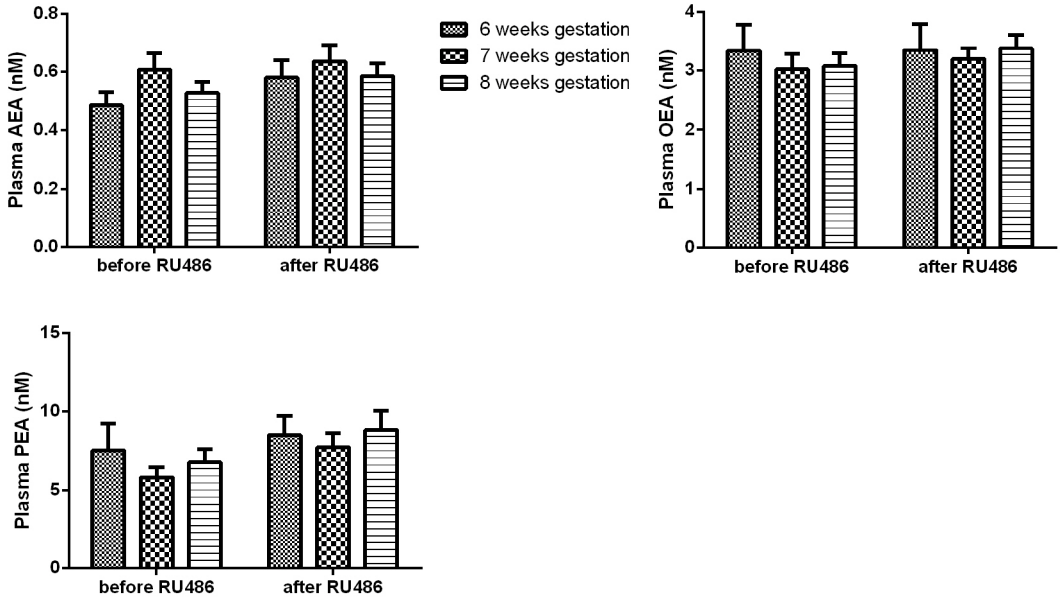

Figure 2.15: Plasma NAE levels and gestational age of women undergoing MTOP. There was no significant difference in Plasma AEA, OEA and PEA levels before and after the administration of RU486 in women with different gestational ages (Two-way ANOVA with Bonferroni post hoc test).

2.3.7 NAE Concentrations in Women attending for STOP and MTOP

For this arm of the study 36 women who attended for MTOP and 36 who had STOP were recruited. They were all White women with a BMI<30 kg/m^2 who were matched for age, BMI and gestational age. Their demographic characteristics are shown in Table 2.12. Plasma NAE levels from the clinic and when they attended the ward were compared. Plasma AEA, OEA and PEA levels did not change significantly between the two groups (Figure 2.16).

Table 2.12: Demographics of matched women undergoing MTOP and STOP

	MTOP (n=36)	STOP (n=36)
Age (years)	26 (16-43)	27 (16-42)
BMI (kg/m^2)	23.1 (19.8-29.6)	23.5 (18.1-29.4)
Gestation in clinic (days)	52 (42-62)	55 (38-65)
Gestation on ward (days)	57 (50-63)	61 (53-67)

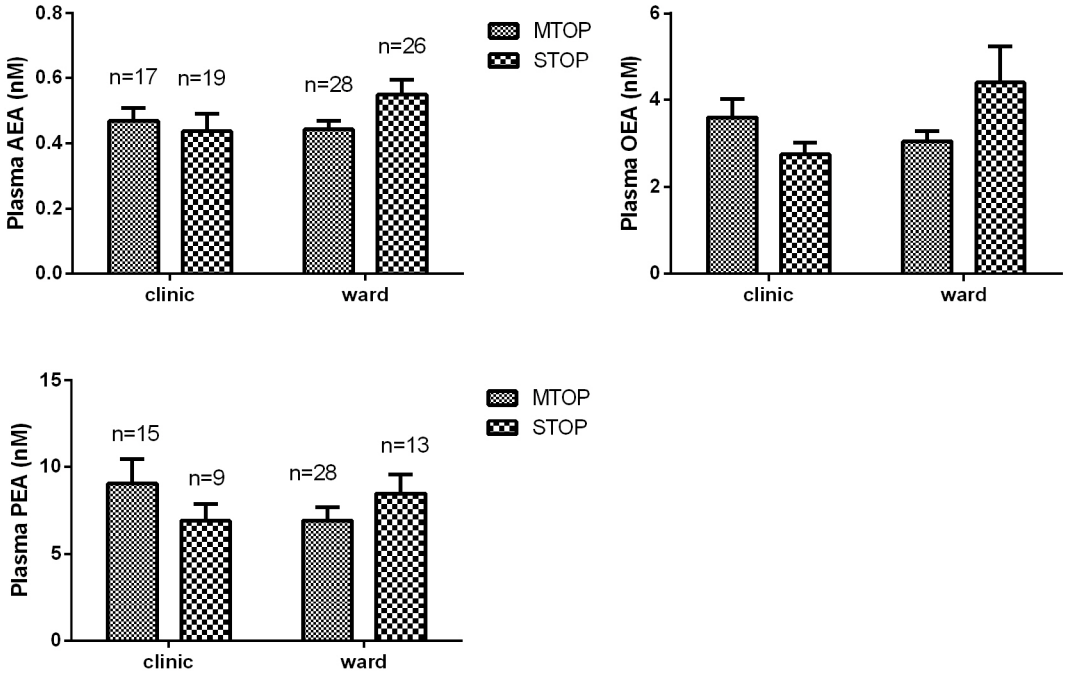

Figure 2.16: Plasma NAE levels of women attending for MTOP or STOP.
There was no significant difference in the plasma NAE levels ($p>0.05$) (Two-way ANOVA test with Bonferroni post hoc test).

2.3.8 Plasma Leptin Levels in Women undergoing MTOP

Typical results for a standard curve for human leptin are shown in Table 2.13 and Figure 2.17.

Table 2.13: Standard curve for human leptin

Standard Human Leptin (pg/ml)	Optical Density (450nM)
1000	2.37
500	1.3
250	0.7
125	0.36
62.5	0.22
31.2	0.17
15.6	0.14
0	0.1

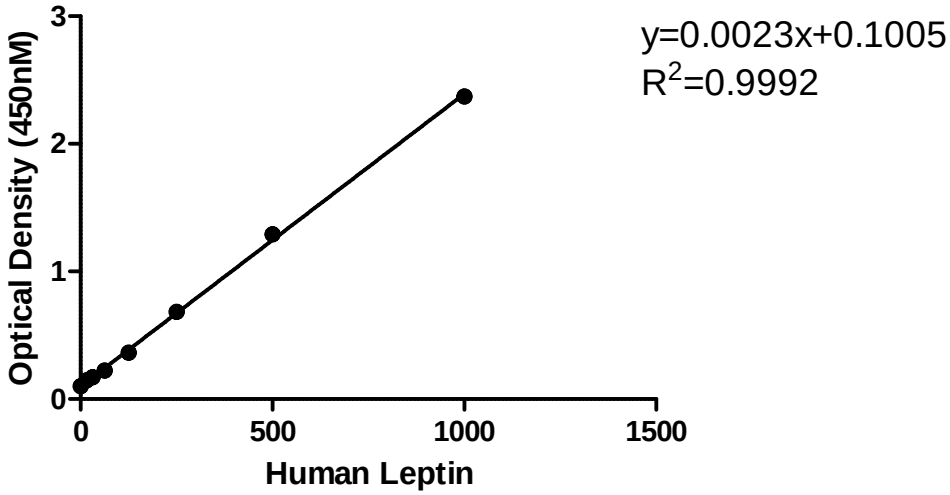

Figure 2.17: Standard curve of human leptin

Forty-nine women had additional blood samples taken for the analysis of leptin levels. The demographic data of these patients are shown in Table 2.14. There was no significant difference in serum leptin levels between White and Asian women (p=0.139) (Figure 2.18). No significant correlation between serum leptin levels and gestation (p=0.66) and or BMI (Figure 2.19) could be established. Overweight and obese White women had significantly higher leptin levels compared to White women with a normal BMI throughout the first trimester (p<0.0001) (Figure 2.18).

Table 2.14: Demographic data of women with leptin results

	Mean (range)	
Ethnic groups	**White n=37**	**Asian n=12**
Age (years)	26 (16-42)	26 (18-33)
BMI (kg/m^2)	24.5 (19.5-35)	22.2 (18-28)
Gestation on ward (days)	54.4 (44-63)	52.2 (44-58)

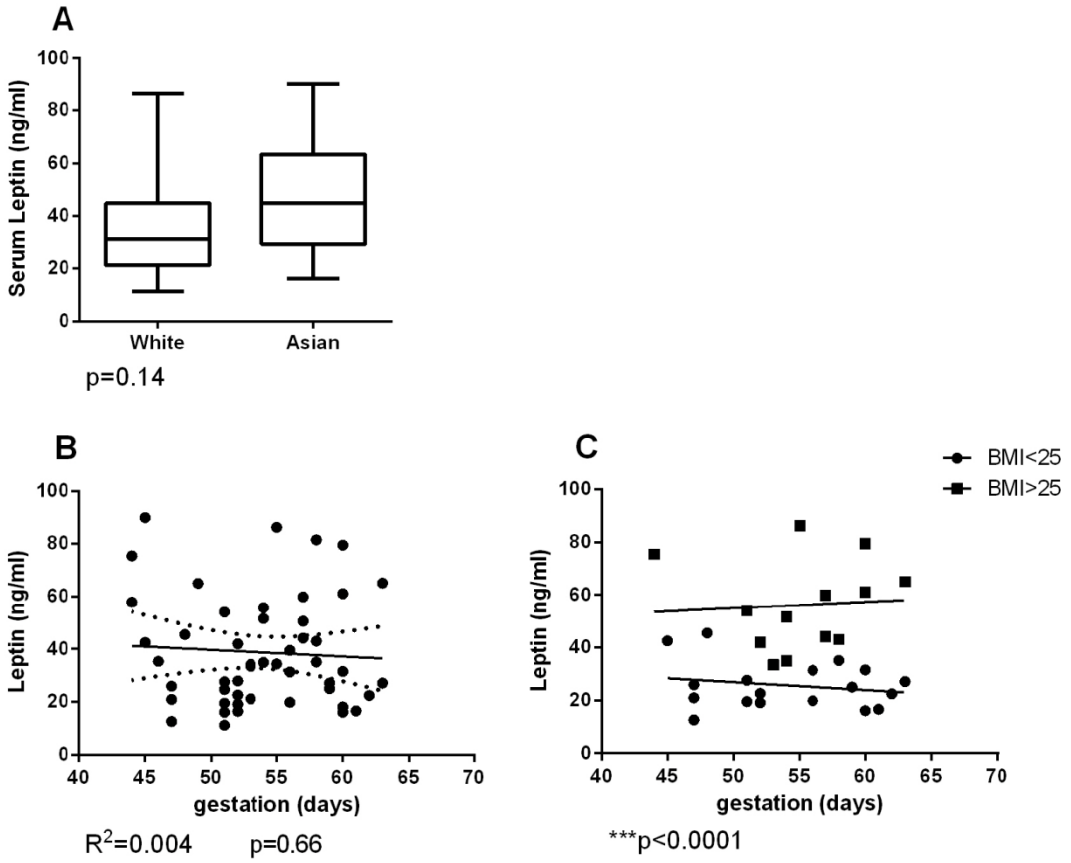

Figure 2.18: Serum leptin levels and ethnicity and gestation.
(A) No significant difference in serum leptin levels was seen in White and Asian pregnant women (Mann-Whitney U-test).
Correlation of leptin levels and gestation: (B) All patients with leptin results (n=49), (C) patients divided according to their BMI with BMI<25 kg/m^2 and BMI>25 kg/m^2. No significant correlation was found between serum leptin levels and gestation (BMI<25 kg/m^2: p=0.42, R^2=0.039; BMI>25 kg/m^2: p=0.82 R^2=0.004). However women with a BMI>25 kg/m^2 had significantly higher serum leptin levels when compared to women with a BMI<25 kg/m^2 (p<0.0001) (linear regression analysis).

There was a significant positive correlation between serum leptin levels and BMI (p<0.0001) as well as plasma AEA levels and BMI (p=0.012) (Figure 2.19). However no significant correlation between serum leptin and plasma AEA, OEA or PEA levels could be identified (Figure 2.20). Women with a BMI<30 kg/m^2 were included in the analysis of leptin levels before and 1 day after RU486 administration. No significant differences were seen in leptin levels before and after RU486 either in the full cohort or when data for White or Asian women were analysed separately (Figure 2.21).

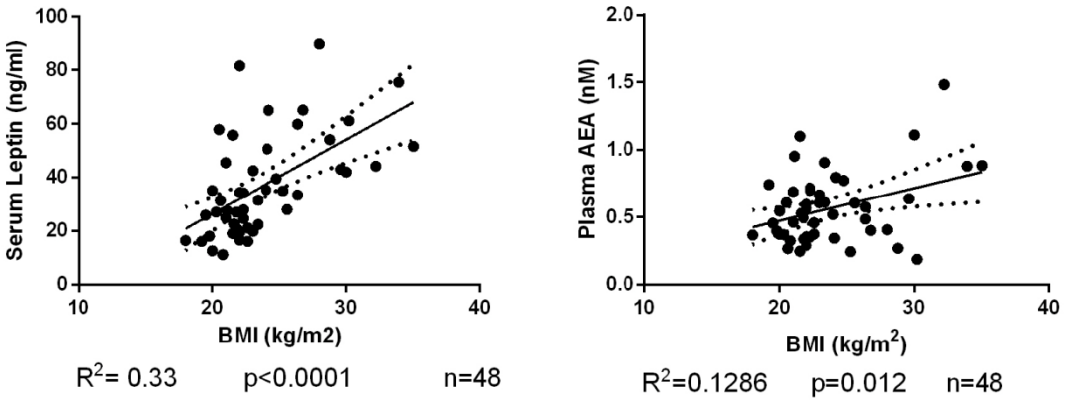

Figure 2.19: Correlation of serum leptin and BMI and plasma AEA and BMI.
There is a positive significant correlation of serum leptin levels and plasma AEA levels with BMI ($p<0.0001$ and $p=0.012$, respectively) (linear regression analysis).

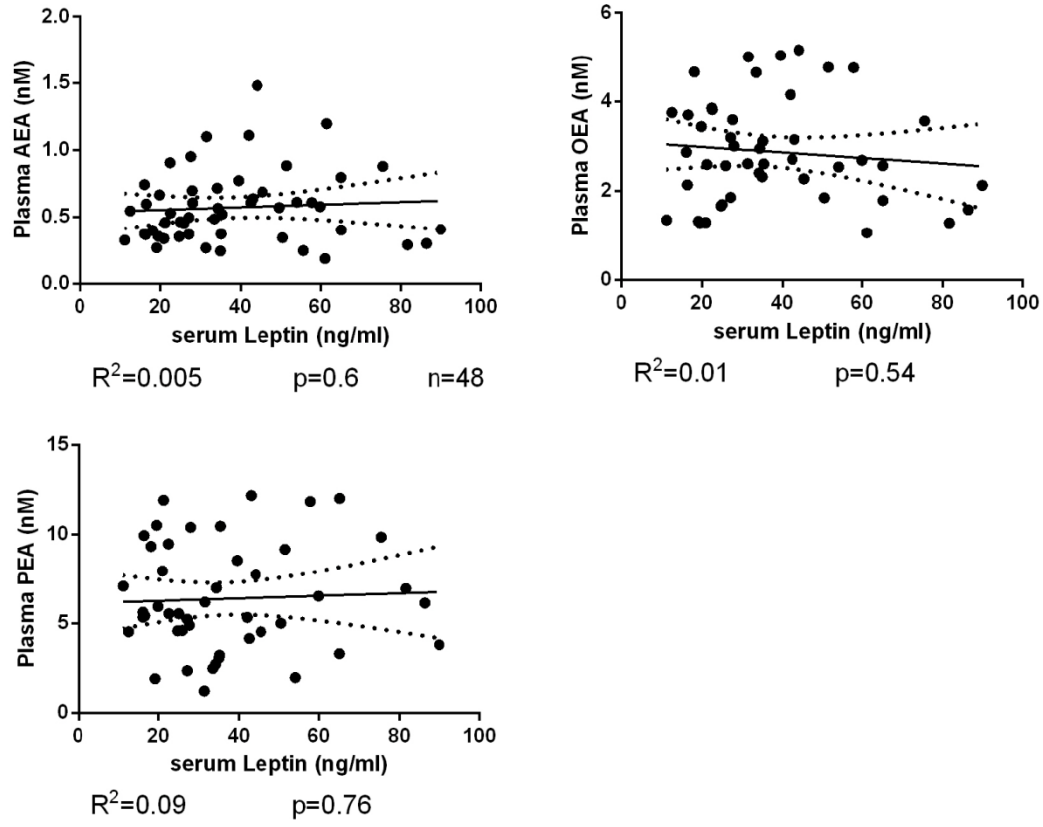

Figure 2.20: Correlation of serum leptin and plasma AEA, OEA and PEA levels.
Plasma AEA, OEA and PEA levels did not show a significant correlation with serum leptin levels (n=48) (linear regression analysis).

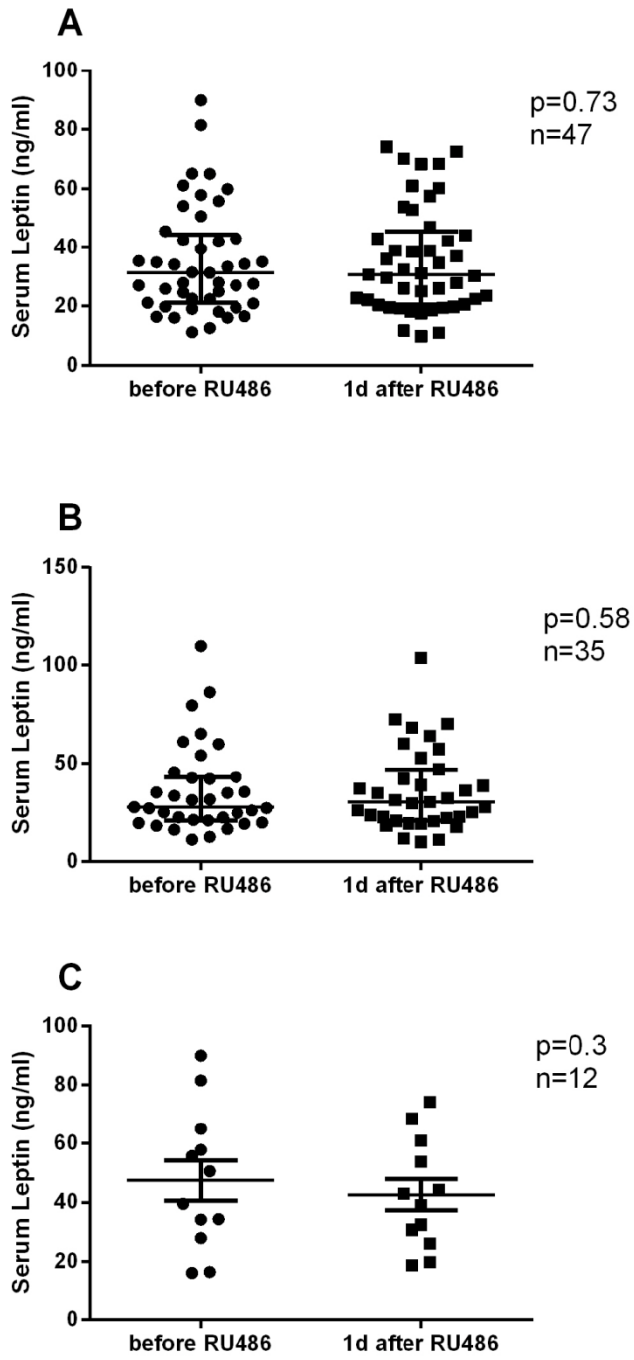

Figure 2.21: Serum leptin levels before and after RU486 in (A) all women (B) White women only and (C) Asian women only.
All women had a BMI <30 kg/m^2. No significant difference in leptin levels before and 1 day after RU486 were identified. (A), (B) Wilcoxon matched pairs signed rank test; (C) paired t-test.

2.4 DISCUSSION

The ECS plays a critical role in implantation and successful pregnancy for example, elevated plasma AEA levels (Habayeb et al. 2008) and reduced FAAH activity in lymphocytes (Maccarrone et al. 2000b) are associated with subsequent spontaneous miscarriage. Plasma AEA levels have been shown to fluctuate throughout pregnancy with lower levels in the first and second trimester, followed by an increase in the third trimester and a 2-4 fold increase with the onset of labour (Habayeb et al. 2004, Lam et al. 2008). In this study, there was no significant correlation between maternal plasma NAE levels and gestational age between 5 and 11 weeks, in contrast to published data where the plasma AEA levels decreased in the first trimester (Habayeb et al. 2004, El-Talatini, Taylor & Konje 2009) and FAAH activity in peripheral lymphocytes are increased in normal healthy pregnancies (Maccarrone et al. 2002). The latter observation suggests AEA levels should decline until 10 weeks and thereafter increase.

The women in this study came from different ethnic backgrounds. When ethnicity was examined as a variable it was found that Black African and Asian women had significantly higher AEA levels compared to White women (Figure 2.4). OEA and PEA levels were also higher in Black African women compared to White women but this was not statistically different. It would seem that the ethnicity should be taken into consideration when interpreting NAE in pregnancy. Even when women of different ethnicity were examined independently a reanalysis of the gestational age data did not show a significant change in NAE levels with increasing gestational age. Interestingly, the Black ethnic group suffer more from spontaneous miscarriages in assisted reproductive technology (ART) cycles than White women with the Black ethnic group being an independent risk factor for failure to achieve a live birth (Feinberg et al. 2006, Seifer, Frazier & Grainger 2008). Similarly, Asian women also have a lower clinical pregnancy rate and decreased live birth rate after ART when compared to Caucasians. Asian ethnicity is regarded as an independent predictor of poor outcome in ART cycles (Purcell et al. 2007). These poor outcomes in Black and Asian women may be associated with their high AEA levels as previous research has demonstrated an association between increased AEA levels and miscarriage (Habayeb et al. 2008). In our study Black African and Asian women had significantly higher AEA levels in normal pregnancies compared to White women. It would therefore be logical to suggest

that ethnicity should be taken into consideration when interpreting NAE in pregnancy. There needs to be caution exercised here as the numbers are small. Although our cohort of patients was from a different geographical location, these ethnicity associated observations warrant further investigation especially around implantation and very early pregnancy.

Another factor influencing plasma NAE levels is the BMI. Obese women with a BMI greater than 30 kg/m^2 had statistically significant higher plasma AEA and OEA levels compared to women with a BMI less than 30 kg/m^2, whereas PEA levels did not significantly change with BMI (Figure 2.5).

Similarly, obese women attending for STOP had higher AEA levels on the ward, in the anaesthetic room and in the recovery room compared to non-obese women. Of note is an increased risk of miscarriage in obese women (Fedorcsak et al. 2004, Boots, Stephenson 2011) and this may also be associated with increased AEA levels. NAEs are known to regulate food intake and energy metabolism (Lambert, Muccioli 2007, Cavuoto, Wittert 2009) (Matias, Di Marzo 2007) with AEA and OEA involved in the regulation of appetite (Hansen, Diep 2009). Obesity elevates EC production by adipocytes (Matias et al. 2007). Our findings are therefore consistent with observations of higher AEA, 2-AG and OEA levels in obese women compared with normal weight women (Engeli et al. 2005, Joosten et al. 2010). Therefore in these studies we considered women with a BMI>30 kg/m^2 separately.

Serial blood sampling in women attending the DSU for STOP has provided insights into how NAE concentrations alter before and after termination of a healthy pregnancy. All women attending for STOP received prostaglandin (PG) for cervical ripening shortly after their arrival on the ward. This was associated with a significant increase in plasma AEA levels. The increase in AEA levels may be a consequence of the prostaglandin causing uterine contractions, since labour is associated with increase in prostaglandin. These findings are in agreement with previous observations that plasma AEA levels increased in spontaneously labouring women compared with non labouring controls at term and also following induction of labour after 37 weeks gestation (Habayeb et al. 2004, Nallendran et al. 2010). Similarly, women at risk of preterm birth presenting with Braxton Hicks myometrial contractions also showed higher AEA levels if they delivered preterm compared with women who continued with the pregnancy

until term (Nallendran et al. 2009). Ex vivo myometrium from pregnant women relaxed on exposure to AEA (Dennedy et al. 2004), an effect which has been confirmed in the ERG laboratory using primary human myometrial cells and a human myometrial cell line (Brighton et al. 2009). The importance of prostaglandin for the elevation of AEA was intimated by the lack of a significant rise in AEA concentration in pregnant women attending for STOP who did not receive prostaglandin prior to surgery. Similar observations were observed in non-pregnant women undergoing surgery. However, the number of patients in these studies was small.

OEA levels increased more than AEA levels in women attending for STOP. This may also be related to the prostaglandin administration, but OEA levels also increased significantly in the women undergoing STOP without the administration of prostaglandin. No increase was seen in non-pregnant women undergoing minor gynaecological procedures therefore suggesting the rise in OEA may be related to the loss of products of conception but independent of prostaglandin. Further studies are necessary to understand the relationship between increases in OEA and pregnancy loss.

One explanation for these changes is that women attending hospital, especially for surgery, be stressed and endocannabinoid signalling is regulated by acute stress (Hill, McEwen 2010, Finn 2010). This could potentially affect the levels measured here. Hohmann et al. demonstrated an immediate and transient increase in 2-AG and AEA in the periaquaductal grey of rats after a brief exposure to a food shock stress (Hohmann et al. 2005). Rats also showed decreased AEA and increased 2-AG levels in most limbic brain structures following stress (Hill et al. 2007). Human studies have not shown a significant effect of stress on serum AEA, but 2-AG levels significantly increased following administration of TSST (Trier Social Stress Test) while PEA and OEA significantly declined during the stress recovery phase (Hill et al. 2009). These latter studies suggest that it is less likely that the increased AEA levels in the anaesthetic room may be caused by acute stress.

Non pregnant women attending for minor gynaecological surgery had no significant changes in AEA, OEA and PEA concentrations from the time of admission on to the ward to just before induction in the anaesthetic and in the recovery rooms. The anaesthetic propofol was used for induction and maintenance of general anaesthesia in all the women. Propofol is highly protein-bound and mainly metabolised in the liver

(Favetta et al. 2002). It works through the potentiation of gamma aminobutyric acid (GABA) A receptor activity (Krasowski et al. 2001) and by blocking sodium channels (Haeseler et al. 2008). It is commonly used for sedation as well as general anaesthesia due to its characteristic rapid onset and recovery. Recent findings suggest that the endocannabinoid system may contribute to the anaesthetic action of propofol (Fowler 2004). Patel et al. observed that propofol inhibits FAAH in mice after intraperitoneal administration and thereby increases AEA levels (Patel et al. 2003). Increased AEA blood levels during orthopaedic surgery under total intravenous anaesthesia with propofol were noticeable at 10, 20 and 40 min after induction (Schelling et al. 2006). However, a more recent study on endocannabinoids and anaesthesia with propofol on 14 women and 14 men showed decreased AEA plasma levels and could not confirm previous findings (Jarzimski et al. 2012). In this study, no increase in AEA plasma levels were seen following the onset of anaesthesia, however no blood was collected until 15 min following surgery and cessation of anaesthesia (the STOP procedure itself takes about 10 min). It may be that AEA levels were not increased in these samples after cessation of propofol as it is a short acting agent and may have a transient effect upon AEA. However, further research needs to be undertaken to explain the precise effects of propofol on the ECS.

In terms of the effect of RU486 on the ECS published studies predicted that there should be a rise in AEA levels following the administration of RU486. RU486 binds to the P4 receptor (PR) and blocks its activity. *In-vitro* experiments demonstrated that P4 stimulates FAAH activity and expression in human lymphocytes at the transcriptional and translational level. The P4/PR complex enhances the transcription of FAAH gene and RU486 fully reverses this effect (Maccarrone et al. 2001). FAAH was also up-regulated by Th2-type cytokines and down-regulated by Th1-type cytokines (Maccarrone et al. 2001). High FAAH activity in lymphocytes is paralleled by low plasma AEA levels. In the study group, AEA and PEA levels significantly increased one day after RU486 administration. Peak plasma concentrations of RU486 are reached 1-3 hours following oral administration and therefore another blood sample was taken in a subgroup of patients one hour after RU486 administration. However, this did not show any significant changes in AEA, OEA or PEA levels in the plasma. This indicates that the effect of RU486 on NAE levels may take more than one hour.

In a subgroup of women undergoing MTOP an additional blood sample was collected after passing products of conception. The timing was on average about 3-4 hours after the prostaglandin administration. No significant changes in NAE levels could be identified. This could be explained by the short half-life and the short time to achieve peak concentrations of prostaglandin. Ideally it would have been more informative to take more blood samples at different time periods to see serial changes in AEA levels. Unfortunately it was not possible to apply this as it was outside of the remit of the ethics for these studies.

Studies have demonstrated an interaction between the ECS and the cytokine leptin in the regulation of reproduction (Maccarrone et al. 2003, Maccarrone et al. 2005). Together with sex steroid hormones, they form the endocannabinoid-hormone-cytokine network to regulate human fertility (Maccarrone et al. 2002, Karasu et al. 2011).

In this study, leptin levels in South Asian women were higher than in White women but this was not significantly different. The Asian women recruited were all from South Asia. A previous study of ethnicity and leptin levels in 1176 women and men with a mean age of 50 years found significantly higher leptin levels in South Asians when compared to Chinese and Europeans (Mente et al. 2010). This difference was still present when male participants were excluded. The study by Lilja et al. confirmed these findings and also excluded seasonal leptin variations as an explanation for the observed ethnic differences (Lilja et al. 2010). A different study investigating 183 premenopausal women with an average age of 43 years showed that East Asian (Japanese, Chinese, and Filipino) women had significantly lower leptin levels when compared to White women (Conroy et al. 2011).

Leptin levels correlate with BMI in non pregnant (Considine et al. 1996) and pregnant adults (Highman et al. 1998). The findings here also confirmed the positive correlation between BMI and serum leptin levels. During pregnancy leptin levels increase dramatically due to increased adipose tissues and placenta production (Hauguel-de Mouzon, Lepercq & Catalano 2006). Leptin levels in early pregnancy (12-14 weeks) are significantly increased when compared to that in pregravid women (Highman et al. 1998). A correlation between early gestation (6- 9 weeks) and leptin levels was absent in this study, even when normal and obese women were examined separately. It may be due to the narrow window of gestation. Leptin levels in overweight and obese women

were higher compared to levels in women with a normal BMI throughout early pregnancy. This finding is in accordance with the results of Misra et al. (Misra, Trudeau 2011). An explanation for the findings here could be that at an early gestation between 6-9 weeks maternal adipose tissue has a higher contribution to leptin production than placenta.

Leptin also has a relationship with the ECS, it enhances FAAH expression and thereby reduces AEA levels in human lymphocytes as mentioned in Chapter 1 (Maccarrone et al. 2003). Furthermore, P4 activates FAAH synergistically with leptin (Maccarrone et al. 2003). Against this background the plan was to identify whether there is a relationship between plasma AEA levels and serum leptin levels in pregnant women between 6 and 9 weeks gestation. These results did not show a significant relationship between plasma AEA, OEA or PEA and leptin levels. Furthermore, leptin levels did not change after the administration of RU486 unlike plasma AEA levels. This is contradictory to another study where a decrease of leptin levels was observed when taken pre- RU486 and pre- misoprostol (Honkanen et al. 2005). What would be interesting is, to measure FAAH activity in lymphocytes before and after the administration of RU486 in order to establish any *in-vivo* interactions between leptin, P4 and AEA.

These modest changes in plasma NAE levels raise the interesting question of where these NAE are produced. One hypothesis is that changes in plasma NAE levels after RU486 administration may be a consequence of changes to the ECS present in first trimester trophoblast. The following chapter investigates the ECS in the first trimester trophoblast in relation to the anti-progesterone RU486.

Chapter 3

Localisation and Quantification of the Endocannabinoid System and Modulation by Mifepristone in Human First Trimester Placental Tissues

3.1 INTRODUCTION

In Chapter 2 it was shown that plasma AEA levels in healthy women in the first trimester pregnancy are significantly increased one day after the administration of the antiprogesterone RU486 (Figure 2.10). This finding indicates that there may be a relationship between the endocannabinoid system (ECS) and progesterone (P4) and that any changes to this delicate endocannabinoid-hormone network may lead to pregnancy loss. Previous studies have shown that P4 is involved in maintaining endocannabinoid (EC) levels (Maccarrone et al. 2002, Karasu et al. 2011). P4 stimulates FAAH in human T lymphocytes and thereby decreasing AEA levels (Maccarrone et al. 2001). In women with threatened miscarriage low plasma AEA levels are associated with normal ongoing pregnancies, whereas higher plasma AEA levels are observed in those who subsequently miscarried (Habayeb et al. 2004).

It has been shown that the ECS is expressed in the first trimester placenta and has a function in trophoblast proliferation (Habayeb et al. 2008, Taylor et al. 2011). Although the localisation and expression of the ECS in viable trophoblast has been studied before, there are no data available on the ECS in trophoblast after the administration of RU486 in women undergoing medical termination of pregnancy (MTOP). It is therefore important to examine the ECS in trophoblast after the administration of RU486 in order to gain a better understanding of how the ECS is involved in the regulation of early pregnancy and to explain the relationship between the ECS and P4 in trophoblast.

The aims of this part of the study are therefore

(1) to quantify *N*-acylethanolamide (NAE) levels in trophoblast, decidua and membranes from viable 1st trimester pregnancies from women undergoing medical termination of pregnancy (MTOP) and surgical termination of pregnancy (STOP) and to compare them to the plasma levels and

(2) to study the expression of NAPE-PLD, FAAH, CB1, CB2 and TRPV1 in trophoblast from women undergoing MTOP and STOP.

3.2 MATERIAL AND METHODS

3.2.1 Measurement of NAE Levels in Tissue by UHPLC-MS/MS

3.2.1.1 Subjects

Women attending the Leicester Royal Infirmary at the University Hospital of Leicester NHS Trust for medical termination (study group) or surgical termination (control group) of pregnancy were recruited. All patients gave written informed consent. The study was approved and conducted according to the guidelines of the Leicestershire and Rutland Local Research Ethics Committee (Appendix 4).

3.2.1.2 Tissue Collection

Tissue samples were collected immediately after either surgical or medical termination of pregnancies. Excess blood was removed by repeated washing with ice cold sterile phosphate-buffered saline. Initially, products of conception from terminations of pregnancies were kept on ice and transported to the Histology department of the Leicester Royal Infirmary. The tissues were separated into trophoblast, decidua and membrane, flash-frozen in liquid nitrogen and stored separately at -80°C until further analysis. In later studies, tissues from terminations of pregnancies were collected in Kimble vials with RNA*later* an RNA Stabilization Reagent and transported to the laboratory at room temperature. The tissue in RNA*later* was then left for at least 24 hours at 4°C before being stored at -80°C until further analysis.

3.2.1.3 Tissue Preparation

The frozen tissue (trophoblast, decidua and membrane) was ground to a fine powder in a mortar and pestle and collected into Kimble vials. After weighing the disrupted tissue (around 100mg), 1ml phosphoric acid together with 12.5 pmol/g AEA-d8, 12.5 pmol/g OEA-d2 and 25 pmol/g PEA-d4 internal standards were added. The samples were vortexed thoroughly and diluted with 1 ml deionized H_2O. All samples were then homogenized with an Ultra Turrax homogenizer and then centrifuged at 3000 rpm for 15 min at 4°C. The supernatant was transferred to a clean Kimble vial and re-centrifuged at 3000 rpm for 15 min at 4°C.

3.2.1.4 Extraction of AEA, OEA and PEA from Tissue

Solid phase extraction (SPE) was used to extract the NAE from human tissue (Marczylo et al. 2009). Extraction from tissues was performed in the same way as that from human plasma (see section 2.2.9). Quantification of AEA, OEA and PEA was achieved using Ultra high Performance Liquid Chromatography- Tandem Mass Spectrometry (UHPLC-MS/ MS, Waters Ltd) (Lam et al. 2008, Marczylo et al. 2009, Marczylo et al. 2010).

3.2.1.5 Statistical Analysis

Statistical analysis of the data was performed using GraphPad Prism version 5.00 for Windows (Graph Pad Software, San Diego California USA). Data of groups that were not normally distributed were compared using Mann-Whitney U-test and for normally distributed groups the Student's t-test was used. Parametric data were analysed by Pearson correlation and non-parametric data by Spearman correlation. The level of significance was set at $p<0.05$. A power analysis based on previous work by the Endocannabinoid Research Group and that of other published data (Maccarrone et al. 2002) with $\alpha = 0.05$ and $\beta = 0.2$ showed that a minimum of 6 subjects were required in both the MTOP and STOP groups.

3.2.2 Immunohistochemistry

3.2.2.1 Tissue Collection

Trophoblast was collected immediately after surgical or medical termination of pregnancies and fixed in 10% neutral-buffered formalin for 4 days, dehydrated through 70% and 99% industrial methylated spirits (IMS) and cleared with Xylene before embedding in paraffin wax for immunohistochemistry. This was performed in a LEICA ASP3000 automated vacuum tissue processor. The blocks were cut into 4 μm thick sections, picked up on a vectabond coated slide and stored at room temperature until further use.

3.2.2.2 Immunostaining

Immunohistochemistry was undertaken for NAPE-PLD, FAAH, CB1 and CB2. Myometrial biopsies obtained from hysterectomy samples for benign reasons acted as a positive control. The primary antibodies and the dilutions are shown in Table 3.1.

Negative controls were rabbit IgG from Vector Laboratories (Peterborough, UK, Catalogue number X0903) and normal rabbit serum from Dako (Glostrup, Denmark; Catalogue number: X0902). They were used at the same concentrations as for the primary antibodies.

The Avidin-Biotin blocking kit was obtained from Vector Laboratories (Catalogue number: SP2001). An ABC detection system (ABC Elite, Vector Laboratories; Catalogue number: PK-6100 series) was used with 3, 3'-diaminobenzidine (DAB, Vector laboratories; Catalogue number: Sk4100) to identify immunoreactive complexes for anti-NAPE-PLD, anti-FAAH anti-CB1 and anti-CB2 antibodies (Taylor et al. 2011). The secondary antibody was biotinylated goat anti-rabbit antibody from DAKO (Catalogue number: E 0432) and used at a dilution of 1:400. DPX mounting medium was from BDH (Poole, Dorset, UK).

Table 3.1: Primary antibodies and their dilutions used for Immunohistochemistry.

Antibody	Company	Product Number	Type	Dilution
NAPE-PLD	Abcam	ab 119259	rabbit polyclonal	1:1000
FAAH	Abbiotec	200189	rabbit polyclonal	1:100
CB1	Sigma-Aldrich	C1108	rabbit polyclonal	1:500
CB2	Abcam	ab3561	rabbit polyclonal	1:100

3.2.2.2.1 Immunohistochemical Protocol for NAPE-PLD, FAAH and CB2

Figure 3.1 shows the pathway for the immunohistochemical studies. Briefly, paraffin embedded tissue sections were dewaxed in xylene and rehydrated in graded alcohol and water. Endogenous peroxidase activity was blocked with 6% hydrogen peroxide (H_2O_2) in water for 10 min. Slides were then washed in running tap water for 5 min, followed by TBS (Tris-buffered saline = 2.4g Tris Base, 8.7g NaCl, pH 7.5 in 1 litre of distilled water (dH_2O)) containing 1ml of Triton-X-100 (TBS/ 0.1% Triton-X-100) for another 5 min. Vectastain blocking serum was prepared by adding 150µl of normal goat serum to 10ml of TBS/ 0.1% Triton-X-100/ 3% BSA. 100µl of Vectastain blocking serum was applied to each slide and left in a humid chamber for 20 min at room temperature. The slides were drained and the sections blotted dry and avidin (100µl) added for 15 min. Avidin was made up by 200µl of avidin stock and 1ml of Vectastain blocking serum. After that the slides were rinsed briefly with TBS 0.1% Triton-X-Buffer and biotin (100µl) was added for 15 min. Biotin was made up of 200µl biotin stock and 1ml of normal serum. Excess liquid was drained from each slide. Primary antibody, normal rabbit serum or Ig G diluted as mentioned in Table 1 (100µl) was applied to the slides and incubated overnight at 4°C in a humid chamber. The following day the slides were washed in TBS/ 0.1% Triton-X-100 for 20 min and 100µl of Vectastain secondary biotinylated anti-rabbit antibody was applied and left in a humid chamber for 30 min at room temperature. Following a 20 min wash with TBS/ 0.1% Triton-X-100 the slides were incubated with ABC Elite solution (Vector Laboratories) for 30 min at room temperature and washed again with TBS/ 0.1% Triton-X-100 for 20 min. Afterwards 3,3'-diaminobenzidine (DAB) (Vector Laboratories) (100µl) was applied to each slide for 5 min at room temperature and the slides were washed in distilled water for 5 min before counterstaining with Mayer's haematoxylin (Sigma-Aldrich) for 1 min. Thereafter the slides were washed under running tap water for 5 min and then dehydrated through graded ethanol, cleared in xylene and mounted in DPX mounting medium (BDH Poole, Dorset). Slides were visualised at 10x, 20x or 40x magnification on an Axioplan transmission microscope and images obtained with a Sony analogue camera connected to Axiovision image capture and processing software (Axiovision version 4.4, Carl Zeiss Ltd, Welwyn Garden City, Hertfordshire, UK).

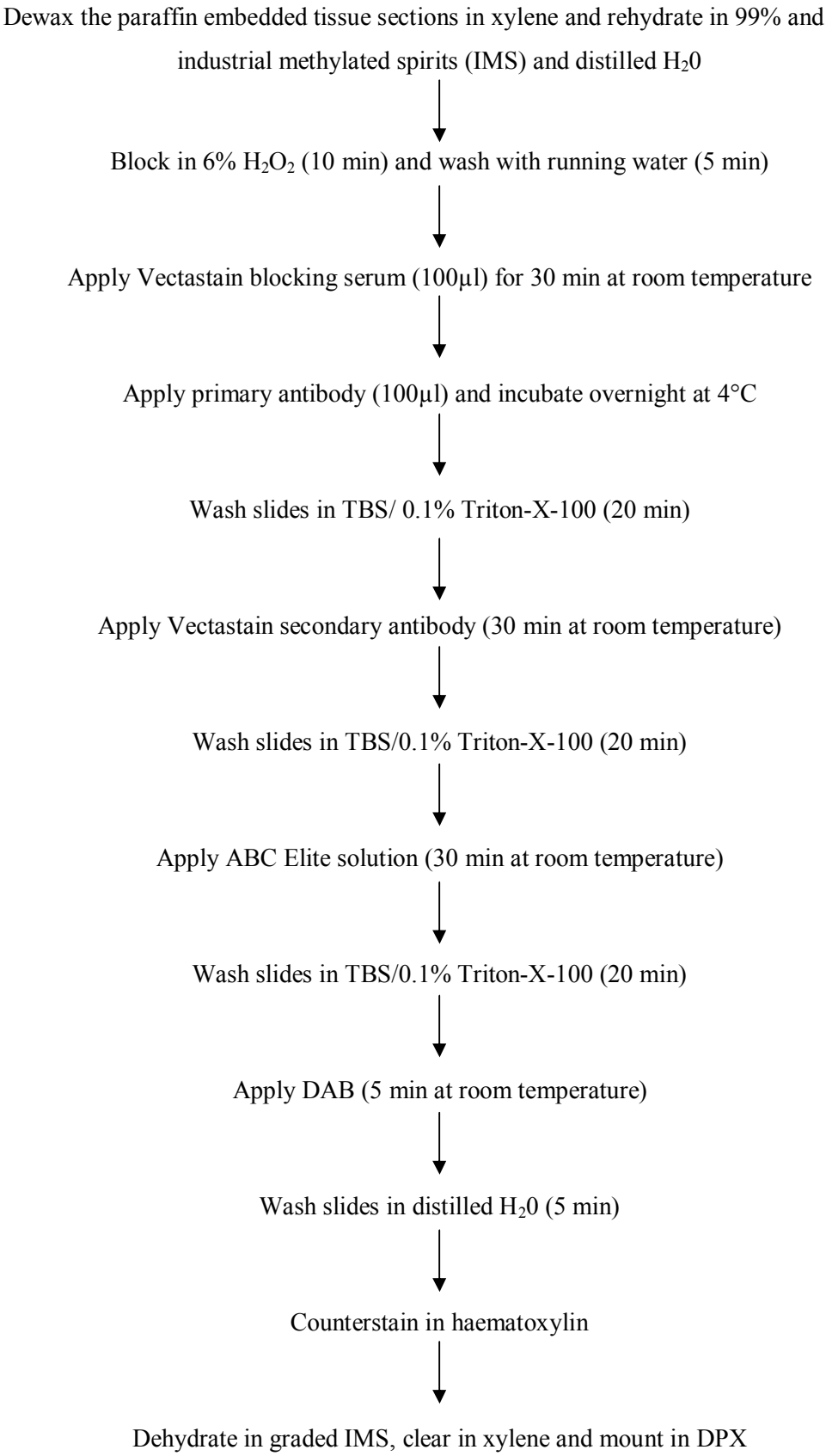

Figure 3.1: Flow diagram for the immunohistochemical protocol for NAPE, FAAH and CB2

3.2.2.2.2 Immunohistochemical Protocol for CB1

After dewaxing and washing of the slides, microwave antigen retrieval was performed with 10mM citric acid buffer (pH 6.0). The slides were next placed in 500 ml citric acid buffer and then in a microwave for 10 min at 700 watts and thereafter cooled down for 20 min. Afterwards the slides were placed in water for 5 min. Endogenous peroxidase activity was blocked with 6% H_2O_2 for 10 min. Slides were then washed under running tap water followed by Tris-buffered saline with 0.1% bovine serum albumin (TBA) each for 5 min. 200µl of 1:10 Normal Goat Serum (NGS) in TBA was applied on each slide and left for 20 min at room temperature. The slides were drained and avidin blocking solution (200µl/ml in 10% NGS/ TBA) applied for 15 min and thereafter washed in TBA for 5 minutes. The slides were drained and a biotin blocking solution (200µl/ml 10% NGS/ TBA) applied for 15 min. After draining the slides, 200µl of primary CB1 rabbit antibodies diluted at 1:500 in either blocking solution (10%NGS/ TBA), NGS or Ig G were added and the slides incubated in a humid chamber overnight at 4°C. Slides were washed in TBA for 20 min; biotinylated goat anti-rabbit antibody (Dako, Denmark) (200µl) diluted to 1:400 in TBS was applied to each slide for 30 min at room temperature, followed by another wash in TBA for 20 min. Thereafter ABC Elite reagent (Vector Laboratories) (200µl) was applied according to the manufacturer's instructions for 30 min followed by a wash in TBA for 20 min and application of 3, 3'-diaminobenzidine (Vector Laboratories) on each slide for 5 min. The slides were then washed in distilled water for 5 min, counterstained by immersion in Mayer's haematoxylin (Sigma-Aldrich) for 1 min and washed under running tap water for 5 min. Thereafter the slides were dehydrated through graded ethanol, cleared in xylene and mounted in DPX mounting medium (BDH Poole, Dorset).

3.2.2.3 Image Capture and Evaluation of Immunostaining

Trophoblast areas (cytotrophoblast and syncytiotrophoblast) of 6 samples of MTOP and 5 samples of STOP were assessed by histomorphometric analyses. The samples were from pregnancies at 7-8 weeks gestation. Images were captured using an Axioplan microscope (Carl Zeiss, Hertfordshire. UK) and a DXC-151P colour video camera (Sony CCD/RGB).10-13 randomly selected fields per slide at 400x magnification were captured during normal daylight and medium neutral filters with the lamp set at 6,400K

(Taylor et al. 2010). The levels of immunostaining in cytotrophoblast and syncytiotrophoblast were analysed using an unbiased histoscore method within the Imagescope software package version 10.2.2.2319 (Aperio Technologies Inc., Vista, CA. USA). The area of interest was outlined using the positive pen tool of Imagescope and then analysed using the positive pixel count algorithm version 9.1. The pixel count was calculated automatically and shown as red for strong positive, orange for positive, yellow for weak positive and blue for negative staining (Figure 3.2).

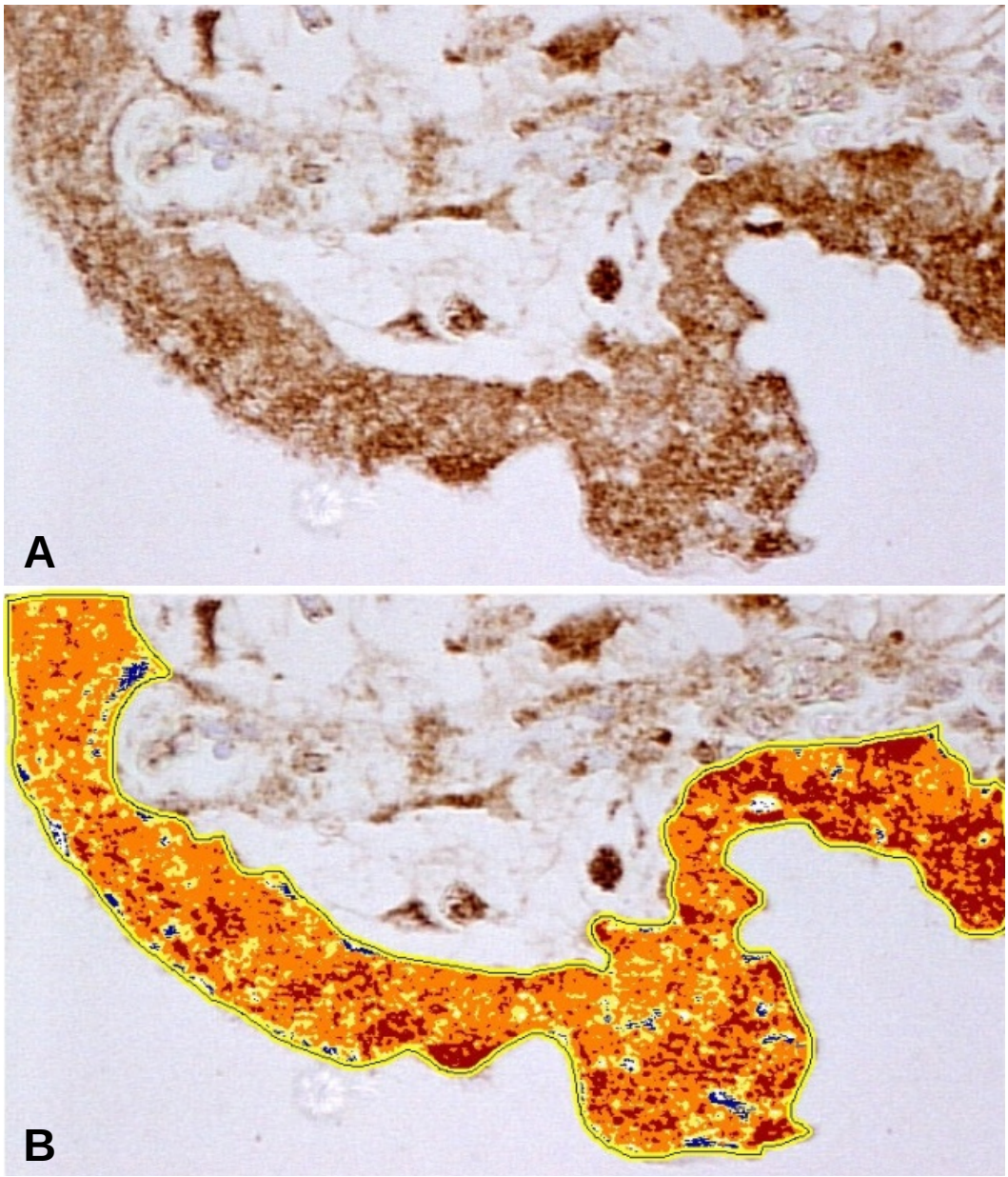

Figure 3.2: A) FAAH immunostained trophoblast slide, B) Pixel count of above slide using Imagescope software.
The area of interest is outlined. The different colours represent the staining intensities as follows: red- strong positive, orange- positive, yellow-weak positive and blue- negative staining.

The total number of pixels (NT) was determined by adding the positive and negative ones. A score of 0 was assigned to unstained areas, 1 to weakly stained areas, 2 to moderate stained and 3 to strongly stained areas as determined by the software. The immunohistochemical score (H-score) was calculated as follows:

$$\text{H-score} = 3x\ Nsp/NT + 2x\ Nnp/NT + 1x\ Nwp/NT + 0x\ Nn/NT$$

Nsp= total number of strong positive

Nnp= total number of normal positive

Nwp= total number of weak positive

Nn= total number of negative

3.2.2.4 Statistical Analysis

Statistical Analysis of the data was performed using GraphPad Prism version 5 for Windows (GraphPad Software, San Diego California, USA). The histomorphometric data were normally distributed and the groups were compared using the unpaired student t-test with $p<0.05$ being set as significant.

3.2.3 Polymerase Chain Reaction (PCR)

3.2.3.1 Principles of Reverse Transcription PCR (RT-PCR)

PCR is able to calculate the starting quantity of DNA after exponential amplification. Following RNA extraction, digestion of contaminating genomic DNA from the sample occurs, so that the genomic DNA does not get amplified at the PCR. The RNA samples are then reverse transcribed to create copy DNA (cDNA) from messenger RNA (mRNA). The resulting single cDNA is used as a template for subsequent PCR amplifications. RT^+ reactions contain the reverse transcriptase enzyme whereas in RT^- reactions the enzyme is omitted. The RT^- sample is used as a negative control during the PCR. A positive signal may indicate genomic DNA contamination.

During the PCR, double stranded DNA is converted to single stranded DNA upon which the enzyme DNA polymerase acts and binds deoxyribonucleotides to build the complementary sequence for the single strand DNA. Two sets of DNA oligonucleotide primers are designed to bind to the specific area of DNA with the genetic code of interest

PCR involves repeated thermal cycles, each one with 3 stages (Figure 3.3). During the first stage of each cycle high temperatures lead to denaturation where the double-stranded DNA separates. This allows specific oligonucleotide primers to bind to the strands at a lower temperature in the second stage called hybridisation or annealing. In the final extension stage complimentary bases are added in a 5' to 3' direction with the help of heat stable DNA polymerase. The DNA template quantity is exponentially amplified.

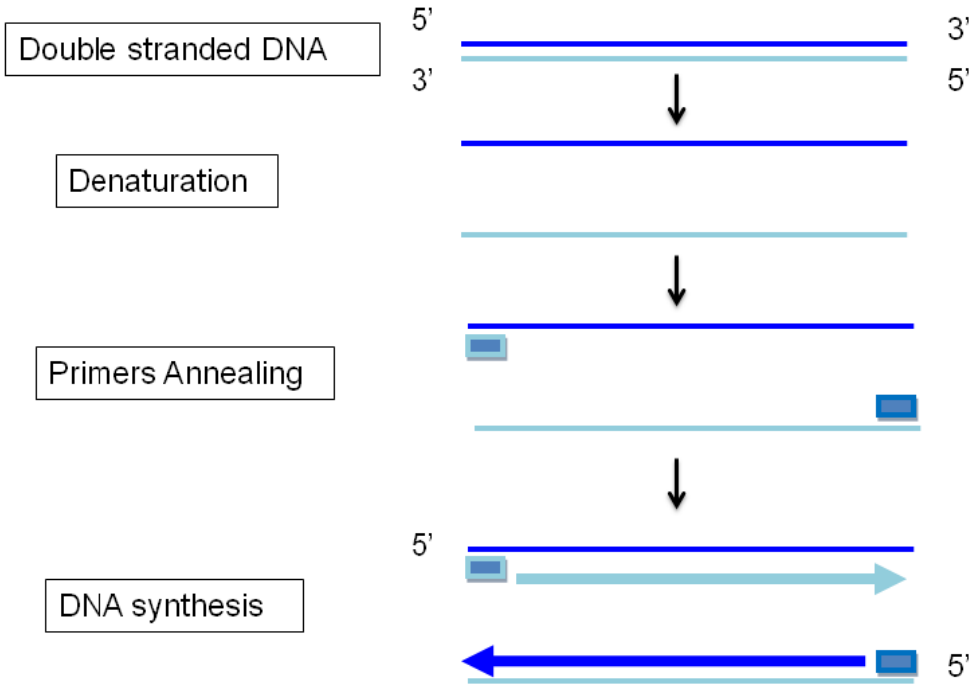

Figure 3.3: Schematic drawing of one PCR cycle.
Following the separation of double stranded DNA in high temperatures (denaturation), specific primers bind to the single strands (annealing) and DNA synthesis with the heat stable DNA polymerase occurs.

3.2.3.2 Principles of quantitative Real Time PCR (qRT-PCR)

The Real Time PCR thermal cycler can measure the relative quantity of DNA after each cycle by a fluorescent probe (e.g. SYBR green, Taqman). The fluorescence is plotted against the number of cycles which results in an amplification curve (Figure 3.4). During the exponential phase, a doubling of the DNA template takes place if the reaction is 100% efficient. When the starting materials (template, Oligonucleotides, primers, fluorescent probe) are getting low, the linear phase of reaction begins and by the plateau phase, the reaction has ended (Figure 3.4). There is a detection threshold, below which the amplicon (PCR product) DNA cannot be distinguished from the background noise. The cycle at which this discrimination occurs is called the threshold cycle (Ct). The Ct value is dependent on the starting quantity of template cDNA: high initial concentrations will have low Ct values.

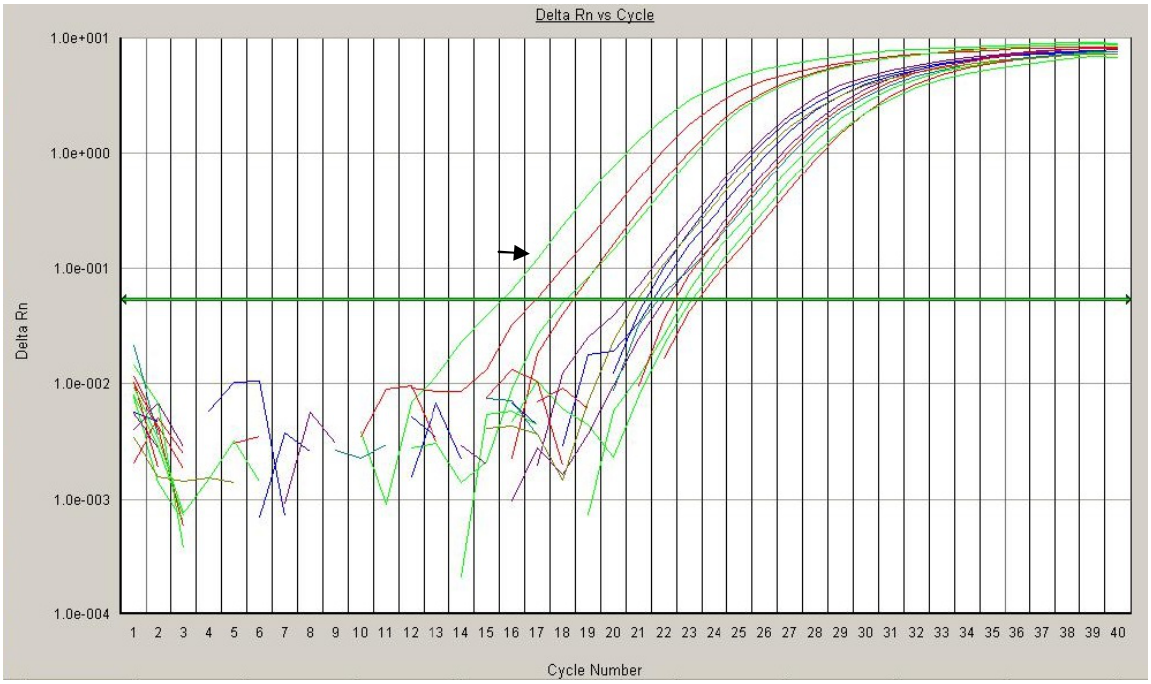

Figure 3.4: Example of amplification curve.
The first plot from the left (turquoise, arrow) has the highest amount of template and therefore the lowest numbers of cycles are necessary to reach the fluorescence detection level (green line). The last plot (purple) has the lowest initial template and therefore requires the highest number of amplification cycles to pass the detection threshold.

3.2.3.3 PCR Quantification

To get relative quantification, the Ct value of a gene of interest (GOI) is normalised against an endogenous control gene/ reference gene (RG). It is important to choose the reference gene very carefully, as it should remain constant during the experiment.

The ΔΔCt Method is an approximation one which assumes that the efficiencies of the GOI and reference genes are similar.

The highest Ct value of the GOI is identified and subtracted from all other Ct values of the same GOI in order to obtain a delta Ct value (Δ Ct) as seen in the following formula:

$$\Delta\, Ct = Ct\ GOI\ max - Ct\ GOI$$

Then the following equation was applied:

$$fold\ change = 2\text{^} - \Delta Ct$$

All the data are presented relative to the expression of the sample with the lowest expression.

And to identify the fold change in GOI expression relative to the reference gene (RG), the following formula was applied if the PCR reaction was 100% efficient:

$$Ratio\ of\ GOI\ expression = \frac{\text{fold change in GOI expression}}{fold\ change\ in\ RG\ expression}$$

Another way of quantifying mRNA levels using real-time PCR is the Pfaffl method (Pfaffl 2001) where the equation incorporates the PCR efficiencies of the reference gene (RG) and gene of interest (GOI) and thereby increases the accuracy in the final relative fold value. This formula is demonstrated below:

$$ratio = \frac{Efficiency\ (GOI)\Delta CT\ GOI\ (control - treated)}{Efficiency\ (RG)\Delta CT\ RG\ (control - treated)}$$

There are two main fluorescent probes for quantitative real-time PCR: SYBR Green and Taqman probe.

SYBR Green

SYBR Green is a DNA specific dye, which fluoresces when it binds to double stranded DNA (Schneeberger et al. 1995). The specificity of the amplified products can be checked by analysis of a dissociation curve/ melting curve, whereby a single sharp fall in fluorescence is seen. A melting curve is performed by slowly increasing the temperature from e.g. 65°C to a high temperature. At a low temperature all PCR products are double stranded and bound to SYBR Green, which leads to fluorescence, whereas at a higher temperature PCR degrades and the fluorescence rapidly falls. The change in fluorescence with time is measured against the temperature and plotted resulting in a curve with a peak at the respective melting temperature (Tm). If the Tm is lower than that for a specific PCR product, primer-dimers (primers annealing to themselves) may have formed. The formation of non specific products leads to a dissociation curve with stepped or gradual falls in fluorescence (Wittwer et al. 1997, Lutfalla, Uze 2006). A disadvantage is the nonspecific binding of SYBR Green to any double stranded DNA and therefore production of dimeric primer compounds. Contaminating genomic DNA should be minimised.

Taqman ™ PCR

Taqman probes are fluorogenic hydrolysis probes with a fluorophore tag at the 5' end of the probe and a quencher tag at the 3' end. They work through 5' exonuclease activity of Taq polymerase (Heid et al. 1996). When the two tags are close, no fluorescence signals occur. During the complementary extension the probe is hydrolysed and the fluorophore released. Taqman probes are very specific and only bind to the target of interest (Figure 3.5).

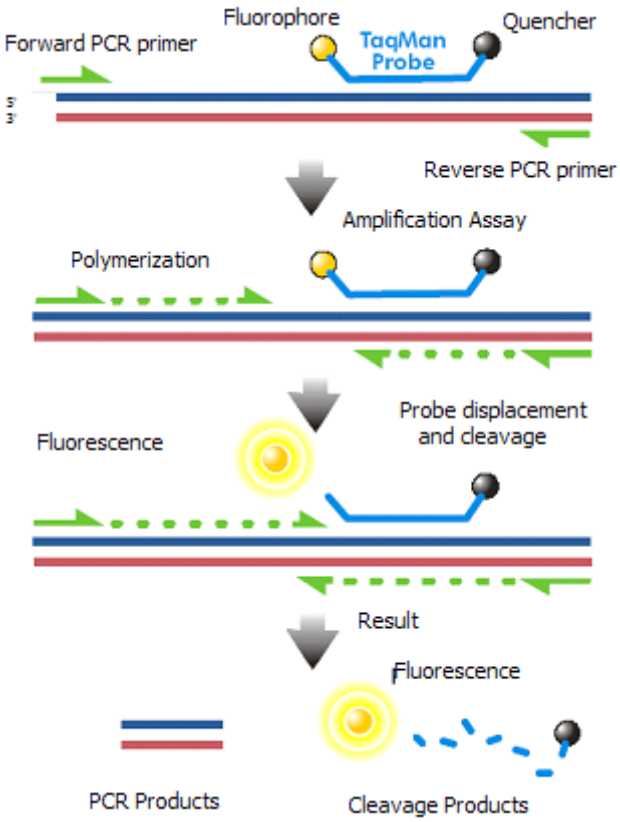

Figure 3.5: Schematic drawing of the process of amplification and probe cleavage using Taqman® probe system.
The Taqman Probe binds to single stranded DNA and the Taq polymerase extends the primer to synthesise DNA. The 5' to 3' exonuclease activity of the polymerase degrades the probe and fluorophore gets released. The fluorescence is then measured by the RT-PCR thermal cycler (Adapted from Wikipedia image).

3.2.3.4 Primer Design

A primer consists usually of not more than 30 nucleotides and is a starting point for DNA synthesis. Primer sequences need to be selected for the specific region of DNA. In order to avoid the possibility of contaminating genomic DNA being amplified in place of RNA during PCR, the primers were designed to cover exon-exon junctions. Exons are the DNA sequences that are transcribed into mRNA whereas introns are found in between exons and are not transcribed. In this way the genomic DNA containing the intron will not be able to compliment the primer (Figure 3.6). Primers against the genes of interest were designed using Primer Express version 2.0.0 (Applied Biosystems, Warrington, UK) and ordered from Sigma Aldrich (Poole, UK).

