An Exploration into the Role of the Endocannabinoid System in Endometrial Receptivity

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by

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ABSTRACT

While it is clear that the control of implantation is multifactorial, one emerging component that appears to be important in the success or failure of embryo implantation is the endocannabinoid system.

The primary ligand of the endocannabinoid system (ECS) is anandamide (AEA) which is synthesised by a *N*-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) and degraded by fatty acid amide hydrolase (FAAH). A careful balance in the activities of NAPE-PLD and FAAH is required to ensure the appropriate levels of AEA are available during implantation.

The purpose of this thesis was to explore further the role of the ECS, specifically its role in uterine receptivity using both *in-vivo* and *in-vitro* models.

In-vivo models were used to study the expression of the ECS by measuring plasma concentrations of AEA, along with OEA and PEA (two other ligands of the ECS). A statistically significant change in plasma PEA concentrations was noted when comparing urine pregnancy test results. No significant differences in either AEA or OEA plasma concentrations were demonstrated.

In-vitro models were used to investigate the interactions between galectin 3, integrin β3 and the ECS. The results show that while galectin 3 did not have any effect on the ECS, up-regulation of the expression of integrin β3 in receptive endometrial cells both increased the expression of FAAH and decreased the expression on NAPE-PLD in a dose-dependent manner. However, no effect was demonstrated in non-receptive endometrial cells, suggesting that integrin β3 expression, in collaboration with the ECS, plays an important role in endometrial receptivity.

Overall, this work gives us further insight into the immensely complex processes involved in ensuring the endometrium is receptive to an embryo. Most importantly, it has suggested a link between the expression of integrin β3 and the enzymes of the ECS that is absent in the non-receptive endometrium.

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LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AA	arachidonic acid
Abh4	alpha/beta-hydrolase 4
ACU	assisted conception unit
AEA	*N*-arachidonyl ethanolamine, anandamide
2-AG	2-arachidonoylglycerol
ANOVA	analysis of variance
ART	assisted reproductive technology
BMI	body mass index
CB1	cannabinoid receptor type 1
CB2	cannabinoid receptor type 2
cDNA	copy DNA
COX2	cyclooxygenase 2
CRD	carbohydrate recognition domains
CSF	colony stimulating factor
Δ9-THC	Δ9 –tetrahydrocannabinol
DEPC	diethylpyrocarbonate
dH2O	distilled water
DNase	deoxyribonuclease
E2	17β-estradiol
ECS	endocannabinoid system
EEC	endometrial epithelial cells
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ERG	Endocannabinoid Research Group
ET	embryo transfer
ETDA	ethylene diamine tetra-acetic acid
EVT	extravillous trophoblast
FAAH	fatty acid amide hydrolase
FABP	fatty acid binding protein
GAPDH	glyceraldehyde-3-phospate dehydrogenase
hCG	human chorionic gonadotrophin
HEC 1A	human endometrial adenocarcinoma 1A cells
HFEA	Human Fertilisation and Embryology Authority
HLA	human leukocyte antigen
HOXA10	Homeobox A10
HOXA11	Homeobox A11
HPLC	high-performance liquid chromatography
ICM	Inner cell mass
ICSI	intra-cytoplasmic sperm injection
IGFBP-1	insulin-like growth factor binding protein
IL	interleukin
IFNγ	interferon gamma
IVF	in vitro fertilisation
KIRs	killer-cell immunoglobulin-like receptors
LH	luteinising hormone
LIF	leukaemia inhibitory factor
FSH	follicle stimulating hormone
Gal	galectin

GnRH	gonadotrophin releasing hormone
hCG	human chorionic gonadotrophin
MHC	major histocompatibility complexes
mRNA	messenger ribonucleic acid
MS	mass spectrometer
NAE	*N*-acylethanolamine
NICE	National Institute for Health and Care Excellence
NAPE	*N*-arachidonoyl phosphatidylethanolamine
NAPE-PLD	N-arachidonoyl phosphatidylethanolamine hydrolysing phospholipase D
NK cells	natural killer cells
NO	nitric oxide
OEA	*N*-oleoylethanolamide
OR	oocyte retrieval
PCR	polymerase chain reaction
PEA	*N*- palmitoylethanolamide
PG	prostaglandin
P4	progesterone
PRA	progesterone receptor A
PRB	progesterone receptor B
QC	quality control
qRT-PCR	quantitative reverse transcription polymerase chain reaction
R&D	Research and Development
RNA	ribonucleic acid
RNAse	ribonuclease
ROUT	regression and outlier removal
RT	reverse transcription
SD	standard deviation
SNAP	S-nitroso-N-acetylpenicillamine
TCR	T cell receptor
TNF-b	tumour necrosis factor beta
TRPV1	transient receptor potential vanilloid 1
Th1	T helper 1 cell
Th2	T helper 2 cell
Th17	T helper 17 cell
TGF	tumour growth factor
Treg	T regulatory cells
UHPLC	ultra high-performance liquid chromatography
UPLC-MS/MS	ultra-performance liquid chromatography tandem mass-spectrometry
uNK cells	uterine natural killer cells

1 Introduction

The National Institute for Health and Care Excellence (NICE) defines infertility as "failure to conceive after regular unprotected sexual intercourse for 12 months in the absence of known reproductive pathology". Implantation failure is considered a major cause of infertility in otherwise healthy women, with inadequate uterine receptivity considered to be responsible for approximately two thirds of implantation failures and problems with the embryo itself being responsible for the remainder. In fact, it has been estimated that only 30% of eggs that contact sperm result in successful human term pregnancies (Lim and Wang 2010).

For the majority of infertile couples, procedures such as *in-vitro* fertilisation (IVF) or intracytoplasmic sperm injection (ICSI) offer their only hope of having a baby. Data from the Human Fertilisation and Embryology Authority (HFEA) show that success rates following these procedures for UK couples over the last decade has remained around 25% after embryo transfer. Failure to significantly improve pregnancy rates is due largely to poor understanding of the precise physiological mechanisms regulating implantation.

1.1 Normal Process of Implantation

Implantation has three stages; apposition, adhesion and penetration. During apposition, the blastocyst comes into close proximity with the endometrial luminal surface and a dialogue between the polarised trophoblast and luminal epithelial cells of the endometrium is initiated. When the blastocyst comes into close contact with the endometrium, it interacts with bleb-like protrusions that have developed on the apical surface of the endometrium (Usadi et al. 2003). These blebs, known as pinopodes, appear to be progesterone dependent and their development is linked to the plasma mid-luteal increase in progesterone level (Achache and Revel 2006, Stavreus-Evers et al. 2001). Endometrial pinopode development is also associated with an increased expression of leukaemia inhibitory factor (LIF) and its receptor (Aghajanova et al. 2003), as well as the integrin $\alpha V\beta 3$ (Lessey et al. 1992), and is dependent on the activation of the homeobox transcription factor, HOXA-10 (Bagot et al. 2001). Although the exact role of pinopodes remains unknown, it seems that they are the preferred sites of embryo-endometrial interactions and blastocyst attachment has been shown to occur above endometrial pinopodes that have undergone cycle-dependent morphological changes (Achache and Revel 2006). These changes are only present for a maximum of two days of the menstrual cycle (Rashid et al. 2011), usually 2-4 days after ovulation in women, and 24-36 hours after ovulation in mice. As apposition completes, microvilli on both trophoblast and endometrial surfaces interdigitate and the pinopodes are withdrawn.

Following apposition, the blastocyst needs to adhere tightly to the endometrium, otherwise it will detach and 'float' around the uterus. During this period of adhesion, the microvilli disappear and glycoproteins (especially the integrins and cadherins) are produced, leading to increased cell-to-cell contact over a large surface area. The role of these glycoproteins is to firmly attach the blastocyst to the endometrial epithelial cell surface (Paria et al. 2002).

The final stage of implantation, penetration, involves contraction of microfilaments in the trophoblast, which permits the blastocyst to migrate between endometrial cells. At the same time, a newly specialised cell layer generated from the underlying trophoblast layer, the syncytiotrophoblast, forms and synthesis of syncytiotrophoblast-specific proteins

(including human chorionic gonadotrophin (hCG)) begins. This promotes two main events: the establishment and maintenance of the corpus luteum (in the ovary) and the development of decidual cells, which 'pump' out high levels of insulin-like growth factor binding protein 1 (IGFBP-1) and prolactin to support the on-going pregnancy.

To ensure that implantation occurs before the nutrients obtained from the uterine secretions become inadequate, these steps tend to happen when the blastocyst is relatively small, i.e. when only a few trophoblast cells are involved in making contact with the maternal epithelium.

At a point 5-7 days after ovulation there is a noticeable increase in mucin, glycogen and glycoproteins in the glandular lumen and by 9-10 days following ovulation there is marked stromal differentiation whereby the cells around the spiral arteries become plump and glycogen rich. Concomitantly, there is associated oedema and localised changes in the intercellular matrix, with progressive sprouting and in-growth of capillaries (vasculogenesis) (Matsumoto et al. 2002). These changes are known as the "primary decidualisation reaction" – a process that is particularly marked in primates and rodents. If pregnancy occurs, the decidual changes in the stroma become much more extensive and can be clearly divided into 3 discrete layers: decidua compacta (comprising of decidualised stroma and non-secretory glands, decidua spongiosa (found below the decidua compacta with dilated secretory glands) and the basal layer (undifferentiated cells which regenerate following menstruation/parturition).

As the trophoblastic tissue invades further into the uterine glandular tissue and decidual tissue, the adjacent cells are destroyed, primarily through apoptosis. This results in the release of primary metabolic substrates, which are taken up by the growing conceptus (acting as a "yolk reservoir"). The goal of this trophoblastic invasion is to find the uterine spiral arteries and plug them to prevent the inflow of maternal blood into the developing implantation site, because too much oxygen at this stage (and the free radicals arising from that oxygen) could damage the developing embryo.

1.2 Placentation

Following fertilisation and rapid cell division, the conceptus in successful pregnancies needs a controlled supply of oxygen and metabolic substrates in order to grow and develop (Berlanga et al. 2011). Initially it obtains these from uterine secretions; primarily the endometrial glands with some material produced by the endometrial stroma that transforms into the decidua (Carter and Enders 2004). These organic molecules and ions are supplied through specific transport mechanisms, while the exchange of oxygen and carbon dioxide is diffusional. There is, however, a limit to the size that a free-living conceptus can reach before these supplies become inadequate to maintain survival. At this critical point, a suitable mechanism for supplying these nutrients must be established for implantation to be successful (Rossant and Cross 2001).

The exact point in the reproductive cycle at which this occurs varies from mammal to mammal, but one common theme is that the implanted conceptus needs to develop its own blood supply through the generation of a local vascular system. It is through this vasculogenesis that essential metabolites at the extra-embryonic surface are exchanged and subsequently distributed throughout the conceptus' own developing tissues (Moore 2012). Simultaneously, waste products are returned to the maternal system and excreted by the mother. To do this, mammals have developed a unique organ that regulates this process, called the placenta. The main functional cell in the early placenta, the trophoblast, develops as the conceptus reaches the blastocyst stage.

Evolution of viviparity (the bearing of live young) in vertebrates, including fish, reptiles and mammals, occurred to provide protection of offspring from cold, inhospitable environments and from predators. The spectrum of viviparity ranges from a mother simply holding yolky eggs in her body until they hatch (ovoviviparity) to the development of a complex placenta that extracts nutrients from the mother (Luckett 1976).

At the simplest level, a placenta is formed when fetal membranes become closely attached to the uterine wall facilitating exchange of gases, nutrients and waste products. The transition from ovoviviparity to viviparity started with the evolution of the anamniote embryo. Here, the embryo developed outside of the mother, but it was necessary that this

occurred in water (or a moist habitat). Evolution from the anamniote to the amniote egg included the development of four extra-embryonic membranes; the yolk sac, amnion, chorion and allantois. These provided an interface between the embryo and its immediate environment and allowed eggs to be laid on dry land, while still enabling gas diffusion and waste excretion as well as nutrient provision. In the majority of mammals, the eggshell was subsequently lost, and only minor modifications were required for the evolution of the placenta we see today (Moffett and Loke 2006).

In the mouse and human, the formation of the placenta begins when the embryo reaches the "blastocyst stage" where there is organisation of the cells into an outer layer (which will form the trophoblast), a blastocoelic fluid and an inner cell mass (ICM) (Moffett and Loke 2006, Soares et al. 2012). Whilst the ICM goes on to form the embryonic disc (which will eventually give rise to the definitive structures of the fetus), the outer layer penetrates the maternal endometrium, becoming embedded in the uterine stroma. This occurs 6-7 days after ovulation and at this point a layer of "trophoectoderm" cells encircle the blastocyst. By day 8, the trophoblast has differentiated into two layers, the cytotrophoblast (a single celled, inner layer) and the syncytiotrophoblast (a thickened outer layer that invades the endometrial stroma). The syncytial later penetrates between uterine epithelial cells. The initial development of the uteroplacental circulation occurs once the syncytiotrophoblast have invaded the maternal venous capillaries (Nel-Themaat and Nagy 2011).

After this initial phase of implantation, trophoblast cells further differentiate into two discreet entities: villous and extravillous cells. The villous trophoblast eventually becomes chorionic villi and functions to transport oxygen and nutrients from the mother to the child. In contrast, the extravillous trophoblast migrates deep into the uterine mucosa (as far as the myometrium) (Yoshinaga 2010).

In all species, the trophoblast cells are always the outermost layer of fetal cells overlying an inner core of mesenchyme and fetal capillaries, although one of the most obvious differences between species is the extent of invasion of trophoblast cells into the uterus. This can range from no invasion, as seen in horses and whales, to very extensive invasion, as seen in humans, rodents and rabbits.

The least invasive form is epitheliochorial placentation, where trophoblast cells are in direct apposition with the surface epithelial cells of the uterus and there is no trophoblast-cell invasion beyond this layer. A slightly more invasive form is endotheliochorial placentation, where the uterine epithelium is breached and trophoblast cells are in direct contact with endothelial cells of maternal uterine blood vessels. The most invasive form is haemochorial placentation, where maternal uterine blood vessels are infiltrated by trophoblast cells causing rupture and release of blood into the intervillous space resulting in the syncytiotrophoblast being bathed in blood. The different forms of placentation are depicted in Figure 1-1.

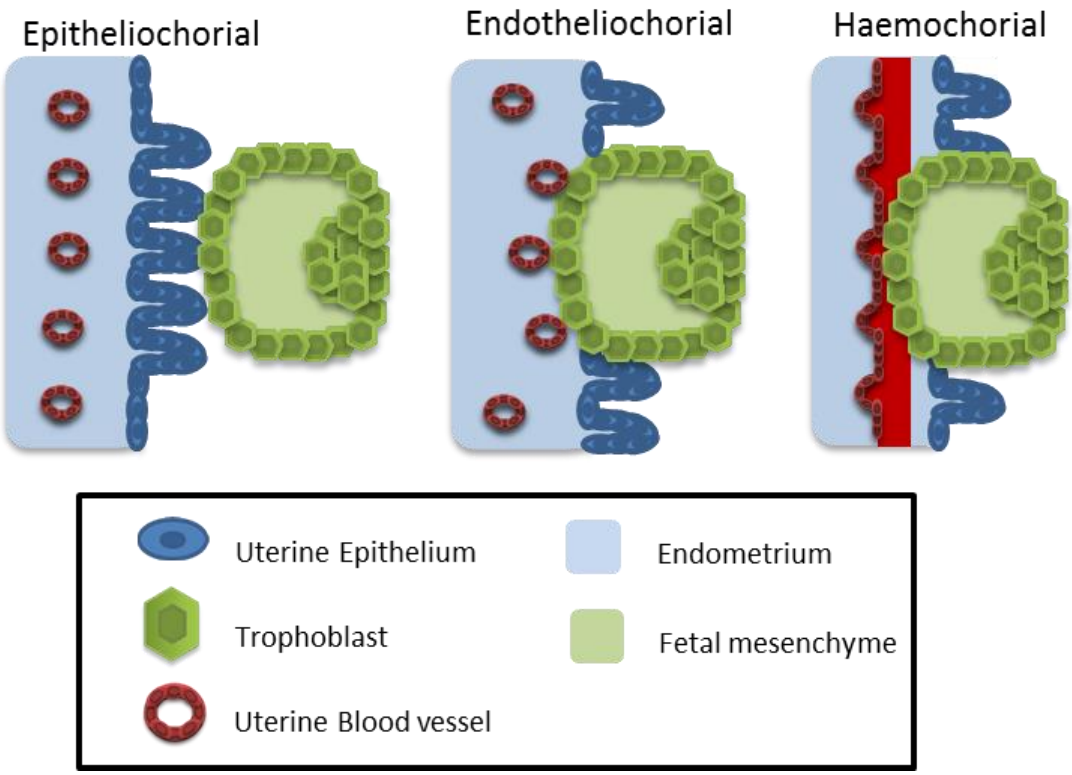

Figure 1-1 Different forms of placentation

Figure 1-1 shows the different degrees of placentation, ranging from the least invasive form (epitheliochorial placentation) where trophoblast cells are in direct apposition with the surface epithelial cells of the uterus with no invasion, to the most invasive form (haemochorial placentation), where maternal uterine blood vessels are infiltrated by trophoblast cells causing rupture and release of blood into the intervillous space.

With such diversity in placentation, it is obvious that the mechanisms for increasing the blood flow to the feto-placental unit are completely different. In epitheliochorial placentation, for example, this is achieved by the expansion of the size of the vascular bed in the uterus by angiogenesis, whereas in haemochorial placentation, there is lowering of resistance in the vessels of the placental bed caused by modification of the walls of pre-existing arteries, resulting in an increased low-pressure blood flow system (Moffett, Loke 2006).

While both humans and mice have haemochorial placentae, one fundamental difference between human and rodents is the depth to which the epithelium is invaded by the blastocyst. Human (as well as chimpanzee and guinea pig) placentae implant interstitially; that is to say, the conceptus invades the stoma so deeply that the surface epithelium becomes restored over it. When comparing the depth of placental invasion for all mammals with interstitial implantation, human placentae are found to be particularly invasive. In contrast, rodents, cats, dogs and the rhesus monkey conceptuses implant eccentrically; the stroma is only partially invaded and the conceptus continues to project to varying degrees into the uterine lumen.

The overall effect though is that haemochorial placentae provide the fetus with easy access to nutrients directly from the maternal blood. However, the disadvantage of this form of placentation is that the mother and fetus are no longer separated by an intact layer of epithelial cells allowing exposure of the trophoblast cells to potential allogenic immune responses by the mother (Hannan et al. 2011).

1.3 Immune responses to implantation

It therefore follows that as well as allowing exchange of gases, nutrients and waste products, another important function of the placenta is the regulation of the maternal immune response thereby ensuring the fetal semi-allograft is tolerated during pregnancy (Lee et al. 2011). Once the basement membrane of the endometrium is breached, trophoblast cells will have direct contact with decidual cells, as well as other components of stromal tissue, such as fibroblasts, macrophages and T cells, and because of this, trophoblast cells are presumed to be key in preventing rejection of the fetal semi-allograft (Lee et al. 2011, Bambang et al. 2012). In primates, trophoblast cells encounter the maternal immune system in two ways – (i) the villous trophoblast cells interact with the maternal blood and (ii) the extravillous trophoblast (EVT) cells interact with the uterine tissue. The ability to evade the maternal immune response is critical for assisted reproductive technology (ART), which uses donated embryos; in these cases the fetus is a complete allograft, with no maternal genes whatsoever, yet the pregnancy is still able to establish without an increased rate of miscarriage (Saito et al. 2007).

1.3.1 Major Histocompatibility Complex

The important components of the immune system in this regard are the major histocompatibility complexes (MHC) class I and II. These are responsible for presenting peptide antigens to antigen receptors on surveillance immune cells (King et al. 1998). MHC class I proteins are expressed on all nucleated somatic cells and can be divided into two broad groups; classical and non-classical. Classical MHC class I genes are highly polymorphic and encode proteins that are expressed in most somatic cells. They present peptides from intracellular pathogens ("non-self"), along with "self" cells, to T cell receptors (TCRs) on T lymphocytes. Non-classical class I genes have diverse functions, but one important function is to act as ligands for inhibitory leukocyte receptors, including receptors encoded within the natural killer complex. In contrast, MHC class II proteins are only expressed on professional antigen presenting cells (e.g. dendritic cells, macrophages and B cells) (Parham et al. 2012).

Classical MHC Class I and II genes are the most polymorphic genes in the human genome. This high degree of variation is thought to have arisen through pathogen-driven selection and thus provides increased protection from multiple pathogens.

In humans, the MHC system is also known as the human leukocyte antigen (HLA) system (Parham et al. 2012). Focussing on its involvement in implantation, the repertoire of trophoblast HLA class I expression is unique (Chaouat 2001, Moffett and Loke 2006), as shown in table 1-1. There is no expression of highly polymorphic HLA-A or HLA-B molecules, which function mainly as T cell ligands. There is, however, expression of oligomorphic non-classical HLA class I molecules, HLA-G and HLA-E (Saito et al. 2007, Parham et al. 2012). The only polymorphic HLA molecule expressed is HLA-C, and this is predominantly expressed in extravillous trophoblast. This lack of polymorphism is thought to offer the embryo some protection from the maternal immune system.

Table 1-1 The number of different variant of HLA Class I molecules

Isotype	Number of Variants
HLA-A	1243
HLA-B	1737
HLA-C	**884**
HLA-E	**3**
HLA-F	4
HLA-G	**15**

Highlighted are the HLA molecules expressed by the placenta.

1.3.2 Cytokines

For an immune response mediated by T cell activation to occur, several interactions between the antigen and the TCR have to take place in a co-ordinated manner. Firstly, the antigen must be presented to the T cell as a peptide fragment within the groove of the MHC molecule. This is necessary because the TCR will only recognise the combined shape of the foreign antigen together with self-MHC; free antigen will have no effect. For some time it was believed that CD8+ (cytotoxic) T cells recognised antigen with class I MHC and CD4+ (helper) T cells were more limited and could only be stimulated by cells that bear MHC class II (Parham et al. 2012). The class I MHC molecule is present on all nucleated somatic cells (Davies 2007) and so nearly all cells can present to cytotoxic T cells. In contrast, class II MHC molecules expression is limited to antigen presenting cells (APCs)(Parham et al. 2012). However, it has been shown that HLA-G antigens are able to activate CD4+ as well as CD8+ T cells (van der Meer et al. 2007).

Triggered CD4+ T cells, also known as T-helper cells, are involved in orchestrating the immune response (Piccinni 2010). They cannot directly destroy their target, but recognise specific foreign antigen and proceed to activate other parts of the system which can eradicate it. T-helper cells have been categorised into three major functional subpopulations based on their pattern of cytokine production: T-helper 1 (Th1) and T helper 2 (Th2) and the more recently discovered T-helper 17 (Th17). Table 1-2

summaries the actions of the three classes of T-helper cells, namely the cytokines produced along with the specific immune cells that are activated and responses initiated.

Table 1-2 The cytokines produced by T-helper cells and their effects.

	Cytokines produced	Cells activated by cytokines	Response initiated
Th1	IL-2 IL-3 INFγ TNF-β	Cytotoxic T cells Natural Killer cells Macrophages	Cell mediated Inflammatory
Th2	IL-4 IL-5 IL-6 IL-10	B cells	Antibody release
Th17	IL-17 IL-21 IL-22 IL-26	Neutrophils	Autoimmune based inflammation

Another key component of the immune system involved in embryo implantation are T regulatory cells (also known as Treg cells), which are CD4+ cells that co-express CD25 (and so are sometimes referred to as CD4+CD25+ T cells) (Bambang et al. 2012). The normal response of activated Treg cells are able to suppress CD4+ (and CD8+) T cell responses and thus reduce the number of Th1 and Th2 cytokines produced (Thornton 2005). Women with recurrent miscarriages and unexplained infertility have been both found to have decreased levels of Treg cells (Sasaki et al. 2004, Jasper et al. 2006).

Chaouat et al. (2007) concluded that while Th1-type cytokines (pro-inflammatory) appear to be key determinants of the attachment and adhesion stages of implantation, an excess of pre-implantation Th1 cytokines might induce early pregnancy loss. Th1 cytokines include interferon gamma (IFNγ) and transforming growth factor beta (TGF-β), both of which are known to play important roles in enhancing implantation (Lee et al. 2011). IFNγ has been shown to stimulate changes to the vasculature of the endometrium, that in turn, improves implantation success, while TGF-βs are well known to be immuno-suppressive and also to act on both leukaemia inhibitory factor (LIF; see below) and interleukin-6 (IL-6) pathways. IL-6 (along with interleukin-1 (IL-1)) exerts a strong stimulatory effect on endometrial expression of corticotrophin releasing factor (CRF), which is known to induce decidualisation of endometrial stromal cells (Ferrari et al. 1995). Female rats treated with antalarmin, a CRF receptor antagonist, showed a marked reduction in implantation and live embryos (Makrigiannakis et al. 2001).

While able to demonstrate the importance of the pro-inflammatory Th1-type cytokines, Chaouat et al. (2002) did not demonstrate a mandatory requirement for Th2-type cytokines (anti-inflammatory) in the implantation process. Further supporting the notion that implantation is a pro-inflammatory condition, Zhou et al. (2008) and Barash et al. (2003) have both demonstrated that wounding the endometrium with a biopsy catheter actually seems to improve embryo implantation (Revel 2009). Th17 may play a role in inflammation but its role in implantation is not yet fully defined (Nakashima et al. 2010a, Nakashima et al. 2010b, Saito et al. 2010, Lee et al. 2011).

Conversely, for stages of pregnancy after implantation, Th1 cytokines are thought to compromise pregnancy success, whereas Th2 cytokines (by inhibiting Th1 responses) promote allograft tolerance and therefore may improve fetal survival (Piccinni 2010, Lim et al. 2000, Lee et al. 2011). As with many of the components involved in implantation, there are no “good” or “bad” cytokines, but instead it is the balance of production in conjunction with timing of production that affect all stages of pregnancy.

1.3.3 Natural Killer Cells

NK cells are part of the lymphoid lineage, and like T and B lymphocytes, can be divided into several sub-populations. The NK cells present in the uterus (uNK) are phenotypically and functionally different from those present in the systemic circulation (King et al. 1998, Lee et al. 2011). uNK cells are the main immune maternal cells present in the decidualised endometrium prior to and during the establishment of the placenta in species with invasive haemochorial placentation (Moffett and Loke 2006); it has been estimated that up to 60-80% of the peri-implantation uterus stroma in mice and humans is comprised of uNK cells, which is more lymphocytes than are present in some lymph nodes (Chaouat et al. 2007, King et al. 1998)! NK cells are activated by cytokines produced by Th1 cells.

NK cells display killer-cell immunoglobulin-like receptors (KIRs) that become activated continuously by the recognition of endogenous self-MHC, and this activation inhibits lysis. Lack of MHC (i.e. in a "non-self" cell) deactivates KIR and thus activates NK cells to destroy the MHC negative cell (Parham et al. 2012). Although very few cells express HLA-G (trophoblast and thymus), all NK cells express the HLA-G binding KIR, KIR2DL4 (Moffett and Loke 2006). This helps prevent human fetal rejection by NK cells as the trophoblast becomes less sensitive to NK-medicated lysis (although 100% inhibition of NK medicated lysis has not been observed) (Chaouat 2001).

Uterine NK cells secrete several angiogenic factors including angiopoietin 2 and vascular endothelial growth factor (VEGF), which in turn stimulate the thickening of the vascular walls to ensure successful implantation (Lee et al. 2011). While mice lacking uNK cells are fertile, they display inadequate uterine vascular remodelling during pregnancy, poor decidualisation and low fetal weight, highlighting the importance of uNK cells in implantation (Colucci et al. 2011). These findings have been investigated further, reaching the conclusion that in NK+/+ mice, IFNγ activates NK cells which in turn stimulates vascular changes as detailed above, whereas in NK-/- mice, IFNγ stimulates the vascular changes directly.

In mice, the presence of uterine NK cells in the media of the arteries indicated that they might have a direct physiological role in regulating the blood pressure and flow in the

placenta (Moffett and Loke 2006). Mouse uNK cells might also indirectly modify blood flow through an effect on trophoblast cell behaviour. It is not yet clear whether uterine NK cells have the same role or use the same molecular mechanisms in mice and humans (Moffett and Loke 2006).

1.4 The window of implantation

Implantation can only occur successfully during the "window of implantation"; a critical time period when both the uterine environment is receptive to blastocyst implantation and the blastocyst is in an "active" state.

The window of implantation varies greatly between different species, for example, In humans it is considered to be 5-9 days after ovulation, i.e. in the middle of the luteal phase of the normal menstrual cycle (days 20-24) (Wilcox et al. 1999) and spans a three-day period, whereas in mice it lasts for 24 hours and occurs on day 4 of their cycle.

For the human uterus to achieve a "receptive state", it first requires a pre-ovulatory increase in estrogen secretion (Norwitz et al. 2001), primarily in the form of 17β-estradiol (E2), to stimulate proliferation and differentiation of uterine epithelial cells. Next, it needs a continuous production of progesterone (by the corpus luteum) to enable further proliferation and differentiation of the E2-primed stromal cells into decidual cells (Salamonsen et al. 2003). High levels of progesterone also inhibit gonadotrophin releasing hormone (GnRH) secretion by the hypothalamus, resulting in a reduction of luteinising (LH) and follicle stimulating hormone (FSH) and thus preventing further ovulation. With successful implantation, hCG secreted by the embryo ensures the corpus luteum continues to secrete progesterone until the placenta is able to support the pregnancy itself.

The uterus of the mouse achieves a receptive state in much the same way as the human uterus; by the co-ordinated effects of estrogen and progesterone (Song et al. 2000). In contrast, the ovulatory cycle of small rodents, such as mice and rats, is considered to be an "incomplete cycle" in that ovulation occurs every 4-5 days (Caligioni 2009) but the corpus luteum formed is not fully functional, and its inhibitory effect on the secretion of GnRH (and thus LH and FSH) only occurs following mechanical stimulation of the cervix (Milligan 1975, Smith et al. 1975).

Studies have identified both estrogen (ERα and ERβ) and progesterone (PRA and PRB) receptors in the endometrium. Both glandular and stroma cell expression of ERs increase

until ovulation and then gradually decline until it is undetectable by the mid-luteal phase of the cycle. The expression of PRs in both the stroma and glands also increases during the proliferation phase, and is maximal at the time of ovulation, followed by a rapid fall in levels found in the glands but minimal change in the expression of receptors in the stroma (Lessey et al. 1988, Snijders et al. 1992, Mylonas et al. 2004, Young 2013). In pregnancy, there continues to be an absence of expression of ERs or PRs in the glandular epithelial cells, but expression of PRs has been noted in stromal cells and in the endothelial and medial cells of spiral arteries (Loke and King 1995, Perrot-Applanat et al. 1994).

When considering the uterine environment, it is worth mentioning that humans have a menstrual cycle and the mouse has an estrous cycle. Both are under the influence of the hypothalamic-pituitary axis, but while humans shed the endometrial lining at the end of each cycle, mice reabsorb the endometrium if conception does not occur during that cycle. Interestingly, decidualisation occurs in humans in the absence of an embryo (although the extent of the reaction is enhanced by implantation) whereas the presence of an embryo is necessary for the same reaction in rodents (Salamonsen et al. 2003). This suggests that slightly different mechanisms are in place during the period of implantation in mice and women. While progesterone has been shown as necessary for decidualisation, Das et al. (2009) demonstrated that estrogen is not essential for this process, but it is critical for uterine angiogenesis during this time.

The receptive uterus can only accept an "active" blastocyst. Paria et al. (1993) demonstrated that it is a metabolite of E2 that causes the blastocyst to become activated for implantation. This estrogen, 4-OH-E2, is formed by aromatic hydroxylation of phenolic estrogen (produced by the ovary) at C-4 and activates dormant blastocysts via generation of prostaglandins (with the induction of uterine COX-2 at the sites of blastocyst apposition).