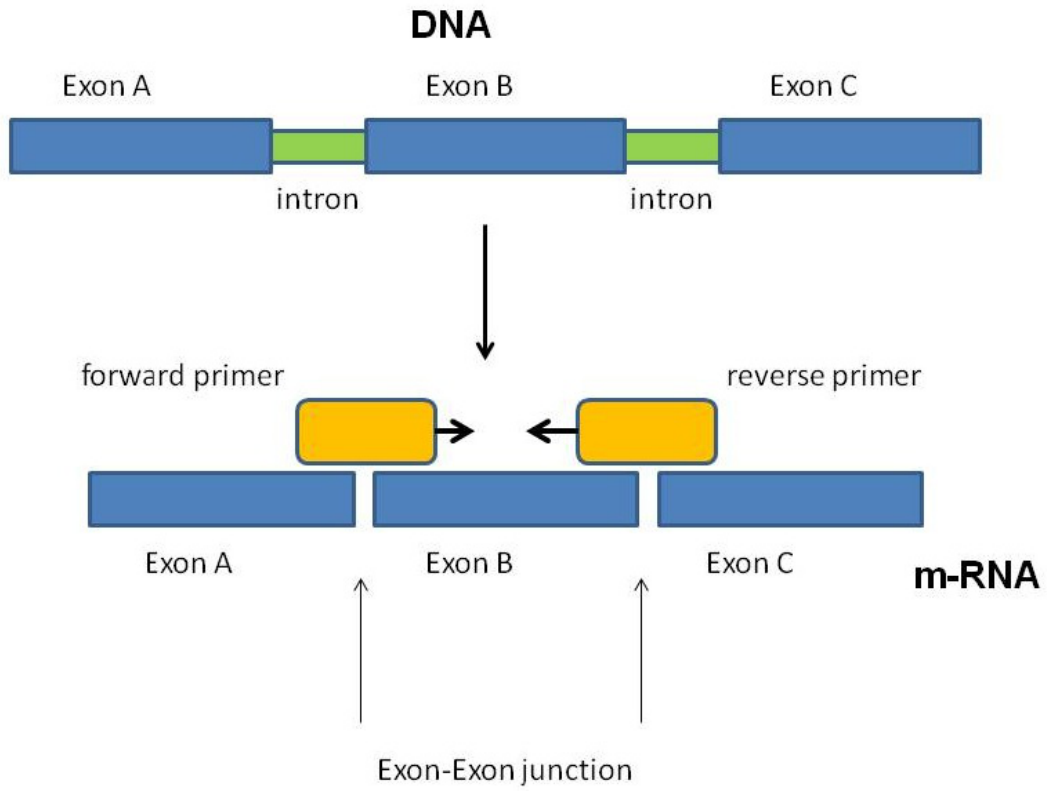

Figure 3.6: Schematic drawing of Primer Design.
The DNA consists of exons and introns. The exons are transcribed into m-RNA. The primers chosen are covering exon-exon junctions in order to avoid DNA amplification from the introns.

The sequences of the primer pairs investigated for determination of NAPE-PLD, FAAH, TRPV1, CB1 and CB2 mRNA/cDNA are summarized in Table 3.2.

Table 3.2: Primer sequences for PCR amplification.

Gene		Primer Sequences
NAPE-PLD_01	Forward	5'-AAGAGATAGGAAAAAGATTTGGACCTT
	Reverse	5'-CTGGGTCTACATGCTGGTATTTCA
NAPE-PLD_02	Forward	5'-CAAACAAAGAAATCTATGGCAATTCA
	Reverse	5'-ATCTTCAGCGTTAAGTCCGTATCTC
FAAH_01	Forward	5'-CATGCTCTGGAGACCCTGTCA
	Reverse	5'-CCAGCAGTCCTTTAAGCCATTG
FAAH_02	Forward	5'-GGAGACCAAACAGAGCCTTGAG
	Reverse	5'-CTGAAGAGCCCACCTGTTGAC
TRPV1_01	Forward	5'-GCTGGAGGTGATCGCCTACA
	Reverse	5'-AGGCAGTAGACCAGGAAGTTGAA
TRPV1_02	Forward	5'-GAAGCCGTTGCTCAGAATAACTG
	Reverse	5'-AGCATGGCTTTCAGCAGACA
CB1_01	Forward	5'-TGCTGAACTCCACCGTGAAC
	Reverse	5'-TCCCCCATGCTGTTATCCA
CB1_02	Forward	5'-CCCCTGGCCTATAAGAGGATTG
	Reverse	5'-TGGGAAAATGTCTGAGCAAACA
CB2_01	Forward	5'-TGGCAGCGTGACTATGACCTT
	Reverse	5'-CCACGGGTGAGCAGAGCTT
CB2_02	Forward	5'-GCCCAGCCACCCACAAC
	Reverse	5'-GCTATCTCTGTCACCCAGCATTC

NAPE-PLD, *N*-arachidonylphosphatidylethanolamine phospholipase 1; FAAH, fatty acid amine hydrolase; TRPV1, transient receptor potential vanilloid type 1; CB1, Endocannabinoid receptor 1; CB2, Endocannabinoid receptor 2.

3.2.3.5 Tissue Homogenisation

Human trophoblast was collected in RNA later RNA stabilisation reagent from women undergoing MTOP and women undergoing STOP. The tissue was stored at -80°C until analysis. About 40mg of tissue was ground to a fine powder under liquid nitrogen using a mortar and pestle. The tissue powder was then transferred to a 1.5ml sterile (DNase/RNase free) Eppendorf tube and lysis buffer added. At this stage RNA extraction was performed or the homogenate was stored at -80°C for several months.

3.2.3.6 RNA Extraction Methods

Two different ways of RNA extraction were used to isolate total RNA from human trophoblast: (a) the phenol: chloroform organic extraction, (b) the Qiagen miRNeasy mini kit (Qiagen, Crawley/West Sussex/UK).

A) Phenol:Chloroform organic Extraction

1 ml of a phenol/guanine thiocyanate solution (TRI reagent, Sigma) was used as a lysis buffer for the tissue and the tissue homogenized using Ultra-Turrax T8 for 40 s. The samples were incubated at room temperature for 5 min. 200µl of 1-bromo-3-chloro-propane (Sigma B9673) was added. It is claimed to lessen genomic DNA contamination of the RNA preparation compared to Chloroform which is usually used. The eppendorf tube was vortexed and then centrifuged at 10,000 x g for 15 min at 4°C. Three distinct liquid phases form in the tube: a top clear phase containing RNA, a white interphase with DNA and a red/pink phase containing protein. The upper aqueous phase (about 600µl) was transferred to a fresh tube and 600µl of 70% isopropanol was added. This was mixed and incubated at room temperature for 10 min to precipitate RNA. The sample was then centrifuged at 10,000 x g for 10 min at 4°C. The supernatant was aspirated and discarded. The RNA pellet was washed with 1 ml of ice cold 75% ethanol, gently vortexed and centrifuged at 10,000 x g for 5 min. The previous step was again repeated. The final ethanol wash was poured off and the sample left uncovered for 20-30 min so that the remaining trace of ethanol could evaporate. The pellet containing RNA was resuspended in 50µl of RNase-free water and stored at -80°C for later use.

The concentration of RNA was calculated by recording the absorbance at 260nm using the NanoDrop™ 8000 spectrophotometer (Thermo Scientific, Wilmington, USA). RNA quantity and integrity was checked with the Agilent 2100 Bioanalyzer (Agilent Technologies, Berkshire, UK). More details about the Bioanalyzer are presented in Chapter 3.2.3.7.

DNase treatment

TURBO™ DNase (Applied Biosystems) was used to degrade genomic DNA present in total RNA. 10µg of RNA was included in each reaction and the reaction mixture (Table 3.3) was incubated for 30 min at 37°C. The DNase activity was stopped by adding 5µl of the inactivation reagent. This was mixed and allowed to stand for 3 min at room temperature and then centrifuged at 10,000xg for 2 min at room temperature to sediment the inactivation substance. The supernatant containing the RNA was removed into a fresh tube and stored at -80°C until reverse transcription.

Table 3.3: Reaction volumes for TURBO™ DNase treatment.

Component	Volume per reaction
10xbuffer	5µl
DNase I	1µl
RNase-free water	44µl

B) RNA Extraction using the Qiagen miRNeasy Mini Kit (silica gel based purification method)

700µl of QIAzol Lysis Reagent was added to the powdered tissue. The homogenate was left at room temperature for 5 min to promote the dissociation of nucleoprotein complexes. Then 140µl 1 bromo3 chloropropane was added to the homogenate and the tube was shaken vigorously. The tube was left at room temperature for 3 min and then centrifuged for 15 min at 12000 x g at 4°C. This separated the sample into 3 phases. The upper clear phase (350µl) containing nucleic acids was transferred to a new tube and 525µl of 100% ethanol was added and mixed by pipetting up and down. 700µl of the sample were pipetted into a miRNeasy Mini spin column in a 2 ml collection tube. At this stage the RNA binds to the membrane and phenol and other reagents were washed away. The tube was centrifuged at 8000 x g for 15 s at room temperature and the flow-through discarded. On column DNase digestion was performed to remove any contaminating DNA. Lyophilized DNase I (1500 Kunitz units) was dissolved in 550µl of RNase-free water in order to prepare the DNase I stock solution, divided into aliquots and stored at -20°C. 350µl RWT Buffer was pipetted onto the miRNeasy Mini column and centrifuged at 8000 x g for 15 s at room temperature and the flow-through discarded. 10µl DNase I stock solution was added to 70µl RDD buffer and mixed gently by inverting the tube. The mix (80µl) was pipetted directly onto the column and left at room temperature for 15 min. This was followed by washes with RWT buffer (350µl) and RPE buffer (500µl) for 15 s at 8000 x g at room temperature. The flow-through was always discarded. Another 500µl of RPE buffer was added onto the column and centrifuged for 2 min. After that the miRNeasy Mini spin column was transferred to a new 1.5 ml collection tube and 50µl of RNase-free water was added onto the column. This was centrifuged at 8000 x g for 1 min at room temperature to elute the RNA. This was stored at -80°C until further analysis or transported on ice for RNA quantification.

RNA samples from the organic extraction with phenol-chloroform can frequently be contaminated with proteins, ethanol and phenol-chloroform as well as genomic DNA. Furthermore this extraction has to be carried out in fume hood and adds more time to the procedure. The column RNA extraction method does not require the use of toxic organic solvents; it is simple, less time consuming with a good RNA yield and minimal

protein contamination. Digestion with DNAse is important to remove any DNA traces that can give false positive results in qRT-PCR and increase the background noise.

3.2.3.7 RNA Quantity and Quality Assessment

The RNA integrity can be assessed by various methods:

- Gel optical density (OD) measurement
- OD measurement via Nano-Drop
- Agarose gel-electrophoresis
- Bioanalyzer 2100 (Agilent Technologies, USA)

The quality and quantity assessment with a spectrophotometer uses the wave lengths 260nm for specific nucleic acids and 280nm for specific proteins. The quantity is assessed on the OD of 260, and the quality by the ratio of $OD_{260/280}$ with a value >1.8 for good RNA quality (Manchester 1996). The advantage of the NanoDrop is that it uses only 1µl of your sample. All samples selected for qRT-PCR had $OD_{260/280}$ absorbance ratios greater than 1.9.

Another method involves gel electrophoresis with RNA either stained with SYBR Green dye or ethidium bromide (Le Pecq, Paoletti 1966, Bonini, Hofmann 1991, Bustin, Nolan 2004). Clear ribosomal bands at 18S and 28S confirm RNA integrity and a ratio of 28S: 18S band of 2.0 is considered to be high quality RNA whereas smearing of the bands are signs of degradation. However, this method is subjective and there is no numerical output for unbiased quality assessment. Furthermore ethidium bromide is mutagenic.

RNA samples were run on a 1.5% Tris Borate EDTA (TBE) agarose gel and were stained with ethidium bromide according to the following protocol.

The recipe for 2 litres of 10x TBE is: 218g Tris Base, 110g Boric acid, 9.3g EDTA. The ingredients are dissolved in 1.9 litre of distilled water (pH 8.3) and made up to 2 litres with distilled water. Then 100 ml of 10x TBE are mixed with 900 ml of distilled water for the preparation of 1x TBE.

For 1.5% TBE agarose gel 0.75g agarose was added to 50 ml 1xTBE Buffer and swirled to mix. The agarose solution was heated in the microwave for 1 min on full power and then cooled down to ~65°C. After that it was poured onto a gel support, the comb was inserted and allowed to cool down for about one hour. In the meantime the samples and the RNA ladder were prepared.

1µg RNA was added to RNase free water up to a volume of 10µl into a microfuge tube and mixed before adding 3µl loading buffer. The loading buffer gives colour and density to the sample. It is negatively charged in neutral buffers. The loading buffer consists of 25mg bromophenol blue, 4g sucrose and water up to 10ml. It is stored at 4°C. 1µl of the RNA ladder was diluted with 9µl RNase free water and 3µl loading buffer and mixed. The RNA ladder and the samples were denatured at 70°C for 3 min and cooled down at 4°C for 2 min in a PTC 225 Peltier Thermalcycler and then loaded onto the gel submerged in 1xTBE (running buffer). Electrophoresis was performed at 80V until the bromophenol blue dye migrated two-thirds of the gel length. As RNAs are negatively charged they migrate to the anode during electrophoresis.

Thereafter, the gel was transferred into a plastic container filled with tap water containing 0.5 µg/µl ethidium bromide. The plastic container was left on a shaker for 30 min to stain the gel. Then the fluid was discarded and the gel washed with water. The gel was visualised and an image taken in a UV Transilluminator (302nm).

The Agilent 2100 Bioanalyzer (Agilent Technologies, Berkshire, UK) uses lab-on-chip technologies to access the integrity and quantity of extracted RNA. RNA samples on a chip get electrophoretically separated and detected via laser induced fluorescence. Integrity is assessed by the visualisation of 18S and 28S ribosomal RNA bands. Signs of degradation are an elevated threshold baseline and a decreased 28S:18S ratio (Schroeder et al. 2006) (Figure 3.7). High intensity 18S and 28S rRNA peaks are seen with intact RNA (Figure 3.8). A broad band shows DNA contamination.

Samples get an RNA integrity number (RIN) from 1 to 10: score 1 means highly degraded RNA and score 10 represents completely intact RNA. This facilitates comparison of samples. In human tissue RIN scores can vary due to problems with tissue collection and sampling, handling and storage and RNase enzymatic activity (Fleige et al. 2006). In solid tissue a RIN score between 6 and 8 are considered as reliable RNA (Strand et al. 2007). Studies have reported a good correlation between

RIN values and qRT-PCR results (Fitzpatrick et al. 2002, Fleige et al. 2006). A RIN score >6 is a suitable RNA to justify further analysis (Fitzpatrick et al. 2002, Strand et al. 2007). Therefore all samples included in this study had intact RNA without DNA contamination and with a RIN score>6.

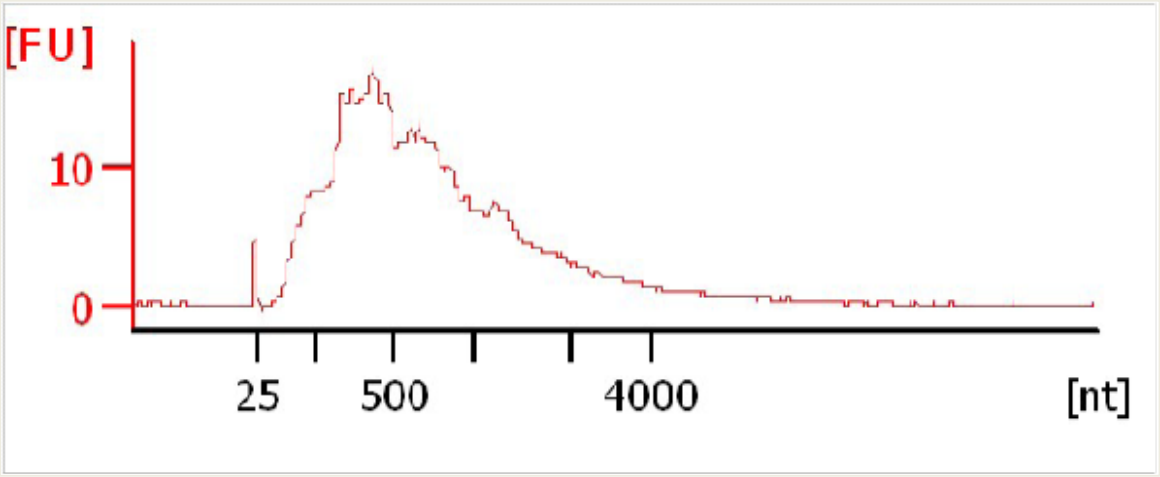

Figure 3.7: Electropherogram of micro-capillary electrophoresis from RNA showing a degraded sample with a shift towards shorter fragments (RIN=2).

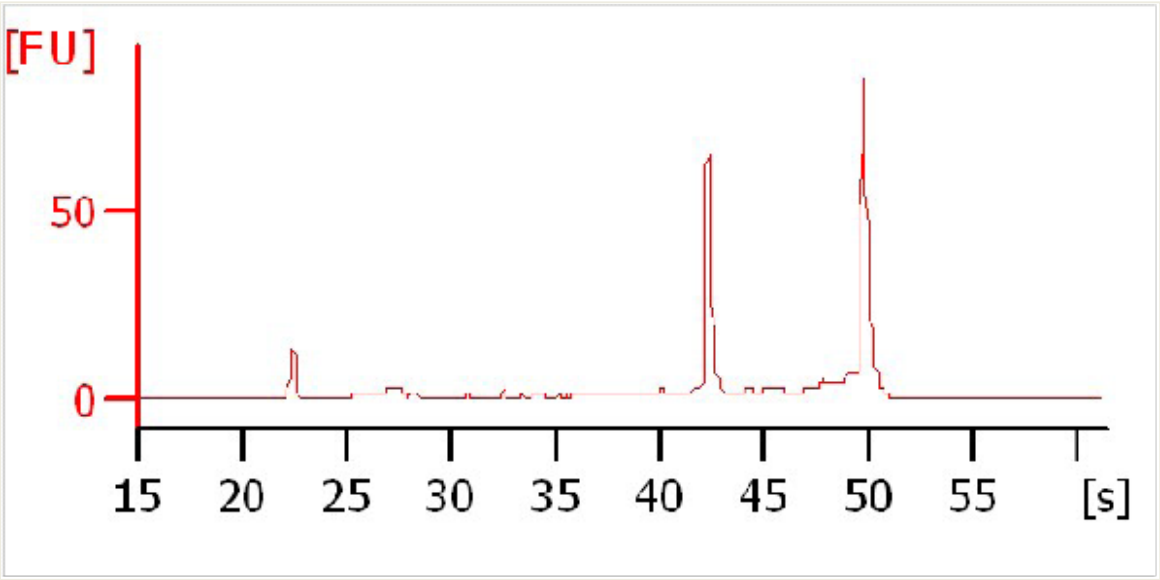

Figure 3.8: Electropherogram of micro-capillary electrophoresis from RNA showing intact, high quality RNA with intense peaks at 18S and 28S representing rRNA and a small peak at 5S RNA (RIN=9.2).

We used 200ng of RNA sample with the Agilent RNA 6000 Nano kit. The samples were heated at 70°C for 2 min for denaturation and then transferred to ice. We prepared 12 samples per chip according to the manufacturer's instructions. In brief, 65µl of the RNA 6000 Nano gel and 1µl of RNA 6000 Nano dye concentrate were mixed and centrifuged at 13000 x g for 10 min at room temperature. The Nano chip was placed on the chip priming station and 9µl of the gel-dye mix was pipetted in the well marked with G and the chip priming station closed. A plunger was pressed down for 30s and the priming station reopened. Then 9µl of the gel-dye mix was added to 2 wells marked with G and 5µl of the Nano marker was pipetted to all 12 sample wells and the well marked with a ladder. Finally, 1µl of the ladder was put into the well marked with a ladder and 1µl of sample into the 12 sample wells. The chip was vortexed for 1min at 2400 rpm and run in the Agilent 2100 bioanalyzer within 5 min. The results were available within 30 min. Samples with RIN scores ≥6 were included for further analysis.

3.2.3.8 Reverse Transcription and cDNA Synthesis

Reverse transcription of 2µg DNase-treated RNA was performed using the high-capacity cDNA reverse transcription kit (Applied Biosystems). The 20µl reaction volume consisted of 10µl of 2x RT buffer, 1µl of 20x RT enzyme and 2µg of total RNA diluted with RNase/ DNase free water to a volume of 9µl. A no RT control was used by replacing 1µl 20x RT enzyme with 1µl RNase/ DNase free water in order to determine any genomic DNA contamination. The samples were mixed and placed in a thermal cycler with the following programme:

Step1:	37°C	60 min
Step2:	95°C	5 min
Step3:	4°C	indefinite

The samples were then stored at -20°C until PCR was performed.

3.2.3.9 Quantitative Real Time PCR

Quantitative real time PCR (qRT-PCR) reactions were run using SYBR Green fluorescence probes. The 25µl reaction mix for custom designed primers contained 12.5µl SYBR Green PCR Master Mix (Applied Biosystems; AmpliTaq Gold® DNA polymerase, dNTPs, PCR buffer, $MgCl_2$, passive reference dye (ROX) and SYBR Green I dye), 0.45µl (500nM) forward primer, 0.45µl reverse primer, 10.6µl RNase/DNase free water and 1µl cDNA template (Table 3.4). The 20µl reaction mix for the reference gene consisted of 10µl SYBR Green PCR Master Mix (Applied Biosystems), 300nM of resuspended reference gene primer mix, 8µl of RNase/DNase free water and 1µl of diluted cDNA (10x diluted cDNA from samples) (Table 3.5).

Table 3.4: Components for the PCR Reaction.

Component	Volume (µl)
SYBR Green	12.5
Forward Primer (500nM)	0.45
Reverse Primer (500nM)	0.45
RNase/DNase free water	10.6
cDNA	1

Table 3.5: Mastermix for the assessment of reference genes.

Component	Volume (µl)
SYBR Green	10
Primer (300nM)	1
RNase/DNase free water	8
cDNA	1

The reaction mix was centrifuged at 1000 rpm for 5 min at 4°C. Reactions were run in duplicate or triplicate using an ABI PRISM® 7000 real-time PCR system (Applied Biosystems, Warrington, UK). RT- samples were used to detect any genomic DNA contamination. A 96 well optically clear microtitre plate (Applied Biosystems) was utilized for analysis. The plate was spun down in a centrifuge before it was loaded into the real time PCR machine. PCR conditions started with a denaturation step of 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min for denaturation, annealing and primer extension. A dissociation curve was generated and melting curve analysis was performed to confirm the presence of specific products. In order to check for the PCR efficiency of the two primer pairs for the analysis of the ECS serial cDNA dilutions were made and a standard curve of Ct values was generated for each primer pair. The log of input cDNA was plotted against the Ct value and the PCR efficiency calculated according to the following formula:

Efficiency = 10 ^ (-1/slope of standard curve).

3.2.3.10 Selection of Reference Genes

To normalise the expression of the GOI, expression levels are usually assessed in relation to a reference gene which should have stable gene expression. Twelve reference genes were tested. It is important to choose the correct reference gene(s) for the specific experiments as it should not be affected by the experiment. Commonly used reference genes, like GAPDH and ß-actin, can vary across different tissues and with different experimental settings and therefore may be inappropriate for normalisation of gene expression (Tricarico et al. 2002). There is no universal reference gene and therefore selection of these genes should be undertaken for each experimental setting to get an accurate normalisation (Vandesompele et al. 2002). To find the appropriate reference gene or genes for the tissues in the experiment here the geNorm™ Reference Gene Selection Kit with 12 reference genes (PrimerDesign, Southhampton, UK) was employed. The reference genes are listed in Table 3.6.

Table 3.6: 12 human reference genes tested for human trophoblast tissue with their Genbank number and function.

Reference gene	Abbreviation	Genbank Number	Function
ß-actin	ACTB	NM_001101.3	Cytoskeletal structural protein
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	NM_002046.3	Oxireductase in glycolysis and gluconeogenesis
Ubiquitin C	UBC	NM_021009.5	Protein degradation
ß-2-microglobulin	B2M	NM_004048.2	Beta chain of major histocompatibility complex class I molecules
Phospholipase A2	YWHAZ	NM_001135699.1	Signal transduction by binding to phosphorylated serine residues on a variety of signalling molecules
Splicing factor 3a, subunit1	SF3A1	NM_200094.1	Involved in mRNA splicing
18S rRNA gene	18S	NR_003286.2	Small ribosomal subunit
Cytochrome c-1	CYC1	NM_001916.3	Subunit of electron transport chain
Eukaryotic translation initiation factor 4A, isoform-2	EIF4A2	NM_001967.3	Binds mRNA to the ribosome
Succinate dehydrogenase complex	SDHA	NM_004168.2	Electron transporter in the TCA cycle and respiratory chain
Topoisomerase (DNA) 1	TOP1	NM_003286.2	Enzyme to regulate winding of DNA
ATPsynthase	ATP5B	NM_001686.3	Catalysis ATP synthesis

The data obtained from the qRT-PCR were used to evaluate the gene stability with two freely available and downloadable software programmes GeNorm (version 3.4, Center for Medical Genetics, Ghent University, Belgium. http://medgen.ugent.be/genorm) and Normfinder (version 0953, Molecular Diagnostics Laboratory, Dept of Clinical Biochemistry, Aarhus University Hospital, Aarhus, Denmark, www.mdl.dk/publicationsnormfinder/). GeNorm ranks the reference genes according to their stability and calculates the variation of reference gene stability in order to establish the optimal number of reference genes necessary for normalisation. The M-value is defined as the average pair wise variation of a gene with all other control genes. Genes with the lowest M-value are the most stable genes (Vandesompele et al. 2002). Normfinder looks at the intra and inter group variation of the sample subgroups and calculates a gene expression stability value to each reference gene (Andersen, Jensen & Orntoft 2004). The most stable reference genes are used to normalise the Ct values.

The ΔΔCt relative quantification method was used for analysis (Livak, Schmittgen 2001), where the Ct value of the samples of interest are expressed relative to the Ct value of the reference genes (see above). When more than one reference gene is employed for normalisation, the geometrical mean of the Ct values of the different reference genes is used for normalising these results.

3.2.4 Western Blotting

Western Blotting was used for qualitative and semi-quantitative analysis of protein expression of the endocannabinoid enzymes NAPE-PLD and FAAH in human trophoblast.

3.2.4.1 Protein Extraction

Trophoblast tissue stored in RNA later was weighed and the homogenization buffer (20 mM HEPES buffer, 2 mM EDTA, 10 mM KCl, 1.5 mM $MgCl_2$; 1:500 dilution) was added together with Protease Inhibitors benzamide, pepstatin, leupeptin and phenylmethylsulfonyl fluoride (PMSF) (1:1000) to inhibit protease activity. The tissue was then homogenized using an Ultra Turrax homogenizer for 30 s. The homogenates

were then centrifuged at 3000 rpm for 10 min at 4°C, the supernatant was decanted to a fresh tube and re- centrifuged at 13000 rpm for 30 min at 4°C. The supernatants were taken and total protein quantification performed or stored at -80°C until required.

3.2.4.2 Protein Quantification

Protein was quantified using the Bio-Rad Protein Assay (Bradford Reagent, Catalogue number: B6916, Sigma) according to the manufacture's protocol. Bovine serum albumin (BSA, Catalogue number: A2153, Sigma) was used as a protein standard at concentrations of 0.06, 0.125, 0.25, 0.5,1 and 2µg/µl (Table 3.7). The samples were diluted to 1:20 with 0.1M NaOH and 20µl of the diluted sample or standard in duplicate was assayed with 1ml dye reagent. The absorbance was detected at 595 nm in a spectrophotometer after 15 min of incubation at room temperature. The absolute protein concentration was calculated from the standard curve created from the serial dilution of BSA and the absorbance of their protein concentration.

Table 3.7: BSA Standard Curve

Volume of BSA	Volume of 0.1M NaOH (µl)	Amount of Protein
2mg BSA	1000	2 mg/ml
300 µl of 2mg/ml	300	1 mg/ml
300 µl of 1mg/ml	300	0.5 mg/ml
300 µl of 0.5mg/ml	300	0.25 mg/ml
300 µl of 0.25mg/ml	300	0.125 mg/ml
300 µl of 0.125mg/ml	300	0.06 mg/ml
0	300	0

3.2.4.3 Preparation of the Gel

Polyacrylamide gels (1.5 mm thick) were used for the separation of protein. 10% running gel and 5% stacking gel were prepared as summarized in Table 3.8.

Table 3.8: Composition of SDS-polyacrylamide gels.

Components (ml)	Running Gel (10%)	Stacking Gel (5%)
H_2O	7.9	8.25
30% Acrylamide Mix	6.7	1.95
1.0 M Tris (pH 6.8)	0	1.5
10%SDS	0.2	0.12
10% Ammonium Persulfate	0.2	0.12
TEMED	0.008	0.012
1.5 M Tris (pH 8.8)	5.0	0
Total Gel Volume	20	12

TEMED and 10% ammonium persulfate (APS) were added just before pouring the gel in order to start polymerisation. 0.5ml of isopropanol was added on top of the running gel to ensure a level interface between the two gels. The resolving gel set within 20 min and the isopropanol was removed. TEMED and 10% APS were then added to the stacking gel before pouring on top of the running gel and allowing to polymerize with the 1.5 mm comb in place to set the wells required for loading samples.

3.2.4.4 Separation of Proteins by SDS-polyacrylamide Gel

40µg of sample lysate in sample buffer was denatured at 100°C for 5 min and loaded into each well. The positive controls were kindly provided by Drs. Jon Willets and Anthony Taylor (Reproductive Sciences Section, Department of Cancer Studies and Molecular Medicine, University of Leicester). Positive controls were human recombinant NAPE-PLD and FAAH proteins prepared from HEK-293 cells transfected with expression plasmids containing human cDNA for NAPE-PLD (Okamoto et al. 2004a) and FAAH (Giang, Cravatt 1997). Proteins were denatured using the anionic detergent 1x sodium dodecyl sulphate (SDS) buffer (Tris HCL 100 mM, EDTA 1%, NaCl 200 mM, Igepal 1.25%, SDS 0.2% w/v). SDS breaks the three dimensional structure of proteins and coats them with negative charges. The samples were centrifuged and the supernatant aliquoted, snap-frozen in liquid nitrogen and stored at -

80°C. 30 µg of the protein suspension was added to SDS sample buffer (final concentration 63 mM Tris, pH 6.5, 100 mM dithiothreitol, 1% SDS, 11.6% glycerol and 0.02% bromophenol blue) and loaded into a well. 10µl of pre-stained SDS marker (Catalogue number: SDS7B2, Sigma) and 7.5µl of biotinylated protein marker (Catalogue number: 7727S, Cell Signalling) were loaded to one well. The buffers used for Western Blotting are listed in Table 3.9. The tank was filled with running buffer. The gel was run at 140 Volts (Minigel apparatus (BioRad)) for 1- 2 h until the coloured marker had just run off of the gel.

Table 3.9: Composition of buffers used for Western Blotting.

Buffer	Reagent	Volume/ Weight
2x Sample Buffer (pH 6.8) (10ml)	0.125M Tris	2.5ml
	4% SDS	4ml
	20% Glycerol	2ml
	10% 2-Mercaptoethanol	1ml
	Bromophenol Blue	0.01g
	distilled H_2O	0.5ml
Running Buffer (1L)	stock	100ml
	distilled H_2O	900ml
Transfer Buffer (pH 8.3) (1L)	25mMTris	3g
	191mM Glycine	14.4g
	Methanol	200ml
Washing Buffer (TBS-T) (pH 7.5) (1L)	5M NaCl	30ml
	1M Tris	20ml
	Tween 20	500µl
	distilled H_2O	Made up to 1L
Semi-dry blotting buffer (1L)	Tris base	5.8g
	Glycine	2.9g
	SDS 0.037%w/v	0.37g
	Methanol 20%	200mls
	distilled H_2O	Made up to 1L

3.2.4.5 Protein Transfer and Blocking

A nitrocellulose membrane was cut to the size of the gel, soaked in methanol for 30 s and distilled water for 4 min and then washed in semi-dry blotting buffer. Two filter papers (Catalogue number: 1703968, Bio-Rad) cut to the same size as the gel were also washed in semi-dry buffer. One filter paper was placed on the wet semi-transfer plate with the nitrocellulose membrane on top of it. The gel and the second filter paper were put on top of the nitrocellulose membrane (Catalogue number: EP4HY00010, Anachem) (Figure 3.9). Air bubbles were removed. It is important not to dry out the membrane during transfer. The lid was placed on the sandwich and a potential difference of 21V applied for 25 min to transfer proteins from the gel to the nitrocellulose membrane.

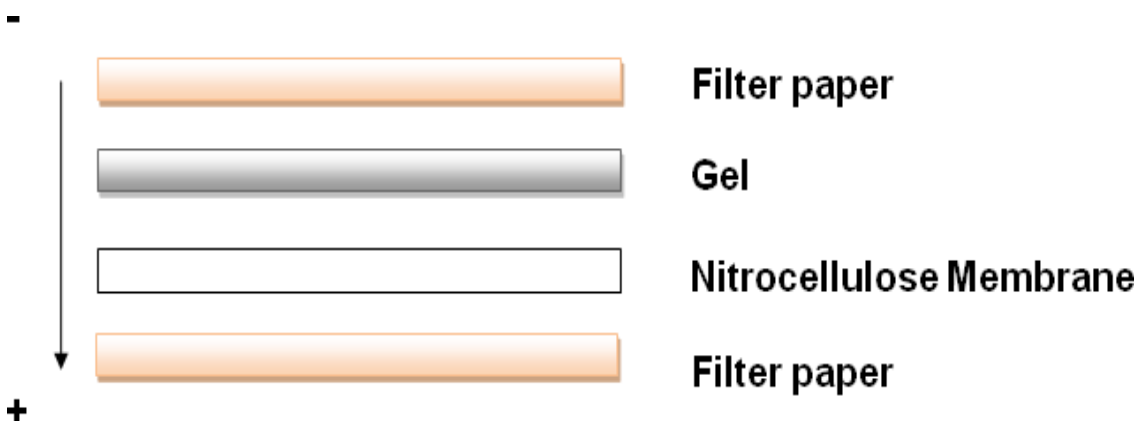

Figure 3.9: Schematic representation of protein transfer assembly.
The nitrocellulose membrane and gel are sandwiched by two filter papers. The current enables to transfer proteins from the gel to the nitrocellulose membrane.

3.2.4.5.1 Immuno-detection of Proteins

After protein transfer the membrane was washed in blocking solution (5% non-fat milk in TBS-T) for 60 min at room temperature on a rocker in order to remove any non specific binding. Thereafter the membrane was incubated with the diluted primary antibody overnight at 4°C. The primary antibodies and their dilutions are listed in Table 3.10.

The following day the membrane was washed in TBS-T for 45 min and then incubated with secondary antibody and anti biotin horseradish peroxidise-linked antibody (1:2000) in blocking solution for 60 min on a rocker. This was followed by washes with TBS-T. All the secondary antibodies and the reagents for Western Blotting are detailed in Table 3.11 and Table 3.12.

Table 3.10: Primary antibodies used in Western Blotting.

Antibody	Company	Product Number	Type	Dilution
Anti- NAPE-PLD	Cayman	10306	Rabbit polyclonal	1:250
Anti- NAPE-PLD	Abcam	77474	Rabbit polyclonal	1:500
Anti-FAAH	Cayman	101600	Rabbit polyclonal	1:500
Anti-FAAH	Abbiotec	100189	Rabbit polyclonal	1:500
Anti-FAAH	Santa Cruz	Sc-26428	Goat polyclonal	1:100
Anti-CB1	Cayman	10006590	Rabbit polyclonal	1:100
Anti-TRPV1	Santa Cruz	Sc-12498	Goat polyclonal	1:100
Anti-TRPV1	Tocris	2233	Rabbit polyclonal	1:1000
Anti- ß-Tubulin	Santa Cruz	Sc-55529	Mouse monoclonal	1:100
Anti-GAPDH	Abcam	9484	Mouse monoclonal	1:4000

Table 3.11: Secondary antibodies used in Western Blotting

Antibody	Company	Product Number	Dilution
Anti rabbit IgG/HRP	Sigma	A6154	1:1000
Donkey anti-Goat IgG/HRP	Santa Cruz	SC-2020	1:10000
Anti-mouse IgG/HRP	Sigma	A4416	1:1000
Anti biotin horseradish peroxidise linked antibody	Cell Signalling	7075	1:2000

Table 3.12: Reagents for Western Blotting

Reagent	Company	Product Number
Benzamide	Sigma	135828
Pepstatin	Sigma	77170
Leupeptin	Sigma	L2884
PMSF	Sigma	78830
Trizma Base	Sigma	T6066
Isopropanol	Sigma	109827
Ultrapure Protoflowgel arylamide	Flow Bioscience	H16996
TEMED	Sigma	T7024
SDS	Fisher	BPE166-100
Methanol	Fisher	M/4000/PB17
Tween 20	Fisher	BPE337-500
DTT	Sigma	D9163
Ultra Pure 10x Tris/Gly/SDS (Running buffer)	Geneflow	B9-0032
Ammonium persulfate (APS)	Sigma	215589
Bromophenol blue	Sigma	B5525

3.2.4.6 Optimization of Antibodies for Western Blotting

The Western Blotting method was optimized using different dilutions for each of the primary and secondary antibodies. The anti-CB1 antibody was abandoned as it yielded several bands at the expected molecular weight. This was consistent with the published observations of Grimsey et al. using a number of commercially available anti-CB1 antibodies (Grimsey et al. 2008). No TRPVR1 was detected using the anti-TRPV1 antibody and after several attempts with different dilutions this part of the experiments with this antibody was abandoned. A summary of the optimization process can be seen in Table 3.13.

Table 3.13: Optimization of primary antibodies.

Primary Antibody	Dilution	Secondary Antibody	Dilution	Result
Anti-NAPE-PLD (Cayman)	1:250	anti-rabbit	1:1000	Good bands
Anti-NAPE-PLD (Abcam)	1:500	anti-rabbit	1:1000	No bands
Anti-FAAH (Abbiotec)	1:500	anti-rabbit	1:1000	Very light bands with background noise
Anti-FAAH (Santa Cruz)	1:100	anti-goat	1:1000	Very light bands
Anti-FAAH (Cayman)	1:500	anti-rabbit	1:1000	Good bands
Anti-CB1 (Cayman)	1:100	anti-rabbit	1:1000	No bands
Anti-TRPV1 (Santa Cruz)	1:100	anti-goat	1:20000	Light bands
Anti-TRPV1 (Tocris)	1:100	anti-rabbit	1:20000	no bands

3.2.4.7 Chemiluminescence Detection

Chemiluminescence is the emission of light due to a chemical reaction. It is based on the horseradish peroxidase (HRP) catalysed oxidation of luminol in the presence of peroxide. The light emission is a result of the excitation decay of the luminol. Chemical enhancers like phenol are used to enhance the light emission to be much prolonged and very intense (Figure 3.10). The reagents A and B from the enhanced Chemiluminescence (ECL) Detection kit (Catalogue Number: RPN 2109, GE Healthcare, UK) were prepared in a 1:1 solution and pipetted onto a glass plate. The membrane was placed face downwards onto the glass plate and incubated for 5 min. The membrane was then wrapped in saran wrap and exposed to X-Ray film for various time durations depending on the band intensity. The film (Catalogue Number: 28906844, GE Healthcare, UK) was developed using an AGFA Curix 60 film developer. Signal intensities were quantified by using the GeneGnome image analysis system and software (Syngene, Cambridge, UK).

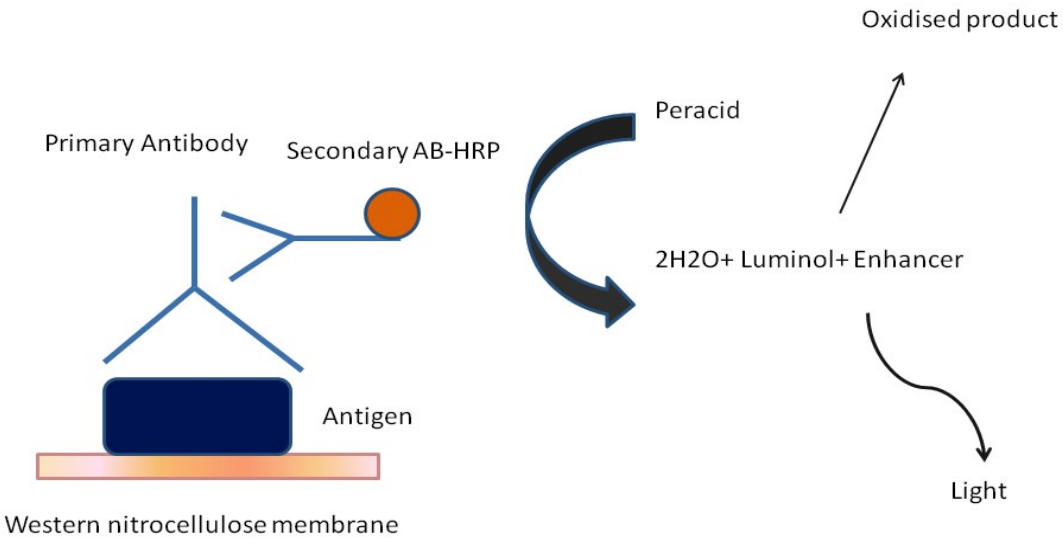

Figure 3.10: Schematic overview of principles of chemiluminescence detection.
The primary antibody is attached to the antigen on the membrane. The secondary antibody is linked with horseradish peroxidase (HRP). The oxidation of luminol by horseradish peroxidase (HRP) in the presence of an enhancer results in light emission which can be captured by X-ray film.

3.2.4.8 Loading Control for Western Blotting

Two primary antibodies (anti-β-Tubulin and anti-GAPDH) were checked to identify a suitable control protein to assess loading accuracy. These proteins have expression levels that remain unaltered under various conditions and therefore the expression levels of the loading control do not change in different samples. Using this method verifies that all the lanes have been equally loaded. This is important for confirmation of any changes in the expression levels of a protein in different samples. β -Tubulin is a protein present in the whole cell and cytoplasm with a molecular weight of 55kD. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is also found in the whole cell and cytoplasm and plays a role in glycolysis. The molecular weight of GAPDH is 40kD.

Different dilutions of primary and secondary mouse antibody were used to optimize β–Tubulin and GAPDH detection (Table 3.14). As anti-GAPDH worked better with the samples the use of GAPDH as the loading control was continued.

Table 3.14: Dilutions of antibodies used for loading control.

Primary Antibody	Dilution	Secondary Antibody	Dilution	Result
Anti-β-Tubulin	1:500	anti-mouse	1:1000	Too much background
Anti-β-Tubulin	1:500	anti-mouse	1:2000	Too much background
Anti-β-Tubulin	1:100	anti-mouse	1:5000	Bands too light
Anti-GAPDH	1:4000	anti-mouse	1:1000	Bands clearly visible

3.2.4.9 Data Analysis and Statistics

Statistical analysis was done using GraphPad Prism 5.0. The unpaired t-test (two groups of variables) and one way ANOVA (for more than two groups of variables) followed by Bonferroni ad hoc test were used for data with Gaussian distribution. For non-parametric data the Mann Whitney U-test (for two groups of variables) or Kruskal Wallis Test (for more than two groups of variables) were used. All the tests were two-tailed and differences were considered to be statistically significant for a p-value<0.05.

3.3 RESULTS

3.3.1 NAE Levels in Tissues from TOP

3.3.1.1 NAE Levels in Trophoblast

Trophoblast tissue obtained following 17 MTOP and 13 STOP as controls were anlaysed for AEA, OEA and PEA levels. Levels of the three NAE in trophoblast were 7.24±1.06, 26.34±2.28 and 76.36±5.21 pmol/g (mean ± SEM) for AEA, OEA and PEA respectively. There was a negative correlation between gestational age and NAE levels in trophoblast of the pooled data set (MTOP and STOP combined); this was significant for OEA and PEA (AEA: R^2=0.11, p=0.068; OEA: R^2=0.156, p=0.031; PEA: R^2=0.202, p=0.016) (Figure 3.11). However, there was no significant correlation between gestational age and NAE levels in trophoblast when the samples were sub-divided into MTOP (AEA: R^2=0.00036, p=0.942; OEA: R^2=0.037, p=0.457 and PEA R^2=0.126, p=0.177) (Figure 3.12) and STOP (AEA: R^2=0.0588, p=0.42; OEA: R^2=0.0085, p=0.764 and PEA R^2=0.8, p=0.348) (Figure 3.12).

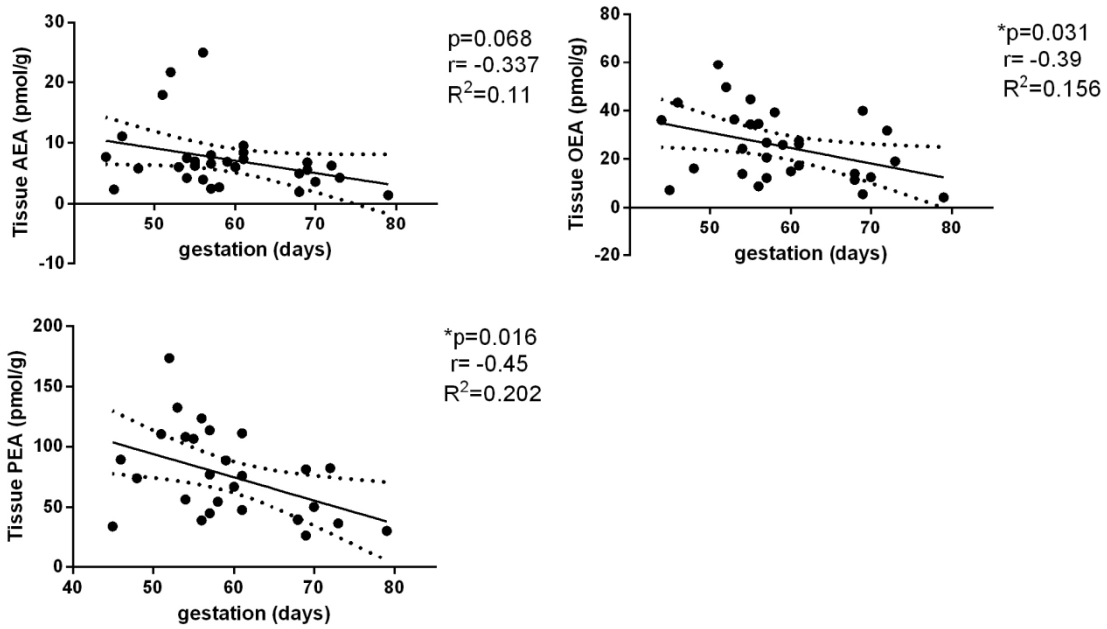

Figure 3.11: Correlation between NAE levels in first trimester trophoblast and gestation (n=30).
Significance was determined by Spearman Correlation for AEA and PEA and Pearson Correlation for OEA (r= correlation coefficient).

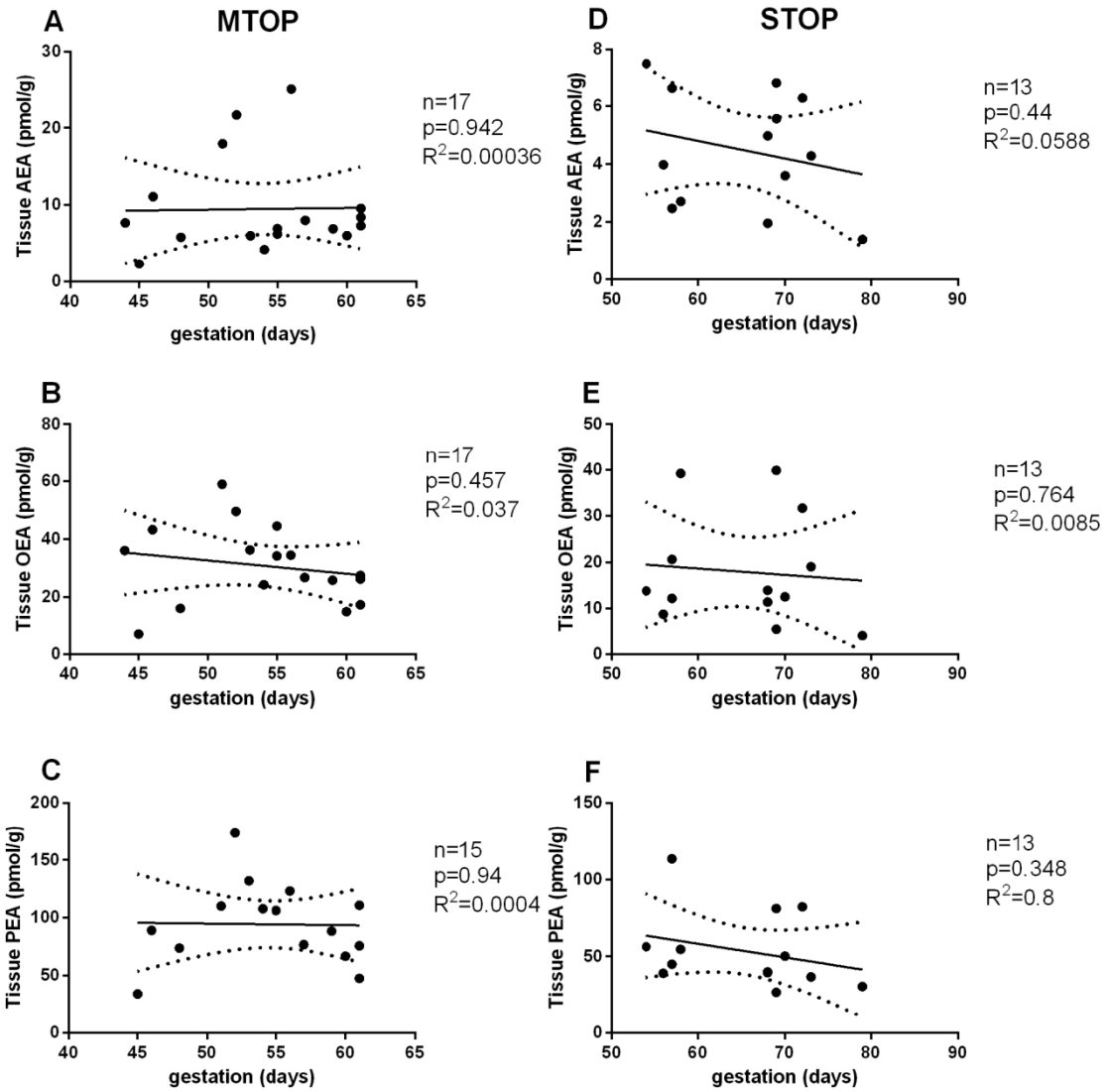

Figure 3.12: Correlation between NAE levels in trophoblast after MTOP, STOP and gestation.
Spearman Correlation was used for (A, D-F)) and Pearson Correlation for (B-C) (linear regression analysis).

When NAE levels in trophoblast were related to plasma obtained from the same patients after TOP there was no significant correlation (Figure 3.13). When these were sub-divided into MTOP and STOP groups, there was a significant negative correlation between OEA levels in trophoblast and plasma in the MTOP group (p=0.025, R^2=0.33): high trophoblast levels were correlated with low plasma OEA levels. However, there was no significant relationship for AEA and PEA (Figure 3.14).

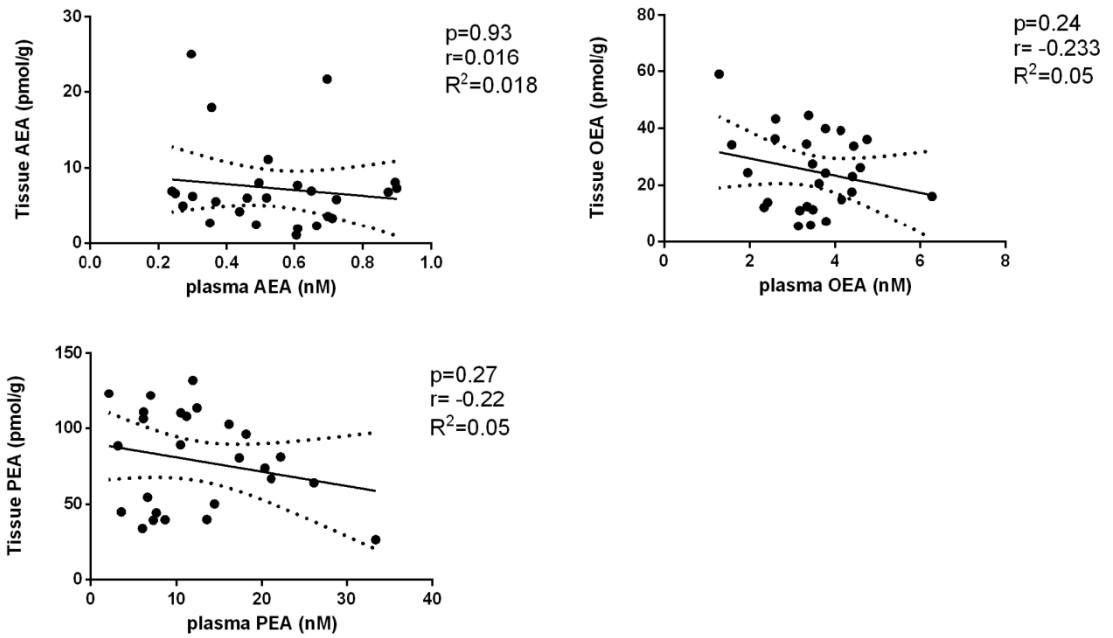

Figure 3.13: Correlation between NAE levels in trophoblast and plasma levels of MTOP and STOP combined (n=26).
Spearman correlation was used for AEA levels and Pearson Correlation for OEA and PEA levels.

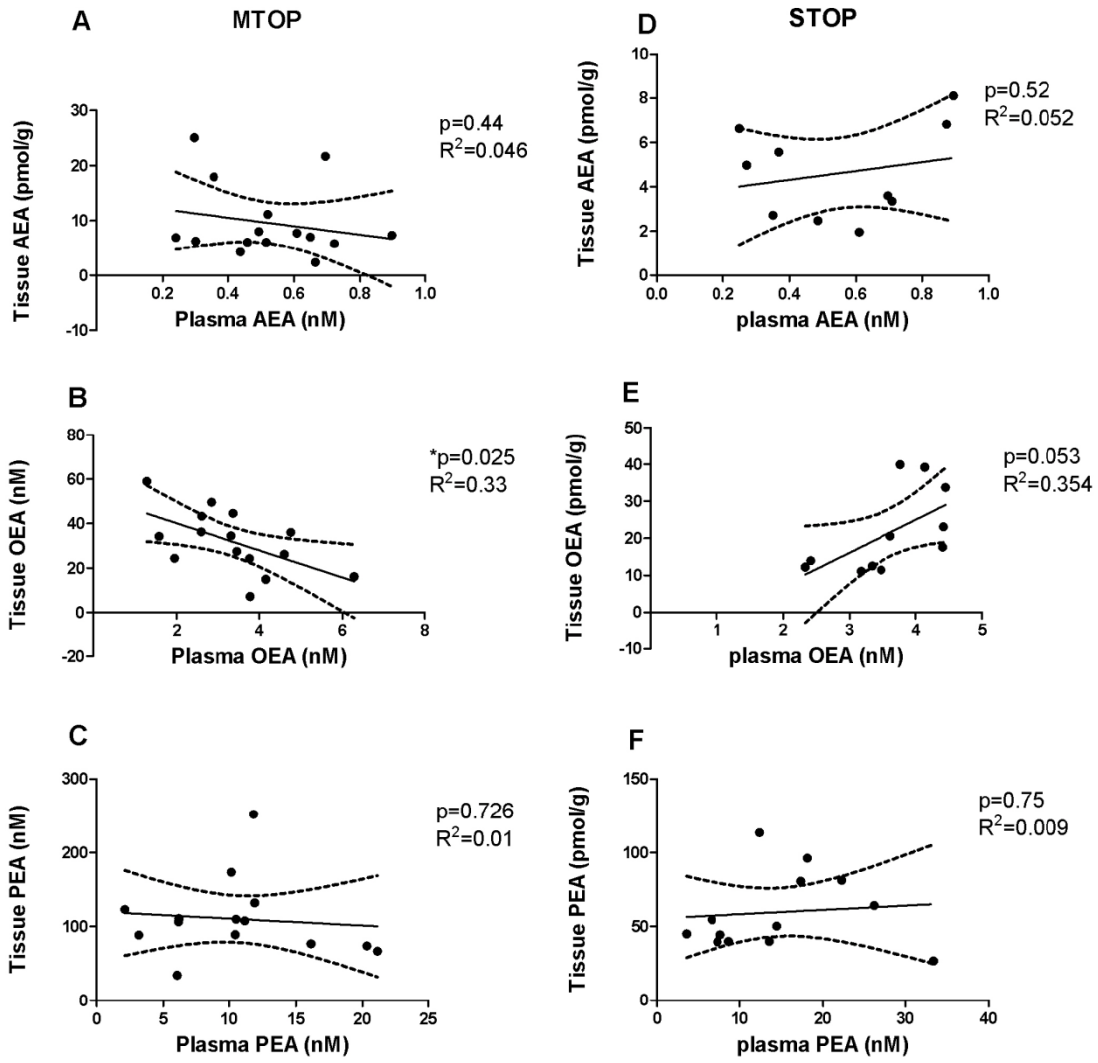

Figure 3.14: NAE levels in trophoblast and plasma from women undergoing MTOP (n=15) and STOP (n=11).

A significant correlation could only be established between OEA levels in trophoblast and plasma (p=0.025, R^2=0.33) (B). The significance was calculated by Spearman (A) and Pearson Correlation (B-F).

In order to compare NAE levels in trophoblast from MTOP and STOP, a matched analysis was performed of 13 MTOP and 9 STOP samples. The demographic data are shown in Table 3.15. Trophoblast AEA, OEA and PEA levels were significantly higher after MTOP when compared to STOP ($p<0.0062$, $p<0.016$ and $p<0.0029$) (Figure 3.16).

Table 3.15: Demographic data of women with MTOP and STOP.

	MTOP (n=13)	STOP (n=9)	p-value
Age (years)	28 (20-39)	24 (17-35)	p=0.14
BMI (kg/m^2)	23.3 (20.6-30)	24.1 (19.2-29.4)	p=0.54
Ethnicity	White n=10 Asian n=3	White n=7 Asian n=2	
Gestation (days)	57 (51-61)	62 (54-69)	p=0.08

The number of samples (n) is given for each group. Data presented as a mean (range). Mann-Whitney U-test was used for the analysis of BMI and gestation whereas student's t-test was applied for the analysis of age.

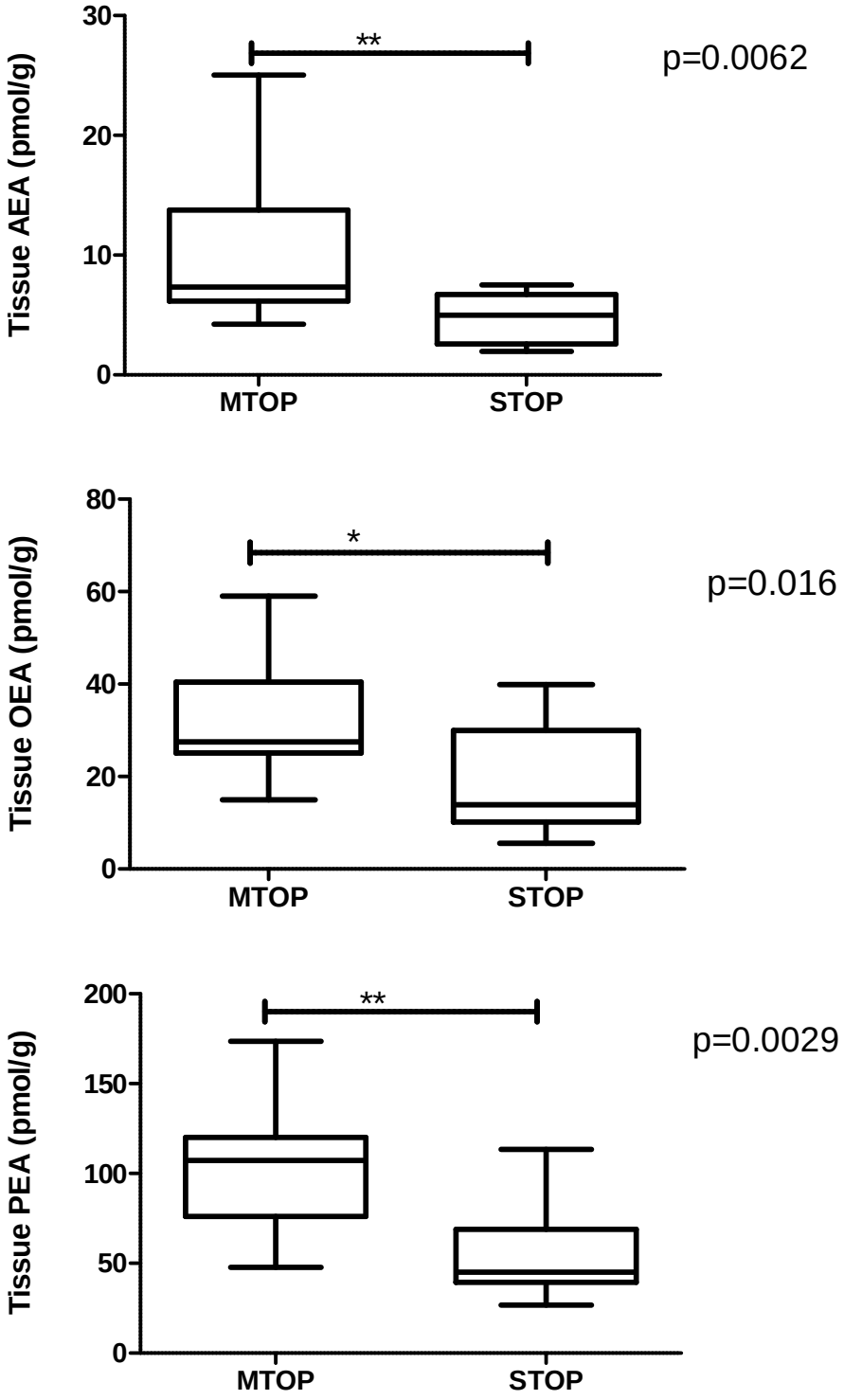

Figure 3.15: NAE levels from trophoblast after MTOP and STOP.
Trophoblast NAE levels were significantly increased in MTOP (n=13) compared to STOP (n=9). The boxplot is representing lower quartile, median and upper quartile with whiskers from minimum to maximum. The Mann-Whitney U-test was used for AEA and OEA and the unpaired Students t-test for PEA.

3.3.1.2 NAE Levels in Trophoblast, Decidua and Membrane

NAE levels were quantified in fetal membranes and decidua obtained from a cohort of volunteers who had TOP. The demographic data can be found in Table 3.16. NAE levels in trophoblast from MTOP were the highest followed by membrane and decidua. AEA and PEA levels in trophoblast from MTOP were significantly higher than in decidua ($p=0.0059$ and $p=0.438$) (Figure 3.16). In the STOP samples, the differences in levels were less distinct between the different tissues; only OEA levels in trophoblast were significantly higher than in decidua ($p=0.035$) (Figure 3.16).

Table 3.16: Demographic data of women undergoing MTOP and STOP and providing membrane, trophoblast and decidua for UHPLC analysis.

	Membrane		Trophoblast		Decidua	
	MTOP	**STOP**	**MTOP**	**STOP**	**MTOP**	**STOP**
Age (years)	21 (19-29)	25 (18-39)	27(18-39)	25 (18-35)	25 (20-32)	26 (19-39)
BMI (kg/m^2)	21.5 (18-25)	22.9 (19-29.4)	23.3 (18-30)	22.7 (18.4-29.4)	21.9 (18-25.6)	22 (19.2-23.7)
Ethnicity						
White	n=4	n=12	n=11	n=10	n=3	n=9
Asian	n=1	n=1	n=4	n=1	n=1	n=0
Mixed Asian/ White		n=2		n=2		n=1
Gestation (days)	51 (45-56)	65 (57-80)	54 (45-61)	66 (57-80)	54 (48-62)	68 (57-80)

Data is presented as a mean (range).

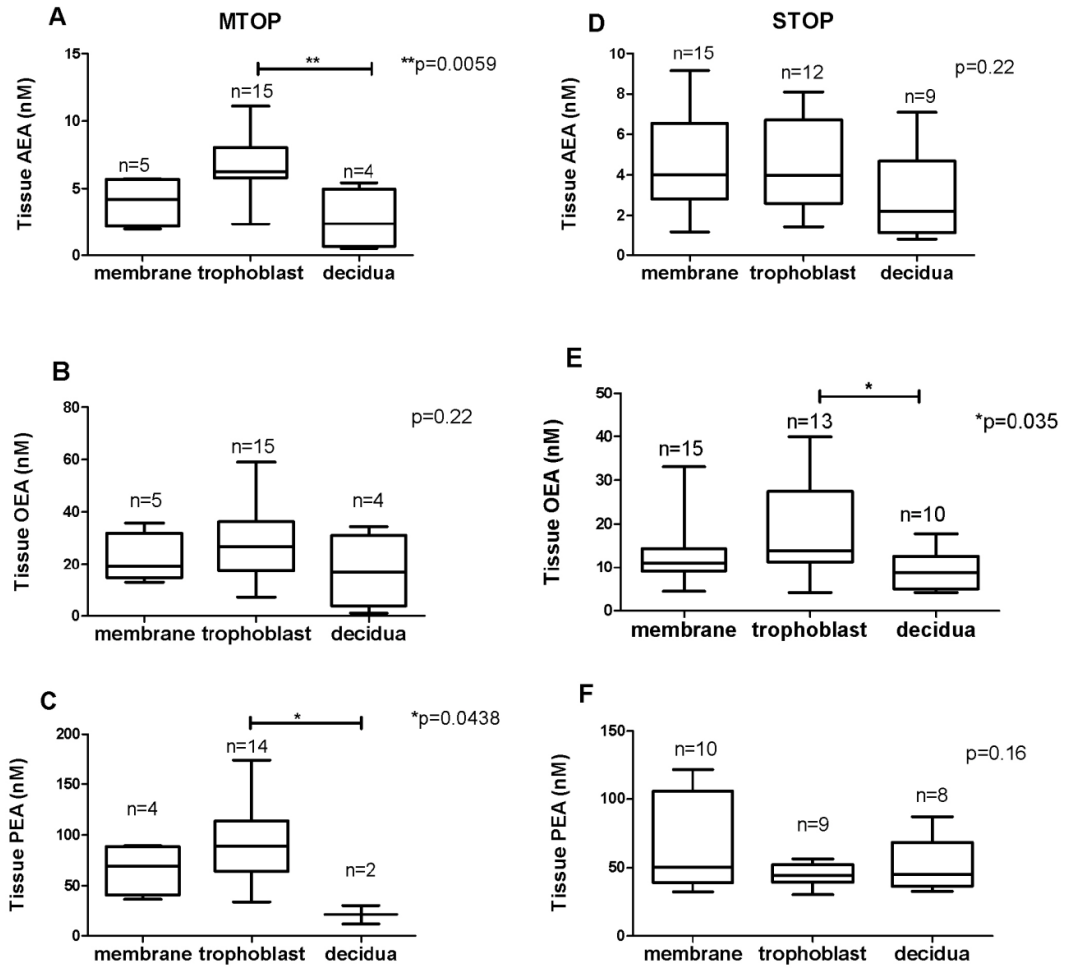

Figure 3.16: NAE levels in membrane, trophoblast and decidua tissues obtained from women undergoing MTOP (A-C) and STOP (D-F).
The boxplot is representing lower quartile, median and upper quartile with whiskers from minimum to maximum. Statistical differences between tissues were investigated using one way ANOVA with Bonferroni post-hoc test.

3.3.2 Immunohistochemistry

Immunoreactive staining for the cannabinoid enzymes NAPE-PLD and FAAH as well as the cannabinoid receptors CB1 and CB2 were demonstrated in healthy 1st trimester trophoblast as shown previously (Helliwell et al. 2004, Habayeb et al. 2008, Taylor et al. 2011).