It is known that elimination of pre-implantation E2 secretion results in implantation failure (Das et al. 2009), but in these situations the blastocyst can sometimes remain dormant in the quiescent uterine lumen. As well as being demonstrated under laboratory conditions (Wang et al. 2006), it can also be observed in nature. Suppression of endogenous estrogens in rats that are suckling the previous litter prevents further

pregnancies, a process known as facultative delayed implantation. In many species (e.g. badger, elephant seal, stoat and brown bear) an obligatory delayed implantation is necessary to allow the animals to mate in the summer when the adults are well fed and in their prime, but for the pregnancy to be delayed to allow parturition to occur in the following summer when there will be plentiful food supplies available (Moffett and Loke 2006).

Highlighting the interplay between the endometrium and blastocyst, Paria et al. (1993) demonstrated that the "window of implantation" in the mouse uterus remains open for less than a day when exposed to "active" blastocysts, and for an even a shorter time when exposed to "dormant" blastocysts. Even after activating dormant blastocysts with E2, the "window of implantation" was shorter when compared with blastocysts that had remained active throughout the implantation process. They also demonstrated that unless the endometrium was receptive (that is to say, it had been primed with progesterone and then activated with E2) active blastocysts failed to implant.

Progesterone plays an equally important role in successful implantation, and progesterone-receptor antagonists readily induce abortion, if given before seven weeks of gestation (Peyron et al. 1993). Early luteal phase administration of mifepristone (an anti-progestogen) induces desynchronisation of endometrial development and repression of glandular secretory differentiation and vascular maturation (Marions and Danielsson 1999). It has also been shown that treatment with mifepristone during the luteal phase of the menstrual cycle affects levels of prostaglandin F2α, prostaglandin PGE2 and COX in the human endometrium (Gemzell-Danielsson and Hamberg 1994, Nayak et al. 1998). In addition, pinopode development is closely linked to progesterone concentrations (Stavreus-Evers et al. 2001).

While the importance of E2 and progesterone in implantation are best understood, they are far from the sole components influencing the success of this process. It is well documented that many components secreted by both the embryo and the endometrium play an important part in implantation, and embryo-endometrial interactions start before the embryo even attaches to the endometrial epithelial surface. For example, it is known that the embryo secretes frucosylated oligosaccharides, such as ghrelin and LIF, and the

endometrium expresses receptors for these ligands. Similarly, the endometrium secretes heparin-binding EGF-like growth factor (HB-EGF), insulin-like growth factor and LIF that all interact with receptors expressed by the embryo (Aghajanova 2010).

The medium in which pre-implantation embryos have be cultured has been shown to contain a variety of other active substances, including tumour growth factor (TGF) α and β, platelet-derived growth factor, colony-stimulating factor (CSF) 1, interleukin-1, interleukin-6, prostaglandin E2 and platelet activating-factor (Stewart and Cullinan 1997), all of which play a role in implantation. Another family that has been shown to play a part in this role is the endocannabinoid system.

1.5 Endocannabinoid System

Endocannabinoids have been identified as playing an important role in successful implantation (Habayeb et al. 2002, Taylor et al. 2007, Sun and Dey 2008, Maccarrone 2009, Bambang et al. 2010). They are unsaturated fatty acid derivatives of arachidonic acid that act as endogenous ligands for cannabinoid receptors that also bind exocannabinoids/ phytocannabinoids from the *Cannabis sativa* plant.

Preparations of the *Cannabis sativa* plant are some of the most commonly used illegal drugs and exposure to these has long been associated with adverse pregnancy outcomes, including miscarriage and prematurity (Park et al. 2004). Δ9–tetrahydrocannabinol (Δ9-THC) for example, the main psychoactive component in marijuana, inhibits ovulation by suppressing FSH and the pre-ovulation FSH surge (Ayalon et al. 1977), possibly through the hypothalamic inhibition of GnRH secretion. Mice treated with Δ9-THC demonstrate several defects in embryo development and implantation, such as oviductal retention, asynchronous development and signs of apoptosis in embryos and implantation failure (Paria et al. 2001).

Following the identification of a receptor that binds to exogenous cannabinoids, such as Δ9-THC, the identity of an endogenous ligand was sought. Arachidonylethanolamide (also known as anandamide, AEA) was discovered in the 1990s (Devane et al. 1992), followed quickly by the discovery of another endocannabinoid, 2-arachidonoylglycerol (2-AG). AEA and 2-AG are the most studied cannabinoid receptor agonists.

While exploring the importance of endocannabinoids with regards to implantation, it is pertinent to mention the involvement of *N*-acylethanolamines (NAEs). The two endocannabinoids, AEA and 2-AG, are both derivatives of arachidonic acid and 2-AG possesses an ester while AEA is an *N*-acylethanolamine. Other members of the NAE family have been identified, including *N*-palmitoylethanolamine (PEA), *N*-oleoylethanolamine (OEA), *N*-stearoylethanolamine (SEA) and *N*-linoleoylethanolamide (Hanus et al. 1993, Wang and Ueda 2009).

1.5.1 Cannabinoid Receptors

There are currently two well-documented cannabinoid receptors. The first, CB1, was first described and characterised in the rat brain (Devane et al. 1988). It has since been shown that human CB1 shares 97.3% sequence identity with the rat CB1, and 100% identity within the transmembrane regions (Gérard et al. 1991). The second cannabinoid receptor, CB2, was first identified in splenic cells and was shown to share 48% identity with CB1 (with 68% identity within the trans-membrane regions) (Munro et al. 1993, Habayeb et al. 2002).

It is now known that both CB1 and CB2 are expressed in a multitude of tissues other than the brain and spleen, but for the purpose of this thesis the discussion will be limited to their expression in the reproductive tract. While in humans it has been shown that CB1 and CB2 are expressed in the fallopian tubes and uterus (Taylor et al. 2010, Gebeh et al. 2012), in murine models only CB1 is expressed (Das et al. 1995). Animal studies have also shown that both CB1 and CB2 are expressed in pre-implantation embryos; CB1 mRNA is primarily detected from the four-cell stage through to the blastocyst stage, whereas CB2 is present from the one-cell through to the blastocyst stage (Paria et al. 1995, S K Das et al. 1995, Wang et al. 2004). Unfortunately, due to ethical considerations, the same studies have not been conducted on human embryos.

Both CB1 and CB2 are G protein-coupled receptors with seven transmembrane domains, and belong to the Gi/o family (Sun and Dey 2008, Wang et al. 2003, Wang and Ueda 2009). Binding of endocannabinoids to either of these receptors can trigger a whole host of signalling cascades, including regulation of Ca^{2+} channels, stimulation of mitogen-activated protein kinase (MAPK) and activation of cytosolic phospholipase A_2 (cPLA2) (Wang et al. 2003, Sun and Dey 2008, Wang and Ueda 2009). They also have some opposing actions, for example, CB1 causes activation of inducible nitric oxide synthase (iNOS) whereas CB2 causes inhibition of the same pathway (Sordelli et al. 2011). The signalling cascades triggered by binding to CB1 and CB2 all have an impact on implantation.

There is also emerging evidence of a possible third endocannabinoid receptor, known as CB3 or GPR55 (McPartland et al. 2006). CB3 has been shown to bind endocannabinoids at an extracellular site (as do CB1 and CB2) but shares low sequence homology (10-15%) with the classical CB1 and CB2 (Maccarrone 2009). Agonist binding to CB3 triggers activation of the small GTPase proteins phoA, rac and cdc44 which, once activated, interact with adenylyl cyclase and thus join the G protein signalling cascade (Ross 2009). With regards to early pregnancy events, CB3 has linked to the decidual remodelling process necessary for placental development (Fonseca et al. 2011).

A "non-cannabinoid" receptor for AEA has also been identified (Van Der Stelt and Di Marzo 2004) called the transient receptor potential vanilloid 1 (TPRV1), which is a ligand gated, six-trans-membrane spanning protein that is activated by molecules derived from plants, such as capsaicin (the pungent component of "hot" red peppers). Although a number of studies Ralevic et al. 2001, Andersson et al. 2002, Jerman et al. 2002, Lam et al. 2005) have suggested a physiological role for AEA as a TRPV1 agonist, the effects seem to be limited to the nervous system, and not related to the reproductive system, even though it is present in the uterus, decidua and trophoblast.

AEA and its interaction with the cannabinoid receptor CB1 is believed to play an important role in facilitating successful embryo implantation (Yang et al. 1996b, Paria et al. 1998, Paria and Dey 2000) (aka absence of CB1 results in implantation failure), whereas Paria et al. (1998) did not find the same involvement of CB2. While 2-AG is also known to interact with the cannabinoid receptors, its exact role is still under investigation (Battista et al. 2012).

1.5.2 Synthesis

Endocannabinoids were considered to be generated 'on demand' from long chain polyunsaturated fatty acid precursors and can act in both an autocrine or paracrine fashion (Guo et al. 2005), as well as following release into the lymphatic system/blood stream (Taylor et al. 2007). However, recent evidence suggests that they are stored and transported around the cell by fatty acid binding proteins (Kaczocha et al. 2009).

N-acylphosphatidylethanolamine (NAPEs) are thought to be the precursors for NAEs. When considering the synthesis of AEA, the main enzyme involved in this process is NAPE-PLD, a type-D phospholipase (PLD), which hydrolyses NAPE to AEA (Guo et al. 2005). However, NAPE-PLD knockout mice have been shown to have wild-type levels of AEA in their brains, with only a moderate decrease in levels of OEA and PEA, highlighting the possibility of additional pathways responsible for NAE (especially AEA) synthesis. These pathways were later identified; the first involving the serine hydrolase ABHD4, and the second involving the phosphatase, protein tyrosine phosphatase PTPN22 (Simon and Cravatt 2006, Muccioli 2010). Figure 1-2 is a schematic representation of the synthesis of NAE.

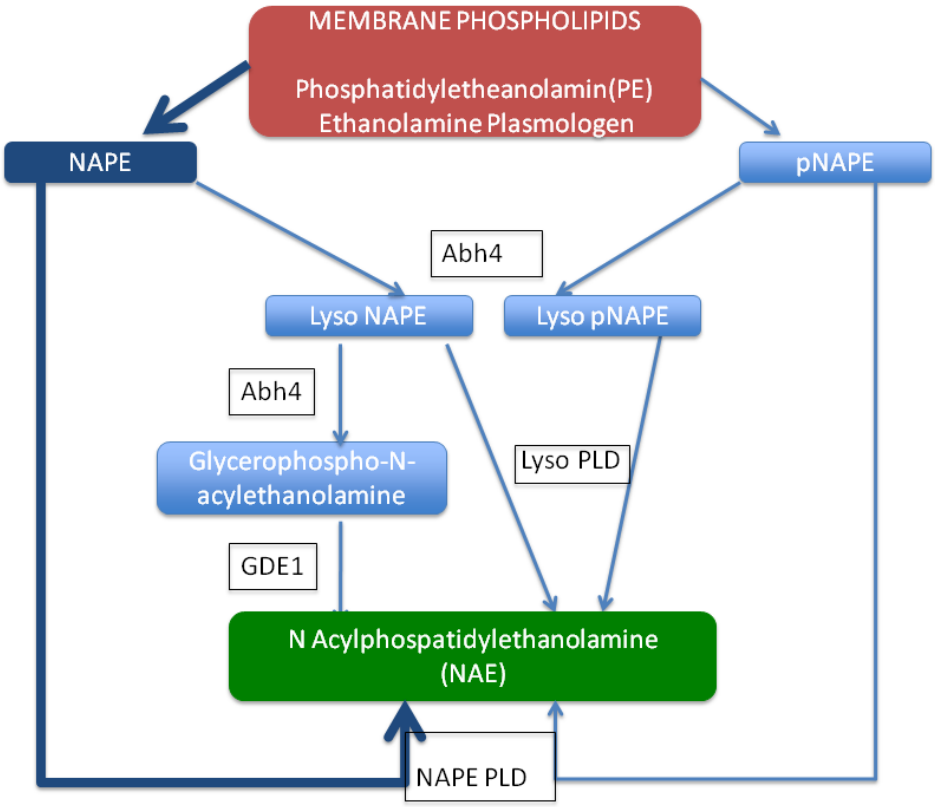

Figure 1-2 Proposed pathways for the synthesis of NAEs, with NAPE-PLD thought to be the main route

A schematic representation of the various proposed routes of NAE synthesis.
Key: Red boxes are precursors; Blue boxes are intermediate compounds; green boxes indicate the final product; White boxes are the enzymes involved. Thicker arrows indicate the dominant pathways to NAE synthesis in most cells. NAPE = N-acyl phosphatidylethanolamine; pNAPE = N-acylethanolamine plasmalogen; Lyso NAPE = 1-alkenyl-2-hydroxy-glycero-3-phospho (N-acyl) ethanolamines; Abh4 = alpha/beta-hydrolase 4; GDE1 = Glycerophosphodiesterase; NAPE PLD = N-acyl phosphatidylethanolamine-specific phospholipase D.

1.5.3 Degradation

Anandamide is catabolised into ethanolamine and arachidonic acid (the precursor for prostaglandin synthesis). The first enzyme identified as being involved in the degradation of AEA was fatty acid amide hydrolase (FAAH) and Maccarrone et al. (2001) have shown that lymphocyte FAAH is stimulated by progesterone and Th2 cytokines. Most studies investigating this enzyme have been conducted in mice, with murine FAAH sharing 80% sequence identify with human FAAH (Giang and Cravatt 1997).

A gene that encodes a second FAAH ("FAAH-2") enzyme has been found in multiple primate genomes, marsupials and more distantly related vertebrates but interestingly has not been identified in the mouse or rat genome (Wei et al. 2006). The original FAAH (FAAH-1) and FAAH-2 share ~20% sequence identity across their entire primary structure (Wei et al. 2006). Unlike FAAH-1, FAAH-2 is localised in cytosolic lipid droplets, and is less efficient at hydrolysing NAEs. It has been found to have a high expression in peripheral tissues and because of this it has been suggested that FAAH-2 might have a "rescue role" in hydrolysing NAEs upon FAAH-1 inactivation (Muccioli 2010).

Following the demonstration that blastocyst culture medium does not have the ability to hydrolyse AEA, it has been proposed that the blastocyst produces a "FAAH activator" rather than directly expressing FAAH. The exact nature of this FAAH activator is still unknown; its activity is fully neutralised by lipases, but not by phospholipases A2, C or D, DNAse or RNAse and for this reason, it is suspected to be a lipid. However, it is not thought to be any of the lipids known to be released by blastocysts, such as platelet-activating factor, leukotriene B4 or prostaglandins E2 and F2 (Maccarrone et al. 2004).

It has been postulated by several authors that COX-2 may play a role in the degradation of AEA (Yu et al. 1997, Kozak and Marnett 2002, Karasu et al. 2011). Yu et al. (1997) have shown that human cyclooxygenase 2 (hCOX-2) binds to, and oxidizes AEA. They further demonstrated that the products of metabolism in this pathway are a unique form of eicosanoid, called prostaglandin E2 ethanolamide (PGE2-EA), disparate from the metabolites of arachidonic acid (AA). The same experiments performed with hCOX-1 did not yield PGE2. They also showed that COX-2 oxidised AEA at 60-85% of the rates

seen with the oxidation of AA by the same enzyme, utilizing a similar mechanism. In addition, it has also been shown (in the mouse brain) that COX-2 and FAAH compete to metabolise AEA (Glaser and Kaczocha 2010).

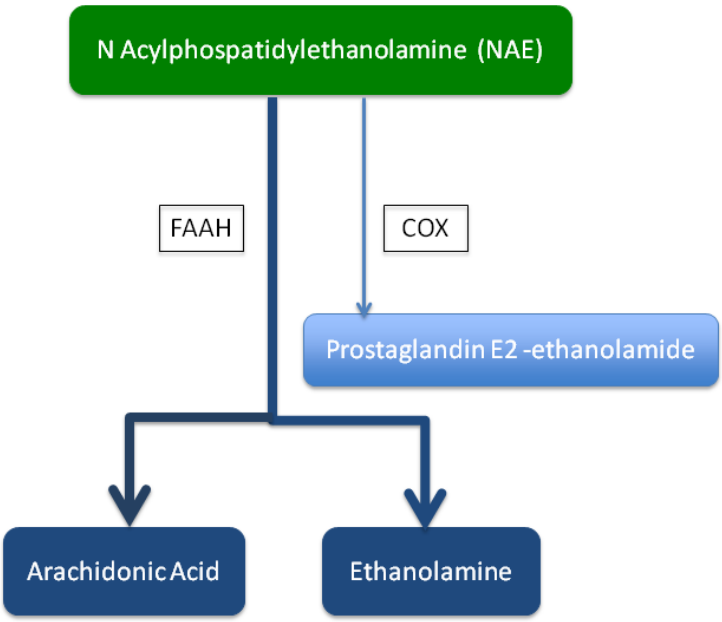

Figure 1-3 A schematic representation of the degradation of NAEs

A schematic representation of the various proposed routes of NAE degradation.
Key: Green box is precursor; Blue boxes are intermediate indicate the final product; White boxes are the enzymes involved. Thicker arrows indicate the dominant pathways to NAE degradation in most cells. FAAH = Fatty Acid Amide Hydrolase; COX 2 = Cyclo-oxygenase 2

1.5.4 N-acylethanolamines

As already discussed, AEA is an *N*-acylethanolamine (NAE). Other members of the NAE family have been identified, including *N*-palmitoylethanolamine (PEA), *N*-oleoylethanolamine (OEA), *N*-stearoylethanolamine (SEA) and *N*-linoleoylethanolamide (Hanus et al. 1993, Wang and Ueda 2009).

In the context of reproduction, OEA and PEA (along with AEA) have all been identified in human seminal plasma, mid-cycle oviductal fluid, follicular fluid, amniotic fluid, milk and fluids from malignant ovarian cysts (Schuel et al. 2002). In addition to their presence in these fluids, it is believed that PEA has both anti-inflammatory activity (Lambert et al. 2002) and is involved in pain sensation (Calignano et al. 1998), while OEA has an anti-anorexic effect (Rodriguez de Fonseca et al. 2001).

Despite having a very similar structure to AEA, and sharing the same synthesis and degradation pathways, OEA has negligible affinity for either the CB1 or CB2 receptor (Bradshaw and Walker 2005). When considering PEA (which is also synthesised by NAPE-PLD and hydrolysed by FAAH), it too exhibits poor affinity for CB1 or CB2, but its anti-nociceptive effects can be blocked by a CB2 antagonist (Calignano et al. 1998), further highlighting their similarities. AEA, OEA and PEA have all been shown to bind to two "non cannabinoid" receptors, transient receptor potential cation channel subfamily V member 1 (TPRV1) and peroxisome proliferator-activated receptor α (PPAR-α) (Ahern 2003, Suardiaz et al. 2007, Ezzili et al. 2010). However, these receptors will not be discussed any further in this thesis as neither of them have been shown conclusively to play a role in embryo implantation.

Although both PEA and OEA have been linked to reproductive failure or success in mice and women, this effect is thought to occur through a so-called "entourage effect". It is thought that both PEA and OEA compete with AEA for degradation by FAAH, thereby potentiating AEA activity (Bambang et al. 2010).

1.5.5 Transport across plasma membranes

While there is increasing evidence pertaining to the synthesis, degradation and actions of anandamide, the method by which AEA traverses the plasma membrane still remains a controversial topic.

It is currently believed that the mechanisms for synthesis and degradation of AEA are located within the cell yet the receptors upon which it acts are extracellular (Sun and Dey 2008). After synthesis, AEA needs to cross the plasma membrane to reach the target receptors, and then re-cross the plasma membrane before it can be broken down (Muccioli 2010). Unlike most neurotransmitters, which are hydrophilic, and freely diffusible inside the cytosol, AEA is an uncharged lipid that is generally insoluble in an aqueous environment (Glaser et al. 2005, Kaczocha et al. 2009). In addition to crossing the plasma membrane, AEA also needs to transverse the cytosol in order to be metabolised as FAAH has been shown by immunohistochemistry to be localised in the endoplasmic reticulum (ER).

One argument is that FAAH facilitates passive diffusion of AEA across the membrane by creating a concentration gradient (Glaser et al. 2005, Kaczocha et al. 2009) although further studies have suggested that it is unlikely to be the sole factor required for transport to occur (Day et al. 2001). An alternative suggestion for AEA transport is that it occurs by a carrier mediated process (Hillard et al. 1997, McFarland and Barker 2004, Sun and Dey 2008) in which a carrier protein binds to AEA and transports it across the plasma membrane.

Once inside the cell, in both of these suggested methods, AEA needs to traverse the cytosol to interact with FAAH. One proposed mechanism for traversing the cytosol is by binding with fatty acid binding proteins (FABPs) acting as an intracellular carrier for AEA, transporting it from the plasma membrane to the ER. To this effect, it has been reported that albumin and heat shock protein (Hsp) 70 also function as AEA carriers (Oddi et al. 2009).

A third mechanism for crossing the plasma membrane involves the endocytic uptake of anandamide, which is then presented to FAAH in a vesicle (thus negating the need for a carrier method across the cytosol) (McFarland, Barker 2004, Fowler 2012, Kaczocha, Glaser et al. 2009, McFarland and Barker 2004).

What is clear is that at the time of writing is that we are far from understanding the exact method by which AEA is transported and it is likely to remain a topic of heated debate for the foreseeable future.

1.6 Other key components involved in uterine receptivity

1.6.1 Prostaglandins

Prostaglandins (PGs) are small lipid molecules that regulate numerous processes in the body (Harris et al. 2002). The precursor for PGs is arachidonic acid, which is released from membrane phospholipids by phospholipase A2. Arachidonic acid is then converted to prostaglandin H_2 (PGH_2) by COX-1 or COX-2, and then PGH2 is converted into a variety of prostaglandins by cell-specific prostaglandin synthases.

Prostaglandin E_2 (PGE_2), produced by many cells of the body, is probably the most widely studied of the prostaglandins (Harris et al. 2002) and has been closely linked to the success of the implantation process (Holmes and Gordashko 1980). Two important roles PGE_2 plays in this are stimulation of trophoblast development (Chan 1991) and increased endometrial vascular permeability (Evans and Kennedy 1978). More recently, it has been shown that COX-2-derived prostaglandins (including PGE_2) influence uterine angiogenesis during decidualisation by differentially regulating VEGF and angiopoietin signalling cascades (Matsumoto et al. 2002).

As well as increasing vascular permeability and stimulating trophoblast development, prostaglandins have also been linked to the inflammatory response. It has become clear that prostaglandins can both promote and inhibit inflammation, depending on several factors including the level of immune cell activation and the presence of other mediators (Tilley et al. 2001).

Cyclooxygenase (COX) enzymes are the rate-limiting enzymes involved in the conversion of arachidonic acid to prostaglandins, and are found in three isoforms COX-1, COX-2, and the more recently identified COX-3 (Chakraborty et al. 1996, Chandrasekharan et al. 2002). COX-1 and -2 are encoded by two separate genes and exhibit distinct cell-specific expression, regulation and sub-cellular localisation, yet share similar structural and kinetic properties (Lim et al. 1997) whereas COX-3 is encoded by the same gene as COX-1 (Chandrasekharan et al. 2002). COX-1 is a constitutive enzyme located in the endoplasmic reticulum of most mammalian cells (Chakraborty et al. 1996)

and PGs synthesised here exit the cell via G protein-coupled cell surface receptors. In contrast, COX-2 is an inducible enzyme (Kujubu et al. 1991) that is undetectable in most normal tissues, becoming abundant in active macrophages and other cells at sites of inflammation (Lee et al. 1992), and is primarily associated with the nuclear envelope (Lim et al. 1997). COX-2 is induced in a variety of cell types by diverse stimuli including cytokines, growth factors, mitogens and tumour promoters (Kujubu et al. 1991, Lee et al. 1992) in response to cell activation processes, such as growth, differentiation and inflammation (Ristimaki et al. 1994). Chandrasekharan et al. (2002) showed that COX-3 shares all of the catalytic features and important structural features of COX-1 and COX-2, but with significantly diminished activity.

1.6.2 Leukaemia Inhibitory Factor (LIF)

LIF is a pleiotropic cytokine that regulates various cellular functions including proliferation and differentiation. Its production is boosted by progesterone and endometrial expression of LIF is decreased following treatment with the antiprogestin mifepristone (Cameron et al. 1997, Marions and Danielsson 1999).

LIF is a member of the IL-6 family (Diedrich et al. 2007, Markert et al. 2011). This family shares a common receptor for signal transduction, resulting in considerable overlap among the cytokines (Song et al. 2000) and low levels of IL-6 have been associated with recurrent miscarriages (Lim et al. 2000). Both the human embryo and the endometrium secrete LIF (Cameron et al. 1997, van der Gaast et al. 2009), and both possess LIF receptors (Aghajanova 2010), suggesting that the embryo-endometrial interactions start before the embryo even attaches to the endometrial epithelial surface.

1.6.3 HOXA-10 and HOXA-11

Two other components identified as imperative for successful implantation are the homeobox genes HOXA10 and HOXA11. Hox genes (HOX in humans) have a similar 183 base pair sequence, termed the homeobox (McGinnis et al. 1984). The Hox genes are shared by all animals and are involved in body axis patterning (Eun Kwon and Taylor

2004a). The mouse and human express a very similar pattern of Hox genes, with a total of 39 HOX genes arranged in four parallel clusters A, B, C and D. With regards to the reproductive tract, HOXA9 to HOXA13 play a critical role in the paramesonephric duct developing into the oviduct, uterus, cervix and upper part of the vagina, respectively (Eun Kwon and Taylor 2004).

While most tissues only express Hox genes during the embryonic period, the reproductive tract in women re-establishes Hox gene expression following puberty where this may underlie the plasticity of the endometrium, facilitating the menstrual cycle and pregnancy (McGinnis et al. 1984).

1.6.4 Galectins

Galectins (gal) are a family of proteins defined by their binding specificity for β-galactoside sugars (Camby et al. 2006). There are currently 15 described galectins, numbered 1 to 15 (Yang et al. 2008). The galectins already identified have carbohydrate recognition domains (CRDs) that are responsible for carbohydrate binding. Most galectins have one CRD, while gal-4, -6, -8, -9 and -12 have two CRDs. Gal-3 is unique as its CRD is connected to a non-lectin N terminal region (Camby et al. 2006, Blidner and Rabinovich 2013).

Unlike the majority of lectins, galectins are not membrane bound, but soluble proteins with both intra- and extra-cellular functions. They have distinct but overlapping distributions and are found primarily in the cytosol, as well as occasionally in the nucleus. They belong to a group of proteins that do not have a signal sequence and thus can be secreted and function outside of the cell (Yang et al. 2008).

Galectins have been implicated in a vast array of intracellular processes, but are best known for their involvement in cell-to-cell adhesion, apoptosis, influencing an immune response and tumour formation (Liu 1993, Perillo et al. 1995, Yang et al. 1996a, Colnot et al. 1998, Colin 2001, Fischer et al. 2011). It has been proposed that the reason galectins are involved in such a wide variety of processes is because they are able to recognise multiple glycoconjugates (Liu 2000).

Galectins -1, -2, -3, -4, -7, -8, -9 and -12 all have orthologues in human, mouse, rat and other mammalian species. When considering the two most studied of the galectins, gal-1 and -3 (Almkvist and Karlsson 2002), it has been shown that there is 87% identity when mouse and human variants are compared (Leffler et al. 2004).

The intracellular actions of both galectin-1 and -3 have also been implicated in several processes necessary for successful embryo implantation. Although many ligands (e.g. LIF, HOXA, COX-2) have been identified as essential for successful embryo implantation, this has not been shown to be the case with galectin-1 and -3. However, while gal-1 and gal-3 -/- mice are able to fulfil all the "functions" required under laboratory conditions, it has been suggested that the absence of these proteins may well be detrimental for the population in the long run (Leffler et al. 2004, von Wolff et al. 2005).

1.6.5 Integrins

The term "integrin" is used to describe a family of heterodimeric, non-covalently bound α and β adhesion receptors that mediate cell attachment to extracellular matrix (ECM) proteins and cell-to-cell interactions (Lessey et al. 1992, Jones and Walker 1999, Nardo L.G et al. 2002, Yoshimura 2002). Integrins are promiscuous and tend to recognise several ligands, rather than being specific for one or two (Ruoslahti and Pierschbacher 1987, Hemler 1990, Nardo L.G et al. 2002). Common cell surface ECM proteins linked to integrins include vitronectin, fibronectin, collagen and laminin (Sueoka et al. 1997) as well as both galectin-1 and -3 (Furtak et al. 2001, Moiseeva et al. 2003, Fischer et al. 2005, Lei et al. 2009).

The expression of integrins in the endometrium varies throughout the menstrual cycle, and this has been studied extensively using immunohistochemistry (Sueoka et al. 1997). The variation is noted both in the timing of expression, as well as which endometrial cells express the different integrins. While it has been shown that many integrins are expressed by the endometrium (Lessey et al. 1992, Bischof et al. 1993, Sillem et al. 1997, Lessey et

al. 2000) only a few have been highlighted as playing an important role in uterine receptivity. These include α4β1 and αvβ3.

Despite a link being made between the expression of certain integrins and uterine receptivity, there remains debate surrounding the effects of estrogen (E2) and progesterone (P4) on the expression of integrins. For example, Yoshimura et al. (2002) concluded that treatment of endometrial stromal cells with both E2 and P4 increased the expression of β1. However, Sillem et al. (1997) reached the opposite conclusion, namely the expression of integrins was not affected by E2 or P4. This is obviously an area that needs clarification so that the precise regulatory roles of E2 and P4 can be ascertained.

1.7 Involvement of key components in uterine receptivity

1.7.1 Endocannabinoids

1.7.1.1 Expression within the reproductive tract

A careful balance in the activities of NAPE-PLD and FAAH is required to ensure the appropriate levels of anandamide are available during implantation (Guo et al. 2005, Paria et al. 1996). This "anandamide tone" is crucial for successful implantation to occur (Wang et al. 1999). In 1997, Schmid and co-workers (Schmid et al. (1997)) demonstrated that a high level of anandamide inhibits the development of mouse two-cell embryos into blastocysts and zona-hatching of blastocysts from eight-cell embryos *in-vitro*. Also, mouse blastocysts exposed in culture to low levels of AEA (7nM) exhibited accelerated trophoblast differentiation and outgrowth, whereas inhibition of trophoblast differentiation was observed at higher (28nM) doses of AEA (Wang et al. 2006). Similar studies on human blastocysts have not yet been performed.

The work of Sun et al. (2010) further supports this notion of "anandamide tone" being important because they demonstrated that both CB1-/- and FAAH -/- mice had a significant reduction in litter size, suggesting that both high and low levels of AEA have a negative effect on pregnancy. Furthermore, Guo et al. (2005) demonstrated that the murine uterine epithelium is a major source of AEA prior to implantation and showed that NAPE-PLD activity correlates with AEA levels, whereas higher expression of NAPE-PLD was noted in a "non-receptive" uterus when compared to a uterus that was "receptive" to implantation. Interestingly, they showed that NAPE-PLD expression was significantly lower at the implantation site when compared to the peri-implantation site, and proposed that the embryo might play a role in regulating these levels.

It has been demonstrated that the pre-implantation blastocyst expresses NAPE-PLD at all stages of development and this is found in both the cytoplasm and nucleus of the pre-implantation embryo, whereas FAAH protein is only expressed in embryos beyond the two cell stage and limited to the outer cells of the morula and trophoectoderm (Battista et

al. 2008). Wang et al. (2006) surmised that the increasing levels and localised expression of FAAH in morulae and blastocysts suggest that FAAH-mediated anandamide degradation functions as a protective mechanism against the negative effects that high levels of AEA have on implantation. A summary of the activity of various components of the endocannabinoid system is shown in Table 1-3.

It is not only the absolute levels of AEA that affect the success of implantation, but also the presence or absence of receptors. Wang et al. (2006) demonstrated that the absence of the CB1 receptor in embryos results in asynchronous development, while absence of maternal CB1 receptors results in oviductal retention of the embryo. Conversely, wild type mice subjected to high levels of methanandamide (a stable AEA analogue) or natural THC showed pregnancy loss, with the embryos also retained in the oviduct.