Human myometrium was used as a positive control as it is known to express NAPE-PLD and FAAH and the cannabinoid receptors CB1 and CB2 (Brighton et al. 2009). Rabbit IgG and normal goat serum (NGS) were used as negative controls for secondary antibody non-specific binding.

Histomorphometric analysis was performed on trophoblast samples from MTOP (n=6) and STOP (n=5), as described in section 3.2.2.4. The samples were all from gestational age 8 - 9 weeks. The demographic data of the patients are shown in Table 3.17.

Table 3.17: Demographic data of women with MTOP and STOP and histomorphometric analysis of trophoblast tissue.

	MTOP (n=6)	STOP (n=5)	p-value
Age	24 (18-43)	26.8 (19-37)	0.36
BMI	21.50 (19.8-25.3)	21.42 (19.8-23.6)	0.94
Gestational age	58.3 (54-62)	59.2 (54-70)	0.78

Data is presented as mean (range). Significance was calculated using Mann-Whitney U-test for age and gestational age and unpaired Student's t-test for BMI.

3.3.2.1 NAPE-PLD

Immunostaining of NAPE-PLD was detected in 1st trimester trophoblast after both STOP and MTOP. It was mainly localised in the cytotrophoblast and syncytiotrophoblast layers. There was also nuclear staining in the cytotrophoblast. Some nuclei of the mesenchymal core also showed positive staining for NAPE-PLD (Figure 3.17 and 3.18). NAPE-PLD immunoreactivity was more intense in the cytotrophoblast and syncytiotrophoblast of tissues obtained from STOP compared to that from MTOP (Figure 3.19).

3.3.2.2 FAAH

Immunoreactive FAAH was localised in the mesenchymal core, cytotrophoblast and syncytiotrophoblast layers of 1st trimester trophoblast. It was present throughout the cytoplasm of the cells with some nuclear staining (Figure 3.17 and 3.18). Trophoblast obtained after MTOP showed an increased FAAH expression when compared to that after STOP, but this was not statistically significant (Figure 3.19).

3.3.2.3 CB1

CB1 immunoreactivity was present in 1st trimester trophoblast collected after both STOP and MTOP. It was detected in the cytoplasm of syncytiotrophoblast, in the cytoplasm and nuclei of cytotrophoblast, in the mesenchymal core and the endothelial cells of the blood vessels. However, there was no CB1 immunostaining in either fetal blood cells or maternal plasma cells (Figure 3.17 and Figure 3.18). CB1 expression in trophoblast collected after MTOP was stronger when compared to tissue collected after STOP, but this was not statistically significant (Figure 3.19).

3.3.2.4 CB2

CB2 was also detected in trophoblast from 1st trimester pregnancies. However the staining was weak when compared to CB1 staining in trophoblast. CB2 immunoreactivity was demonstrated in cytoplasm of cytotrophoblast and syncytiotrophblast and the mesenchymal core (Figure 3.17 and Figure 3.18). The expression of CB2 was significantly increased in trophoblast collected after MTOP when compared to that collected after STOP ($p=0.031$) (Figure 3.19).

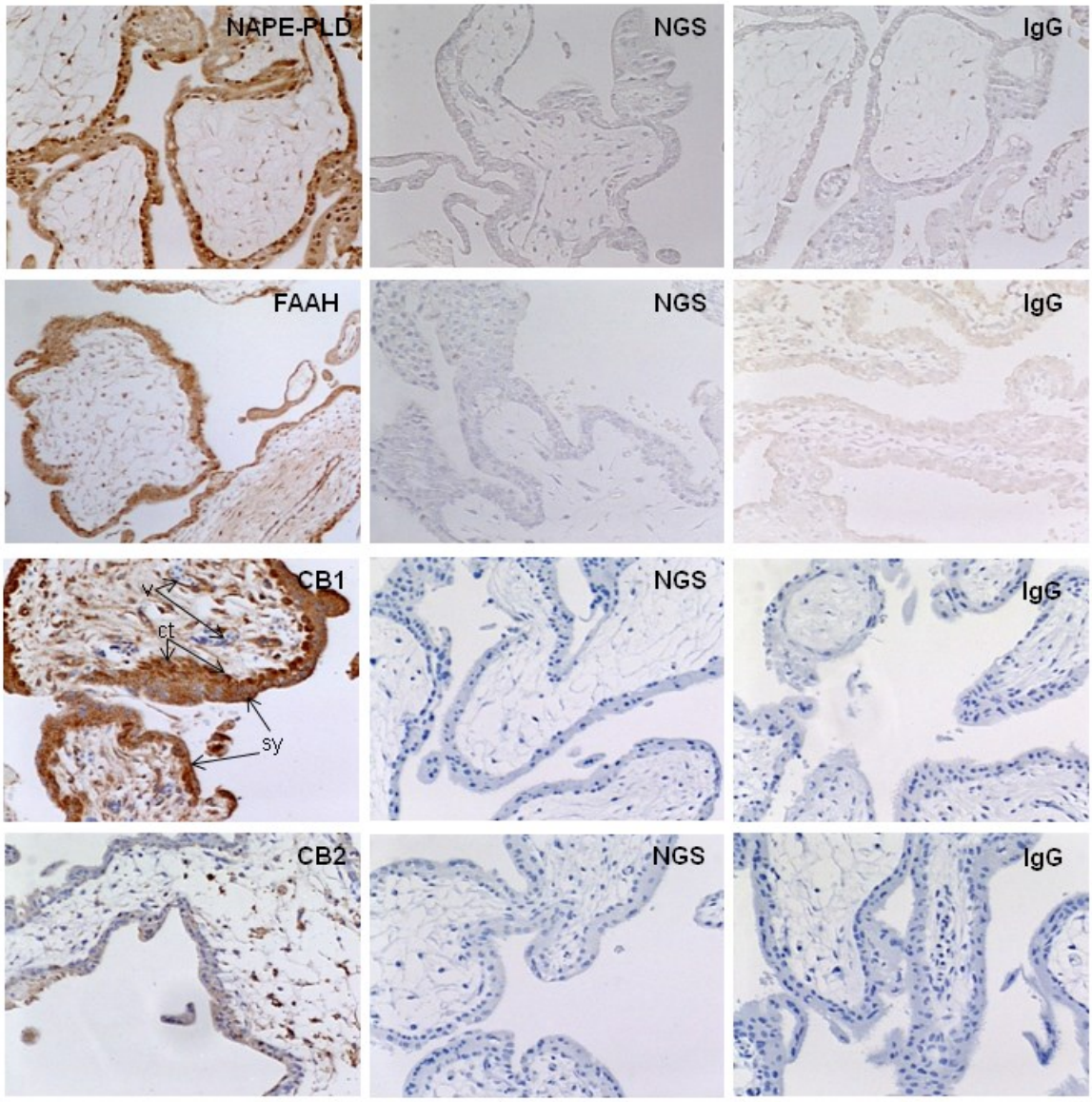

Figure 3.17: Specificity of the ECS antibodies.
Staining of NAPE-PLD, FAAH, CB1 and CB2 is illustrated in first trimester trophoblast following STOP. NAPE-PLD was found in the cytoplasm and some nuclei of syncytiotrophoblast (sy) and cytotrophoblast (ct). FAAH was also present in both syncytiotrophoblast and cytotrophoblast. CB1 was observed in cytoplasm of syncytiotrophoblast and nuclei and cytoplasm of cytotrophoblast. There was no CB1 staining in fetal blood vessels (v). CB2 was visible in the cytoplasm of syncytiotrophoblast and cytotrophoblast. Normal goat serum (NGS) (middle panel) and rabbit IgG isotype (right panel) were used as negative controls at equivalent concentrations. Images are representative of trophoblast samples at 8 and 9 weeks gestation. All images were captured at 200x magnification.

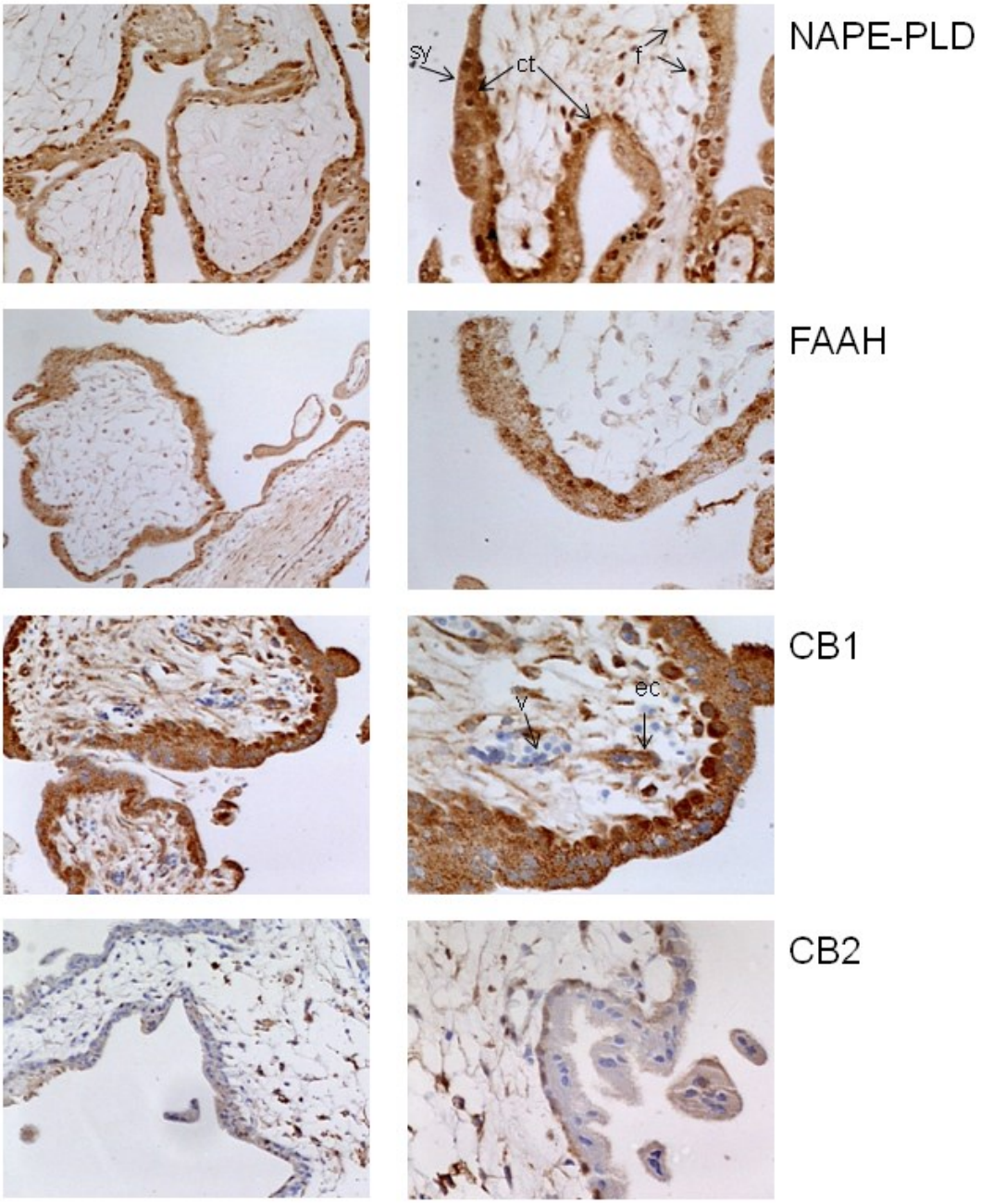

Figure 3.18: Immunoreactivity of NAPE-PLD, FAAH, CB1 and CB2 in first trimester trophoblast.

Immunostaining for NAPE-PLD was detected in cytoplasm and some nuclei of syncytiotrophoblast (sy) and cytotrophoblast (ct) and fibroblast (f) cells. FAAH immunoreactivity was confined to the syncytiotrophoblast and cytotrophoblast. CB1 staining was seen in the cytoplasm of the syncytiotrophoblast and the cytoplasm and nuclei of cytotrophoblast. It was also detected in the cytoplasm of fetal endothelial cells (ec). CB2 was visible in the cytoplasm of the syncytiotrophoblast and the cytotrophoblast. All the tissues are from surgical termination of pregnancies at 8 weeks gestation. Images are representative of trophoblast samples at 8 and 9 weeks gestation. Images on the left panel were taken at 200x magnification and images on the right panel were captured at 400x magnification.

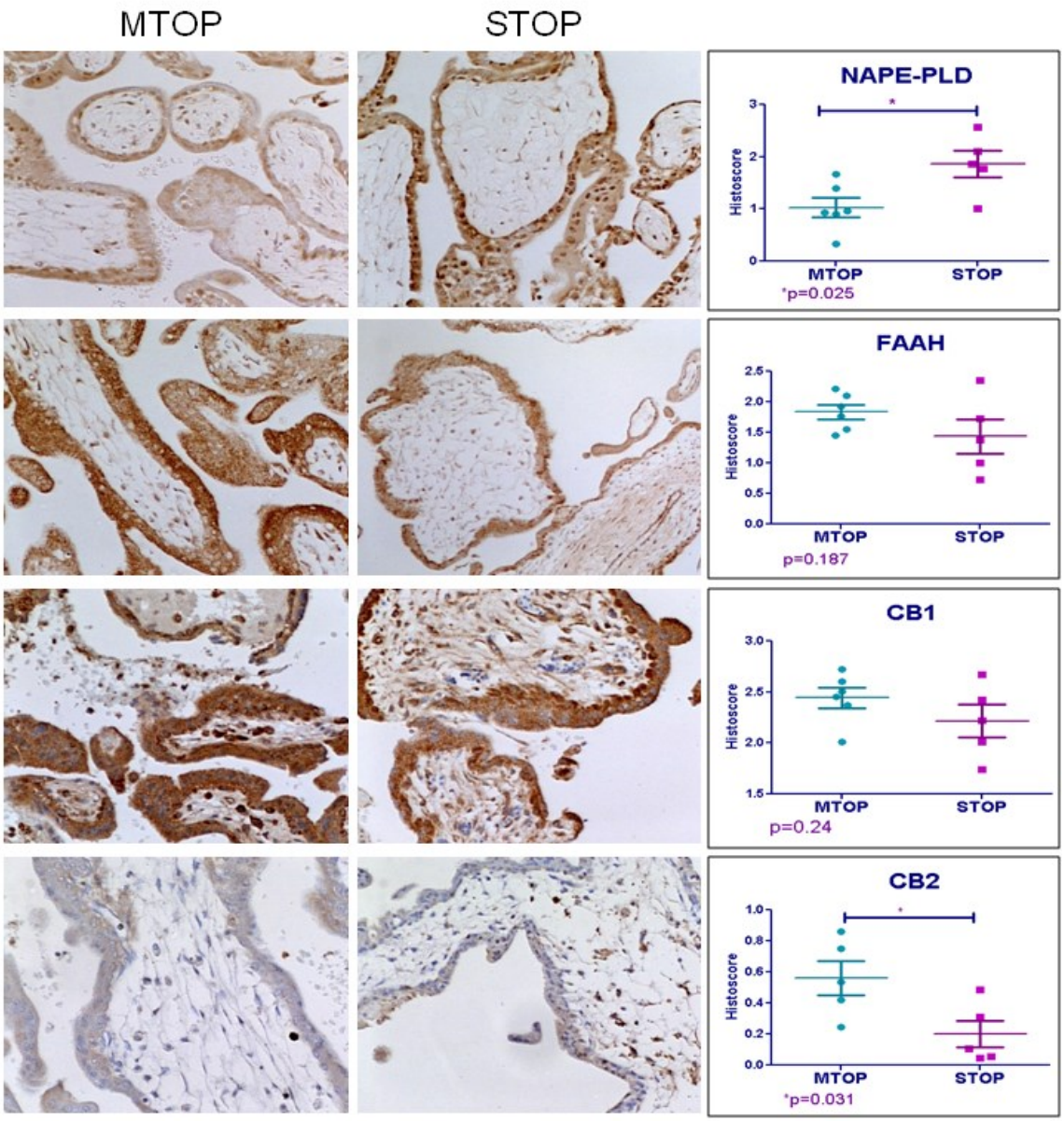

Figure 3.19: Immunohistological localisation and staining intensities of NAPE-PLD, FAAH, CB1 and CB2 in first trimester trophoblast collected after medical and surgical termination of pregnancies.

Images were taken at 200x magnification and are representative of trophoblast samples from 8 and 9 weeks gestation. The histoscore value for each antigen is shown as mean ± SEM. Each histoscore consists of 10 random fields/ slide for 6 MTOP and 5 STOP samples. NAPE-PLD expression was significantly decreased in trophoblast from MTOP when compared to STOP. FAAH and CB1 expression did not change significantly. CB2 expression in tissue from MTOP was significantly higher when compared to STOP. Unpaired student's t-test was used to compare the two groups.

3.3.3 PCR

3.3.3.1 Agarose Gel Electrophoresis for RNA Quality Assessment

The first tissue samples were collected and transported on dry ice to the Histopathology department, where they were separated into decidua, trophoblast and fetal membranes (see section 3.2.1.2), flash frozen in liquid nitrogen and stored at -80°C until further analysis. RNA was then extracted and assessed for its quality using Agarose gel electrophoresis. Unfortunately all the samples were degraded as can be seen by the smearing and absence of defined bands on Figure 3.20. Therefore all further tissues collected following termination of pregnancies were immediately stored in RNA*later*. The RNA integrity of the samples in RNA*later* was checked with the Agarose gel electrophoresis which confirmed intact RNA in all samples but one sample in lane 5 (Figure 3.20). Further RNA quality assessment was performed with the Bioanalyser.

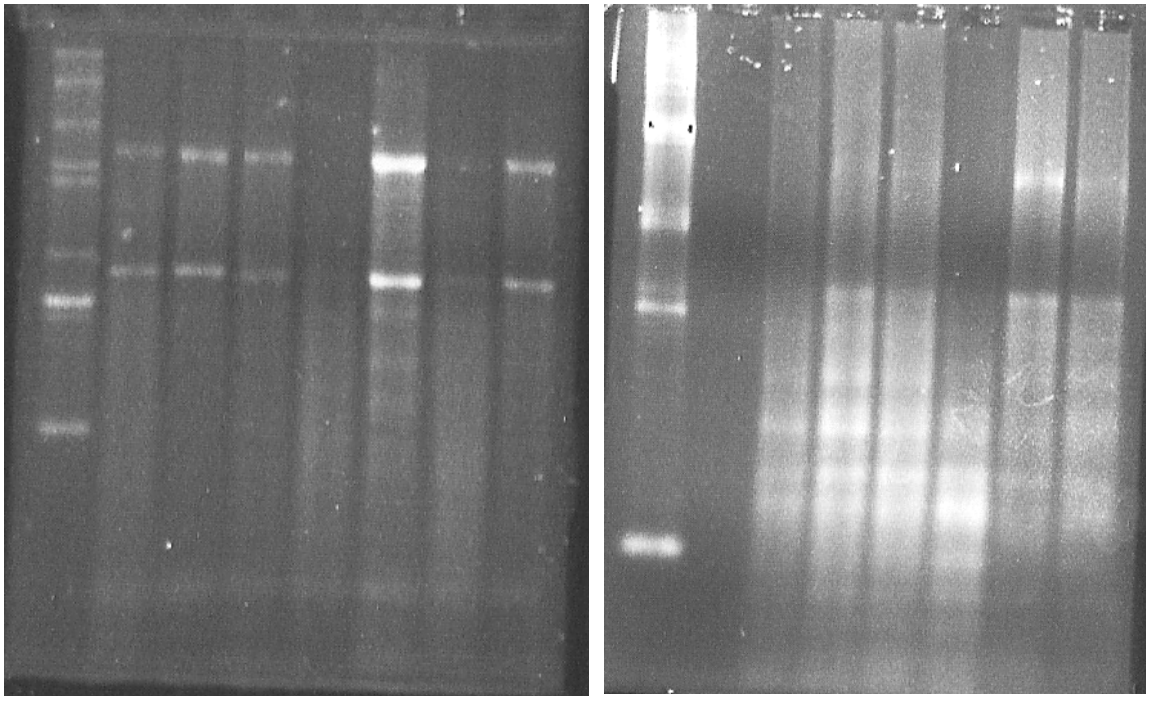

Figure 3.20: Agarose gel electrophoresis of samples collected with (A) and without (B) RNA*later*.
A. Lane 1= ladder, lanes 3-4 trophoblast samples from MTOP, lanes 5-8 trophoblast samples from STOP. B. Lane 1= ladder, lanes 2-5 and 7-8 trophoblast samples from MTOP, lane 6 trophoblast sample from STOP.

3.3.3.2 Comparison of two different RNA Extraction Methods

RNA extraction was performed on three samples from tissues collected following STOP with the Qiagen miRNeasy mini kit and the phenol-chloroform extraction method. The RNA integrity was checked with the Bioanalyser and the results are presented in Table 3.18.

The RIN scores with both extraction methods confirmed a good integrity of RNA that could be used for qRT-PCR. The membrane tissue used for RNA extraction with the Qiagen miRNeasy mini kit was very small and therefore only a very small RNA yield was available and this was not enough to determine an RIN score. Further RNA extraction was performed with the Qiagen miRNeasy mini kit as it is simpler and less time consuming than the organic extraction.

Table 3.18: RNA yield and RIN score in STOP tissues after RNA extraction using the Qiagen miRNeasy mini kit and the phenol-chloroform extraction.

tissue	Qiagen miRNeasy mini kit		Phenol- chloroform extraction	
	RNA concentration (ng/µl)	RIN score	RNA concentration (ng/ µl)	RIN score
trophoblast	816.53	7.7	685.17	7.5
decidua	320.99	7.5	116.95	7.4
membrane	21.04	ND	1109.21	8.6

ND= not determined

3.3.3.3 Dissociation Curves

Dissociation curves were produced for all the PCR products in order to detect any non specific cDNA amplification. The dissociation curves for all genes analysed were specific with a single peak at the melting temperature of the PCR product. Dissociation curves for NAPE-PLD and FAAH qRT-PCR reactions are shown in Figures 3.21 and 3.22, respectively.

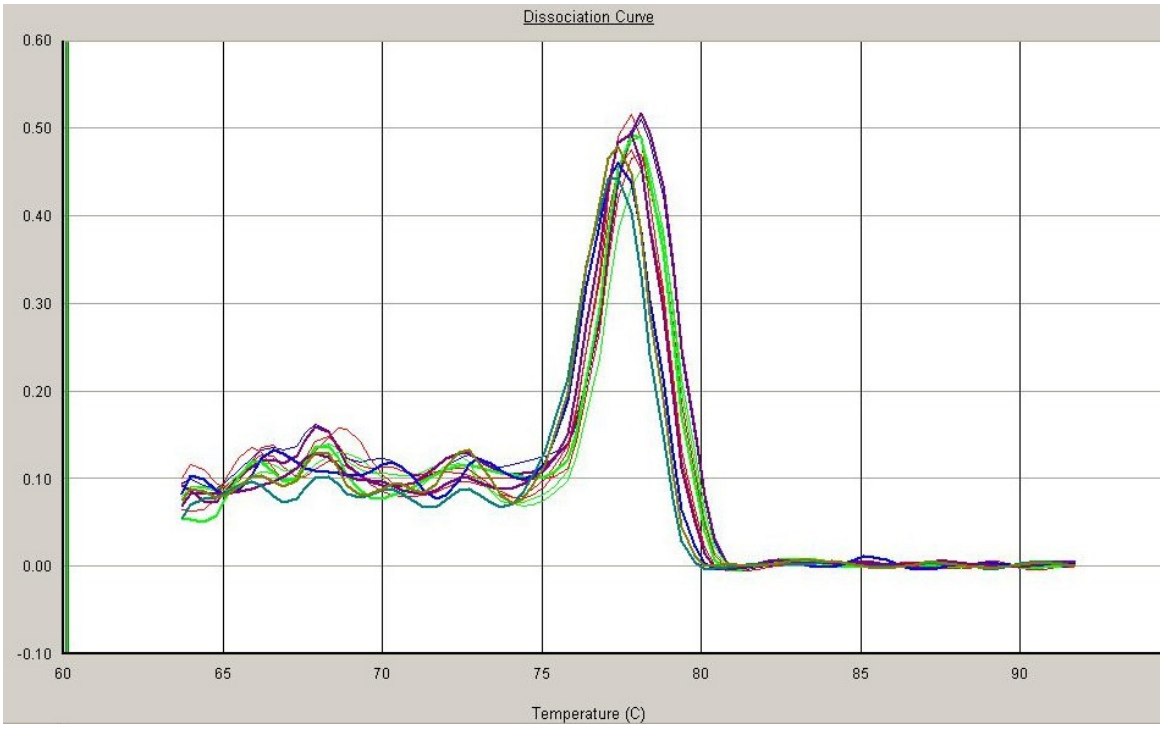

Figure 3.21: NAPE-PLD qRT-PCR dissociation curve on trophoblast tissues obtained from MTOP and STOP.
All tissues were stored in RNA later. There is a single sharp peak at the melting temperature of NAPE-PLD at 78°C.

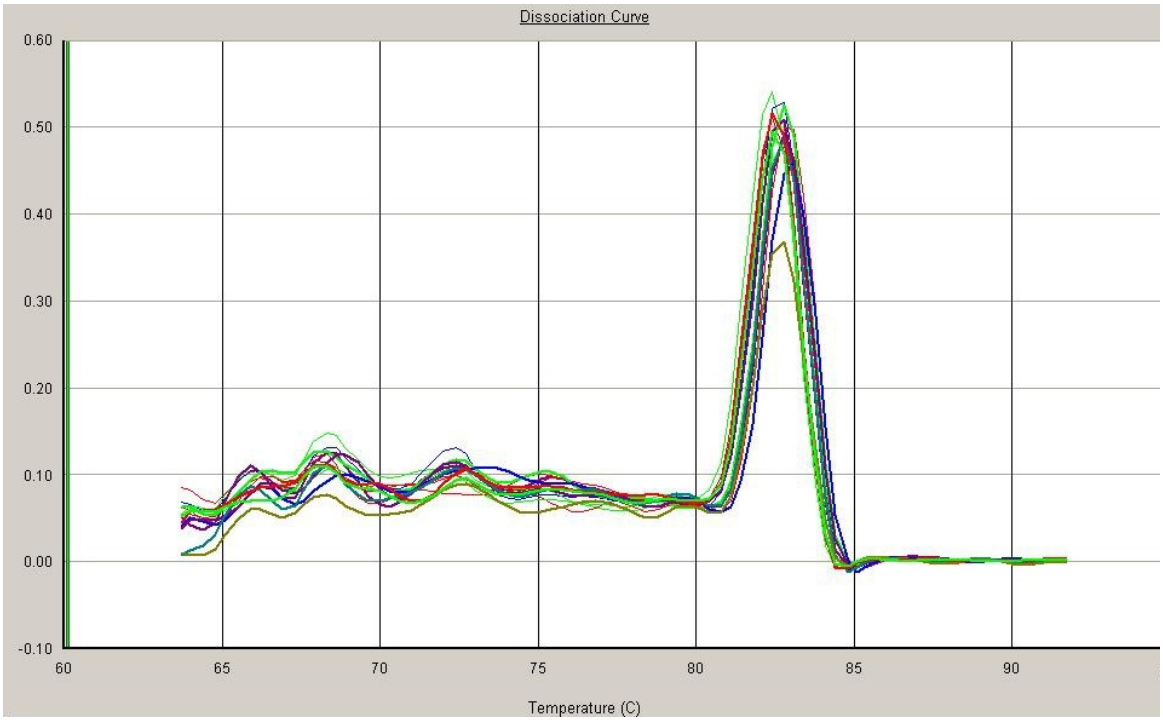

Figure 3.22: FAAH dissociation curve on tissues from MTOP and STOP trophoblast. All the tissues were stored in RNA later. There is a single sharp peak at the melting temperature of FAAH at 83°C.

3.3.3.4 Identification of Reference Genes most suited for Normalisation of Data from MTOP and STOP Trophoblast

To find the appropriate reference gene or genes for normalisation of qRT-PCR data obtained from the tissues the geNorm^{TM} Reference Gene Selection Kit (PrimerDesign, Southhampton, UK) was used. 12 reference genes were tested in 12 human trophoblast samples. The Ct values showed a variation of expression. The lowest Ct values were found with the reference gene 18S, which means it is a highly expressed gene in the trophoblast and the highest Ct values were present with the reference gene SF3A1 (Figure 3.23). The Ct values from the qRT-PCR were converted to relative gene expression values and analysed using the programs geNorm and Normfinder (see section 3.2.3.10).

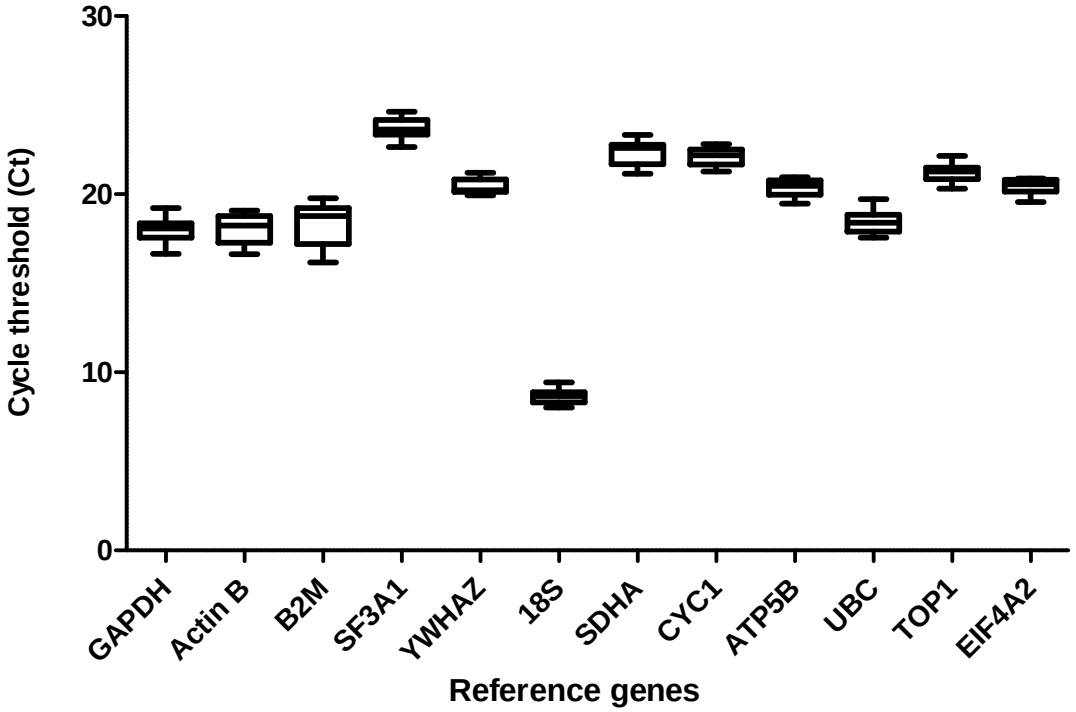

Figure 3.23: The Ct values of 12 reference genes tested in 12 human trophoblast samples.

The lowest Ct values were measured in the reference gene 18s and the highest Ct values were seen with the reference gene SF3A1. The data are presented as boxplots whereby the box shows the upper and lower quartiles and the median; and the ends of the whiskers represent the minimum and maximum values.

3.3.3.4.1 GeNorm Analysis of Housekeeping Gene Expression in First Trimester Trophoblast

GeNorm calculates the average gene expression stability known as the M-value of a reference gene and ranks the reference genes. The lower the M-value the more stable the gene. The ranking order of the reference genes for first trimester trophoblast from MTOP and STOP were (least stable to most stable): B2M> Actin B> SDHA> SF3A1> UBC> 18S, TOP1> GAPDH> YWHAZ> CYC1> ATP5B and EIF4A2 (Figure 3.24). In addition, the geNorm analysis also determines the optimal number of reference genes for this data set by normalisation with the pairwise variation ($V_{n/n+1}$) analysis between consecutively determined normalisation factors of n and n+1 genes (Vandesompele et al. 2002) (Figure 3.25). At a cut-off value of below 0.15, no additional reference gene is required. In this study the V2/3 value was 0.102 and therefore the top 2 reference genes were required for normalisation and these were ATP5B and EIF4A2.

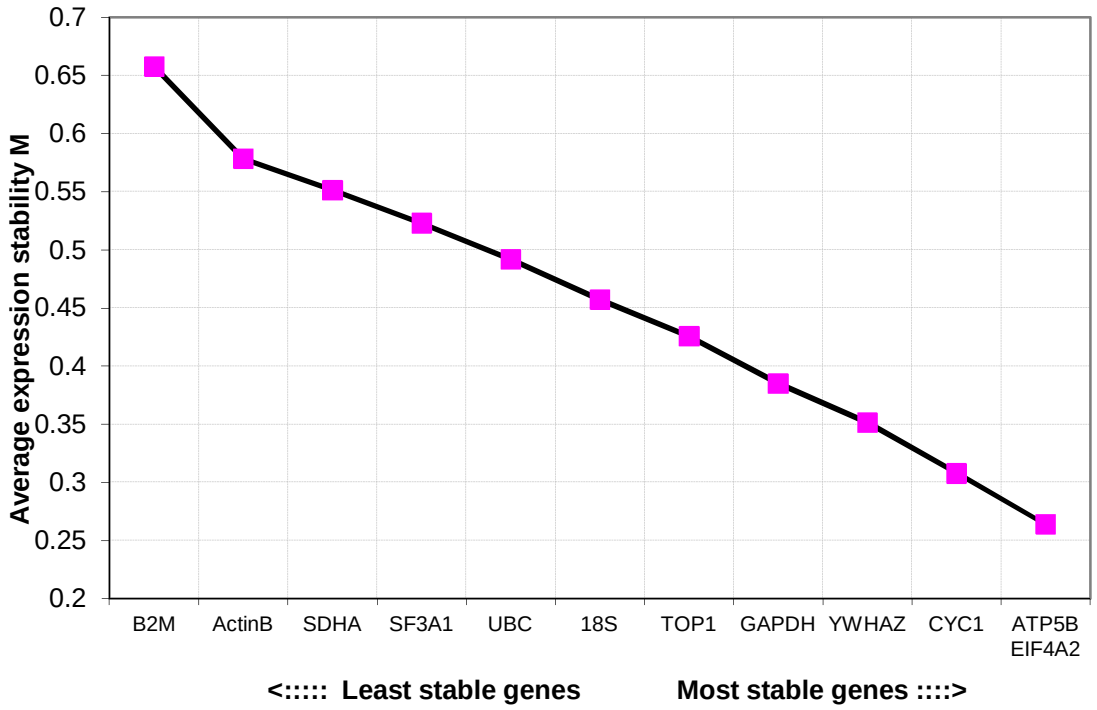

Figure 3.24: Average expression of stability values of the 12 reference genes for human first trimester trophoblast (n=12) using the geNorm analysis. Results are according to the output file of the geNorm software.

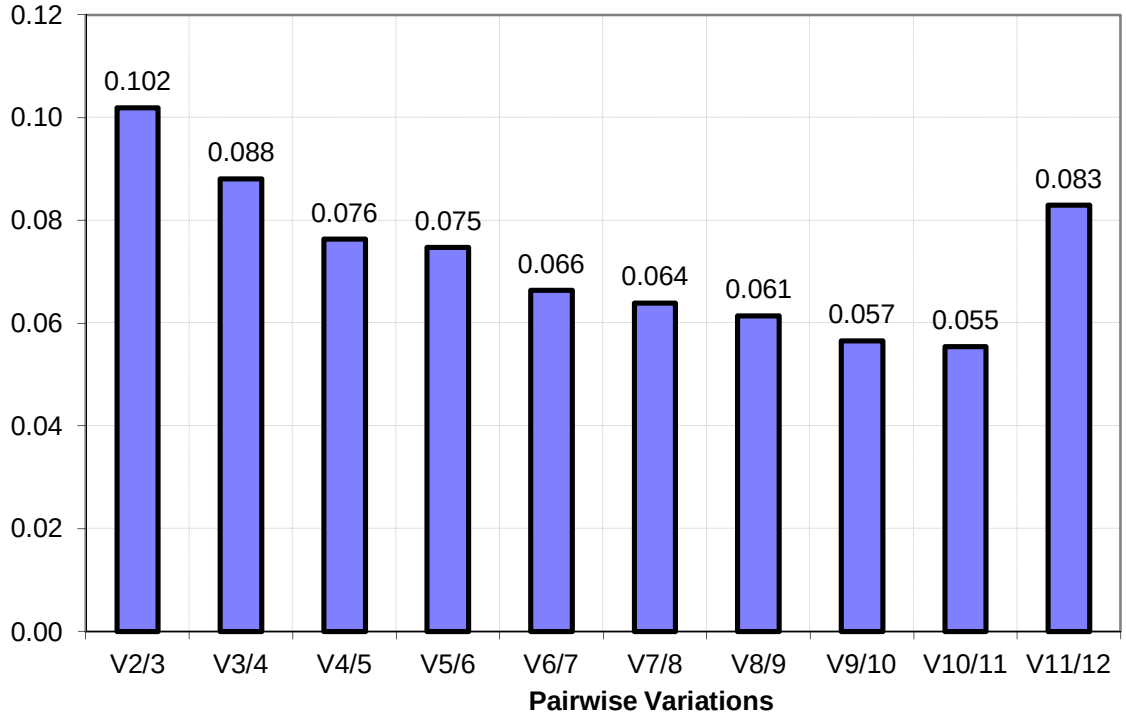

Figure 3.25: Determination of the optimal number of reference genes for normalisation of trophoblast qRT-PCR with the pairwise variation (V) calculated by geNorm. Results are according to the output file of the geNorm software following analysis of human first trimester trophoblast (n=12).

3.3.3.4.2 Normfinder Analysis of Reference Gene Expression in First Trimester Human Trophoblast

The Normfinder program ranks the genes according to their intragroup (Figure 3.26) and intergroup (Figure 3.27) expression variation as a measure of their expression stability. The best single gene was EIF4A2 with a stability value of 0.05 and the best combination of two genes involved ATP5B and EIF4A2 with a stability value of 0.04 (Table 3.19). They were followed by the following less stable genes: GAPDH, CYC1, 18S, YWHAZ, SDHA, Actin B, TOP1, B2M, UBC and SF3A1 (Table 3.20).

Table 3.19: Best reference gene and best combination of reference genes
For normalisation in human trophoblast tissues (n=12) obtained following MTOP and STOP according to the Normfinder program.

Best gene	EIF4A2
Stability	0.05
Best combination of two genes	**ATP5B and EIF4A2**
Stability value for the combination of two genes	0.04

Table 3.20: Stability value of reference genes in trophoblast tissues (n=12) obtained following MTOP and STOP as calculated by the Normfinder program.

Gene name	Stability value
EIF4A2	0.05
ATP5B	0.056
GAPDH	0.074
CYC1	0.085
18S	0.091
YWHAZ	0.095
SDHA	0.100
Actin B	0.110
TOP1	0.113
B2M	0.122
UBC	0.122
SF3A1	0.128

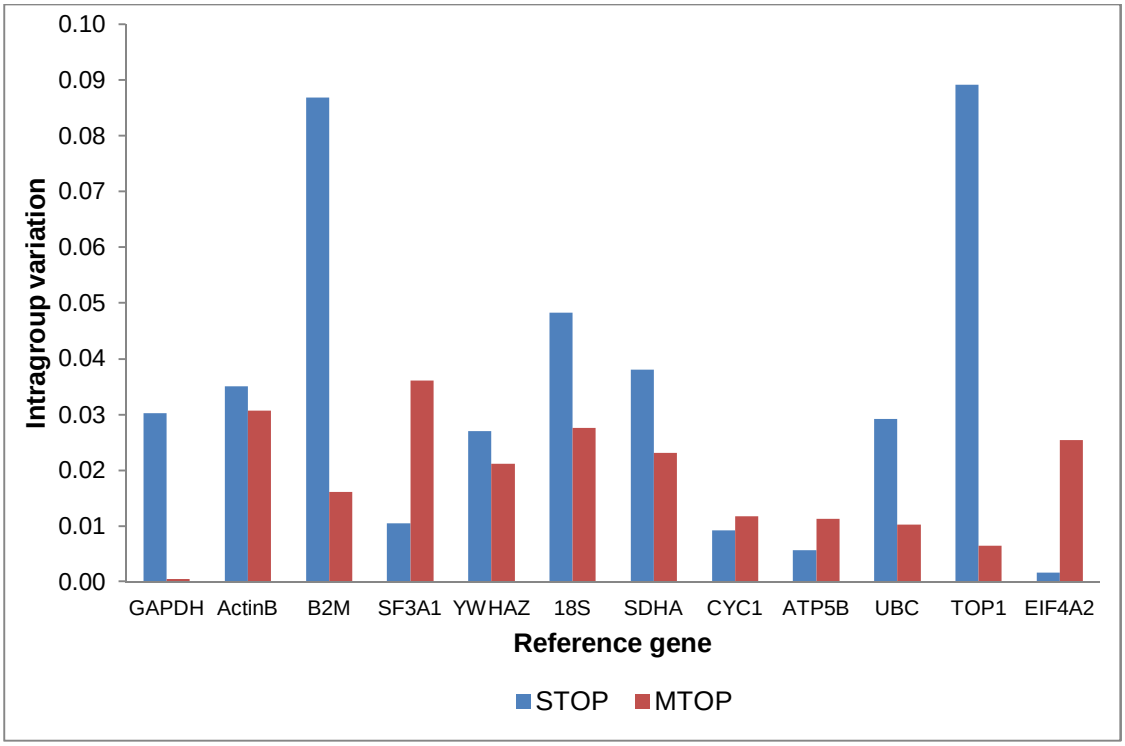

Figure 3.26: Intragroup variation of the Ct values obtained for reference genes in trophoblast following MTOP and STOP according to Normfinder.

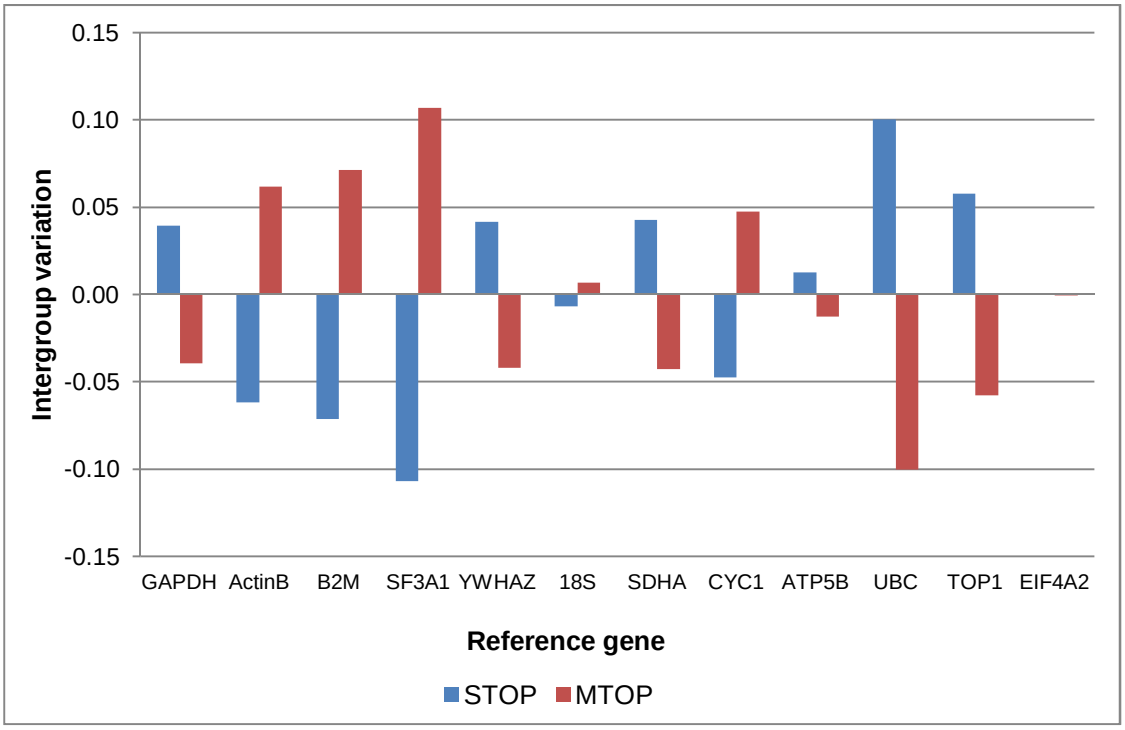

Figure 3.27: Intergroup variation of the reference genes in trophoblast tissues following MTOP and STOP (n=12) calculated by the Normfinder program**.**

3.3.3.5 qRT-PCR Primer Efficiencies

Initial characterisation of the primers was undertaken to optimise the primer concentrations for each target cDNA. Optimal primer concentrations were determined by serial dilutions and were 2µg. To calculate the primer efficiencies of each primer pair standard curves were constructed (Figure 3.28 A, B). Therefore serial dilutions of the template trophoblast cDNA and PCR were performed. The obtained Ct values and the template quantity showed a linear correlation (Figure 3.28). PCR efficiency was calculated from the gradient of the standard curve according to the formula (see section 3.2.3.3). All standard curves showed good regression analysis (R^2>0.98). The PCR efficiencies of the primers were between 105% and 109% (Table 3.21).

The PCR efficiencies for reference gene primer pairs were calculated in the same way (Figure 3.29). The PCR efficiencies for ATP5B and EIF4A2 were 99% and 101% (Table 3.22).

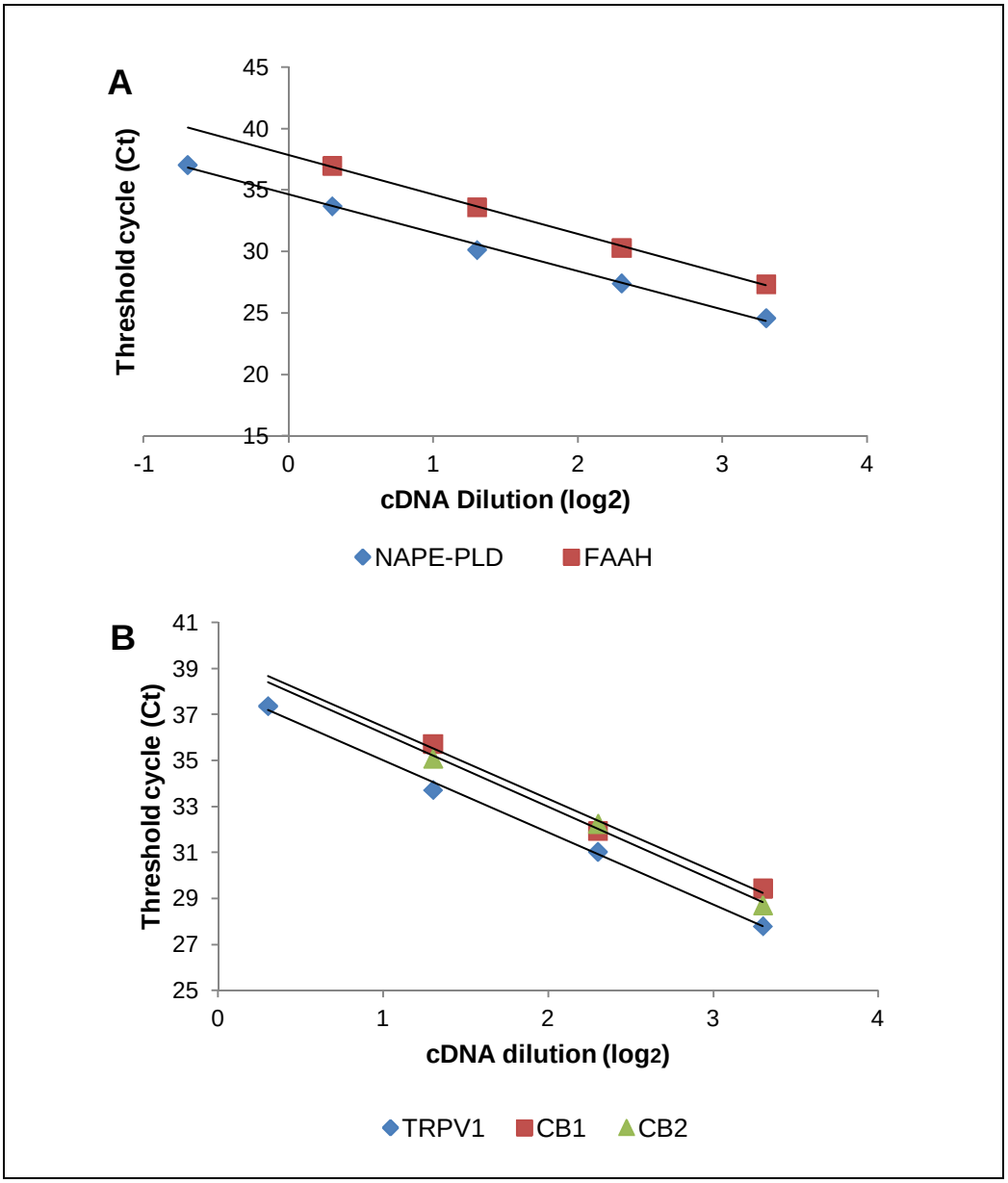

Figure 3.28: Efficiencies of the qRT-PCR primers for NAPE-PLD and FAAH (A) and the receptors TRPV1, CB1 and CB2 (B).
The PCR efficiency was calculated from the slope of the linear curve generated by serial dilution of the template cDNA.

Table 3.21: PCR efficiency for the primers.

Gene	Standard Curve Equation	R^2 value	Primer Efficiency (%)
NAPE-PLD	y = -3.119x + 34.644	0.997	109
FAAH	y = -3.2195x + 37.857	0.999	105
TRPV1	y = -3.14x + 38.145	0.996	108
CB1	y = -3.145x + 39.617	0.986	108
CB2	y = -3.185x + 39.352	0.996	106

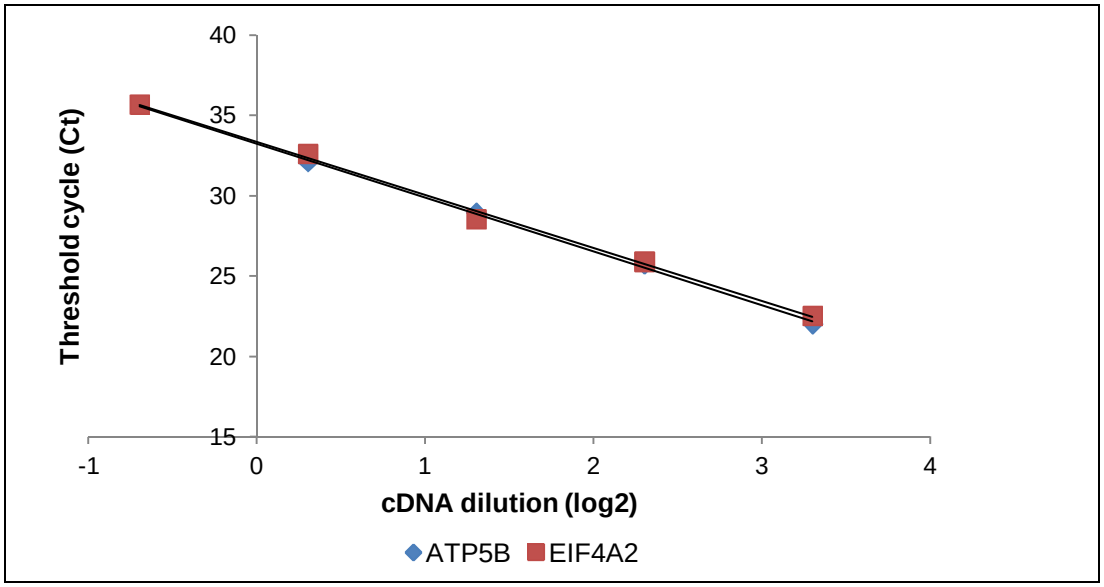

Figure 3.29: Efficiency of primer pairs for qRT-PCR of the reference genes ATP5B and EIF4A2.
The PCR efficiency was calculated from the slope of the linear curve.

Table 3.22: PCR efficiency of the reference genes ATP5B and EIF4A2.

Gene	Standard curve equation	R^2 value	Primer Efficiency (%)
ATP5B	y = -3.567x + 33.755	0.995	99
EIF4A2	y = -3.300x + 33.356	0.997	101

3.3.3.6 Real-time quantitative RT-PCR

Trophoblast tissue was collected from women undergoing MTOP (n=11, study group) and STOP (n=11, control group). These were matched for gestation at the time of the procedure, BMI and ethnicity. The demographic data of the patients is shown in Table 3.23.

Table 3.23: Demographic data of patients undergoing MTOP and STOP that were employed for qRT-PCR analysis of ECS.

		MTOP n=11	STOP n=11	p-value
Ethnicity	**White**	n=9	n=9	
	Asian	n=2	n=1	
	Mixed		n=1	
Age		27 (17-41)	25 (17-33)	0.39
BMI (kg/m^2)		23.2 (19.5-30)	23.7 (20.5-26)	0.68
Gestation (days)		53 (47-61)	57 (52-61)	0.09

Data are presented as mean (range).

3.3.3.6.1 Expression of NAPE-PLD, FAAH, TRPV1, CB1 and CB2 in Trophoblast

Transcript levels of NAPE-PLD, FAAH, TRPV1, CB1 and CB2 were detected in all trophoblast specimens collected from healthy first trimester pregnancies following MTOP and STOP.

A. NAPE-PLD Expression

The trophoblast transcript levels for NAPE-PLD in trophoblast obtained from MTOP were significantly higher when compared to trophoblast from STOP (p=0.0006). The mean NAPE-PLD expression in trophoblast from MTOP was 1.5 whereas the mean expression in trophoblast from STOP was 0.7 (Figure 3.30).

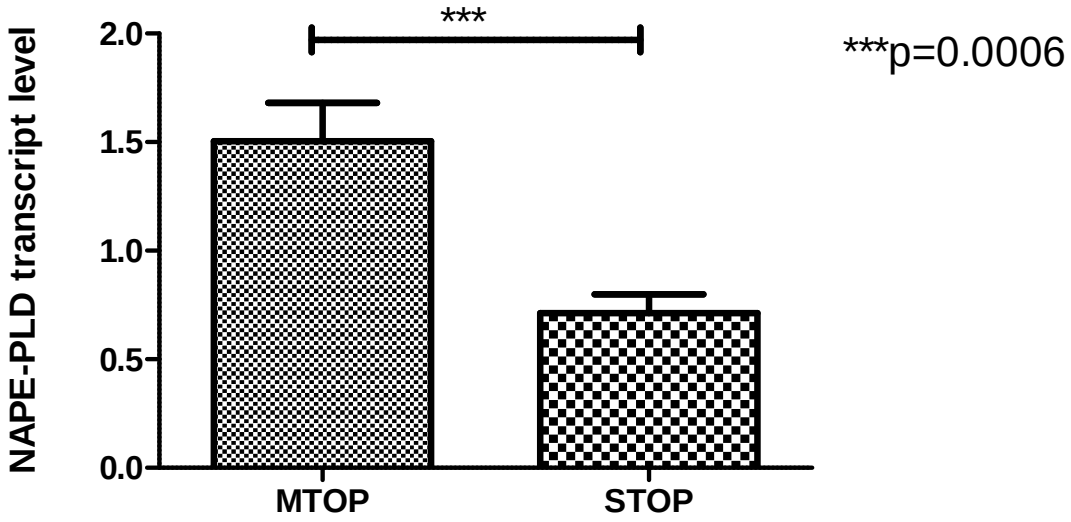

Figure 3.30: Normalised NAPE-PLD transcript levels in first trimester trophoblast (n=11).
Significance was calculated using unpaired Student's t-test. Data are presented as mean ± SEM.

B. FAAH Expression

FAAH transcript levels in trophoblast obtained after MTOP were significant higher when compared to trophoblast from STOP (p=0.021). The mean FAAH expression was 1.34 in trophoblast from MTOP and 0.62 in trophoblast from STOP (Figure 3.31).

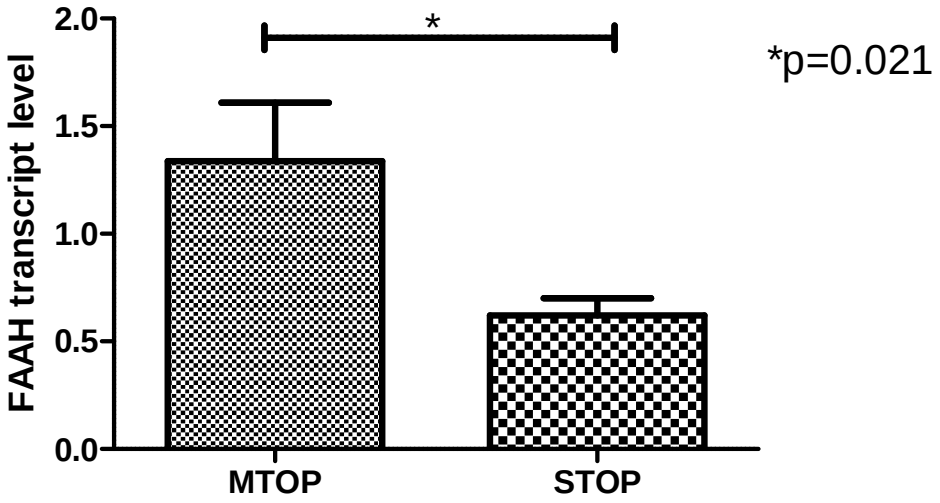

Figure 3.31: FAAH transcript levels in first trimester trophoblast following MTOP and STOP**.**
Statistical analysis was performed by unpaired Student's t-test (n=11) and data are presented as mean ± SEM.

C. TRPV1 Expression

TPRV1 expression varied hugely in the trophoblast after MTOP. The mean normalised expression was 1.76 with a range of 0.33 to 4.7. The TRPV1 expression in tissue from STOP was more consistent with a mean of 0.77 and a range of 0.25 and 1.78. Significantly higher TRPV1 mRNA levels were identified in tissues from MTOP compared to STOP (p=0.042) (Figure 3.32).

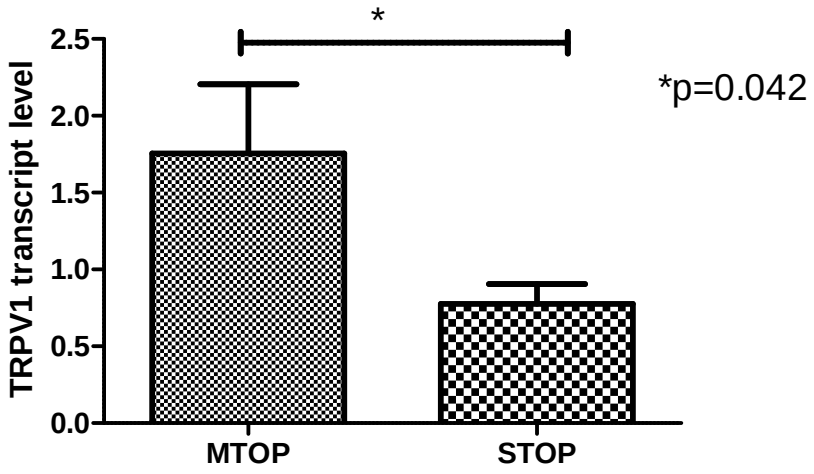

Figure 3.32: TRPV1 transcript levels in first trimester trophoblast following MTOP and STOP (n=11).
Data were analysed by unpaired Student's t-test and are presented as mean ± SEM.

D. CB1 Expression

The relative CB1 transcript levels fluctuated extensively in the trophoblast of MTOP. The mean normalised expression was 1.6 and ranged from 0.25 to 4.1. The CB1 expression in trophoblast after STOP was also broad but was still significantly lower than in MTOP with a mean of 0.5 and a range of 0.05 to 1.48 (p=0.03) (Figure 3.33).

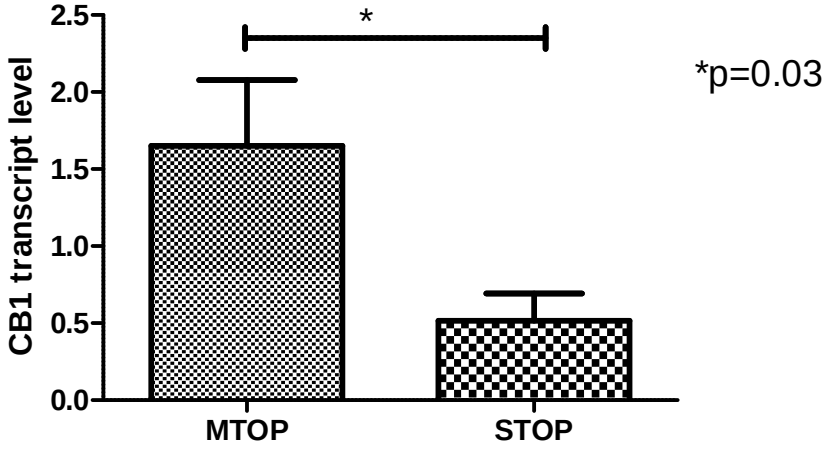

Figure 3.33: Normalised CB1 transcript levels in first trimester trophoblast following MTOP and STOP (n=11).
Data were analysed by unpaired Student's t-test and are presented as mean ± SEM.

E. CB2 Expression

The expression of CB2 mRNA in first trimester trophoblast from STOP was low compared to from MTOP. Here a mean relative expression of 0.94 with a range of 0.57 to 1.39 was identified in STOP trophoblast whereas the relative expression in trophoblast from MTOP ranged considerably from 0.29 to 8.1 and the mean was 2.35. However, there was statistically no significant difference in the CB2 expression of trophoblast from MTOP and STOP (p=0.18) (Figure 3.34).

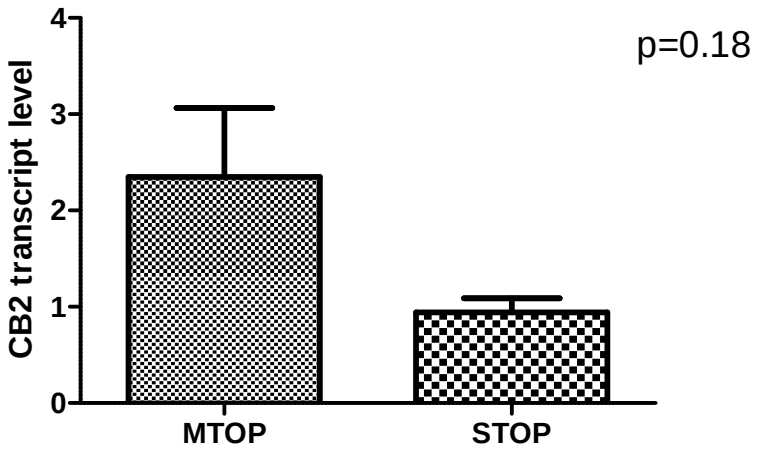

Figure 3.34: Normalised CB2 transcript levels in first trimester trophoblast samples obtained following MTOP and STOP (n=11).
Data were compared using unpaired Student's t-test and are presented as mean ± SEM.

An overview of the ECS transcript levels in trophoblast obtained after MTOP and STOP are shown in Figure 3.35.

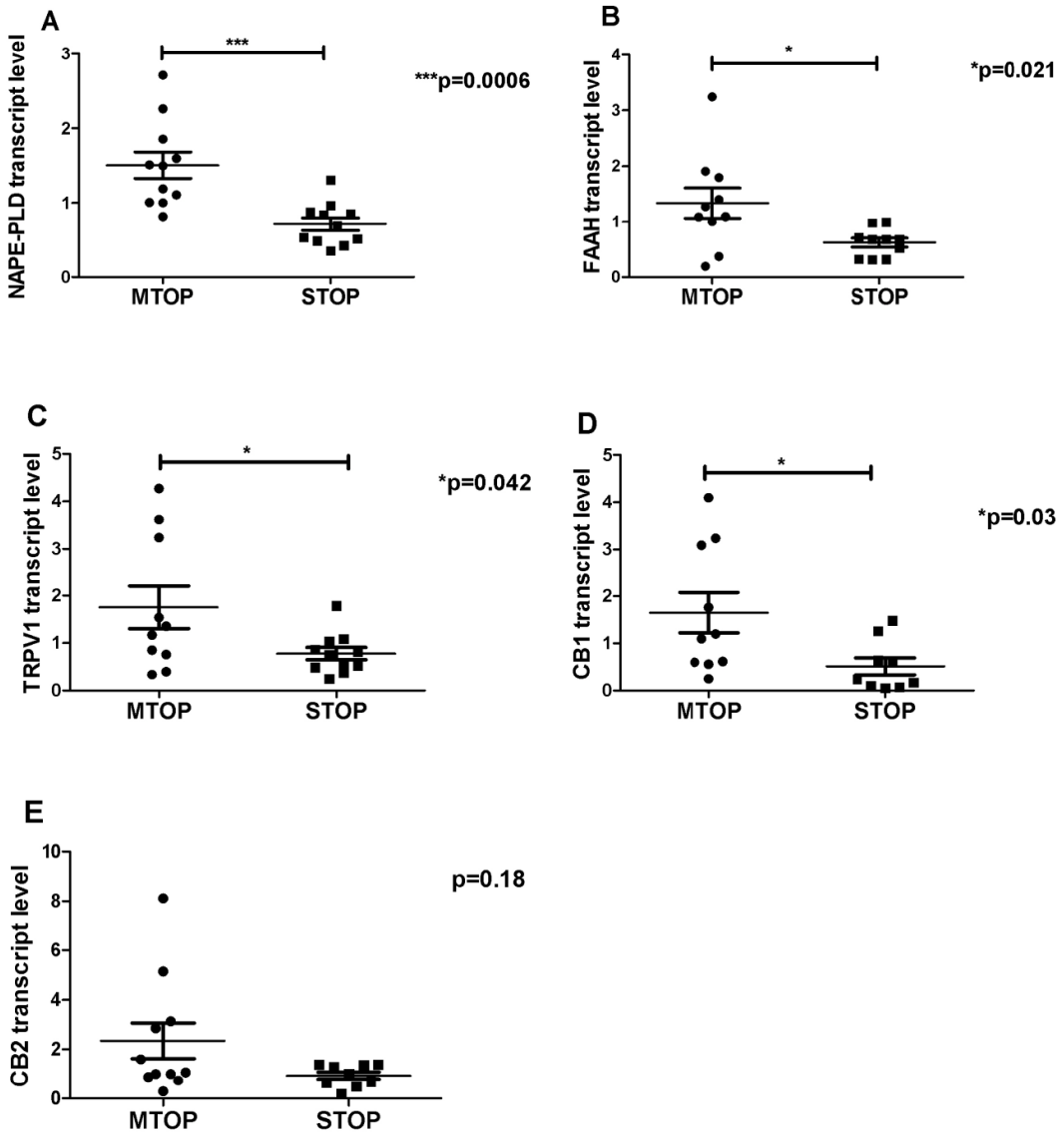

Figure 3.35: ECS transcript levels in trophoblast of MTOP and STOP.
Transcript levels of the enzymes (A) NAPE-PLD and (B) FAAH and the receptors (C) TRPV1, (D) CB1 and (E) CB2. Data are presented as mean ± SEM and were analysed by unpaired Student's t-test.