Guo et al. (2005) demonstrated that on days one to four of mouse pregnancy, NAPE-PLD is primarily expressed in the luminal and glandular epithelium of the uterus (when compared to the stroma), indicating that the epithelium is the major source of uterine anandamide prior to implantation. However, on days five to seven of pregnancy, expression of NAPE-PLD is primarily restricted to the luminal and glandular epithelium of the inter-implantation region, with very low expression in the luminal epithelium close to the implanting blastocyst. With this result, they suggested there is a potential role of the implanting embryo in regulating uterine NAPE-PLD expression and anandamide levels. The effects of the various components of the endocannabinoid system in implantation is summarised in Table 1-4. At this point it is worth noting that all of the data described above is the result of work with animal models as due to obvious ethical considerations it has not been possible to perform the same investigations with human subjects.

Table 1-3 Activity of various components of the endocannabinoid system in implantation, as identified in animal models.

	1 cell	2 cell	4 cell	Blastocyst	Oviduct	Implantation site	Peri-implantation site
CB1				**			
CB2							
NAPE-PLD					Higher in isthmus than ampulla	Low Levels	High Levels
FAAH				**	Higher in ampulla than isthmus	High Levels	Low Levels

** Limited to trophectoderm

Shaded areas = active
Blank areas = no activity demonstrated

(Data from: (Paria et al. 1995, Das et al. 1995, Wang et al. 2004, Guo et al. 2005, Battista et al. 2008))

Table 1-4 Effect of the various components of the endocannabinoid system in implantation (as found in murine studies)

		Embryo	Trophoblast	Endometrium
NAPE - PLD	High Levels	Associated with dormant blastocyst		Found in non-receptive uterus
	Low Levels	Associated with active blastocyst		Found in receptive uterus
AEA	High Levels	Inhibited development	Inhibited Differentiation	Found at inter-implantation sites
	Low Levels	Supports development	Accelerated Differentiation	Found at site of embryo implantation
FAAH	High Levels	Thought to be associated with low AEA levels and so beneficial to implantation		Found at site of embryo implantation
	Low Levels	Causes delayed, asynchronous development of pre-implantation embryo		Found at inter-implantation sites

(Data from: (Paria et al. 1996, Schmid et al. 1997, Wang et al. 1999, Guo et al. 2005, Wang et al. 2006a))

That is not to say that the role of the ECS has not been explored in humans. A recent study by El-Talatini et al. (2009) followed trends in plasma AEA levels from oocyte retrieval (OR) to embryo transfer (ET) in IVF/ICSI patients and showed that patents who successfully became pregnant had a significant fall in their plasma AEA levels between OR to ET whereas the non-pregnant group did not show that drop. In addition, El-Talatini et al. (2010) suggested that during the follicular phase of the menstrual cycle, FSH might be the main controlling factor regulating plasma AEA levels, whereas during ovulation, LH and estrogen may regulate implantation and early pregnancy AEA levels.

It is well known that the ovarian sex steroids, estradiol and progesterone, both influence the endocannabinoid system. Progesterone has been shown to stimulate the activity of FAAH in humans and it has been found that ovariectomised animals have significantly reduced levels of FAAH when compared to controls (Maccarrone et al. 2001b, Battista et al. 2008). Conversely, high levels of estrogen and/or progesterone down-regulate the expression on NAPE-PLD (Guo et al. 2005).

1.7.1.2 Effects on the immune system

For some time it has been known that exogenous cannabinoids have an inhibitory effect on the immune system, weakening both humoral and cell mediated immunity as well as cellular defences against infective agents (Matias et al. 2002). The inhibitory effects of the cannabinoids on the immune system have, however, been shown to be transient (Pandey et al. 2009) and there is down regulation of cannabinoid receptors when the immune cells are active. This is important as it allows the immune system to be activated during infections.

There is increasing evidence that while cannabinoids with a micromolar concentration have an inhibitory effect on the immune system *in-vitro*, cannabinoids may have a stimulatory effect on the immune system with a nanomolar concentration (Croxford and Yamamura 2005). Furthermore, Croxford et al. (2005) have estimated that concentrations in the micromolar range are at least 10 fold higher than observed in the blood of marijuana smokers.

Work with cannabinoids has shown that THC regulates the expression of Th1 and Th2 cytokine mRNA. It has also been shown that as well as influencing the expression of Th1 and Th2 cytokines, THC also caused suppression of NK cell cytotoxicity (Newton et al. 1994, Srivastava et al. 1998, Klein et al. 2000, Zhu et al. 2000, Yuan et al. 2002, Newton et al. 2004).

Similar findings have also been shown with endocannabinoids. When considering the effect on T cells, AEA has the ability to either inhibit or induce the expression of both Th1 and Th2 cytokines (Pandey et al. 2009). AEA has also been shown to inhibit migration of T lymphocytes (Ribeiro et al. 2010). In addition, there is evidence to suggest that low doses of AEA can both inhibit T lymphocyte proliferation and lipopolysaccharide-induced expression of pro-inflammatory mediators via CB2 mediated mechanisms (Ross et al. 2000, Cencioni et al. 2010) as well as increasing the production of anti-inflammatory cytokines such as IL-10 and TGF-β (Pandey et al. 2009, Krishnan and Chatterjee 2012).

It is known that peripheral lymphocytes play a critical role in human embryo implantation. The addition of AEA to human lymphocytes *in-vitro* inhibits the release of LIF, further highlighting the close interaction between the many components involved in supporting early pregnancy. FAAH is also thought to be a key player in the lymphocyte-mediated control of the hormone-cytokine network at the fetal-maternal interface. Maccarrone et al. (2001b) demonstrated that lymphocyte FAAH activity was significantly lower in patients who failed to become pregnant or became pregnant and then went on to miscarry when compared to those who became pregnant (Maccarrone et al. 2000, Maccarrone et al. 2001b).

As well as the endocannabinoid system having the ability to influence the immune system, the immune system has the ability to influence the endocannabinoid system. For example, Maccarrone et al. (2001a) have shown that IL-4 and IL-10 increased progesterone-stimulated FAAH activity whilst IL-12 and IFN-γ reduced FAAH activity and protein expression in lymphocytes.

1.7.2 Prostaglandins

Implantation requires the tight regulation of prostaglandin production. It has been shown that the concentrations of prostaglandins in the human decidua in early pregnancy are lower than those in the endometrium at any stage of the menstrual cycle (Abel et al. 1980, Maathuis and Kelly 1978), and the administration of exogenous prostaglandins induces abortion in all species and at any gestation (Abel et al. 1980, Jaschevatzky et al. 1983).

Studies examining the timing of COX-1 and COX-2 expression with respect to implantation have demonstrated the expression of the COX-1 gene in the uterine epithelium at the time of generalised oedema, whereas expression of the COX-2 was limited to the luminal epithelial cells exclusively surrounding the blastocyst at the time of the attachment reaction (Chakraborty et al. 1996).

COX-1 is expressed in murine uterine epithelial cells in the morning of D4 of pregnancy, but the expression becomes undetectable by the time of the attachment reaction (Chakraborty et al. 1996). Interestingly, and in contrast to the knowledge that exogenous prostaglandins induce abortion, COX-2 is expressed in the luminal epithelium and underlying stromal cells, solely at the sites of the blastocyst attachment reaction, and only in the presences of an active blastocyst, suggesting that the blastocyst produces a soluble and localised factor important for implantation success (Lim et al. 1997, Marions and Danielsson 1999). It has been shown that COX-1 (-/-) females preserve their fertility, whereas the absence of COX-2 results in infertility (Lim et al. 1997).

The endometrial production of COX-1 decreases in response to rising progesterone and estradiol levels in the mid luteal phase of the menstrual cycle whereas the endometrial content of COX-2 is unaffected by steroid hormones (Marions and Danielsson 1999). However, mifepristone decreases the expression of both COX-1 and COX-2 in the endometrium, suggesting that both genes are regulated by progesterone (Marions and Danielsson 1999). Further supporting the notion that COX-2 activity is influenced by the menstrual cycle are studies conducted by Sirois et al. (1992) who looked at a "second PG synthase" (PGSi aka COX-2) and showed that both LH and FSH, (at concentrations mirroring the pre-ovulatory surge), as well as GnRH can induce this enzyme's activity.

COX-2 has been shown to be present at the implantation sites (Chakraborty et al. 1996). Wang et al. (2007) demonstrated that COX-2 protein is present in the luminal epithelium, but not the glandular epithelium or myometrium on D1 of murine pregnancies. By D4 the expression is undetectable. Following implantation (D5), COX-2 expression was exclusively induced in the luminal epithelium and sub-epithelial stromal cells at the anti-mesometrial pole surrounding the implanting blastocyst. Chakraborty et al. (1996) demonstrated that while COX-2 gene is expressed at the anti-mesometrial pole on D4-5, its expression is switched to the mesometrial pole from D6 onwards. They concluded that PGs produced at this site by COX-2 are involved in angiogenesis for the establishment of placenta and postulated that a blastocyst-derived growth factor/cytokine may be responsible for this. These findings support the work of Parr et al. (1988) who investigated the occurrence of "prostaglandin synthase" throughout the implantation period. They noted that while prostaglandin synthase was not detected on D1 or pregnancy, by D5 there was strong staining in the luminal epithelium of the implantation site (more so than at the inter-implantation sites) as well as on blastocysts from D5-D7.

Further emphasising the importance of COX-2 in embryo implantation are studies that show COX-2 -/- mice to be infertile. Lim et al. (1997) demonstrated that COX-2 -/- mice have a reduced incidence of ovulation, and none of the oocytes released by mice that did successfully ovulate were fertilized. In addition, they showed that wild type blastocysts failed to implant in the uteri of COX-2 -/- mice.

1.7.3 Leukaemia Inhibitory Factor

LIF was the first cytokine to be shown to be absolutely necessary for implantation, as LIF knockout mice (even when impregnated with LIF +/+ embryos) displayed total implantation failure, a phenomenon that could be resolved with continuous injection of recombinant LIF (Stewart et al. 1992). However, there are LIF "over producers" among sterile women, so not all infertility is due to LIF deficiency, and "too much" LIF in the luminal fluid is a predictor of poor IVF-ET outcome (Ledee-Bataille et al. 2002).

LIF also plays a role in both the adhesive and invasive phases of implantation due to its anchoring effect on the trophoblast and regulation of trophoblast differentiation. Pinopodes have been shown to release LIF containing vesicles into the uterine lumen (Kabir-Salmani et al. 2005).

LIF has been shown to modulate trophoblast invasiveness and affect immune tolerance by controlling human HLA-G expression of invasive cytotrophoblast cells during implantation (Bamberger et al. 2000, van der Gaast et al. 2009, Aghajanova 2010).

As already discussed, LIF is a member of the IL-6 family; a family which is included in the Th2 cytokine group. Again, as already discussed, implantation is a "pro-inflammatory" phenomenon. In the vast majority of cases, a pro-inflammatory response is mediated by Th1 cytokines rather that Th2. However, it has been shown that the IL-6 cytokines have the ability to exert a "pro-inflammatory" response (Gabay 2006). It there should not come as a surprise that LIF also supports this process. In addition, Linker et al. (2008) found that LIF -/- mice had impaired IFN-γ (a Th1 cytokine) production.

1.7.4 Homeobox A10 and A11 (HOXA10 and HOXA11)

HOXA10 and HOXA11 are considered to be essential for implantation, and disruption in either results in "uterine factor" infertility in mice (Eun Kwon and Taylor 2004b). Although such mice ovulate normally and produce viable pre-implantation embryos, they are unable to support implantation. Furthermore, embryos from HOXA10 (-/-) mice successfully implant in pseudo-pregnant wild mice, whereas wild-type embryos do not

implant in HOXA-10 or HOXA-11 deficient uteri. In addition, targeted disruption of HOXA-11 also results in reduced LIF secretion.

HOXA-10 expression is up-regulated by the presence of either estrogen or progesterone, and significantly up-regulated in the presence of both estrogen and progesterone simultaneously. At the time of implantation, levels of HOXA-10 and HOXA-11 both rise dramatically. It has also been demonstrated that in ectopic pregnancies, HOXA-10 expression is increased at the site of implantation in the oviduct, with the level of expression almost mirroring the levels found in the endometrium with normal intrauterine pregnancies (Salih and Taylor 2004), further supporting the notion that successful implantation is a result of a complex interplay between many different components.

Sarno et al. (2009) demonstrated a decreased expression of HOXA-10 and HOXA-11 in women experiencing a spontaneous miscarriage when compared to patients undergoing a surgical termination of a healthy pregnancy, showing that these two molecules are possibly as important in human pregnancy as they are in mouse pregnancy.

It has also been suggested that blastocysts express an as yet unidentified soluble factor that causes an up regulation of HOXA-10 expression. This further supports the theory that the embryo may be responsible for increasing the receptivity of the adjacent endometrium with an ultimate aim of improving its chances of successful implantation (Eun Kwon and Taylor 2004).

1.7.5 Galectins

Galectin-1 and -3 are both expressed within the endometrium (von Wolff et al. 2005). However, their spacio-temporal expression has been shown to differ. While Gal-1 is expressed in stromal and decidual cells, gal-3 is predominantly expressed in endometrial glandular tissue (although it is also found in stromal cells, macrophages and occasionally in vascular endothelial cells) (Brustmann et al. 2003). In addition, while the expression of both gal-1 and gal-3 significantly increases during the secretory phase of the menstrual cycle, it has been shown that gal-1 is also expressed during the proliferative phase.

As already discussed, implantation can only occur successfully during the "window of implantation"; a critical time period under the control of ovarian steroids. Interestingly, the expression of both gal-1 and gal-3 are under the control of ovarian steroids and this regulation also correlates with the window of implantation (Choe et al. 1997, von Wolff et al. 2005, Yang et al. 2011, Tirado-González et al. 2013).

The final stage of implantation, penetration, is often compared to that seen with invasion of normal tissue by tumours, and there is strong evidence linking galectins to some of the processes adopted by tumours. For example, gal-1 has been shown to play a role in tumour angiogenesis (Yang et al. 2008) and an increased expression of gal-3 has been found in malignant endometrial cells when compared to benign cells (Brustmann et al. 2003). In addition, Matarrese et al. (2000) showed that gal-3 over expression resulted in tumours that were more adhesive to endothelial cells and the extracellular matrix. The adhesive properties of gal-3 have also been shown by Lei et al. (2009). Furthermore, the same team demonstrated that gal-3 enhanced cell-to-cell adhesion is mediated by integrin β3 (but not integrin β1).

However, there is some inconsistency in findings relating to gal-3's apoptotic effect. While Brustmann et al. (2003) and Matarrese et al. (2000) have shown that gal-3 inhibits apoptosis in tumours, Yang et al. (2011) showed that gal-3 induces apoptosis of endometrial cells and inhibits proliferation, (both effects occurring in a dose-dependent manner).

There is mounting evidence that galectins influence the immune system. It has been shown that gal-1 induces a Th2 immune response and increased gal-1 expression (or administration) causes the immune response to shift in the Th2 direction (Yang et al. 2008). Furthermore, gal-1 deficient mice produce significantly more Th1 cytokines, such as IFNγ, than normal mice (Toscano et al. 2007). In addition, it has been shown that gal-1 suppresses Th1 and Th17 mediated immune responses. Ri-Yao Yang et al. (2008), Blois et al. (2007) and Toscano et al. (2007) have all shown that Th1 cells are susceptible to gal-1 induced apoptosis, while Th2 cells are not. Overall, the available evidence suggests that gal-1 induces a shift from a Th1 response to a Th2 response (Blois et al. 2007, Rabinovich et al. 2007).

As already discussed, the cytokines produced by activated Th1 cells (IL-2, IL-3, INFγ and TNF-β) are thought to be "pro-inflammatory" while the majority of Th2 cytokines (IL-4, IL-5, and IL-10) are thought to be "anti-inflammatory", and implantation is believed to be a pro-inflammatory condition. In the context of galectins, while gal-1 suppresses chronic inflammation, gal-3 has been proposed to be a powerful pro-inflammatory signal (Rabinovich et al. 2002, Saito et al. 2010). These data suggest that gal-3 could have a key role to play during blastocyst implantation.

1.7.6 Integrins

As already discussed, the expression of integrins α4β1 and αvβ3 have been linked to the window of implantation. Integrin α4β1 interacts with fibronectin, while αvβ3 is a vitronectin receptor. It has been suggested that both play a role in cell-to-cell adhesion (Sueoka et al. 1997).

During the early proliferative phase, β1 expression is restricted to glandular epithelium, whereas by the mid secretory phase there is marked move to expression in stromal cells (Yoshimura 2002). In addition, it has been shown that α4β1 is expressed by decidual cells (Sueoka et al. 1997) and levels remain high during early pregnancy (Yoshimura 2002), where α4β1 has been detected in the endometrial epithelial cells of fertile women, but not in those with unexplained infertility. The loss of α4β1 expression corresponds with the closure of the "window of implantation" and it has been suggested that decreased expression of α4β1 might be linked to unexplained infertility.

In contrast, αvβ3, has been found to be a marker of luteal phase endometrial glandular maturity (Damario et al. 2001), appearing from post ovulatory days 6-8 with increasing expression as the cycle continues (Sueoka et al. 1997, Creus et al. 2002). Its expression during this time, which corresponds with the "window of implantation", has also been linked to endometrial pinopode development (Lessey et al. 1992, Lessey 2003) and aberrant expression of αvβ3 has been linked to infertility (Lessey and Castelbaum 1995, Creus et al. 1998, Yoshimura 2002). Conversely, Thomas et al. (2003) demonstrated that

endometrial integrin expression is the same in patients with proven fertility, those undergoing intra-cytoplasmic sperm injection (ICSI - a technique used when the cause of infertility is related to male factors) and those undergoing *in-vitro* fertilisation (IVF - a technique used when the sperm quality is normal i.e. infertility is due to female factors).

Nikzad et al. (2010) have shown a statistically significant increase in the amount of gal-3 at the same time as this increased expression of αVβ3. In addition, they showed that the expression of both gal-3 and αVβ3 was significantly higher in pinopodes (which are predominately found at the implantation zone) compared to adjacent tissue.

Following the work of Noyes (1950), it became common practice to describe the endometrium was described as either "in phase" or "out of phase" based on its histological appearance and corresponding point of the menstrual cycle. For some time it has been assumed this influenced the success or failure of the implanting embryo although there is increasing evidence to suggest this may not be the case (Achache and Revel 2006, Coutifaris et al. 2004, Creus et al. 1998). When considering the involvement of integrins, Lessey et al. (2000) concluded that the pattern of expression of integrins could not be used to date the endometrium. Creus et al. (2002) also showed that αvβ3 expression (along with the expression of pinopodes) occurred at specific times of the menstrual cycle, regardless of whether the endometrium was in or out of phase. These findings support work carried out by the same team in the past (Creus et al. 1998).

1.8 Interaction between the key components

As already discussed, LIF, HOXA-10 and COX-2 are all essential for implantation and/or decidualisation. It has, however, been noted that while COX-2 expression is abnormal in LIF(-/-) mice, uterine LIF expression is normal in COX-2(-/-) mice. Similarly, COX-2 expression is also reduced in HOXA-10(-/-) mice. Conversely, HOXA-10 expression is normal in LIF(-/-) mice, and LIF expression is normal in HOXA-10(-/-) mice. From this information, Song et al. (2000) concluded that information from the LIF and HOXA-10 signalling pathways might converge on the COX-2 pathway to control implantation, further supporting the notion that implantation is the consequence of a complex

interaction of many factors, including a specific cross-talk between the blastocyst and the uterine endometrium.

It has been postulated by several authors that COX-2 may play a role in the degradation of AEA (Yu et al. 1997, Kozak and Marnett 2002, Karasu et al. 2011). Yu et al. (1997) have shown that human cyclooxygenase 2 (hCOX-2) binds to and oxidizes AEA. They further demonstrated that the products of metabolism in this pathway are a unique form of eicosanoid, Prostaglandin E_2 Ethanolamide (PGE_2-EA), which is disparate from the metabolites of Arachidonic Acid (AA). The same experiments performed with hCOX-1 did not yield PGE_2. They also showed that COX-2 oxygenated AEA at 60-85% of the rates seen with the oxygenation of AA by the same enzyme, utilizing a similar mechanism. In addition, it has also been shown (in the mouse brain) that COX-2 and FAAH complete to metabolise AEA (Glaser and Kaczocha 2010).

Both AEA and COX-2 levels are influenced by the sex steroids. El-Talatini et al. (2009, 2010) showed that during the follicular phase of the menstrual cycle, FSH might be the main controlling factor regulating plasma AEA levels, whereas during ovulation, LH and estrogen appear to regulate implantation and early pregnancy AEA levels. Sirois et al. (1992) studied a second PG synthase (PGSi aka COX-2) and showed that (at concentrations mirroring the pre-ovulatory surge) both LH and FSH, as well as GnRH can induce this enzyme's activity.

While all of the components above have been shown to play an important role in embryo implantation, the interactions between the components appear to be equally important. Figures 1-4, 1-5 and 1-6 illustrate the interactions already known, and the effect (either enhancing or inhibiting) these have on expression.

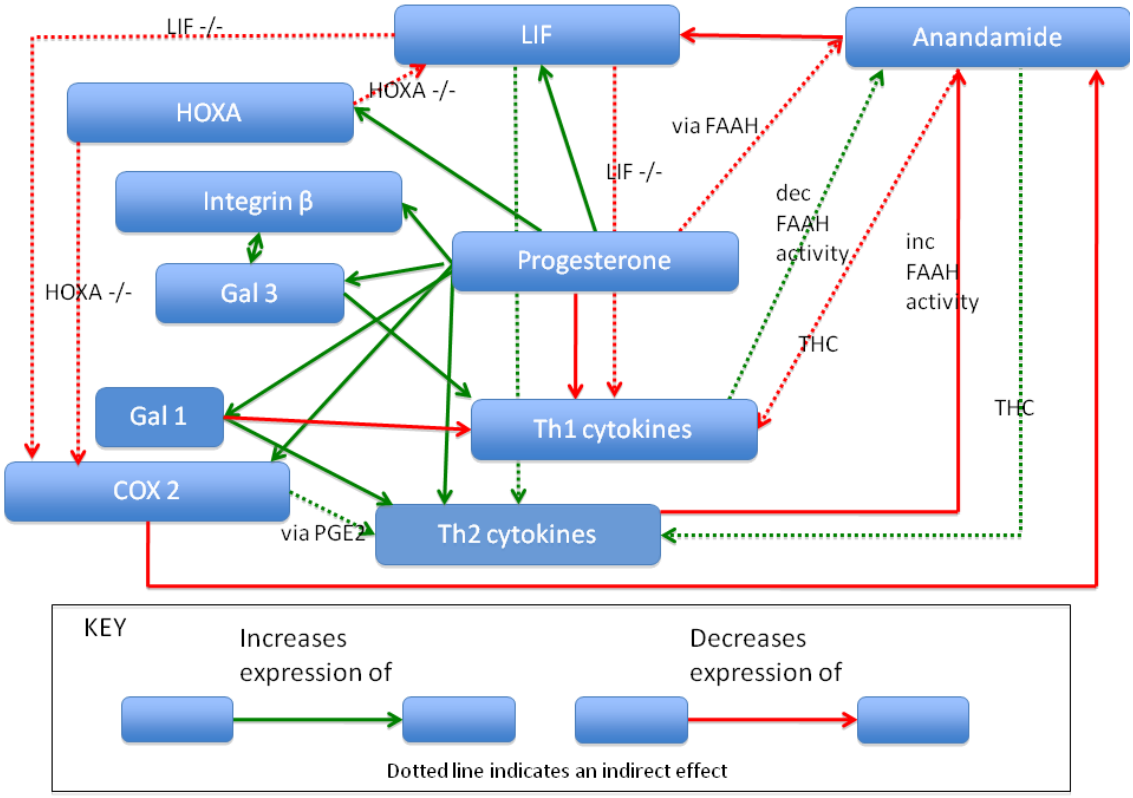

Figure 1-5 The interaction between the key components involved in implantation

While quite a complex diagram, its purpose is to highlight just how intertwined all the components are. It also highlights that progesterone has been shown to influence (either with a positive or negative effect) all of the key implantation components discussed thus far.

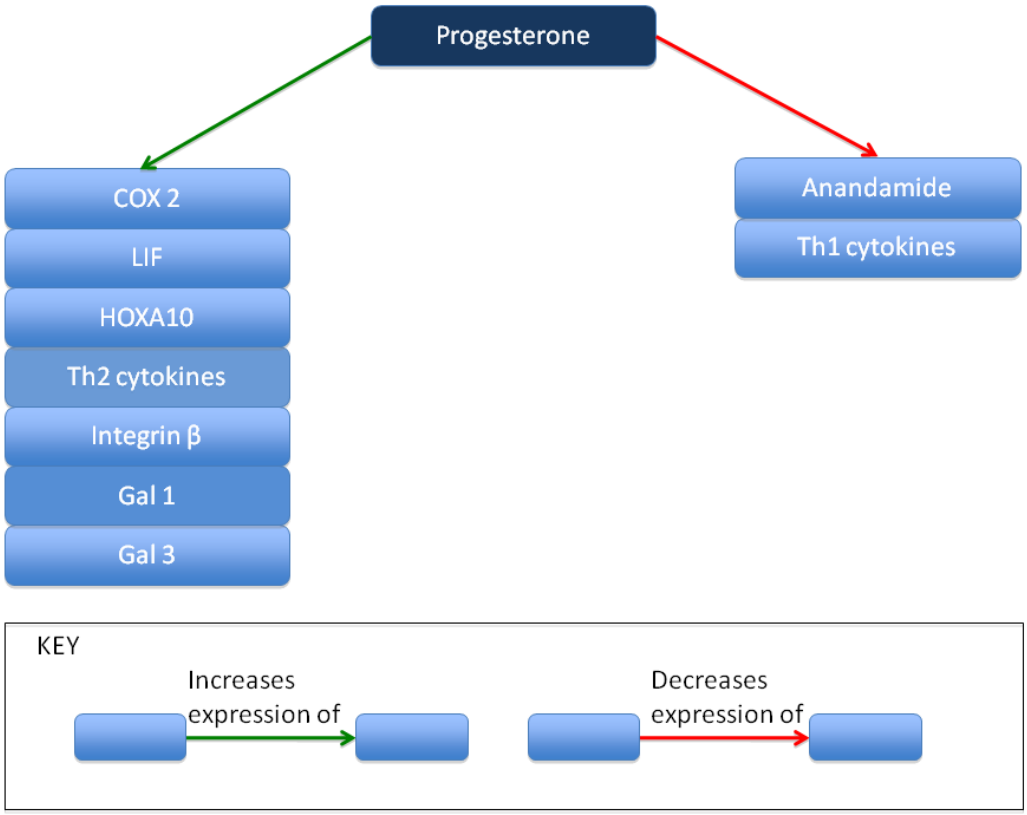

Figure 1-6 The interaction of progesterone and other key components involved in endometrial receptivity

Figure 1-5 focuses on the interaction progesterone has on other key components involved in endometrial receptivity, and shows that while it increases the expression of most components, it decreases the expression of AEA and Th1 cytokines.

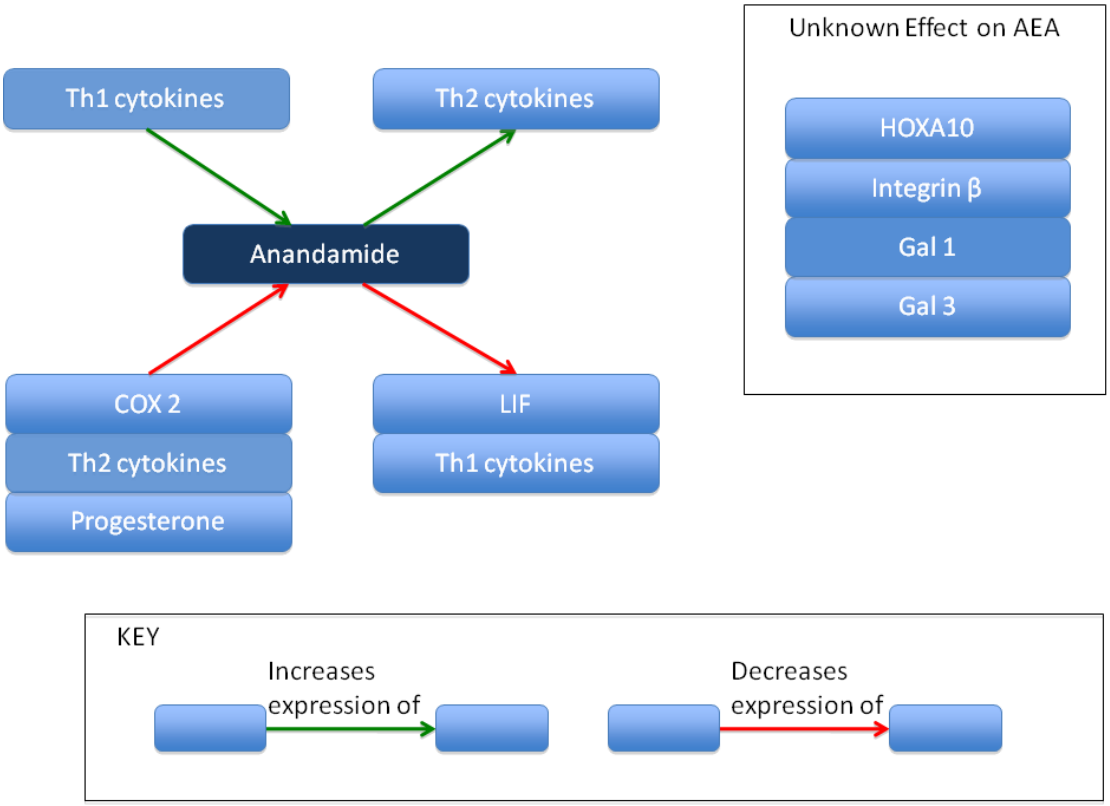

Figure 1-7 The role of anandamide and the other key components involved in endometrial receptivity

Figure 1-6 focuses specifically on the interaction anandamide has with other key components of endometrial receptivity.

While current links have been made between key components of implantation and AEA, the same link has not been investigated for the key components and other NAEs. It is already suspected that both OEA and PEA have an "entourage effect" on levels of AEA, and so it would not be unreasonable to assume they also play a role in implantation itself.

As well as detailing what is known about the interaction between components, Figure 1-6 also highlights what is not yet known. Focusing on the role of the ECS in implantation, one obvious deficit in our current knowledge is the presence (or absence) of a link between AEA and the galectins, integrins and HOXA genes.

1.9 Hypotheses and Aims

At the beginning of this chapter I stated:

"Failure to significantly improve pregnancy rates is due largely to poor understanding of the precise physiological mechanisms regulating implantation."

While this statement is accurate, it may be misinterpreted to mean that little work has been performed to find the answers. In fact, the complete opposite is true and many significant advances have been made into our understanding of the factors controlling the implantation process, but unfortunately we are still some way from solving the problem entirely. From work already described, what is obvious is that the control of uterine receptivity is complex, multi-factorial, and the result of multiple interactions between several key components. While some areas have been investigated extensively, relatively little is known about the endocannabinoid system. Although the endocannabinoid system is proving to be an exciting component of this process, the majority of data available has primarily come from animal studies. The work available from human studies is limited, and in some places incomplete. Not only are the data often incomplete, but data from animal models are not always congruent with what happens in the human uterus (Melford et al. 2014). I therefore plan to explore the role of the endocannabinoid system in human uterine receptivity in a little more detail.

The knowledge that already exists from the available studies in early pregnancy events led to the following hypotheses:

1. The ECS plays an important role in human endometrial receptivity

2. The ECS does this by interacting with other components that are known to influence endometrial receptivity

3. The concentrations of the NAEs will be associated with successful implantation, and that

4. Other NAEs, such as OEA and PEA, also play an important role in endometrial receptivity.

5. The expression of NAPE-PLD and FAAH in the human endometrium differs between the implantation and inter-implantation zones

6. There will be a direct correlation between plasma and endometrial concentrations of the NAEs

These hypotheses will be tested using the following experiments:

1. Endometrial cells will be cultured in the presence and absence of both galectin 3 and integrin β3. The relative expression of NAPE-PLD and FAAH, along with the resulting concentrations of NAEs found in the culture medium, will then be evaluated.

2. Any differences noticed in experiment #1 will be explored further by comparing the effect in receptive and non-receptive endometrial cell lines.

3. The plasma concentrations of AEA from patients undergoing assisted reproduction techniques (ART) will be used to guide the timing of embryo transfer. This will be achieved by delaying embryo transfer until plasma AEA concentrations have fallen by 40% of more. The pregnancy outcome will then be recorded.

4. Plasma OEA and PEA concentrations in patients undergoing ART will be measured during treatment and linked to clinical outcome.

5. Endometrial biopsies will be taken from the implantation and inter-implantation zones of patients undergoing surgical termination of pregnancy and the relative levels of NAPE-PLD and FAAH transcripts determined.

6. Plasma and endometrial biopsies will be taken from healthy, fertile, women and concentrations of NAEs compared.

2 The effects of galectin 3 on the endocannabinoid system – feasibility study

2.1 Introduction

In Chapter 1, the basis of the theory that endometrial receptivity is dependent upon the interaction of several ligands and receptors and that although the involvement of some of these interactions are known, the interactions between the ECS and galectins 1 and 3, the integrins β1 and β3 and HOXA proteins are poorly understood or not known at all was set out.

Because of financial and time constraints associated with this project, it was not possible to explore the interactions between all of the above factors, and so investigations were limited to one compound, galectin 3 (gal-3). The reason for this was mainly because a previous member of the endocannabinoid research group had started investigating the role of gal-3, having posed the question, "does the ECS affect expression of gal-3 in the placenta?" and was getting some interesting results. Thus, it was this work that led to the choice of gal-3 as the compound of interest.

Previously, the role of gal-3 and uterine receptivity to embryo implantation was purposed by Yang et al. (2012) where they concluded that gal-3 expression supports successful embryo implantation. They showed that higher levels of gal-3 are expressed by the endometrium of pregnant mice (when compared to their non-pregnant controls) and that there was a reduction in embryo implantation in gal-3 "knock down" mice. These data were supported by the observations that gal-3 expression increases during the window of implantation (Choe et al. 1997, Tirado-González et al. 2013, von Wolff et al. 2005, Yang et al. 2011) and its expression is higher in the implantation zone when compared to that of the inter-implantation zone (Nikzad et al. 2010). Furthermore, a role for gal-3 in implantation is suggested from the proposal that gal-3 provides a powerful pro-inflammatory signal (Rabinovich et al. 2002, Saito et al. 2010) that is required for successful embryo implantation.

By identifying that gal-3 expression mirrors the actions of some ECS components, whilst also providing an inflammatory response in the endometrium and placenta, it was hypothesised that "there is a link between gal-3 and the ECS, and that gal-3 influences the expression of the ECS at the implantation site".

However, before committing too much time (and money) to this hypothesis, it was vital to first conduct a pilot study looking to answer the question, "does galectin 3 have any effect on the expression of the ECS in the endometrium?". To do this PCR was used to quantify the expression of some components of the ECS expressed by Ishikawa cells cultured with increasing concentrations of gal-3. The rationale for using this cell line is given at 2.1.2.

2.1.1 Choice of PCR technique

The two most common methods of quantitative PCR either use SybrGreen probes or Taqman probes. SYBR Green is a DNA specific dye which preferentially binds to double stranded DNA. The resulting DNA-dye-complex absorbs blue light (λ_{max} = 497 nm) and emits green light (λ_{max} = 520 nm). An increase in DNA product during PCR therefore leads to an increase in fluorescence intensity and is measured at each cycle, thus allowing DNA concentrations to be quantified.

In contrast, TaqMan probes are more specific, and only bind to the gene of interest. They consist of a fluorophore covalently attached to the 5'-end of the oligonucleotide probe and a quencher at the 3'-end. At rest, the fluorophore and quencher are in close proximity and so no signal is detected. However, during the amplification stage of PCR, the exonuclease actions of Taq polymerase causes the quencher to be cleaved from the probe and thus a fluorescent signal is emitted (which is then detected).

The advantage of SYBR Green based PCR is that it can detect lower levels of DNA than the Taqman technique and it is also more cost effective because specialised reagents are not required. The disadvantage is that it lacks the specificity of the Taqman technique as all double stranded DNA, regardless of source is detected. To resolve the latter issue,

melting point analysis of the products is routinely added to the PCR protocol. Following discussion with my supervisor, the decision was made to use SYBR Green probes for this study.

2.1.2 Rational for using Ishikawa cells

When investigating the endometrium, a common laboratory technique is to use cell culture. Cell culture allows the study of interactions between endometrial cells and various components *in-vitro*, and is reliably reproducible.

Ideally, the cells extracted from an endometrial biopsy would be the model of choice. However, the volume of sample obtained from biopsy techniques (e.g. Pipelle® sampling) is small, and culturing the cells is notoriously difficult, both in ensuring all components of the tissue are cultured and producing tissue that can be maintained in culture medium for more than a few hours (Stavreus-Evers et al. 2003). In addition, tissue obtained from different patients would invariably display patient to patient variability. One solution to these problems is to use cell lines – endometrial cells that can be rapidly and repeatedly cultured *in-vitro*.

The choice of cell line is important because of the need to ensure the functions of the line you chose are similar to those found in *in-vivo* or *ex vivo* cultured endometrial cells. One such cell line that has been identified with these characteristics is the Ishikawa cell line (Castelbaum et al. 1997, Hannan et al. 2010). Features possessed by both Ishikawa and *in-vivo* endometrial cells include the expression of estrogen, progesterone, androgen and luteinising hormone receptors (*in-vivo* endometrial cells also express chorionic gonadotrophin receptors) and the expression of the integrins α2β1, α3β1, α6β4 and αvβ3 (Castelbaum et al. 1997). All these features mean Ishikawa cells are considered to be a good model of an adhesive endometrium.

Until recently there were several alternative options for "receptive" cell lines. One of the most popular choices were endometrial cancer cell line 1 (ECC-1) cells, that like Ishikawa cells have been shown to express estrogen, progesterone and androgen receptors, and so again have been considered to be a good model of an adhesive endometrium (Hannan et

al. 2010). However, recently Korch et al. (2012) have shown that ECC-1 cells are in fact a variant of Ishikawa cells and as such no longer useful as an alternative model for experiments comparing Ishikawa and ECC-1 cells.

Another choice of endometrial cell line previously used to study embryo implantation was Human Embryonic Stem (HES) cells (Hannan et al. 2010), as again they express estrogen, progesterone and androgen receptors. However, Korch et al. (2012) have shown that these are actually a variant of HeLa cervical cancer cells and so once again no longer considered a suitable model.

That said, Ishikawa cells are not the only cell line deemed suitable to mimic a receptive endometrium. A valid alternative choice is the RL95-2 cell line. This cell line is thought to be highly adhesive, and so is often used for implantation studies. However, Hannan et al. (2010) showed one feature of Ishikawa cells that sets them above RL95-2 cells, when they identified that Ishikawa cells express LIF receptors, whereas RL95-2 cells do not. This is important because both LIF and its receptor are known to be important in endometrial receptivity. It is for these reasons that Ishikawa cells were chosen rather than RL95-2 cells for the following studies.

2.2 Methods

2.2.1 Treatment of cells

Ishikawa cells (5×10^5) were seeded into a 6-well plate and cultured in 2ml of culture medium (DMEM:F12 with Glutamax, 10% Fetal Calf Serum and 1% penicillin/streptomycin) in an incubator maintained at 37°C with 5% CO_2 in air for 24 hours to achieve 50% confluence.

Once the cells had reached 50% confluence, 1ml of culture medium was collected from each well, snap frozen in liquid nitrogen, and stored at -80°C for later analysis of AEA/OEA/PEA concentrations. The remaining culture medium was discarded and replaced with medium augmented with different concentrations of gal-3 (0 to 40 ng/ml). The doses of gal-3 used are based on the work of Yang et al. (2011).

The cells were then re-incubated for a further 48 hours. At this point, an additional 1ml of culture medium was collected from each well and again snap frozen to facilitate later analysis of endocannabinoid concentrations. The remaining medium was discarded and total cellular RNA extracted and analysed (see section 2.2.4).

2.2.2 Extraction and measurement of NAEs from culture medium

The extraction and analysis of NAEs were performed as described in Chapter 7, with 0.5ml of culture medium being analysed instead of 0.5ml of plasma.

2.2.3 Extraction and analysis of cell RNA

The extraction and analysis of total cellular RNA is a multistep process that requires careful consideration and aptitude. Due to cost implications, the cheapest methods were employed.

2.2.4 RNA extraction (phenol/chloroform method)

After the cell medium was removed from the cell, 1ml of Trizol reagent (Invitrogen, Loughborough, UK) was added to each well and incubated at room temperature for 10 minutes. At this point the cells were dislodged from the culture surface with the aid of a cell scraper and transferred to an Eppendorf tube, to which 0.5ml of chloroform was added and the two liquids combined by vigorous shaking. Following centrifugation for 15 minutes at 7000rpm (maintained at 4°C), the top aqueous layer was transferred to a clean Eppendorf tube and 0.5ml of isopropanol added and the contents vigorously mixed on a vortexer for 1 minute before being allowed to stand for 10 minutes at room temperature. To precipitate the RNA, the tubes were centrifuged for 10 minutes at 10000rpm (again maintained at 4°C). The supernatant was discarded and the RNA pellet washed with 0.5ml of 75% ethanol:25% DEPC-treated dH_2O. Following a further centrifugation, (5 minutes at 13000rpm at 4°C), the supernatant was discarded, tubes inverted over paper tissues to air dry for 7 minutes and any remaining ethanol/water wiped away with a dry tissue. The resulting pellet was dissolved in 100μL of DEPC-treated dH_2O at 56°C for 5 minutes, and the dissolved RNA collected at the bottom of the tube by centrifugation for 2 minutes at 14000rpm (maintained at 4°C) before being stored at -80°C for later analysis.

2.2.5 DNAse-1 treatment of RNA sample

To ensure that genomic DNA contamination was removed, samples were treated with DNase-1. Whilst frozen samples were thawed slowly on ice, a master mix of DNase-1 was made as shown in table 2-1:

Table 2-1 The ingredients used to make a DNAse-1 mastermix

	Volume per sample (µL)
DNase 1 buffer	15
RNase-free DNase 1	10
RNasin	3.15
DEPC treated H_2O	21.85

The volumes were measured for n+1 samples

Once the master mix was prepared, 50 µL was added to each sample, vortexed thoroughly and then incubated at 37°C for 60 minutes. The reaction was stopped by adding 50µL phenol:chloroform:isoamyl alcohol (Phenol:$CHCl_3$:IAA, 25:24:1) to each sample followed by vigorous mixing on a vortex mixer for 1 minute and centrifugation for 2 minutes at 13000 rpm at 4°C. The supernatant was transferred to a fresh Eppendorf tube and 160µL $CHCL_3$:IAA (24:1) added. The samples were again vortex mixed for 1 minute and centrifuged for 2 minutes at 13000 rpm at 4°C. The supernatant was once again transferred into a fresh Eppendorf tube and 100µL of isopropanol was added to each sample, vortex mixed for 20 seconds and then stored overnight at -20°C. The samples were then thawed, centrifuged for 5 minutes at 14000 rpm at 4°C to collect the RNA. The RNA pellet was washed with 75% ethanol: 25% DEPC-treated water, air-dried for 7 minutes and re-suspended in 50µl of DEPC-treated water. After collecting the dissolved RNA at the bottom of the tube at 14000 rpm for 2 minutes, the amount and quality of the purified RNA was assessed.

2.2.6 RNA Quantity and Quality assessment

RNA quality and quantity was assessed using an Ultrospec 2100 Spectrophotometer. To achieve this, 5µL of RNA was added to 55µL of DEPC-dH_2O, and the absorbances at 260nm, 280nm, the 260/280 ratio and concentration of RNA in µg/µL were simultaneously recorded. These values were then used to calculate the volume of sample RNA needed for 1µg of pure RNA, based on the idea that 1 AU260 = 40µg/mL of pure RNA and that the 260/280 ratio of pure RNA = 2.0.

2.2.7 Reverse Transcription

Reverse transcription was performed using the Avian Myeloblastosis Virus Reverse Transcriptase (AMV RT) method. To convert the mRNA to cDNA, 1µg of RNA was combined with sufficient DEPC-treated H_20 to create a volume of 15.38µL and then 9.62µL of master mix (Table 2-2) was added to each sample and centrifuged for 5 seconds at 14000 rpm to collect at the bottom of the Eppendorf tube.

Table 2-2 Mastermix ingredients for the AMV-RT reaction

	Volume per sample (µL)
AMV-RT (5x) buffer	5
AMV-RT enzyme	0.5
RNasin	0.62
Anchored oligo dT primers	1
100mM dNTPs	2.5

Sufficient mastermix was made of n+1 reactions

The samples were then transferred to the thermal cycler for the RT reaction. The conditions for RT were 42°C for 60 min. The reaction was stopped by heating to 95°C for 2 min and then the reaction was held at 4°C. The resultant cDNA was then stored at -20°C for PCR analysis.

2.2.8 Polymerase Chain Reaction (PCR)

In this project, the cDNA produced following extraction of RNA from Ishikawa cells treated with gal-3 was analysed using HiGreen (Thermo Scientific, Waltham, Massachusetts USA), which is a variant of SYBR Green, in a Roche Lightcycler 2. After a HiGreen qPCR mastermix was created (Table 2-3), 19µL of mastermix was transferred to a glass PCR capillary tube and combined with 1µL cDNA. The capillaries were placed in the Lightcycler carousel and centrifuged twice at 1400rpm for 15 seconds to collect the reactants at the bottom of the capillaries.

Table 2-3 Components of HiGreen qPCR master mix.

	Volume used per sample (µL)
2x mastermix	10
PCR grade dH_2O	7
5' primer (10pmol/µL)	1
3' primer(10pmol/µL)	1

Sufficient mastermix was made to n+2 (one for the water control)

The Lightcycler settings for the annealing and extension stages varied depending on the primer used in this study, and are as shown in table 2-4.

Table 2-4 Primer sequences and PCR protocol

	Primer Sequence	**(5'-3')**	**PCR Settings**	**(denature / anneal**	**/ extend)**
Gene	**Sense**	**Antisense**	**Temperature(°C)**	**Time (sec)**	**Number of cycles**
GAPDH	ATGACCCCTTCATTGACC	GAAGATGGTGATGGGATTTC	95/60/72	10/5/13	50
CB1	TGCTGAACTCCACCGTGAAC	TCCCCCATGCTGTTATCCA	95/62/72	12/7/15	60
CB2	TGGCAGCGTGACTATGACCTT	CCACGGGTGAGCAGAGCT	95/62/72	12/7/15	60
NAPE-PLD	AAGAGATAGGAAAAAGATTTGGACCTT	CTGGGTCTACATGCTGGTATTTCA	94/58/72	30/30/120	40
FAAH	TGGAAGTCCTCCAAAAGCCCA	TGTCCATAGACACAGCCCTTCA	94/58/72	12/9/12	50

2.3 Results

2.3.1 Concentration of NAEs in culture medium

To investigate the effects of gal-3 on the ECS, its effect on the concentration of the ligands AEA, OEA and PEA found in culture medium were first examined.

2.3.1.1 AEA

The data showed that there was marked variability in the mean concentrations of AEA expressed by cells prior to treatment with gal-3 (Figure 2-1a). However, statistical analysis (two-way ANOVA, Tukey's multiple comparison tests) indicated that any differences were not statistically significant. Similarly, there was no statistically significant change between post-treatment of AEA concentrations (two-way ANOVA, Tukey's multiple comparison tests) or with any single concentration of gal-3 (two-way ANOVA, Sikak's multiple comparison test). However, as the area of interest was analysing the change in AEA, rather than absolute values, it therefore was useful to also examine the mean % change in AEA concentrations.

Figure 2-1b shows that although there were differences in AEA concentrations in the culture medium, there was no consistent correlation between the concentration of gal-3 and the production of AEA. There was a clear increase in the concentrations of AEA in the untreated cells and reduced expression at 5, 20, 30 and 40 ng/ml gal-3, but perversely an enhanced 70% increase in AEA concentrations at 10 ng/ml. These data suggest that AEA production by Ishikawa cells is not affected by gal-3.

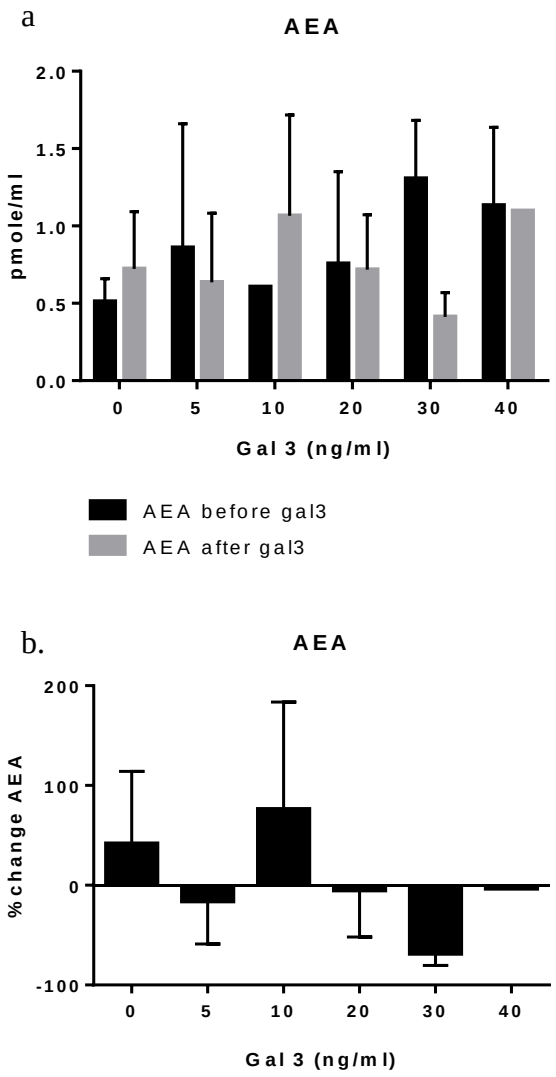

Figure 2-1 The effect of galectin 3 on anandamide (AEA) concentrations secreted into the culture medium

Ishikawa cells were exposed to the indicated concentrations of gal-3 for 48 hours. AEA was extracted from the culture medium before and after treatment and measured using UHPLC-MS/MS.

a) The data are presented as the mean ± SD AEA concentrations; (n= 3 times in triplicate). Wilk's-Shapiro test, Kilmagorov-Smirnoff test and d'Agostini test all confirmed that the data followed Gaussian distribution ($p<0.0001$ for all three tests). The changes observed are not statistically significantly different; two-way ANOVA with Tukey's HSD test and Sikak multiple comparison test.

b) The data are presented as the % change in AEA concentrations comparing pre and post treatment culture medium; (n= 3 times in triplicate). They did not show any correlation when analysed using Pearson's correlation analysis (Pearson's coefficient $r= -0.4893$; $p = 0.3246$).

2.3.1.2 OEA

As with AEA, there was marked variability in the concentrations of OEA produced by cells prior to treatment with gal-3 (Figure 2-2a) that was not statistically significant (two-way ANOVA, Tukey's multiple comparison tests). Similarly, there was no statistically significant change between post-treatment production of OEA (two-way ANOVA, Tukey's multiple comparison tests), nor in the expression of OEA at any single concentration of gal-3 (two-way ANOVA, Sikak's multiple comparison test). The mean % change in OEA concentrations (Figure 2-2b) showed that there was no consistent correlation between the concentration of gal-3 and OEA production.

Unlike the production of AEA which was increased in the control cultures, OEA was decreased by 24% in the control cultures. Although OEA concentrations initially increased at 5 and 10 ng/ml, there was a sudden change to decreased production at 20, 30 and 40 ng/ml, which is in keeping with AEA production at the same concentrations of gal-3. However, because the data were not statistically significantly different, the conclusion must be that OEA production by Ishikawa cells is unaffected by gal-3.

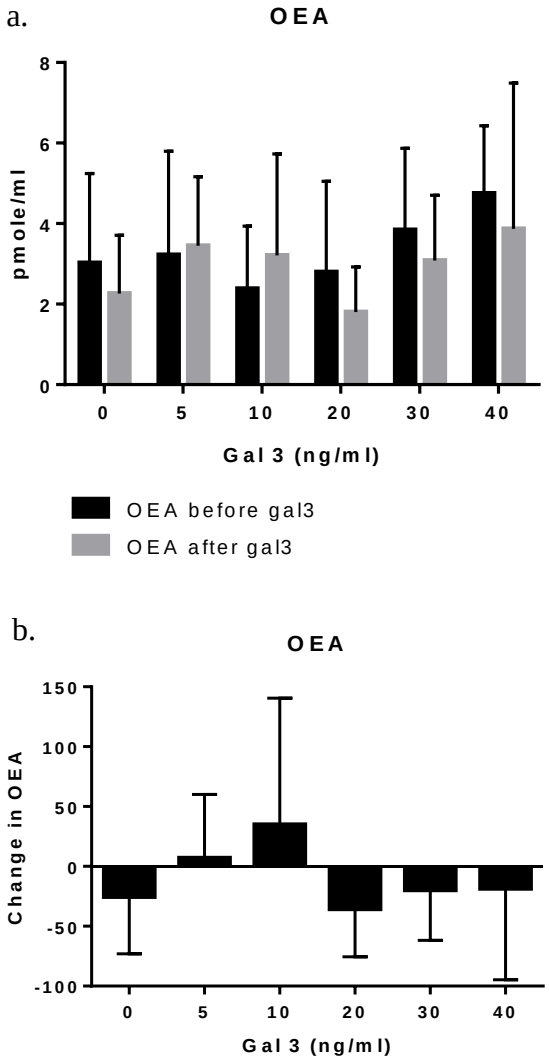

Figure 2-2 The effect of galectin 3 on oleoylethanolamide (OEA) concentrations secreted into the culture medium

Ishikawa cells were exposed to the indicated concentrations of gal-3 for 48 hours.OEA was extracted from the culture medium before and after treatment and measured using UHPLC-MS/MS.

a. The data are presented as the mean ± SD OEA concentrations; (n= 3 times in triplicate). Wilk's-Shapiro test, Kilmagorov-Smirnoff test and d'Agostini test all confirmed that the data followed Gaussian distribution ($p<0.0001$ for all three tests). The changes observed are not statistically significantly different; two-way ANOVA with both Tukey's and Sidak's multiple comparison tests.

b) The data are presented as the % change in OEA concentrations comparing pre and post treatment culture medium; (n= 3 times in triplicate). They did not show any correlation when analysed using Pearson's correlation analysis (Pearson's coefficient r= -0.3301; p = 0.5228).

2.3.1.3 PEA

Once again, there was a marked variability in the concentration of PEA produced by cells prior to treatment with gal-3 (Figure 2-3a) that was not statistically significantly different (two-way ANOVA, Tukey's multiple comparison tests). As with AEA and OEA concentrations, there was no statistically significant change between post-treatment production of PEA (two-way ANOVA, Tukey's multiple comparison tests). In addition, there was no statistically significant change in the production of PEA and any single concentration of gal-3 (two-way ANOVA, Sikak's multiple comparison test). Furthermore, the % change in mean PEA concentrations (Figure 2-3b) showed that there was a significant inverse correlation between the concentration of gal-3 and the production of PEA.

As with the production of AEA (and in contrast to OEA), production of PEA was increased in the control cultures. In keeping with AEA and OEA production, there was decreased production of PEA at 30 and 40 ng/ml.

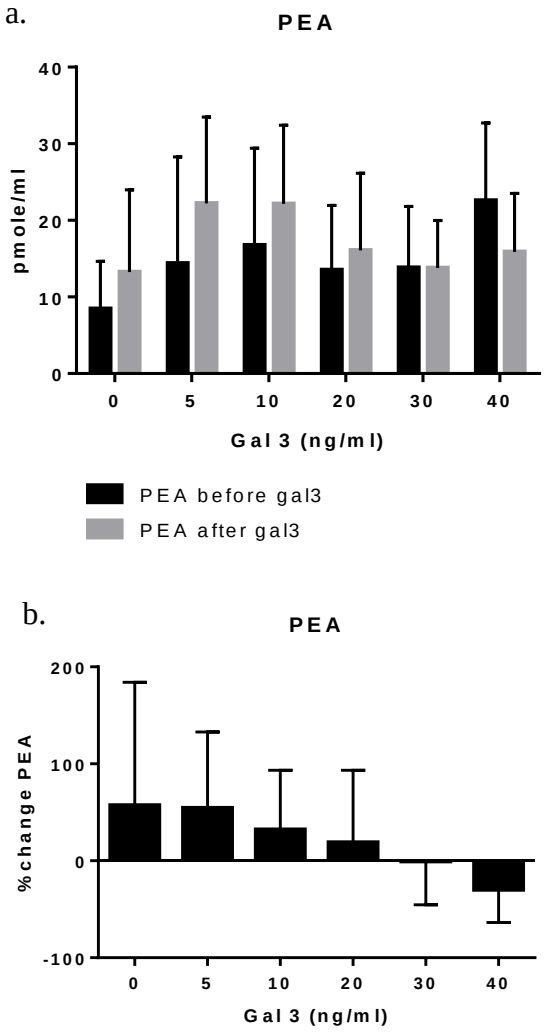

Figure 2-3 The effect of galectin 3 on palmitoylethanolamide (PEA) concentrations secreted into the culture medium

Ishikawa cells were exposed to the indicated concentrations of gal-3 for 48 hours. PEA was extracted from the culture medium before and after treatment and measured using UHPLC-MS/MS.

a. The data are presented as the mean ± SD PEA concentrations; (n= 3 times in triplicate). Wilk's-Shapiro test, Kilmagorov-Smirnoff test and d'Agostini test all confirmed that the data followed Gaussian distribution ($p<0.0001$ for all three tests).The changes observed are not statistically significantly different; two-way ANOVA with both Tukey's and Sidaks multiple comparison tests.

b. The data are presented as the mean % change in PEA concentrations; (n= 3 times in triplicate). This showed negative correlation when analysed using Pearson's correlation analysis (Pearson's coefficient $r=-0.9902$; $p = 0.001$).

2.3.2 Relative amounts of the key components of the ECS

The ECS consists of more than just the ligands and so the effect of gal-3 on the relative amounts of mRNA of the key enzymes and receptors that regulate the levels and actions of AEA, OEA and PEA was examined.

2.3.2.1 NAPE-PLD

Treatment of Ishikawa cells with a concentration of 5ng/ml of gal-3 resulted in a statistically significant rise in the relative amounts of NAPE-PLD (Figure 2-4). Although there appeared to be minor fluctuations in the relative amounts of NAPE-PLD at other concentrations of gal-3, those fluctuations were not statistically significant.

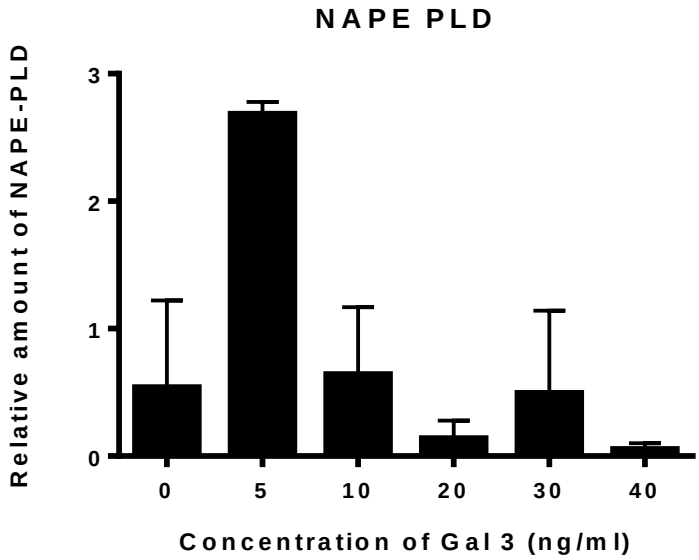

Figure 2-4 The effect of galectin 3 on relative amounts of the enzyme N-acylphosphatidylethanolamine-phospholipase D (NAPE-PLD) mRNA.

Ishikawa cells were exposed to the indicated concentrations of gal-3 for 48 hours. The relative amount of NAPE-PLD mRNA (compared against the "house-keeping gene" GAPDH) was then calculated following HiGreen based qPCR.

The data are presented as the mean ± SD relative amounts of NAPE-PLD (n= 3). The changes observed were statistically significantly different; one-way ANOVA p=0.0009. Tukey's multiple comparison test confirmed statistical significant between 5ng/ml of gal-3 and all other concentrations ($p < 0.01$ for 0, 10 and 30ng/ml and $p < 0.01$ for 20 and 40ng/ml).

2.3.2.2 FAAH

Treatment of Ishikawa cells with gal-3 appeared to increase the relative amounts of FAAH at all concentrations (Figure 2-5). Most notably, there appeared to be an almost 4 fold increase in FAAH expression when comparing 0 and 20ng/ml of gal-3. However, none of the increases in relative amounts of FAAH were found to be statistically significant.

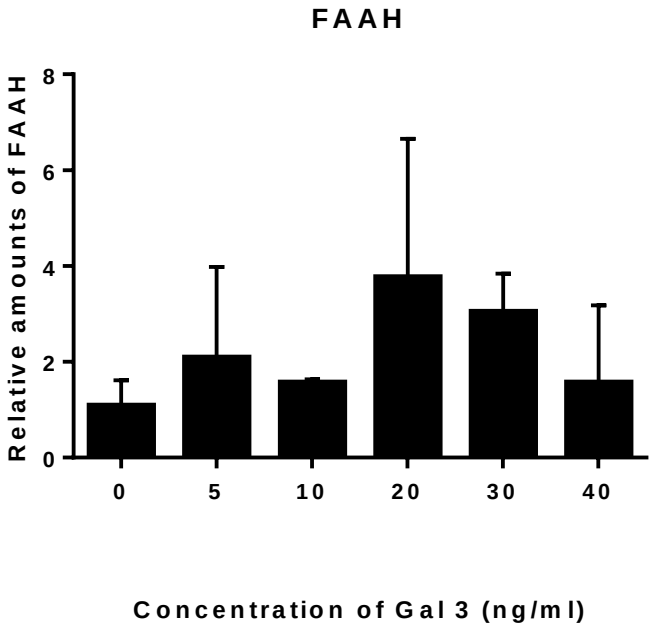

Figure 2-5 The effect of galectin 3 relative amounts of the enzyme Fatty Acid Amide Hydrolase (FAAH) mRNA.

Ishikawa cells were exposed to the indicated concentrations of gal-3 for 48 hours. The relative amount of FAAH mRNA (compared against the "house-keeping gene" GAPDH) was then calculated following HiGreen based qPCR.

The data are presented as the mean ± SD relative amounts of FAAH (n= 3). The changes observed are not statistically significantly different; one-way ANOVA p=0.4859.

2.3.2.3 CB1

Figure 2-6 shows there is no clear relationship between the concentration of gal-3 used to treat Ishikawa cells with and the relative amounts of CB1 mRNA produced. At 5, 10 and 30ng/ml there appeared to be an increase it CB1 receptor transcript levels when compared to that of the untreated cultures (0ng/ml), whereas at 20 and 40ng/ml there appeared to be a decrease. This finding is supported by a lack of statistical significance when analysed with one-way ANOVA.

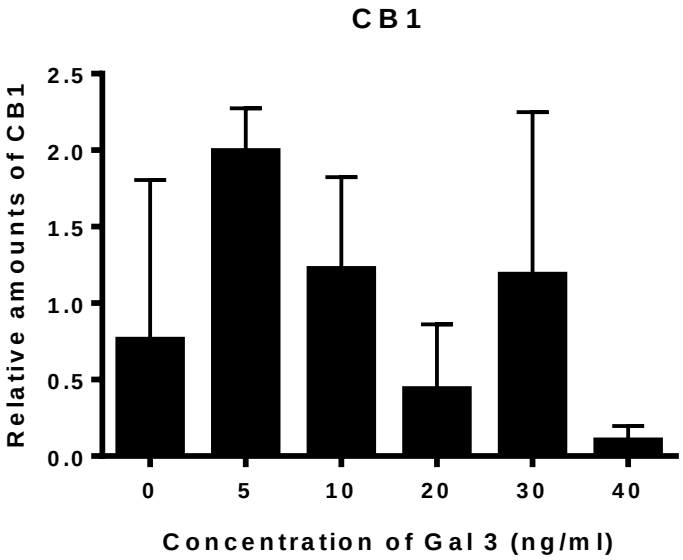

Figure 2-6 The effect of galectin 3 on the relative amounts of Cannabinoid Receptor 1 (CB1) mRNA.