3.3.3.6.2 Expression of the ECS in Trophoblast from different Ethnicities

Since the endocannabinoid levels in plasma were different in the various ethnic groups (section 2.3.3) these experiments were undertaken to identify whether there is an altered expression of the ECS in trophoblast from different ethnicities. Therefore trophoblast from women undergoing STOP with White (n=7) and Asian ethnicity (n=5) were collected for qRT-PCR analysis. There was not enough tissue from women from other ethnicities hence they were excluded. Trophoblast samples were matched for gestation, BMI and age and the demographic data is in Table 3.24.

qRT-PCR analysis was performed on the first trimester trophoblast from the above volunteers. The relative expression levels of trophoblast NAPE-PLD, FAAH, TRPV1, CB1 and CB2 did not alter significantly between White and Asian women (p=0.73, p=0.97, p=0.54, p=0.39 and p=0.56) (Figure 3.36).

Table 3.24: Demographic data of White and Asian women undergoing qRT-PCR analysis of the ECS in trophoblast following STOP.

	White (n=7)	Asian (n=5)	p-value
Age	28 (19-38)	26 (21-30)	0.36
BMI	24.3 (20-29)	22.5 (18.3-25)	0.35
Gestation	61 (58-69)	62 (56-69)	0.87

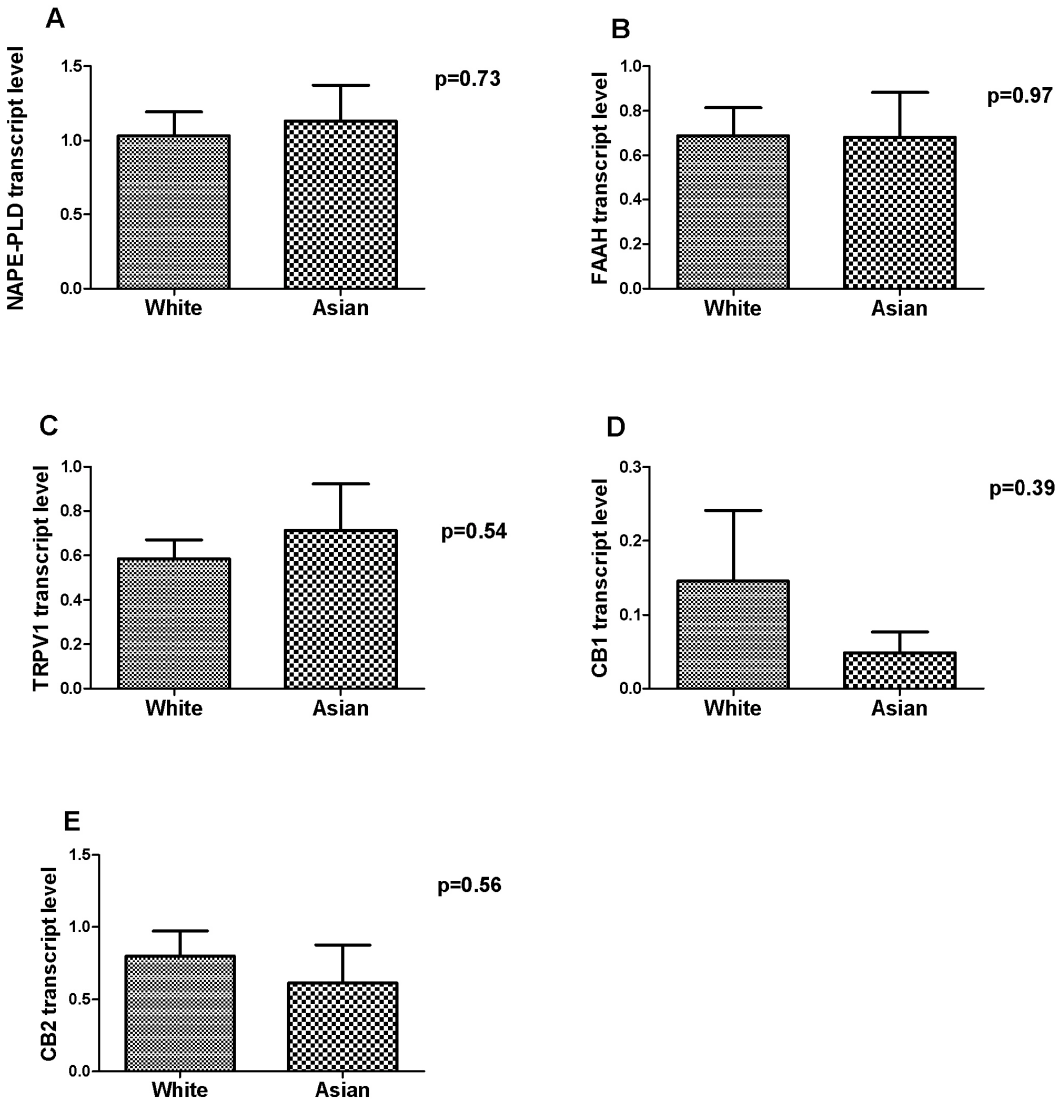

Figure 3.36: qRT-PCR analysis of the ECS transcript levels in first trimester trophoblast from White (n=7) and Asian (n=5) women who underwent STOP.
Data are presented as mean ± SEM and were analysed by unpaired Student's t-test.

3.3.4 Analyses of NAPE-PLD and FAAH Protein Levels in First Trimester Trophoblast by Western Blotting

Western blotting for NAPE-PLD and FAAH proteins was carried out on trophoblast from 16 patients in total undergoing MTOP and STOP. 40µg of sample lysates were separated on both a 10% and a 5% SDS-PAGE gel, transferred to nitrocellulose membrane and immunoblotted with anti-NAPE-PLD and anti-FAAH antibodies (see section 3.2.4.3). GAPDH was used as a loading control.

Typical Western blots for NAPE-PLD and FAAH protein expression in trophoblast after MTOP and STOP are shown below (Figure 3.37 and Figure 3.38).

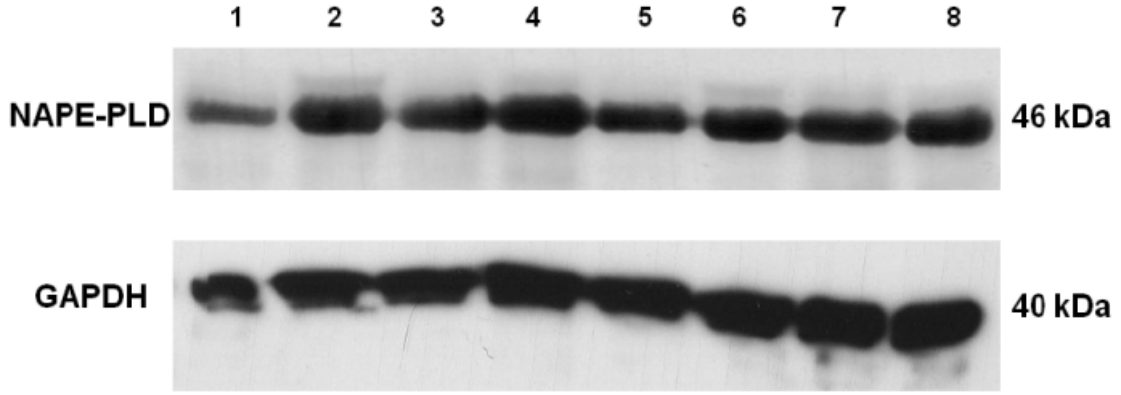

Figure 3.37: Typical Western blot showing protein expression of NAPE-PLD and GAPDH in trophoblast after MTOP and STOP.
Lanes 1, 3, 5, 7= STOP trophoblast, Lanes 2, 4, 6, 8= MTOP trophoblast.

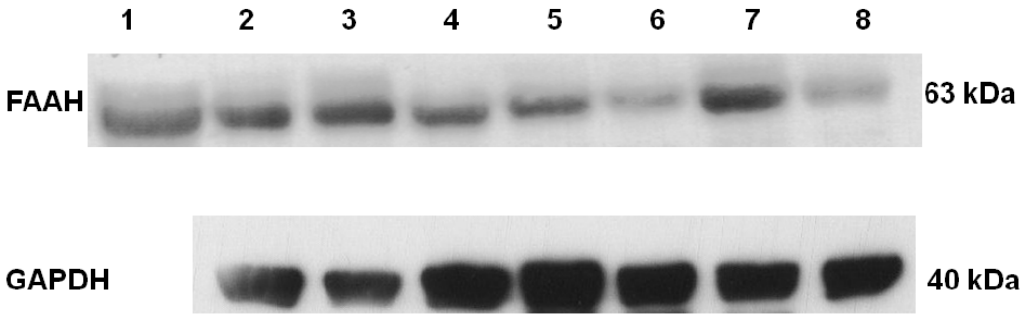

Figure 3.38: Typical Western blot showing protein expression of FAAH and GAPDH in trophoblast after MTOP and STOP.
Lanes 1, 3, 5, 7 = trophoblast samples after MTOP, lanes 2, 4, 6, 8 trophoblast samples after STOP

Semi quantitative densitometric analyses of NAPE-PLD and FAAH protein bands showed that there were no significant differences in protein expression of neither NAPE-PLD nor FAAH in trophoblast after MTOP and STOP (p=0.66 and p=0.27) (Figure 3.39).

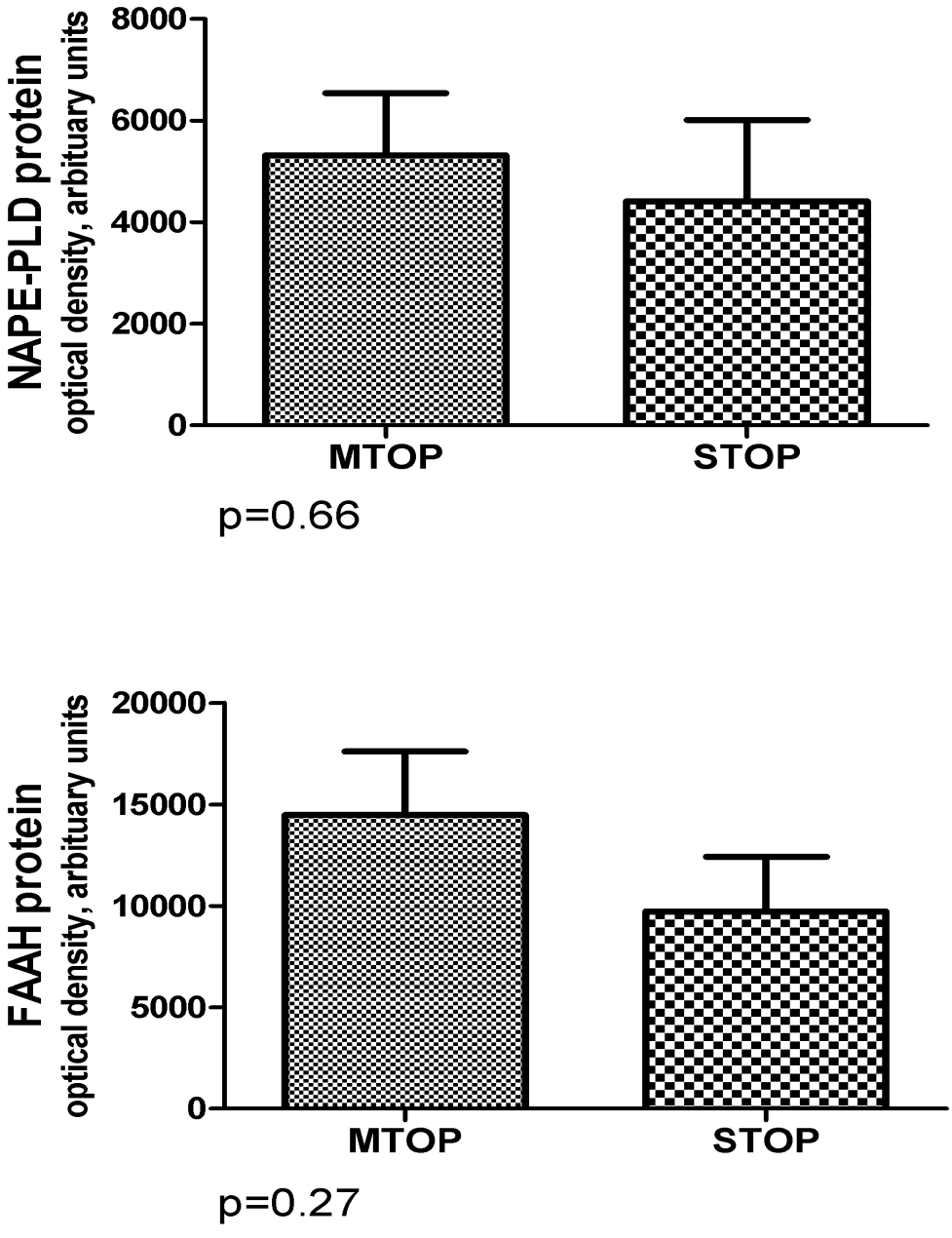

Figure 3.39: Densitometric analysis of NAPE-PLD and FAAH protein expression in trophoblast after MTOP and STOP.
Data analysis was performed by Student's t-test and data are presented by mean ± SEM (n=8).

3.4 DISCUSSION

These results demonstrate that elements of the ECS in trophoblast from MTOP are altered when compared to trophoblast from STOP. Tissue levels of AEA, OEA and PEA were significantly higher in trophoblast from MTOP than STOP. Plasma AEA and PEA levels were also significantly higher in women undergoing MTOP than STOP as described in Chapter 2. To my knowledge this is the first study looking into NAE levels in trophoblast following MTOP. The results support the hypothesis that the P4 receptor antagonist RU486 reduces the up-regulation of FAAH by P4 and thereby increases AEA levels in trophoblast. Looking at NAE levels in trophoblast, decidua and membrane, the highest NAE levels were found in trophoblast. Significantly higher AEA and PEA levels were identified in trophoblast after MTOP when compared to decidua. Significantly higher OEA levels were observed in trophoblast after STOP when compared to decidua. Low P4 receptor expression in human first trimester decidua after MTOP and very low P4 receptor expression in trophoblast after MTOP and STOP have been reported (Milne et al. 2005, Hill et al. 1990). This could explain why AEA levels in trophoblast were higher than in decidua. However, the numbers in these studies, especially of deciduas were small and the results would need to be verified with a bigger sample size.

OEA levels in plasma and trophoblast had a significant negative correlation ($p=0.025$), but this was not the case with AEA and PEA, the other members of the NAE family. The lack of consistently significant correlation between plasma and trophoblast NAE levels implies that the regulation of EC synthesis and degradation in the trophoblast and blood are independent as has previously been suggested in the mouse and the rat (Wang et al. 2007, Fonseca et al. 2010). In addition these observations suggest that NAE produced by the trophoblast in the first trimester pregnancy may not contribute significantly to circulating NAE levels in plasma.

NAPE-PLD, FAAH, CB1 and CB2 were localised in the trophoblasts from women after STOP in agreement with previous observations in trophoblast from first trimester pregnancies (Helliwell et al. 2004, Habayeb et al. 2008) and also in trophoblast from MTOP (Taylor et al. 2011). The presence of all the components of the ECS in trophoblast supports the belief that the ECS plays a role in early pregnancy maintenance. Immunoreactive NAPE-PLD protein was identified in fetal membranes,

cytoplasm and the nuclei of the cytotrophoblast and syncytiotrophoblast. However, another study had showed immunoreactive NAPE-PLD mainly in nuclei of cytotrophoblast cells in trophoblast from STOP and in nuclei of cytotrophoblast and syncytiotrophoblast from MTOP (Taylor et al. 2011). Nuclear staining of NAPE-PLD has also been found in rat decidual cells (Fonseca et al. 2010) and in the normal human ovary (El-Talatini et al. 2009). The presence of NAPE-PLD in the nucleus is interesting as AEA is produced on demand from membrane precursors. It may suggest that the enzyme in the nucleus is not active or that AEA is synthesised within the cell. In this study NAPE-PLD staining intensities were significantly lower in trophoblast from MTOP when compared to STOP. This is in contrast to the study by Taylor et al. where no significant differences between MTOP and STOP were present (Taylor et al. 2011).

Immunoreactive FAAH was also mainly in the cytoplasm of cytotrophoblast and syncytiotrophoblast as seen in previous studies (Habayeb et al. 2008, Taylor et al. 2011). Trophoblast from MTOP demonstrated non-significantly higher staining intensities of FAAH when compared to STOP. This has also been observed in a previous study (Taylor et al. 2011).

The pattern of CB1 immunoreactivity was similar to that previously published work showing immunoreactive staining in the syncytiotrophoblast and cytotrophoblast layers, in the mesenchymal core and in endothelial cells of the blood vessels (Taylor et al. 2011). However, nuclear staining in cytotrophoblast layers was demonstrated in the studies here. There is now increasing evidence for a functional intracellular role for G-protein-coupled receptors (Gobeil et al. 2006, Jiang, Benovic & Wedegaertner 2007, Goetzl 2007, Boivin et al. 2008). It is thought that receptors on the cell surface are responsible for immediate effects whereas intracellular receptors are involved in long-term responses (Marrache et al. 2005). This could explain the presence in CB1 staining in the nucleus and cytoplasm of trophoblasts. CB1 signal intensity was stronger in trophoblast from MTOP when compared to STOP, but this was not significantly different. In another study a decrease of CB1 signal intensity was observed in trophoblast from MTOP, but this was also not significantly different (Taylor et al. 2011). In contrast to the finding here no CB1 was identified in first trimester placenta in the study of Helliwell et al. (Helliwell et al. 2004). However, this could be related to the different antibody used in that study.

CB2 immunoreactivity in trophoblast showed a similar distribution to previous studies and was detected in syncytiotrophoblast and cytotrophoblast, in the mesenchymal core and endothelial cells of the blood vessels (Habayeb et al. 2008, Taylor et al. 2011). CB2 staining intensities significantly increased in trophoblast from MTOP compared to STOP. These findings are similar to those from a previous study that examined ECS in human trophoblast after miscarriages, MTOP and STOP (Taylor et al. 2011). AEA inhibits trophoblast cell proliferation via the CB2 receptor (Habayeb et al. 2008). Elevation of CB2 in MTOP trophoblast may be an indication that part of the mechanism involved in RU486-induced MTOP is an inhibition of cell proliferation via CB2; however, this hypothesis requires considerable further investigation.

To the best of my knowledge this is the first study evaluating the choice of reference gene for normalisation of qRT-PCR data in human first trimester trophoblast. In other qRT-PCR studies of the ECS, one reference gene was been randomly selected without validation. However, some commonly chosen reference genes can vary with different cell types, time or the experimental design (Sturzenbaum, Kille 2001). Therefore, validation of reference genes is now recommended by the MIQE guidelines (Bustin et al. 2009). The MIQE guidelines propose a minimum amount of information regarding qRT-PCR experiments so that studies with inconsistent and misleading results can be avoided, including the use of multiple validated reference genes instead of a single reference gene to minimise bias and give more accurate results. In the experiments here the two reference genes ATP5B and EIF4A2, as indicated by both geNorm and Normfinder, were most stably expressed in these trophoblast samples and therefore were most suitable for normalisation.

Reference gene selection has previously been assessed in human placenta from normal pregnancies and those with fetal growth restriction, preeclampsia and gestational diabetes (Meller et al. 2005, Murthi et al. 2008, Lanoix et al. 2012). Six reference genes were measured in the placenta from pregnancies with fetal growth restriction and the best reference genes were GAPDH, 18sRNA and YWHAZ (Murthi et al. 2008). In contrast, from 7 reference genes investigated in placenta from pregnancies with preeclampsia and gestational diabetes the most suitable reference genes were TBP, SDHA and YWHAZ. In a separate study of preeclampsia and gestational diabetes TOP1, HPRT and PPIA were the best reference genes to assess preeclamptic women and GAPDH, YWHAZ and PPIA were best for placentas from women with gestational

diabetes (Meller et al. 2005, Lanoix et al. 2012). All these studies used placenta from the last trimester. This tissue is different from first trimester trophoblast and therefore it would not necessarily be expected to generate the same optimal reference genes. However, the findings here demonstrate that reference gene stability needs to be checked for each experimental condition in order to avoid using inappropriate reference genes which may lead to inaccurate results. The use of geNorm and Normfinder facilitates the selection of the most appropriate reference genes.

Testing the RNA for integrity is especially important given the variability in the handling of human tissue. The integrity of RNA is also crucial for good quality qRT-PCR studies. Degraded RNA yields lower gene expression giving unreliable and potentially misleading results. Initially there were problems with RNA degradation in a significant number of tissue samples as the time from collecting the tissue to its being snap-frozen in liquid nitrogen varied. Therefore the collection protocol was modified to immediate storage in RNA*later*® and thereby sufficiently reduced RNA degradation.

With the optimal housekeeping genes identified the analysis of transcript levels of ECS by qRT-PCR was then undertaken. In addition to the changes in immunoreactivity of the ECS proteins in trophoblast following RU486 administration, qRT-PCR investigations also revealed changes in mRNA expression. This experiment showed that NAPE-PLD, FAAH, TRPV1 and CB1 mRNA were significantly increased in trophoblast from MTOP when compared to STOP. CB2 mRNA transcript levels were also higher in trophoblast from MTOP, but this was not statistically significant. The most significant increase was in the mRNA expression of the AEA synthesising enzyme NAPE-PLD followed by the AEA degrading enzyme FAAH. The higher AEA levels identified in plasma and trophoblast of women undergoing MTOP may be as a result of the up-regulation of NAPE-PLD. CB1 and CB2 mRNA expressions in trophoblast from MTOP were also increased suggesting the elevated AEA levels following MTOP may be amplified by the concurrently amplified receptor levels. Amplified endocannabinoid signalling is known to induce growth arrest or apoptosis; for example in the study of Habayeb et al. high AEA levels prevented trophoblast cell proliferation in BeWo cells by a CB2-dependent mechanism (Habayeb et al. 2008). BeWo choriocarcinoma cells have many characteristics of trophoblast cells and therefore they are widely used to study placental cellular signalling. Furthermore, lower levels of AEA accelerated trophoblast differentiation in mice whereas higher AEA levels inhibited trophoblast

differentiation via a CB1-dependent mechanism (Wang et al. 1999). Taken together these results suggest that the elevated ligand and receptor levels following RU486 administration inhibit cell growth and differentiation which may comprise at least in part the mechanism of RU486-induced termination of pregnancy. A difference in the mRNA expression of the ECS in trophoblast from White and Asian women undergoing STOP could not be determined in contradiction to the differences in plasma NAE levels.

Protein levels of the enzymes NAPE-PLD and FAAH determined by Western blotting are consistent with the qRT-PCR observations of transcript levels. An increased protein expression of NAPE-PLD and FAAH was observed in trophoblast from women undergoing MTOP when compared to STOP, but this was not statistically significant.

A limitation of the study was the way of tissue collection. Women undergoing MTOP passed the tissue in a non sterile bowl which led to a time delay in tissue collection. Women undergoing STOP had a surgical vacuum extraction, whereby pregnancy tissue can get mashed and that may have an effect on the purity of the tissues. However, as all the tissues were handled the same way, any effect would be the same in tissues collected after MTOP or STOP.

This chapter has offered new insights into an understanding of the possible relationship between the ECS and P4 in the human trophoblast. The anti-progesterone RU486 resulted in high AEA levels in trophoblast and also increased the mRNA and protein expression of the enzymes NAPE-PLD and FAAH. It is known that high AEA levels exhibit an inhibition of trophoblast proliferation and differentiation via CB2 (Habayeb et al. 2008) and via CB1 (Wang et al. 1999), respectively. These experiments have provided insights into the mechanism of action of RU486 in relation to the ECS, however, further work is required to fully appreciate and define these interactions.

Chapter 4

The Effect of Timing and Dose of intraperitoneal Anandamide (AEA) Administration in Rat

4.1 INTRODUCTION

Fertility depends on the synchronized development of the blastocyst and a receptive uterus, which depends on the regulation of steroid hormones, growth factors and lipid mediators. Several other factors are thought to be involved in this synchronized process and one of this is the endocannabinoid system. The latter includes the endocannabinoid system which plays an important role in implantation whereby local AEA levels are considered crucial. In mice, lower AEA levels are observed at implantation sites compared with inter-implantation sites (Schmid et al. 1997). The AEA tone, which is vital for successful implantation at each site, is maintained by the differential expression of AEA synthesizing enzyme NAPE-PLD and the AEA degrading enzyme FAAH. Furthermore, lower AEA levels in the blastocyst accelerated differentiation and outgrowth whereas higher AEA levels inhibit blastocyst differentiation (Wang et al. 1999, Wang et al. 2003).

On the basis of these findings it is hypothesised that the exogenous administration of anandamide around the time of implantation maybe expected to interfere with this process. As it is unethical to test this hypothesis in human volunteers, the rat model was used instead. The rat is frequently used as a model to study pregnancy-related questions as implantation occurs via trophoblast invasion and vascular remodelling, similar to that in human pregnancies (Caluwaerts et al. 2005, Vercruysse et al. 2006).

Rats have a 4-5 days menstrual cycle and can breed throughout the year. The rat cycle consists of the proestrus phase with a duration of 12-14 h when follicles start growing, the estrus phase when the female is sexually receptive ("in heat") for 25-27 h, the metestrus phase when the corpora lutea are formed and last for within 6-8 h and the diestrus phase when the corpora lutea are producing progesterone for 55-57 h (Mandl 1951). If no pregnancy occurs, the corpora lutea regresses. If pregnancy occurs, the length of gestation is usually 21 days. The first day of pregnancy is considered to be when spermatozoa are present in the vaginal smear. The fertilised eggs undergo several mitotic divisions resulting in blastocyst formation. The blastocyst consists of the inner cell mass that develops to an embryo and the trophectoderm that develops to the placenta. The blastocysts migrate via the oviduct to the uterus. Rats have an elongated uterus consisting of the right and left horn. This enables the rat to have multiple implantations. The horns of the uterus join together and open into the vagina. Rat

implantation occurs 5 days after fertilisation and involves blastocyst embedding within the endometrium (Mayer, Nilsson & Reinius 1967). At the site of blastocyst attachment the underlying antimesometrial stromal cells differentiate to decidual cells (Mayer, Nilsson & Reinius 1967). A few days later, on day 8, the mesometrial stromal cells differentiate into decidual cells. They are small with a single nucleus whereas the antimesometrial decidual cells are large with multiple nuclei. On day 10 decidual cells begin to regress by programmed cell death from the antimesometrial site of initial attachment to the mesometrial site of the definitive haemochorial placenta to accommodate the developing embryo (Gu et al. 1994). The involvement of AEA in decidual regression has been shown in rat primary decidual cells (Fonseca, Correia-da-Silva & Teixeira 2009).

The rat placenta starts developing after blastocyst attachment. Trophoblast cell invasion is endovascular and interstitial towards the mesometrial triangle also referred to as metrial gland, which is a rich vascular site and an extension from the mesometrial decidua (Vercruysse et al. 2006). There are two regions in the chorioallantoic placenta: the basal zone and the labyrinth zone. The basal zone, also called junctional zone, is comprised of giant trophoblast cells, spongiotrophoblast and glycogenic trophoblast cells and is located at the maternal-placental interface. The labyrinth zone is situated at the fetal interface and is comprised of giant trophoblast cells and syncytiotrophoblasts (Figure 4.1). The giant trophoblast and spongiotrophoblast cells have endocrine functions whereas the glycogen cells serve as an energy reservoir (Davies, Glasser 1968, Soares et al. 1996).

In this chapter the hypothesis was tested in an animal experiment where exogenous AEA was given around the time of implantation to determine whether it reduced the number of viable pregnancies in the rat. Changes to the ECS after AEA administration were also investigated. For this purpose, AEA, OEA and PEA levels were quantified in plasma, decidua and placenta from the rats using UHPLC-MS/MS. Furthermore, the expression profiles of NAPE-PLD and FAAH in implantation sites were examined by immunohistochemistry (IHC), transcript levels of AEA metabolizing enzymes (NAPE-PLD and FAAH) and AEA receptors (CB1, CB2 and TRPV1) were analysed at implantation and inter-implantation sites by qRT-PCR while protein levels of NAPE-PLD and FAAH were evaluated at the implantation and inter-implantation sites by Western Blotting.

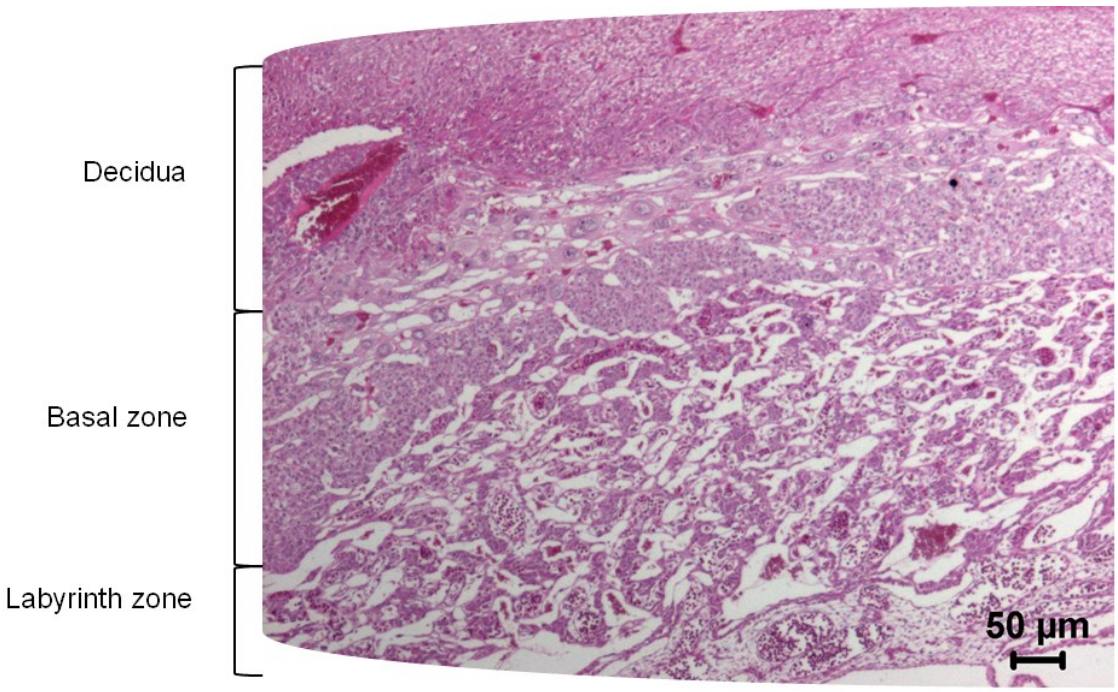

Figure 4.1: Schematic representation of the rat fetal-maternal interface on day 14 of pregnancy.
The fetal-maternal interface consists of the chorioallantoic placenta and decidua. The placenta is formed by the basal (junctional) and labyrinth zone.

4.2 MATERIAL AND METHODS

4.2.1 Animal Experiment

Nulliparous Wistar rats weighing 200–250 g (Charles River Laboratories, Barcelona, Spain) were housed in a temperature controlled room with standard 12 h/12 h light/dark cycle in the animal care facility of the institution in Oporto University, Portugal. The animals had access to food and water ad libitum. All procedures involving animals were conducted in accordance with the guidelines of the Ethics Committee of the Institute of Molecular and Cellular Biology, Oporto University, Porto, Portugal.

A vaginal smear was performed daily at around 10:00 a.m. to determine the stage of oestrus cycle. They were examined under the microscope and vaginal cytology was used to determine the oestrus stage by a commonly used method described by Baker (Baker 1979).

A preliminary experiment was performed to determine whether the AEA dose reaches target tissue and whether levels are maintained sufficient to prevent implantation. Therefore, three female rats in the oestrus stage were given an intraperitoneal injection of AEA (5 mg/kg). One rat did not receive an injection and was anaesthetised at 0 min. The other rats were anaesthetised at 30, 60 or 120 min after the AEA injection and intracardial blood was collected into EDTA tubes for the quantification of AEA, OEA and PEA. After the sacrifice, uterine horns and tubes were collected for NAE analysis and stored at -80°C.

In the main experiments, female rats in the oestrus stage were mated. The following morning a vaginal smear was taken to check for the presence of spermatozoa. A positive smear was used to identify day 1 of pregnancy. The pregnant rats were then randomly allocated into groups of treated and control animals.

In an initial experiment rats were treated either with daily intraperitoneal (i.p.) injections of AEA (0.2 mg/kg) or with the vehicle saline only on days 2, 3 and 4 of pregnancy. Another group of rats received a single injection of AEA (0.5 mg/kg) or saline only as a vehicle on day 4 of pregnancy.

A second experiment involved a single 500µl intraperitoneal injection of 200µg AEA (40µl of stock solution) (1mg/kg AEA) diluted in 460µl propylene glycerol and NaCl (1:1). The vehicle consisted of 500µl propylene glycerol and NaCl (1:1). The pregnant rats received the injection on either day 2, day 4 or day 6 of the pregnancy (Figure 4.2). Pregnant rats were randomly allocated to the treatment groups and each group comprised 6 animals. The vehicle was chosen according to a previous study (Wenger et al. 1997).

All rats were sacrificed on day 14. Gestational day 14 was chosen as it corresponds to the time when the placenta is fully developed. The uterus was collected and washed in phosphate-buffered saline (PBS) to remove excess blood. Each gestational sac was dissected intact. Implantation and resorbed sites (based on size and gross appearance), if present, were counted in each uterine horn (Figures 4.3 and 4.4). The implantation units, placentas and maternal tissues were inspected grossly for change in colour and shape. Percent resorption was determined by dividing the number of resorbed fetuses by the total number of implantations (including viable and resorbed fetuses).

The following investigations were performed in the rats from the second experiment only.

After anaesthesia intracardial blood was collected into EDTA tubes for the quantification of AEA, OEA and PEA. After the animals were sacrificed, implantation, inter-implantation and reabsorbed units were fixed in 10% (v/v) buffered formalin for immunohistochemistry (IHC). Implantation sites were separated into myometrium, decidua and placenta and stored in RNA*later* for qRT-PCR together with the inter-implantation sites or homogenized for Western blotting. Decidua and placenta collected for NAE analysis were stored at -80°C until analysis.

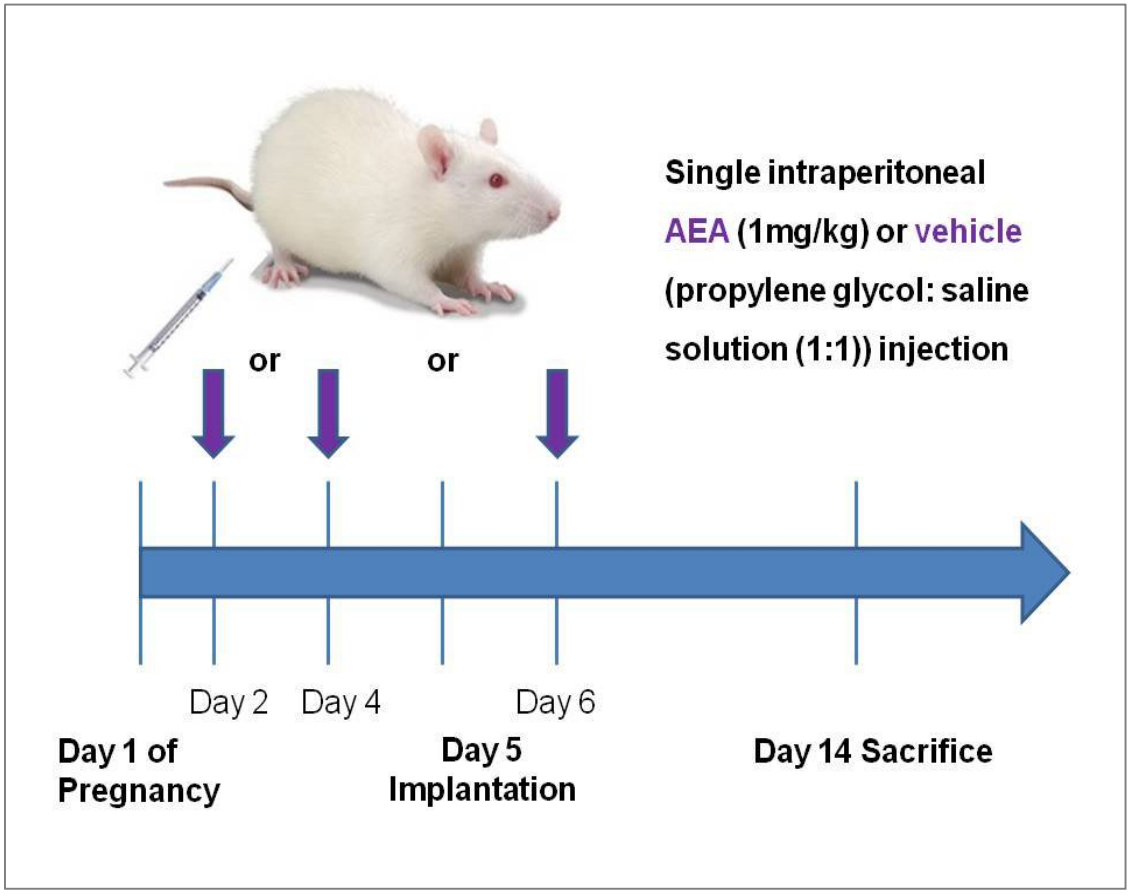

Figure 4.2: Experimental design.
Rats received a single intraperitoneal injection of either AEA (1mg/kg) or vehicle (propylene glycol: saline solution (1:1) on day 2 or day 4 or day 6 of pregnancy. They were sacrificed on day 14.

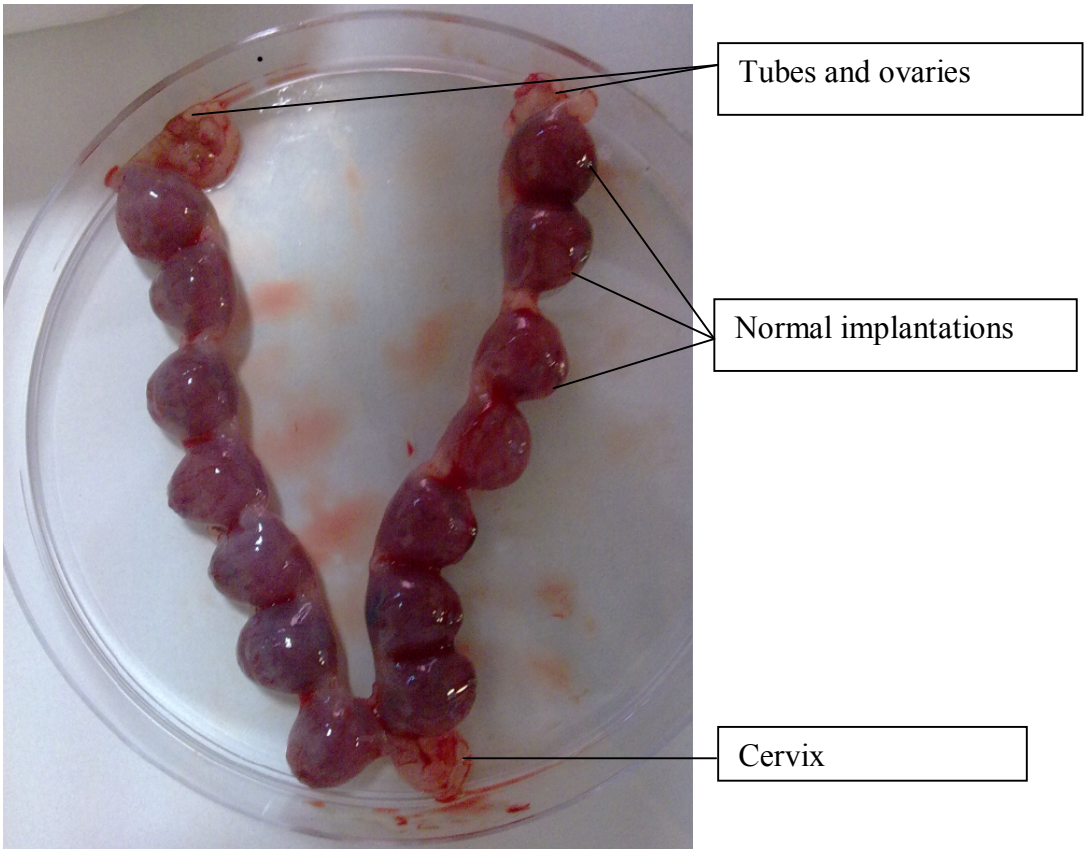

Figure 4.3: Rat pregnant uterine horns with normal implantations.
The rat uterus consists of two uterine horns with multiple implantation sites.

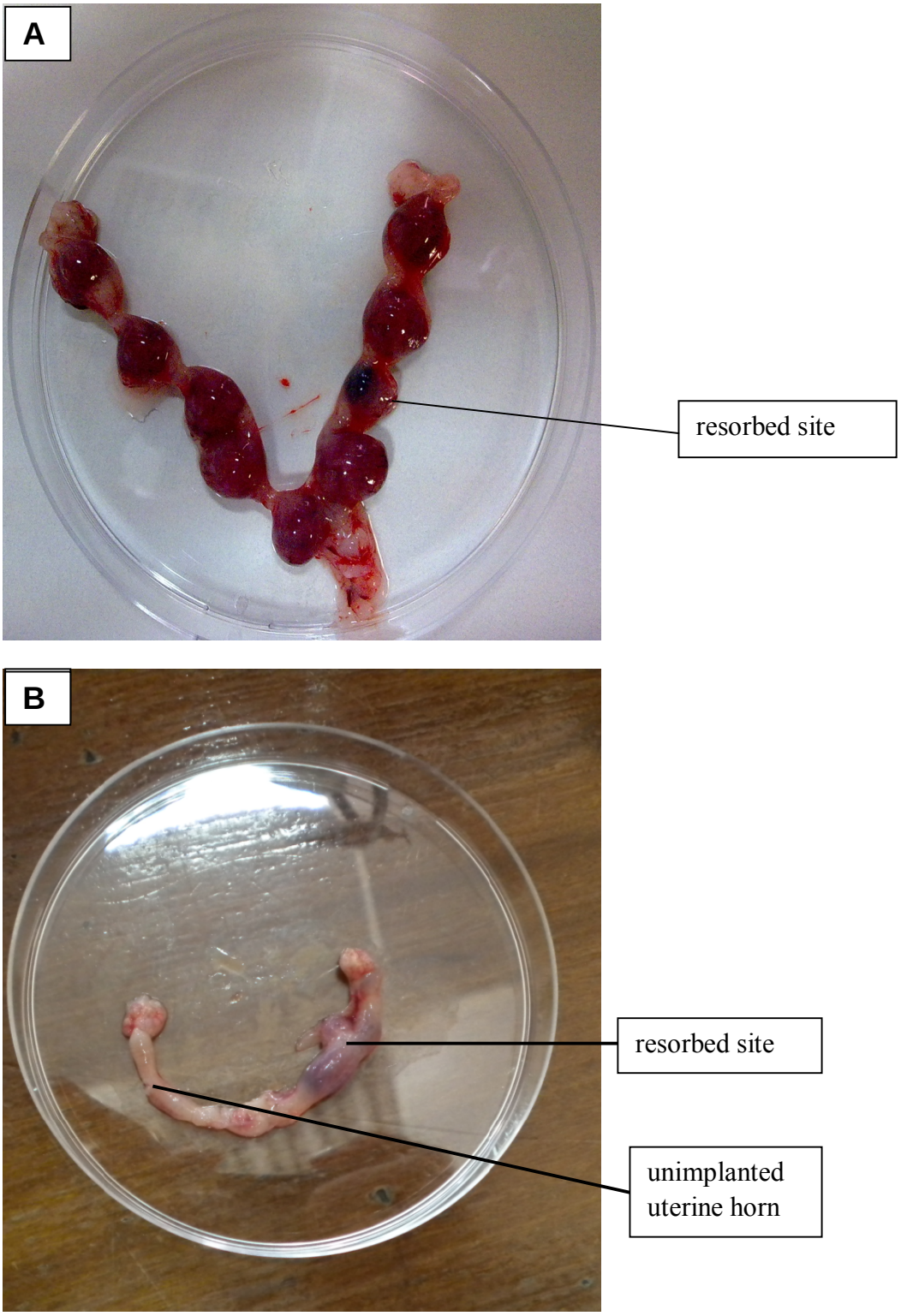

Figure 4.4: Examples of rat uterine horns from day 4 (A) and day 6 (B) treated rats.

(A) The uterine horn of a day 4 treated rat showed reduced number of normal implantation sites and also one resorbed site. (B) One day 6 treated rat had no implantation sites in one uterine horn and one resorbed site in the other uterine horn.

4.2.2 Extraction of AEA, OEA and PEA from Rat Plasma and Tissue

Whole blood was centrifuged at 1200 x g for 15 min at 4°C to separate plasma from the red blood cells. Plasma was transferred into clean Eppendorf tubes and stored at -80°C until further analysis.

The tissue (decidua and placenta) preparation was handled the same as for the human tissue as described in section 3.2.1.3.

The extraction of AEA, OEA and PEA from rat plasma and tissue was by solid phase as described in section 2.2.9 and quantified using the UHPLC-MS/MS (section 2.2.12).

4.2.2.1 Statistical Analysis of Tissue and Plasma Levels of NAEs

Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (Graph Pad Software, San Diego California USA). Normally distributed groups were compared using one way ANOVA with Bonferroni post-hoc test. Data that did not follow the Gaussian distribution were compared using the Mann-Whitney U Test for 2 groups or Kruskal Wallis Test with Dunn's Multiple Comparison Test for multiple groups. Correlations were made using Pearson Correlation for normally distributed data and Spearman correlation for data that did not follow the Gaussian distribution. The level of significance was set at $p<0.05$. All numerical data are shown as mean ± SEM.

4.2.3 Immunohistochemistry

4.2.3.1 Animals

Rats received a single intraperitoneal injection of AEA (1mg/kg) or vehicle (propylene glycol: saline) on day 2 or day 4 or day 6 of pregnancy. They were sacrificed on day 14 and the uteri were collected for immunohistochemistry.

4.2.3.2 Tissue Collection and Handling

Implantation, inter-implantation and resorbed units were fixed in 10% (v/v) buffered formalin for 48 h. Afterwards they were processed for routine paraffin histology as explained in Figure 4.5. The implantation sites were embedded in paraffin such that all sections were transverse. Serial sections (4 μm) were taken and placed on slides coated with aminopropyl-triethoxysilane (Sigma Chemical Co, St. Louis, USA). The slides were stored at room temperature until further use.

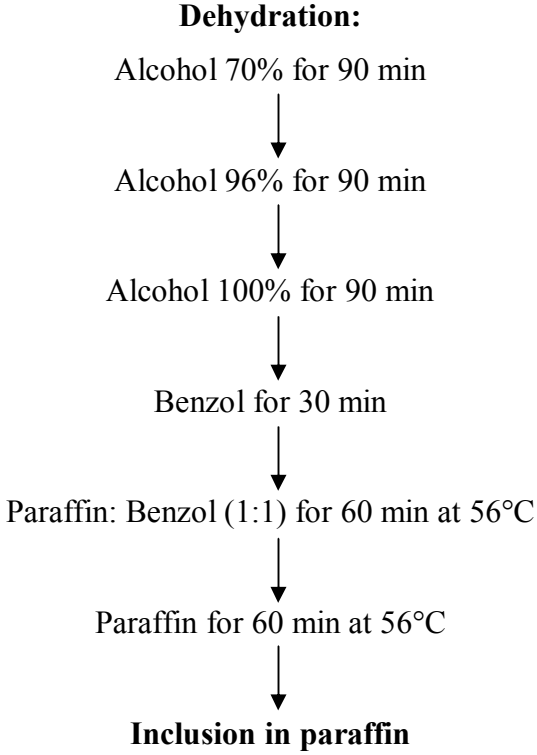

Figure 4.5: Flow diagram for the preparation of blocks.
Tissues fixed in formalin were dehydrated in graded alcohol and benzol. Then they were placed in a 1:1 mixture of paraffin and benzol and thereafter in paraffin only at 56°C. Finally the tissues were included in paraffin and left at room temperature.

4.2.3.3 Immunostaining

Slides were dewaxed, rehydrated and stained with standard haematoxylin and eosin (H&E) to determine the morphology and histology.

Localisation of NAPE-PLD and FAAH was by an avidin-biotin alkaline phosphatase complex immunohistochemical technique (Vectastain ABC kit, Vector laboratories, CA, USA). The slides were dewaxed, rehydrated and blocked with normal blocking serum. The slides were then incubated overnight with the rabbit primary anti-rat NAPE-PLD antibody (dilution 1:100) (catalog no. 10005430; Cayman Chemicals) or goat primary anti-rat FAAH antibody (dilution 1:100) (catalog no. sc-26427; Santa Cruz) at 4°C. The following day, the slides were washed with PBS and incubated with diluted biotinylated secondary antibody for 30 min. After another wash they were incubated with Vectastain ABC-AP reagent for 30 min as per kit instructions. The immunoreactive complexes for anti- NAPE-PLD and anti-FAAH were identified with Sigma Fast Red tablets (Sigma). The slides were washed under tap water for 6 min, counterstained with Mayer's haematoxylin solution (Sigma diagnostics) and mounted in an Aqua-Mount improved medium (BDH Laboratory Supplies, Poole, UK).

As negative controls, blocking peptides were used whereby the primary antibody was incubated with an excess of the corresponding blocking peptides for NAPE-PLD (Cayman Chemicals, Catalogue number: 10009737) and FAAH (Santa Cruz, Catalogue number: sc 26427p). The primary antibody binds to the blocking peptide and gets neutralized. This helps to evaluate the specificity of antibody reactivity in immunohistochemistry.

Images were taken at 100x, 200x or 400x magnification on a Nikon Eclipse E400 microscope (Japan) attached to a Leica DC 300F digital camera with the Leica Qwin Lite y 2.8 image capture system.

4.2.3.4 Image Capture and Evaluation of Immunostaining

Implantation sites from three day 4 and five day 6 treated as well as six control rats were assessed by histomorphometric analyses. 10-13 randomly selected fields per slide at 400x magnification were captured. The levels of immunostaining were analysed

using an unbiased histoscore method within the Imagescope software package version 10.2.2.2319 (Aperio Technologies Inc., Vista, CA, USA). A detailed description was given in Chapter 3.2.2.4.

4.2.3.5 Statistical Analysis of Immunohistochemical Analyses

Statistical analysis of the data was performed using GraphPad Prism version 5 for Windows (GraphPad Software, San Diego, California, USA). The groups were compared using Kruskal Wallis Test with Dunn's Multiple Comparison Test and a p-value<0.05 was set as significant.

4.2.4 Polymerase Chain Reaction (PCR)

4.2.4.1 Primer Design

The primers against the genes of interest were designed using the Primer Express version 2.0.0 (Applied Biosystems, Warrington, UK) and ordered from Sigma Aldrich (Poole, UK).

The sequences of the primers for NAPE-PLD, FAAH, TRPV1, CB1 and CB2 are summarized in Table 4.1.

Table 4.1: Primer sequences for PCR amplification.

Gene		Primer Sequences (5'-3')
NAPE-PLD_01	Forward	5'CCTGCTTTTGAAGAGATTGGAAA
	Reverse	5'TCATAAACCACCTTGGCTCATAAG
NAPE-PLD_02	Forward	5'CCAGAGGATGCTGTAAGGATTCA
	Reverse	5'GGCGGCTCTAAGTAATGCTCAT
FAAH_01	Forward	5'TCCTGCTGAAGCCTCTGTTTC
	Reverse	5'AGCTGACCGAGGACGCATA
FAAH_02	Forward	5'CGGCAGTGCAGCTTTCTCTT
	Reverse	5'CACAGTAGAGCTTTCAGGCATAGC
TRPV1_01	Forward	5'TTTCAGGGTGGACGAGGTAAA
	Reverse	5'TGCCTGGGTCCTCGTTGA
TRPV1_02	Forward	5'TGGACAGCTACAGTGAGATACTTTTCTT
	Reverse	5'CCATGGCCAGGGAGAACA
CB1_01	Forward	5'CAAGCACGCCAACAACACA
	Reverse	5'TCTTAACGGTGCTCTTGATGCA
CB1_02	Forward	5'TCGACAGGTACATATCCATTCACA
	Reverse	5'GGAGAGGCAACACAGCGATT
CB2_01	Forward	5'ATAAGCAGGAGTTGGGAGGAGACT
	Reverse	5'CTGAATCTGCCAGAGACAGCAT
CB2_02	Forward	5'CTCCAGGGCCTCGGATCT
	Reverse	5'GGACCAGAGTCTCAGCCTCTGT

4.2.4.2 Tissue Collection and Homogenisation

Placenta, decidua, myometrium from implantation sites, resorbed sites and inter-implantation sites were collected into RNA*later* and stored at -80°C until analysis. About 40 mg of tissue was ground to a fine powder under liquid nitrogen using a mortar and pestle and transferred to a 1.5 ml sterile (DNase/RNase free) eppendorf tube and lysis buffer added. The homogenate was either stored at -80°C or RNA extraction performed with the Qiagen miRNeasy mini kit (Qiagen, Crawley/, West Sussex, UK) (see section 3.2.3.6).

4.2.4.3 RNA Quantity and Quality Assessment

The RNA quantity and quality was assessed with a Nanodrop ND-1000 UV spectrophotometer (Nanodrop technologies, Wilmington, DE, USA) and a Bioanalyzer 2100 (Agilent Technologies, USA). All samples selected for qRT-PCR had OD $_{260/280}$ absorbance ratios greater than 1.9 and the RIN score above 7.

4.2.4.4 Reverse Transcription and cDNA Synthesis

Reverse transcription and cDNA synthesis were performed in the same way as described for the human tissues as detailed in section 3.2.3.8.

4.2.4.5 Quantitative Real Time PCR

SYBR Green fluorescence probes were used for the qRT-PCR reactions. cDNA from rat placenta, decidua, myometrium, inter-implantation sites and resorbed sites were used as templates. The 25µl final reaction volume for qRT-PCR of the designed primers consisted of 12.5µl 2x SYBR Green PCR Master Mix (Applied Biosystems), 0.2µl (500nM) forward primer, 0.2µl (500nM) reverse primer, 11.1µl of RNase/DNase free water and 1µl of diluted cDNA (10x diluted from samples) (Table 4.2). The 20µl reaction mix for the reference gene investigations included 10µl SYBR Green PCR Master Mix (Applied Biosystems), 300nM of resuspended reference gene primer mix,

8µl of RNase/DNase free water and 1µl of diluted cDNA (Table 4.3). More details are described at section 3.2.3.9.

Table 4.2: Components for the qRT-PCR Reaction

Component	Volume (µl)
SYBR Green	12.5
Forward Primer (500nM)	0.2
Reverse Primer (500nM)	0.2
RNase/DNase free water	11.1
cDNA	1

Table 4.3: Mastermix for the reference genes

Component	Volume (µl)
SYBR Green	10
Primer (300nM)	1
RNase/DNase free water	8
cDNA	1

4.2.4.6 Selection of Reference Genes

The geNorm™ Reference Gene Selection kit with 12 reference genes (PrimerDesign, Southampton, UK) was used to identify the appropriate reference genes for the tissues used in this experiment. The reference genes are listed in Table 4.4. The data from the qRT-PCR were used to calculate gene stability with two freely available and downloadable software programmes geNorm (version 3.4, Center for Medical Genetics, Ghent University, Belgium. http://medgen.ugent.be/genorm) and Normfinder (version 0953, Molecular Diagnostics Laboratory, Dept of Clinical Biochemistry, Aarhus University Hospital, Aarhus, Denmark, www.mdl.dk/publicationsnormfinder/). More details are described in section 3.2.3.10.

The quantification of mRNA level was undertaken with the Pfaffl method (Pfaffl 2001) where the equation incorporates the PCR efficiencies of the reference gene (RG) and gene of interest (GOI) and thereby increases the accuracy in the final relative fold value. This formula is demonstrated below:

$$ratio = \frac{Efficiency\ (GOI)\Delta CT\ GOI\ (control - treated)}{Efficiency\ (RG)\Delta CT\ RG\ (control - treated)}$$

Table 4.4: Reference genes tested in rat tissues with their Genbank number and function.

Name of Reference gene	Abbreviation	Genbank number	Function
18S Ribosomal RNA	18S	NR_046237	Small ribosomal subunit
ATP synthase	ATP5B	NM_134364	Catalysis ATP synthesis
Topoisomerase 1	TOP1	NM_022615	Enzyme to regulate DNA winding
Malate dehydrogenase 1	MDH1	NM_033235	Enzyme in citric acid cycle catalyzes conversion of malate to oxaloacetate
Cytochrome c-1	CYC1	NM_001130491	Subunit of electron transport chain
Calnexin	CANX	NM_172008	Protein of endoplasmatic reticulum, assists in protein folding
Ribosomal protein L13a	RPL13A	NM_173340	Structural component of the large 60s ribosomal subunit
Tyrosine 3-monooxygenase	YWHAZ	NM_013011	Catalyses conversion of amino acid L-tyrosine to dihydroxyphenylalanine
Beta-2-microglobulin	B2M	NM_012512	Beta chain of major histocompatibility complex class I molecules
Ubiquitin C	UBC	NM_017314	Protein degradation
Glyceraldehydes-3-phosphate deyhrogenase	GAPDH	NM_017008	Oxidoreductase in glycolysis and gluconeogenesis
Beta actin	ACTB	NM_031144	Cytoskeletal structural protein

4.2.4.7 Statistical Analysis of qRT-PCR

Statistical analysis of the data was performed using GraphPad Prism version 5 for Windows (GraphPad Software, San Diego, California, USA). The groups were compared using Kruskal Wallis Test with Dunn's Multiple Comparison Test and two factors were analysed using Two-Way ANOVA with Bonferroni ad-hoc test. A p-value<0.05 was set as significant. The data are expressed as mean ± SEM.

4.2.5 Western Blotting

Protein expression of NAPE-PLD and FAAH in rat placenta, decidua, myometrium and inter-implantation sites was analysed by Western Blotting.

4.2.5.1 Protein Extraction

Tissue was collected and weighed. This was then homogenized with a Potter homogenizer in a homogenizing buffer of 20 mM HEPES buffer, 2 mM EDTA, 10 mM KCl, 1.5 mM $MgCl_2$ (1:2), 1 mM phenylmethylsulphonylfluoride (PMSF) and aprotinin (1:100). The homogenate was centrifuged at 700 x g for 10 min at 4°C, the supernatant removed into a new tube and re-centrifuged at 12000 x g for 30 min at 4°C. The supernatant was stored at -80°C for later analysis.

4.2.5.2 Protein Quantification

Protein was quantified using the Bio-Rad Protein Assay kit (Bradford Reagent, Catalogue number: B6916, Sigma) according to the manufacture's protocol (see section 3.2.4.2).

4.2.5.3 Separation of Proteins, Protein Transfer and Detection

1.5 mm polyacrylamide gels (10% running gel and 5% stacking gel) were used for the separation of protein. The preparation of the gels is described at section 3.2.4.3.

40µg of sample lysate in sample buffer was denatured at 100°C for 5 min and loaded into each well. The positive controls were homogenates of rat brain. The proteins were separated by electrophoresis and transferred onto a nitrocellulose membrane as described at section 3.2.4.5. The membranes were incubated with primary rabbit anti-rat polyclonal antibodies against NAPE-PLD (dilution 1:250) (Cayman, product number 10306) and FAAH (dilution 1:500) (Cayman, product number 101600) overnight at 4°C. The following day the membranes were washed, incubated with the secondary antibody (1:1000) and anti-biotin horseradish peroxidase-linked antibody (1:2000) (3.2.4.5). The membranes were exposed to X-Ray films and the bands were visualised by chemiluminescence (see section 3.2.4.7). The signal intensities were quantified by densitometry. Unfortunately, it was not possible to quantify the protein content of CB1 and TRPV1 in rat tissue using Western blotting. The primary antibodies used are listed in Table 3.14 in section 3.2.4.6. GAPDH was used as a loading control in rat tissue. β – Tubulin did not appear as a clear band and so normalisation with this marker protein was abandoned (section 3.2.4.8).

4.2.5.4 Statistical Analysis of Western Blotting

Statistical analysis was performed using Two-way repeated measures ANOVA with Bonferroni post test (GraphPad Prism version 5.0, GraphPad Software, San Diego, CA, USA). A p-value of $p<0.05$ was considered as statistically significant. The data are expressed as mean ± SEM.

4.3 RESULTS

4.3.1 NAE levels in Plasma and Uterus of Female Rats

Plasma and uterine AEA levels were increased at 30, 60 and 120 min after intraperitoneal AEA injection compared to a rat without AEA injection (Figure 4.6).

The maximum concentration was reached within 30 min. However, plasma and uterine OEA and PEA did not change after AEA injection (Figure 4.7).

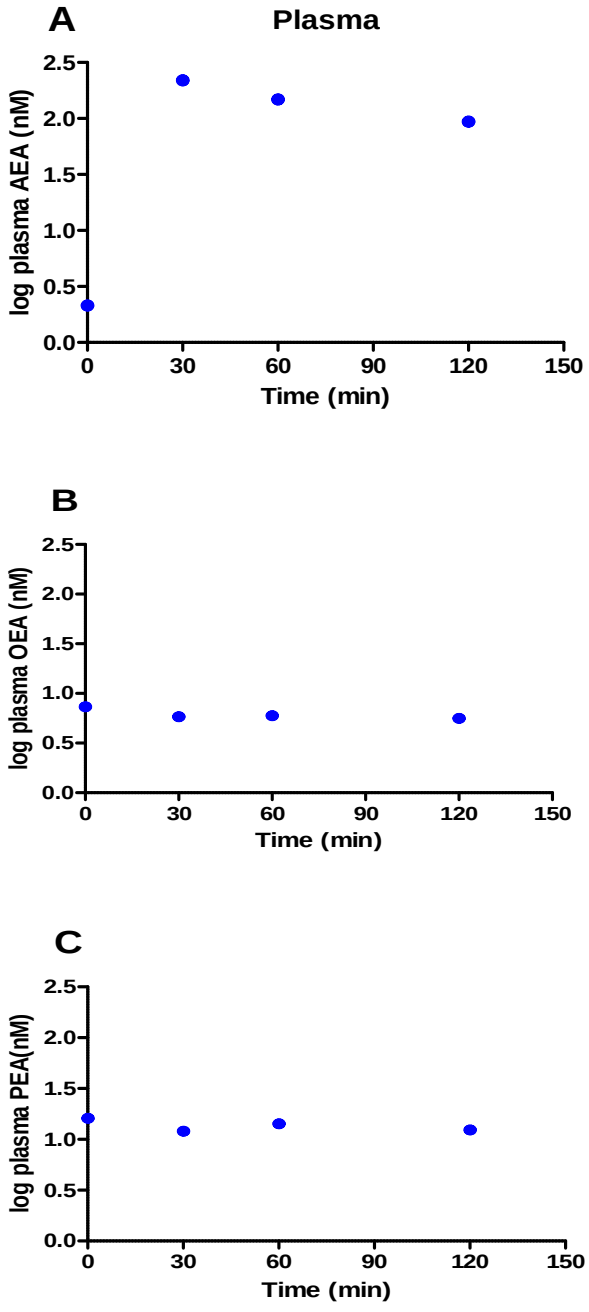

Figure 4.6: Plasma AEA, OEA and PEA levels in female rats after intraperitoneal AEA injection (5mg/kg) (n=4).

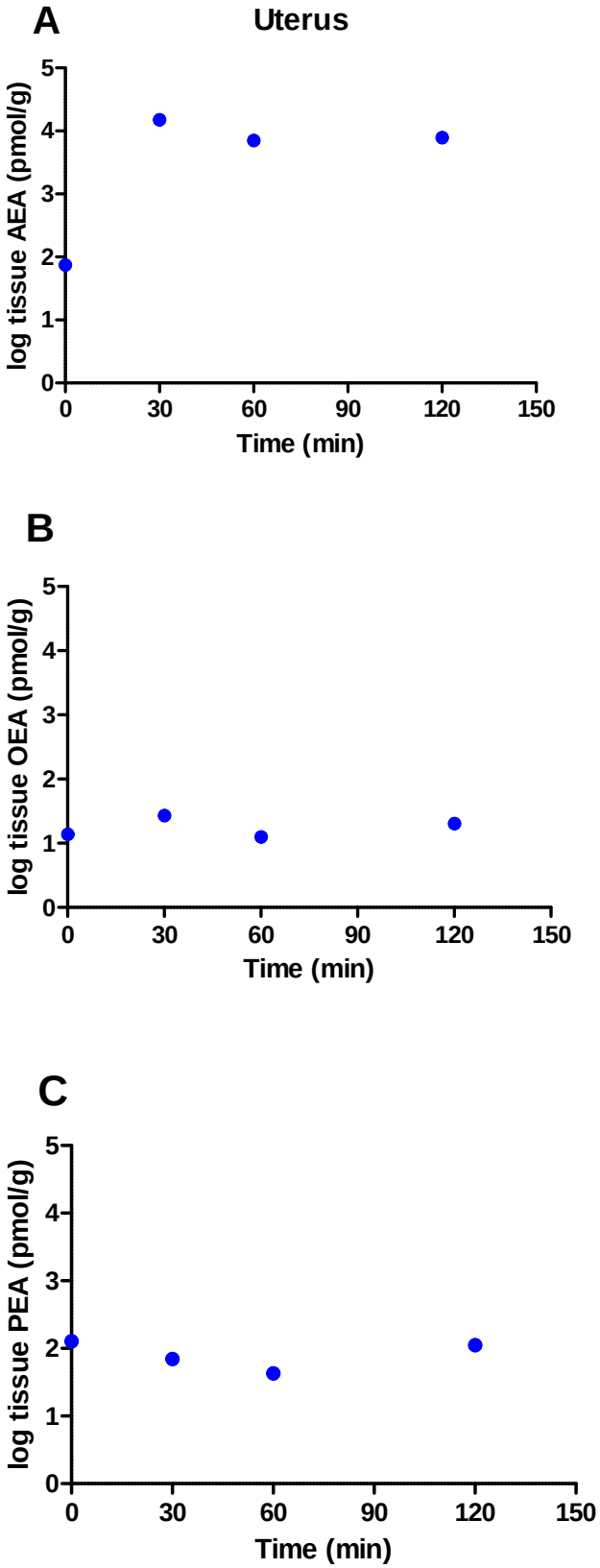

Figure 4.7: Uterine AEA, OEA and PEA levels in female rats after intraperitoneal AEA injection (5mg/kg) (n=4).

4.3.2 Effect of exogenous AEA on the Number of Implantation and Resorbed Units

In the initial experiment three rats received either a single injection of AEA (0.5 mg/kg) or normal saline only as a control on day 4 of pregnancy. Rats in the treated group showed a significant reduction in the number of normal implantation sites ($p=0.049$) (Figure 4.8).

The other experiment involved daily injections of AEA (0.25 mg/kg) or vehicle normal saline on day 2, day 3 and day 4. Here again a reduction in the number of normal implantation sites with a concurrent increase in resorbed units was observed in treated rats (Figure 4.8).

As the reduction in implantation sites with the single injection was more marked compared to the daily injections a decision was taken to continue the experiments with a single injection of AEA. However, in an attempt to improve efficacy, the dose of AEA was increased to 1 mg/kg.