Ishikawa cells were exposed to the indicated concentrations of gal-3 for 48 hours. The relative amount of CB1 mRNA (compared against the "housekeeping gene" GAPDH) was then calculated following HiGreen based qPCR.

The data are presented as the mean ± SD relative amount of CB1 (n= 3). The changes observed are not statistically significantly different; one-way ANOVA p=0.1066.

2.3.2.4 CB2

Figure 2-7 shows relative stable amounts of CB2 mRNA in Ishikawa cells, regardless of the concentration of gal-3 they were exposed to. While there appeared to be a 50% reduction in the amount of CB2 mRNA produced by cells treated with 10ng/ml gal-3, this observation was not statistically significant.

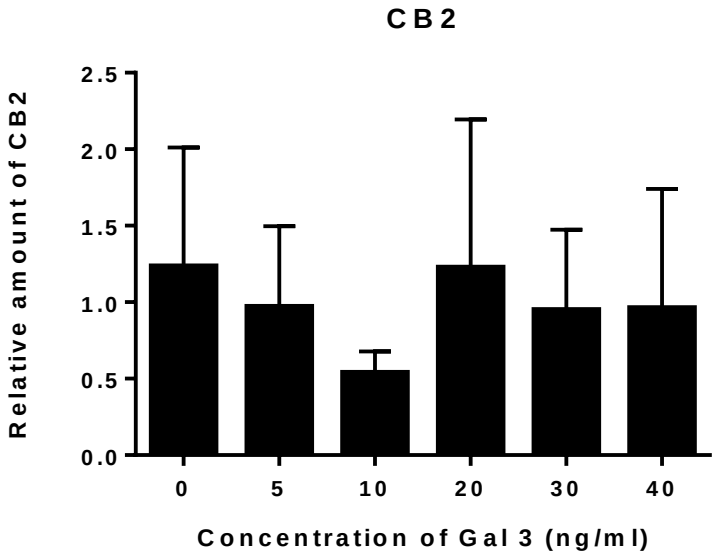

Figure 2-7 The effect of galectin 3 on the relative amounts of Cannabinoid Receptor 2 (CB2) mRNA

Ishikawa cells were exposed to the indicated concentrations of gal-3 for 48 hours. The relative amounts of CB2 mRNA (compared against the "house-keeping gene" GAPDH) was then calculated following HiGreen based qPCR.

The data are presented as the mean ± SD relative amount of CB2 (n= 3). The changes observed are not statistically significantly different; one-way ANOVA p=0.1276.

2.4 Discussion

The rationale behind this feasibility study was to identify whether the initial hypothesis that galectin 3 may influence the ECS was worth pursuing. While the small sample size here does not contain enough data to provide meaningful statistical results, it does identify some statistical trends that warrant further exploration of the effects on galectin 3 on the ECS. The graphical trends identified are that low levels of gal-3 appear to increase the amount of NAPE-PLD mRNA whereas higher levels of gal-3 increase the amount of FAAH mRNA and reduce the production of the NAEs. While differences in the amounts of the enzymes' mRNA (NAPE-PLD and FAAH) were noted, no such differences were noted in the amount of the receptors' mRNA (CB1 and CB2) suggesting that no signalling expression coupling between gal-3 receptors and CB1/CB2 transcription exists in the Ishikawa cells. By extension, this suggests that as gal-3 activity in the implantation site is modulated, there will no alteration of CB receptor expression at that site. It is noteworthy at this point out that these data could be the first documented evidence that CB1 and CB2 receptors are expressed by Ishikawa cells.

The findings in the above pilot study correlate with past work produced by the endocannabinoid research group. Previously, Taylor et al. (2010) studied the expression of CB1, CB2, NAPE-PLD and FAAH throughout the menstrual cycle. They found that while the endometrium expressed both CB1 and CB2 throughout the cycle, neither receptor's immunoreactivity was affected by the phase of the cycle. This suggests that neither CB1 nor CB2 play a crucial role in uterine receptivity. This work showed that galectin 3 does not affect the expression of CB1 or CB2, which is not unexpected knowing that galectin 3 has been linked to the success or failure of embryo implantation.

In contrast, Taylor et al. (2010) showed that the immunoreactivity of both NAPE-PLD and FAAH varied throughout the menstrual cycle, with FAAH immunoreactivity higher in the mid-proliferative gland and mid-secretory stroma, whilst NAPE-PLD immunoreactivity was down-regulated in the secretory epithelial gland compared to the proliferative epithelial gland (and unaffected in the stroma).

It is already known that the expression of NAPE-PLD and FAAH varies when the implantation zone and the inter-implantation zone in the mouse model of implantation are compared, with low levels of FAAH and high levels of NAPE-PLD in the inter-implantation sites and the opposite in the implantation site Guo et al. (2005). It is also known that galectin 3 plays an important (although not vital) role in successful embryo implantation. In light of the observations presented from this pilot study, it was postulated that galectin 3 may play an important role in influencing the expression of NAPE-PLD and FAAH, and as such, influence the success of embryo implantation by modulating the expression of NAPE-PLD and FAAH in the human implantation site. With this hypothesis in mind, it was decided to expand the feasibility study into a full study.

3 The effects of galectin 3 on the endocannabinoid system – full study

Whilst the Ishikawa cells were cultured following the same method as described in the feasibility study, it was necessary to change the method used for the extraction and analysis of the mRNA expressed in this project. In the feasibility study, the mRNA was extracted using a phenol/chloroform method and the cDNA produced from this was analysed using HiGreen (a variant of SYBR Green) based PCR. This method gave some interesting results, as described in Chapter 4. However, on review of these results, it was felt that the Ct values for GAPDH were much higher than expected, and time was taken to optimise PCR conditions (including buying new reagents, altering the volume of primer of cDNA used and using different batches of the cell line). During this time, the Ct values for GAPDH worsened rather than improved. Eventually it was concluded that the lightcycler was likely to be responsible for the poor results and that a newer machine should be used. Unfortunately, none of the newer machines within the Department were compatible with HiGreen and so the method for quantifying cDNA was changed to TaqMan based PCR. This coincided with a change of supervision, resulting in a decision to also change the RNA extraction method to one using a Turbo DNA free kit (described below).

As already discussed, the advantage of SYBR Green based PCR is that it can detect lower levels of DNA than Taqman and is more cost effective. However, SYBR Green can also detect non-specific PCR products, such as single-stranded DNA, as well as the gene of interest which can potentially interfere with, or prevent, accurate quantification of the intended target sequence. Conversely, Taqman probes are more expensive but are more specific to the PCR products they detect.

3.1 Methods

3.1.1 Cell culture

Culture of Ishikawa cells was conducted as described in the Chapter 4, although to ensure that meaningful amounts of data were obtained, 6 wells were treated with each concentration of gal-3 (rather than the 3 wells used in the feasibility study).

3.1.2 Extraction and analysis of cellular RNA

As already discussed, following a change in supervision, RNA extraction was performed using a Turbo DNA free kit.

3.1.3 RNA Quantity and Quality assessment

The method used to analyse the quantity and quality of RNA produced changed for this stage of the project from the Ultrospec 2100 spectrophotometer used in the feasibility study to a Nanodrop spectrophotometer for this part. While both assess RNA quality and quantity using optical density (OD) measurement, the Ultrospec 2100 Spectrophotometer requires 5µL of RNA whereas the Nanodrop spectrophotometer only uses 1µL.

3.1.4 DNAse treatment of RNA sample

DNA contamination was removed from each sample using Turbo-DNAse (Thermo Fisher Scientific, Rugby, United Kingdom). To achieve this, 5µg of RNA (volume calculated using quantification results) was added to an microfuge tube containing 1µL Turbo-DNAse, 5µL of 10x buffer and dH2O to make a final reaction volume of 50µL. The mixture was then incubated for 30 minutes at 37°C. Following this, 5µL of inactivation buffer was added and after 3 minutes of mixing at room temperature, the samples were centrifuged for 90 seconds (10000g at 4°C). Finally, the supernatant was removed and transferred to a fresh microfuge tube.

3.1.5 Reverse Transcription (RT) with Multiscribe kit

One µg of RNA was combined with 10µL of "+ reaction" master mix (Table 3-1) and centrifuged for 5 seconds.

Table 3-1 Components of "+ reaction" mastermix

	Volume per sample (µL)
RT buffer (10x) buffer	2
Multiscribe reverse transcriptase	1
RNAse inhibitor	1
Random primers (10x)	2
100mM dNTPs (25x)	0.8
PCR grade water	3.2

The same method was employed to make the negative controls, using 1µg of RNA combined with 10µL of "- reaction" mastermix (Table 3-2).

Table 3-2 Components of "- reaction" mastermix (negative control)

	Volume per sample (µL)
RT buffer (10x) buffer	2
RNAse inhibitor	1
Random primers (10x)	2
100mM dNTPs (25x)	0.8
PCR grade water	4.2

The samples were then transferred to the thermocycler for both the +RT and –RT reactions. First, the samples were held at 25°C for 10 minutes to allow the primers to anneal to the RNA template. Next, the RT reaction occurred while the samples were heated to 37°C for 120 min. Finally, the reaction was stopped by heating to 85°C for 5

min and kept on hold at 4°C. The resultant cDNA was then stored at -20°C while awaiting future analysis by PCR.

3.1.6 Polymerase Chain Reaction

In this project, the cDNA produced following extraction of RNA from Ishikawa cells treated with gal-3 was analysed using Taqman probes in a StepOne qPCR system. After a Taqman qPCR mastermix was created (Table 3-3), 18μL of mastermix was transferred to a plastic PCR reaction strip and combined with 2μL cDNA. Following brief centrifugation (to remove any bubbles) the strips were placed in the StepOne qPCR system.

Table 3-3 Components of Taqman qPCR mastermix.

	Volume per sample (μL)
TaqMan Gene expression MasterMix	10
GAPDH (housekeeper gene)	1
Gene of interest	1
PCR grade water	6

Sufficient mastermix was made to n+2 (n = samples + duplicates + non-template controls).

3.2 Results

3.2.1 Effect of Galectin 3 on Ishikawa cells

3.2.1.1 Concentration of NAEs in culture medium

The "full study" results of treating Ishikawa cells with gal-3 (Figure 3-1a) are the total opposite of those found in the "feasibility study" (Figure 2-1a), as it does not appear that treatment of Ishikawa cells with any concentration of gal-3 had a significant difference in the concentration of AEA produced, although statistical analysis revealed that treatment with 10ng/ml of gal-3 resulted in a statistically significant increase in the concentration of AEA.

Figure 3-1b shows that although there were differences in AEA concentrations in the culture medium, there was no consistent correlation between the concentration of gal-3 and the production of AEA. In contrast to the feasibility study, in the full study there was a slight reduction in the concentration of AEA in the untreated cells, followed by an increase in AEA concentrations at 5 and 10ng/ml gal-3. This was followed by a reduced expression at 20 and 30 ng/ml gal-3, followed by a slight increase in expression at 40ng/ml. These data support the conclusion suggested in Chapter 4, namely that AEA production by Ishikawa cells is not affected by gal-3.

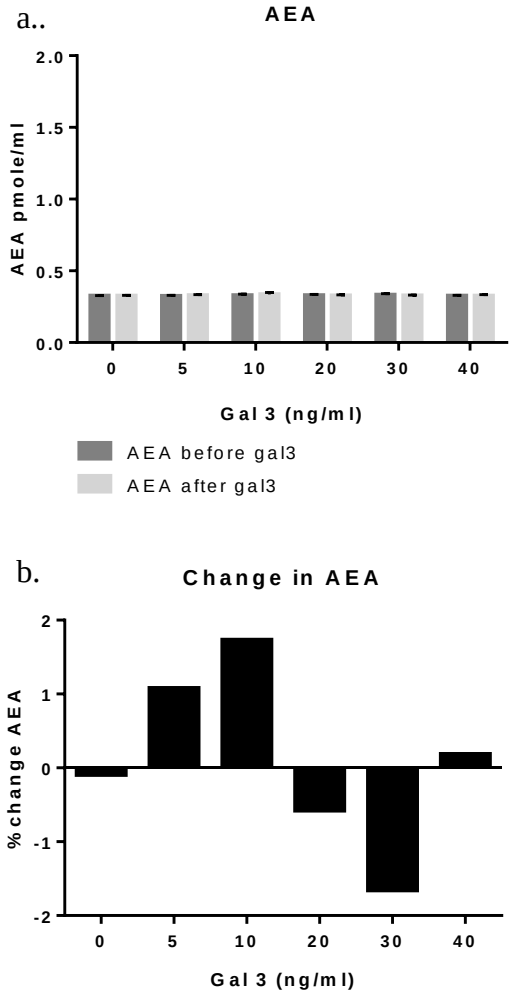

Figure 3-1 The effect of galectin 3 on anandamide (AEA) concentrations secreted into the culture medium

Ishikawa cells were exposed to the indicated concentrations of gal-3 for 48 hours. AEA was extracted from the culture medium before and after treatment and measured using UHPLC-MS/MS.

a. The data are presented as the mean ± SD AEA concentrations; (n= 6 times in triplicate). Wilk's-Shapiro test, Kilmagorov-Smirnoff test and d'Agostini test all confirmed that the data followed Gaussian distribution ($p<0.0001$ for all three tests). While the changes observed pre- and post-treatment at each concentration were not statistically significantly different (two-way ANOVA with Sikak multiple comparison test), there was a statistically significant difference between to post dose concentration of AEA in the culture medium of cells treated with 10ng/ml gal-3 when compared to the control ($p < 0.01$, two-way ANOVA with Tukey's multiple comparison test).

b. The data are presented as the % change in mean AEA concentrations; (n= 6 times in triplicate). It did not show any correlation when analysed using Pearson's coefficient ($r=0.4632$, $p = 0.2146$). (NB – extraction of AEA from cells treated with 40ng/ml only generated 3 data points, and so cannot confidently be assessed).

As with AEA, it appears that there was limited variability in the concentration of OEA produced by cells prior to treatment with gal-3 (Figure 3-2a), however, statistical analysis (two-way ANOVA, Tukey's multiple comparison tests) again showed that there was a statistically significant difference in OEA concentrations for cells treated with all concentrations of gal-3. In addition, there was a statistically significant difference when comparing post treatment concentrations of OEA in Ishikawa cell with the control (two-way ANOVA, Sikak's multiple comparison test). As with AEA, this was the complete opposite to what was found in the feasibility study.

Unlike the data collected in the feasibility study, Figure 3-2b shows that there was a reduction in OEA expression by all cells, including the controls. However, there was correlation between the feasibility study and the full study, in that concentrations of OEA in cells treated with 5 and 10ng/ml of gal-3 were higher than the control, whereas the concentration of OEA in cells treated with 20ng/ml or more of gal-3 was lower than that found in the culture medium of the control cells. This was confirmed by statistical analysis, where Pearson's coefficient confirms there was an inverse correction between the concentration of gal-3 and the change in OEA concentration.

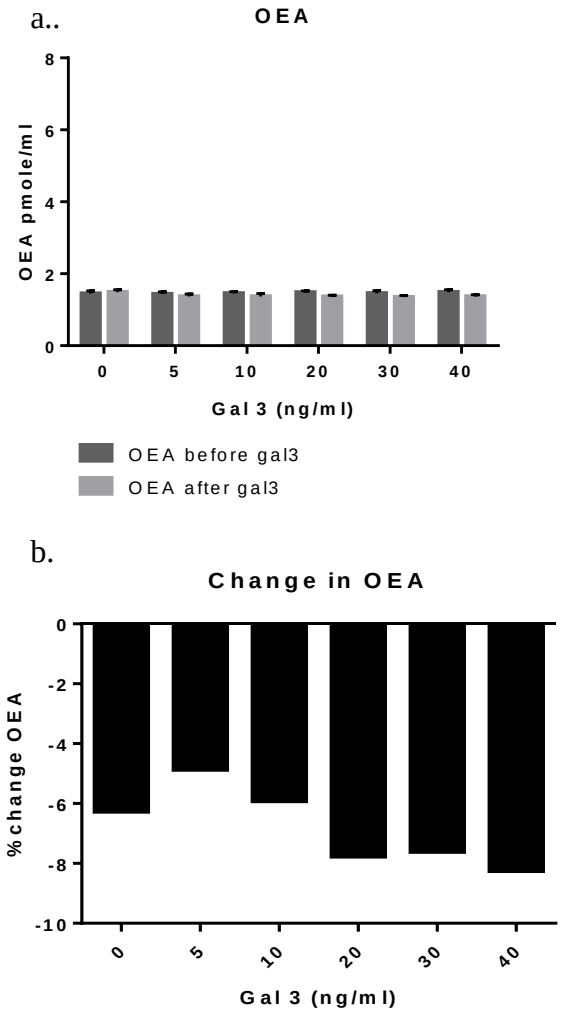

Figure 3-2 The effect of galectin 3 on oleoylethanolamide (OEA) concentrations secreted into the culture medium

Ishikawa cells were exposed to the indicated concentrations of gal-3 for 48 hours. OEA was extracted from the culture medium before and after treatment and measured using UHPLC-MS/MS.

a. The data are presented as the mean ± SD OEA concentrations; (n= 6 times in triplicate). Wilk's-Shapiro test, Kilmagorov-Smirnoff test and d'Agostini test all confirmed that the data followed Gaussian distribution ($p<0.0001$ for all three tests). There was a statistically significant difference when comparing pre- and post-treatment concentrations of OEA for all treated cells ($p<0.01$ for 5ng/ml, $p<0.001$ for 10ng/ml and $p<0.0001$ for 20, 30 and 40ng/ml; two-way ANOVA, Sikak's multiple comparison test). In addition, there was a statistically significant difference when comparing post-treatment concentrations of OEA in Ishikawa cells when compared with the control ($p<0.0001$ for all concentrations of gal-3; two-way ANOVA, Tukey's multiple comparison test).

b. The data are presented as the % change in mean OEA concentrations; (n= 6 times in triplicate). It also showed an inverse correlation when analysed using Pearson's coefficient ($r=-0.8496$, $p=0.032$).

In keeping with the findings of the feasibility study, it appears that there was marked variability in the concentration of PEA expressed by cells prior to treatment with gal-3 (Figure 3-3a). However, unlike the feasibility study, this finding was supported by statistical analysis (two-way ANOVA, Tukey's multiple comparison tests). As with the changes in OEA concentrations, there was a statistically significant difference between post treatment production of PEA (two-way ANOVA, Tukey's multiple comparison tests); however, this occurred at 5ng/ml (whereas with OEA it was at 0ng/ml). In addition, there was a statistically significant change in the expression of PEA in cells treated with 5ng/ml of gal-3 (two-way ANOVA, Sikak's multiple comparison test).

Again, as with the feasibility study, Figure 3-3b shows that there was significant inverse correlation between the concentration of gal-3 and the expression of PEA, and this was confirmed by statistical analysis. As with the production of AEA (and in contrast to OEA), production of PEA was increased in the control cultures. In keeping with AEA and OEA production, there was decreased production of PEA at 30 and 40 ng/ml.

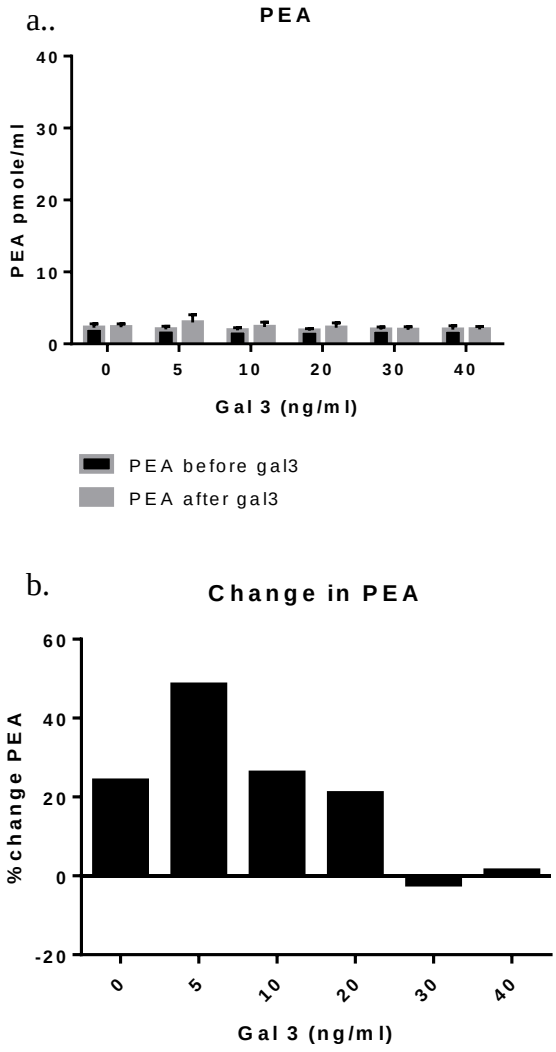

Figure 3-3 The effect of galectin 3 on palmitoylethanolamide (PEA) concentrations secreted into the culture medium

Ishikawa cells were exposed to the indicated concentrations of gal-3 for 48 hours. PEA was extracted from the culture medium before and after treatment and measured using UHPLC-MS/MS.

a. The data are presented as the mean ± SD PEA concentrations; (n= 6 times in triplicate). Wilk's-Shapiro test, Kilmagorov-Smirnoff test and d'Agostini test all confirmed that the data followed Gaussian distribution ($p<0.0001$ for all three tests). There was a statistically significant difference when comparing pre- and post-treatment concentrations of PEA for cells treated with 5ng/ml gal-3 ($p<0.0001$; two-way ANOVA, Sikak's multiple comparison test). In addition, there was a statistically significant difference in the concentration of PEA expressed by cells treated with 5ng/ml when comparing post treatment concentrations of PEA with all other cell groups ($p<0.05$ for 10ng/ml, $p<0.01$ for 0 and 20ng/ml and $p<0.0001$ for 30 and 40ng/ml of gal-3; two-way ANOVA, Tukey's multiple comparison test).
b. The data are presented as the % change in mean PEA concentrations; (n= 6 times in triplicate). It showed an inverse correlation when analysed using Pearson's coefficient ($r=-0.8212$, $p=0.0451$).

3.2.1.2 Relative amounts of the ECS enzymes

As shown in Figure 3-4, treatment of Ishikawa cells with a concentration of 5ng/ml of gal-3 resulted in a statistically significant increase in the relative amounts of NAPE-PLD mRNA. Although there appears to be minor fluctuations in the relative expression of NAPE-PLD at other concentrations of gal-3, there was no statistical evidence to support this. This suggests that gal-3 affects the relative amounts of NAPE-PLD mRNA in an inverse relationship.

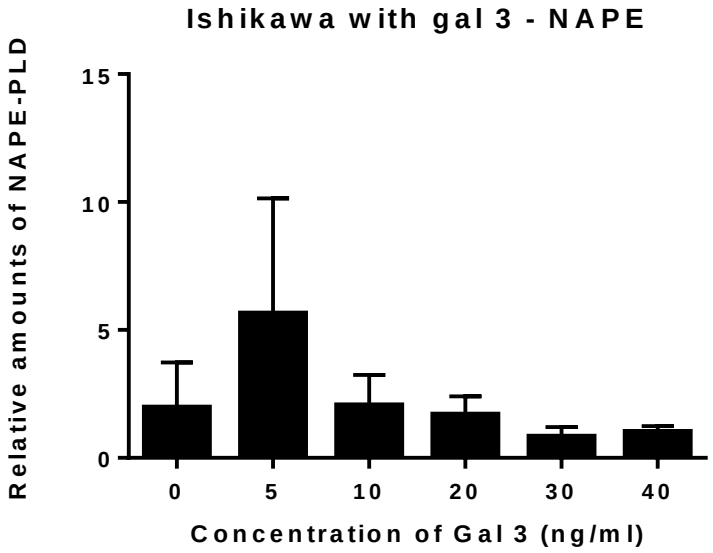

Figure 3-4 The effect of galectin 3 on relative amounts of N-acylphosphatidylethanolamine-phospholipase D (NAPE-PLD) mRNA

Ishikawa cells were exposed to the indicated concentrations of gal-3 for 48 hours. The relative amount of NAPE-PLD mRNA (compared against the "house-keeping gene" GAPDH) was then calculated following TaqMan based PCR.

The data are presented as the mean ± SD relative amounts of NAPE-PLD (n= 6). As with the feasibility study, the data are statistically significantly different; one-way ANOVA p=0.0007. Tukey's multiple comparison test confirmed statistical significance between cells treated with 5ng/ml of gal-3 and those treated with 20, 30 and 40ng/ml ($p < 0.05$ for 20ng/ml and $p < 0.01$ for 30 and 40ng/ml).

As shown in Figure 3-5, treatment of Ishikawa cells with higher concentrations of gal-3 (30 and 40ng/ml), appeared to increase the relative amounts of FAAH mRNA by over 100%. However, none of the increases in relative amounts of FAAH mRNA were found to be statistically significant.

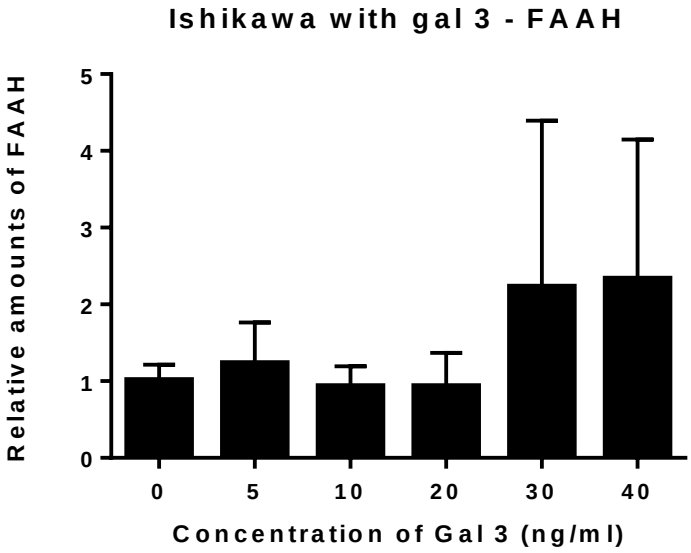

Figure 3-5 The effect of galectin 3 on relative amounts of Fatty Acid Amide Hydrolase (FAAH) mRNA

Ishikawa cells were exposed to the indicated concentrations of gal-3 for 48 hours. The relative amount of FAAH mRNA (compared against the "house-keeping gene" GAPDH) was then calculated following TaqMan based PCR.

The data are presented as the mean ± SD relative amounts of FAAH (n= 6). The changes observed are not statistically significantly different; one-way ANOVA p=0.1376.

3.2.2 Correlation between ECS enzymes and NAE concentration

As already discussed, it is thought that OEA and PEA provide an "entourage effect" to the actions of AEA by competing for degradation by FAAH. In Figure 3-6, it was shown that AEA concentrations remained pretty static, regardless of the relative FAAH expression. The same was seen for OEA concentrations. However, the concentration of PEA fell as the relative amounts of FAAH mRNA increased. This change was not statistically significant (Pearson's correlation; $r= -0.2418$, $p= 0.1900$).

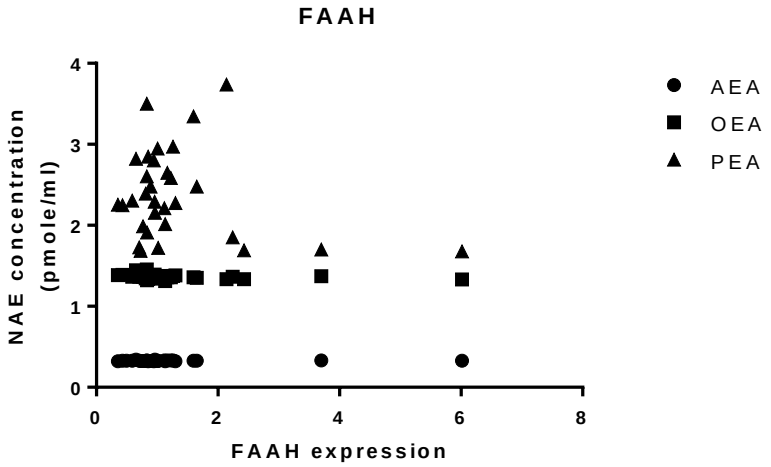

Figure 3-6 The effect of the amount of Fatty Acid Amide Hydrolase (FAAH) mRNA levels on the concentration of various NAEs.

Ishikawa cells cultured with various concentrations of gal-3 for 48 hours, and then both the culture medium and cells were collected. AEA, OEA and PEA were extracted from the culture medium and their concentrations were calculated using UPLC MS/MS. The relative amount of FAAH mRNA was calculated from the cell's RNA using Taqman PCR.

The concentrations of the various NAEs were then plotted against the relative amounts of FAAH mRNA of the cells from the same well. The data are presented individual data points. The correlation of each NAE against the relative amount of FAAH mRNA was then calculated using Pearson's correlation. None of the results were statistically significant (AEA p= 0.6128, OEA p= 0.1109, PEA p= 0.1900).

The described “entourage effect” related to competition for degradation by FAAH. However, having already discussed the importance of “anandamide tone” the next step was to see if the “entourage effect” also relates to synthesis by NAPE-PLD. Figure 3-7 shows very similar findings to Figure 3-6; while concentrations of AEA and OEA remain relatively unchanged despite increasing amounts of NAPE-PLD mRNA, concentrations of PEA increased with increasing amounts of enzyme mRNA.

Interestingly, this change in PEA concentration related to the relative amount of NAPE-PLD mRNA was statistically significant (Pearson’s correlation, $r= 0.7014$, $p= <0.0001$).

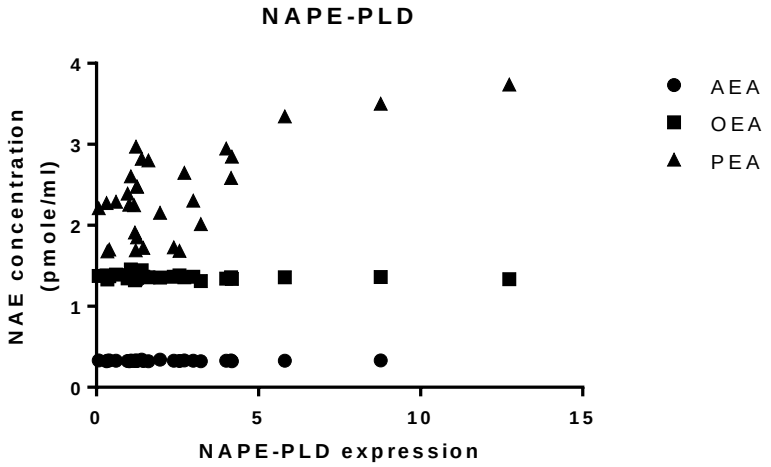

Figure 3-7 The effect of the amount of N-acylphosphatidylethanolamine-phospholipase D (NAPE-PLD) mRNA on the concentration of various NAEs

Ishikawa cells cultured with various concentrations of gal-3 for 48 hours, and then both the culture medium and cells were collected. AEA, OEA and PEA were extracted from the culture medium and their concentrations were calculated using UPLC MS/MS. The relative amount of NAPE-PLD mRNA was calculated from the cell's RNA using Taqman PCR.

The concentrations of the various NAEs were then plotted against the relative amount of NAPE-PLD mRNA of the cells from the same well. The data are presented individual data points. The correlation of each NAE against the amount of NAPE-PLD mRNA amounts was then calculated using Pearson's correlation. The results for AEA and OEA were not statistically significant (AEA p= 0.9137, OEA p= 0.1259). However, the correlation between the amount of NAPE-PLD mRNA and PEA concentration was statistically significant (r= 0.7104, p= <0.0001).

3.3 Discussion

In this study it was shown that treating Ishikawa cells with varying concentrations of galectin 3 had statistically significant effects on the production of OEA and PEA with an inverse relationship. In addition, a negative correlation between the concentration of gal-3 Ishikawa cells are treated with and the subsequent relative amount of NAPE-PLD mRNA was demonstrated. These findings together support a hypothesis that low concentrations of gal-3 (<10ng/ml) promote NAPE-PLD expression and thus enhance the production of the NAEs OEA and PEA.

In contrast to NAPE-PLD, there was no statistically significant change in the relative amount of FAAH mRNA with varying concentrations of gal-3 Ishikawa cells were exposed to. However, the concentration of both OEA and PEA fell below that of the control when treated with higher concentrations of gal-3. The same finding may also be true for AEA. However, extraction of AEA from cells treated with 40ng/ml only generated 3 data points, and so cannot confidently be assessed.

At this point it is pertinent to clarify an important assumption made, and that it that the relative amounts of NAPE-PLD or FAAH mRNA measured equates to the amount of active enzyme. However, it is known that RNA transcription does not necessarily lead to protein expression, and protein expression does not necessarily leave to enzyme activity. It is therefore important that the reader is made aware that in these experiments the data referit refers to relative amounts of mRNA measured.

In this study the suggested "entourage effect" that OEA and PEA have on AEA's concentration was also considered. Here a statistically significant ($p<0.001$) correlation between amounts of NAPE-PLD mRNA and PEA concentration was demonstrated and graphically it appeared that a correlation between FAAH and PEA existed (although this was not supported statistically). There was no statistically significant correlation between NAPE-PLD or FAAH and OEA. The "entourage effect" implies that both PEA and OEA are involved in the regulation of AEA production by competing for degradation. Overall, the results suggest that there is a closer link between the ECS enzymes and PEA

production than OEA production, but further work would be necessary to identify if it is PEA (or the combined effects of PEA and OEA) that affect AEA production.

A decrease in the concentration of NAEs following an increase in gal-3 concentrations suggests an increase in its degradation, and cannot be explained by the decreased amount of NAPE-PLD mRNA at the higher concentrations of gal-3. It therefore seems logical that the decrease in NAE concentrations must be a result of increased expression of FAAH at higher concentrations of gal-3.

These results, however, do not support this hypothesis, and lead to consideration of whether another component was involved in enhancing the expression of FAAH when cells are treated with gal-3. It has already been suggested that there is a bi-directional interaction between integrin β3 and gal-3 (Lei et al. 2009). It was therefore decided to investigate this phenomenon further, to try to determine if the changes in NAPE-PLD and FAAH expression (and their subsequent effects on NAE concentrations) seen are as a result of gal-3, integrin β3 or a combination of both.

4 The effects of galectin 3 and integrin β3 on the endocannabinoid system

4.1 Introduction

Gal-3 is known to enhance pathways known to be important for successful endometrial receptivity and is a natural ligand for the plasma membrane receptor integrin β3 (Yang et al. 2012). Binding of gal-3 to integrin β3 (but not integrin β1) has been shown to enhance endometrial cell-to-cell adhesion (Lei et al. 2009).

The expression of integrin β3 in the endometrium, or more specifically αvβ3, coincides with the "window of implantation" and has also been linked to endometrial pinopode development (Lessey et al. 1992, Lessey 2003). It is known that integrin β3 mediates fibronectin binding activity of peri-implantation mouse blastocysts (Schultz and Armant 1995). Furthermore, aberrant expression of αvβ3 has been linked to infertility and abnormal embryo implantation (Lessey and Castelbaum 1995, Creus et al. 1998, Yoshimura 2002).

It has been suggested that there is a bi-directional interaction between integrin β3 and gal-3, with Lei et al. (2009) demonstrating that a decrease in expression of integrin β3 in gal-3 knock-down cells (suggesting that gal-3 supports the expression of integrin β3) occurs, and that blocking integrin β3 enhanced the effects of exogenous gal-3 on cell proliferation and adhesion.

In Chapter 3 it was shown that the concentration of gal-3 appears to affected the relative amounts of both NAPE-PLD and FAAH mRNA (although with no statistical significance for FAAH), and considering all of the information above, it seemed logical to expand the project to identify whether or not the effects shown were a result of gal-3 action, integrin β3 expression or a combination of both factors.

4.1.1 Investigating the effects of galectin 3 (without integrin β3)

To investigate the effects of gal-3 (without any additional effects of integrin β3) it was necessary to block the integrin receptor. To achieve this, cells were treated with cilengitide (Figure 4-1), which is a commercially available agent that blocks αvβ3 (Gilbert et al. 2012, Nisato et al. 2003, Reardon and Cheresh 2011)) with an IC50 of 4.1nM.

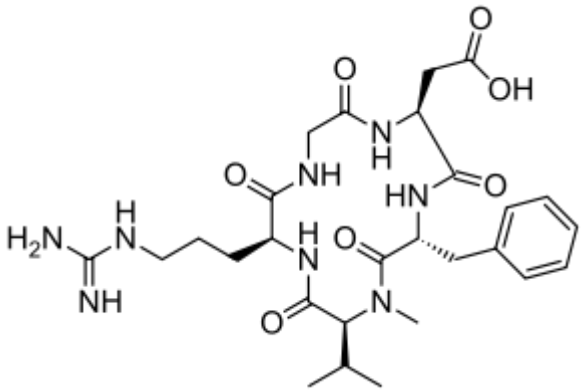

Figure 4-1 The chemical structure of cilengitide, a cyclic RGD pentapeptide

Unfortunately, it was not possible to find any past work detailing the concentration of cilengitide needed to completely block the actions of integrin β3, and the paper identifying the IC50 of cilengitide did not state the concentration needed to completely block the actions of integrin β3. However, work conducted by Maurer et al. (2009) showed that concentrations of up to 10μM cilengitide did not have a significant effect on cell adhesion or viability, whereas work by Lautenschlaeger et al. (2013) showed that concentrations of 20μM did affect cell viability. As the aim here was to completely block the actions of integrin β3 without causing cell toxicity, it was decided to use the highest dose of cilengitide demonstrated to be safe (i.e. 10μM).

With integrin β3 blocked, the cells were treated with the same concentrations of gal-3 as in the previous study (0 to 40ng/ml) (Yang et al. 2011).

4.1.2 Investigating the effects of integrin β3 (without galectin 3)

To investigate the effects of integrin β3 (without any additional effects of gal-3), a decision was made to artificially upregulate the expression of the receptor using S-nitroso-N-acetylpenicillamine (SNAP). This was because Lee (2000) demonstrated that SNAP up-regulates the expression of integrin β3 in a dose-dependent manner, and so cells were treated with increasing SNAP concentrations, as described in their paper (i.e. 0μM to 2000μM).

4.2 Methods

Ishikawa cells (5×10^5) were seeded into a 6-well plate and cultured in 2ml of culture medium (DMEM:F12 with Glutamax, 10% Fetal Calf Serum and 1% penicillin/streptomycin) in an incubator maintained at 37°C with 5% CO_2 in air, for 24 hours to achieve 50% confluence. Once the cells had reached 50% confluence, 1ml of culture medium was collected from each well, snap frozen in liquid nitrogen, and stored at -80°C for later analysis of AEA/OEA/PEA concentrations. The remaining culture medium was discarded and replaced with medium augmented with different concentrations of either cilengitide (10µM) + gal-3 (0 to 40ng/ml) or S-nitroso-N-acetylpenicillamine (0-2000µM). The cells were then re-incubated for a further 48 hours. At this point, an additional 1ml of culture medium was collected from each well and snap frozen to facilitate later analysis for endocannabinoid concentrations. The remaining medium was discarded and total cellular RNA extracted and quantified (see Chapter 3 for full description of the methods used).

4.3 Results

The data presented in this section relate to effects on NAE expression, cell growth and the relative amounts of endocannabinoid modulating enzyme transcript produced.

4.3.1 Effects on NAE concentration

Unfortunately it was not possible to obtain any results from the analysis of the culture medium collected during these experiments. The ULPC developed a mechanical fault, and as such measurements of the NAEs in the cell culture samples were not possible. In a financially conscious environment, it was decided that repairing the ULPC was not financially viable, and unfortunately a suitable replacement UPLC was not available. The decision therefore was made to abandon this aspect of the project, and to focus solely on the effects of gal-3 and integrin β3 on the expression of NAPE-PLD and FAAH.

4.3.2 Effects of galectin 3 (without integrin β3)

4.3.2.1 Effects on cell growth

One unexpected effect of treating cells with cilengitide was an effect on cell growth. As already discussed, the decision was made to treat cells with 10μM of cilengitide as it had already been shown by Maurer et al. (2009) that this concentration did not affect cell viability. However, the cells treated with cilengitide did not adhere to the plastic of the culture flask, and instead clumped together and were free floating in the culture medium, as shown in Figure 4-2.

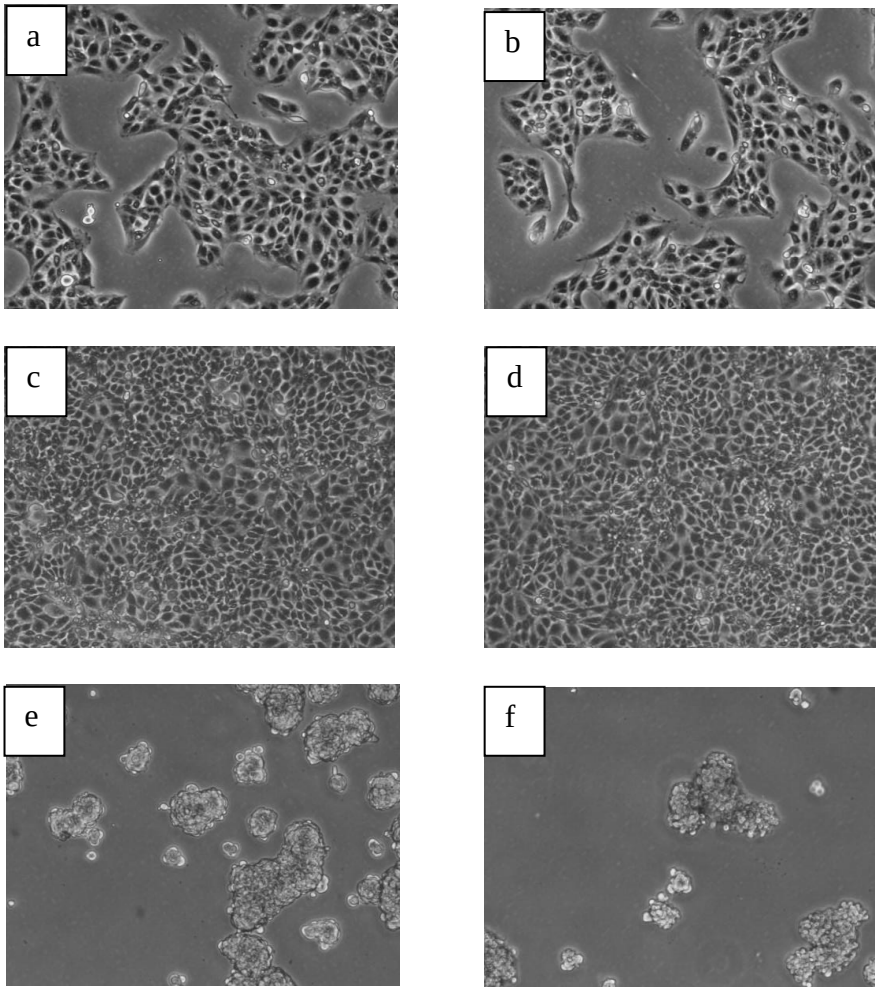

Figure 4-2 Morphology and growth of Ishikawa cells (x100 Magnification) cultured with the following components added to the culture medium

a. 20ng/ml galectin 3 (after 24 hours in culture)
b. 20ng/ml galectin 3 (after 48 hours in culture)
c. 500µL SNAP (after 24 hours in culture)
d. 500µL SNAP (after 48 hours in culture)
e. cilengitide and 20ng/ml galectin 3 (after 24 hours in culture)
f. cilengitide and 20ng/ml galectin 3 (after 48 hours in culture)

While the morphology and growth of Ishikawa cell cultures following 24 and 48 hours in culture with gal-3 and SNAP was as expected, cells treated with cilengitide were free floating and clustered together.

4.3.2.2 Effects on enzyme expression

Figure 4-3 shows that the expression of NAPE-PLD transcript (relative to GAPDH) by cultured Ishikawa cells was not significantly affected by increasing concentrations of gal-3. This is in stark contrast to data presented in Chapter 3, where gal-3 had a significant effect. From these data it was concluded that gal-3 in isolation does not affect the expression of NAPE-PLD and requires the actions of β3-integrins.

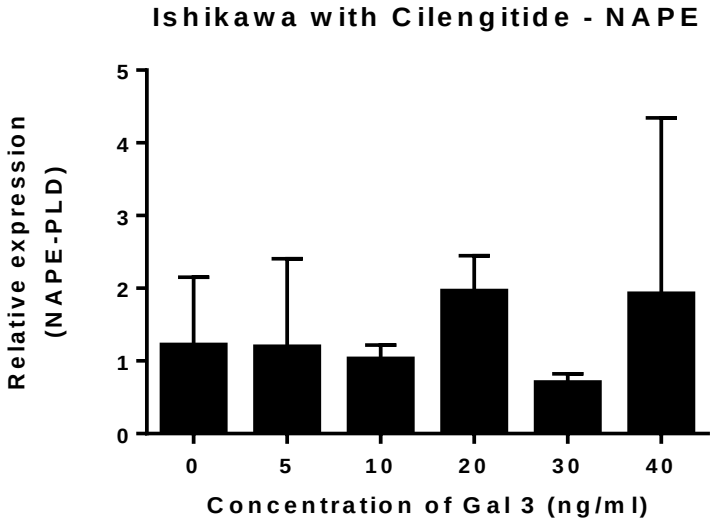

Figure 4-3 The effect of galectin 3 (without involvement of integrin β3) on relative amounts of N-acylphosphatidylethanolamine-phospholipase D (NAPE-PLD) mRNA

Ishikawa cells were exposed to the indicated concentrations of gal-3 and with 10μM of cilengitide for 48 hours. The relative amount of NAPE-PLD mRNA (compared against the "house-keeping gene" GAPDH) was then calculated following TaqMan based PCR.

The data are presented as the mean ± SD relative amounts of NAPE-PLD (n=6). Unlike previous studies, data here was not statistically significantly different; one-way ANOVA p=0.5391.

Figure 4-4 shows that the relative amounts of FAAH mRNA produced by cultured Ishikawa cells was also not affected by increasing concentrations of gal-3. Unlike the NAPE-PLD data, this was exactly the same as the results presented in Chapter 3. From this it was concluded that gal-3 in isolation does not affect the expression of FAAH.

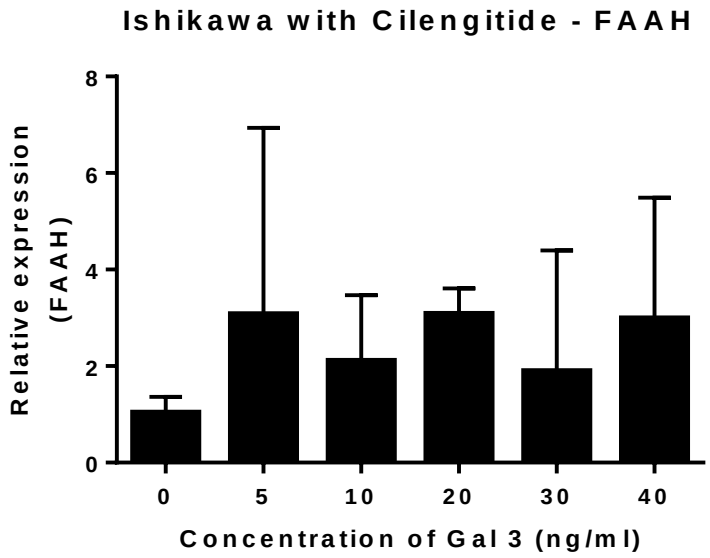

Figure 4-4 The effect of galectin 3 (without the involvement of integrin β3) on relative amounts of Fatty Acid Amide Hydrolase (FAAH) mRNA.

Ishikawa cells were exposed to the indicated concentrations of gal-3 along with 10μM of cilengitide for 48 hours. The relative amount of FAAH mRNA (compared against the "house-keeping gene" GAPDH) was then calculated following TaqMan based PCR.

The data are presented as the mean ± SD relative amounts of FAAH (n=6). The changes observed are not statistically significantly different; one-way ANOVA p=0.5453.

4.3.3 Effects of integrin β3 (without gal-3)

In contrast to the data presented in Figure 4-3, the data in Figure 4-5 shows a clear inverse and significant correlation between the concentration of SNAP and the amount of NAPE-PLD mRNA. There was a statistically significant difference between lower concentrations of SNAP (0 and 50μM) and the higher concentrations (1000 and 2000μM). Higher concentrations of SNAP reduced amounts of NAPE-PLD mRNA in relation to the control.

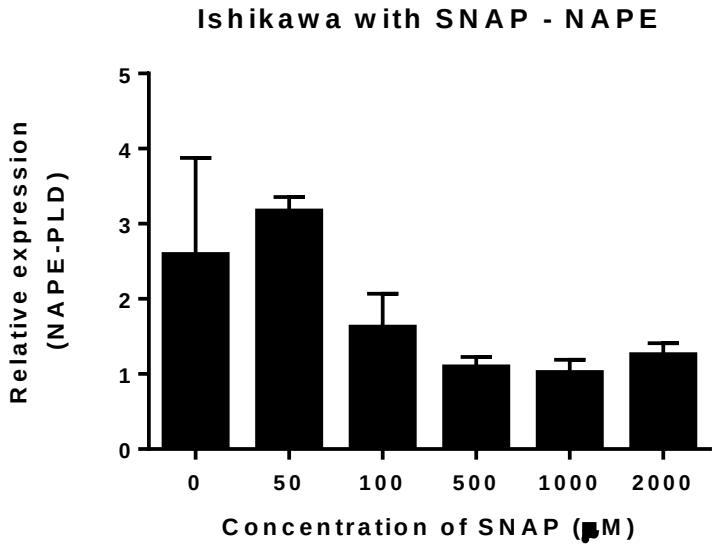

Figure 4-5 The effect of integrin β3 (without involvement of gal-3) on the relative amounts of N-acylphosphatidylethanolamine-phospholipase D (NAPE-PLD) mRNA.

Ishikawa cells were exposed to the indicated concentrations of SNAP for 48 hours. The relative amount of NAPE-PLD mRNA (compared against the "house-keeping gene" GAPDH) was then calculated following TaqMan based PCR.

The data are presented as the mean ± SD relative amounts of NAPE-PLD (n=6). Unlike previous studies, data here was statistically significantly different; one-way ANOVA $p<0.0001$. Further analysis revealed statistically significant differences between 0 µM and 500, 1000 and 2000 µM SNAP ($p<0.01$ for 500 and 1000µM, $p<0.05$ for 2000µM; Tukey's multiple comparison test) and between 50 µM and 100, 500, 1000 and 2000µM SNAP ($p<0.01$ for 100µM, $p<0.0001$ for 500 and 1000µM, $p<0.001$ for 2000µM; Tukey's multiple comparison test).

Figure 4-6 shows that as the concentration of SNAP increases, so does the relative amount of FAAH mRNA. This finding is also supported statistically, with a strong statistical significance when the data were analysed using one-way ANOVA. The only concentration of SNAP that showed a statistically significant difference from the control was the 2000μM. However, there was a significant difference between the highest concentration of SNAP (2000μM) and all other concentrations of SNAP except 1000μM. Unlike other experiments conducted in this project, this is the first statistically significant change in the amount of FAAH mRNA. This further supported the suggestion that it is the effects of integrin β3, not gal-3 that influences the expression of some ECS components.

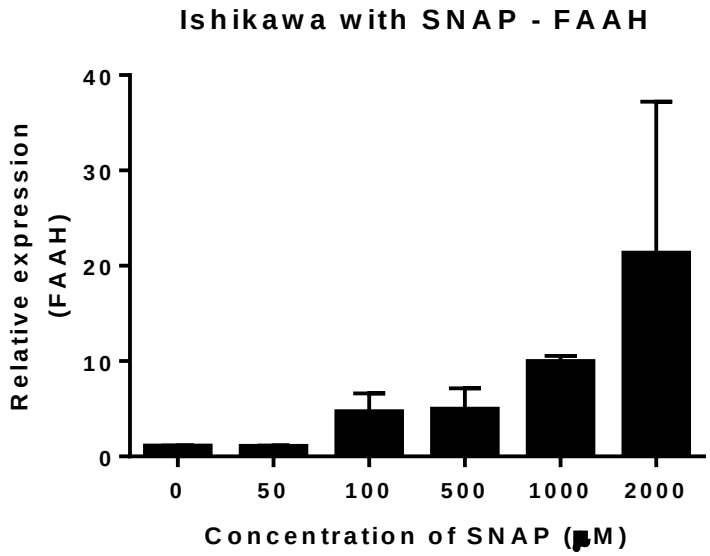

Figure 4-6 The effect of integrin β3 (without the involvement of gal-3) on relative amounts of Fatty Acid Amide Hydrolase (FAAH) mRNA.

Ishikawa cells were exposed to the indicated concentrations of SNAP for 48 hours. The relative amount of FAAH mRNA (compared against the "house-keeping gene" GAPDH) was then calculated following TaqMan based PCR.

The data are presented as the mean ± SD relative amounts of FAAH (n=6). The changes observed were statistically significantly different; one-way ANOVA $p<0.0001$. Further analysis revealed statistically significant differences between 2000μM and 0, 50, 100 and 500μM SNAP ($p<0.001$ for 0 and 50μM, $p<0.01$ for 100 and 500μM SNAP; Tukey's multiple comparison test).

4.4 Discussion

In Chapter 3, it was shown that galectin 3 plays an important role in influencing the amount of NAPE-PLD mRNA produced and also had an effect on the resultant concentration of the NAEs OEA and PEA (along with a small effect on the concentration of AEA). Based on the changes of the NAE concentrations at higher doses of gal-3, it was also suspected that it also affected the amount of FAAH mRNA, but this was not shown to be statistically significant. In this Chapter however, the original hypothesis has been disproved, by showing that gal-3 in isolation had no demonstrable effect on the amount of NAPE-PLD or FAAH mRNA produced.

As already described in the results section, Ishikawa cells treated with cilengitide did not adhere to the culture flask, and instead grew as free-floating clusters. A direct result of the cells not adhering to the culture flask was that it was harder to collect the cells and they needed separating from the culture medium by centrifugation. While every attempt was made to collect all of the cells, it is likely that some cells were lost during this procedure. Analysis of the RNA collected showed a poor concentration (0.1124-0.5384 µg/µL), which suggests that either the cellular proliferation rate was affected by cilengitide, not all cells were collected, or both events were involved. While the concentration of RNA collected was poor, the quality or RNA collected from cell culture was good (260/280 ratios ranging from 1.78-1.90), which supports the finding of Maurer et al. (2009) who found that cilengitide did not affect cell viability**.** It is unlikely however that the low concentrations of RNA collected will have influenced the results obtained in this Chapter because a weight (1µg) rather than a volume of RNA was used for reverse transcription and so the poor concentration of RNA would have been corrected by this.

It is known from the work of Lei et al. (2009) that blocking integrin β3 is known to enhance gal-3's proliferative and adhesive effects. It is unlikely that the results here are skewed because of this property. This is because the proliferative and adhesive effects of gal-3 are the very properties that highlight gal-3 as supportive in uterine receptivity, and "anandamide tone" (provided by the balance of NAPE-PLD and FAAH) is also considered to be beneficial to uterine receptivity. It was therefore concluded that any exaggeration of gal-3's properties related to uterine receptivity would also exaggerate the

processes necessary to provide the appropriate "anandamide tone" (by modifying the expression of NAPE-PLD and FAAH accordingly) and so would make any changes even more significant. As this was not the case, there was confidence in the conclusion that gal-3 in isolation does not influence the expression of either NAPE-PLD or FAAH.

While it was shown that treatment of Ishikawa cells with SNAP significantly up-regulates the amount of FAAH mRNA, what still needs to be determined is whether these differences are as a direct result of the action of SNAP, or the results of the increased expression of integrin β3. SNAP spontaneously releases nitric oxide (NO) under physiological conditions (Lee 2000). However, investigating the role of NO in embryo implantation has reached mixed conclusions. Zhang et al. (2005) found that low concentrations of NO enhanced embryo implantation while higher concentrations inhibited it. The work of Barroso et al. (1998) showed that low concentration of NO had no effect on embryo development or implantation, whereas higher concentrations were detrimental to both.

Saxena et al. (2000) found that the peri-implantation uterus showed increased expression of inducible nitric oxide synthase (iNOS) when compared to the post-implantation uterus. Furthermore, Purcell et al. (1999) showed that the expression of iNOS varies between the implantation and inter-implantation zones, with the highest expression within the implantation zone.

With evidence suggesting that NO plays an important role in endometrial receptivity, it is plausible that the changes seen in the amounts of NAPE-PLD and FAAH mRNA are a result of the increased availability of NO, rather than integrin β3 as suggested in this hypothesis. However, the results in Chapter 3 show a statistically significant change in the amount of NAPE-PLD mRNA following the combined effects of gal-3 and integrin β3, without the added effects of NO. The same was not seen when integrin β3 was inhibited. This would suggest that integrin β3 has the ability to modulate the expression of the ECS enzymes. Further work is needed to establish whether the results shown in Figure 4-6 are the result of enhanced integrin β3 expression, NO production or a combination of both.

While disproving the original hypothesis was disheartening, it was instead established that rather than gal-3 influencing the ECS, it is in fact integrin β3 (+/- the superimposed effects of NO). It has been shown that up regulating the expression of integrin β3 by using S-nitroso-N-acetylpenicillamine (SNAP) results in a corresponding up regulation in the expression of FAAH, whilst simultaneously down regulating the expression of NAPE-PLD. It has also been shown these changes are associated with a much stronger statistical significance than the changes shown in Chapters 2 and 3. As already discussed, further work is needed to establish that these changes are purely the effects of enhancement of integrin β3's expression, or a combined effect of enhancement of integrin β3's expression and production of NO. There is also a possibility that the results obtained in Chapter 3 were 'damped' because of the lack of effect exerted by gal-3. These data are consistent with the observed data in a pilot study where increased FAAH expression and reduced plasma AEA levels were associated with increased pregnancy success (Maccarrone et al. 2000, Maccarrone et al. 2001, El-Talatini et al. 2009, Rapino et al. 2014) and that increased plasma AEA was detrimental to on-going pregnancy (Habayeb et al. 2008, Taylor et al. 2011).

From animal studies, it is known that the expression of both NAPE-PLD and FAAH varies at the implantation zone and inter-implantation zone, and this is vital in providing the required "anandamide tone" to enable successful implantation. The results of this study suggest that integrin β3 may also play a role in maintaining this "anandamide tone" – with low levels of integrin β3 encouraging NAE synthesis (by enhancing NAPE-PLD expression) and high levels of integrin β3 encouraging NAE degradation (by enhancing FAAH expression).

As set out at the beginning of the thesis, the goal was to explore the role that the endocannabinoid system plays in embryo implantation, with specific focus on the effects the ECS has on uterine receptivity. Having identified that integrin β3 appears to affect the expression of both NAPE-PLD and FAAH, one obvious question is "do the observed effects of integrin β3 on the expression of NAPE-PLD and FAAH have any impact on uterine receptivity?"

Ishikawa cells were selected as a suitable model for studying the "receptive" endometrium. Therefore, to explore the question posed in the paragraph above it was

necessary to identify the effects of integrin β3 on the ECS using a "non-receptive" endometrial model. This will be examined in the next Chapter.

5 The effects of integrin β3 on the endocannabinoid system expression when considering endometrial receptivity

5.1 Introduction

Having demonstrated that treating Ishikawa cells with SNAP has a statistically significant effect on the amounts of both NAPE-PLD and FAAH mRNA (Chapter 4), the next key step to address was whether or not this is a feature expressed by all endometrial epithelial cells, and if it may play a role in uterine receptivity.

Due to the problems described in Chapter 4, it was not possible to explore the expression of NAEs in the culture medium and so the project was limited to the expression of NAPE-PLD and FAAH transcripts in a non-receptive epithelial cell line.

5.1.1 Choice of cell line

As already discussed in the previous Chapter, Ishikawa cells have been identified as one of the most suitable cell lines to use when investigating embryo implantation because of their adhesive nature and their expression of estrogen and progesterone receptors (Castelbaum et al. 1997). In contrast, HEC-1A cells have been identified (Hannan et al. 2010) as a good model for mimicking a "non-receptive" endometrium because, although they possess both estrogen and progesterone receptors (but not androgen), they are poorly adhesive. With specific relevance to this project, both Ishikawa (Castelbaum et al. 1997) and HEC-1A cells (Wadehra et al. 2005) are known to express integrin β3. It therefore seemed logical to use HEC-1As as a model for a "non-receptive" endometrium to investigate the effects of integrin β3 on the expression of NAPE-PLD and FAAH.

5.2 Methods

The culture of HEC-1A cells differed from that of Ishikawa cells, in that a different medium was used (McCoy's 5a Medium Modified with 10% Fetal Calf Serum). For the following experiments, HEC-1A cells (5×10^5) were seeded into a 6-well plate and cultured in 2ml of culture medium and maintained at 37°C with 5% CO_2 for 24 hours to achieve 50% confluence. At this point, the culture medium was discarded and replaced with medium augmented with different concentrations of S-nitroso-N-acetylpenicillamine (SNAP, 0-2000 µM). The cells were then re-incubated for a further 48 hours. At this point, the total cellular RNA extracted and quantified (as described in Chapter 4) and the relative levels of NAPE-PLD and FAAH transcripts measured using quantitative RT-PCR.

5.3 Results

As stated above, measurement of NAEs in the culture medium was not possible and so this section focuses totally on the expression of NAPE-PLD and FAAH transcript levels.

5.3.1 Cell Growth

In keeping with the work using Ishikawa cells, there was no significant difference in the growth or morphology of HEC-1A cells cultured with either 20ng/ml gal-3 or 500µL SNAP added to their culture medium (as shown in Figure 5-1).

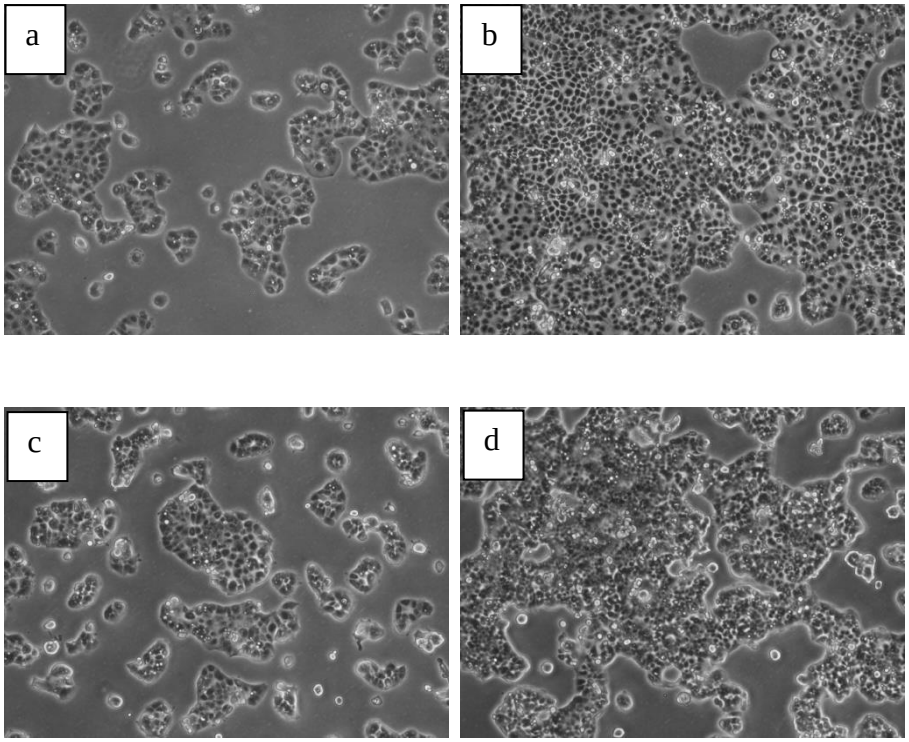

Figure 5-1 Morphology and growth of HEC-1A cells (x100 Magnification) cultured with the following components added to the culture medium

a. *untreated (after 24 hours in culture)*
b. *untreated (after 72 hours in culture)*
c. *500µL SNAP (after 24 hours in culture)*
d. *500µL SNAP (after 72 hours in culture)*

5.3.2 Effects of SNAP on enzyme production

Unlike data from my work in Chapter 4 (using Ishikawa cells), Figure 5-2 does not show any effect of increasing concentrations of SNAP on the relative amounts of NAPE-PLD mRNA produced by HEC-1A cells. The mean relative amount of NAPE-PLD mRNA remained around 1.0 (0.80-1.52), and there was no significant change from this value. From these data it can be concluded that SNAP (and by extrapolation, integrin β3 expression) does not affect the production of NAPE-PLD mRNA in HEC-1A cells, and that NAPE-PLD expression remained constant regardless of SNAP treatment.