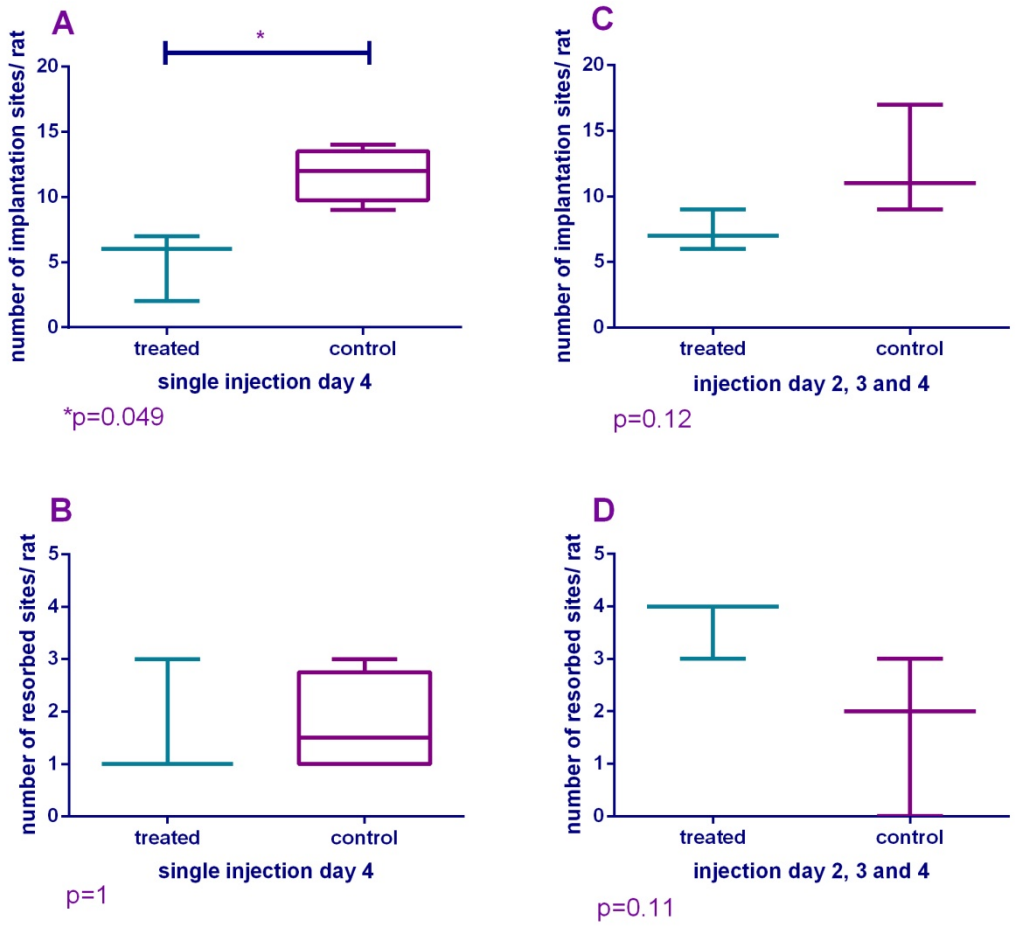

Figure 4.8: Number of normal implantation sites and resorbed units after a single injection (A, B) and after daily injections (C, D) of AEA or vehicle.
(A)There was a significant reduction in the number of implantation sites after single dose of AEA (0.5 mg/kg) on Day 4 ($p<0.049$). (B) The number of resorbed units after a single injection of AEA (0.5mg/kg) or vehicle did not differ. (C) The number of implantation sites was reduced (D) and the number of resorbed sites was increased in rats with daily AEA (0.25 mg/kg) injections compared to the control (Mann-Whitney U test). The results are shown as minimum, median and maximum.

The next experiment consisted of six rats in each treatment group (day 2, day 4 and day 6) and the control group; the AEA or vehicle was given as a single intraperitoneal injection. After sacrifice, the uteri were examined. One rat treated with AEA on day 4 had 14 small, non viable, implantation sites and was excluded and one rat treated with AEA on day 6 had no implantations despite a positive sperm swab but was also excluded as an outlier.

Rats treated with AEA on days 4 and 6 had a 14% and 33% decrease in the number of normal implantation sites, respectively compared to control animals. The left uterine horn, close to the site of injection consistently had fewer implantation sites than the right uterine horn (Figure 4.9 and Table 4.5).

AEA treated rats also showed more resorbed sites compared to the control group. The percentage of resorption was highest after AEA treatment on day 6, one day after implantation (Figure 4.10).

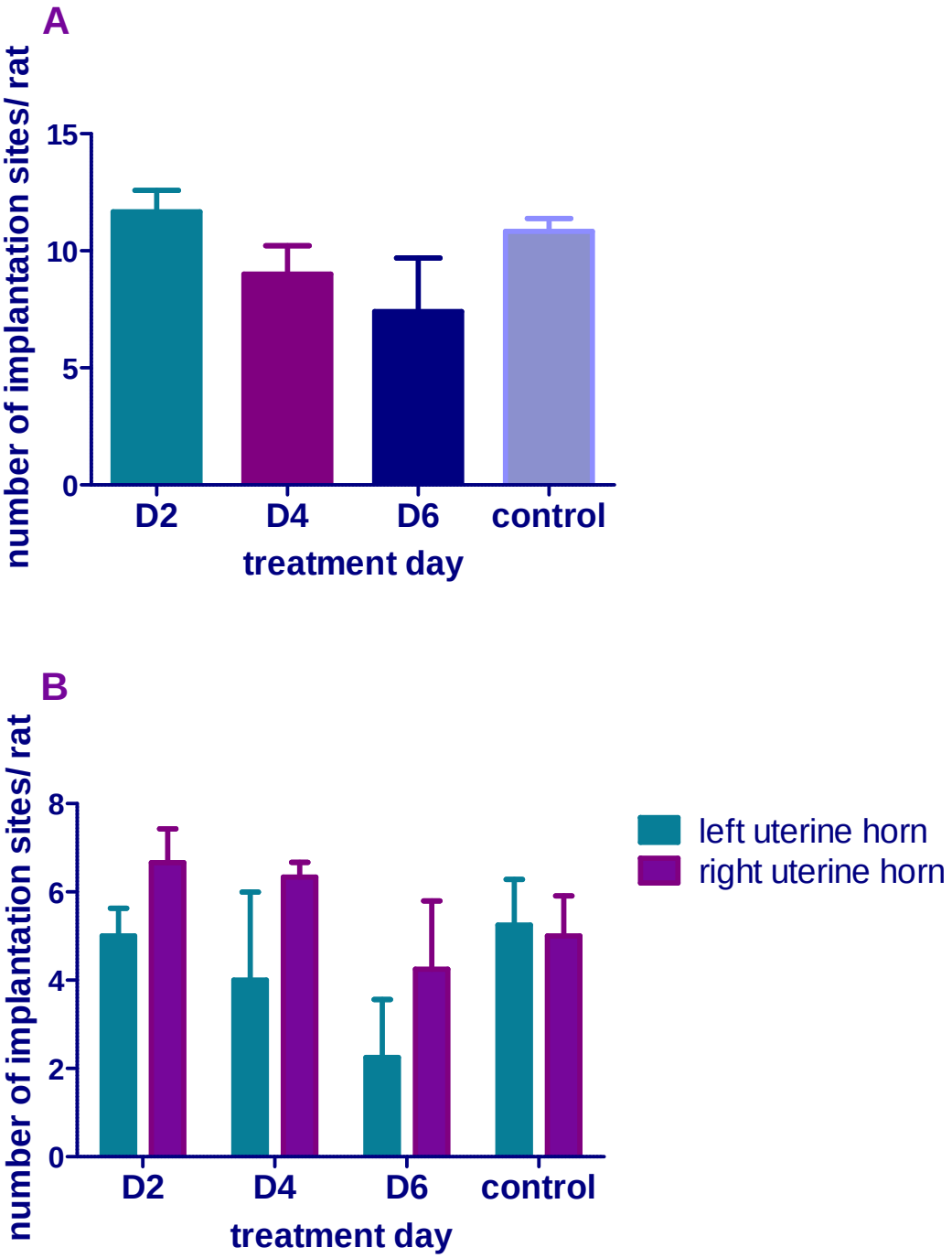

Figure 4.9: Number of normal implantation sites (A) in both horns and (B) in the left and right uterine horn of treated and control rats.
(A) Day 6 treated rats showed reduced implantation sites when compared to the control. (B) The left uterine horns in all treated rats had reduced implantation sites compared to the right horns. The results are illustrated as mean ± SEM.

Table 4.5: Number of implantation sites and percentage change in number of successful implantation sites after AEA treatment.

	Number of implantation sites (mean ± SEM)		Percentage of change in implantation sites compared to control animals	
	Left uterine horn	**Right uterine horn**	**Left uterine horn**	**Right uterine horn**
Day 2 (n=6)	5.0±0.6	6.7±0.8	-4.8 %	+33.3 %
Day 4 (n=3)	4.0±2.0	6.3±0.3	-23.8 %	+26.7 %
Day 6 (n=4)	2.3±1.3	4.3±1.5	**-57.1 %**	-15.0 %
Control (n=4)	5.3±1.0	5.0±0.9		

The left uterine horn of treated animals contained less implantation sites than the control, whereby the biggest reduction in implantation sites were observed in Day 6 treated rats.

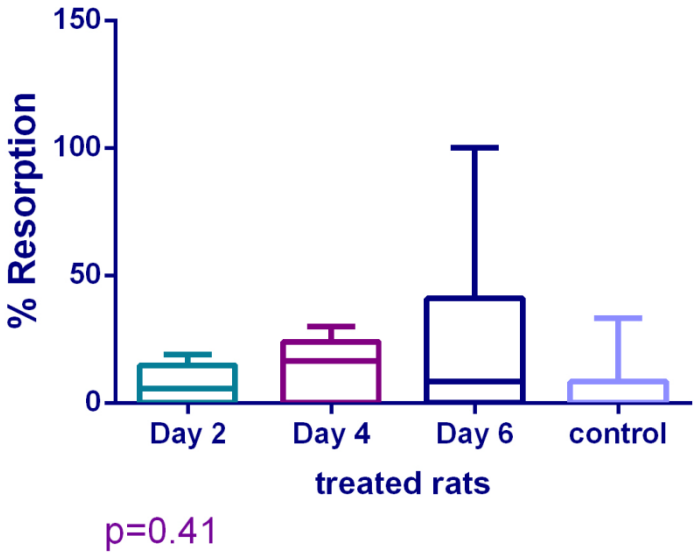

Figure 4.10: Effect of intraperitoneal injection of AEA on resorption rate.
A higher percentage of resorption was observed in treated rats compared to the control rat, whereby day 6 treated rats showed the highest percentage of resorption (Kruskal-Wallis Test with Dunn's Multiple Comparison Test). The results are presented as boxplots whereby the box shows the upper and lower quartiles and the median and the end of the whisker represent the maximum value.

4.3.3 NAE levels in Plasma and Tissue

4.3.3.1 NAE Levels in Plasma at Gestation Day 14

NAE plasma levels were measured from rats treated with AEA on day 2 (n=6), day 4 (n=5), day 6 (n=4) and control (n=6) rats at sacrifice on day 14. Plasma AEA levels in day 2 treated rats were significantly higher than day 6 treated rats. There was no significant difference in OEA or PEA levels between treated and control rats (Figure 4.11).

Plasma AEA levels showed a significant positive correlation with the number of normal implantation sites as well as with the total number of implantation sites including normal implantation and resorbed sites ($p<0.0091$; $r=0.58$ and $p=0.023$; $R2=0.26$) (Figure 4.12 A and D). However, there was no correlation between either plasma OEA or PEA levels with the number of implantation sites (Figure 4.12).

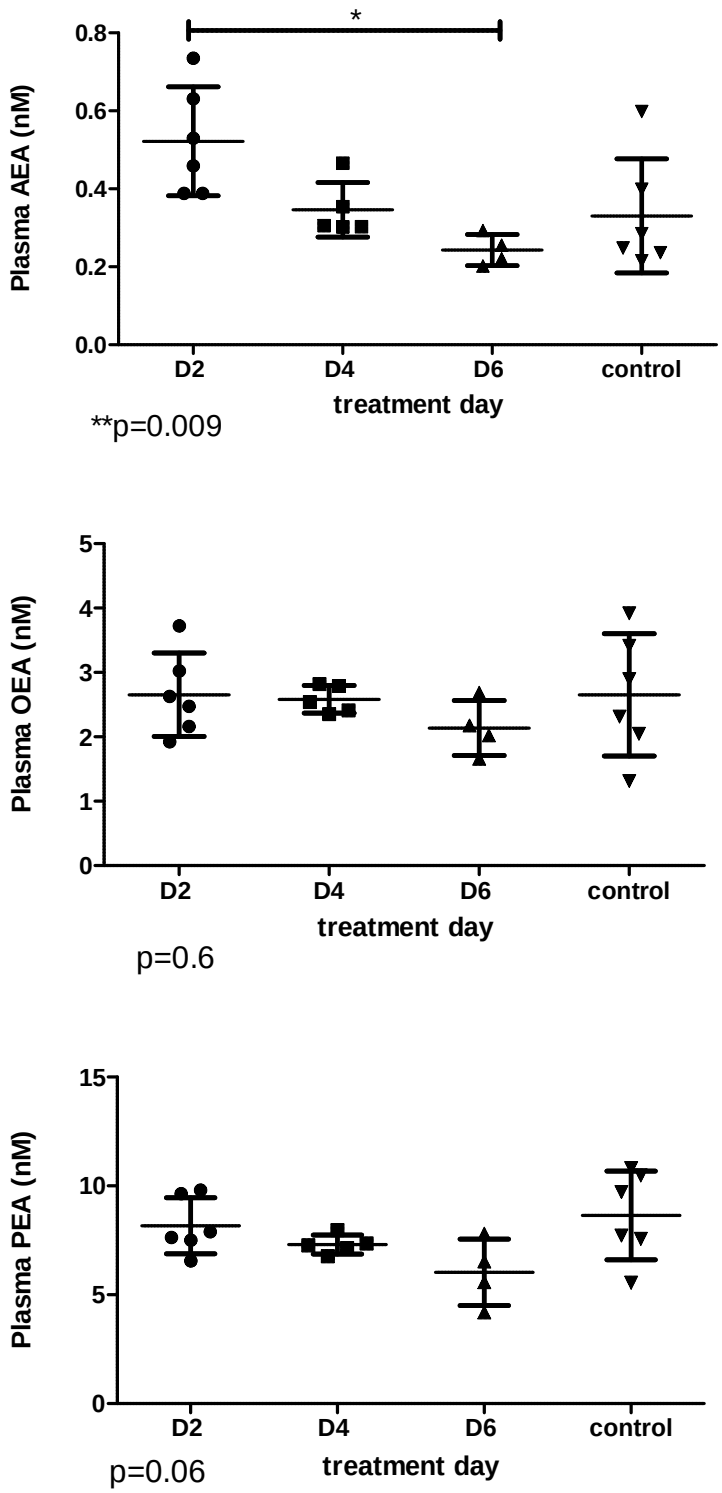

Figure 4.11: Gestation day 14 plasma NAE levels in D2 (n=6), D4 (n=5), D6 (n=4) AEA-treated and control rats (n=6).
Plasma AEA levels on gestation day 14 of day 2 treated rats were significantly higher than day 6 treated rats (p=0.009). No significant differences were detected in OEA and PEA levels of treated and control rats (One-way ANOVA test with Bonferroni post-hoc test). The results are described as mean ± SEM.

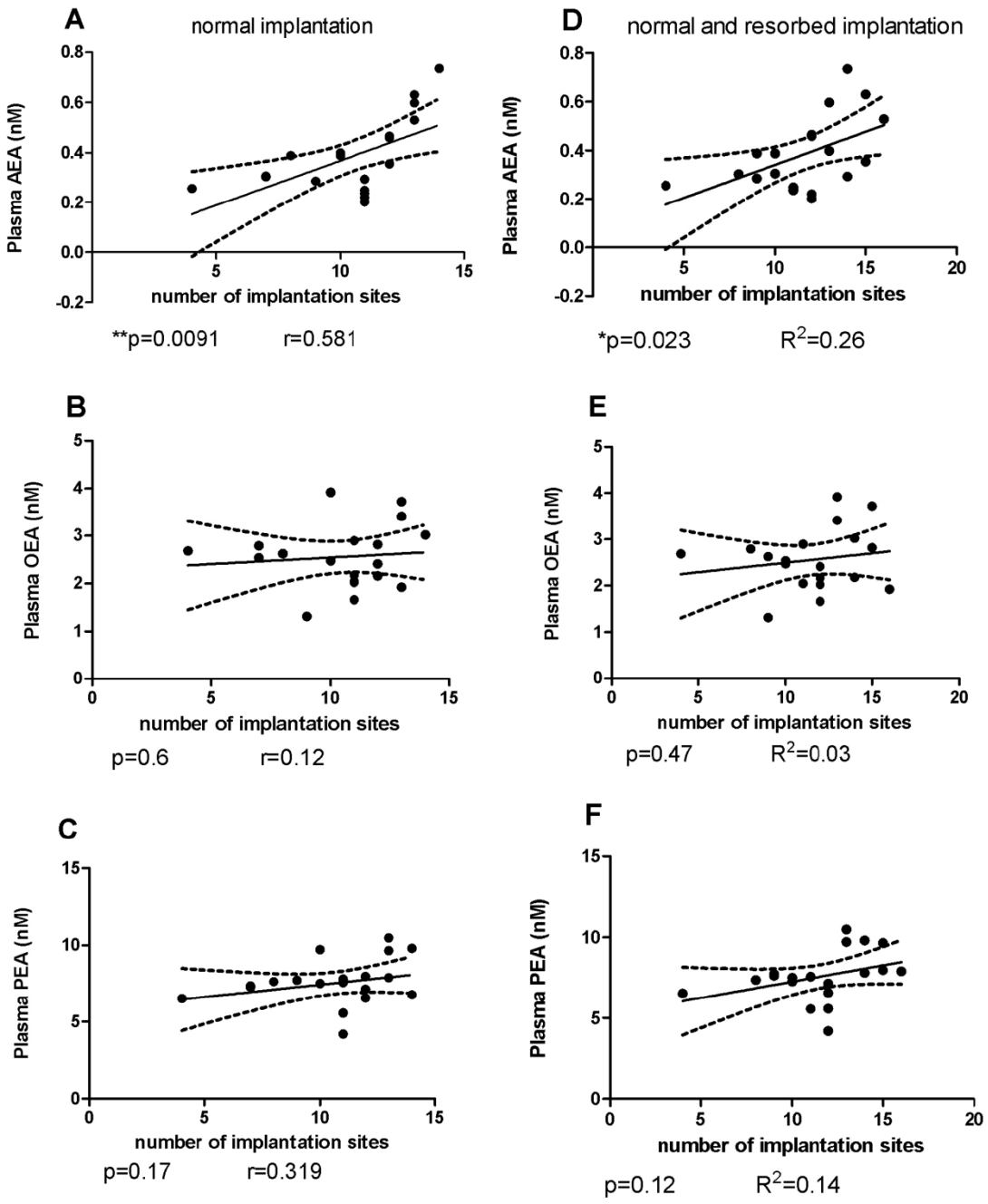

Figure 4.12: Correlation between gestation day 14 plasma NAE concentration and number of normal (A-C) and total (normal and resorbed) (D-F) implantation sites.
There is a positive correlation between AEA plasma levels and number of normal implantation sites (A) (p=0.0071) and number of all implantation sites (D) (p=0.023). Plasma OEA and PEA levels did not show a correlation with number of implantation sites (B, C, D and F) (A-C Spearman Correlation and D-F Pearson Correlation).

4.3.3.2 Day 14 NAE Levels in Rat Trophoblast and Decidua

NAE levels in decidua from treated animals were higher when compared to the controls, but this was only statistically significant for PEA levels in day 6 treated rats ($p=0.03$) (Figure 4.13). In placental tissue there were no statistically significant differences in AEA, OEA and PEA levels between treated and control rats. However, the highest placental NAE levels were found in day 4 treated rats, but this was only significant for AEA when compared to day 2-treated rats ($p=0.017$) (Figure 4.13).

When NAE levels in decidua and placenta were compared, no significant differences were seen with the untreated group. In day 4 treated rats, AEA levels in placenta were significantly higher than decidua ($p<0.05$); there were no significant differences for OEA and PEA levels in placenta and decidua (Figure 4.13).

There was a positive, significant correlation between PEA levels in the placenta and decidua but not for AEA and OEA levels in these tissues (Figure 4.14).

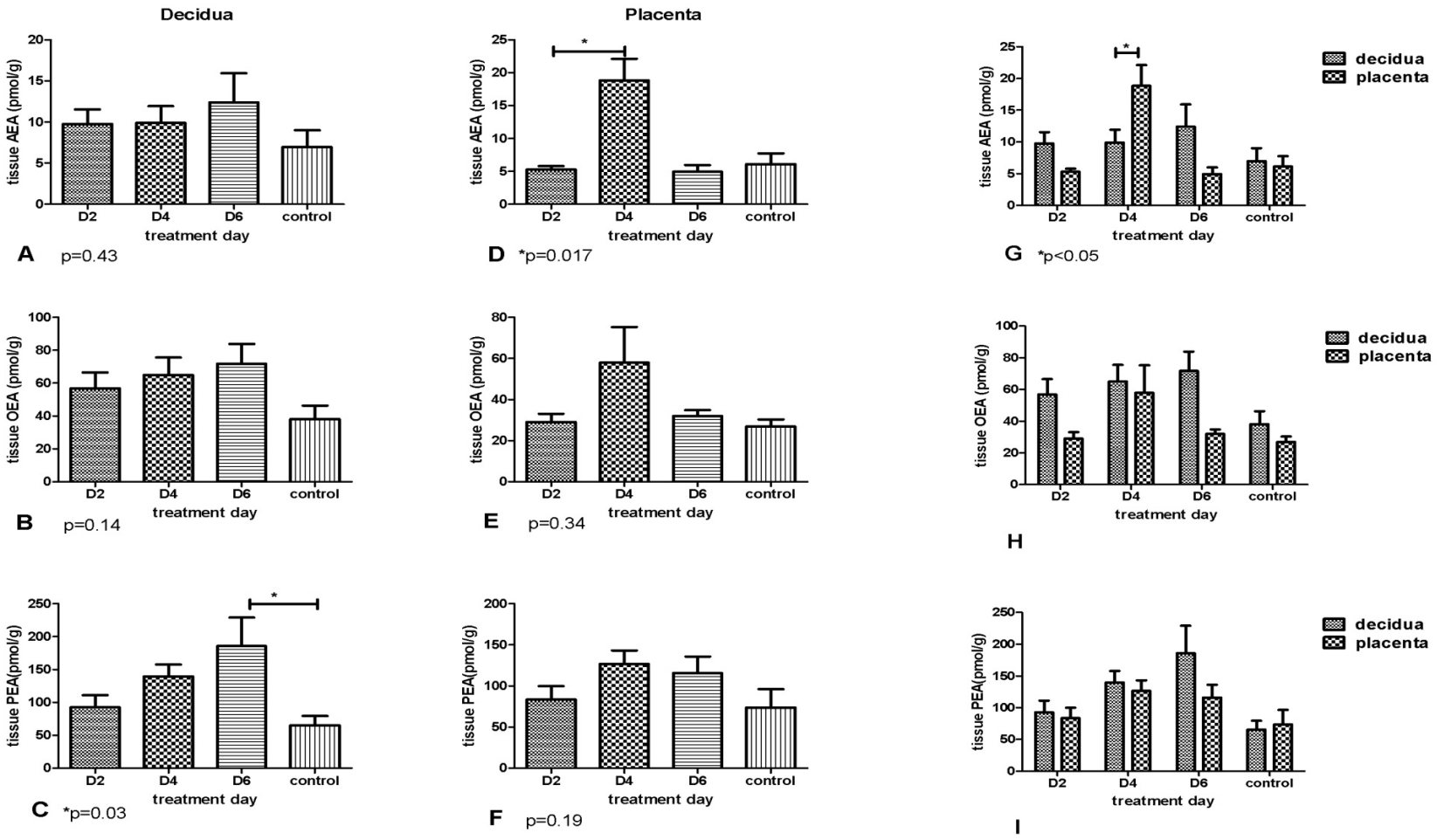

Figure 4.13: AEA (A,D,G), OEA (B,E,H) and PEA (C,F,I) levels in decidua (A-C), placenta (D-F) and both decidua & placenta (G-I) at gestation day 14.
A-F: Kruskal-Wallis Test with Dunn's Multiple Comparison Test, G-I: Two-way ANOVA with Bonferroni post-hoc test.

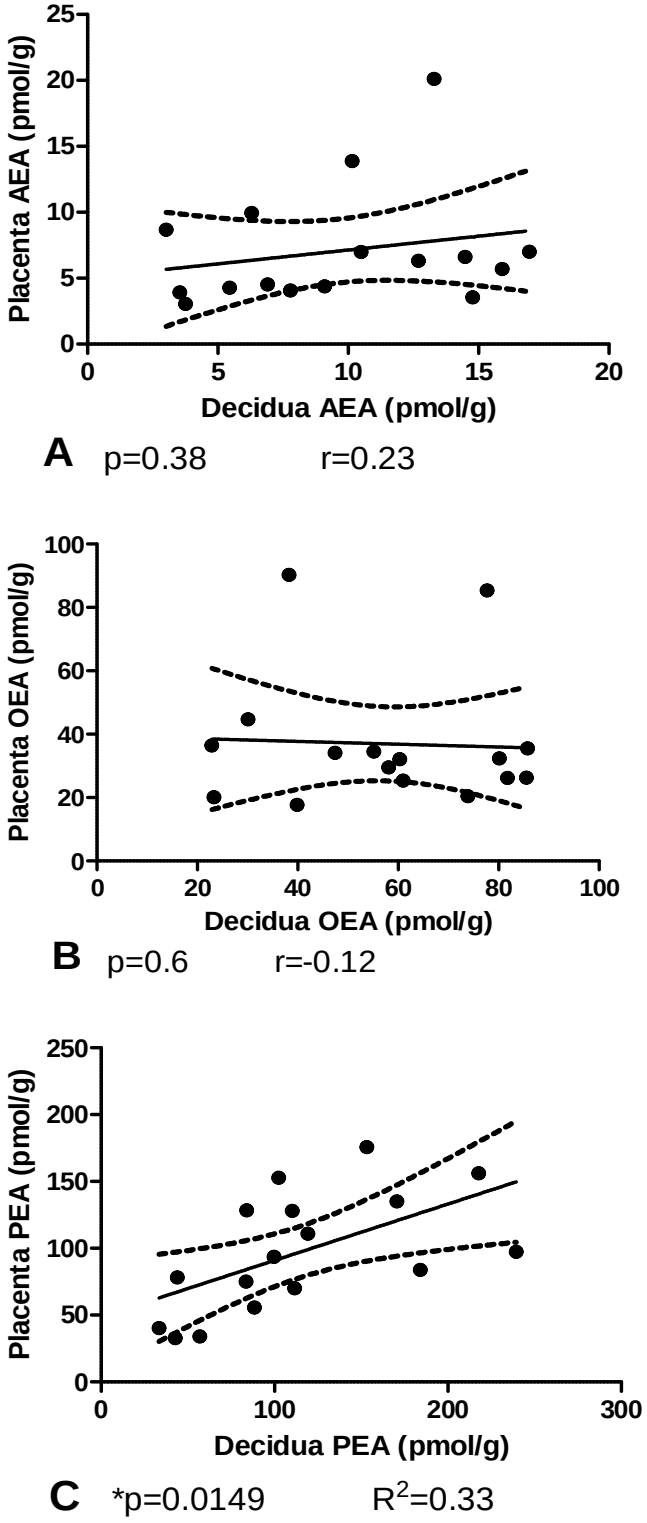

Figure 4.14: Correlation of NAE levels in decidua and placenta at gestation day 14.
A significant correlation between PEA levels in placenta and decidua was observed (p=0.0149), but not in AEA and OEA levels (A-B: Spearman Correlation with Spearman coefficient (r) and C: Pearson Correlation).

4.3.3.3 Comparison of NAE Levels in Plasma and Tissue at Gestation Day 14

There were no significant correlations in AEA, OEA and PEA levels on gestational day 14 between plasma and either decidua or placenta (Figure 4.15).

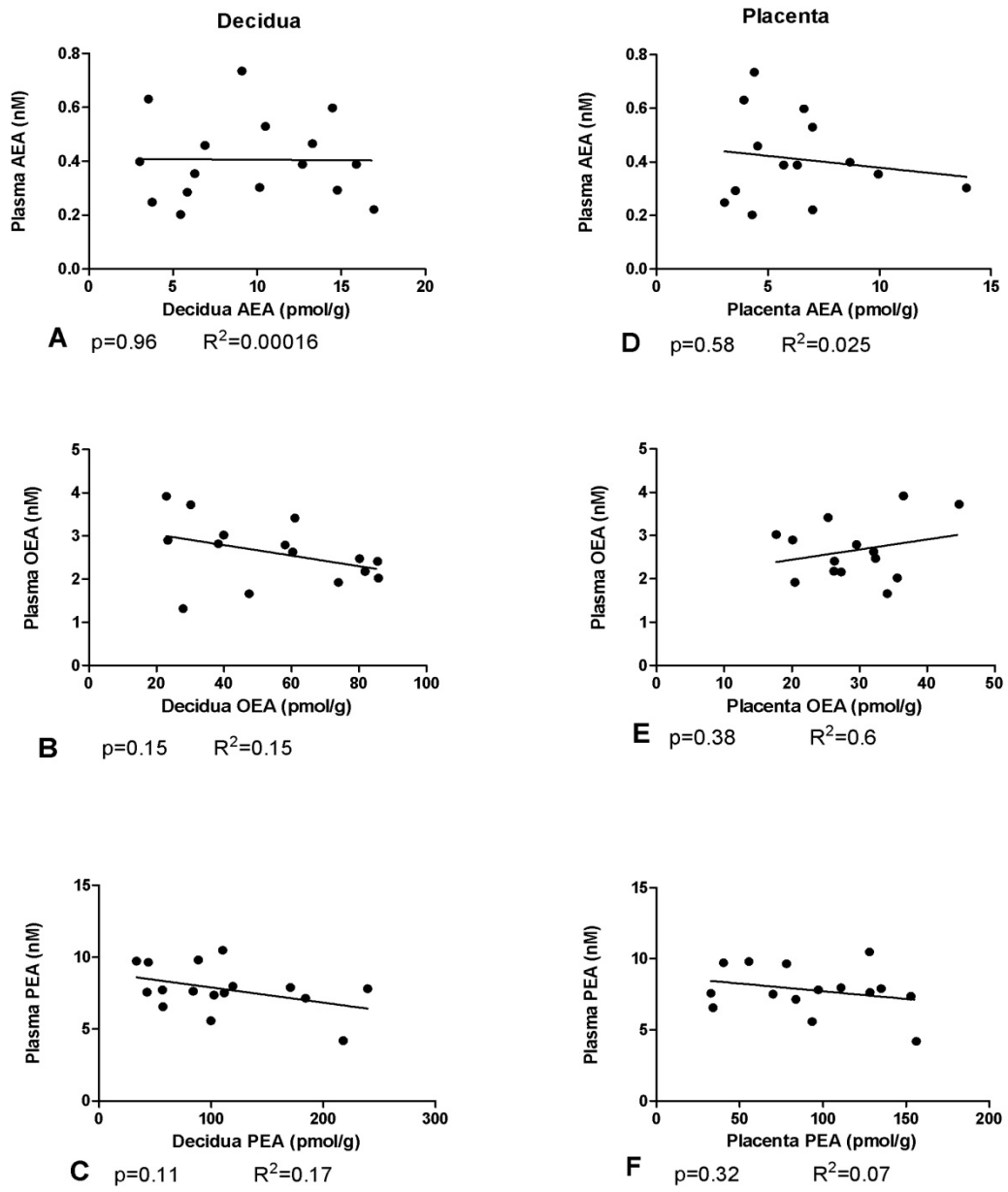

Figure 4.15: Correlation of AEA, OEA and PEA levels in plasma and decidua (A-C) and plasma and placenta (D-F) on gestational day 14.
No significant correlation between NAE levels in plasma and decidua or placenta was observed (Pearson Correlation).

4.3.4 Immunohistochemistry

The spatiotemporal expression of the enzymes NAPE-PLD and FAAH in rat pregnancy has been established (Fonseca et al. 2010). Here the localisation of NAPE-PLD and FAAH on day 14 of pregnancy in rats pre-treated with AEA on day 4 and day 6 of pregnancy and control rats was characterized.

4.3.4.1 NAPE-PLD

NAPE-PLD immunostaining was present in implantation and resorbed units. After binding the primary NAPE-PLD antibody with excess blocking peptide no tissue staining was seen and specific immunostaining for the antibody NAPE-PLD was confirmed (Figure 4.16).

In control animals NAPE-PLD was expressed in decidual cells (mesometrial decidua) and on the fetal side, only the giant trophoblast cells revealed positivity in the basal zone. In contrast, the entire labyrinth zone was positive with immunoreactivity in syncytiotrophoblast and glycogen trophoblast cells (Figure 4.17). The endotrophoblast cells were negative.

In day 4 treated animals, NAPE-PLD was localised in decidual cells with cytoplasm and nuclear staining similar to that of the controls. Endotrophoblast cells were also negative (Figures 4.17 and 4.18). Decidual cells in resorbed units were positive although with less intensity than normal implantation units (Figure 4.19).

The fetal side revealed differences, particularly in the basal zone, which was immunoreactive to this enzyme (Figure 4.17 and 4.18). Immunostaining in the basal zone showed that giant trophoblasts cells were immunopositive, and within the labyrinth zone, the spongiotrophoblasts were also positively stained revealing nuclear expression.

Day 6 treated animals exhibited the same pattern of NAPE-PLD expression as observed in day 4 treated animals, though the placental architecture was more disorganized. Interestingly, NAPE-PLD exhibited notable membrane expression in giant trophoblast cells in control implantation units, whereas in treated animals no differentiable membrane staining was observed (Figures 4.17 and 4.19). In resorbed sites decidual cells were only positive for NAPE-PLD near to the resorbed embryo (Figure 4.19).

There was no significant difference in staining intensities of NAPE-PLD in normal implantation sites of day 4, day 6 treated and control rats (Figure 4.20).

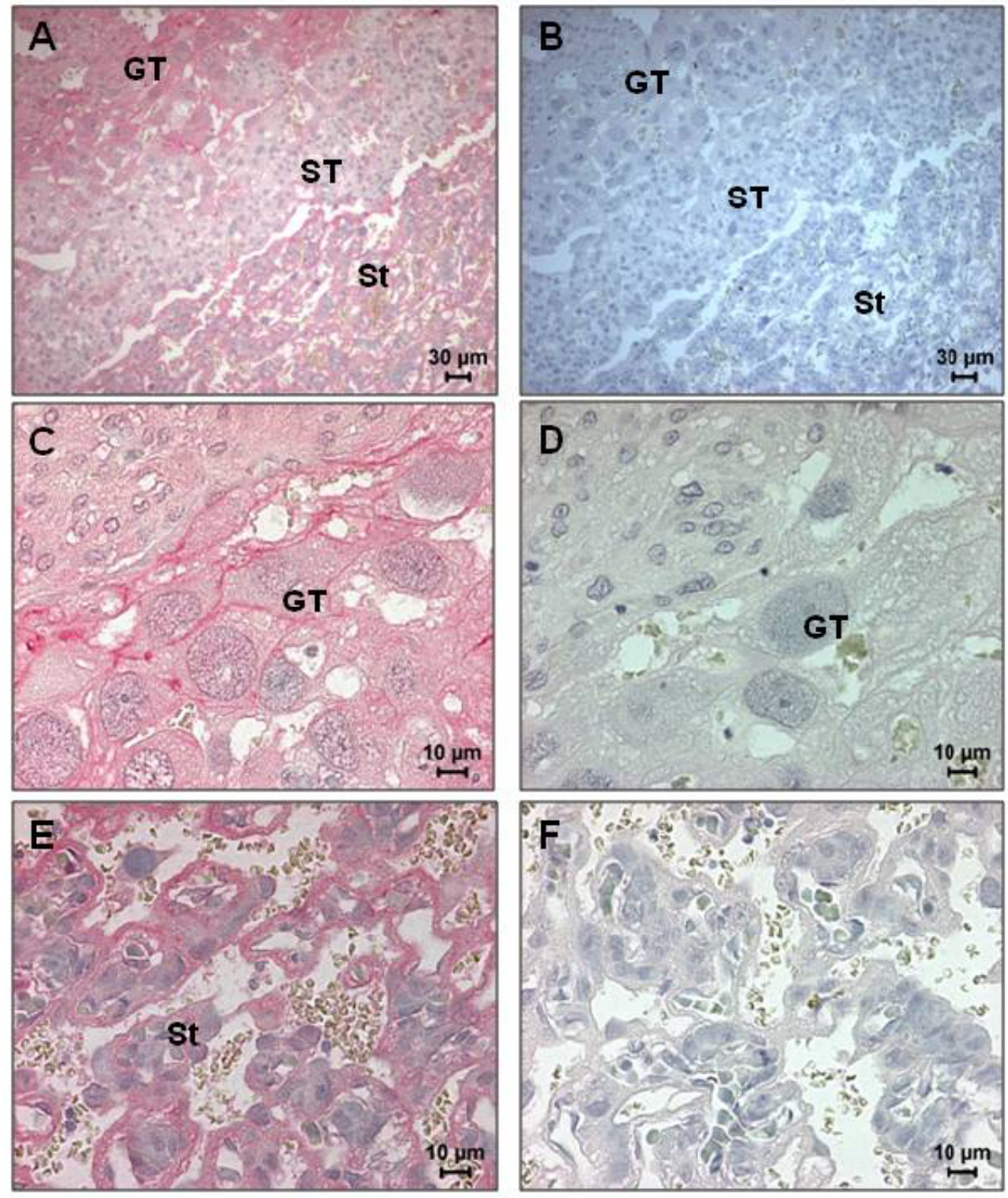

Figure 4.16: Specificity of polyclonal antibody NAPE-PLD.
Immunoreactive NAPE-PLD on rat implantation sites (A, C, E) and negative control (B, D, F). (A) NAPE-PLD expression in giant trophoblast (GT), spongiotrophoblast (ST) and syncytiotrophoblast (St). Higher magnification of giant trophoblast cells (C, D) and of syncytiotrophoblast cells (E, F).

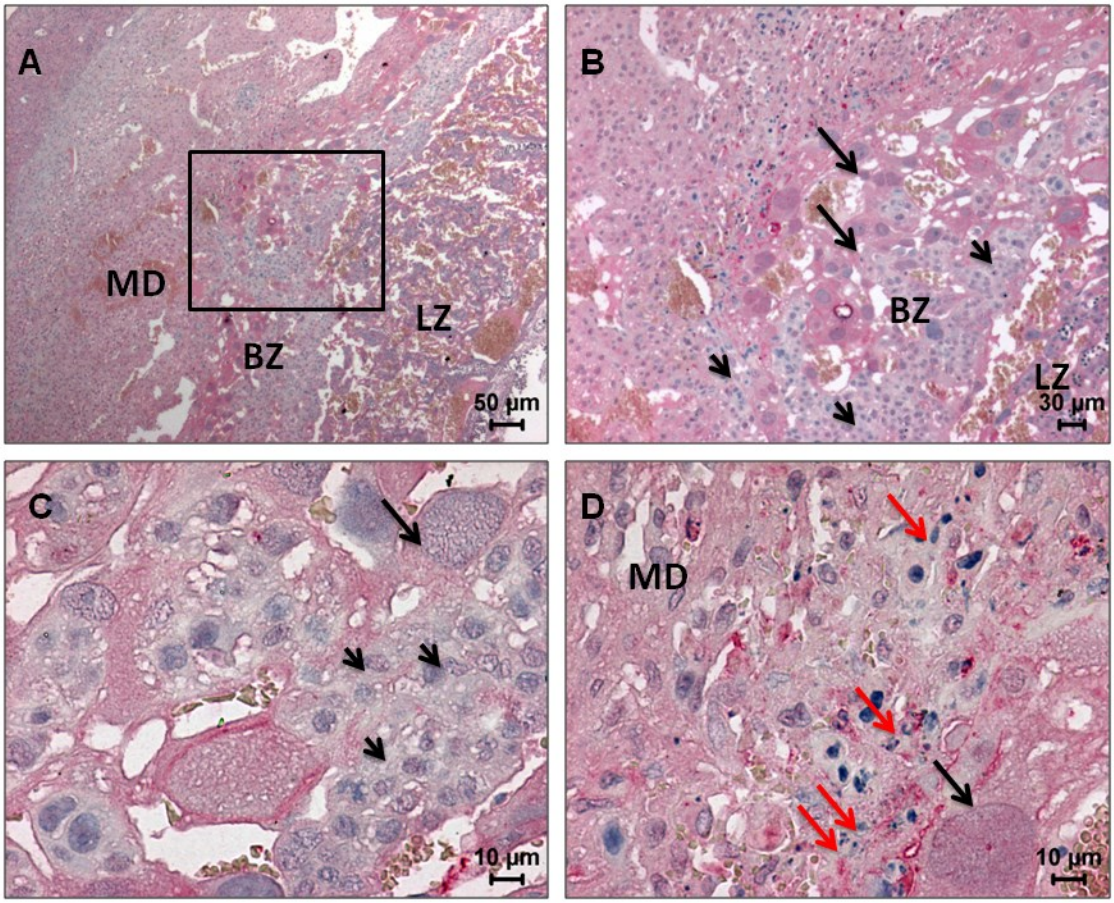

Figure 4.17: Immunoreactive NAPE-PLD localisation in implantation sites of control rats.
(A) NAPE-PLD expression in decidual cells of the mesometrial pole (MD), basal zone (BZ) and labyrinth zone (LZ) of the placenta, (B) high magnification of the square indicated in A showing the positive giant trophoblast cells (arrows) and the negative spongiotrophoblast cells (arrow heads), (C) giant trophoblast cells (arrows) were immunoreactive to NAPE-PLD whereas spongiotrophoblast cells (arrow heads) were negative (D) Apoptotic bodies (red arrows) were seen within the mesometrial pole. BZ=basal zone.

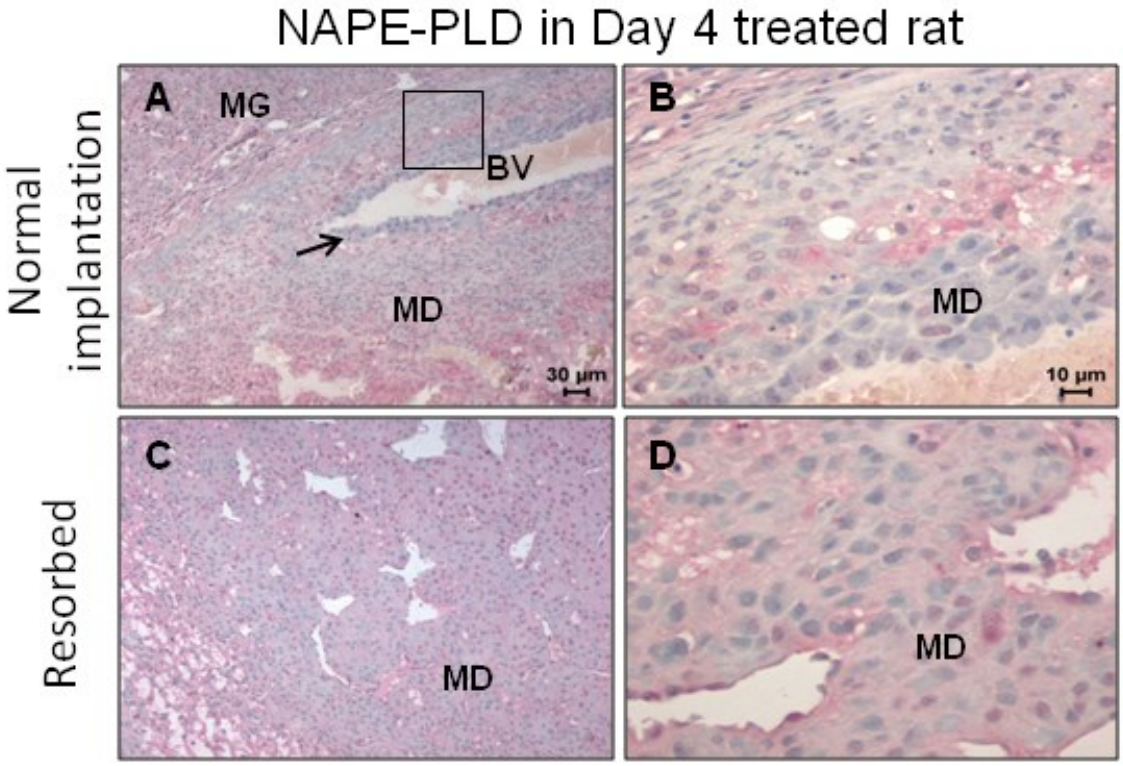

Figure 4.18: NAPE-PLD localisation in normal implantation and resorbed sites after day 4 treatment.
(A) and (B) decidual cells were positive for NAPE-PLD whereas endotrophoblast cells were negative (arrow), (C) and (D) NAPE-PLD staining in decidual cells of resorbed sites; MD: mesometrial decidua; MG: metrial gland.

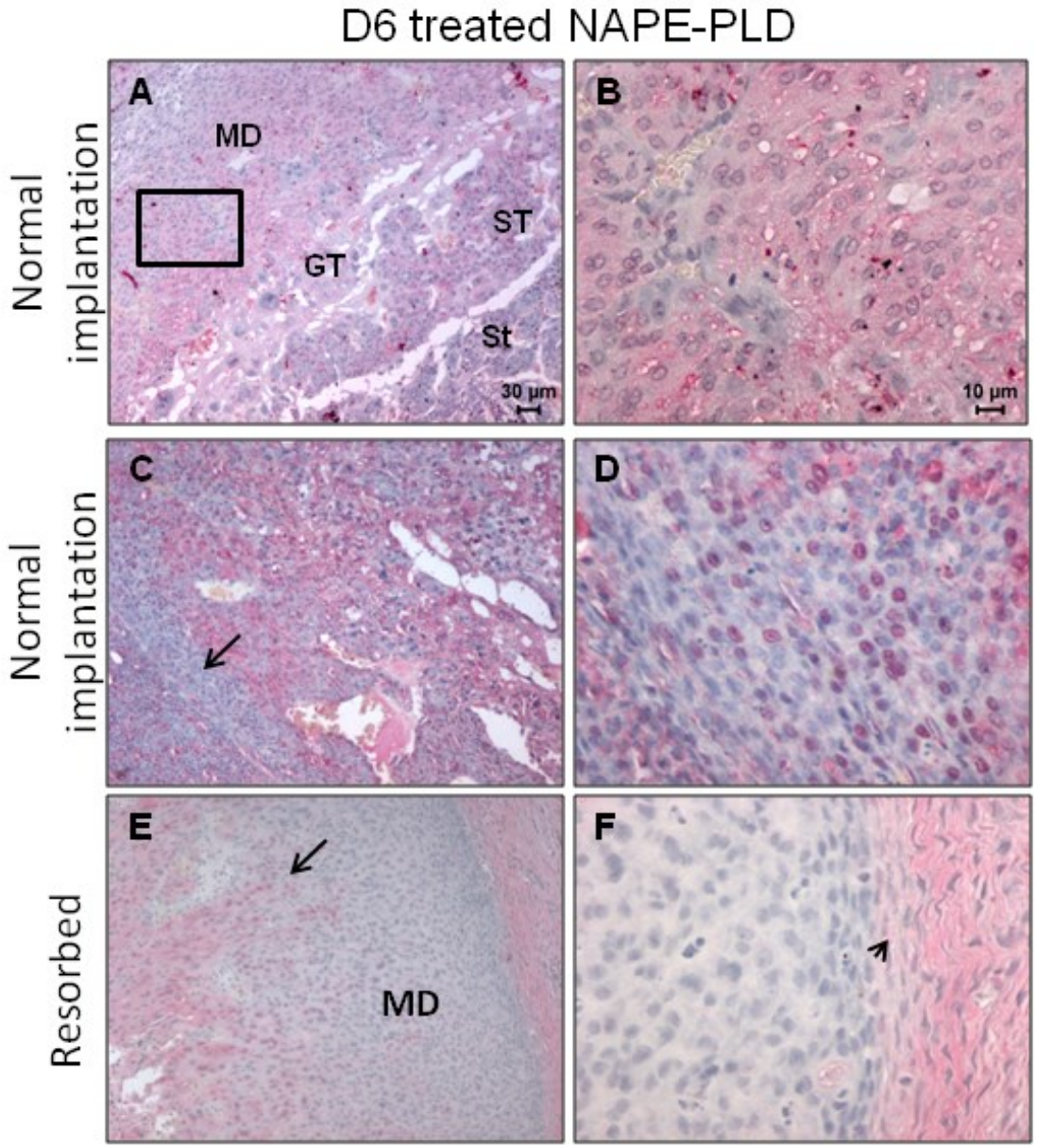

Figure 4.19: NAPE-PLD localisation in normal implantation and resorbed sites after Day 6 treatment.
(A) and (B) NAPE-PLD staining in cytoplasm and nucleus of decidual cells, (C) and (D) nuclear staining of decidual cells (arrow) to NAPE-PLD, (E) positive staining of decidual cells near the resorbed embryo (arrow), (F) positive staining of the muscle (arrowheads). MD: mesometrial decidua; GT: giant trophoblast; ST: spongiotrophoblast, St: syncytiotrophoblast.

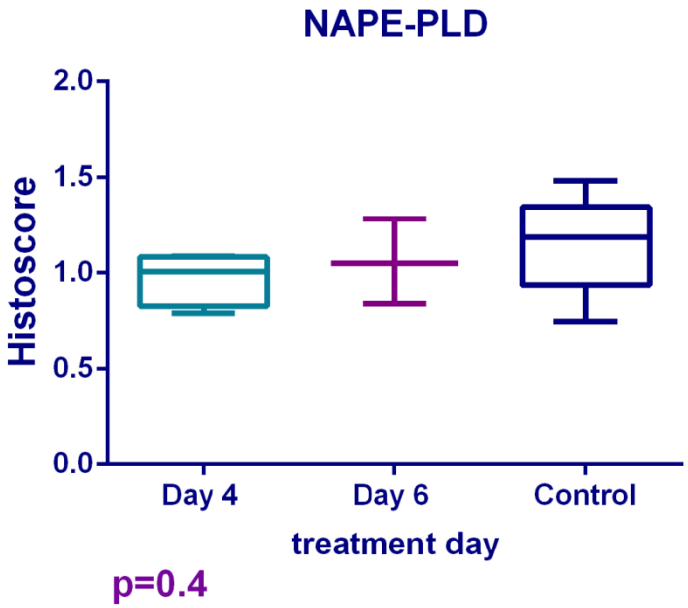

Figure 4.20: Staining intensities of NAPE-PLD in normal implantation sites of day 4 and day 6 AEA-treated and control rats.
No significant changes in NAPE-PLD levels in placenta and decidua of treated and control rats were detected (Kruskal Wallis Test with Dunn's Multiple Comparison Test). The results are presented as boxplots whereby the box shows the upper and lower quartiles and the median and the end of the whiskers represent the minimum and maximum value.

4.3.4.2 FAAH

FAAH immunostaining was present in implantation and resorbed units. Specific immunostaining using the anti-FAAH antibody was identified. No staining was seen when the blocking peptide was incorporated as a negative control (Figure 4.21).

Control animals exhibited FAAH expression in decidual cells and giant trophoblast cells as well as the labyrinth zone of the placenta. However, spongiotrophoblast cells were immuno-negative to anti-FAAH antibodies (Figure 4.22).

Implantation units from animals treated on day 4 revealed FAAH expression in decidual and giant trophoblast cells. Immunostaining in the basal zone showed that giant trophoblasts were immunopositive. Within the labyrinth zone, some spongiotrophoblasts were also positive. Contrary to the control animal, endothelial cells were also positive for FAAH immunostaining (Figures 4.22 and 4.23).

In the animals treated on day 6, the implantation units generally demonstrated less expression of FAAH than implantation sites from controls. Endothelial cells were negative. Decidual cells adjacent to the muscle layer were immunonegative for FAAH. Also in the normal unit of day 6 treated rats, basal placenta was positive whereas the labyrinth zone was negative. Spongiotrophoblast cells and giant trophoblast cells revealed nuclear expression, while syncytiotrophoblast cells were negative (Figures 4.21 and 4.23).

However, there was no significant difference in staining intensities of FAAH between implantation sites of neither day 4 nor day 6 treated and control rats (Figure 4.24).

The resorbed sites showed less FAAH staining intensity than the normal implantation sites. Resorbed units from AEA-treated animals exhibited the same abnormalities and expression patterns observed with resorbed units from control animals (Figure 4.23).

Overall, it was found that in AEA-treated rats, histopathological changes in FAAH expression were more prominent on the maternal side when compared with the fetal side of the placenta. This pattern of expression is in marked contrast to the pattern obtained with NAPE-PLD expression.

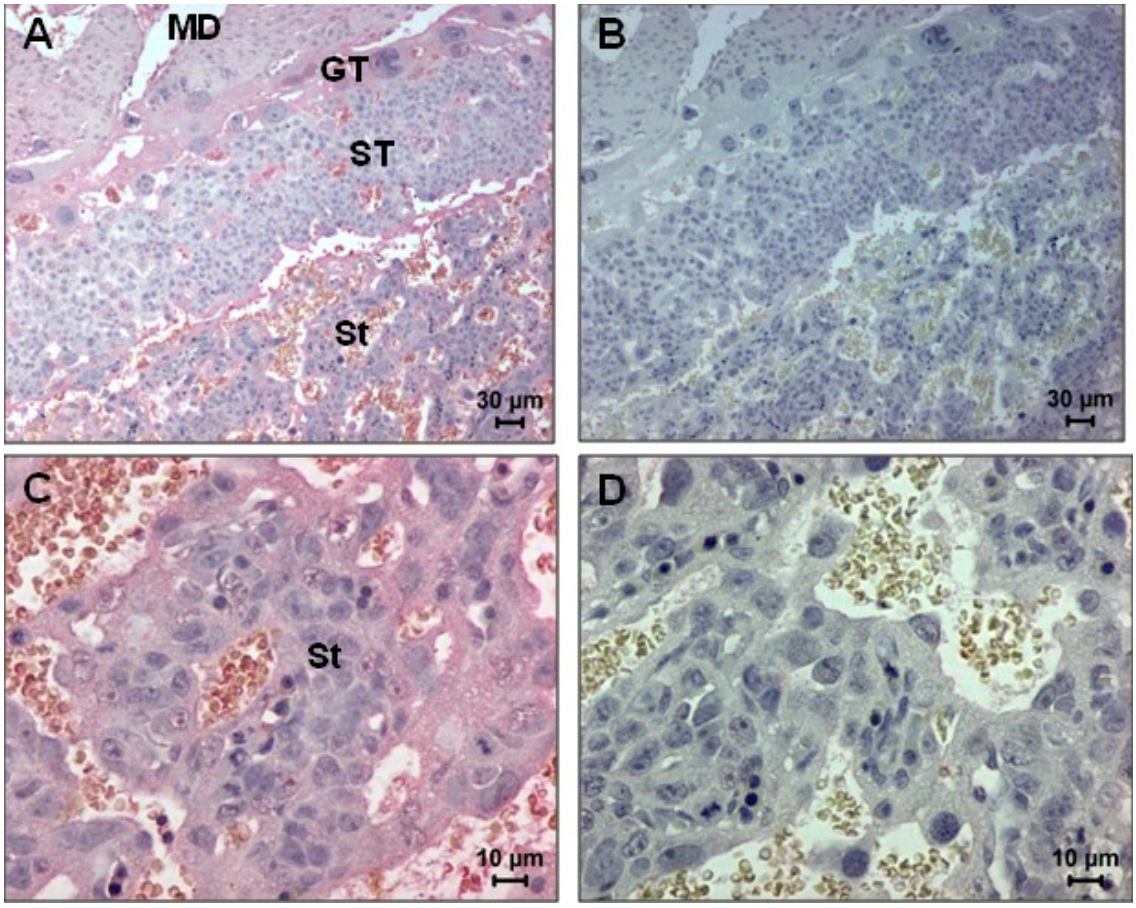

Figure 4.21: Specificity of polyclonal anti- FAAH antibody.
Immunoreactive FAAH on rat implantation sites (A, C) and negative control (B, D). (A) Mesometrial decidual cells (MD) and giant trophoblast cells (GT) were immunopositive whereas spongiotrophoblast cells (ST) were immunonegative for FAAH. (C) Higher magnification of (A) showing FAAH expression in syncytiotrophoblast (St). (D) Higher magnification of (B).

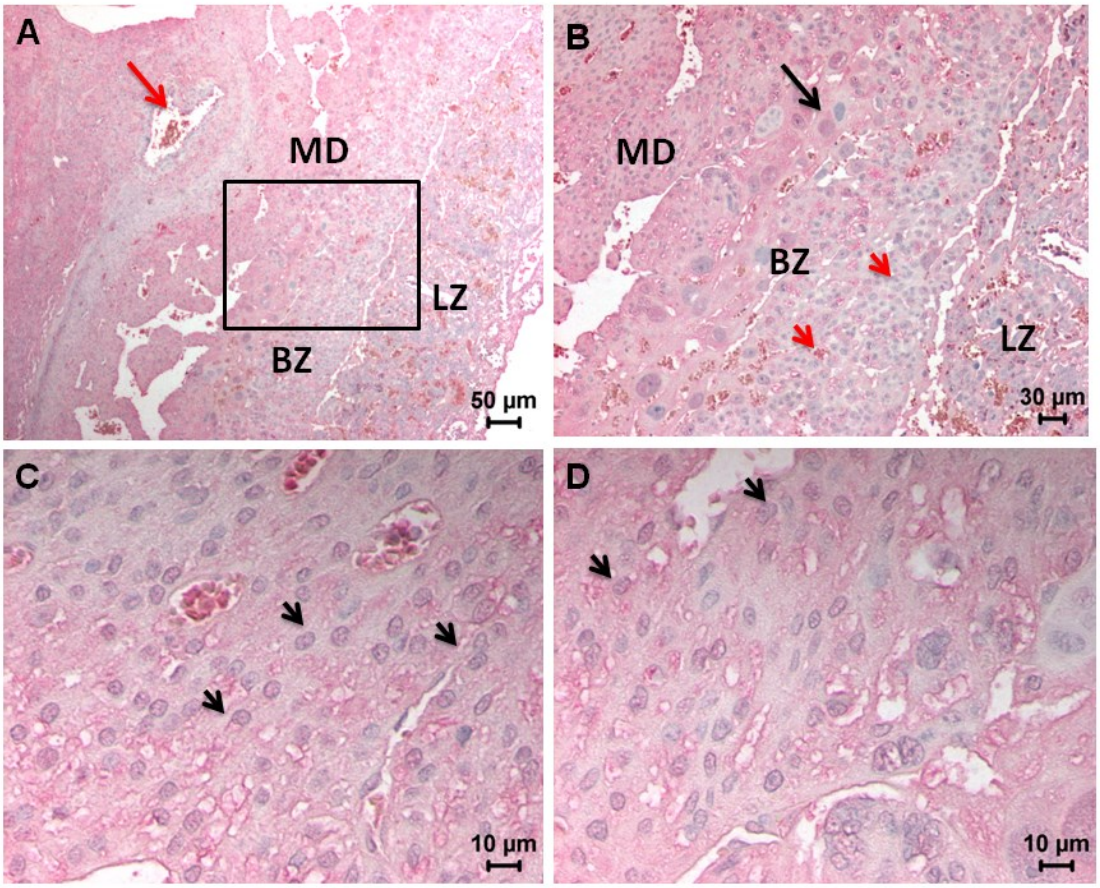

Figure 4.22: Immunoreactive FAAH localisation in implantation sites from control rats.

(A) FAAH expression in decidual cells of the mesometrial pole (MD), giant trophoblast cells and labyrinth zone (LZ) of the placenta, (B) Higher magnification of the square in (A). Spongiotrophoblast cells (red arrow heads) and giant trophoblast cells (arrow) were immunopositive to FAAH, (C) and (D) FAAH expression in decidual cells (arrow heads). BZ=basal zone, red arrow= spiral artery.

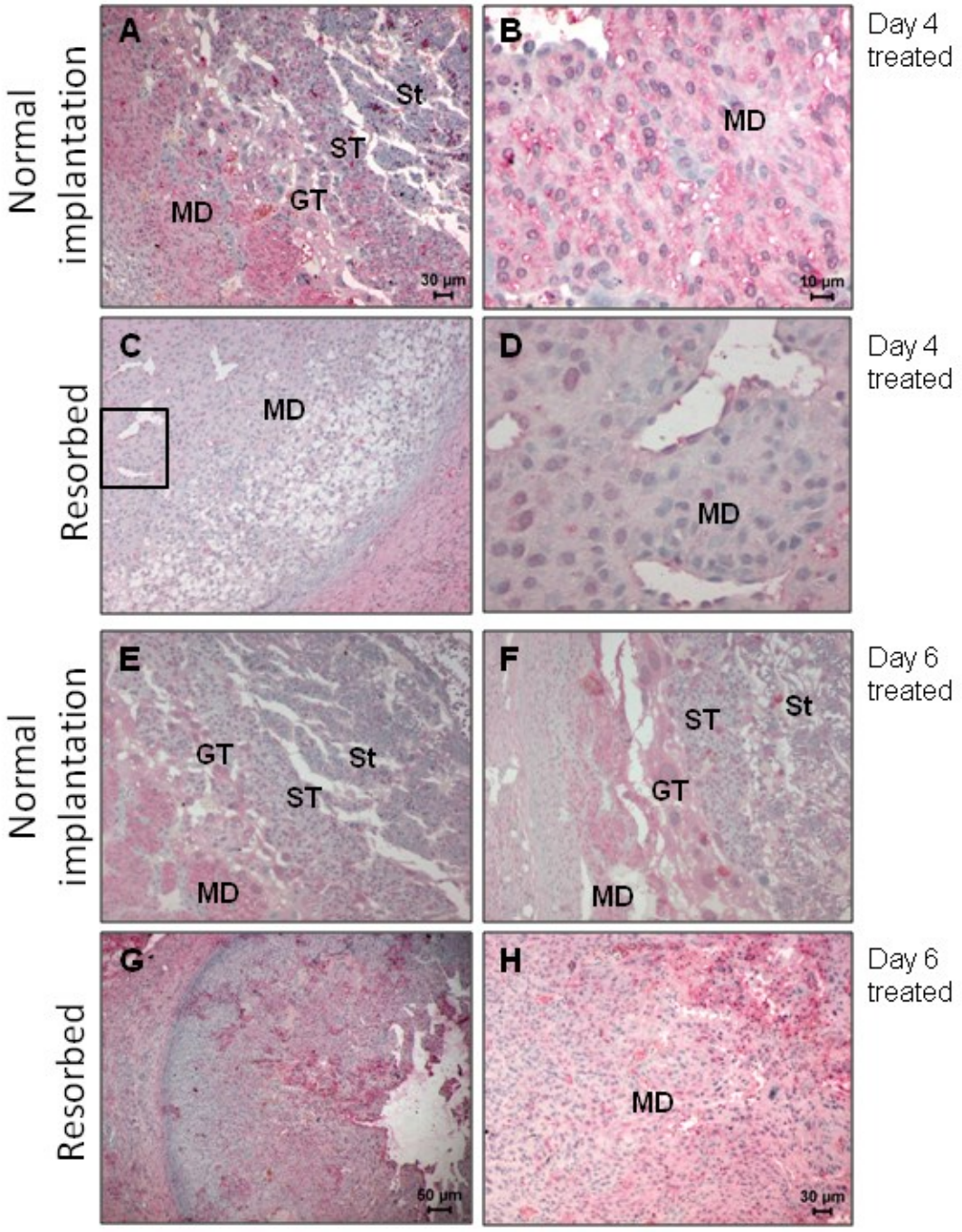

Figure 4.23: Immunoreactive FAAH in implantation sites of day 4 and day 6 treated rats.

(A) decidual cells, giant trophoblast (GT) and spongiotrophoblast (ST) cells were positive for FAAH whereas syncytiotrophoblast (St) cells were immunonegative, (B) positive staining of decidual cells (MD) in higher magnification, (C-D) decidual cells in resorbed sites expressed less FAAH intensity than normal implantation sites, (E) syncytiotrophoblast cells were immunonegative to FAAH, (F) decidual cells close to the myometrium were immunonegative whereas the decidual cells close to the labyrinth zone were immunopositive, (G) resorbed site with FAAH staining in decidual cells and (H) a higher magnification of FAAH staining in decidual cells.

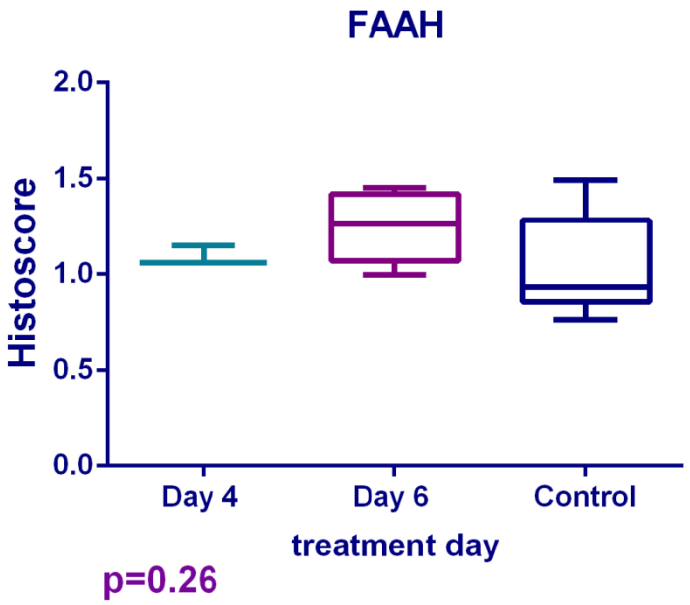

Figure 4.24: Staining intensities of FAAH in normal day 14 implantation sites of controls and rats treated with AEA on gestational day 4 and day 6.
There was no significant change in FAAH expression in trophoblast after AEA treatment (Kruskal Wallis Test with Dunn's Multiple Comparison Test). The results are shown as boxplots whereby the box shows the upper and lower quartiles and the median and the end of the whiskers represent the minimum and maximum value.

4.3.5 PCR

4.3.5.1 Identification of suitable Reference Genes

The geNorm Reference Gene Selection Kit with 12 reference genes was used to identify suitable reference genes for normalisation of qRT-PCR data from rat placenta, decidua, myometrium and inter-implantation sites. More details can be found in section 3.3.3.4.

4.3.5.1.1 Reference Gene Selection in Rat Placenta

The reference genes were tested on 10 samples of rat placenta. No data was obtained for the reference gene MDH suggesting this mRNA is not expressed in rat placenta. The Ct values for the remaining genes varied as shown in Figure 4.25. These values were converted to relative gene expression values and then transferred to the programs geNorm and Normfinder for analysis of the data.