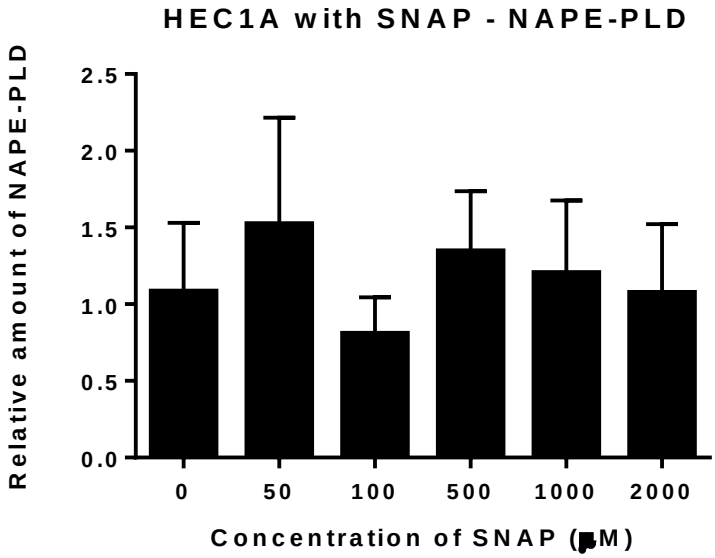

Figure 5-2 The effect of integrin β3 (without involvement of gal-3) on relative amount of N-acylphosphatidylethanolamine-phospholipase D (NAPE-PLD) mRNA

HEC-1A cells were exposed to the indicated concentrations of SNAP for 48 hours. The relative amount of NAPE-PLD mRNA (compared against the "house-keeping gene" GAPDH) was then calculated following TaqMan based PCR.

The data are presented as the mean ± SD relative amounts of NAPE-PLD (n= 6). Unlike previous studies, data here were not statistically significantly different; one-way ANOVA p = 0.1862.

Again, unlike data from Ishikawa cells, Figure 5-3 shows that there was no effect of increasing concentrations of SNAP on the relative amounts of FAAH mRNA produced by HEC-1A cells. The mean relative amounts of FAAH mRNA remained around 0.05 (0.015-0.065) suggesting that HEC-1A cells express very little FAAH in comparison to GAPDH (the house-keeping gene) and their expression of FAAH is constant despite treatment with SNAP. From these data it was concluded that SNAP (and by extrapolation, integrin β3 expression) does not affect the expression of FAAH in HEC-1A cells.

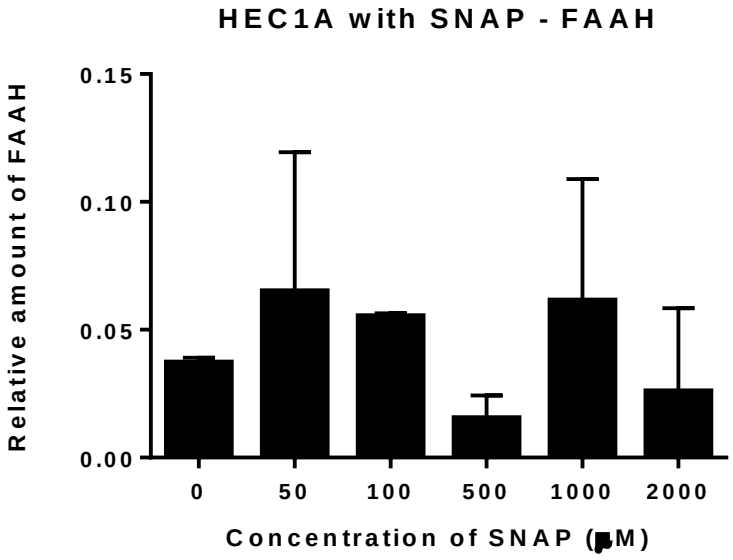

Figure 5-3 The effect of integrin β3 (without the involvement of gal-3) on the relative amount of Fatty Acid Amide Hydrolase (FAAH) mRNA

HEC-1A cells were exposed to the indicated concentrations of SNAP for 48 hours. The relative amount of FAAH mRNA (compared against the "house-keeping gene" GAPDH) was then calculated following TaqMan based PCR.

The data are presented as the mean ± SD relative amounts of FAAH (n= 6). The changes observed are not statistically significantly different; one-way ANOVA p=0.2170.

5.4 Discussion

In Chapter 4, it was demonstrated that increasing concentrations of SNAP affected both the amounts of NAPE-PLD (by negative correlation) and FAAH (by positive correlation) mRNA by Ishikawa cells. Ishikawa cells were selected as they are a good model of a receptive endometrium.

In this Chapter, the data failed to show any correction between increasing concentrations of SNAP and the amounts of NAPE-PLD or FAAH mRNA by HEC-1A cells (HEC-1A cells were selected as a good model of a non-receptive endometrium).

These results are extremely interesting, because they show that while the amounts of NAPE-PLD and FAAH can be modified by increasing concentrations of SNAP in receptive cells, the same does not occur in non-receptive cells, and so raises that possibility that these differences might play a role in endometrial receptivity. The role of the ECS and uterine receptivity is explored further in Chapter 6.

6 Endocannabinoid system enzyme expression and endometrial receptivity

Having shown a difference in the effect of SNAP on the amounts of ECS enzyme transcript produced by receptive and non-receptive endometrial cells, the amounts of mRNA for the ECS enzymes in receptive and non-receptive cells was further explored.

6.1 Results

6.1.1 Relative amounts of FAAH mRNA produced by Ishikawa cells under different conditions

In Chapter 4, a striking change in the relative amount of FAAH mRNA in Ishikawa cells treated with SNAP was identified. How this compared to the relative amount of FAAH mRNA in Ishikawa cells treated with gal-3 and cilengitide is shown in Figure 6-1.

As is clearly demonstrated in Figure 6-1 (after paying careful attention to the y axis scale) it is clear that the relative amount of FAAH mRNA was significantly increased in Ishikawa cells treated with concentrations of SNAP ≥100μM, culminating with a relative expression 10x greater in cells treated with 2000 μM SNAP than in cells treated with gal-3 or cilengitide as well.

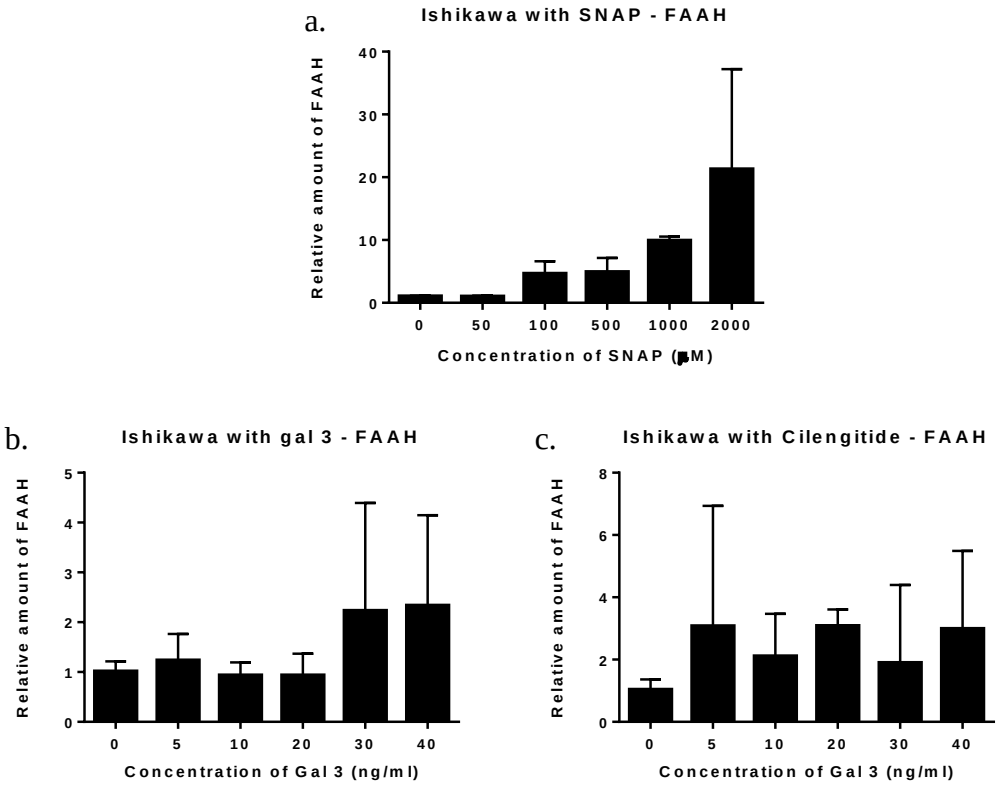

Figure 6-1 The relative amount of FAAH mRNA in Ishikawa cells treated with either SNAP, gal-3 or gal-3 + cilengitide.

A comparison of the relative amount of FAAH mRNA produced by Ishikawa cells treated with:

a. SNAP
b. Gal-3
c. Gal-3 + Cilengitide

(see Figures 4-6, 3-5 and 4-4 for full description)

6.1.2 Relative amounts of NAPE-PLD mRNA produced by Ishikawa cells in different conditions

Having identified such a dramatic difference in the relative amounts of FAAH mRNA in Ishikawa cells treated with SNAP, I sought to explore if the same differences were present for NAPE-PLD (Figure 6-2). In contrast to the relative amounts of FAAH mRNA (Figure 6-1), there was no difference in the relative amounts of NAPE-PLD mRNA.

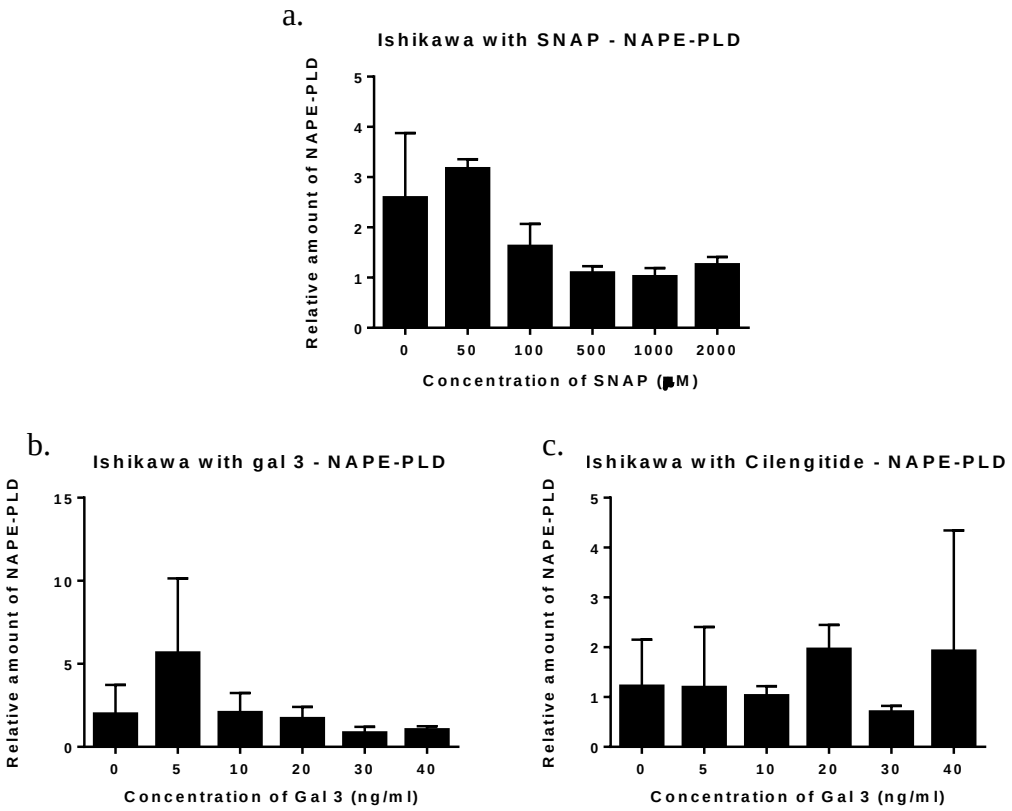

Figure 6-2 The relative amounts of NAPE-PLD mRNA in Ishikawa cells treated with either SNAP, gal-3 or gal-3 + cilengitide

A comparison of the relative amounts of NAPE-PLD mRNA by Ishikawa cells treated with:

a. SNAP
b. Gal-3
c. Gal-3 + Cilengitide

(see Figures 4-5, 3-4 and 4-3 for full description)

6.1.3 Comparison of relative amounts of ECS enzymes' mRNA by Ishikawa and HEC-1A cells

Figure 6-3 shows that relative amount of NAPE-PLD mRNA was within a fairly narrow range for both Ishikawa cells (between 1- and 3-fold) and HEC-1A cells (between 0.6- and 1.5- fold), whereas the relative amount of FAAH mRNA varied quite significantly. When considering the relative amount of FAAH mRNA in HEC-1A cells, it is interesting to note that it was almost negligible (0.015-0.065). Conversely, while the relative amount of FAAH mRNA was approximately 1 at lower concentrations of SNAP (0 and 50μM), it rose to as much as over 20-fold at the highest concentration.

These findings lead to the suggestion that FAAH may play a more significant role in achieving uterine receptivity than NAPE-PLD.

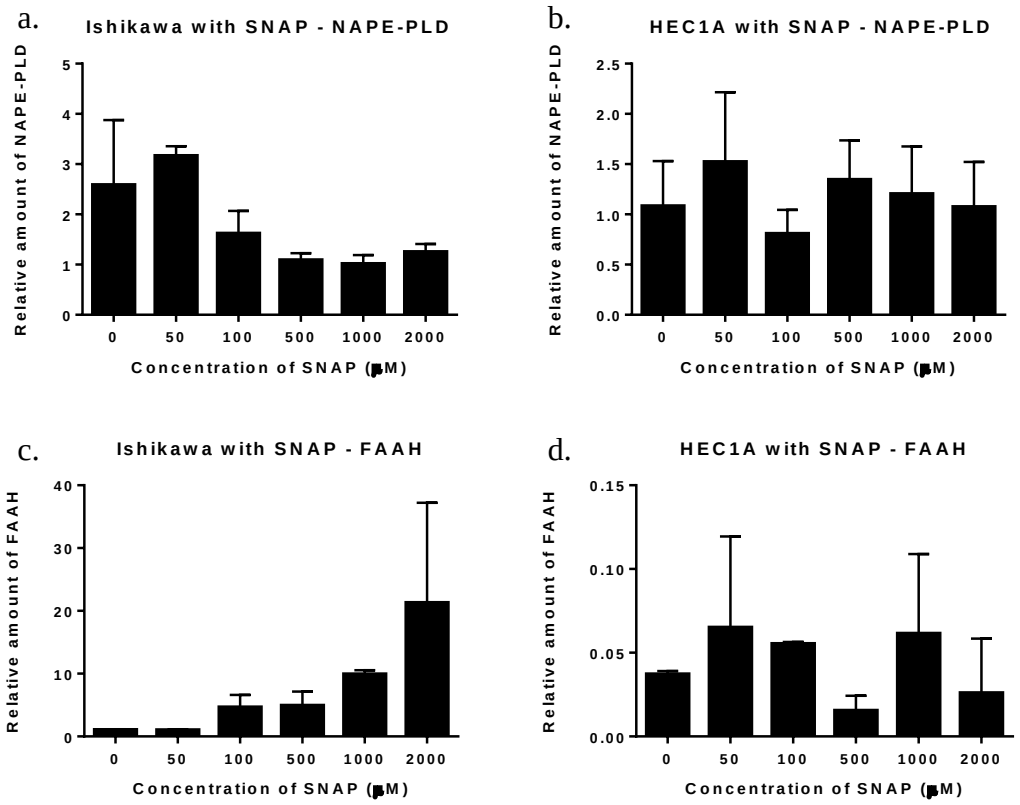

Figure 6-3 A comparison of the relative amounts of NAPE-PLD and FAAH mRNA in Ishikawa and HEC-1A cells

Note the differing y axes.

- *a. Ishikawa and NAPE-PLD mRNA amounts*
- *b. HEC-1A and NAPE-PLD mRNA amounts*
- *c. Ishikawa and FAAH mRNA amounts*
- *d. HEC-1A and FAAH mRNA amounts*

(See Figures 4-5, 5-2, 4-6 and 5-3 for full description)

6.1.4 Comparison of the ratio ECS enzymes in Ishikawa and HEC-1A cells

Having identified such a strong correlation between the up-regulation in the amount of FAAH mRNA and SNAP concentration, along with a statistically significant difference in the amount of NAPE-PLD mRNA, the next step was to see if there was any correlation between enzyme expression by comparing the ratio of mean relative amounts of FAAH:NAPE-PLD against SNAP concentration (Figure 6-4). In keeping with the results in Figure 6-3, the ratio of FAAH:NAPE-PLD showed a strong dose dependent correlation (Pearson's correlation; $r=0.9844$, $p<0.0001$) for Ishikawa cells, but no correlation for HEC-1A cells ($r= 0.1295$, $p=0.4835$).

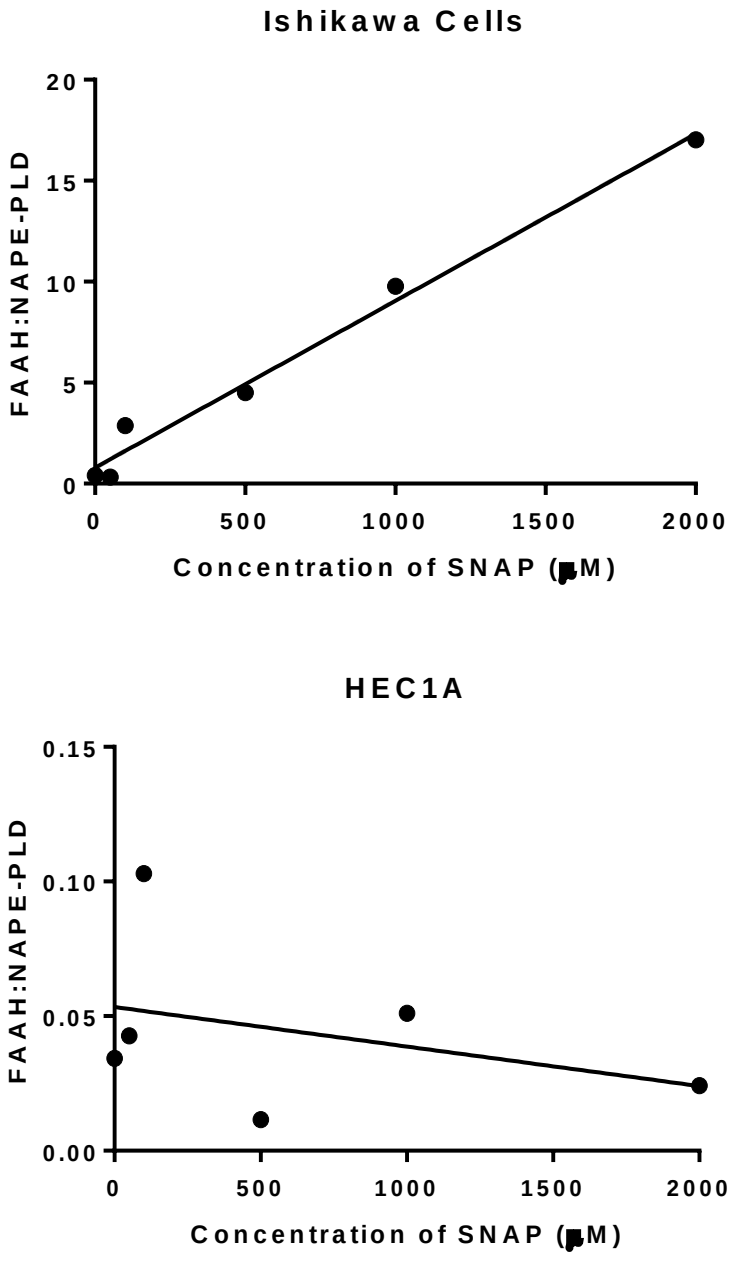

Figure 6-4 Comparing the ratio of FAAH against NAPE-PLD in Ishikawa and HEC-1A cells at increasing concentrations of SNAP.

The data are presented as a single plot for each FAAH:NAPE-PLD ratio against SNAP concentration, along with a regression line, for Ishikawa (upper) and HEC-1A (lower) cells. Data was analysed using Pearson's correlation. The ratio of the amount of ECS enzyme mRNA in Ishikawa cells showed strong correlation to SNAP concentration ($r= 0.9844$, $p <0.0001$), whereas there was no correlation between the FAAH:NAPE-PLD ratio and SNAP concentrations in HEC-1A cells ($r= 0.1295$, $p= 0.4835$).

6.1.5 Expression of the ECS enzymes in untreated Ishikawa and HEC-1A cells

Finally, to further investigate the role of the ECS and endometrial receptivity, the relative amounts of NAPE-PLD and FAAH mRNA in untreated Ishikawa and HEC-1A cells (Figure 6-5) was examined. The data used came from Chapters 4 and 5, and compared the "untreated cells" of the experiments where all other wells were treated with SNAP. The amount of both NAPE-PLD and FAAH mRNA was higher in Ishikawa cells than HEC-1A cells, and these differences were both statistically significant (NAPE-PLD $p= 0.0228$, FAAH $p= <0.0001$).

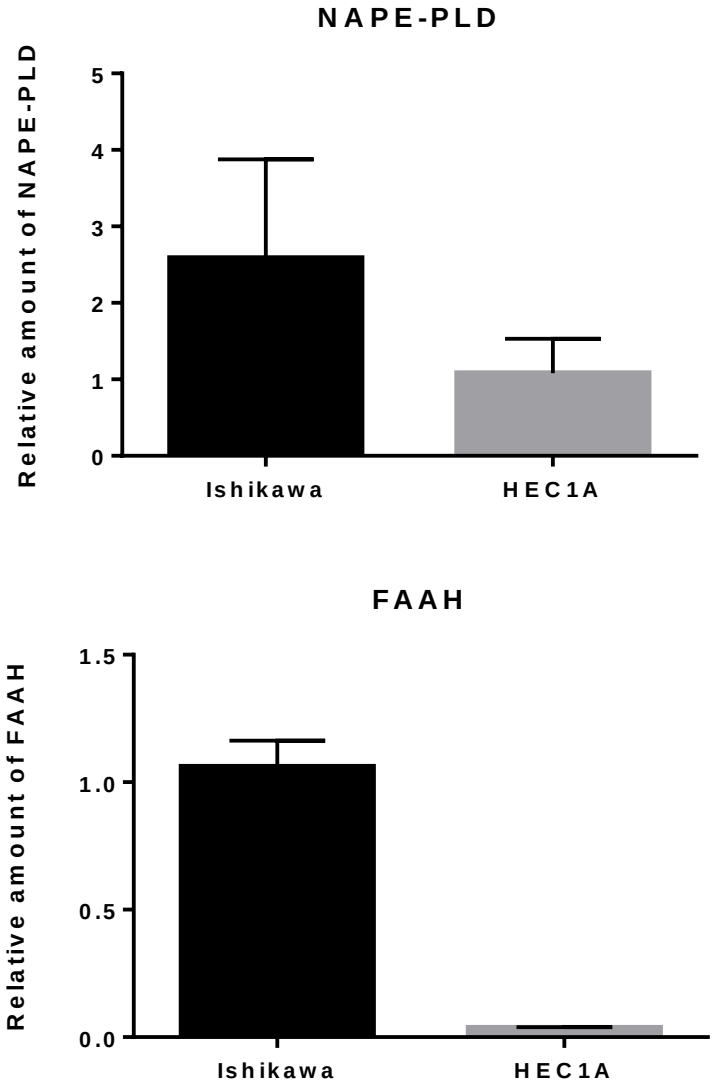

Figure 6-5 Comparing the amount of NAPE-PLD and FAAH mRNA in untreated Ishikawa and HEC-1A cells

Ishikawa and HEC-1A cells were cultured for 48 hours, and then the relative amount of a) NAPE-PLD mRNA and b) FAAH mRNA (compared against the "house-keeping gene" GAPDH) was then calculated following TaqMan based PCR.

The data are presented as the mean ± SD relative amounts of a) NAPE-PLD and b) FAAH. Both results showed a statistically significant difference (using a Student's unpaired t-test); NAPE-PLD p= 0.0228, FAAH p= <0.0001.

6.2 Discussion

The basis of an "anandamide tone" has been discussed repeatedly throughout this report, as has its importance in providing a suitable environment for embryo implantation. This Chapter has explored how the expression of the key enzymes responsible for anandamide tone varies in receptive and non-receptive cell lines. What is clear from this work, whether it is the result of integrin β3, NO, or both, the effects on the expression of the ECS are limited to receptive cell lines, and by extrapolation to receptive epithelial cells of the endometrium. As far as is known, this is the first documented evidence that integrin β3 expression appears to play a role in helping to maintain a suitable anandamide tone.

As already discussed, SNAP spontaneously produced nitric oxide which in turn increases integrin β3 expression in rats (confirmed by immunohistochemistry) (Lee et al. 2000). In addition, Purcell et al. (1999) showed that the expression of iNOS varies between the implantation and inter-implantation zones, with the highest expression at the implantation zone. It is known from animal studies that NAPE-PLD and FAAH gene expression and activity (confirmed using both qPCR and Northern blot hybridisation for quantification of expression and enzyme assays to demonstrate activity) also differs between the implantation and inter-implantation zones (Guo et al. 2005) which raises questions as to whether the expression of integrin β3 also varies between the implantation and inter-implantation zones.

Sadly, this work does not fully support the findings of Guo et al. (2005), who demonstrated a higher expression of NAPE-PLD in the "non-receptive" uterus when compared to a uterus that was "receptive" to implantation. However, when comparing the ratio of NAPE-PLD:FAAH in receptive and non-receptive cell lines, this is 10x higher in the non-receptive cells (2.43 in Ishikawa cells vs 29.13 in HEC-1A cells). This further supports the notion of "anandamide tone", as it is the ratio of the enzymes (and thus the overall concentration) that is important, rather than their absolute expression.

As well as considering the effects of SNAP on receptive and non-receptive cell lines, the expression of NAPE-PLD and FAAH in untreated Ishikawa and HEC-1A cells was also explored. The relative amounts of both enzymes' transcripts were higher in receptive

cells than non-receptive cells, and these differences were statistically significant. This finding was emphasised by cells treated with SNAP, where the effects of NO or integrin β3 (or both) exerted a much greater effect on the relative amount of FAAH mRNA by Ishikawa cells than that of NAPE-PLD.

As already discussed in the introduction, both NAPE-PLD and FAAH play key roles in maintaining an appropriate "anandamide tone"; NAPE-PLD by ensuring adequate synthesis and FAAH by ensuring timely degradation. It is also already known that an appropriate "anandamide tone" is necessary for successful embryo implantation. When comparing the amounts of NAPE-PLD, there is very little difference between the amounts found in receptive and non-receptive cells. However, when comparing the amounts of FAAH mRNA, receptive cells have 10x the amount of non-receptive cells. This suggests that FAAH may be more influential than NAPE-PLD in maintaining the "anandamide tone" necessary for endometrial receptivity.

7 IMPLANT Study

7.1 Introduction

The Endocannabinoid Research Group had already investigated the role of the ligands of the ECS and pregnancy outcomes, and found that high plasma concentrations of AEA are detrimental to the implanting embryo (Habayeb et al. 2008, El-Talatini et al. 2009, Taylor et al. 2011). In Chapter 6 a statistically significant difference in the expression of the ECS enzymes between untreated receptive and non-receptive cell types was demonstrated. A marked alteration in the FAAH:NAPE-PLD ratio in the receptive endometrial cell in response to SNAP was also shown. The work leads to the suspicion that the concentrations of the ligands that FAAH and NAPE-PLD act upon may be altered in the endometria of women who have a successful pregnancy to a greater effect than in the endometria of women whose pregnancy fails to implant. Since FAAH greatly outstrips NAPE-PLD in the receptive endometrium and thus by extension in plasma of women in the receptive window, then AEA levels should fall. In a non-receptive endometrium then NAPE-PLD predominates and so you might expect AEA levels to increase in the non-receptive woman.

Investigating early pregnancy events using spontaneous conceptions would be virtually impossible, and so patients undergoing assisted conception are generally used as a substitute. The plasma concentration of AEA in patients undergoing IVF and ICSI have already been explored by El-Talatini et al. (2009) who measured trends of plasma AEA concentrations during early fertility treatment. They found that a fall of approximately 40% in plasma AEA concentrations between the day of oocyte retrieval (OR) to the day of embryo transfer (ET) was associated with successful embryo implantation, as evidenced by increasing plasma hCG concentrations. However, this was only a pilot study and so only included data from a small number of patients. Nevertheless, the data lead the endocannabinoid research group to suspect that the change in plasma AEA concentrations could be predictive of successful infertility treatment and the IMPLANT study was devised. In the IMPLANT study design, the day of ET would be delayed until the patient's plasma AEA concentration fell by 40% or more, on the proviso that embryo

quality was sufficient to potentially delay the transfer until day 5 (as assessed by the attending embryologist) and then pregnancy outcome would be assessed.

A power calculation was performed prior to commencing the projects, with an aim of improving IVF success rates by 40%. This concluded that 362 would need to be recruited into the study.

7.2 Materials and Method

7.2.1 Subjects

Patients who were undergoing either *in-vitro* fertilisation (IVF) or intra-cytoplasmic sperm injection (ISCI) treatment for infertility were recruited at the Assisted Conception Unit at Leicester Royal Infirmary. All volunteers gave written informed consent to take part in the study. The study was approved and conducted according to the guidelines of the East Midlands (Leicester) local Research Ethics Committee.

7.2.1.1 Inclusion Criteria

- Non-smoker
- BMI between 19 and 24 Kg/m^2
- Written informed consent

7.2.1.2 Exclusion Criteria

- Presence of a systemic disease such as diabetes, hypertension, etc.
- Recent (within last 6 months) use of or exposure to *Cannabis sativa* (marijuana)

7.2.2 Recruitment, randomisation and timing of blood sample collection

7.2.3 Initial study design

Patients were approached during the "follicle tracking" stage of their ART and given verbal and written information about the study, and any questions they had were answered. Those happy to be involved, were then recruited into the study, with recruitment occurring on the day of OR. Patients recruited into the study were asked to donate blood samples for the measurement of plasma AEA.

7.2.3.1 Randomisation of patients

Once recruited, patients were randomised into one of two groups using computer software randomisation tool (www.sealedenvelope.com). The control group continued with the standard ART protocol, whereby the morphology of the embryo was assessed and graded by a member of the embryology team and the day of ET (either 3 or 5 days after OR) was decided based on this finding (with a repeat blood sample taken at the time of ET for scientific interest). In comparison, the plan for the study group was to have a second blood test taken on day 3 after OR, and this was to be considered along with the morphology of the embryo to guide when ET should take place. Unless there was a strong clinical reason [for example: (a) poorly developing embryos that were unlikely to survive past day 3 or (b) too many "good" embryos making it impossible to identify the "best" embryo without leaving time for further growth] then the decision for the day of ET was to be made based on the measured concentration of plasma AEA on day 3: a fall of ≥40% resulting in an ET on day 3, whereas a fall of <40% resulting in a delay in ET until day 5 (the plan is shown graphically/schematically in Figure 7.1).