We inferred from the data obtained from the geNorm and Normfinder analyses that YWHAZ, RPL13A and GAPDH were the most stably expressed reference genes in rat placenta and utilised these 3 most stably expressed reference genes for the accurate normalisation of the expression of genes of interest.

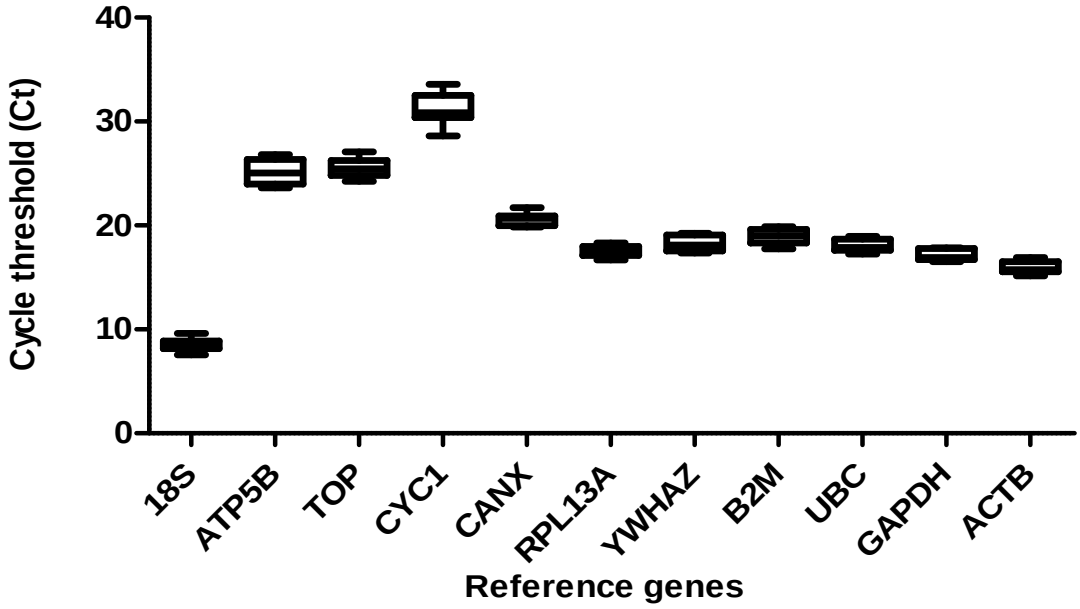

Figure 4.25: Ct values of 11 reference genes in rat placenta (n=10).
The lowest Ct value was measured in 18s and CYC1 had the highest Ct values. The Boxplot is representing lower quartile, median and upper quartile with Whiskers from minimum to maximum.

4.3.5.1.2 Decidual Reference Gene Identification

The Ct values in rat decidua varied with the different reference genes with 18s being the lowest (Figure 4.26). The relative gene expression values were used then for the calculations in geNorm and Normfinder. When the results from geNorm and Normfinder were compared the 3 best reference genes for normalisation of rat decidua were ATP5B, CANX and GAPDH.

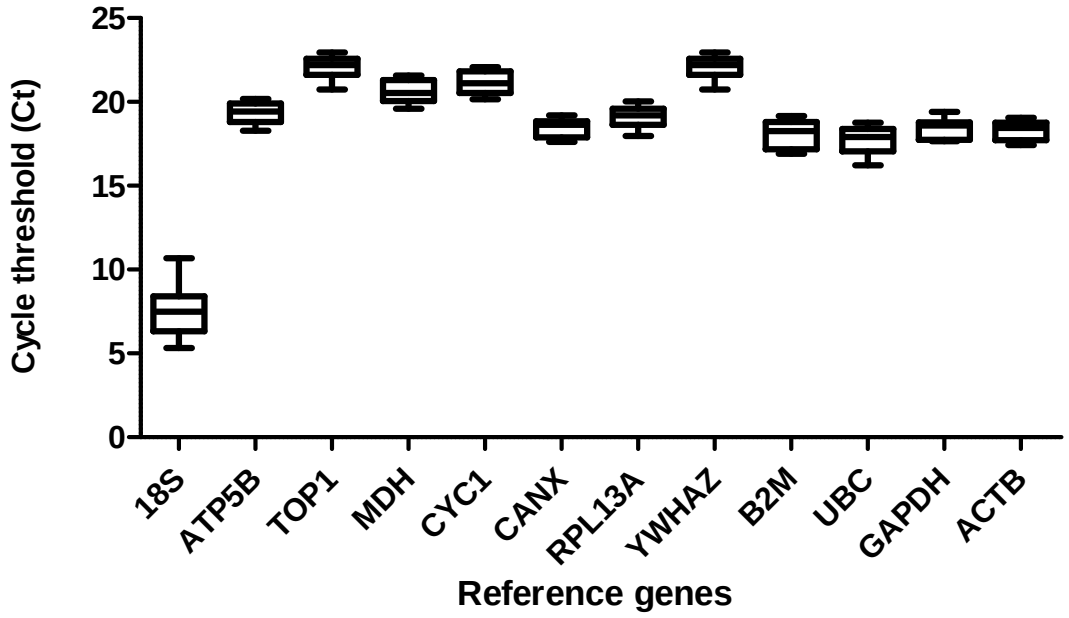

Figure 4.26: Ct values of 12 different reference genes in rat decidua (n=10).
The lowest Ct value was measured in 18s and YWHAZ had the highest Ct values. The Boxplot is representing lower quartile, median and upper quartile with whiskers from minimum to maximum.

4.3.5.1.3 Identification of Suitable Reference Genes for qRT-PCR Normalisation in Myometrium

The stable expression of 12 reference genes was tested in 12 samples of rat myometrium. The Ct values are shown in Figure 4.27. When the results from geNorm and Normfinder were compared the 3 best reference genes for normalisation of rat myometrium were RPL13A, GAPDH and UBC.

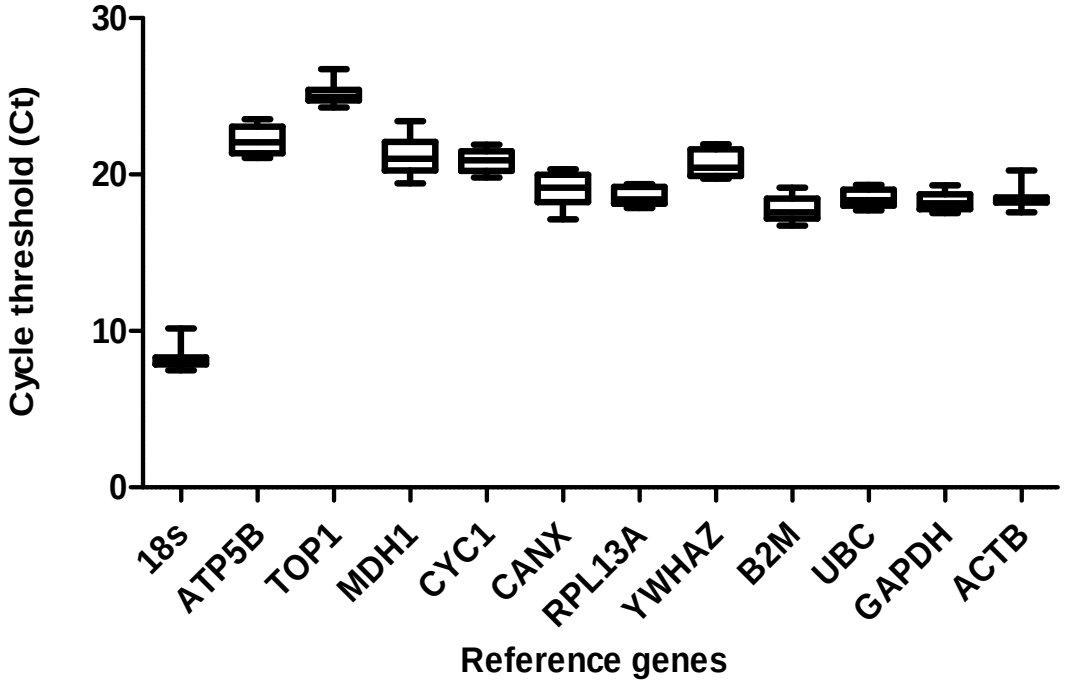

Figure 4.27: Ct values of rat myometrium (n=12) with 12 different reference genes. The lowest Ct value was measured in 18s and TOP1 had the highest Ct values. The boxes present the lower and upper quartiles with medians, whiskers present the ranges.

4.3.5.1.4 Identification of Suitable Reference Genes in Rat Inter-implantation Site

The stable expression of the 12 reference genes was tested in 10 samples of rat inter-implantation sites. The Ct values varied with the different reference genes (Figure 4.28) but most were very stably expressed in this tissue. Overall the three best reference genes were YWHAZ, RPL13A and B2M.

In Table 4.6 is a summary of the optimal reference genes combinations for the different rat tissue.

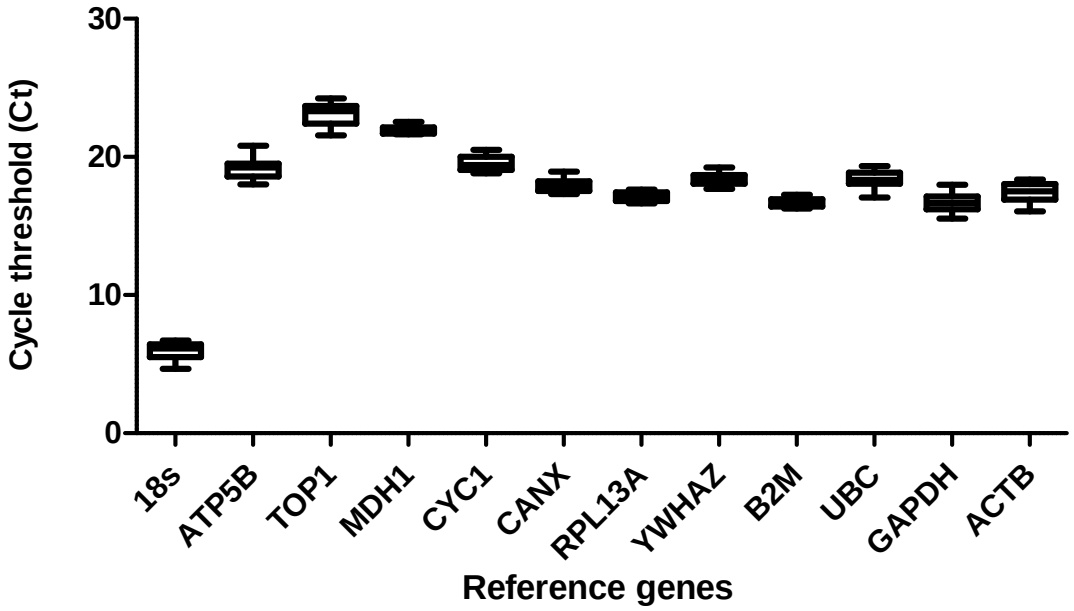

Figure 4.28: Ct values of 12 different reference genes expressed in rat inter-implantation sites (n=10).
The lowest Ct value was measured in 18s and CYC1 had the highest Ct values. The boxes present the lower and upper quartiles with medians, whiskers present the ranges.

Table 4.6: Optimal reference genes for normalisation of qRT-PCR data from different rat tissues

Rat tissue	Reference genes
Placenta	YWHAZ, RPL13A, GAPDH
Decidua	CANX, GAPDH, ATP5B
Myometrium	RPL13A, GAPDH, TOP1
Inter-implantation	YWHAZ, RPL13A, B2M

4.3.5.2 PCR Efficiencies

Standard curves were produced to calculate the primer efficiencies as described in section 3.3.3.5 (Figure 4.29). Two different primer pairs for each primer were evaluated and the PCR efficiencies are shown in Table 4.7. The primers in bold were selected for the examination of qRT-PCR expression of the ECS.

The PCR efficiencies for the reference genes were also calculated from the standard curves (Figure 4.29) and they are shown in Table 4.8.

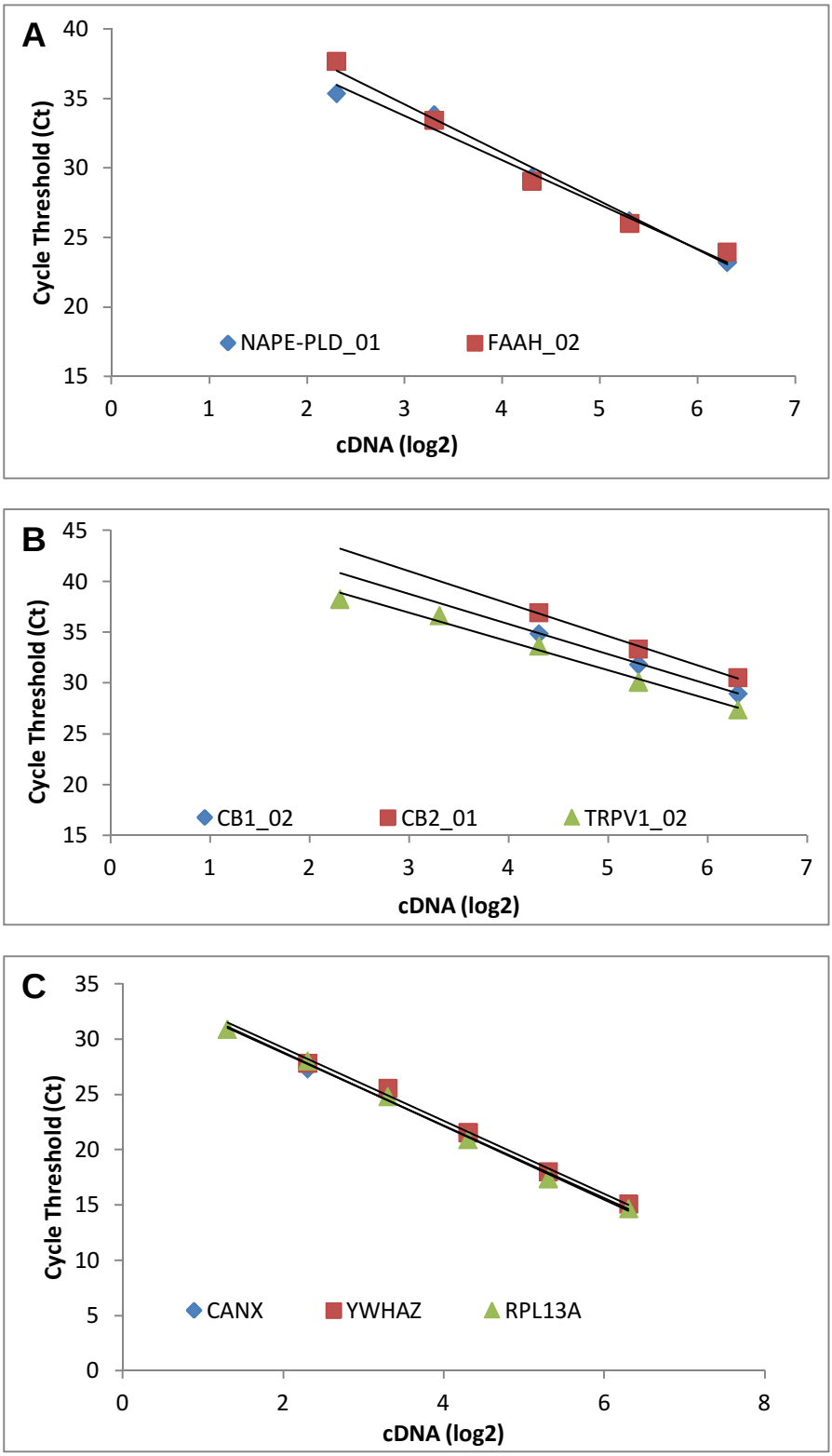

Figure 4.29: Efficiencies of the qRT-PCR primers.
PCR efficiencies for (A) NAPE-PLD and FAAH (B) CB1, CB2 and TRPV1 and (C) reference genes CANX, YWHAZ and RPL13A. The PCR efficiency was calculated from the slope of the linear curve generated by serial dilutions of template cDNA.

Table 4.7: PCR efficiencies for the qRT-PCR primers used to access the ECS.

Gene	Standard curve equation	R^2 value	Primer Efficiency (%)
NAPE-PLD_01	**y = -3.1833x + 43.313**	**0.985**	**105.8**
NAPE-PLD_02	y = -3.07x + 42.001	0.999	111.7
FAAH_01	y = -3.072x + 43.122	0.983	111.6
FAAH_02	**y = -3.4875x + 45.046**	**0.98**	**93.5**
TRPV1_01	y = -3.5435x + 48.147	0.989	91.5
TRPV1_02	**y = -3.126x + 46.968**	**0.998**	**108.9**
CB1_01	y = -2.475x + 43.903	0.995	153
CB1_02	**y = -2.9575x + 47.588**	**0.999**	**117.8**
CB2_01	**y = -3.1925x + 50.559**	**0.992**	**105.7**
CB2-02	y = -2.205x + 46.701	0.998	184

Genes in bold were used for the experiments.

Table 4.8: PCR efficiencies for the reference genes.

Gene	Standard curve equation	R^2 value	Primer Efficiency (%)
RPL13A	y = -3.339x + 35.487	0.997	99.3
YWHAZ	y = -3.3025x + 35.819	0.994	100.8
CANX	y = -3.2825x + 35.294	0.989	101.6

4.3.5.3 Investigations of ECS mRNA Expression in Day 14 Pregnant Rats by quantitative Real-Time PCR

4.3.5.3.1 Expression of NAPE-PLD, FAAH, CB1, CB2 and TRPV1 in Placenta and Decidua

The expression of NAPE-PLD mRNA in the day 14 placenta was reduced in all AEA-treated rats when compared to controls but this was not statistically significant. Placental transcript levels of FAAH and the receptors CB1, CB2 and TRPV1 did not change significantly in AEA-pretreated rats (Figure 4.30).

In decidua, NAPE-PLD transcript levels did not alter significantly in AEA-treated rats compared to controls whereas FAAH and CB1 levels were lower in the AEA-treated rats, but this difference did not reach statistical significance. No difference was seen in CB2 and TRPV1 transcript levels between AEA-treated and control rats (Figure 4.30).

The expression levels of NAPE-PLD in placenta was significantly raised compared to the expression in decidua in day 4 AEA-treated rats ($p<0.05$) (Figure 4.31). This was consistent with the increased AEA levels observed in day 14 placental tissue in day 4 AEA-treated rats (Figure 4.13G). FAAH and CB2 transcript levels in decidua and placenta did not differ significantly. In control rats, significantly increased CB1 transcript levels were observed in decidua compared to placenta whereas in all AEA-treated rats CB1 transcript levels were down-regulated in decidua ($p<0.01$). The expression of TRPV1 was also significantly higher in decidua compared to placenta in control rats ($p<0.05$). This increased expression was also observed in day 2 and day 4 AEA-treated rats, but not in day 6 AEA-treated rats ($p<0.05$) (Figure 4.31).

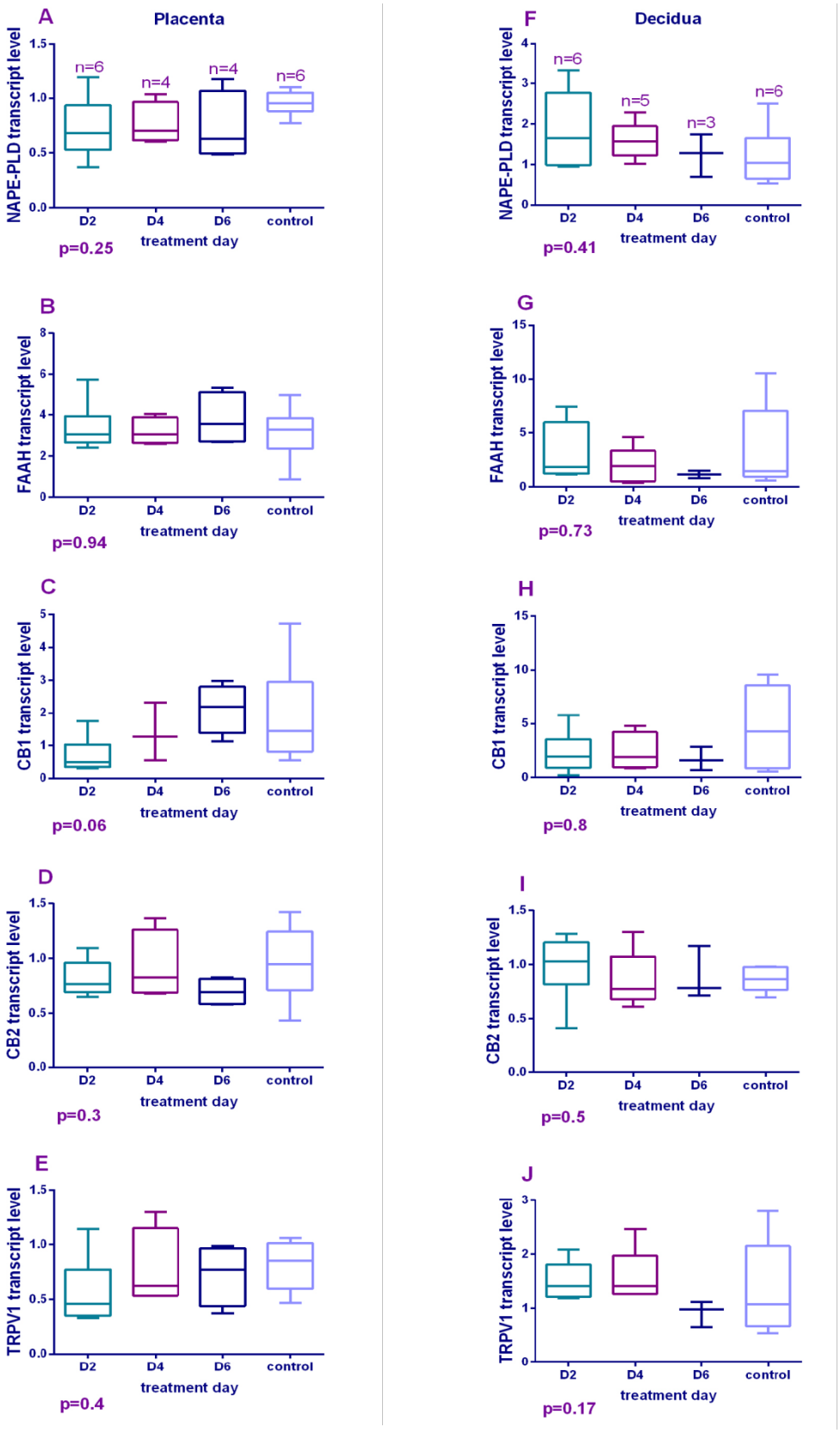

Figure 4.30: Transcript levels of NAPE-PLD (A, F), FAAH (B, G), CB1 (C, H), CB2 (D, I) and TRPV1 (E, J) in placenta and decidua.
(Kruskal Wallis Test with Dunn's Multiple Comparison Test). The boxes present the lower and upper quartiles with medians, whiskers present the ranges.

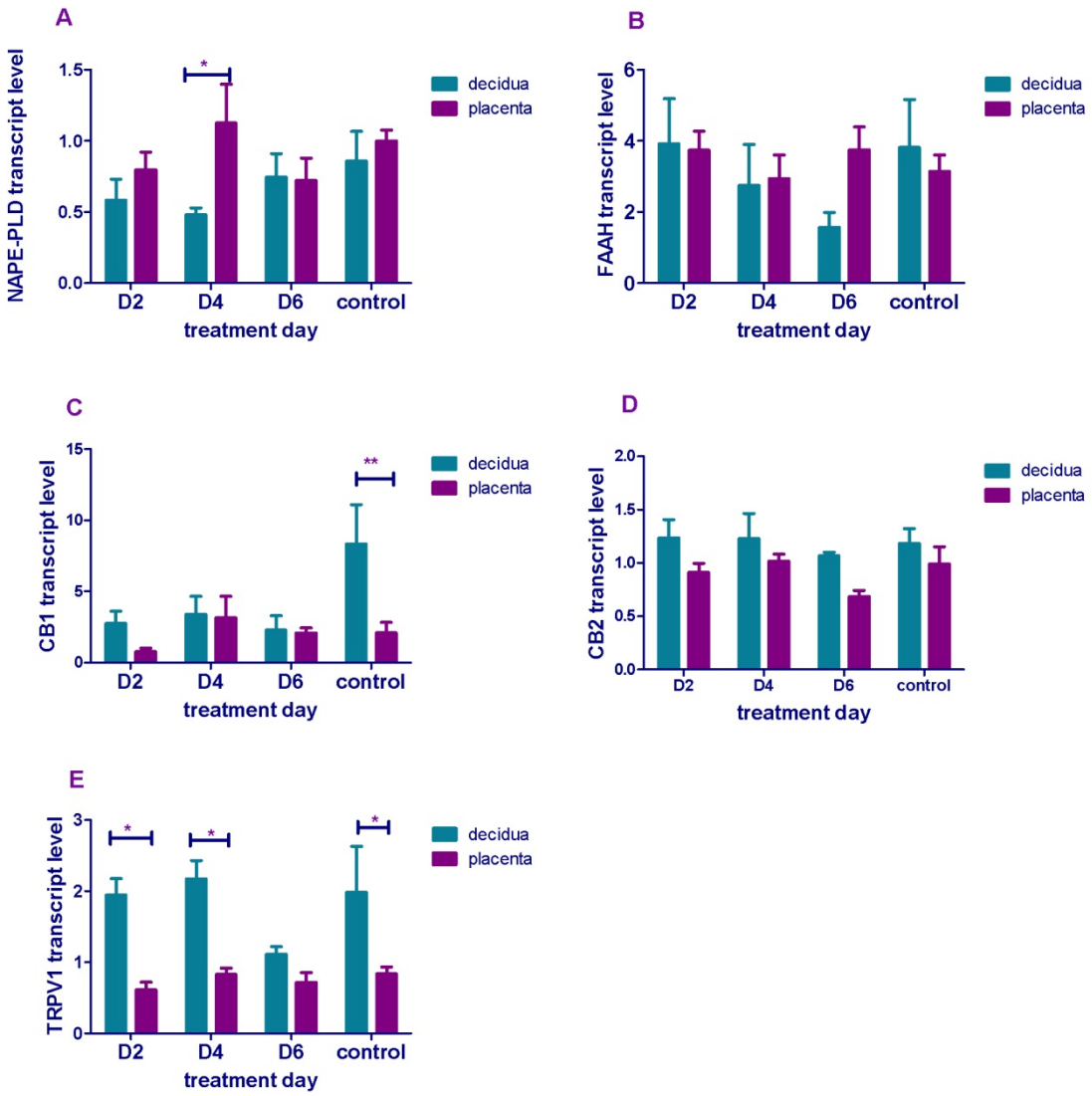

Figure 4.31: Endocannabinoid system transcript levels in day 14 decidua and placenta of AEA treated and control rats.

(A) Day 4 AEA-treated rats had significantly higher NAPE-PLD expression in placenta than decidua (*$p<0.05$), (B) FAAH transcript levels in placenta were higher than in decidua of day 6 AEA-treated rats, but not significantly, (C) high CB1 transcript levels in decidua of control rats were found when compared to placenta (**$p<0.01$). CB1 expression in decidua was down-regulated in all AEA-treated rats, (D) no significant changes in transcript levels of CB2 were seen in either decidua or placenta in neither AEA treated nor control rats, (E) TRPV1 expression in decidua of day 2, day 4 AEA-treated and control rats were significantly higher than placenta (*$p<0.05$) (Two-Way ANOVA with Bonferroni post test). Results are described as mean ± SEM.

4.3.5.3.2 Expression of NAPE-PLD, FAAH, CB1, CB2 and TRPV1 in Myometrium

Transcript levels of NAPE-PLD, FAAH, CB1, CB2 and TRPV1 did not change significantly after AEA treatment when compared to control rats (Figure 4.32).

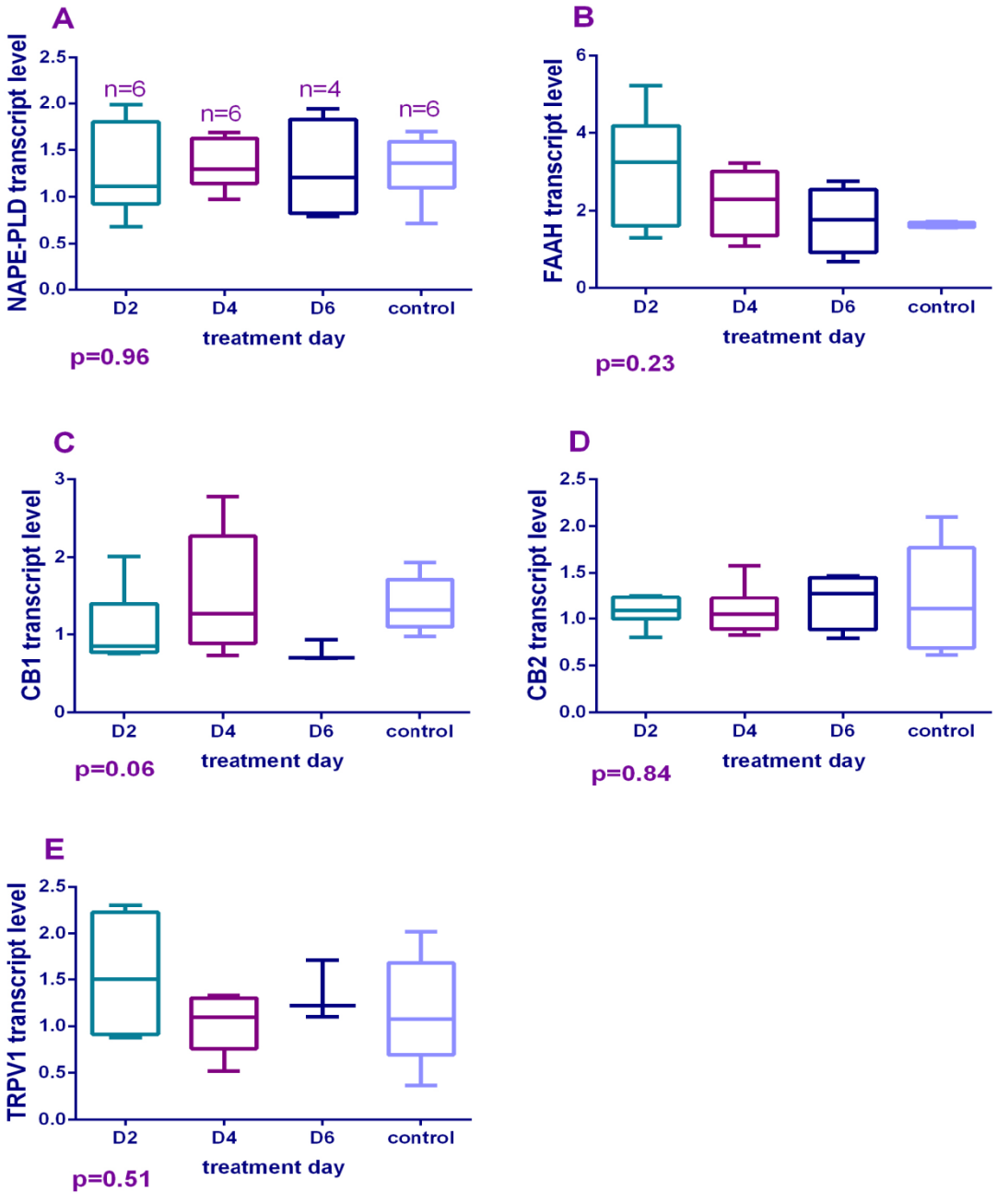

Figure 4.32: Endocannabinoid system transcript levels in myometrium of AEA treated and control rats.
(A) NAPE-PLD, (B) FAAH, (C) CB1, (D) CB2 and (E) TRPV1 (Kruskal Wallis Test with Dunn's Multiple Comparison Test). The boxes present the lower and upper quartiles with medians, whiskers present the ranges.

4.3.5.3.3 Expression of NAPE-PLD, FAAH, CB1, CB2 and TRPV1 in Inter-implantation Sites

FAAH and CB1 transcript levels at day 14 inter-implantation sites were increased after AEA treatment when compared to that in the control rats, but this achieved statistical significance only for day 4 treated rats ($p<0.05$) (Figure 4.33). NAPE-PLD and TRPV1 transcript levels were also higher in AEA treated rats when compared to control rats, but these changes did not reach statistical significance. The expression of CB2 in day 2 and day 4 AEA-treated rats were also increased in comparison to that in control rats, but once again without statistical significance (Figure 4.33).

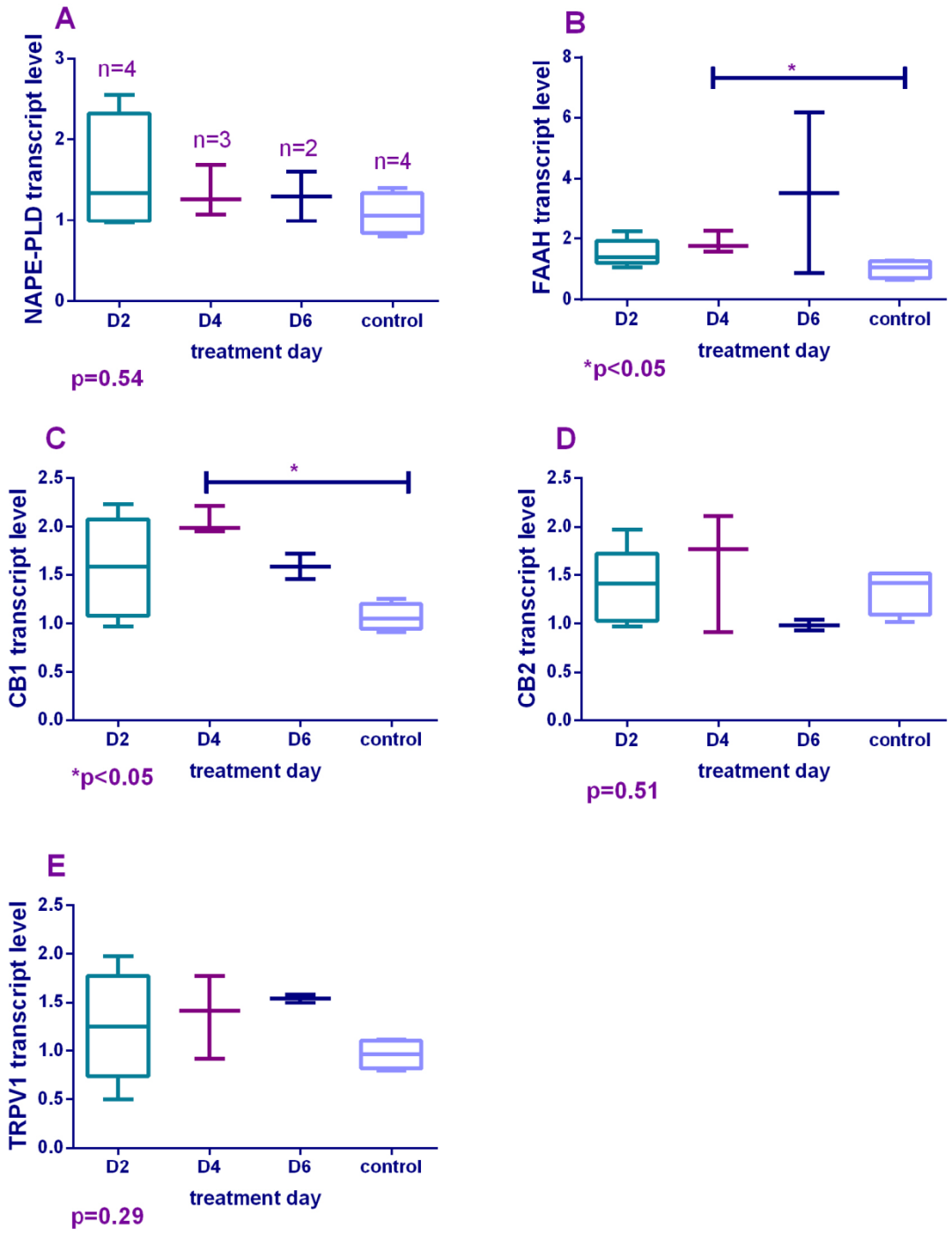

Figure 4.33: Endocannabinoid system transcript levels in inter-implantation sites of AEA treated and control rats.
(A) NAPE-PLD transcript levels did not significantly change after AEA treatment, (B) FAAH transcript levels in day 4 treated rats were significantly higher than in control (*p<0.05), (C) CB1 expression in day 4 treated rats were significantly higher than the control (*p<0.05), (D) no significant differences in CB2 expression of treated and control rats were seen, (E) TRPV1 transcript levels were increased in treated rats compared to control, but not significantly (Kruskal Wallis Test with Dunn's Multiple Comparison Test). The boxes present the lower and upper quartiles with medians, whiskers present the ranges.

4.3.5.3.4 Comparison of Expression Levels of NAPE-PLD, FAAH, CB1, CB2 and TRPV1 between different Tissues in Control and AEA-treated Rats

Suitable reference genes for the comparison of expression levels in implantation and inter-implantation sites were identified using geNorm and Normfinder software. These were RPL13A, YWHAZ and CANX.

An investigation of components of the ECS in untreated animals showed that CB1-mRNA expression was significantly higher in the decidua compared to placenta, myometrium (implantation) and inter-implantation sites ($p<0.001$), whereas CB2 transcript levels were significantly higher at the inter-implantation sites compared with myometrium ($p<0.05$). Consistent with previous observations that a reduction in local AEA concentration is critical for implantation and pregnancy maintenance, FAAH expression was highest in placenta and was significantly higher than at the inter-implantation sites ($p<0.05$). NAPE-PLD mRNA expression was higher in decidua than in placenta or myometrium (implantation) and inter-implantation sites, but this did not reach statistical significance. TRPV1 expression in decidua and myometrium was higher than in placenta and inter-implantation sites, but this was not significantly different (Figure 4.34).

AEA treatment changed the expression of these enzymes and receptors. A significant down-regulation of CB1 expression in decidua of day 2, day 4 and day 6 treated rats was observed ($p<0.001$, $p<0.01$ and $p<0.001$) compared with controls. In contrast, CB2 mRNA expression was significantly higher in inter-implantation sites than in the decidua of day 4 treated rats ($p<0.05$) (Figure 4.34). NAPE-PLD mRNA expression was elevated in decidua of AEA-treated rats ($p>0.05$) compared with placenta ($p<0.05$). FAAH transcript levels in the placenta remained higher than at the inter-implantation sites in AEA-treated animals, though significant differences were only observed in day 2 treated rats ($p<0.05$) (Figure 4.34). TRPV1 mRNA expression was significantly lower in the placenta than in the decidua and myometrium ($p<0.05$ and $p<0.01$, respectively) (Figure 4.34).

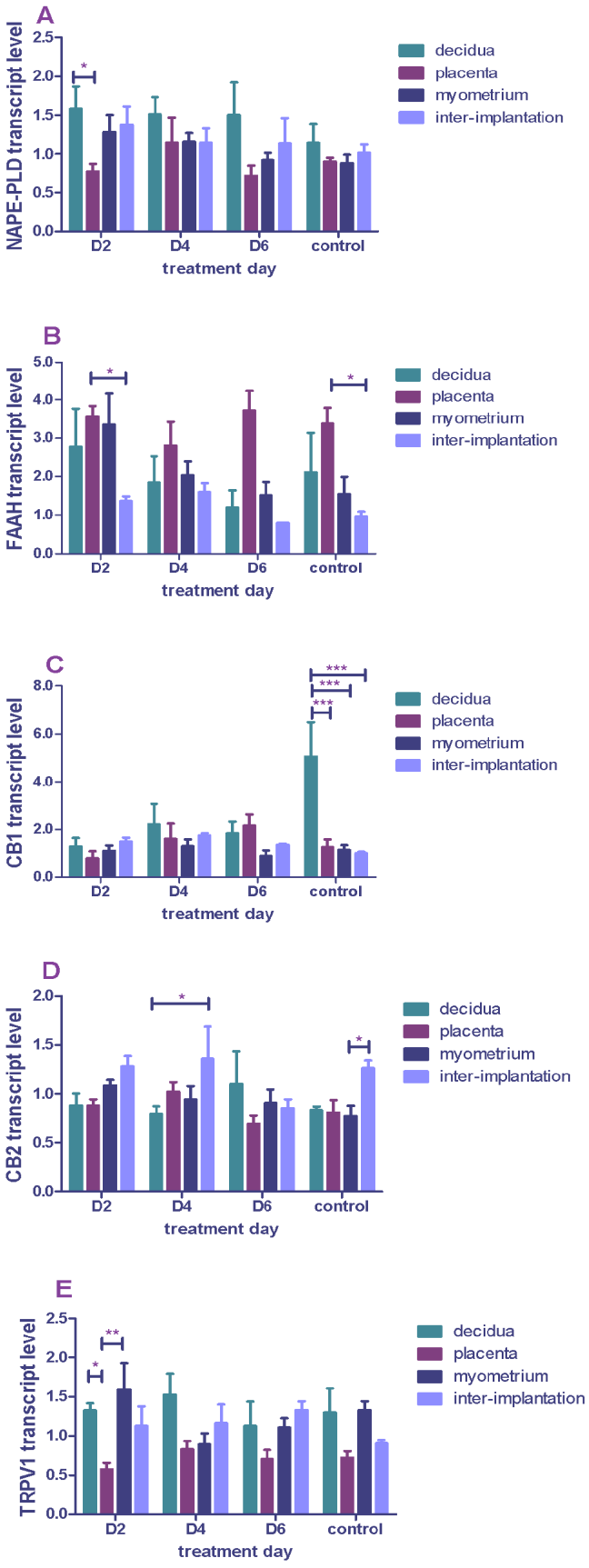

Figure 4.34: Endocannabinoid system transcript levels of implantation and inter-implantation sites in AEA treated and control rats.

(A) NAPE-PLD expression in placenta of day 2 treated rats were significantly lower than in decidua (*$p<0.05$), (B) FAAH expression in inter-implantation sites of day 2 treated rats and control were significantly lower than in placenta ($p<0.05$), (C) CB1 transcript levels in decidua of controls were significantly higher than in placenta, myometrium and inter-implantation sites (***$p<0.001$), (D) Increased CB2 transcript levels were observed in inter-implantation sites than myometrium in control rats and decidua in day 4 treated rats (*$p<0.05$), (E) TRPV1 transcript levels in placenta were significantly lower than in decidua (*$p<0.05$) and myometrium (**$p<0.01$) in day 2 treated rats (Two-Way ANOVA with Bonferroni post test). Results are presented as means ± SEM.

4.3.6 Western Blotting Analysis

Western blotting analysis of the protein levels of the enzymes NAPE-PLD and FAAH was performed on rat decidua, placenta, myometrium (implantation site) and inter-implantation sites from day 4 and day 6 AEA-treated and control rats. GAPDH was used as a loading control.

NAPE-PLD and FAAH proteins were detected at both implantation and inter-implantation sites of gestation day 14 rats. In placenta of day 4 AEA-treated rats FAAH protein was expressed less compared to decidua, myometrium and inter-implantation sites (Figure 4.35). However, densitometric analysis did not reveal any significant difference in FAAH protein expression between day 4 treated and control rats (Figure 4.37). This is in agreement with the mRNA results presented in section 4.3.4.3.4. The protein levels of FAAH in the decidua of day 6 AEA-treated rats were significantly higher than that at the inter-implantation sites ($p<0.05$) (Figure 4.36). In the control group, FAAH protein expression in decidua was significantly lower than at the inter-implantation sites ($p<0.05$) (Figure 4.37).

There were no significant differences in NAPE-PLD expression at the implantation and inter-implantation sites in neither AEA-treated nor control rats, which agrees with the transcript results presented in section 4.3.4.3.4 (Figures 4.35, 4.36 and 4.37).

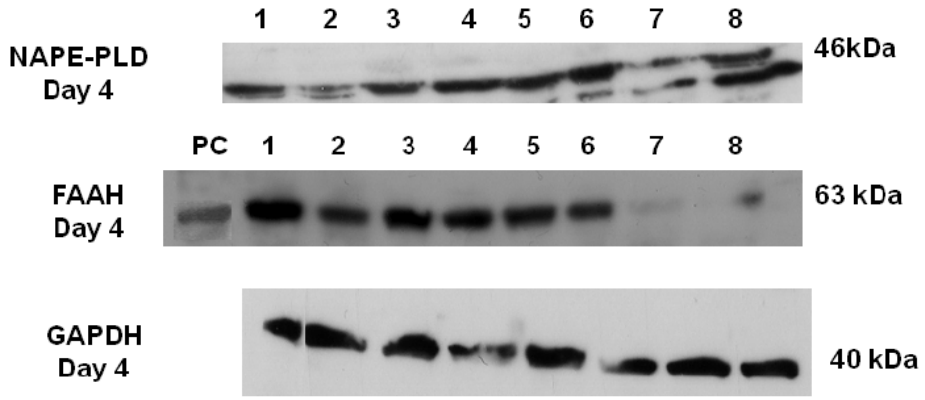

Figure 4.35: Protein expression of NAPE-PLD, FAAH and GAPDH in implantation and inter-implantation sites of day 4 AEA-treated and control rats.
Representative western blots for NAPE-PLD, FAAH and GAPDH proteins. Brain homogenates were used as positive controls (PC).

1=decidua, AEA-treated, 2=control decidua, 3=inter-implantation site, AEA-treated, 4=control inter-implantation site, 5=myometrium, AEA-treated, 6=control myometrium, 7=control placenta, 8=placenta, AEA-treated.

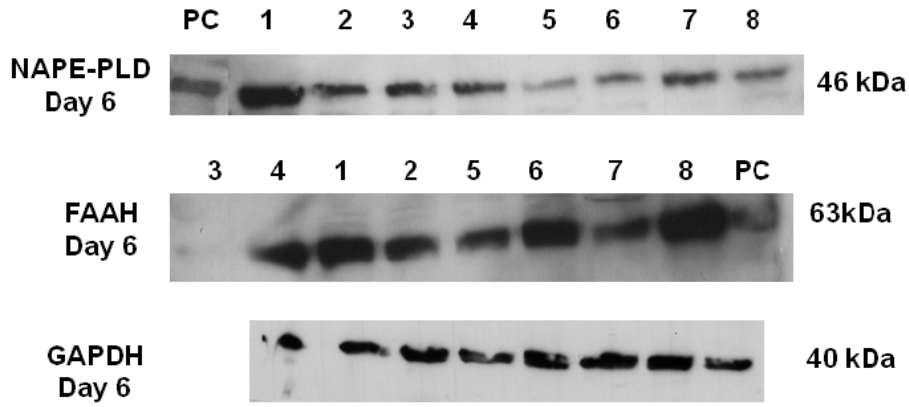

Figure 4.36: Protein expression of NAPE-PLD, FAAH and GAPDH in implantation and inter-implantation sites of day 6 treated and control rats.
Representative western blots for NAPE-PLD, FAAH and GAPDH proteins. Brain homogenates were used as positive controls (PC).

1=decidua, AEA-treated, 2=control decidua, 3=myometrium, AEA-treated, 4= control myometrium, 5=placenta, AEA-treated, 6=control placenta, 7=inter-implantation site, AEA-treated, 8= control inter-implantation site.

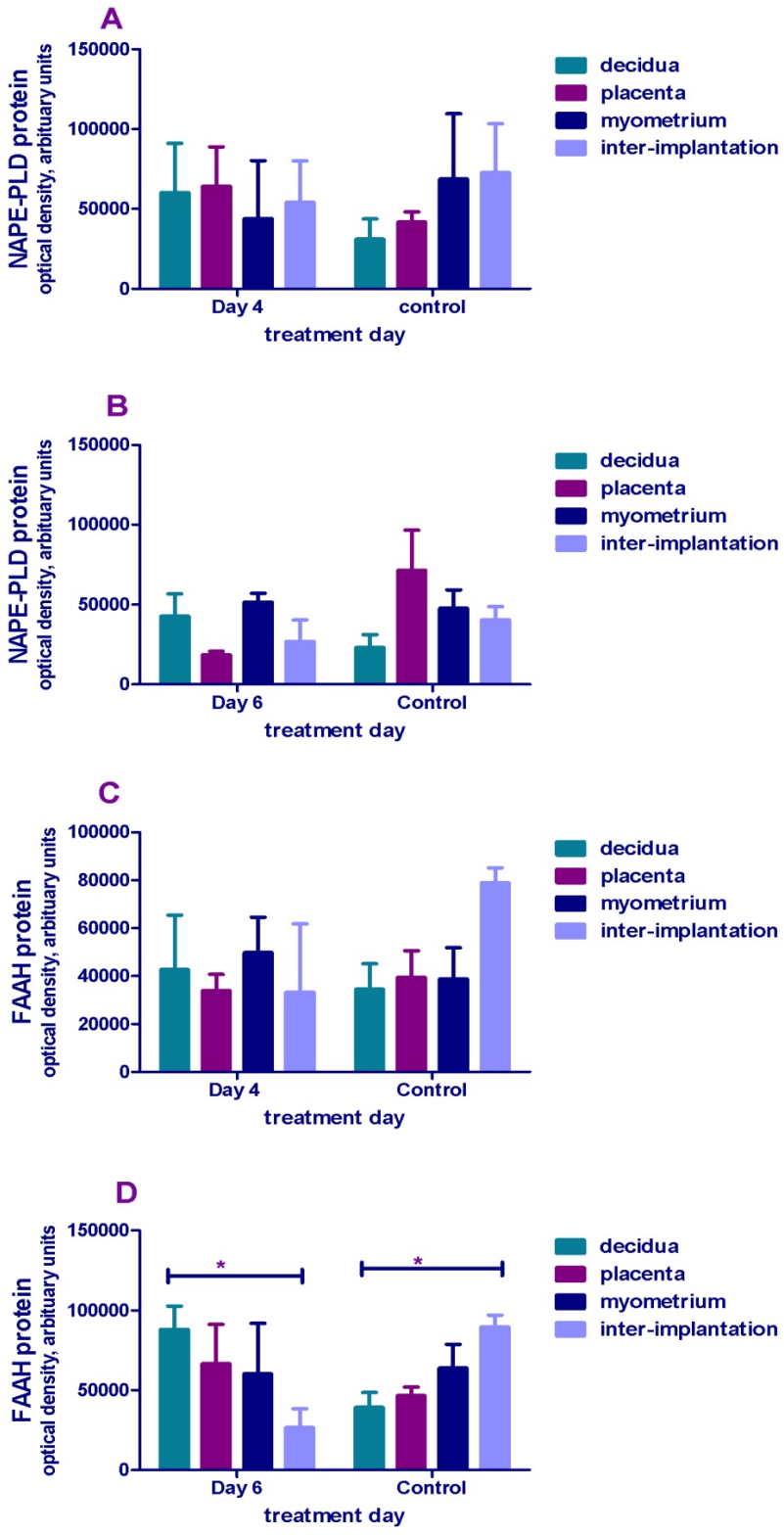

Figure 4.37: Densitometric analysis of NAPE-PLD and FAAH protein expression. The analysis is in implantation and inter-implantation sites of day 4 (A, C) and day 6 AEA-treated (B, D) and control rats (n=3). (A-B) There were no significant differences in NAPE-PLD protein levels between implantation and inter-implantation sites from day 4 and day 6 AEA-treated and control rat, (C) FAAH protein levels of implantation and inter-implantation sites in day 4 AEA-treated and control rats did not significantly

differ, (D) whereas FAAH protein levels in decidua were significantly higher than at the inter-implantation site of day 6 AEA-treated rats and in the controls decidua protein levels were significantly lower than at the inter-implantation sites (Two-way repeated measures ANOVA with Bonferroni post test; *$p<0.05$). Results are shown as means ± SEM.

4.4 DISCUSSION

Implantation, embryo development and pregnancy maintenance are a series of complex processes, involving maternal, fetal and placental factors. The ECS has been identified as an important mediator in reproduction (Battista et al. 2007, Battista et al. 2008a, Karasu et al. 2011, Bari et al. 2011). A successful implantation depends on a normal pre-implantation embryo development to a blastocyst competent for implantation and a receptive endometrium (Paria, Wang & Dey 2002). This involves a finely tuned endocannabinoid signalling system (Paria, Wang & Dey 2002). Previous studies in mice highlighted the role of AEA in the process of implantation (Schmid et al. 1997, Guo et al. 2005). High AEA levels are detrimental for implantation and these high AEA levels via CB1 have a direct inhibitory effect on mouse blastocyst development (Schuel 2006). Differentiation of trophectoderm cells are regulated by CB1 mediated endocannabinoid signalling and aberrant signalling can adversely change placentation leading to retarded fetal development, mid-gestational demise and compromised pregnancy outcome in mice (Sun et al. 2010). These findings suggest that maternal and fetal tissues/ organs are differentially responsible for local AEA levels.

In this study the effect of AEA administration around the time of implantation in rats on the number of ongoing pregnancies was investigated. The rat model allows us to study implantation and early pregnancy events, which are not accessible through studies with human pregnancies. Nevertheless, caution needs to be used when extrapolating results to the human. For example, rats are multiparous and have a shorter gestation than human with a fully functioning placenta of only one week (Fonseca et al. 2012). However, rats exhibit haemochorial placentation and trophoblast invasion like in the human and therefore are good animal models to study those events (Caluwaerts et al. 2005).

As a tight regulation of AEA concentration is crucial for successful implantation (Schmid et al. 1997) a detrimental effect on implantation after AEA treatment was anticipated. Indeed a reduction in implantation sites following AEA administration near the time of implantation was observed. Interestingly the number of implantation sites was more markedly reduced in the left compared to the right uterine horn. An explanation for this observation could be that AEA concentrations are much higher at

the site of AEA injection which was in the left side of the lower abdomen. Higher AEA levels are therefore predicted to be reached at the left uterine horn compared to the right uterine horn. These results are in concordance with previous studies that showed harmful effects of high AEA during the implantation process (Schmid et al. 1997, Paria et al. 2001).

High AEA or Δ^9 THC levels via CB1 receptor inhibited pre-implantation embryo development to blastocyst *in-vitro* in mice (Paria, Das & Dey 1995, Yang, Paria & Dey 1996, Paria et al. 1998). AEA treatment was also able to decrease blastocyst zona hatching *in-vitro* and continuous infusion of a synthetic cannabinoid ligand prevented implantation *in-vivo* in mice (Schmid et al. 1997). Furthermore, exposure of blastocysts to AEA in culture inhibited trophoblast differentiation while low AEA levels accelerated it (Wang et al. 1999). These findings suggested that if exogenous AEA alters local AEA levels of the embryo and uterus it may impede successful implantation.

In this study an increase in the number of resorbed sites after exogenous AEA treatment around the time of implantation was also observed. AEA treatment may have interrupted normal embryo development which resulted in a higher number of resorbed sites. A rat does not abort but resorbs an imperfect conceptus. This is in line with a previously reported clinical study whereby women with a threatened miscarriage and high plasma AEA levels subsequently miscarried (Habayeb et al. 2008). AEA treatment also had an effect on the histomorphological structure of the placenta which was more disorganised in day 6 than day 4 AEA-treated rats. Histomorphological changes in cultured rat primary decidual cells have been reported after exposure to high doses of AEA (10μM) which cause apoptosis (Fonseca, Correia-da-Silva & Teixeira 2009).

Here, high plasma AEA levels were found on gestational day 14 in rats treated with AEA on day 2, but not those treated on day 4 or day 6. OEA and PEA plasma levels on day 14 of gestation were not different in treated and control rats. This suggests that exogenous AEA has no long lasting effect on plasma OEA and PEA levels. Of note is that plasma AEA levels increased with the number of implantations at day 14. This positive correlation was more evident when only normal implantation sites were included. However, tissue AEA, OEA and PEA levels in placenta and decidua did not

show any correlation with the total number of implantation sites. These results suggest that each implantation has closely regulated local AEA levels but that each implanted embryo contributes to the circulating blood AEA pool.

There was no significant correlation between plasma and tissue (placenta and decidua) AEA, OEA and PEA levels. In deed Fonseca et al. could not also establish a correlation between NAE plasma and decidua levels (Fonseca et al. 2010). These observations suggest that EC levels in placenta and decidua are more likely to be regulated locally by metabolizing enzymes as has been shown in mice and rat (Wang et al. 2007, Fonseca et al. 2010).

AEA, OEA and PEA levels in the decidua of AEA-treated rats were higher than in control rats, especially when AEA was given around the day of implantation (either day 4 or day 6). PEA levels in decidua of day 6 treated rats were significantly higher when compared to controls. Placental AEA, OEA and PEA levels were also higher in rats treated with AEA on day 4. These results taken together suggest that intraperitoneal injections of AEA on either day 4 or day 6 of pregnancy have a long lasting effect on NAE levels in the placenta and decidua up to at least gestation day 14. Tissue AEA, OEA and PEA levels in decidua and placenta of control rats did not significantly differ from each other, but day 4 AEA-treated rats demonstrated a significantly higher AEA level in the placenta than decidua. In order to understand how the metabolizing enzymes may be involved in the regulation of AEA levels, qRT-PCR and Western blot analyses were performed.

It is established that the selection of stable reference genes for qRT-PCR is necessary in order to obtain reliable gene expression data under different experimental conditions (Bustin et al. 2009). However, testing for the expression stability of reference genes is not routinely performed and arbitrarily chosen reference genes are used without any prior validation process. The most commonly used reference genes for qRT-PCR in rat placenta are either ß-Actin or GAPDH (Asami-Miyagishi et al. 2004, Lips et al. 2005, Xu, Knipp & Cook 2005, Fonseca et al. 2010). The use of several reference genes is also preferred to a single reference gene as it gives more accurate and reliable results, because changes in expression of one housekeeping gene will have less effect on the normalised expression values of the gene of interest. The optimal number of reference genes should also be experimentally established to provide optimal normalisation of data (Vandesompele et al. 2002). In the studies in this Chapter geNorm and Normfinder

software were used to analyse the stability of candidate reference genes for the placenta, decidua, myometrium and inter-implantation sites of the rat. There was a good correlation between the results obtained with the two software tools. The optimal number of reference genes used in this experiment was three. The best reference genes for rat placenta were YWHAZ, RPL13A and GAPDH; the reference genes for the decidua were ATP5B, CANX and GAPDH; for myometrium were RPL13A, GAPDH and UBC and for the inter-implantation YWHAZ, RPL13A and B2M were identified as the most stably expressed reference genes. The optimal reference genes varied between the different tissues. This demonstrates the importance of the selection of stable reference genes in order to obtain accurate results. Interestingly the most widely used reference gene in PCR investigations in rat placenta ß-Actin (ACTB) was not identified in the best 5 reference genes here for any of the tissues when geNorm and Normfinder software were used. This observation suggests that ACTB is not stably expressed in these tissues and as such the results of previous investigations normalised with ACTB are questionable. No previous study has analysed the stability of reference genes in rat implantation and inter-implantation sites. These results here suggest that reference gene stability is variable in these tissues and the intergroup variability also changes dramatically for different genes suggesting the expression of these reference genes are altered by the AEA pre-treatment. Consequently, reference genes should be optimised for each tissue in any given experiment. The use of both geNorm and Normfinder assists in selecting the reference genes for a given experiment.

The genes of interest were normalised with the 3 most stable reference genes following both geNorm and Normfinder analyses in order to obtain our results. Where optimal genes did not agree between the two analyses algorithms, the genes with the best three averaged rankings were selected. The differential gene expression in implantation and inter-implantation sites suggests that the ECS plays a role in implantation as well as fetoplacental development. Differences between ECS expression at the implantation and inter-implantation sites are complex. CB1 expression in decidua was significantly higher compared to that at the inter-implantation sites in the control group, whereas CB2 expression was significantly higher at the inter-implantation sites. These observations suggest differential expression of cannabinoid receptors for directing embryo implantation. Exogenous AEA treatment has a multifaceted effect on the expression of the ECS components at gestational day 14. TRPV1 was up-regulated at

the inter-implantation sites while CB1 expression was down-regulated in decidua. Of note is the fact that AEA has been said to induce programmed cell death or apoptosis via CB1 or TRPV1 depending on the cell type (Maccarrone et al. 2000a, Maccarrone, Finazzi-Agro 2003, Contassot et al. 2004). In cultured rat decidual cells AEA caused apoptosis via CB1 in a dose and time-dependent manner; low AEA concentrations (10μM) induced apoptosis via CB1 whereas high AEA concentrations (25μM) had a marked effect on cell viability and morphology which was not reversed by cannabinoid or vanilloid antagonists (Fonseca, Correia-da-Silva & Teixeira 2009). These results show that AEA treatment deranges CB1 expression in decidua and thereby may affect the process of decidualisation or even decidua regression. Interestingly, low CB1 expression was demonstrated in fallopian tubes and decidualised endometrium of women with ectopic pregnancies compared to intrauterine pregnancies (Horne et al. 2008).

To my knowledge this is the first study where TRPV1 mRNA levels have been identified in rat myometrium and inter-implantation sites. A previous study demonstrated TRPV1 mRNA and protein in rat decidua and placenta (Fonseca et al. 2009, Fonseca et al. 2012). Immunohistochemistry also localised TRPV1 protein in the mesometrial decidua and uterine natural killer cells (uNK), in the giant trophoblast cells and a few spongiotrophoblast cells and some signalling was observed in the smooth muscle layer of normal implantation sites on day 14 (Fonseca et al. 2009). Furthermore, a spatiotemporal expression of TRPV1 receptor was demonstrated and it has been suggested that this receptor could be involved in the induction of apoptosis in decidual cells (Fonseca et al. 2009). It was shown here that there is a higher TRPV1 transcript level in decidua compared to placenta in control and treated rats. The differential expression of TRPV1 at the implantation and inter-implantation sites implies an involvement of TRPV1 in the process of implantation.

Additionally, it was found that the expression of NAPE-PLD mRNA in placenta of day 4 AEA-treated rats was significantly higher than in the decidua while FAAH transcript levels in decidua and placenta were not different. This could explain the raised AEA levels in day 14 placenta following AEA treatment on day 4. This is in keeping with a previous study on mice, where uterine AEA was shown to be regulated by the spatiotemporal expression of NAPE-PLD and FAAH (Guo et al. 2005, Wang et al.

2007). When implantation and inter-implantation sites at day 14, NAPE-PLD transcript and protein levels were compared, there was no significant difference in the levels of day 4 and day 6 AEA-treated rats as well as controls. FAAH transcript levels at the inter-implantation sites were lower than at the implantation sites, which is consistent with previously reported studies in mice where lower FAAH transcript levels and higher AEA levels were found at the inter-implantation sites (Schmid et al. 1997, Paria et al. 1999). However, FAAH protein levels at the inter-implantation sites were higher than at the implantation sites of the control group. It is well known that a lack of concordance between mRNA and protein concentrations can occur (Gygi et al. 1999), especially if the protein is part of a protein complex (Schmidt et al. 2007). The presence of mRNA does not mean that it will be translated into protein. The variation between mRNA and protein concentrations can be attributed to post-transcriptional and post-translational regulation. High protein concentrations can be due to frequent transcription, high mRNA stability, frequent translation and high protein stability.

Immunohistochemistry showed that NAPE-PLD was located throughout the mesometrial decidua, giant trophoblast cells of the basal zone and the entire labyrinth zone of the placenta, a finding consistent with that from a previous study (Fonseca et al. 2009). Interestingly, there was no membrane staining for NAPE-PLD in the giant trophoblast cells in the treated rats. FAAH expression was also found in the decidua and placenta except for the spongiotrophoblast cells of control rats, whereas in treated rats spongiotrophoblast were immuno-positive probably to protect the embryo from high AEA levels. These results suggest that AEA treatment has an effect on the local expression of the metabolising enzymes in the placenta and decidua.

Immunohistochemistry of resorbed sites from AEA-treated and untreated rats revealed that NAPE-PLD was located in decidual cells close to the embryo while FAAH expression was reduced in decidual cells of resorbed sites when compared to normal implantation sites. This could lead to high localised AEA levels at resorbed sites which are linked to the abnormal pregnancies. Low FAAH expression has also been found in first trimester trophoblast of women with a spontaneous miscarriage (Trabucco et al. 2009) and high plasma AEA levels were observed in women who miscarried (Habayeb et al. 2008). The changes in expression of NAPE-PLD and FAAH in resorbed sites also

confirm that dysregulation of the ECS has a detrimental effect on pregnancy outcome (Sun et al. 2010).

Altogether the results show that AEA levels need to be tightly regulated for a successful pregnancy and that AEA administration around the day of implantation (day 4 and day 6) causes a derangement of the ECS leading to a reduction in implantation and an increase in resorbed sites.

Further studies are necessary to establish whether higher doses of AEA can completely inhibit implantation. It may be worth considering the use of AEA together with a FAAH inhibitor in order to inhibit AEA degradation and increase AEA activity or to use methanandamide a synthetic AEA which has a longer half-life and higher potency than AEA (Abadji et al. 1994).Further studies also need to investigate shortcomings of these studies. For example, the concentrations of AEA reaching the implantation site during the implantation window and the effects this has on the ECS at the time of implantation are unknown. This should be investigated to decide whether an alternative dosing route or regimen is necessary to increase the efficacy of AEA-induced termination/failure to implant. Furthermore, because previous studies have linked AEA to preterm birth and miscarriage, to fully evaluate the effects of the AEA-treatment pregnancies should be allowed to progress to term such that incidences of live birth, premature birth, birth weight etc. can be investigated.

Chapter 5

General Discussion and Future Outlook

5.1 GENERAL DISCUSSION

Numerous studies have identified the importance of the ECS in human reproduction, e.g. in the regulation of embryo implantation and pregnancy maintenance via a finely tuned ECS (Paria, Wang & Dey 2002). Manipulation of the ECS may interrupt implantation which could be clinically used as a new form of post coital contraception. The hypothesis tested in this thesis was that exogenous AEA will prevent implantation. The first aim of the studies was therefore to investigate the relationship between P4 and AEA in early pregnancy. Thus the effect of the anti-progesterone RU486 on plasma AEA levels was studied and presented in Chapter 2 and on the ECS in trophoblast in Chapter 3. The second aim was to explore the effect of exogenous AEA on implantation success in a rat model and this is presented in Chapter 4.

In Chapter 2 the effect of the anti-progesterone drug RU486 on plasma AEA levels in women undergoing MTOP was examined. *In-vitro* experiments have previously shown an enhancement of FAAH gene transcription by P4 in human lymphocytes which was fully reversed by RU486 (Maccarrone et al. 2001). The question here was whether RU486 has a similar effect *in-vivo*. As expected significantly increased plasma AEA levels one day following the administration of anti-progesterone RU486 but not one hour following RU486 was found. This delayed effect of RU486 poses a number of questions about the mechanism of recovery demonstrated *in-vitro*. RU486 is both a progesterone and glucocorticoid receptor antagonist given orally as an abortifacient so may act in multiple mechanisms. Peak plasma levels of RU486 are reached 1-3 h after dosing therefore insufficient time may have passed for RU486 to reach the target organ and reverse the effects of P4 on FAAH gene expression. Finally, it is unclear at this time, where plasma AEA is derived from in women during the first trimester of pregnancy and whether these cells are targeted by RU486.

The interpreting of plasma NAE results may be influenced by a number of factors including ethnicity and maternal BMI. Significantly higher plasma AEA levels were found in Asian and Black African women when compared to White women. Moreover obese women (BMI>30 kg/m^2) also had significantly higher AEA levels compared to women with a BMI<30 kg/m^2. Interestingly, Asian and Black African women have lower clinical pregnancy and live birth rates after ART compared to Caucasians

(Feinberg et al. 2006, Purcell et al. 2007, Seifer, Frazier & Grainger 2008). Furthermore, obese women are at greater risk of a miscarriage after a spontaneous pregnancy or ART (Fedorcsak et al. 2004, Brewer, Balen 2010, Boots, Stephenson 2011). These population differences in pregnancy outcome may be a consequence of genetic predisposition or lifestyle choices that may in turn alter AEA concentrations. The poor pregnancy outcomes may be related to these increased plasma AEA levels as an association between increased plasma AEA levels and miscarriage have been established (Habayeb et al. 2008). However, further studies are required to evaluate the relevance of AEA in the susceptibility of these populations to pregnancy failure.

The cytokine leptin is associated with obesity through regulation of energy intake and expenditure but is also important for pregnancy and is involved in the regulation of the ECS. In Chapter 2 the relationship between leptin, progesterone and plasma AEA levels in healthy pregnant women was studied. There was a significant positive correlation between serum leptin levels and BMI as previously described (Highman et al. 1998). Serum leptin levels did not alter one day after RU486 administration unlike plasma AEA levels and no significant correlations were observed between serum leptin and plasma NAE levels. However, another study identified a decrease in leptin levels after RU486 administration but before misoprostol (Honkanen et al. 2005). They measured leptin levels by immunoradiometric assay kits (IRMA) whereas ELISA kits were used here. Further work is required to give further insight into *in-vivo* interactions between leptin, P4 and AEA.

In Chapter 3 experiments on the effect of RU486 on the ECS in trophoblast were presented. This is the first study to measure NAE levels in trophoblast following medical termination of pregnancy (MTOP). AEA levels were significantly higher in trophoblast obtained after MTOP than after surgical termination of pregnancy (STOP) supporting the hypothesis that RU486 reduces the up-regulation of FAAH by P4. Furthermore, AEA levels in trophoblast obtained after MTOP were significantly higher than in decidua. There was no significant correlation between plasma and trophoblast AEA levels, suggesting an independent regulation of plasma and the trophoblast AEA levels as shown in the mouse and rat (Wang et al. 2007, Fonseca et al. 2010). The source of plasma AEA is as yet unconfirmed but may be white blood cells. Regulation

of ECS in trophoblast and white blood cells may be markedly different. Immunohistochemistry confirmed the localisation of NAPE-PLD, FAAH, CB1 and CB2 in first trimester trophoblast as demonstrated before (Taylor et al. 2011), confirming trophoblast as a suitable site for AEA synthesis, degradation and activity. The presence of components of the ECS in trophoblast supports its critical role in the establishment and maintenance of early pregnancy. Interestingly immunoreactive NAPE-PLD was lower and FAAH higher in trophoblast from MTOP when compared to that from STOP. As P4 is known to up-regulate FAAH expression in peripheral lymphocytes (Maccarrone et al. 2001) it had been expected that FAAH expression in trophoblast from MTOP will be low. However, FAAH expression in MTOP was increased, though not significantly, as reported in a previous study (Taylor et al. 2011). In addition, our sample size was small; trophoblast from only six samples was examined; hence a drawback of the experiments. It may be worth increasing the sample size. Immunoreactive CB2 was significantly increased in tissues from MTOP when compared to that from STOP confirming the data of Taylor et al (Taylor et al. 2011). This suggests that reduced CB2 expression is an important factor influencing early pregnancy. AEA via CB2 inhibits trophoblast cell proliferation (Habayeb et al. 2008) and this may explain why expression levels are kept low in ongoing pregnancy and suggests elevation of CB2 with resultant inhibition of trophoblast cell proliferation plays a role in MTOP.

Furthermore, the mRNA expression of the ECS was examined by qRT-PCR in trophoblast following MTOP and STOP. This is the first study to evaluate the optimum reference genes for normalisation of human first trimester trophoblast using the software programs geNorm and Normfinder. The reference genes identified here were ATP5B and EIF4A2. Analysis of ECS transcript levels revealed significantly increased NAPE-PLD, FAAH, CB1 and TRPV1 transcript levels in trophoblast from MTOP when compared to that from STOP. CB2 levels were also increased, though this was not statistically significant. Protein levels of the AEA metabolizing enzymes NAPE-PLD and FAAH respectively were also increased in trophoblast from MTOP compared to that from STOP, but this was not statistically significant. High tissue AEA levels were identified together with raised NAPE-PLD and FAAH levels. However, NAPE-PLD

transcript levels were greater than FAAH transcript levels leading to a net increase of AEA. Another possibility could be the involvement of other synthesis enzymes.

Other studies have observed that high AEA levels inhibit trophoblast cell differentiation in mice via CB1 (Wang et al. 1999) and trophoblast proliferation in BeWo cells via CB2 (Habayeb et al. 2008). The results in this thesis suggest that the increased AEA levels in trophoblast following RU486 may inhibit cell growth and differentiation via an increase in cannabinoid receptors. This may indeed be part of the mechanism for the effectiveness of a medical termination of a pregnancy. Here, it was demonstrated for the first time that the anti-progesterone RU486 indeed increases AEA levels not only in plasma but also in trophoblast. These results confirm the importance of the endocannabinoid-hormone network at the maternal-fetal interface and that a deregulation of this network by the anti-progesterone RU486 leads to fetal loss.

Further studies are necessary to investigate the effect of ethnicity and BMI on NAE levels. A larger cohort of pregnant women of different ethnicities ensuring that adequate numbers from each ethnic group are recruited will help to address the hypothesis that NAE levels vary in different ethnic groups. Additionally, the measurement of FAAH activity may contribute to a better understanding of the *in-vivo* relationship between AEA, P4 and leptin as all of these products are essential for a successful pregnancy. In this study it was demonstrated that mRNA levels of TRPV1 in human trophoblast are differentially expressed in tissues from MTOP and STOP. These findings suggest a contribution of this receptor to early pregnancy. However, further research into the role of TRPV1 in trophoblast is required. Furthermore, there is a need for highly specific antibodies for cannabinoid receptors to specifically detect protein levels for Western blot and immunohistochemical analyses.

In Chapter 4 the effect of high exogenous AEA administration on rat implantation and pregnancy when given around the time of implantation (day 5) was examined. An increase in AEA levels in plasma and the uterus was expected to prevent implantation. Indeed a reduction in the number of implantation sites after intraperitoneal AEA administration (1mg/kg) on day 4 or day 6 of pregnancy with less implantation sites on the left uterine horn compared to the right one was observed. The left uterine horn was

closer to the site of AEA injection and therefore higher AEA levels may have reached this site and caused a more profound effect on implantation. Uterine AEA levels are important in regulating implantation by synchronizing trophoblast differentiation and uterine receptivity (Wang, Dey & Maccarrone 2006). In mice low AEA levels are beneficial to implantation whereas high levels are detrimental (Wang, Dey & Maccarrone 2006). Furthermore, low AEA levels are found at implantation sites whereas high AEA levels are present at inter-implantation sites in mice (Schmid et al. 1997). Here, a detrimental effect of high AEA administration on implantation in rat was shown. In addition, an effect of AEA treatment on embryo development was also observed. There was an increase in the number of resorbed sites after exogenous AEA on day 4 and day 6 of pregnancy. AEA treatment also caused a more disorganised placental structure when examined histomorphologically. This was more profound in day 6 than day 4 AEA-treated rats. Overall, AEA treatment in the rats around the time of implantation reduced the number of successful implantations. Even when implantation occurred, embryo development was affected or the placental structure was disturbed potentially impairing a fully functional placenta.

Interestingly plasma AEA levels significantly increased with the total number of implantations (normal and resorbed) and this increase was more marked with normal implantation sites. These findings suggest that the placenta may be involved in the origin of plasma AEA levels. However, further studies are necessary to understand this relationship.

In AEA-treated rats, NAE levels in the decidua were higher than in the controls whereas placental NAE levels increased mainly in day 4 treated rats. In the control group, tissue NAE levels in the placenta and decidua were similar while in day 4 treated rats placental AEA levels were significantly higher than decidua levels. The results demonstrate that AEA treatment around the time of implantation still has an effect on tissue NAE levels on day 14 of the pregnancy, the day the animals were sacrificed.

Immunohistochemistry of rat implantation sites confirmed the presence of NAPE-PLD and FAAH in placenta and decidua as described previously (Fonseca et al. 2009). Day 6

treated rats displayed a more disorganized placental structure than day 4 treated rats. Of note was the absence of NAPE-PLD staining of the membrane in giant trophoblast cells and the presence of FAAH staining in spongiotrophoblast in treated rats probably to protect the embryo from high AEA levels. This is the first study to demonstrate the localisation of NAPE-PLD and FAAH at resorbed sites in rat with NAPE-PLD being present in decidual cells close to the embryo and reduced FAAH levels in the decidua when compared to normal implantation sites. This would have resulted in increased AEA levels in the tissue which is linked with abnormal pregnancies (Sun et al. 2010). Another study also detected low FAAH expression in first trimester trophoblast of women with a spontaneous miscarriage (Trabucco et al. 2009).

qRT-PCR with validated reference genes was used to demonstrate transcript levels of NAPE-PLD, FAAH, CB1, CB2 and TRPV1 at implantation and inter-implantation sites of treated and control rats. This is the first time to evaluate reference genes in rat placenta, decidua, myometrium and inter-implantation site using the software programs geNorm and Normfinder. The optimal number of reference genes was identified as three. The best reference genes for the placenta were YWHAZ, RPL13A and GAPDH, for decidua ATP5B, CANX and GAPDH, for rat myometrium RPL13A, GAPDH and UBC and for inter-implantation YWHAZ, RPL13A and B2M. Interestingly the reference gene ß-Actin commonly used for normalisation in rat placenta was not selected here because it was routinely found to be a gene with one of the lower gene expression stability profiles. This emphasises again the importance of validation of reference genes for each tissue in an experiment to obtain accurate results as previously described (Bustin et al. 2009). Differences between ECS expression in tissues at the implantation and inter-implantation sites are complex. In control rats low CB1 expression and high CB2 expression at inter-implantation sites suggest a differential expression of cannabinoid receptors to direct embryo implantation. After AEA treatment CB1 expression in day 14 decidua was down-regulated which may have affect the process of decidualisation or decidua regression. This is the first study describing TRPV1 transcript levels in myometrium and inter-implantation sites. A previous study proposed the involvement of this receptor in the induction of apoptosis in decidual cells (Fonseca et al. 2009) and here higher transcript levels in the decidua than placenta were identified. In day 4 AEA-treated rats, NAPE-PLD expression in the day 14 placenta was higher than in the decidua while FAAH transcript levels were not

different. This will result in increased AEA levels in the placenta as seen earlier in the data here. It is known that AEA levels are regulated by the spatiotemporal expression of NAPE-PLD and FAAH (Guo et al. 2005, Wang et al. 2007). No significant difference was seen in NAPE-PLD transcript and protein levels at the implantation and inter-implantation sites of day 4, day 6 treated rats and control. FAAH transcript levels were lower at the inter-implantation than implantation sites in treated and control rats which are in concordance with findings in mice studies where low FAAH levels and high AEA were found at the inter-implantation sites (Schmid et al. 1997, Paria et al. 1999). FAAH protein levels at the inter-implantation sites in day 6 treated rats showed the same pattern whereas in the control group FAAH protein levels were higher at the inter-implantation than implantation sites. The discrepancy between transcript and protein levels can be due to post-transcriptional and post-translational regulation.

In summary the studies have demonstrated in Chapter 4, a detrimental effect of exogenous AEA given around the time of implantation on implantation and resorption and furthermore a derangement of the ECS following AEA administration, which was still present on day 14 of pregnancy.

5.2 FUTURE OUTLOOK

In the future it would be interesting to explain this work on the components of the endocannabinoids at the rat implantation and inter-implantation sites in treated and control rats as listed below:

- UHPLC-MS/MS to determine NAE levels at inter-implantation sites
- IHC of CB1, CB2 and TRPV1 on implantation sites
- IHC of NAPE-PLD, FAAH, CB1, CB2 and TRPV1 on inter-implantation sites
- Western-Blotting of CB1, CB2 and TRPV1 on implantation and inter-implantation sites

The studies here have shown that manipulating the ECS interrupts implantation as suggested in the hypothesis. In order to consider this mechanism as a new post coital contraceptive method more research in this area has to be done which could include:

- Administration of AEA on the day of implantation (day 5)
- Increasing the dose of AEA
- Using a different vehicle
- Using AEA in combination with a FAAH inhibitor to increase the effect of AEA
- Using Methanandamide, a synthetic analogue of AEA with a longer half life
- Using a different form of administration e. g. intravaginal

Overall this research confirmed the importance of the ECS in female reproductive events and that any dysregulation of this delicate system leads to adverse pregnancy outcomes.

Appendix 1

Publications arising from the thesis

1. **The role of sex steroid hormones, cytokines and the endocannabinoid system in female fertility**

   Karasu T, Marczylo TH, Macarrone M, Konje JC. Human Reproduction Update 2011;17 (3): 347-61.

2. **From fertilisation to implantation in mammalian pregnancy-modulation of early human reproduction by the endocannabinoid system**

   Bambang K, Karasu T, Gebeh A, Taylor AH, Marczylo TH, Lam PMW, Willets JM, Konje JC. Pharmaceuticals 2010; 3: 2910-2929.

3. **Endocannabinoids and pregnancy.**

   Taylor AH, Amoako AA, Bambang K, Karasu T, Gebeh A, Lam PMW, Marczylo TH, Konje JC. Clinica Chimica Acta 2010; 411:921-930.

Appendix 2

Oral presentations arising from the thesis

1. **Determination of reference genes for use with first trimester trophoblast**

   RCOG World Conference, Liverpool, June 2013

2. **Changes to the Endocannabinoid System after RU486 administration**

   RCOG World Conference Liverpool, June 2013

3. **The effect of RU486 on the modulation of anandamide and leptin levels**

   RCOG World Conference Liverpool, June 2013

4. **The effect of exogenous Anandamide on implantation**

   EAOGS, Cambridge, October 2012

5. **The effect of Anandamide administration on implantation**

   Gynaecology Visiting Society Winter Meeting, Leicester, October 2012

6. **Exogenous Anandamide administration decreases successful implantation**

   ESHRE, Istanbul, Turkey, July 2012 (nominated for the Best Basic Science Award for oral presentation)

7. **The Endocannabinoid System in Early Human Pregnancy**

   Reproductive Sciences Section Seminar, University of Leicester, Leicester, May 2012

8. **The Endocannabinoid System in Fertility Control**

   Departmental Meeting, University Hospitals of Leicester, Leicester, January 2011

Appendix 3

Poster Presentations arising from the thesis

1. **The effect of BMI and ethnicity on plasma anandamide and lymphocyte anandamide hydrolase activity in early pregnant women**

   RCOG World Conference, Liverpool, June 2013

2. **The role of ethnicity and BMI on plasma Anandamide levels**

   EAOGS, Cambridge, October 2012

3. **Anandamide and leptin levels in women undergoing medical termination of pregnancy**

   Congress of the European Society of Contraception and Reproductive Health, Athens, Greece, July 2012

4. **Endocannabinoid levels in women undergoing medical termination of pregnancy**

   Congress of the European Society of Contraception and Reproductive Health, Athens, Greece, July 2012

5. **The 2-Arachidonylglycerol-Metabolising Enzyme (MAGL) and Receptor Targets (CB1 and CB2) are up-regulated in miscarried trophoblast tissues**

   Annual Scientific Meeting of the Society for Gynaecologic Investigations, San Diego, USA, March 2012

6. **The effect of exogenous administration of Anandamide on pregnancy**

   Annual Scientific Meeting of the Society for Gynaecologic Investigations, San Diego, USA, March 2012

7. **The effect of exogenous administration of anandamide on implantation**

   Annual Scientific Meeting of the Society for Gynaecologic Investigations, San Diego, USA March2012

8. **Modulation of the endocannabinoid System after exogenous administration of anandamide**

   Annual Scientific Meeting of the Society for Gynaecologic Investigations, San Diego, USA 2012

9. **Changes to the Endocannabinoid System after RU486 administration**

   Annual Scientific Meeting of the Society for Gynaecologic Investigations, San Diego, USA 2012

10. **Is there an effect of exogenous prostaglandin on plasma anadamide levels?**

    Annual Scientific Meeting of the Society for Gynaecologic Investigations, Miami, USA, March 2011

11. **The effect of ethnicity on plasma anandamide levels**

    Annual Scientific Meeting of the Society for Gynaecologic Investigations, Miami, USA, March 2011

12. **The role of BMI on plasma anandamide levels**

    Annual Scientific Meeting of the Society for Gynaecologic Investigations, Miami, USA, March 2011

13. **Is there an effect of exogenous prostaglandin on plasma anandamide levels?**

    Blair Bell Meeting, London, November 2010

14. **Does ethnicity have an effect on plasma anandamide levels**

    Blair Bell Meeting, London, November 2010

Appendix 4

Copy of Ethics Committee approval letter, patient information leaflets and consent forms

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

Telephone: 01159123344
Facsimile: 01159123300

13 June 2006

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

Dear Professor Konje,

Full title of study: **The Role of Endogenous Cannabinoids (chemicals similar to cannabis but produced naturally in the human body)in Reproduction**

REC reference number: **06/Q2501/49**

Thank you for your letter of 02 June 2006, responding to the Committee's request for further information on the above research and submitting revised documentation.

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

Confirmation of ethical opinion

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

Conditions of approval

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

Approved documents

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

Document	*Version*	*Date*
Application	1	24 February 2006
Investigator CV	1	23 February 2006
Protocol	2	23 May 2006
Peer Review		
Letter of invitation to participant – V menstrual cycle studies	2	23 May 2006
Letter of invitation to participant – V undergoing gynea surgery	2	23 May 2006
Letter of invitation to participant – Non-P V with non-surgical gynae problem	2	23 May 2006
Letter of invitation to participant - STOP V	2	23 May 2006

Letter of invitation to participant – V spontaneous miscariage	2	23 May 2006
Letter of invitation to participant – V with suspected or diagnosed ectopic pregnancy	2	23 May 2006
Letter of invitation to participant – Early pregnancy complications	2	23 May 2006
Letter of invitation to participant – Pregnant V - Longitudinal study	2	23 May 2006
Letter of invitation to participant - Pregnant V at booking	2	23 May 2006
Letter of invitation to participant – Pregnant V mid-trimester Blood tests	2	23 May 2006
Letter of invitation to participant - 28 weeks pregnant	2	23 May 2006
Letter of invitation to participant - 36 weeks pregnant	2	23 May 2006
Letter of invitation to participant - preterm labour	2	23 May 2006
Letter of invitation to participant - Normal labour	2	23 May 2006
Letter of invitation to participant - Emergency CS	2	23 May 2006
Letter of invitation to participant - Elective CS	2	23 May 2006
Participant Information Sheet: Pregnant V in normal labour	2	23 May 2006
Participant Information Sheet: Pregnant V in preterm labour	2	23 May 2006
Participant Information Sheet: Pregnant V at 36 weeks of pregnancy	2	23 May 2006
Participant Information Sheet: Pregnant V at 28 weeks of pregnancy	2	23 May 2006
Participant Information Sheet: Pregnant V at mid-trimester blood tests	2	23 May 2006
Participant Information Sheet: Pregnant V undergoing elective caesarean section	2	23 May 2006
Participant Information Sheet: Pregnant V undergoing emergency caesarean section	2	23 May 2006
Participant Information Sheet: V with surgical (STOP) Gynaecological problem	2	23 May 2006
Participant Information Sheet: Non-P V with non-surgical Gynaecological problem	2	01 June 2006
Participant Information Sheet: Non- P V undergoing Gynaecological Surgery	2	23 May 2006
Participant Information Sheet: Non-P V for Menstrual Cycly Studies	2	23 May 2006
Participant Information Sheet: Pregnant V at booking	2	23 May 2006
Participant Information Sheet: Pregnant V - longitudinal study	2	23 May 2006
Participant Information Sheet: V with early pregnancy complications	2	23 May 2006
Participant Information Sheet: V with a suspected or diagnosed ectopic pregnancy	2	01 June 2006
Participant Information Sheet: V with the complication of Spontaneous miscarraige	2	23 May 2006
Participant Consent Form: Pregnant V at booking	2	23 May 2006
Participant Consent Form: Pregnant V - longitudinal study	2	23 May 2006
Participant Consent Form: V with early pregnancy complications	2	23 May 2006
Participant Consent Form: V with surgical (STOP) Gynaecological problem	2	23 May 2006
Participant Consent Form: Non-P V with non-surgical Gynaecological problem	2	23 May 2006

Participant Consent Form: Non- P V undergoing Gynaecological Surgery	2	23 May 2006
Participant Consent Form: Non-P V for Menstrual Cycly Studies	2	23 May 2006
Participant Consent Form: Pregnant V undergoing elective caesarean section	2	23 May 2006
Participant Consent Form: Pregnant V undergoing emergency caesarean section	2	23 May 2006
Participant Consent Form: V with a suspected or diagnosed ectopic pregnancy	2	23 May 2006
Participant Consent Form: V with the complication of Spontaneous miscarraige	2	23 May 2006
Participant Consent Form: Pregnant V in normal labour	2	23 May 2006
Participant Consent Form: Pregnant V in preterm labour	2	23 May 2006
Participant Consent Form: Pregnant V at 36 weeks of pregnancy	2	23 May 2006
Participant Consent Form: Pregnant V at 28 weeks of pregnancy	2	23 May 2006
Participant Consent Form: Pregnant V at mid-trimester blood tests	2	23 May 2006
Response to Request for Further Information		02 June 2006
Letter from Dr HJ Porter		11 July 2005
Letter from LJR Brown		11 July 2005
Bupa Foundation Grant		14 October 2005
Letter from LNR1 REC		12 April 2006
Letter to LNR1 REC		08 August 2005

Research governance approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final research governance approval from the R&D Department for the relevant NHS care organisation.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

06/Q2501/49	**Please quote this number on all correspondence**

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

Yours sincerely

Dr Carl Edwards/Ms Linda Ellis
Chair/Co-ordinator

Email: linda.ellis@rushcliffe-pct.nhs.uk

Enclosures: *Standard approval conditions*
Site approval form

Copy to: R&D Department for NHS care organisation at lead site -
University Hospitals Of Leicester NHS Trust

Leicestershire, Northamptonshire & Rutland Research Ethics Committee 1

LIST OF SITES WITH A FAVOURABLE ETHICAL OPINION

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

REC reference number:	06/Q2501/49	**Issue number:**	1	**Date of issue:**	13 June 2006
Chief Investigator:	Professor J C Konje				
Full title of study:	The Role of Endogenous Cannabinoids (chemicals similar to cannabis but produced naturally in the human body)in Reproduction				

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

Principal Investigator	*Post*	*Research site*	*Site assessor*	*Date of favourable opinion for this site*	*Notes (1)*
Professor J C Konje	Professor of Obstetrics and Gynaecology and Honorary Consultant	University Hospitals of Leicester NHS Trust, Leicester Royal Infirmary	Leicestershire, Northamptonshire & Rutland Research Ethics Committee 1	13/06/2006	

Approved by the Chair on behalf of the REC:

..... (Signature of ~~Chair~~/Administrator)

(Delete as applicable)

Linda Ellis ..... (Name)

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

University Hospitals of Leicester NHS
NHS Trust

Leicester Royal Infirmary
Leicester
LE1 5WW

Tel: 0116 254 1414
Fax: 0116 258 5631
Minicom: 0116 258 6878

PATIENT INFORMATION LEAFLET
(Volunteer with a medical (MTOP) gynaecological problem)

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

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

Invitation paragraph

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

Thank you for reading this.

1. What is the purpose of the study?

Endogenous cannabinoids are naturally occurring substances in the body, which are similar to cannabis. Studies in animals have shown that these substances are vital for pregnancy to succeed. High levels are associated with miscarriages but a critical level in the blood is essential for a successful pregnancy. The precise way in which these substances influence human reproduction is not known. While there have been lots of studies on the role of these substances in animal reproduction, very little research has been undertaken in human beings. The main reason for this has been the inability of researchers to measure these substances in the blood. We very recently developed a means of measuring these substances and successfully used the method in a small group of pregnant women and non-pregnant women.

Our results were very encouraging and supported our belief that these substances play a part in successful pregnancies. Despite our results there are several unanswered questions about the exact role of these substances in reproduction. We believe that by defining these questions and looking for answers, we will provide opportunities for improving the outcome of pregnancies especially in women who undergo repeated miscarriages.

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

(a) women during normal pregnancy and
(b) women with different complications of pregnancy

In addition, we would like to investigate:

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

We hope to do this by:

a. measuring the levels of these cannabis-like substances in the blood, and the tissues which we collect
b. studying the way these substances work on the tissues we collect and
c. investigating the genes that control how the body produces them and how they work in the various tissues we collect.

The tissue(s) collected will be used for the research but any left over will be kept in a special bank called the "Female Reproduction Research Tissue Bank" at the University of Leicester. We will ask for your permission to put your tissue(s) into this Bank but before we do this, you will be given an information leaflet about the bank to read and to give us permission by signing a consent form. If you do not want your tissue(s) to be kept in the Tissue Bank, it will be used only for the studies outlined above. Any future research will only be undertaken on the tissues after the Ethics committee has given its approval.

2. Why have I been chosen?

You have been chosen because you are attending the Leicester Royal Infirmary with a gynaecological problem for treatment. We are inviting most women like you to take part in this study.

3. Do I have to take part?

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

4. What will happen to me if I take part?

If you agree to take part, a small amount of blood (one tablespoonful) will be taken from you arm at the time you are having a drip put in your hand (just before you go to sleep) or when the doctors take a blood test from you as you are prepared for surgery. In addition, a small amount of tissue will be taken from the products of pregnancy removed from you at surgery. Since these are not routinely sent to the laboratory, this should have no effect at all on your treatment.

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

5. What do I have to do?

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

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

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

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

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

8. What are the possible disadvantages and risks of taking part?

The only disadvantage to you of this study is the slight discomfort (scratch) you may feel when your blood sample is taken.

9. What are the possible benefits of taking part?

You will not benefit directly from taking part in this study. However, with your help, we may in future be able to help women with either various pregnancy complications or difficulties getting pregnant.

10. What if new information becomes available?

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

11. What happens when the research study stops?

Nothing will happen to you as you will not need to do anything more after this.

12. What if something goes wrong?

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

13. Will my taking part in this study be kept confidential?

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

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

14. What will happen to the results of the research study?

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

15. Who is organising and funding the research?

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

16. Who has reviewed the study?

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

17. For more information, who do I contact?

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

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

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

You will be given a copy of the information sheet and a signed consent form for you to keep.

University Hospitals of Leicester NHS
NHS Trust

Leicester Royal Infirmary
Leicester
LE1 5WW

Tel: 0116 254 1414
Fax: 0116 258 5631
Minicom: 0116 258 6878

Patient name, address, DOB (or ID label)

Patient Identification Number for this trial: _____

CONSENT FORM

(Volunteer with a Medical (MTOP) gynaecological problem)

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

Name of Researcher / Principal Investigator: Professor Justin C Konje

Please initial box

1. I confirm that I have read and understand the information sheet dated 23/05/06 version 2 for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that I may withdraw my consent to my tissue being used at any time without justifying my decision and without affecting my normal care and medical management. ☐
4. I agree to donate the tissue samples as detailed below and allow their use in medical research as described in the Patient Information Leaflet. ☐
5. I understand that the tissue is a gift and that I will not benefit from any intellectual property that results from the use of the tissue. ☐
6. I understand that tissue samples will not be used to undertake any genetic tests whose results may have adverse consequences on my or my families insurance or employment. ☐
7. **I agree / do not agree** to my tissue samples being used to undertake genetic research as described in the patient information leaflet **(Delete as applicable)** ☐

Trust Headquarters, Gwendolen House, Gwendolen Road, Leicester, LE5 4QF
Website: www.uhl-tr.nhs.uk
Chairman Mr. Martin Hindle **Chief Executive** Malcolm Lowe-Lauri

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

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

10. The samples which I hereby consent to donate are:

a. Blood ☐
b. Lining of the womb ☐
c. Wall of the womb ☐
d. Placenta and fetal membranes ☐
e. Pieces of fallopian tubes ☐
f. Muscle tissue ☐
g. Blood vessels ☐
h. Omentum ☐
i. Pieces of ovary (only if being removed or hysterectomy) ☐
j. Pieces of the neck of the womb (cervix) ☐
k. Pieces of the vagina ☐
l. Pieces of fat tissue ☐
m. Tissue from the bladder ☐
n. Any other tissue – Please specify

.....
.....
.....

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

Name of Patient	Date	Signature
Name of Parent/guardian/ legal representative ***(If applicable)***	Date	Signature

Researcher	Date	Signature

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

University Hospitals of Leicester NHS
NHS Trust

Leicester Royal Infirmary
Leicester
LE1 5WW

Tel: 0116 254 1414
Fax: 0116 258 5631
Minicom: 0116 258 6878

PATIENT INFORMATION LEAFLET
(Volunteer with a surgical (STOP) gynaecological problem)

Title of Study:
The role of endogenous cannabinoids (cannabis like compounds produced in the human body in reproduction

Principal Investigator: Professor Justin Konje

The details of Professor Konje are given below in case you wish to contact him.
Reproductive Sciences Section
Department of Cancer Studies and Molecular Medicine
University Hospitals of Leicester NHS Trust
Leicester Royal Infirmary
Leicester, LE2 7LX
Phone: 0116 252 5826
Pager through switchboard.

Invitation paragraph

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

Thank you for reading this.

1. What is the purpose of the study?

Endogenous cannabinoids are naturally occurring substances in the body, which are similar to cannabis. Studies in animals have shown that these substances are vital for pregnancy to succeed. High levels are associated with miscarriages but a critical level in the blood is essential for a successful pregnancy. The precise way in which these substances influence human reproduction is not known. While there have been lots of studies on the role of these substances in animal reproduction, very little research has been undertaken in human beings.

The main reason for this has been the inability of researchers to measure these substances in the blood. We very recently developed a means of measuring these substances and successfully used the method in a small group of pregnant women and non-pregnant women. Our results were very encouraging and supported our belief that these substances play a part in successful pregnancies. Despite our results there are several unanswered questions about the exact role of these substances in reproduction. We believe that by defining these questions and looking for answers, we will provide opportunities for improving the outcome of pregnancies especially in women who undergo repeated miscarriages.

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

(a) women during normal pregnancy and
(b) women with different complications of pregnancy

In addition, we would like to investigate:

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

We hope to do this by:

a. measuring the levels of these cannabis-like substances in the blood, and the tissues which we collect
b. studying the way these substances work on the tissues we collect and
c. investigating the genes that control how the body produces them and how they work in the various tissues we collect.

The tissue(s) collected will be used for the research but any left over will be kept in a special bank called the "Endocannabinoid Tissue Bank" at the University of Leicester. We will ask for your permission to put your tissue(s) into this Bank but before we do this, you will be given an information leaflet about the bank to read and to give us permission by signing a consent form. If you do not want your tissue(s) to be kept in the Tissue Bank, it will be used only for the studies outlined above. Any future research will only be undertaken on the tissues after the Ethics committee has given its approval.

2. Why have I been chosen?

You have been chosen because you are attending the Leicester Royal Infirmary with a gynaecological problem. We are inviting most women like you to take part in this study.

3. Do I have to take part?

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

4. What will happen to me if I take part?

If you agree to take part, a small amount of blood (one tablespoonful) will be taken from you arm at the time you are having a drip put in your hand (just before you go to sleep) or when the doctors take a blood test from you as you are prepared for surgery. In addition, a small amount of tissue will be taken from the products of pregnancy removed from you at surgery. Since these are not routinely sent to the laboratory, this should have no effect at all on your treatment.

5. What do I have to do?

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

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

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

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

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

8. What are the possible disadvantages and risks of taking part?

The only disadvantage to you of this study is the slight discomfort (scratch) you may feel when your blood sample is taken.

9. What are the possible benefits of taking part?

You will not benefit directly from taking part in this study. However, with your help, we may in future be able to help women with either various pregnancy complications or difficulties getting pregnant.

10. What if new information becomes available?

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

11. What happens when the research study stops?

Nothing will happen to you as you will not need to do anything more after this.

12. What if something goes wrong?

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

13. Will my taking part in this study be kept confidential?

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

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

14. What will happen to the results of the research study?

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

15. Who is organising and funding the research?

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

16. Who has reviewed the study?

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

17. For more information, who do I contact?

Professor Justin Konje
Reproductive Sciences Section
Department of Cancer Studies and Molecular Medicine
University of Leicester
Robert Kilpatrick Clinical Sciences Building
Leicester Royal Infirmary
Leicester LE2 7LX

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

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

You will be given a copy of the information sheet and a signed consent form for you to keep.

University Hospitals of Leicester NHS
NHS Trust

Leicester Royal Infirmary
Leicester
LE1 5WW

Tel: 0116 254 1414
Fax: 0116 258 5631
Minicom: 0116 258 6878

Patient name, address, DOB (or ID label)

Patient Identification Number for this trial: _____

CONSENT FORM

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

Please initial box

1. I confirm that I have read and understand the information sheet dated 23/02/06 version 1 for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that I may withdraw my consent to my tissue being used at any time without justifying my decision and without affecting my normal care and medical management. ☐
4. I agree to donate the tissue samples as detailed below and allow their use in medical research as described in the Patient Information Leaflet. ☐
5. I understand that the tissue is a gift and that I will not benefit from any intellectual property that results from the use of the tissue. ☐
6. I understand that tissue samples will not be used to undertake any genetic tests whose results may have adverse consequences on my or my families insurance or employment. ☐
7. **I agree / do not agree** to my tissue samples being used to undertake genetic research as described in the patient information leaflet **(Delete as applicable)** ☐

University Headquarters, Gwendolen House, Gwendolen Road, Leicester, LE5 4QF
Website: www.uhl-tr.nhs.uk
Chairman Mr. Martin Hindle **Chief Executive** Malcolm Lowe-Lauri

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

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

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

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

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

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

BIBLIOGRAPHY

"Randomised controlled trial of levonorgestrel versus the Yuzpe regimen of combined oral contraceptives for emergency contraception. Task Force on Postovulatory Methods of Fertility Regulation", 1998, *Lancet,* vol. 352, no. 9126, pp. 428-433.

Abadji, V., Lin, S., Taha, G., Griffin, G., Stevenson, L.A., Pertwee, R.G. & Makriyannis, A. 1994, "(R)-methanandamide: a chiral novel anandamide possessing higher potency and metabolic stability", *Journal of medicinal chemistry,* vol. 37, no. 12, pp. 1889-1893.

Ahluwalia, J., Urban, L., Bevan, S. & Nagy, I. 2003, "Anandamide regulates neuropeptide release from capsaicin-sensitive primary sensory neurons by activating both the cannabinoid 1 receptor and the vanilloid receptor 1 in vitro.", *European Journal of Neuroscience,* vol. 17, no. 12, pp. 2611-2618.

Andersen, C.L., Jensen, J.L. & Orntoft, T.F. 2004, "Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets", *Cancer research,* vol. 64, no. 15, pp. 5245-5250.

Asami-Miyagishi, R., Iseki, S., Usui, M., Uchida, K., Kubo, H. & Morita, I. 2004, "Expression and function of PPARgamma in rat placental development", *Biochemical and biophysical research communications,* vol. 315, no. 2, pp. 497-501.

Ashok, P.W., Penney, G.C., Flett, G.M. & Templeton, A. 1998, "An effective regimen for early medical abortion: a report of 2000 consecutive cases.", *Human Reproduction,* vol. 13, no. 1O, pp. 2962-2965.

Ashworth, C.J., Hoggard, N., Thomas, L., Mercer, J.G., Wallace, J.M. & Lea, R.G. 2000, "Placental leptin", *Reviews of reproduction,* vol. 5, no. 1, pp. 18-24.

Avelino, A. & Cruz, F. 2006, "TRPV1 (vanilloid receptor) in the urinary tract: expression, function and clinical applications", *Naunyn-Schmiedeberg's archives of pharmacology,* vol. 373, no. 4, pp. 287-299.

Baker, D. (ed) 1979, *Reproduction and Breeding*, Academic Press, New York.

Bambang, K.N., Karasu, T., Gebeh, A., Taylor, A.H., Marczylo, T. H., Willets, J.M. & Konje, J.C. 2010, "From Fertilisation to Implantation in Mammalian Pregnancy-Modulation of Early Human Reproduction by the Endocannabinoid System", *Pharmaceuticals*, vol. 3, no.9, pp.2910-2929.

Bari, M., Battista, N., Pirazzi, V. & Maccarrone, M. 2011, "The manifold actions of endocannabinoids on female and male reproductive events", *Frontiers in bioscience : a journal and virtual library,* vol. 16, pp. 498-516.

Battista, N., Bari, M., Rapino, C., Trasatti, F., D'Agostino, A. & Maccarrone, M. 2007, "Regulation of female fertility by the endocannabinoid system", *Human Fertility,* vol. 10, no. 4, pp. 207-216.

Battista, N., Pasquariello, N., Di Tommaso, M. & Maccarrone, M. 2008a, "Interplay between endocannabinoids, steroids and cytokines in the control of human reproduction", *Journal of neuroendocrinology,* vol. 20, no. Suppl 1, pp. 82-89.

Battista, N., Rapino, C., Di Tommaso, M., Bari, M., Pasquariello, N. & Maccarrone, M. 2008b, "Regulation of male fertility by the endocannabinoid system", *Molecular & Cellular Endocrinology,* vol. 286, no. 1-2 Suppl 1, pp. S17-23.

Baulieu, E.E. 1991, "The steroid hormone antagonist RU486. Mechanism at the cellular level and clinical applications", *Endocrinology & Metabolism Clinics of North America,* vol. 20, no. 4, pp. 873-891.

Ben-Shabat, S., Fride, E., Sheskin, T., Tamiri, T., Rhee, M.H., Vogel, Z., Bisogno, T., De Petrocellis, L., Di Marzo, V. & Mechoulam, R. 1998, "An entourage effect: inactive endogenous fatty acid glycerol esters enhance 2-arachidonoyl-glycerol cannabinoid activity.", *European journal of pharmacology,* vol. 353, no. 1, pp. 23-31.

Bischof, P., Meisser, A. & Campana, A. 2002, "Control of MMP-9 expression at the maternal-fetal interface", *Journal of reproductive immunology,* vol. 55, no. 1-2, pp. 3-10.

Bisogno, T., Melck, D., Bobrov, M.Y., Gretskaya, N.M., Bezuglov, V.V., De Petrocellis, L. & Di Marzo, V. 2000, "N-acyl-dopamines: novel synthetic CB(1) cannabinoid-receptor ligands and inhibitors of anandamide inactivation with cannabimimetic activity in vitro and in vivo.", *Biochemical Journal,* vol. 351, no. Pt 3, pp. 817-824.

Bisogno, T., Melck, D., De Petrocellis, L. & Di Marzo, V. 1999, "Phosphatidic acid as the biosynthetic precursor of the endocannabinoid 2-arachidonoylglycerol in intact mouse neuroblastoma cells stimulated with ionomycin.", *Journal of neurochemistry,* vol. 72, no. 5, pp. 2113-2119.

Bjorbaek, C., Uotani, S., da Silva, B. & Flier, J.S. 1997, "Divergent signaling capacities of the long and short isoforms of the leptin receptor.", *Journal of Biological Chemistry,* vol. 272, no. 51, pp. 32686-32695.

Boivin, B., Vaniotis, G., Allen, B.G. & Hebert, T.E. 2008, "G protein-coupled receptors in and on the cell nucleus: a new signaling paradigm?", *Journal of receptor and signal transduction research,* vol. 28, no. 1-2, pp. 15-28.

Bonini, J.A. & Hofmann, C. 1991, "A rapid, accurate, nonradioactive method for quantitating RNA on agarose gels", *BioTechniques,* vol. 11, no. 6, pp. 708-710.

Boots, C. & Stephenson, M.D. 2011, "Does obesity increase the risk of miscarriage in spontaneous conception: a systematic review", *Seminars in reproductive medicine,* vol. 29, no. 6, pp. 507-513.

Borner, C., Hollt, V. & Kraus, J. 2006, "Cannabinoid receptor type 2 agonists induce transcription of the mu-opioid receptor gene in Jurkat T cells.", *Molecular pharmacology,* vol. 69, no. 4, pp. 1486-1491.

Bouaboula, M., Poinot-Chazel, C., Bourrie, B., Canat, X., Calandra, B., Rinaldi-Carmona, M., Le Fur, G. & Casellas, P. 1995, "Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB1.", *Biochemical Journal,* vol. 312, no. Pt 2, pp. 637-641.

Bouaboula, M., Rinaldi, M., Carayon, P., Carillon, C., Delpech, B., Shire, D., Le Fur, G. & Casellas, P. 1993, "Cannabinoid-receptor expression in human leukocytes.", *European Journal of Biochemistry,* vol. 214, no. 1, pp. 173-180.

Bouloumie, A., Drexler, H.C., Lafontan, M. & Busse, R. 1998, "Leptin, the product of Ob gene, promotes angiogenesis.", *Circulation research,* vol. 83, no. 10, pp. 1059-1066.

Brache, V., Cochon, L., Jesam, C., Maldonado, R., Salvatierra, A.M., Levy, D.P., Gainer, E. & Croxatto, H.B. 2010, "Immediate pre-ovulatory administration of 30 mg ulipristal acetate significantly delays follicular rupture", *Human reproduction (Oxford, England),* vol. 25, no. 9, pp. 2256-2263.

Brewer, C.J. & Balen, A.H. 2010, "The adverse effects of obesity on conception and implantation", *Reproduction (Cambridge, England),* vol. 140, no. 3, pp. 347-364.

Brighton, P.J., McDonald, J., Taylor, A.H., Challiss, R.A., Lambert, D.G., Konje, J.C. & Willets, J.M. 2009, "Characterization of anandamide-stimulated cannabinoid receptor signaling in human ULTR myometrial smooth muscle cells.", *Molecular Endocrinology,* vol. 23, no. 9, pp. 1415-1427.

Bromer, J.G. & Patrizio, P. 2008, "Preservation and postponement of female fertility", *Placenta,* vol. 29, no. Suppl B, pp. 200-205.

Brown, A.J. 2007, "Novel cannabinoid receptors", *British journal of pharmacology,* vol. 152, no. 5, pp. 567-575.

Burrows, T.D., King, A. & Loke, Y.W. 1996, "Trophoblast migration during human placental implantation", *Human reproduction update,* vol. 2, no. 4, pp. 307-321.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J. & Wittwer, C.T. 2009, "The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments", *Clinical chemistry,* vol. 55, no. 4, pp. 611-622.

Bustin, S.A. & Nolan, T. 2004, "Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction", *Journal of biomolecular techniques : JBT,* vol. 15, no. 3, pp. 155-166.

Bygdeman, M. & Swahn, M.L. 1985, "Progesterone receptor blockage. Effect on uterine contractility and early pregnancy.", *Contraception,* vol. 32, no. 1, pp. 45-51.

Cadepond, F., Ulmann, A. & Baulieu, E.E. 1997, "RU486 (mifepristone): mechanisms of action and clinical uses", *Annual Review of Medicine,* vol. 48, pp. 129-156.

Cai, L.Q., Cao, Y.J. & Duan, E.K. 2000, "Effects of leukaemia inhibitory factor on embryo implantation in the mouse.", *Cytokine,* vol. 12, no. 11, pp. 1676-1682.

Caluwaerts, S., Vercruysse, L., Luyten, C. & Pijnenborg, R. 2005, "Endovascular trophoblast invasion and associated structural changes in uterine spiral arteries of the pregnant rat", *Placenta,* vol. 26, no. 7, pp. 574-584.

Campolongo, P., Trezza, V., Palmery, M., Trabace, L. & Cuomo, V. 2009, "Developmental exposure to cannabinoids causes subtle and enduring neurofunctional alterations", *International review of neurobiology,* vol. 85, pp. 117-133.

Castellucci, M., De Matteis, R., Meisser, A., Cancello, R., Monsurro, V., Islami, D., Sarzani, R., Marzioni, D., Cinti, S. & Bischof, P. 2000, "Leptin modulates extracellular matrix molecules and metalloproteinases: possible implications for trophoblast invasion", *Molecular human reproduction,* vol. 6, no. 10, pp. 951-958.

Caterina, M.J. 2003, "Vanilloid receptors take a TRP beyond the sensory afferent", *Pain,* vol. 105, no. 1-2, pp. 5-9.

Cavuoto, P. & Wittert, G.A. 2009, "The role of the endocannabinoid system in the regulation of energy expenditure", *Best Practice & Research Clinical Endocrinology & Metabolism,* vol. 23, no. 1, pp. 79-86.

Charnock-Jones, D.S., Sharkey, A.M., Fenwick, P. & Smith, S.K. 1994, "Leukaemia inhibitory factor mRNA concentration peaks in human endometrium at the time of implantation and the blastocyst contains mRNA for the receptor at this time.", *Journal of Reproduction & Fertility,* vol. 101, no. 2, pp. 421-426.

Chehab, F.F., Lim, M.E. & Lu, R. 1996, "Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin.", *Nature genetics,* vol. 12, no. 3, pp. 318-320.

Chen, J.R., Cheng, J.G., Shatzer, T., Sewell, L., Hernandez, L. & Stewart, C.L. 2000, "Leukemia inhibitory factor can substitute for nidatory estrogen and is

essential to inducing a receptive uterus for implantation but is not essential for subsequent embryogenesis.", *Endocrinology,* vol. 141, no. 12, pp. 4365-4372.

Cheng, L., Kelly, R.W., Thong, K.J., Hume, R. & Baird, D.T. 1993, "The effects of mifepristone (RU486) on prostaglandin dehydrogenase in decidual and chorionic tissue in early pregnancy", *Human reproduction (Oxford, England),* vol. 8, no. 5, pp. 705-709.

Christopoulou, F.D. & Kiortsis, D.N. 2011, "An overview of the metabolic effects of rimonabant in randomized controlled trials: potential for other cannabinoid 1 receptor blockers in obesity", *Journal of clinical pharmacy and therapeutics,* vol. 36, no. 1, pp. 10-18.

Chu, Z.L., Jones, R.M., He, H., Carroll, C., Gutierrez, V., Lucman, A., Moloney, M., Gao, H., Mondala, H., Bagnol, D., Unett, D., Liang, Y., Demarest, K., Semple, G., Behan, D.P. & Leonard, J. 2007, "A role for beta-cell-expressed G protein-coupled receptor 119 in glycemic control by enhancing glucose-dependent insulin release", *Endocrinology,* vol. 148, no. 6, pp. 2601-2609.

Clarke, I.J. & Henry, B.A. 1999, "Leptin and reproduction", *Reviews of reproduction,* vol. 4, no. 1, pp. 48-55.

Clement, K., Vaisse, C., Lahlou, N., Cabrol, S., Pelloux, V., Cassuto, D., Gourmelen, M., Dina, C., Chambaz, J., Lacorte, J.M., Basdevant, A., Bougneres, P., Lebouc, Y., Froguel, P. & Guy-Grand, B. 1998, "A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction.", *Nature,* vol. 392, no. 6674, pp. 398-401.

Conroy, S.M., Chai, W., Lim, U., Franke, A.A., Cooney, R.V. & Maskarinec, G. 2011, "Leptin, adiponectin, and obesity among Caucasian and Asian women", *Mediators of inflammation,* vol. 2011, pp. 253580.

Considine, R.V., Sinha, M.K., Heiman, M.L., Kriauciunas, A., Stephens, T.W., Nyce, M.R., Ohannesian, J.P., Marco, C.C., McKee, L.J. & Bauer, T.L. 1996, "Serum immunoreactive-leptin concentrations in normal-weight and obese humans.", *New England Journal of Medicine,* vol. 334, no. 5, pp. 292-295.

Contassot, E., Tenan, M., Schnuriger, V., Pelte, M.F. & Dietrich, P.Y. 2004, "Arachidonyl ethanolamide induces apoptosis of uterine cervix cancer cells via aberrantly expressed vanilloid receptor-1", *Gynecologic oncology,* vol. 93, no. 1, pp. 182-188.

Correa, F., Mestre, L., Docagne, F. & Guaza, C. 2005, "Activation of cannabinoid CB2 receptor negatively regulates IL-12p40 production in murine macrophages: role of IL-10 and ERK1/2 kinase signaling.", *British journal of pharmacology,* vol. 145, no. 4, pp. 441-448.

Creinin, M.D., Schlaff, W., Archer, D.F., Wan, L., Frezieres, R., Thomas, M., Rosenberg, M. & Higgins, J. 2006, "Progesterone receptor modulator for

emergency contraception: a randomized controlled trial.", *Obstetrics & Gynecology,* vol. 108, no. 5, pp. 1089-1097.

Cristino, L., de Petrocellis, L., Pryce, G., Baker, D., Guglielmotti, V. & Di Marzo, V. 2006, "Immunohistochemical localization of cannabinoid type 1 and vanilloid transient receptor potential vanilloid type 1 receptors in the mouse brain.", *Neuroscience,* vol. 139, no. 4, pp. 1405-1415.

Danielsson, K.G., Swahn, M.L. & Bygdeman, M. 1997, "The effect of various doses of mifepristone on endometrial leukaemia inhibitory factor expression in the midluteal phase--an immunohistochemical study.", *Human Reproduction,* vol. 12, no. 6, pp. 1293-1297.

Das, S.K., Paria, B.C., Chakraborty, I. & Dey, S.K. 1995, "Cannabinoid ligand-receptor signaling in the mouse uterus.", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 92, no. 10, pp. 4332-4336.

Davies, J. & Glasser, S.R. 1968, "Histological and fine structural observations on the placenta of the rat", *Acta Anatomica,* vol. 69, no. 4, pp. 542-608.

De Petrocellis, L., Cascio, M.G. & Di Marzo, V. 2004, "The endocannabinoid system: a general view and latest additions", *British journal of pharmacology,* vol. 141, no. 5, pp. 765-774.

Delage, G., Moreau, J.F., Taupin, J.L., Freitas, S., Hambartsoumian, E., Olivennes, F., Fanchin, R., Letur-Konirsch, H., Frydman, R. & Chaouat, G. 1995, "In-vitro endometrial secretion of human interleukin for DA cells/leukaemia inhibitory factor by explant cultures from fertile and infertile women.", *Human Reproduction,* vol. 10, no. 9, pp. 2483-2488.

Demuth, D.G. & Molleman, A. 2006, "Cannabinoid signalling", *Life Sciences,* vol. 78, no. 6, pp. 549-563.

Dennedy, M.C., Friel, A.M., Houlihan, D.D., Broderick, V.M., Smith, T. & Morrison, J.J. 2004, "Cannabinoids and the human uterus during pregnancy.", *American Journal of Obstetrics & Gynecology,* vol. 190, no. 1, pp. 2-9.

Department of Health 2009, *Abortion Statistics, England and Wales: 2008*. Available: http://www.dh.gov.uk/en/Publicationsandstatistics/Publications/PublicationsStatistics/DH_099285 [2009, 21/05].

Department of Health 2011, *Abortion Statistics, England and Wales*: 2010. Available: http://www.dh.gov.uk/en/Publicationsandstatistics/Publications/PublicationsStatistics/DH_126769

Deroo, B.J. & Korach, K.S. 2006, "Estrogen receptors and human disease", *The Journal of clinical investigation,* vol. 116, no. 3, pp. 561-570.

Devane, W.A., Dysarz, F.A.,3rd, Johnson, M.R., Melvin, L.S. & Howlett, A.C. 1988, "Determination and characterization of a cannabinoid receptor in rat brain.", *Molecular pharmacology,* vol. 34, no. 5, pp. 605-613.

Devane, W.A., Hanus, L., Breuer, A., Pertwee, R.G., Stevenson, L.A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A. & Mechoulam, R. 1992, "Isolation and structure of a brain constituent that binds to the cannabinoid receptor.", *Science,* vol. 258, no. 5090, pp. 1946-1949.

Dey, S.K., Lim, H., Das, S.K., Reese, J., Paria, B.C., Daikoku, T. & Wang, H. 2004, "Molecular cues to implantation", *Endocrine reviews,* vol. 25, no. 3, pp. 341-373.

Di Marzo, V. 1998, "'Endocannabinoids' and other fatty acid derivatives with cannabimimetic properties: biochemistry and possible physiopathological relevance", *Biochimica et biophysica acta,* vol. 1392, no. 2-3, pp. 153-175.

Di Marzo, V., Bisogno, T., De Petrocellis, L., Brandi, I., Jefferson, R.G., Winckler, R.L., Davis, J.B., Dasse, O., Mahadevan, A., Razdan, R.K. & Martin, B.R. 2001a, "Highly selective CB(1) cannabinoid receptor ligands and novel CB(1)/VR(1) vanilloid receptor "hybrid" ligands.", *Biochemical & Biophysical Research Communications,* vol. 281, no. 2, pp. 444-451.

Di Marzo, V., Goparaju, S.K., Wang, L., Liu, J., Batkai, S., Jarai, Z., Fezza, F., Miura, G.I., Palmiter, R.D., Sugiura, T. & Kunos, G. 2001b, "Leptin-regulated endocannabinoids are involved in maintaining food intake.", *Nature,* vol. 410, no. 6830, pp. 822-825.

Dimitriadis, E., White, C.A., Jones, R.L. & Salamonsen, L.A. 2005, "Cytokines, chemokines and growth factors in endometrium related to implantation", *Human reproduction update,* vol. 11, no. 6, pp. 613-630.

Dominguez, F., Remohi, J., Pellicer, A. & Simon, C. 2003, "Human endometrial receptivity: a genomic approach.", *Reproductive Biomedicine Online,* vol. 6, no. 3, pp. 332-338.

Dominguez, F., Yanez-Mo, M., Sanchez-Madrid, F. & Simon, C. 2005, "Embryonic implantation and leukocyte transendothelial migration: different processes with similar players?.", *FASEB Journal,* vol. 19, no. 9, pp. 1056-1060.

Dunglison, G.F., Barlow, D.H. & Sargent, I.L. 1996, "Leukaemia inhibitory factor significantly enhances the blastocyst formation rates of human embryos cultured in serum-free medium.", *Human Reproduction,* vol. 11, no. 1, pp. 191-196.

Dunn, C.L., Kelly, R.W. & Critchley, H.O. 2003, "Decidualization of the human endometrial stromal cell: an enigmatic transformation", *Reproductive Biomedicine Online,* vol. 7, no. 2, pp. 151-161.

el-Refaey, H., Rajasekar, D., Abdalla, M., Calder, L. & Templeton, A. 1995, "Induction of abortion with mifepristone (RU 486) and oral or vaginal misoprostol.", *New England Journal of Medicine,* vol. 332, no. 15, pp. 983-987.

El-Talatini, M.R., Taylor, A.H., Elson, J.C., Brown, L., Davidson, A.C. & Konje, J.C. 2009, "Localisation and function of the endocannabinoid system in the human ovary.", *PLoS ONE [Electronic Resource],* vol. 4, no. 2, pp. e4579.

El-Talatini, M.R., Taylor, A.H. & Konje, J.C. 2010, "The relationship between plasma levels of the endocannabinoid, anandamide, sex steroids, and gonadotrophins during the menstrual cycle.", *Fertility & Sterility,* vol. 93, no. 6, pp. 1989-1996.

El-Talatini, M.R., Taylor, A.H. & Konje, J.C. 2009, "Fluctuation in anandamide levels from ovulation to early pregnancy in in-vitro fertilization-embryo transfer women, and its hormonal regulation.", *Human Reproduction,* vol. 24, no. 8, pp. 1989-1998.

Engeli, S., Bohnke, J., Feldpausch, M., Gorzelniak, K., Janke, J., Batkai, S., Pacher, P., Harvey-White, J., Luft, F.C., Sharma, A.M. & Jordan, J. 2005, "Activation of the peripheral endocannabinoid system in human obesity.", *Diabetes,* vol. 54, no. 10, pp. 2838-2843.

Ernst, M., Inglese, M., Waring, P., Campbell, I.K., Bao, S., Clay, F.J., Alexander, W.S., Wicks, I.P., Tarlinton, D.M., Novak, U., Heath, J.K. & Dunn, A.R. 2001, "Defective gp130-mediated signal transducer and activator of transcription (STAT) signaling results in degenerative joint disease, gastrointestinal ulceration, and failure of uterine implantation.", *Journal of Experimental Medicine,* vol. 194, no. 2, pp. 189-203.

Favetta, P., Degoute, C.S., Perdrix, J.P., Dufresne, C., Boulieu, R. & Guitton, J. 2002, "Propofol metabolites in man following propofol induction and maintenance.", *British journal of anaesthesia,* vol. 88, no. 5, pp. 653-658.

Fedorcsak, P., Dale, P.O., Storeng, R., Ertzeid, G., Bjercke, S., Oldereid, N., Omland, A.K., Abyholm, T. & Tanbo, T. 2004, "Impact of overweight and underweight on assisted reproduction treatment.", *Human Reproduction,* vol. 19, no. 11, pp. 2523-2528.

Fedorcsak, P. & Storeng, R. 2003, "Effects of leptin and leukemia inhibitory factor on preimplantation development and STAT3 signaling of mouse embryos in vitro.", *Biology of reproduction,* vol. 69, no. 5, pp. 1531-1538.

Fedorcsak, P., Storeng, R., Dale, P.O., Tanbo, T. & Abyholm, T. 2000, "Obesity is a risk factor for early pregnancy loss after IVF or ICSI.", *Acta Obstetricia et Gynecologica Scandinavica,* vol. 79, no. 1, pp. 43-48.

Feinberg, E.C., Larsen, F.W., Catherino, W.H., Zhang, J. & Armstrong, A.Y. 2006, "Comparison of assisted reproductive technology utilization and

outcomes between Caucasian and African American patients in an equal-access-to-care setting.", *Fertility & Sterility,* vol. 85, no. 4, pp. 888-894.

Fergusson, D.M., Horwood, L.J., Northstone, K., ALSPAC Study, T. & Avon Longitudinal Study of Pregnancy and,Childhood 2002, "Maternal use of cannabis and pregnancy outcome.", *BJOG: An International Journal of Obstetrics & Gynaecology,* vol. 109, no. 1, pp. 21-27.

Fink, G. 1988, "Oestrogen and progesterone interactions in the control of gonadotrophin and prolactin secretion", *Journal of steroid biochemistry,* vol. 30, no. 1-6, pp. 169-178.

Finn, D.P. 2010, "Endocannabinoid-mediated modulation of stress responses: physiological and pathophysiological significance", *Immunobiology,* vol. 215, no. 8, pp. 629-646.

Fioramonti, J. & Bueno, L. 2008, "Role of cannabinoid receptors in the control of gastrointestinal motility and perception", *Expert review of gastroenterology & hepatology,* vol. 2, no. 3, pp. 385-397.

Fitzgerald, J.S., Busch, S., Wengenmayer, T., Foerster, K., de la Motte, T., Poehlmann, T.G. & Markert, U.R. 2005, "Signal transduction in trophoblast invasion", *Chemical Immunology & Allergy,* vol. 88, pp. 181-199.

Fitzgerald, J.S., Poehlmann, T.G., Schleussner, E. & Markert, U.R. 2008, "Trophoblast invasion: the role of intracellular cytokine signalling via signal transducer and activator of transcription 3 (STAT3)", *Human reproduction update,* vol. 14, no. 4, pp. 335-344.

Fitzpatrick, R., Casey, O.M., Morris, D., Smith, T., Powell, R. & Sreenan, J.M. 2002, "Postmortem stability of RNA isolated from bovine reproductive tissues", *Biochimica et biophysica acta,* vol. 1574, no. 1, pp. 10-14.

Fleige, S., Walf, V., Huch, S., Prgomet, C., Sehm, J. & Pfaffl, M.W. 2006, "Comparison of relative mRNA quantification models and the impact of RNA integrity in quantitative real-time RT-PCR", *Biotechnology Letters,* vol. 28, no. 19, pp. 1601-1613.

Fonseca, B.M., Correia-da-Silva, G., Taylor, A.H., Konje, J.C., Bell, S.C. & Teixeira, N.A. 2009, "Spatio-temporal expression patterns of anandamide-binding receptors in rat implantation sites: evidence for a role of the endocannabinoid system during the period of placental development.", *Reproductive Biology & Endocrinology,* vol. 7, pp. 121.

Fonseca, B.M., Correia-da-Silva, G., Taylor, A.H., Lam, P.M., Marczylo, T.H., Konje, J.C., Bell, S.C. & Teixeira, N.A. 2010, "N-Acylethanolamine Levels and Expression of Their Metabolizing Enzymes during Pregnancy", *Endocrinology*, vol. 151, pp. 3965-3974.

Fonseca, B.M., Correia-da-Silva, G., Taylor, A.H., Lam, P.M., Marczylo, T.H., Konje, J.C. & Teixeira, N.A. 2012, "Characterisation of the endocannabinoid system in rat haemochorial placenta", *Reproductive toxicology (Elmsford, N.Y.),* vol. 34, no. 3, pp. 347-356.

Fonseca, B.M., Correia-da-Silva, G. & Teixeira, N.A. 2009, "Anandamide-induced cell death: dual effects in primary rat decidual cell cultures.", *Placenta,* vol. 30, no. 8, pp. 686-692.

Fonseca, B.M., Correia-da-Silva, G.& Teizeira, N.A. 2012, "The rat as an animal model for fetoplacental development: a reappraisal of the postimplantation period.", *Reproductive Biology*, vol. 12, no.2, pp 97-118.

Fowler, C.J. 2004, "Possible involvement of the endocannabinoid system in the actions of three clinically used drugs", *Trends in pharmacological sciences,* vol. 25, no. 2, pp. 59-61.

Fride, E. 2002, "Endocannabinoids in the central nervous system--an overview", *Prostaglandins Leukotrienes & Essential Fatty Acids,* vol. 66, no. 2-3, pp. 221-233.

Friedman, J.M. & Halaas, J.L. 1998, "Leptin and the regulation of body weight in mammals", *Nature,* vol. 395, no. 6704, pp. 763-770.

Gaillard, R.C., Riondel, A., Muller, A.F., Herrmann, W. & Baulieu, E.E. 1984, "RU 486: a steroid with antiglucocorticosteroid activity that only disinhibits the human pituitary-adrenal system at a specific time of day", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 81, no. 12, pp. 3879-3882.

Gambino, Y.P., Maymo, J.L., Perez-Perez, A., Duenas, J.L., Sanchez-Margalet, V., Calvo, J.C. & Varone, C.L. 2010, "17Beta-estradiol enhances leptin expression in human placental cells through genomic and nongenomic actions", *Biology of reproduction,* vol. 83, no. 1, pp. 42-51.

Garfield, R.E., Blennerhassett, M.G. & Miller, S.M. 1988, "Control of myometrial contractility: role and regulation of gap junctions", *Oxford reviews of reproductive biology,* vol. 10, pp. 436-490.

Gavva, N.R. 2008, "Body-temperature maintenance as the predominant function of the vanilloid receptor TRPV1", *Trends in pharmacological sciences,* vol. 29, no. 11, pp. 550-557.

Gemzell-Danielsson, K. 2010, "Mechanism of action of emergency contraception", *Contraception,* vol. 82, no. 5, pp. 404-409.

Gemzell-Danielsson, K., Bygdeman, M. & Aronsson, A. 2006, "Studies on uterine contractility following mifepristone and various routes of misoprostol", *Contraception,* vol. 74, no. 1, pp. 31-35.

Giang, D.K. & Cravatt, B.F. 1997, "Molecular characterization of human and mouse fatty acid amide hydrolases", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 94, no. 6, pp. 2238-2242.

Giuffrida, A., Rodriguez de Fonseca, F., Nava, F., Loubet-Lescoulie, P. & Piomelli, D. 2000, "Elevated circulating levels of anandamide after administration of the transport inhibitor, AM404.", *European journal of pharmacology,* vol. 408, no. 2, pp. 161-168.

Glaser, S.T., Abumrad, N.A., Fatade, F., Kaczocha, M., Studholme, K.M. & Deutsch, D.G. 2003, "Evidence against the presence of an anandamide transporter.", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 100, no. 7, pp. 4269-4274.

Glasier, A. 2013, "Emergency contraception: clinical outcomes.", *Contraception*, vol.87, pp. 309-313.

Gobeil, F., Fortier, A., Zhu, T., Bossolasco, M., Leduc, M., Grandbois, M., Heveker, N., Bkaily, G., Chemtob, S. & Barbaz, D. 2006, "G-protein-coupled receptors signalling at the cell nucleus: an emerging paradigm", *Canadian journal of physiology and pharmacology,* vol. 84, no. 3-4, pp. 287-297.

Godlewski, G., Offertaler, L., Wagner, J.A. & Kunos, G. 2009, "Receptors for acylethanolamides-GPR55 and GPR119", *Prostaglandins & other lipid mediators,* vol. 89, no. 3-4, pp. 105-111.

Goetzl, E.J. 2007, "Diverse pathways for nuclear signaling by G protein-coupled receptors and their ligands", *FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* vol. 21, no. 3, pp. 638-642.

Gonzalez, R.R., Caballero-Campo, P., Jasper, M., Mercader, A., Devoto, L., Pellicer, A. & Simon, C. 2000, "Leptin and leptin receptor are expressed in the human endometrium and endometrial leptin secretion is regulated by the human blastocyst.", *Journal of Clinical Endocrinology & Metabolism,* vol. 85, no. 12, pp. 4883-4888.

Gonzalez, R.R., Rueda, B.R., Ramos, M.P., Littell, R.D., Glasser, S. & Leavis, P.C. 2004, "Leptin-induced increase in leukemia inhibitory factor and its receptor by human endometrium is partially mediated by interleukin 1 receptor signaling.", *Endocrinology,* vol. 145, no. 8, pp. 3850-3857.

Gorzalka, B.B. & Dang, S.S. 2012, "Minireview: Endocannabinoids and gonadal hormones: bidirectional interactions in physiology and behavior", *Endocrinology,* vol. 153, no. 3, pp. 1016-1024.

Graham, C.H. & Lala, P.K. 1992, "Mechanisms of placental invasion of the uterus and their control", *Biochemistry & Cell Biology,* vol. 70, no. 10-11, pp. 867-874.

Graham, J.D. & Clarke, C.L. 1997, "Physiological action of progesterone in target tissues", *Endocrine reviews,* vol. 18, no. 4, pp. 502-519.

Grimsey, N.L., Goodfellow, C.E., Scotter, E.L., Dowie, M.J., Glass, M. & Graham, E.S. 2008, "Specific detection of CB1 receptors; cannabinoid CB1 receptor antibodies are not all created equal!", *Journal of neuroscience methods,* vol. 171, no. 1, pp. 78-86.

Gu, Y., Jow, G.M., Moulton, B.C., Lee, C., Sensibar, J.A., Park-Sarge, O.K., Chen, T.J. & Gibori, G. 1994, "Apoptosis in decidual tissue regression and reorganization", *Endocrinology,* vol. 135, no. 3, pp. 1272-1279.

Guo, Y., Wang, H., Okamoto, Y., Ueda, N., Kingsley, P.J., Marnett, L.J., Schmid, H.H., Das, S.K. & Dey, S.K. 2005, "N-acylphosphatidylethanolamine-hydrolyzing phospholipase D is an important determinant of uterine anandamide levels during implantation.", *Journal of Biological Chemistry,* vol. 280, no. 25, pp. 23429-23432.

Gutsche, S., von Wolff, M., Strowitzki, T. & Thaler, C.J. 2003, "Seminal plasma induces mRNA expression of IL-1beta, IL-6 and LIF in endometrial epithelial cells in vitro.", *Molecular human reproduction,* vol. 9, no. 12, pp. 785-791.

Guzeloglu-Kayisli, O., Kayisli, U.A. & Taylor, H.S. 2009, "The role of growth factors and cytokines during implantation: endocrine and paracrine interactions", *Seminars in reproductive medicine,* vol. 27, no. 1, pp. 62-79.

Gygi, S.P., Rochon, Y., Franza, B.R. & Aebersold, R. 1999, "Correlation between protein and mRNA abundance in yeast", *Molecular and cellular biology,* vol. 19, no. 3, pp. 1720-1730.

Habayeb, O.M., Taylor, A.H., Bell, S.C., Taylor, D.J. & Konje, J.C. 2008, "Expression of the endocannabinoid system in human first trimester placenta and its role in trophoblast proliferation.", *Endocrinology,* vol. 149, no. 10, pp. 5052-5060.

Habayeb, O.M., Taylor, A.H., Evans, M.D., Cooke, M.S., Taylor, D.J., Bell, S.C. & Konje, J.C. 2004, "Plasma levels of the endocannabinoid anandamide in women--a potential role in pregnancy maintenance and labor?.", *Journal of Clinical Endocrinology & Metabolism,* vol. 89, no. 11, pp. 5482-5487.

Habayeb, O.M., Taylor, A.H., Finney, M., Evans, M.D. & Konje, J.C. 2008, "Plasma anandamide concentration and pregnancy outcome in women with threatened miscarriage.", *JAMA,* vol. 299, no. 10, pp. 1135-1136.

Haeseler, G., Karst, M., Foadi, N., Gudehus, S., Roeder, A., Hecker, H., Dengler, R. & Leuwer, M. 2008, "High-affinity blockade of voltage-operated skeletal muscle and neuronal sodium channels by halogenated propofol analogues.", *British journal of pharmacology,* vol. 155, no. 2, pp. 265-275.

Hamoda, H., Ashok, P.W., Stalder, C., Flett, G.M., Kennedy, E. & Templeton, A. 2004, "A randomized trial of mifepristone (10 mg) and levonorgestrel for emergency contraception", *Obstetrics and gynecology,* vol. 104, no. 6, pp. 1307-1313.

Hamoda, H. & Templeton, A. 2010, "Medical and surgical options for induced abortion in first trimester", *Best practice & research.Clinical obstetrics & gynaecology,* vol. 24, no. 4, pp. 503-516.

Hansen, H.S. & Diep, T.A. 2009, "N-acylethanolamines, anandamide and food intake", *Biochemical pharmacology,* vol. 78, no. 6, pp. 553-560.

Harden, A., Brunton, G., Fletcher, A. & Oakley, A. 2009, "Teenage pregnancy and social disadvantage: systematic review integrating controlled trials and qualitative studies", *BMJ (Clinical research ed.),* vol. 339, pp. b4254.

Hardie, L., Trayhurn, P., Abramovich, D. & Fowler, P. 1997, "Circulating leptin in women: a longitudinal study in the menstrual cycle and during pregnancy.", *Clinical endocrinology,* vol. 47, no. 1, pp. 101-106.

Harper, M.J. 1992, "The implantation window", *Bailliere's Clinical Obstetrics and Gynaecology,* vol. 6, no. 2, pp. 351-371.

Hatch, E.E. & Bracken, M.B. 1986, "Effect of marijuana use in pregnancy on fetal growth.", *American Journal of Epidemiology,* vol. 124, no. 6, pp. 986-993.

Hauguel-de Mouzon, S., Lepercq, J. & Catalano, P. 2006, "The known and unknown of leptin in pregnancy", *American Journal of Obstetrics and Gynecology,* vol. 194, no. 6, pp. 1537-1545.

He, C.H., Shi, Y.E., Ye, Z.H., Zhang, G.G., Jiang, N.X., Van Look, P.F. & Fotherby, K. 1989, "Pharmacokinetic study of orally administered RU 486 in non-pregnant women", *Contraception,* vol. 40, no. 4, pp. 449-460.

Heid, C.A., Stevens, J., Livak, K.J. & Williams, P.M. 1996, "Real time quantitative PCR", *Genome research,* vol. 6, no. 10, pp. 986-994.

Heikinheimo, O. 1989, "Pharmacokinetics of the antiprogesterone RU 486 in women during multiple dose administration", *Journal of steroid biochemistry,* vol. 32, no. 1A, pp. 21-25.

Heikinheimo, O., Kontula, K., Croxatto, H., Spitz, I., Luukkainen, T. & Lahteenmaki, P. 1987a, "Plasma concentrations and receptor binding of RU 486 and its metabolites in humans.", *Journal of steroid biochemistry,* vol. 26, no. 2, pp. 279-284.

Heikinheimo, O., Lahteenmaki, P.L., Koivunen, E., Shoupe, D., Croxatto, H., Luukkainen, T. & Lahteenmaki, P. 1987b, "Metabolism and serum binding of RU 486 in women after various single doses.", *Human Reproduction,* vol. 2, no. 5, pp. 379-385.

Heinrich, P.C., Behrmann, I., Haan, S., Hermanns, H.M., Muller-Newen, G. & Schaper, F. 2003, "Principles of interleukin (IL)-6-type cytokine signalling and its regulation", *Biochemical Journal,* vol. 374, no. Pt 1, pp. 1-20.

Helliwell, R.J., Chamley, L.W., Blake-Palmer, K., Mitchell, M.D., Wu, J., Kearn, C.S. & Glass, M. 2004, "Characterization of the endocannabinoid system in early human pregnancy.", *Journal of Clinical Endocrinology & Metabolism,* vol. 89, no. 10, pp. 5168-5174.

Henshaw, S.K., Singh, S. & Haas, T. 1999, "The incidence of abortion worldwide.", *International Family Planning Perspectives & Digest,* vol. 25, no. Suppl, pp. S30-8.

Henson, M.C. & Castracane, V.D. 2006, "Leptin in pregnancy: an update", *Biology of reproduction,* vol. 74, no. 2, pp. 218-229.

Henson, M.C. & Castracane, V.D. 2000, "Leptin in pregnancy", *Biology of reproduction,* vol. 63, no. 5, pp. 1219-1228.

Henstridge, C.M., Balenga, N.A., Ford, L.A., Ross, R.A., Waldhoer, M. & Irving, A.J. 2009, "The GPR55 ligand L-alpha-lysophosphatidylinositol promotes RhoA-dependent Ca2+ signaling and NFAT activation.", *FASEB Journal,* vol. 23, no. 1, pp. 183-193.

Hickey, M.S., Israel, R.G., Gardiner, S.N., Considine, R.V., McCammon, M.R., Tyndall, G.L., Houmard, J.A., Marks, R.H. & Caro, J.F. 1996, "Gender differences in serum leptin levels in humans.", *Biochemical & Molecular Medicine,* vol. 59, no. 1, pp. 1-6.

Highman, T.J., Friedman, J.E., Huston, L.P., Wong, W.W. & Catalano, P.M. 1998, "Longitudinal changes in maternal serum leptin concentrations, body composition, and resting metabolic rate in pregnancy", *American Journal of Obstetrics and Gynecology,* vol. 178, no. 5, pp. 1010-1015.

Hill, M.N. & McEwen, B.S. 2010, "Involvement of the endocannabinoid system in the neurobehavioural effects of stress and glucocorticoids", *Progress in neuro-psychopharmacology & biological psychiatry,* vol. 34, no. 5, pp. 791-797.

Hill, M.N., McLaughlin, R.J., Viau, V., Gorzalka, B.B. & Hillard, C.J. 2007, "Stress-induced alterations in limbic endocannabinoid content in the rat: correlations to HPA axis activation", *17th Annual Symposium of the International Cannabinoid Research Society,* vol. 23.

Hill, M.N., Miller, G.E., Carrier, E.J., Gorzalka, B.B. & Hillard, C.J. 2009, "Circulating endocannabinoids and N-acyl ethanolamines are differentially regulated in major depression and following exposure to social stress.", *Psychoneuroendocrinology,* vol. 34, no. 8, pp. 1257-1262.

Hill, N.C., Rivera, J., Lopez Bernal, A. & MacKenzie, I.Z. 1990, "The effect of RU 38,486 on progesterone and oestrogen receptor concentrations in the decidua

and placenta in early pregnancy", *Human reproduction (Oxford, England),* vol. 5, no. 4, pp. 464-467.

Hillard, C.J., Edgemond, W.S., Jarrahian, A. & Campbell, W.B. 1997, "Accumulation of N-arachidonoylethanolamine (anandamide) into cerebellar granule cells occurs via facilitated diffusion.", *Journal of neurochemistry,* vol. 69, no. 2, pp. 631-638.

Hillard, C.J. & Jarrahian, A. 2000, "The movement of N-arachidonoylethanolamine (anandamide) across cellular membranes", *Chemistry & Physics of Lipids,* vol. 108, no. 1-2, pp. 123-134.

Hilton, D.J. 1992, "LIF: lots of interesting functions", *Trends in biochemical sciences,* vol. 17, no. 2, pp. 72-76.

Hohmann, A.G., Suplita, R.L., Bolton, N.M., Neely, M.H., Fegley, D., Mangieri, R., Krey, J.F., Walker, J.M., Holmes, P.V., Crystal, J.D., Duranti, A., Tontini, A., Mor, M., Tarzia, G. & Piomelli, D. 2005, "An endocannabinoid mechanism for stress-induced analgesia.", *Nature,* vol. 435, no. 7045, pp. 1108-1112.

Honkanen, H., Ranta, S., Ylikorkala, O. & Heikinheimo, O. 2005, "Effect of antiprogesterone mifepristone followed by misoprostol on circulating leptin in early pregnancy", *Acta Obstetricia et Gynecologica Scandinavica,* vol. 84, no. 2, pp. 134-139.

Hoozemans, D.A., Schats, R., Lambalk, C.B., Homburg, R. & Hompes, P.G. 2004, "Human embryo implantation: current knowledge and clinical implications in assisted reproductive technology", *Reproductive biomedicine online,* vol. 9, no. 6, pp. 692-715.

Horne, A.W., Phillips, J.A.,3rd, Kane, N., Lourenco, P.C., McDonald, S.E., Williams, A.R., Simon, C., Dey, S.K. & Critchley, H.O. 2008, "CB1 expression is attenuated in Fallopian tube and decidua of women with ectopic pregnancy.", *PLoS ONE [Electronic Resource],* vol. 3, no. 12, pp. e3969.

Howlett, A.C., Barth, F., Bonner, T.I., Cabral, G., Casellas, P., Devane, W.A., Felder, C.C., Herkenham, M., Mackie, K., Martin, B.R., Mechoulam, R. & Pertwee, R.G. 2002, "International Union of Pharmacology. XXVII. Classification of cannabinoid receptors", *Pharmacological reviews,* vol. 54, no. 2, pp. 161-202.

Howlett, A.C., Breivogel, C.S., Childers, S.R., Deadwyler, S.A., Hampson, R.E. & Porrino, L.J. 2004, "Cannabinoid physiology and pharmacology: 30 years of progress", *Neuropharmacology,* vol. 47, no. Suppl 1, pp. 345-358.

Human Fertilisation and Embryology Act 1990. London: HMSO;1990. [http://www.legislation.gov.uk/ukpga/1990/37/contents]

Huizink, A.C. 2009, "Moderate use of alcohol, tobacco and cannabis during pregnancy: new approaches and update on research findings", *Reproductive Toxicology,* vol. 28, no. 2, pp. 143-151.

Huizink, A.C. & Mulder, E.J. 2006, "Maternal smoking, drinking or cannabis use during pregnancy and neurobehavioral and cognitive functioning in human offspring", *Neuroscience & Biobehavioral Reviews,* vol. 30, no. 1, pp. 24-41.

Ikawa, M., Inoue, N., Benham, A.M. & Okabe, M. 2010, "Fertilization: a sperm's journey to and interaction with the oocyte", *The Journal of clinical investigation,* vol. 120, no. 4, pp. 984-994.

Jarzimski, C., Karst, M., Zoerner, A.A., Rakers, C., May, M., Suchy, M.T., Tsikas, D., Krauss, J.K., Scheinichen, D., Jordan, J. & Engeli, S. 2012, "Changes of blood endocannabinoids during anaesthesia: a special case for fatty acid amide hydrolase inhibition by propofol?", *British journal of clinical pharmacology,* vol. 74, no. 1, pp. 54-59.

Jiang, X., Benovic, J.L. & Wedegaertner, P.B. 2007, "Plasma membrane and nuclear localization of G protein coupled receptor kinase 6A", *Molecular biology of the cell,* vol. 18, no. 8, pp. 2960-2969.

Joosten, M.M., Balvers, M.G., Verhoeckx, K.C., Hendriks, H.F. & Witkamp, R.F. 2010, "Plasma anandamide and other N-acylethanolamines are correlated with their corresponding free fatty acid levels under both fasting and non-fasting conditions in women", *Nutrition & metabolism,* vol. 7, pp. 49.

Jutras-Aswad, D., DiNieri, J.A., Harkany, T. & Hurd, Y.L. 2009, "Neurobiological consequences of maternal cannabis on human fetal development and its neuropsychiatric outcome", *European Archives of Psychiatry & Clinical Neuroscience,* vol. 259, no. 7, pp. 395-412.

Karasu, T., Marczylo, T.H., Maccarrone, M. & Konje, J.C. 2011, "The role of sex steroid hormones, cytokines and the endocannabinoid system in female fertility", *Human reproduction update,* vol. 17, no. 3, pp. 347-361.

Kathuria, S., Gaetani, S., Fegley, D., Valino, F., Duranti, A., Tontini, A., Mor, M., Tarzia, G., La Rana, G., Calignano, A., Giustino, A., Tattoli, M., Palmery, M., Cuomo, V. & Piomelli, D. 2003, "Modulation of anxiety through blockade of anandamide hydrolysis.", *Nature medicine,* vol. 9, no. 1, pp. 76-81.

Kawamura, K., Sato, N., Fukuda, J., Kodama, H., Kumagai, J., Tanikawa, H., Nakamura, A. & Tanaka, T. 2002, "Leptin promotes the development of mouse preimplantation embryos in vitro.", *Endocrinology,* vol. 143, no. 5, pp. 1922-1931.

Keltz, M.D., Attar, E., Buradagunta, S., Olive, D.L., Kliman, H.J. & Arici, A. 1996, "Modulation of leukemia inhibitory factor gene expression and protein biosynthesis in the human fallopian tube.", *American Journal of Obstetrics & Gynecology,* vol. 175, no. 6, pp. 1611-1619.

Kiess, W., Siebler, T., Englaro, P., Kratzsch, J., Deutscher, J., Meyer, K., Gallaher, B. & Blum, W.F. 1998, "Leptin as a metabolic regulator during fetal and neonatal life and in childhood and adolescence.", *Journal of Pediatric Endocrinology,* vol. 11, no. 4, pp. 483-496.

Kirkham, T.C., Williams, C.M., Fezza, F. & Di Marzo, V. 2002, "Endocannabinoid levels in rat limbic forebrain and hypothalamus in relation to fasting, feeding and satiation: stimulation of eating by 2-arachidonoyl glycerol.", *British journal of pharmacology,* vol. 136, no. 4, pp. 550-557.

Klonoff-Cohen, H.S., Natarajan, L. & Chen, R.V. 2006, "A prospective study of the effects of female and male marijuana use on in vitro fertilization (IVF) and gamete intrafallopian transfer (GIFT) outcomes", *American Journal of Obstetrics and Gynecology,* vol. 194, no. 2, pp. 369-376.

Kojima, K., Kanzaki, H., Iwai, M., Hatayama, H., Fujimoto, M., Inoue, T., Horie, K., Nakayama, H., Fujita, J. & Mori, T. 1994, "Expression of leukemia inhibitory factor in human endometrium and placenta.", *Biology of reproduction,* vol. 50, no. 4, pp. 882-887.

Kozak, K.R., Crews, B.C., Morrow, J.D., Wang, L.H., Ma, Y.H., Weinander, R., Jakobsson, P.J. & Marnett, L.J. 2002, "Metabolism of the endocannabinoids, 2-arachidonylglycerol and anandamide, into prostaglandin, thromboxane, and prostacyclin glycerol esters and ethanolamides.", *Journal of Biological Chemistry,* vol. 277, no. 47, pp. 44877-44885.

Krasowski, M.D., Jenkins, A., Flood, P., Kung, A.Y., Hopfinger, A.J. & Harrison, N.L. 2001, "General anesthetic potencies of a series of propofol analogs correlate with potency for potentiation of gamma-aminobutyric acid (GABA) current at the GABA(A) receptor but not with lipid solubility.", *Journal of Pharmacology & Experimental Therapeutics,* vol. 297, no. 1, pp. 338-351.

Krause, B.J., Hanson, M.A. & Casanello, P. 2011, "Role of nitric oxide in placental vascular development and function", *Placenta,* vol. 32, no. 11, pp. 797-805.

Kulier, R., Kapp, N., Gulmezoglu, A.M., Hofmeyr, G.J., Cheng, L. & Campana, A. 2011, "Medical methods for first trimester abortion", *Cochrane database of systematic reviews (Online),* vol. (11), no. 11, pp. CD002855.

Lage, M., Garcia-Mayor, R.V., Tome, M.A., Cordido, F., Valle-Inclan, F., Considine, R.V., Caro, J.F., Dieguez, C. & Casanueva, F.F. 1999, "Serum leptin levels in women throughout pregnancy and the postpartum period and in women suffering spontaneous abortion.", *Clinical endocrinology,* vol. 50, no. 2, pp. 211-216.

Lahteenmaki, P., Heikinheimo, O., Croxatto, H., Spitz, I., Shoupe, D., Birgerson, L. & Luukkainen, T. 1987, "Pharmacokinetics and metabolism of RU 486.", *Journal of steroid biochemistry,* vol. 27, no. 4-6, pp. 859-863.

Laird, S.M., Tuckerman, E.M., Dalton, C.F., Dunphy, B.C., Li, T.C. & Zhang, X. 1997, "The production of leukaemia inhibitory factor by human endometrium: presence in uterine flushings and production by cells in culture.", *Human Reproduction,* vol. 12, no. 3, pp. 569-574.

Lam, P.M., Marczylo, T.H., El-Talatini, M., Finney, M., Nallendran, V., Taylor, A.H. & Konje, J.C. 2008, "Ultra performance liquid chromatography tandem mass spectrometry method for the measurement of anandamide in human plasma.", *Analytical Biochemistry,* vol. 380, no. 2, pp. 195-201.

Lambert, D.M. & Muccioli, G.G. 2007, "Endocannabinoids and related N-acylethanolamines in the control of appetite and energy metabolism: emergence of new molecular players", *Current Opinion in Clinical Nutrition & Metabolic Care,* vol. 10, no. 6, pp. 735-744.

Lanoix, D., Lacasse, A.A., St-Pierre, J., Taylor, S.C., Ethier-Chiasson, M., Lafond, J. & Vaillancourt, C. 2012, "Quantitative PCR Pitfalls: The Case of the Human Placenta", *Molecular biotechnology, .*

Lauffer, L.M., Iakoubov, R. & Brubaker, P.L. 2009, "GPR119 is essential for oleoylethanolamide-induced glucagon-like peptide-1 secretion from the intestinal enteroendocrine L-cell.", *Diabetes,* vol. 58, no. 5, pp. 1058-1066.

Lavranos, T.C., Rathjen, P.D. & Seamark, R.F. 1995, "Trophic effects of myeloid leukaemia inhibitory factor (LIF) on mouse embryos.", *Journal of Reproduction & Fertility,* vol. 105, no. 2, pp. 331-338.

Lazzarin, N., Valensise, H., Bari, M., Ubaldi, F., Battista, N., Finazzi-Agro, A. & Maccarrone, M. 2004, "Fluctuations of fatty acid amide hydrolase and anandamide levels during the human ovulatory cycle.", *Gynecological Endocrinology,* vol. 18, no. 4, pp. 212-218.

Le Pecq, J.B. & Paoletti, C. 1966, "A new fluorometric method for RNA and DNA determination", *Analytical Biochemistry,* vol. 17, no. 1, pp. 100-107.

Ledee-Bataille, N., Lapree-Delage, G., Taupin, J.L., Dubanchet, S., Frydman, R. & Chaouat, G. 2002, "Concentration of leukaemia inhibitory factor (LIF) in uterine flushing fluid is highly predictive of embryo implantation.", *Human Reproduction,* vol. 17, no. 1, pp. 213-218.

Leite, C.E., Mocelin, C.A., Petersen, G.O., Leal, M.B. & Thiesen, F.V. 2009, "Rimonabant: an antagonist drug of the endocannabinoid system for the treatment of obesity", *Pharmacological Reports: PR,* vol. 61, no. 2, pp. 217-224.

Li, H.Z., Sun, X., Stavreus-Evers, A. & Gemzell-Danielsson, K. 2004, "Effect of mifepristone on the expression of cytokines in the human Fallopian tube.", *Molecular human reproduction,* vol. 10, no. 7, pp. 489-493.

Lilja, M., Rolandsson, O., Shaw, J.E., Pauvaday, V., Cameron, A.J., Tuomilehto, J., Alberti, K.G., Zimmet, P.Z. & Soderberg, S. 2010, "Higher leptin levels in Asian Indians than Creoles and Europids: a potential explanation for increased metabolic risk", *International journal of obesity (2005),* vol. 34, no. 5, pp. 878-885.

Lim, H., Paria, B.C., Das, S.K., Dinchuk, J.E., Langenbach, R., Trzaskos, J.M. & Dey, S.K. 1997, "Multiple female reproductive failures in cyclooxygenase 2-deficient mice.", *Cell,* vol. 91, no. 2, pp. 197-208.

Linne, Y. 2004, "Effects of obesity on women's reproduction and complications during pregnancy", *Obesity Reviews,* vol. 5, no. 3, pp. 137-143.

Lips, K.S., Bruggmann, D., Pfeil, U., Vollerthun, R., Grando, S.A. & Kummer, W. 2005, "Nicotinic acetylcholine receptors in rat and human placenta", *Placenta,* vol. 26, no. 10, pp. 735-746.

Liu, J., Wang, L., Harvey-White, J., Osei-Hyiaman, D., Razdan, R., Gong, Q., Chan, A.C., Zhou, Z., Huang, B.X., Kim, H.Y. & Kunos, G. 2006, "A biosynthetic pathway for anandamide.", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 103, no. 36, pp. 13345-13350.

Livak, K.J. & Schmittgen, T.D. 2001, "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method", *Methods (San Diego, Calif.),* vol. 25, no. 4, pp. 402-408.

Lutfalla, G. & Uze, G. 2006, "Performing quantitative reverse-transcribed polymerase chain reaction experiments", *Methods in enzymology,* vol. 410, pp. 386-400.

Maccarrone, M. 2008, "CB2 receptors in reproduction", *British journal of pharmacology,* vol. 153, no. 2, pp. 189-198.

Maccarrone, M., Bari, M., Battista, N. & Finazzi-Agrò, A. 2002, "Estrogen stimulates arachidonoylethanolamide release from human endothelial cells and platelet activation.", *Blood,* vol. 100, no. 12, pp. 4040-4048.

Maccarrone, M., Bari, M., Di Rienzo, M., Finazzi-Agrò, A. & Rossi, A. 2003, "Progesterone activates fatty acid amide hydrolase (FAAH) promoter in human T lymphocytes through the transcription factor Ikaros. Evidence for a synergistic effect of leptin.", *Journal of Biological Chemistry,* vol. 278, no. 35, pp. 32726-32732.

Maccarrone, M., Bisogno, T., Valensise, H., Lazzarin, N., Fezza, F., Manna, C., Di Marzo, V. & Finazzi-Agrò, A. 2002, "Low fatty acid amide hydrolase and high anandamide levels are associated with failure to achieve an ongoing pregnancy after IVF and embryo transfer.", *Molecular human reproduction,* vol. 8, no. 2, pp. 188-195.

Maccarrone, M., De Felici, M., Bari, M., Klinger, F., Siracusa, G. & Finazzi-Agrò, A. 2000, "Down-regulation of anandamide hydrolase in mouse uterus by sex hormones.", *European Journal of Biochemistry,* vol. 267, no. 10, pp. 2991-2997.

Maccarrone, M., DeFelici, M., Klinger, F.G., Battista, N., Fezza, F., Dainese, E., Siracusa, G. & Finazzi-Agrò, A. 2004, "Mouse blastocysts release a lipid which activates anandamide hydrolase in intact uterus.", *Molecular human reproduction,* vol. 10, no. 4, pp. 215-221.

Maccarrone, M., Di Rienzo, M., Finazzi-Agrò, A. & Rossi, A. 2003, "Leptin activates the anandamide hydrolase promoter in human T lymphocytes through STAT3.", *Journal of Biological Chemistry,* vol. 278, no. 15, pp. 13318-13324.

Maccarrone, M., Falciglia, K., Di Rienzo, M. & Finazzi-Agrò, A. 2002, "Endocannabinoids, hormone-cytokine networks and human fertility", *Prostaglandins Leukotrienes & Essential Fatty Acids,* vol. 66, no. 2-3, pp. 309-317.

Maccarrone, M. & Finazzi-Agro, A. 2003, "The endocannabinoid system, anandamide and the regulation of mammalian cell apoptosis", *Cell Death & Differentiation,* vol. 10, no. 9, pp. 946-955.

Maccarrone, M. & Finazzi-Agrò, A. 2004, "Anandamide hydrolase: a guardian angel of human reproduction?", *Trends in pharmacological sciences,* vol. 25, no. 7, pp. 353-357.

Maccarrone, M., Fride, E., Bisogno, T., Bari, M., Cascio, M.G., Battista, N., Finazzi-Agrò, A., Suris, R., Mechoulam, R. & Di Marzo, V. 2005, "Up-regulation of the endocannabinoid system in the uterus of leptin knockout (ob/ob) mice and implications for fertility.", *Molecular human reproduction,* vol. 11, no. 1, pp. 21-28.

Maccarrone, M., Lorenzon, T., Bari, M., Melino, G. & Finazzi-Agrò, A. 2000a, "Anandamide induces apoptosis in human cells via vanilloid receptors. Evidence for a protective role of cannabinoid receptors.", *Journal of Biological Chemistry,* vol. 275, no. 41, pp. 31938-31945.

Maccarrone, M., Valensise, H., Bari, M., Lazzarin, N., Romanini, C. & Finazzi-Agro, A. 2000b, "Relation between decreased anandamide hydrolase concentrations in human lymphocytes and miscarriage", *Lancet,* vol. 355, no. 9212, pp. 1326-1329.

Maccarrone, M., Valensise, H., Bari, M., Lazzarin, N., Romanini, C. & Finazzi-Agrò, A. 2001, "Progesterone up-regulates anandamide hydrolase in human lymphocytes: role of cytokines and implications for fertility.", *Journal of Immunology,* vol. 166, no. 12, pp. 7183-7189.

Machinal, F., Dieudonne, M.N., Leneveu, M.C., Pecquery, R. & Giudicelli, Y. 1999, "In vivo and in vitro ob gene expression and leptin secretion in rat adipocytes: evidence for a regional specific regulation by sex steroid hormones.", *Endocrinology,* vol. 140, no. 4, pp. 1567-1574.

Magarinos, M.P., Sanchez-Margalet, V., Kotler, M., Calvo, J.C. & Varone, C.L. 2007, "Leptin promotes cell proliferation and survival of trophoblastic cells", *Biology of reproduction,* vol. 76, no. 2, pp. 203-210.

Maheshwari, A., Stofberg, L. & Bhattacharya, S. 2007, "Effect of overweight and obesity on assisted reproductive technology--a systematic review", *Human reproduction update,* vol. 13, no. 5, pp. 433-444.

Mallat, A. & Lotersztajn, S. 2008, "Endocannabinoids and liver disease. I. Endocannabinoids and their receptors in the liver", *American Journal of Physiology - Gastrointestinal & Liver Physiology,* vol. 294, no. 1, pp. G9-G12.

Manchester, K.L. 1996, "Use of UV methods for measurement of protein and nucleic acid concentrations", *BioTechniques,* vol. 20, no. 6, pp. 968-970.

Mandl, A.M. 1951, "The phases of the estrous cycle in the adult white rat.", *J Exp Biol,* vol. 28, pp. 576-584.

Marczylo, T.H., Lam, P.M., Amoako, A.A. & Konje, J.C. 2010, "Anandamide levels in human female reproductive tissues: solid-phase extraction and measurement by ultraperformance liquid chromatography tandem mass spectrometry", *Analytical Biochemistry,* vol. 400, no. 2, pp. 155-162.

Marczylo, T.H., Lam, P.M., Nallendran, V., Taylor, A.H. & Konje, J.C. 2009, "A solid-phase method for the extraction and measurement of anandamide from multiple human biomatrices.", *Analytical Biochemistry,* vol. 384, no. 1, pp. 106-113.

Margetic, S., Gazzola, C., Pegg, G.G. & Hill, R.A. 2002, "Leptin: a review of its peripheral actions and interactions", *International Journal of Obesity & Related Metabolic Disorders: Journal of the International Association for the Study of Obesity,* vol. 26, no. 11, pp. 1407-1433.

Marrache, A.M., Gobeil, F., Zhu, T. & Chemtob, S. 2005, "Intracellular signaling of lipid mediators via cognate nuclear G protein-coupled receptors", *Endothelium : journal of endothelial cell research,* vol. 12, no. 1-2, pp. 63-72.

Matias, I. & Di Marzo, V. 2007, "Endocannabinoids and the control of energy balance", *Trends in Endocrinology & Metabolism,* vol. 18, no. 1, pp. 27-37.

Matias, I., Gonthier, M.P., Petrosino, S., Docimo, L., Capasso, R., Hoareau, L., Monteleone, P., Roche, R., Izzo, A.A. & Di Marzo, V. 2007, "Role and regulation of acylethanolamides in energy balance: focus on adipocytes and beta-cells.", *British journal of pharmacology,* vol. 152, no. 5, pp. 676-690.

Maul, H., Longo, M., Saade, G.R. & Garfield, R.E. 2003, "Nitric oxide and its role during pregnancy: from ovulation to delivery", *Current pharmaceutical design,* vol. 9, no. 5, pp. 359-380.

Mayer, G., Nilsson, O. & Reinius, S. 1967, "Cell membrane changes of uterine epithelium and trophoblasts during blastocyst attachment in rat", *Zeitschrift fur Anatomie und Entwicklungsgeschichte,* vol. 126, no. 1, pp. 43-48.

Maymo, J.L., Perez, A.P., Gambino, Y., Calvo, J.C., Sanchez-Margalet, V. & Varone, C.L. 2011, "Review: Leptin gene expression in the placenta--regulation of a key hormone in trophoblast proliferation and survival", *Placenta,* vol. 32 Suppl 2, pp. S146-53.

McFarland, M.J., Porter, A.C., Rakhshan, F.R., Rawat, D.S., Gibbs, R.A. & Barker, E.L. 2004, "A role for caveolae/lipid rafts in the uptake and recycling of the endogenous cannabinoid anandamide.", *Journal of Biological Chemistry,* vol. 279, no. 40, pp. 41991-41997.

McKinney, M.K. & Cravatt, B.F. 2005, "Structure and function of fatty acid amide hydrolase", *Annual Review of Biochemistry,* vol. 74, pp. 411-432.

McPartland, J.M., Matias, I., Di Marzo, V. & Glass, M. 2006, "Evolutionary origins of the endocannabinoid system.", *Gene,* vol. 370, pp. 64-74.

Mechoulam, R., Ben-Shabat, S., Hanus, L., Ligumsky, M., Kaminski, N.E., Schatz, A.R., Gopher, A., Almog, S., Martin, B.R. & Compton, D.R. 1995, "Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors.", *Biochemical pharmacology,* vol. 50, no. 1, pp. 83-90.

Meller, M., Vadachkoria, S., Luthy, D.A. & Williams, M.A. 2005, "Evaluation of housekeeping genes in placental comparative expression studies", *Placenta,* vol. 26, no. 8-9, pp. 601-607.

Mente, A., Razak, F., Blankenberg, S., Vuksan, V., Davis, A.D., Miller, R., Teo, K., Gerstein, H., Sharma, A.M., Yusuf, S., Anand, S.S., Study of the Health Assessment And Risk Evaluation & Study of the Health Assessment And Risk Evaluation in Aboriginal Peoples Investigators 2010, "Ethnic variation in adiponectin and leptin levels and their association with adiposity and insulin resistance", *Diabetes care,* vol. 33, no. 7, pp. 1629-1634.

Metwally, M., Ledger, W.L. & Li, T.C. 2008, "Reproductive endocrinology and clinical aspects of obesity in women", *Annals of the New York Academy of Sciences,* vol. 1127, pp. 140-146.

Mezey, E., Toth, Z.E., Cortright, D.N., Arzubi, M.K., Krause, J.E., Elde, R., Guo, A., Blumberg, P.M. & Szallasi, A. 2000, "Distribution of mRNA for vanilloid receptor subtype 1 (VR1), and VR1-like immunoreactivity, in the central nervous system of the rat and human.", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 97, no. 7, pp. 3655-3660.

Milne, S.A., Henderson, T.A., Kelly, R.W., Saunders, P.T., Baird, D.T. & Critchley, H.O. 2005, "Leukocyte populations and steroid receptor expression in human first-trimester decidua; regulation by antiprogestin and prostaglandin E analog", *The Journal of clinical endocrinology and metabolism,* vol. 90, no. 7, pp. 4315-4321.

Misra, V.K. & Trudeau, S. 2011, "The influence of overweight and obesity on longitudinal trends in maternal serum leptin levels during pregnancy", *Obesity (Silver Spring, Md.),* vol. 19, no. 2, pp. 416-421.

Moriconi, A., Cerbara, I., Maccarrone, M. & Topai, A. 2010, "GPR55: Current knowledge and future perspectives of a purported "Type-3" cannabinoid receptor", *Current medicinal chemistry,* vol. 17, no. 14, pp. 1411-1429.

Munro, S., Thomas, K.L. & Abu-Shaar, M. 1993, "Molecular characterization of a peripheral receptor for cannabinoids.", *Nature,* vol. 365, no. 6441, pp. 61-65.

Murthi, P., Fitzpatrick, E., Borg, A.J., Donath, S., Brennecke, S.P. & Kalionis, B. 2008, "GAPDH, 18S rRNA and YWHAZ are suitable endogenous reference genes for relative gene expression studies in placental tissues from human idiopathic fetal growth restriction", *Placenta,* vol. 29, no. 9, pp. 798-801.

Nachtigall, M.J., Kliman, H.J., Feinberg, R.F., Olive, D.L., Engin, O. & Arici, A. 1996, "The effect of leukemia inhibitory factor (LIF) on trophoblast differentiation: a potential role in human implantation.", *Journal of Clinical Endocrinology & Metabolism,* vol. 81, no. 2, pp. 801-806.

Nallendran, V., Lam, P.M., Marczylo, T.H., Bankart, M.J., Taylor, A.H., Taylor, D.J. & Konje, J.C. 2010, "The plasma levels of the endocannabinoid, anandamide, increase with the induction of labour", *BJOG : an international journal of obstetrics and gynaecology,* vol. 117, no. 7, pp. 863-869.

Nallendran, V., Lam, P.M., McParland, P.C., Taylor, A.H. & Konje, J.C. 2009, "Prediction of preterm labour among asymptomatic high risk patients using plasma anandamide levels.", *Reprod Sci,* vol. 16, pp. 220.

Nevalainen, T. & Irving, A.J. 2010, "GPR55, a lysophosphatidylinositol receptor with cannabinoid sensitivity?", *Current topics in medicinal chemistry,* vol. 10, no. 8, pp. 799-813.

Nikas, G., Makrigiannakis, A., Hovatta, O. & Jones, H.W.,Jr 2000, "Surface morphology of the human endometrium. Basic and clinical aspects.", *Annals of the New York Academy of Sciences,* vol. 900, pp. 316-324.

Nixon, B., Lu, Q., Wassler, M.J., Foote, C.I., Ensslin, M.A. & Shur, B.D. 2001, "Galactosyltransferase function during mammalian fertilization", *Cells, tissues, organs,* vol. 168, no. 1-2, pp. 46-57.

Norman, J.E., Thong, K.J. & Baird, D.T. 1991, "Uterine contractility and induction of abortion in early pregnancy by misoprostol and mifepristone", *Lancet,* vol. 338, no. 8777, pp. 1233-1236.

Office for National Statistics 2009, , *Office for National Statistics: NHS Maternity Statistics, England*. Available: www.statistics.gov.uk [2009].

Ohishi, T. & Yoshida, S. 2012, "The therapeutic potential of GPR119 agonists for type 2 diabetes", *Expert opinion on investigational drugs,* vol. 21, no. 3, pp. 321-328.

Oka, S., Nakajima, K., Yamashita, A., Kishimoto, S. & Sugiura, T. 2007, "Identification of GPR55 as a lysophosphatidylinositol receptor.", *Biochemical & Biophysical Research Communications,* vol. 362, no. 4, pp. 928-934.

Okamoto, Y., Morishita, J., Tsuboi, K., Tonai, T. & Ueda, N. 2004a, "Molecular characterization of a phospholipase D generating anandamide and its congeners", *The Journal of biological chemistry,* vol. 279, no. 7, pp. 5298-5305.

Okamoto, Y., Morishita, J., Tsuboi, K., Tonai, T. & Ueda, N. 2004b, "Molecular characterization of a phospholipase D generating anandamide and its congeners.", *Journal of Biological Chemistry,* vol. 279, no. 7, pp. 5298-5305.

Overton, H.A., Babbs, A.J., Doel, S.M., Fyfe, M.C., Gardner, L.S., Griffin, G., Jackson, H.C., Procter, M.J., Rasamison, C.M., Tang-Christensen, M., Widdowson, P.S., Williams, G.M. & Reynet, C. 2006, "Deorphanization of a G protein-coupled receptor for oleoylethanolamide and its use in the discovery of small-molecule hypophagic agents.", *Cell Metabolism,* vol. 3, no. 3, pp. 167-175.

Pacher, P. & Steffens, S. 2009, "The emerging role of the endocannabinoid system in cardiovascular disease", *Seminars In Immunopathology,* vol. 31, no. 1, pp. 63-77.

Paria, B.C., Das, S.K. & Dey, S.K. 1995, "The preimplantation mouse embryo is a target for cannabinoid ligand-receptor signaling.", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 92, no. 21, pp. 9460-9464.

Paria, B.C., Deutsch, D.D. & Dey, S.K. 1996, "The uterus is a potential site for anandamide synthesis and hydrolysis: differential profiles of anandamide synthase and hydrolase activities in the mouse uterus during the periimplantation period.", *Molecular Reproduction & Development,* vol. 45, no. 2, pp. 183-192.

Paria, B.C., Ma, W., Andrenyak, D.M., Schmid, P.C., Schmid, H.H., Moody, D.E., Deng, H., Makriyannis, A. & Dey, S.K. 1998, "Effects of cannabinoids on preimplantation mouse embryo development and implantation are mediated

by brain-type cannabinoid receptors.", *Biology of reproduction,* vol. 58, no. 6, pp. 1490-1495.

Paria, B.C., Reese, J., Das, S.K. & Dey, S.K. 2002, "Deciphering the cross-talk of implantation: advances and challenges", *Science,* vol. 296, no. 5576, pp. 2185-2188.

Paria, B.C., Song, H., Wang, X., Schmid, P.C., Krebsbach, R.J., Schmid, H.H., Bonner, T.I., Zimmer, A. & Dey, S.K. 2001, "Dysregulated cannabinoid signaling disrupts uterine receptivity for embryo implantation.", *Journal of Biological Chemistry,* vol. 276, no. 23, pp. 20523-20528.

Paria, B.C., Wang, H. & Dey, S.K. 2002, "Endocannabinoid signaling in synchronizing embryo development and uterine receptivity for implantation", *Chemistry & Physics of Lipids,* vol. 121, no. 1-2, pp. 201-210.

Paria, B.C., Zhao, X., Wang, J., Das, S.K. & Dey, S.K. 1999, "Fatty-acid amide hydrolase is expressed in the mouse uterus and embryo during the periimplantation period", *Biology of reproduction,* vol. 60, no. 5, pp. 1151-1157.

Park, H.Y., Kwon, H.M., Lim, H.J., Hong, B.K., Lee, J.Y., Park, B.E., Jang, Y., Cho, S.Y. & Kim, H.S. 2001, "Potential role of leptin in angiogenesis: leptin induces endothelial cell proliferation and expression of matrix metalloproteinases in vivo and in vitro.", *Experimental & molecular medicine,* vol. 33, no. 2, pp. 95-102.

Patel, S., Wohlfeil, E.R., Rademacher, D.J., Carrier, E.J., Perry, L.J., Kundu, A., Falck, J.R., Nithipatikom, K., Campbell, W.B. & Hillard, C.J. 2003, "The general anesthetic propofol increases brain N-arachidonylethanolamine (anandamide) content and inhibits fatty acid amide hydrolase.", *British journal of pharmacology,* vol. 139, no. 5, pp. 1005-1013.

Perez-Perez, A., Maymo, J., Duenas, J.L., Goberna, R., Calvo, J.C., Varone, C. & Sanchez-Margalet, V. 2008, "Leptin prevents apoptosis of trophoblastic cells by activation of MAPK pathway", *Archives of Biochemistry and Biophysics,* vol. 477, no. 2, pp. 390-395.

Perrier d'Hauterive, S., Charlet-Renard, C., Berndt, S., Dubois, M., Munaut, C., Goffin, F., Hagelstein, M.T., Noel, A., Hazout, A., Foidart, J.M. & Geenen, V. 2004, "Human chorionic gonadotropin and growth factors at the embryonic-endometrial interface control leukemia inhibitory factor (LIF) and interleukin 6 (IL-6) secretion by human endometrial epithelium.", *Human Reproduction,* vol. 19, no. 11, pp. 2633-2643.

Pertwee, R.G. 1997, "Pharmacology of cannabinoid CB1 and CB2 receptors", *Pharmacology & therapeutics,* vol. 74, no. 2, pp. 129-180.

Pertwee, R.G., Howlett, A.C., Abood, M.E., Alexander, S.P., Di Marzo, V., Elphick, M.R., Greasley, P.J., Hansen, H.S., Kunos, G., Mackie, K.,

Mechoulam, R. & Ross, R.A. 2010, "International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB(1) and CB(2)", *Pharmacological reviews,* vol. 62, no. 4, pp. 588-631.

Pertwee, R.G. & Ross, R.A. 2002, "Cannabinoid receptors and their ligands", *Prostaglandins Leukotrienes & Essential Fatty Acids,* vol. 66, no. 2-3, pp. 101-121.

Pfaffl, M.W. 2001, "A new mathematical model for relative quantification in real-time RT-PCR", *Nucleic acids research,* vol. 29, no. 9, pp. e45.

Piccinni, M.P., Beloni, L., Livi, C., Maggi, E., Scarselli, G. & Romagnani, S. 1998, "Defective production of both leukemia inhibitory factor and type 2 T-helper cytokines by decidual T cells in unexplained recurrent abortions.", *Nature medicine,* vol. 4, no. 9, pp. 1020-1024.

Piccinni, M.P. & Romagnani, S. 1996, "Regulation of fetal allograft survival by a hormone-controlled Th1- and Th2-type cytokines", *Immunologic research,* vol. 15, no. 2, pp. 141-150.

Pijnenborg, R., Bland, J.M., Robertson, W.B., Dixon, G. & Brosens, I. 1981a, "The pattern of interstitial trophoblastic invasion of the myometrium in early human pregnancy", *Placenta,* vol. 2, no. 4, pp. 303-316.

Pijnenborg, R., Robertson, W.B., Brosens, I. & Dixon, G. 1981b, "Review article: trophoblast invasion and the establishment of haemochorial placentation in man and laboratory animals", *Placenta,* vol. 2, no. 1, pp. 71-91.

Piomelli, D. 2004, "THC: moderation during implantation.", *Nature medicine,* vol. 10, no. 1, pp. 19-20.

Piomelli, D., Giuffrida, A., Calignano, A. & Rodriguez de Fonseca, F. 2000, "The endocannabinoid system as a target for therapeutic drugs", *Trends in pharmacological sciences,* vol. 21, no. 6, pp. 218-224.

Pollheimer, J. & Knofler, M. 2005, "Signalling pathways regulating the invasive differentiation of human trophoblasts: a review", *Placenta,* vol. 26, no. Suppl A, pp. S21-30.

Porter, A.C., Sauer, J.M., Knierman, M.D., Becker, G.W., Berna, M.J., Bao, J., Nomikos, G.G., Carter, P., Bymaster, F.P., Leese, A.B. & Felder, C.C. 2002, "Characterization of a novel endocannabinoid, virodhamine, with antagonist activity at the CB1 receptor.", *Journal of Pharmacology & Experimental Therapeutics,* vol. 301, no. 3, pp. 1020-1024.

Prabakar, I. & Webb, A. 2012, "Emergency contraception", *BMJ (Clinical research ed.),* vol. 344, pp. e1492.

Practice Committee of the American Society for Reproductive Medicine 2006, "Aging and infertility in women", *Fertility and sterility,* vol. 86, no. 5 Suppl 1, pp. S248-52.

Purcell, K., Schembri, M., Frazier, L.M., Rall, M.J., Shen, S., Croughan, M., Grainger, D.A. & Fujimoto, V.Y. 2007, "Asian ethnicity is associated with reduced pregnancy outcomes after assisted reproductive technology.", *Fertility & Sterility,* vol. 87, no. 2, pp. 297-302.

RCOG 2004, *Royal College of Obstetricians and Gynaecologist (RCOG): The care of women requesting induced abortion evidence- based clinical guideline no7*, RCOG Press, London UK.

Reitman, M.L., Bi, S., Marcus-Samuels, B. & Gavrilova, O. 2001, "Leptin and its role in pregnancy and fetal development--an overview", *Biochemical Society transactions,* vol. 29, no. Pt 2, pp. 68-72.

Richardson, A.R. & Maltz, F.N. 2012, "Ulipristal acetate: review of the efficacy and safety of a newly approved agent for emergency contraception", *Clinical therapeutics,* vol. 34, no. 1, pp. 24-36.

Ross, R.A. 2009, "The enigmatic pharmacology of GPR55", *Trends in pharmacological sciences,* vol. 30, no. 3, pp. 156-163.

Rouzer, C.A. & Marnett, L.J. 2008, "Non-redundant functions of cyclooxygenases: oxygenation of endocannabinoids", *Journal of Biological Chemistry,* vol. 283, no. 13, pp. 8065-8069.

Ryberg, E., Larsson, N., Sjogren, S., Hjorth, S., Hermansson, N.O., Leonova, J., Elebring, T., Nilsson, K., Drmota, T. & Greasley, P.J. 2007, "The orphan receptor GPR55 is a novel cannabinoid receptor.", *British journal of pharmacology,* vol. 152, no. 7, pp. 1092-1101.

Saghatelian, A., Trauger, S.A., Want, E.J., Hawkins, E.G., Siuzdak, G. & Cravatt, B.F. 2004, "Assignment of endogenous substrates to enzymes by global metabolite profiling.", *Biochemistry,* vol. 43, no. 45, pp. 14332-14339.

Schelling, G., Hauer, D., Azad, S.C., Schmoelz, M., Chouker, A., Schmidt, M., Hornuss, C., Rippberger, M., Briegel, J., Thiel, M. & Vogeser, M. 2006, "Effects of general anesthesia on anandamide blood levels in humans.", *Anesthesiology,* vol. 104, no. 2, pp. 273-277.

Schmid, P.C., Paria, B.C., Krebsbach, R.J., Schmid, H.H. & Dey, S.K. 1997, "Changes in anandamide levels in mouse uterus are associated with uterine receptivity for embryo implantation.", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 94, no. 8, pp. 4188-4192.

Schmidt, M.W., Houseman, A., Ivanov, A.R. & Wolf, D.A. 2007, "Comparative proteomic and transcriptomic profiling of the fission yeast Schizosaccharomyces pombe", *Molecular systems biology,* vol. 3, pp. 79.

Schneeberger, C., Speiser, P., Kury, F. & Zeillinger, R. 1995, "Quantitative detection of reverse transcriptase-PCR products by means of a novel and sensitive DNA stain", *PCR methods and applications,* vol. 4, no. 4, pp. 234-238.

Schroeder, A., Mueller, O., Stocker, S., Salowsky, R., Leiber, M., Gassmann, M., Lightfoot, S., Menzel, W., Granzow, M. & Ragg, T. 2006, "The RIN: an RNA integrity number for assigning integrity values to RNA measurements", *BMC molecular biology,* vol. 7, pp. 3.

Schuel, H. 2006, "Tuning the oviduct to the anandamide tone", *The Journal of clinical investigation,* vol. 116, no. 8, pp. 2087-2090.

Seifer, D.B., Frazier, L.M. & Grainger, D.A. 2008, "Disparity in assisted reproductive technologies outcomes in black women compared with white women.", *Fertility & Sterility,* vol. 90, no. 5, pp. 1701-1710.

Sharkey, A.M., King, A., Clark, D.E., Burrows, T.D., Jokhi, P.P., Charnock-Jones, D.S., Loke, Y.W. & Smith, S.K. 1999, "Localization of leukemia inhibitory factor and its receptor in human placenta throughout pregnancy.", *Biology of reproduction,* vol. 60, no. 2, pp. 355-364.

Sharov, A.A., Piao, Y., Matoba, R., Dudekula, D.B., Qian, Y., VanBuren, V., Falco, G., Martin, P.R., Stagg, C.A., Bassey, U.C., Wang, Y., Carter, M.G., Hamatani, T., Aiba, K., Akutsu, H., Sharova, L., Tanaka, T.S., Kimber, W.L., Yoshikawa, T., Jaradat, S.A., Pantano, S., Nagaraja, R., Boheler, K.R., Taub, D., Hodes, R.J., Longo, D.L., Schlessinger, D., Keller, J., Klotz, E., Kelsoe, G., Umezawa, A., Vescovi, A.L., Rossant, J., Kunath, T., Hogan, B.L., Curci, A., D'Urso, M., Kelso, J., Hide, W. & Ko, M.S. 2003, "Transcriptome analysis of mouse stem cells and early embryos.", *Plos Biology,* vol. 1, no. 3, pp. E74.

Sherwin, J.R., Smith, S.K., Wilson, A. & Sharkey, A.M. 2002, "Soluble gp130 is up-regulated in the implantation window and shows altered secretion in patients with primary unexplained infertility.", *Journal of Clinical Endocrinology & Metabolism,* vol. 87, no. 8, pp. 3953-3960.

Shi, Y.E., Ye, Z.H., He, C.H., Zhang, G.Q., Xu, J.Q., Van Look, P.F. & Fotherby, K. 1993, "Pharmacokinetic study of RU 486 and its metabolites after oral administration of single doses to pregnant and non-pregnant women", *Contraception,* vol. 48, no. 2, pp. 133-149.

Shimizu, H., Shimomura, Y., Nakanishi, Y., Futawatari, T., Ohtani, K., Sato, N. & Mori, M. 1997, "Estrogen increases in vivo leptin production in rats and human subjects.", *Journal of Endocrinology,* vol. 154, no. 2, pp. 285-292.

Simon, G.M. & Cravatt, B.F. 2008, "Anandamide biosynthesis catalyzed by the phosphodiesterase GDE1 and detection of glycerophospho-N-acyl ethanolamine precursors in mouse brain.", *Journal of Biological Chemistry,* vol. 283, no. 14, pp. 9341-9349.

Smith, S.K., Charnock-Jones, D.S. & Sharkey, A.M. 1998, "The role of leukemia inhibitory factor and interleukin-6 in human reproduction", *Human Reproduction,* vol. 13, no. Suppl 3, pp. 237-243.

Soares, M.J., Chapman, B.M., Rasmussen, C.A., Dai, G., Kamei, T. & Orwig, K.E. 1996, "Differentiation of trophoblast endocrine cells", *Placenta,* vol. 17, no. 5-6, pp. 277-289.

Stanford, J.B. & Mikolajczyk, R.T. 2002, "Mechanisms of action of intrauterine devices: update and estimation of postfertilization effects", *American Journal of Obstetrics & Gynecology,* vol. 187, no. 6, pp. 1699-1708.

Staton, P.C., Hatcher, J.P., Walker, D.J., Morrison, A.D., Shapland, E.M., Hughes, J.P., Chong, E., Mander, P.K., Green, P.J., Billinton, A., Fulleylove, M., Lancaster, H.C., Smith, J.C., Bailey, L.T., Wise, A., Brown, A.J., Richardson, J.C. & Chessell, I.P. 2008, "The putative cannabinoid receptor GPR55 plays a role in mechanical hyperalgesia associated with inflammatory and neuropathic pain.", *Pain,* vol. 139, no. 1, pp. 225-236.

Stewart, C.L., Kaspar, P., Brunet, L.J., Bhatt, H., Gadi, I., Kontgen, F. & Abbondanzo, S.J. 1992, "Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor.", *Nature,* vol. 359, no. 6390, pp. 76-79.

Strand, C., Enell, J., Hedenfalk, I. & Ferno, M. 2007, "RNA quality in frozen breast cancer samples and the influence on gene expression analysis--a comparison of three evaluation methods using microcapillary electrophoresis traces", *BMC molecular biology,* vol. 8, pp. 38.

Strauss, L.T., Gamble, S.B., Parker, W.Y., Cook, D.A., Zane, S.B., Hamdan, S. & Centers for Disease Control and,Prevention 2006, "Abortion surveillance--United States, 2003.", *Morbidity & Mortality Weekly Report.Surveillance Summaries,* vol. 55, no. 11, pp. 1-32.

Sturzenbaum, S.R. & Kille, P. 2001, "Control genes in quantitative molecular biological techniques: the variability of invariance", *Comparative biochemistry and physiology.Part B, Biochemistry & molecular biology,* vol. 130, no. 3, pp. 281-289.

Substance Abuse and Mental Health Services Administration 2005, , *Substance Abuse and Mental Health Services Administration*. Available: www.samhsa.gov [2005] .

Sugiura, T., Kobayashi, Y., Oka, S. & Waku, K. 2002, "Biosynthesis and degradation of anandamide and 2-arachidonoylglycerol and their possible physiological significance", *Prostaglandins Leukotrienes & Essential Fatty Acids,* vol. 66, no. 2-3, pp. 173-192.

Sugiura, T., Kodaka, T., Nakane, S., Miyashita, T., Kondo, S., Suhara, Y., Takayama, H., Waku, K., Seki, C., Baba, N. & Ishima, Y. 1999, "Evidence

that the cannabinoid CB1 receptor is a 2-arachidonoylglycerol receptor. Structure-activity relationship of 2-arachidonoylglycerol, ether-linked analogues, and related compounds.", *Journal of Biological Chemistry,* vol. 274, no. 5, pp. 2794-2801.

Sugiura, T., Kondo, S., Sukagawa, A., Nakane, S., Shinoda, A., Itoh, K., Yamashita, A. & Waku, K. 1995, "2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain.", *Biochemical & Biophysical Research Communications,* vol. 215, no. 1, pp. 89-97.

Sun, X. & Dey, S.K. 2008, "Aspects of endocannabinoid signaling in periimplantation biology", *Molecular & Cellular Endocrinology,* vol. 286, no. 1-2 Suppl 1, pp. S3-11.

Sun, X., Xie, H., Yang, J., Wang, H., Bradshaw, H.B. & Dey, S.K. 2010, "Endocannabinoid signaling directs differentiation of trophoblast cell lineages and placentation", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 107, no. 39, pp. 16887-16892.

Sun, Y.X., Tsuboi, K., Okamoto, Y., Tonai, T., Murakami, M., Kudo, I. & Ueda, N. 2004, "Biosynthesis of anandamide and N-palmitoylethanolamine by sequential actions of phospholipase A2 and lysophospholipase D.", *Biochemical Journal,* vol. 380, no. Pt 3, pp. 749-756.

Szallasi, A. & Blumberg, P.M. 1999, "Vanilloid (Capsaicin) receptors and mechanisms", *Pharmacological reviews,* vol. 51, no. 2, pp. 159-212.

Tang, O.S., Gemzell-Danielsson, K. & Ho, P.C. 2007, "Misoprostol: pharmacokinetic profiles, effects on the uterus and side-effects", *International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics,* vol. 99 Suppl 2, pp. S160-7.

Tang, O.S., Schweer, H., Seyberth, H.W., Lee, S.W. & Ho, P.C. 2002, "Pharmacokinetics of different routes of administration of misoprostol.", *Human Reproduction,* vol. 17, no. 2, pp. 332-336.

Tartaglia, L.A. 1997, "The leptin receptor", *Journal of Biological Chemistry,* vol. 272, no. 10, pp. 6093-6096.

Taylor, A.H., Amoako, A.A., Bambang, K., Karasu, T., Gebeh, A., Lam, P.M., Marzcylo, T.H. & Konje, J.C. 2010, "Endocannabinoids and pregnancy", *Clinica chimica acta; international journal of clinical chemistry,* vol. 411, no. 13-14, pp. 921-930.

Taylor, A.H., Ang, C., Bell, S.C. & Konje, J.C. 2007, "The role of the endocannabinoid system in gametogenesis, implantation and early pregnancy", *Human reproduction update,* vol. 13, no. 5, pp. 501-513.

Taylor, A.H., Finney, M., Lam, P.M. & Konje, J.C. 2011, "Modulation of the endocannabinoid system in viable and non-viable first trimester pregnancies

by pregnancy-related hormones", *Reproductive biology and endocrinology : RB&E,* vol. 9, pp. 152.

Trabucco, E., Acone, G., Marenna, A., Pierantoni, R., Cacciola, G., Chioccarelli, T., Mackie, K., Fasano, S., Colacurci, N., Meccariello, R., Cobellis, G. & Cobellis, L. 2009, "Endocannabinoid system in first trimester placenta: low FAAH and high CB1 expression characterize spontaneous miscarriage.", *Placenta,* vol. 30, no. 6, pp. 516-522.

Tricarico, C., Pinzani, P., Bianchi, S., Paglierani, M., Distante, V., Pazzagli, M., Bustin, S.A. & Orlando, C. 2002, "Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies", *Analytical Biochemistry,* vol. 309, no. 2, pp. 293-300.

Trussell, J. 1995, "Contraceptive efficacy", *Archives of Dermatology,* vol. 131, no. 9, pp. 1064-1068.

Tsuboi, K., Sun, Y.X., Okamoto, Y., Araki, N., Tonai, T. & Ueda, N. 2005, "Molecular characterization of N-acylethanolamine-hydrolyzing acid amidase, a novel member of the choloylglycine hydrolase family with structural and functional similarity to acid ceramidase.", *Journal of Biological Chemistry,* vol. 280, no. 12, pp. 11082-11092.

Ulmann, A., Silvestre, L., Chemama, L., Rezvani, Y., Renault, M., Aguillaume, C.J. & Baulieu, E.E. 1992, "Medical termination of early pregnancy with mifepristone (RU 486) followed by a prostaglandin analogue. Study in 16,369 women.", *Acta Obstetricia et Gynecologica Scandinavica,* vol. 71, no. 4, pp. 278-283.

Vaisanen-Tommiska, M.R. 2008, "Nitric oxide in the human uterine cervix: endogenous ripening factor", *Annals of Medicine,* vol. 40, no. 1, pp. 45-55.

Van Der Stelt, M. & Di Marzo, V. 2004, "Endovanilloids. Putative endogenous ligands of transient receptor potential vanilloid 1 channels", *European Journal of Biochemistry,* vol. 271, no. 10, pp. 1827-1834.

Van der Stelt, M., van Kuik, J.A., Bari, M., van Zadelhoff, G., Leeflang, B.R., Veldink, G.A., Finazzi-Agro, A., Vliegenthart, J.F. & Maccarrone, M. 2002, "Oxygenated metabolites of anandamide and 2-arachidonoylglycerol: conformational analysis and interaction with cannabinoid receptors, membrane transporter, and fatty acid amide hydrolase.", *Journal of medicinal chemistry,* vol. 45, no. 17, pp. 3709-3720.

Van Look, P.F. & Bygdeman, M. 1989, "Antiprogestational steroids: a new dimension in human fertility regulation", *Oxford reviews of reproductive biology,* vol. 11, pp. 2-60.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. & Speleman, F. 2002, "Accurate normalization of real-time quantitative RT-

PCR data by geometric averaging of multiple internal control genes", *Genome biology,* vol. 3, no. 7, pp. RESEARCH0034.

Vercruysse, L., Caluwaerts, S., Luyten, C. & Pijnenborg, R. 2006, "Interstitial trophoblast invasion in the decidua and mesometrial triangle during the last third of pregnancy in the rat", *Placenta,* vol. 27, no. 1, pp. 22-33.

von Hertzen, H., Piaggio, G., Ding, J., Chen, J., Song, S., Bartfai, G., Ng, E., Gemzell-Danielsson, K., Oyunbileg, A., Wu, S., Cheng, W., Ludicke, F., Pretnar-Darovec, A., Kirkman, R., Mittal, S., Khomassuridze, A., Apter, D., Peregoudov, A. & WHO Research Group on Post-ovulatory Methods of Fertility Regulation 2002, "Low dose mifepristone and two regimens of levonorgestrel for emergency contraception: a WHO multicentre randomised trial", *Lancet,* vol. 360, no. 9348, pp. 1803-1810.

Wang, H., Dey, S.K. & Maccarrone, M. 2006, "Jekyll and hyde: two faces of cannabinoid signaling in male and female fertility", *Endocrine reviews,* vol. 27, no. 5, pp. 427-448.

Wang, H., Guo, Y., Wang, D., Kingsley, P.J., Marnett, L.J., Das, S.K., DuBois, R.N. & Dey, S.K. 2004, "Aberrant cannabinoid signaling impairs oviductal transport of embryos", *Nature medicine,* vol. 10, no. 10, pp. 1074-1080.

Wang, H., Matsumoto, H., Guo, Y., Paria, B.C., Roberts, R.L. & Dey, S.K. 2003, "Differential G protein-coupled cannabinoid receptor signaling by anandamide directs blastocyst activation for implantation.", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 100, no. 25, pp. 14914-14919.

Wang, H., Xie, H. & Dey, S.K. 2008, "Loss of cannabinoid receptor CB1 induces preterm birth.", *PLoS ONE [Electronic Resource],* vol. 3, no. 10, pp. e3320.

Wang, H., Xie, H., Guo, Y., Zhang, H., Takahashi, T., Kingsley, P.J., Marnett, L.J., Das, S.K., Cravatt, B.F. & Dey, S.K. 2006, "Fatty acid amide hydrolase deficiency limits early pregnancy events", *Journal of Clinical Investigation,* vol. 116, no. 8, pp. 2122-2131.

Wang, H., Xie, H., Sun, X., Kingsley, P.J., Marnett, L.J., Cravatt, B.F. & Dey, S.K. 2007, "Differential regulation of endocannabinoid synthesis and degradation in the uterus during embryo implantation.", *Prostaglandins & other lipid mediators,* vol. 83, no. 1-2, pp. 62-74.

Wang, J., Okamoto, Y., Morishita, J., Tsuboi, K., Miyatake, A. & Ueda, N. 2006, "Functional analysis of the purified anandamide-generating phospholipase D as a member of the metallo-beta-lactamase family.", *Journal of Biological Chemistry,* vol. 281, no. 18, pp. 12325-12335.

Wang, J., Paria, B.C., Dey, S.K. & Armant, D.R. 1999, "Stage-specific excitation of cannabinoid receptor exhibits differential effects on mouse embryonic development.", *Biology of reproduction,* vol. 60, no. 4, pp. 839-844.

Wang, J.X., Davies, M. & Norman, R.J. 2000, "Body mass and probability of pregnancy during assisted reproduction treatment: retrospective study.", *BMJ,* vol. 321, no. 7272, pp. 1320-1321.

Wang, J.X., Davies, M.J. & Norman, R.J. 2002, "Obesity increases the risk of spontaneous abortion during infertility treatment.", *Obesity research,* vol. 10, no. 6, pp. 551-554.

Weeks, A.D., Fiala, C. & Safar, P. 2005, "Misoprostol and the debate over off-label drug use", *BJOG : an international journal of obstetrics and gynaecology,* vol. 112, no. 3, pp. 269-272.

Wei, B.Q., Mikkelsen, T.S., McKinney, M.K., Lander, E.S. & Cravatt, B.F. 2006, "A second fatty acid amide hydrolase with variable distribution among placental mammals.", *Journal of Biological Chemistry,* vol. 281, no. 48, pp. 36569-36578.

Wenger, T., Fragkakis, G., Giannikou, P., Probonas, K. & Yiannikakis, N. 1997, "Effects of anandamide on gestation in pregnant rats", *Life Sciences,* vol. 60, no. 26, pp. 2361-2371.

White, J.P., Urban, L. & Nagy, I. 2011, "TRPV1 function in health and disease", *Current Pharmaceutical Biotechnology,* vol. 12, no. 1, pp. 130-144.

Wittwer, C.T., Herrmann, M.G., Moss, A.A. & Rasmussen, R.P. 1997, "Continuous fluorescence monitoring of rapid cycle DNA amplification", *BioTechniques,* vol. 22, no. 1, pp. 130-1, 134-8.

World Health Organisation, Department of Health and Research (ed) 2003, *WHO Safe Abortion: Technical and Policy Guidance for Health Systems*, WHO, Geneva, Switzerland.

Xu, Y., Knipp, G.T. & Cook, T.J. 2005, "Expression of CYP4A isoforms in developing rat placental tissue and rat trophoblastic cell models", *Placenta,* vol. 26, no. 2-3, pp. 218-225.

Yang, Z.M., Paria, B.C. & Dey, S.K. 1996, "Activation of brain-type cannabinoid receptors interferes with preimplantation mouse embryo development.", *Biology of reproduction,* vol. 55, no. 4, pp. 756-761.

Yin, H., Chu, A., Li, W., Wang, B., Shelton, F., Otero, F., Nguyen, D.G., Caldwell, J.S. & Chen, Y.A. 2009, "Lipid G protein-coupled receptor ligand identification using beta-arrestin PathHunter assay.", *Journal of Biological Chemistry,* vol. 284, no. 18, pp. 12328-12338.

Yu, M., Ives, D. & Ramesha, C.S. 1997, "Synthesis of prostaglandin E2 ethanolamide from anandamide by cyclooxygenase-2.", *Journal of Biological Chemistry,* vol. 272, no. 34, pp. 21181-21186.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L. & Friedman, J.M. 1994, "Positional cloning of the mouse obese gene and its human homologue", *Nature,* vol. 372, no. 6505, pp. 425-432.

Zieman, M., Fong, S.K., Benowitz, N.L., Banskter, D. & Darney, P.D. 1997, "Absorption kinetics of misoprostol with oral or vaginal administration.", *Obstetrics & Gynecology,* vol. 90, no. 1, pp. 88-92.

Zygmunt, P.M., Petersson, J., Andersson, D.A., Chuang, H., Sorgard, M., Di Marzo, V., Julius, D. & Hogestatt, E.D. 1999, "Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide.", *Nature,* vol. 400, no. 6743, pp. 452-457.