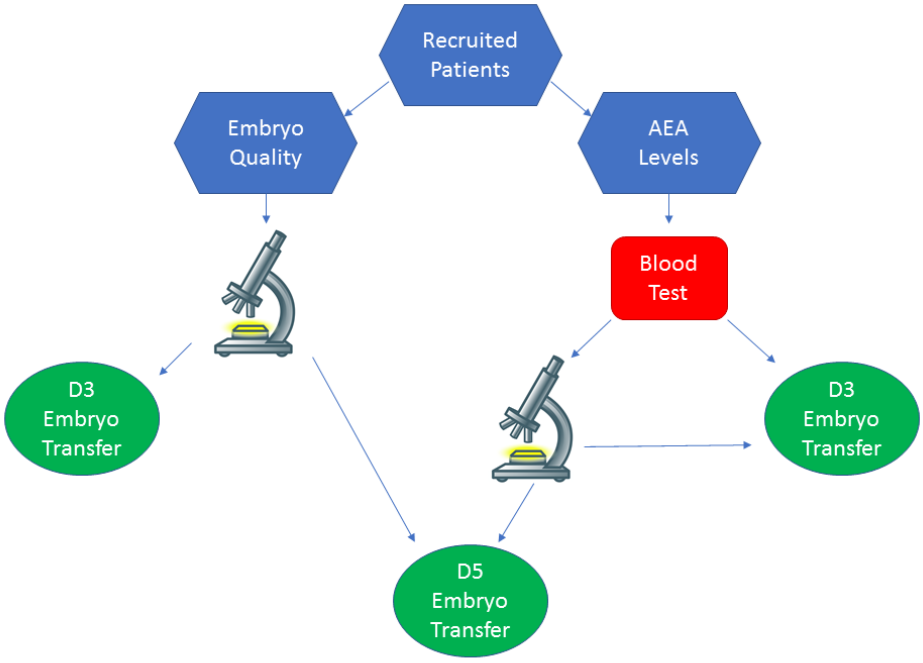

Figure 7-1 Schematic representation of the IMPLANT study

The embryo quality was assessed by the attending embryologist and plasma AEA levels determined by UHPL-MS/MS. Based on results embryo transfer would occur either on day 3 (D3) or day 5 (D5).

7.2.3.2 Extraction of AEA from whole blood

Anandamide (AEA) was extracted from whole blood using a method previously described by Marczylo et al. (2009). Briefly, 7ml of whole blood was obtained using standard venepuncture techniques, into a vial containing EDTA and immediately put on ice and transported to the research laboratory. Blood was then centrifuged at 1200*g* for 30 minutes at 4°C, with all samples entering this step of processing within 1 hour of collection. At that this point, 0.5ml of the supernatant (plasma) was collected and transferred to a Kimble vial where it was diluted to a volume of 1mL by adding 0.5ml of deionised water (dH_2O). The sample was then labelled with 20μL of internal standard (composed of 2.5 pmol/mL AEA-d8, 2.5 pmol/mL OEA-d2 and 5 pmol/mL PEA-d4) and vortexed.

The labelled sample was then loaded into Oasis cartridges that had been preconditioned and equilibrated with 1mL of methanol and 1mL of dH_20, and drawn through at a rate of 1mL/minute using a gentle vacuum. The cartridges were washed with 1mL of 40% aqueous methanol, and the AEA eluted in 1mL of acetonitrile.

This sample was then dried under a gentle stream of nitrogen while being heated at 40°C, reconstituted with 80μL of acetonitrile and transferred to a clean UPLC vial where it was analysed using UPLC-MS/MS.

7.2.3.3 Quantitative analysis of AEA

Quantitative analysis of AEA was also performed using the method previously described by Marczylo et al. (2009), namely with a UHPLC-ESI-MS/MS system (Figure 7-2) which comprised of an Acquity UHPLC coupled to a Quattro Premier tandem mass spectrometer (Waters UK Ltd., Elstree, Herts).

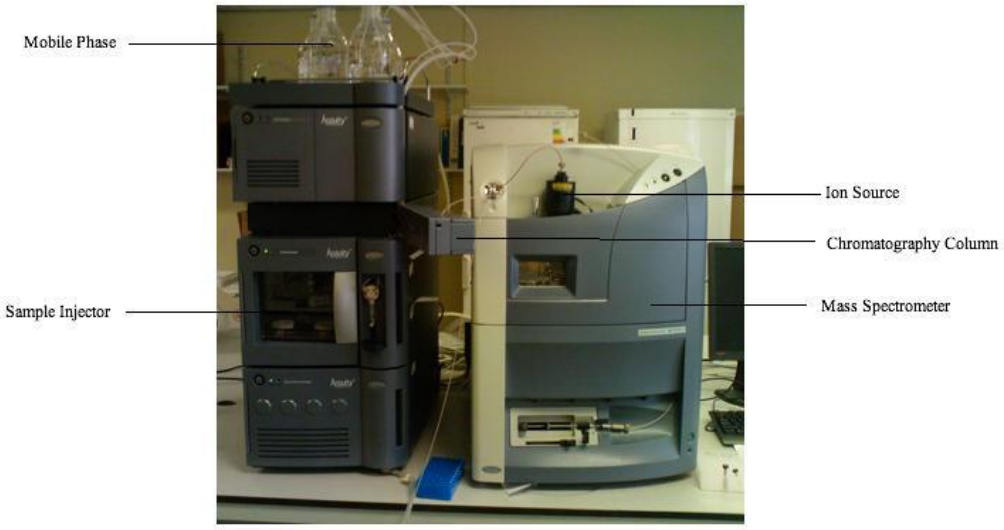

Figure 7-2 Equipment used for quantitative analysis of AEA

The Acquity Ultra-Performance Liquid Chromatography [UPLC] system (left) and Quattro Premier mass spectrometer (right)

Separation was achieved using an Acquity UHPLC BEH C18 (2.1mm × 50mm, 1.7µm) column maintained at 40°C, using the mobile phases A (2 mM ammonium acetate containing 0.1% formic acid, 5% acetonitrile) and B (acetonitrile containing 0.1% formic acid).

LC gradient conditions were: 0–0.5 min, 20% B; 2.5 min, 100% B; 3.5 min, 20% B then re-equilibrated at 20% B until 4.0 min. The flow rate was 0.7 mL/min. Samples were maintained at 4°C prior to injection. Analytes were quantified using electrospray ionization tandem mass spectrometry in positive ion mode (ES+), with source parameters set as follows: capillary voltage of 1kV, cone voltage 21V, source temperature 120°C, desolvation temperature 440°C, cone gas flow 50 L/h and desolvation gas flow 800 L/h. Subsequently, MS/MS conditions for monitoring each precursor $[M+H]^+$ ion comprising entry, collision and exit energies of 6, 16 and 2eV, respectively. Product ions were monitored in selected reaction monitoring (SRM) mode. Mass transitions were AEA (m/z 348.3 > 61.9) and AEA-d8 (m/z 356.4 > 63.0). Injection volumes for samples and standards were 7 µL.

Prior to analysing the concentration of AEA in each plasma sample, seven-point combined AEA calibration curves in triplicate spiked with internal standards were first performed (Figure 7-3a). Peaks from standards were integrated using Masslynx software version 4.1 (Figure 7-3b).

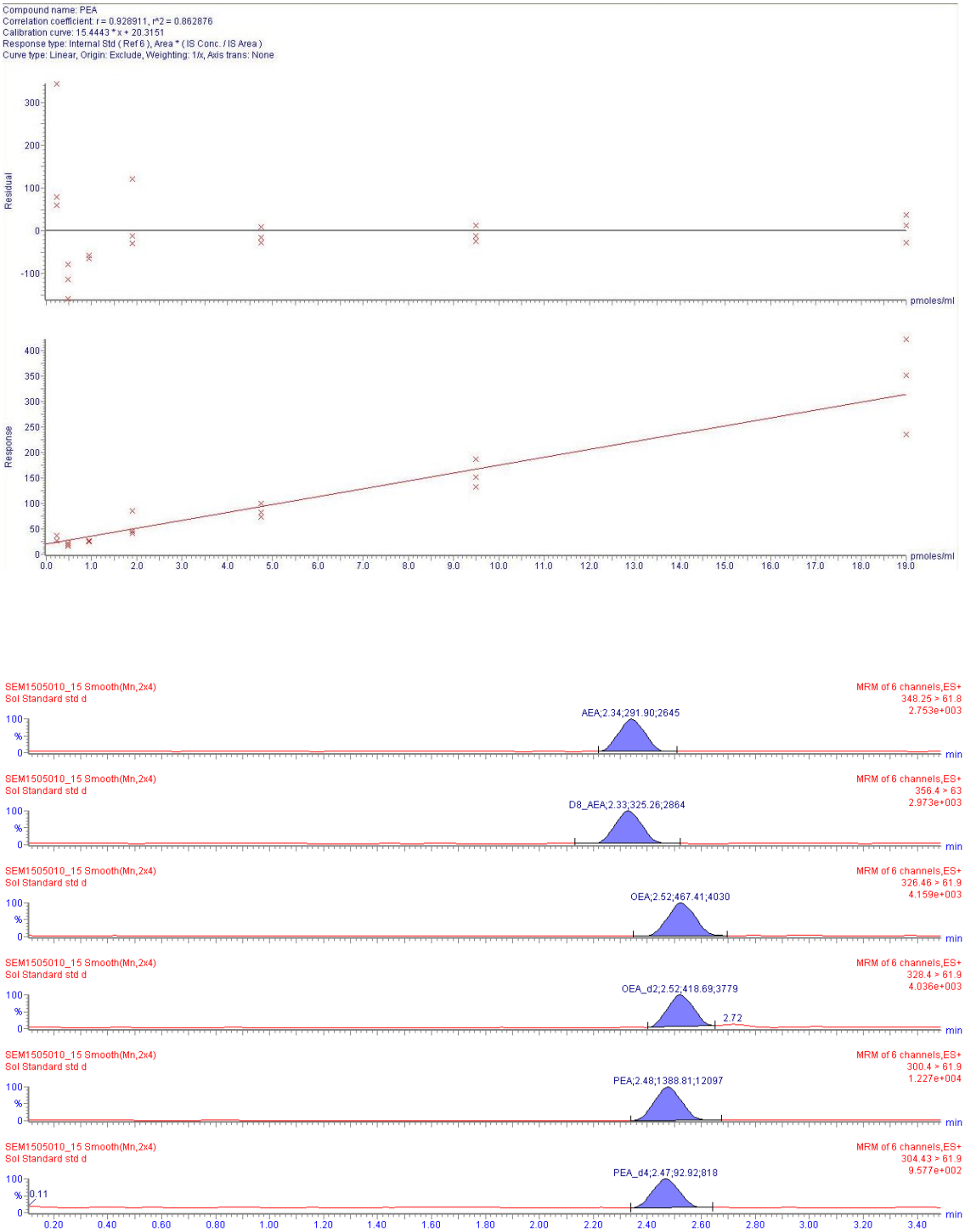

Figure 7-3 An example of the data generated from the UPLC-MS/MS

Figure 7-3a (upper) shows an example of a seven-point combined calibration curves generated.

Figure 7-3b (lower) shows an example of the standard peaks used to generate the standard curve.

The linear regression of the standard curve was calculated by statistical software (GraphPad Prism) and expressed in the format “y = mx + c”; where “y” was the ratio of the area of the d_0 AEA peak against the area of the d_8 peak, “m” was the slope of the line, “x” was the known concentration of AEA in the sample and “c” was the point of intersect on the y axis (Figure 7-4).

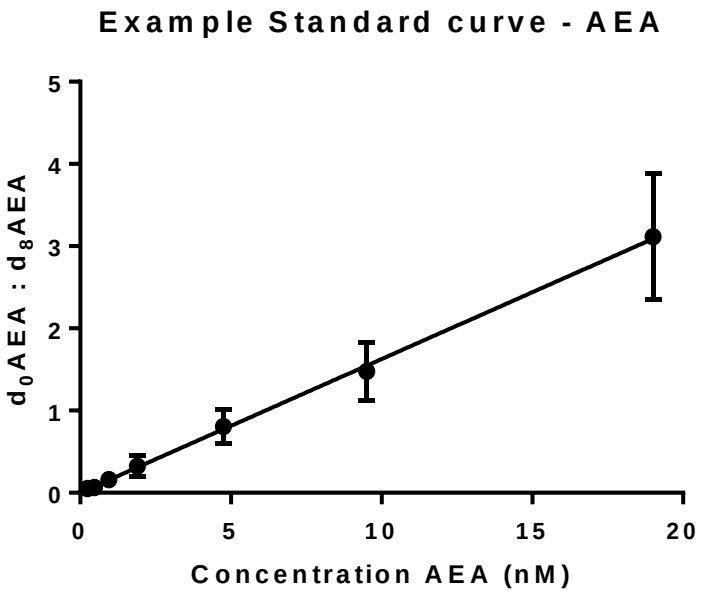

Figure 7-4 An example of the standard curve generated

The data generated from the UPLC using known concentrations of AEA were used to generate a standard curve, which in turn was used to calculate unknown concentrations of AEA in samples.

The linear regression of the standard curve was then used to calculate the concentration of AEA in each sample, with the above equation re-written as x = (y-c)/m, where “x” was the unknown concentration of AEA in the sample, “y” was the ratio of the area of the sample AEA peak against the area of the internal standard peak (Figure 7-5) , “m” was the slope of the line, “x” was the known concentration of AEA in the sample and “c” was the point of intersect on the y axis.

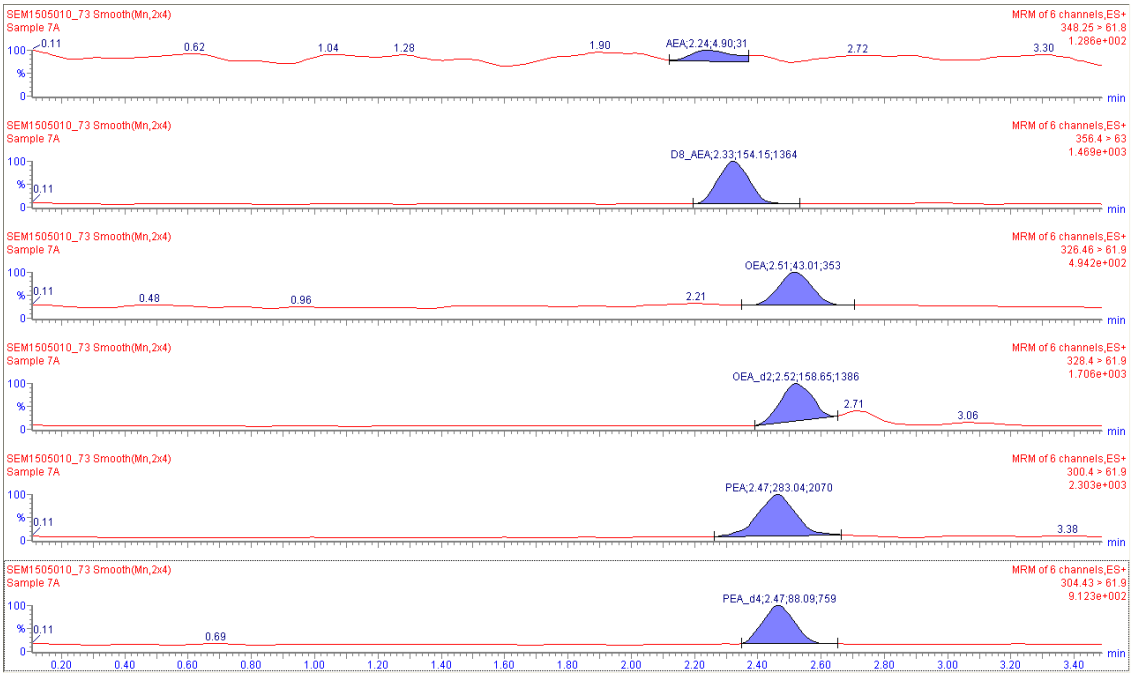

Figure 7-5 An example of the sample and internal standard peaks generated

7.2.4 Problems with initial study design

As already alluded to, there were problems with the initial study design that precluded it from being conducted as originally anticipated and described. These problems can be divided into technical issues (relating to the UPLC-MS/MS) and logistical issues (relating to the process of conducting the study) and are detailed below.

7.2.4.1 Problems with the MS

Measurement of NAEs is now routinely performed using an UPLC-MS/MS, which is an incredibly delicate piece of equipment that is sensitive to any changes to its local environment. It requires specialist knowledge to run and repair it, and unfortunately the member of our department with such knowledge left immediately prior to the start of this project. A new member of staff was employed to help with maintaining UPLC-MS/MS but there was a delay while she became *au fait* with the specific protocols used for analysing NAEs. Because this member of the staff was only allocated to the project for one day a week, it was decided that my supervisor and I should also learn how to analyse samples using the UPLC-MS/MS, and there was a further delay while this took place.

Recruitment into the IMPLANT study started once the team had an understanding of the methods used on the MS. However, this coincided with the MS failing to detect NAEs in any samples, and so further optimisation of the process was needed (including a full clean, service and replacing of all consumables).

Following a short time period when the MS was accurately detecting NAEs, there was a hiatus in recruitment following an audit performed by the R&D team (see below). Approval to restart recruiting coincided with major refurbishment works on the University laboratories, and this further delayed patient recruitment until all the work had been completed. Despite precautions being taken, the delicate instruments within the MS were disturbed as a result of the building work, and again it took a long time (6 months) to resolve this.

Once this had been achieved, recruitment recommenced. Unfortunately, the MS would intermittently develop a small fault, and there was inevitably a delay in its repair. This meant that even after being recruited to the study, it was not always possible to randomise the patients because the results from the MS could not be considered reliable.

Throughout the early stages of recruitment, the MS was plagued with intermittent technical problems, and as such it was never possible to guarantee that the MS would be working on the crucial "day 3" of the patient's treatment. As a consequence, it was concluded that the randomisation of patients was not in their best interests, and so the decision was eventually made to stop randomising patients and purely collect blood samples on OR and ET and analyse them at a later date (i.e. when the MS was actually working!).

7.2.4.2 Problems with conducting the study

As already stated, there was a significant time delay while the team learnt to use the MS, and to solve the multitude of problems it developed. It was decided that while there were problems with the MS it would not be appropriate to randomise patients, and so the samples from patients recruited were used to optimise the study processes. During this time it was observed that there was an unexpected slow recruitment rate. The causes for this were explored and following further education about the project to the staff at the Assisted Conception Unit (ACU) recruitment rates improved.

About 6 months into recruitment, the Endocannabinoid Research Group (ERG) was subjected to an audit by the Research and Development team. This audit raised concerns about aspects of research carried out by previous members of the group, and recruitment for all projects undertaken by the ERG was suspended until this could be rectified. Resolving the issues identified took 3 months, but following additional training the R&D team were happy for the IMPLANT study to continue.

Once R&D approval had been re-instated, concerns were raised by staff in the ACU about the inclusion of patients whose egg collections were on a Wednesday (and so in the study, AEA levels would be used to determine whether embryo transfer took place on a

Saturday). Since there was only a one embryologist available on a given Saturday, the embryology team found including study parameters into the weekend too stressful.

7.2.5 Modified study design

It therefore followed that changes needed to be made to address the concerns raised by the embryologists and so it was agreed that patients having egg collection on a Wednesday would be excluded from the study (limiting recruitment to patients having egg collection on either a Monday or Friday).

As already discussed, the unreliable nature of the MS meant that it was not appropriate to continue randomising patients as described in the initial study design. It therefore decided to limit investigations to expanding on the work of El-Talatini et al. and to increasing the number of patients who had the plasma AEA concentrations compared at the time of OR and ET. Patients were therefore still approached during the follicle tracking stage of their treatment, and then recruited to the study on the day of OR. They had a blood sample collected at this time and then continued with their treatment as planned (day of embryo transfer based on the advice of the embryologist) and had a repeat blood test taken on this day. Plasma was isolated from the whole blood (as described earlier in the Chapter) and then stored at -80°C awaiting analysis (on a day when the MS was working).

7.2.5.1 Measurement of AEA

This was conducted as described earlier, with the only modification being that plasma was frozen following collection, and was thawed and analysed at a later date (when the MS was working).

7.3 Results

A total of 63 patients were recruited into the study but the treatment outcome was not available on 2 of these and so they have been excluded. Of the remaining 61 patients**,** 31 had a positive pregnancy test 2 weeks after ET. Of these 31 patients, 6 were diagnosed with a biochemical pregnancy, 6 miscarried their pregnancy before 12 weeks gestation and the remaining 19 went on to deliver a live baby at 36 or more weeks gestation.

As discussed at length, the MS is an incredibly sensitive piece of equipment, and the retention time for different compounds seemed to constantly change. While consistent results were obtained throughout a run (as confirmed by reproducible standard curves, and constant "Quality Control" values throughout a run), it was not always possible to obtain reproducible values from one run to the next. As a direct result, it was decided that it would not be appropriate to use "absolute values" when analysing the data generated. With this in mind, patient samples were all processed within the same run (that is to say that samples collected at the time of a patient's egg collection (OR) were processed in the same run as the samples collected at the time of the same patient's embryo transfer (ET)) to ensure consistent results per patient. The data presented are therefore percentage change in AEA levels with the value obtained on the day of ET being compared against that obtained on the day of OR. Unfortunately 5 of the samples collected did not generate meaningful data because either a value for OR or ET was not obtained. In addition, after performing ROUT analysis (using GraphPad Prism 6), 3 results were excluded as they were identified as being "outliers". Therefore, measurements from only 54 patients have been included.

7.3.1 Anandamide level and overall outcome of IVF/ICSI treatment

The ultimate outcome to measure when considering ARTs is, "did the patient take a baby home at the end of their treatment?". In relation to this project, that translated into the question, "can a change in plasma AEA levels between OR and ET be used to predict which patients will take a baby home?". The answer is shown in Figure 7-6, where the % change in plasma AEA is plotted against pregnant outcome, where 30% of patients in this study took a baby home at the end of their treatment (Baby n=19 vs No baby n=42). As

can be clearly seen there was no statistically significant correlation between change in plasma AEA concentration and pregnancy outcome ($p= 0.4780$).

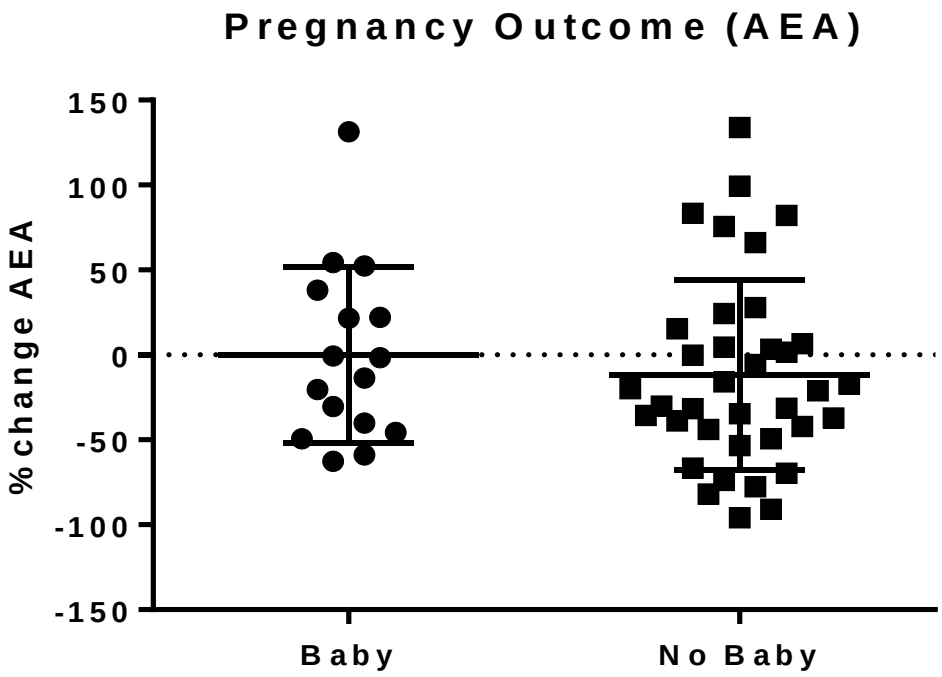

Figure 7-6 The plasma AEA concentration of women undergoing IVF or ICSI in the IMPLANT study and their overall pregnancy outcome

The data show the % change AEA concentration of women who subsequently delivered a baby at term (Baby) or did not become pregnant, miscarried or only had a biochemical pregnancy (No Baby).

The individual data points are plotted together with the mean (-0.21 baby, -11.89 no baby) and SD (51.96 baby, 55.68 no baby). The data are not significantly different; Student's unpaired t-test (p= 0.4780).

7.3.2 Anandamide and urinary pregnancy test result

Having shown that AEA is unlikely to be useful in predicting overall pregnancy outcome, the next question to answer was whether plasma AEA can be used to predict early pregnancy outcomes. Patients undergoing IVF/ICIS return for a pregnancy test (both urinary and plasma hCG) two weeks after their embryo transfer and the question was therefore "can a change in AEA levels between OR and ET be used to predict which patients will have a positive pregnancy test 2 weeks after embryo transfer?". The data in Figure 7-7 show that the number of hCG positive tests was higher than the number of take home babies (n= 31 compared to n= 19), but that there was no statistically significant difference between the % change in AEA concentration in pregnant and non-pregnant groups (p= 0.9575).

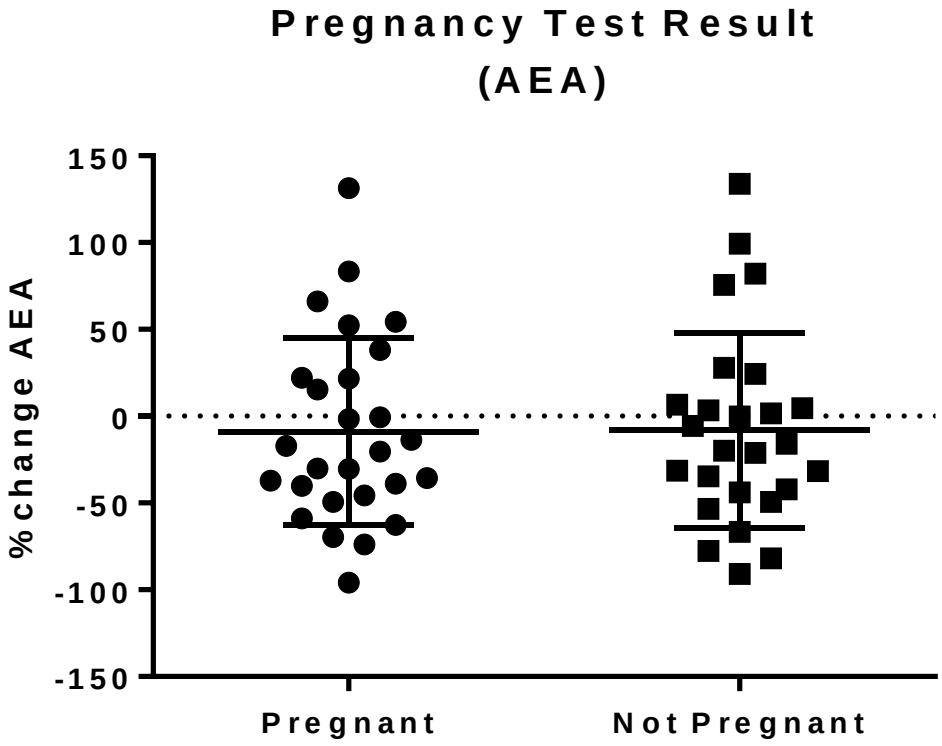

Figure 7-7 The % change in plasma AEA concentration of women undergoing IVF or ICSI in the IMPLANT study and their pregnancy test result

The data show the % change in plasma AEA concentrations and their pregnancy test results (urinary hCG)

The individual data points are plotted together with the mean (-8.76 Pregnant, -7.95 Not Pregnant) and SD (53.66 Pregnant, 56.13 Not pregnant). The change was not statistically significant; Student's unpaired t-test (p= 0.9575).

7.3.3 Anandamide and pregnancy outcome in patients with a "positive" pregnancy test

Exploring the role of AEA in uterine receptivity, the changes in AEA in patients who had a positive urinary pregnancy test 2 weeks after ET was further examined. Following a positive pregnancy test, patients in this study had one of 3 outcomes:

1) Biochemical Pregnancy
2) Spontaneous miscarriage
3) Ongoing pregnancy resulting in a baby being delivered >36 weeks

The changes in plasma AEA levels from OR to ET in these 3 groups, are shown in Figure 7-8. While more than half of the patients with a positive pregnancy test went on to have a successful pregnancy (pregnant n=19 vs biochemical/miscarriage n=12), there was no obvious correlation between changes in AEA levels and the outcome of a positive pregnancy test, even though the median changes in plasma AEA concentrations decreased in the biochemical and miscarriage groups. One-way ANOVA, however, indicated that these small changes were not statistically significantly different ($p= 0.6207$).

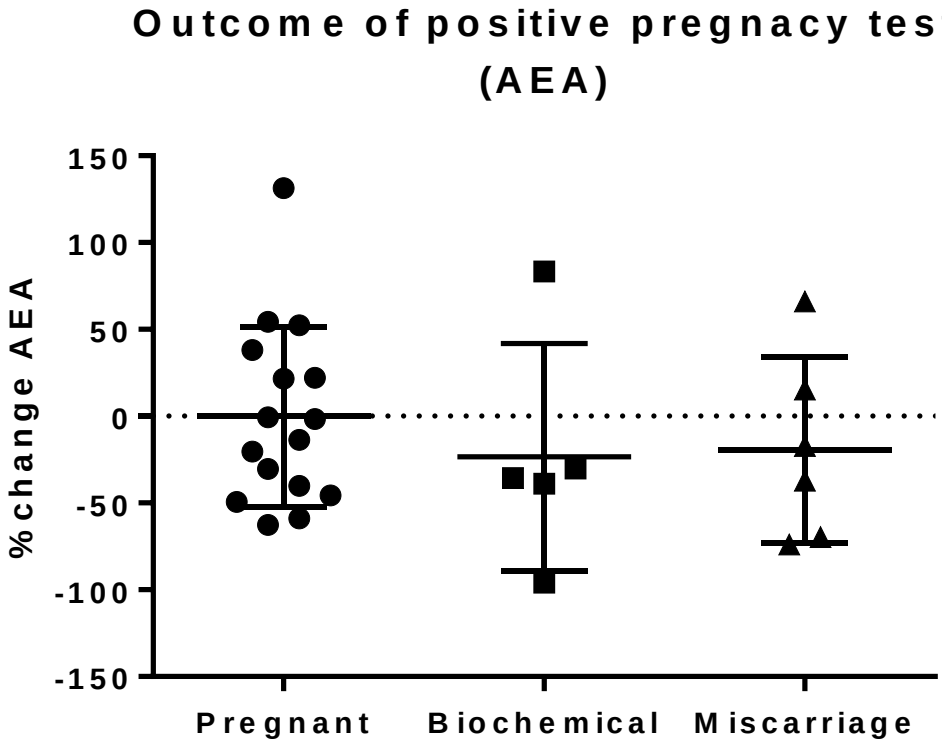

Figure 7-8 The % change in plasma AEA concentration of women undergoing IVF or ICSI in the IMPLANT study and the outcome of a positive pregnancy test

The data show the % change in plasma AEA concentrations and the outcome of their positive pregnancy test.

The individual data points are plotted together with the mean (-0.21 Pregnant, -23.43 Biochemical, -19.34 Miscarriage) and SD (51.96 Pregnant, 65.42 Biochemical, 53.53 Miscarriage). One way ANOVA did not show any statistically significant differences between the three groups (p=0.6207).

7.4 Discussion

At the outset there was strong optimism that findings here would corroborate the promising results from El-Talatini et al. (2009). Sadly this was not the case. While this is disheartening, it highlights the importance of conducting full scale studies (rather than solely relying on pilot data) before adopting a change in clinical practice.

Obviously, when faced with results that differ so dramatically from the work of others (Battista et al. 2008, Habayeb et al. 2008, El-Talatini et al. 2009, Taylor et al. 2011) one has to question what could be the possible cause. When comparing work here to that of El-Talatini et al. (2009), the most striking difference is the method by which AEA was extracted. While El-Talatini et al. (2009) followed the method described by Lam et al. (2008), the method used here was that described by Marczylo et al. (2009). The reason for the change in method was a change in analysis equipment that was incompatible with the chloroform extraction used by El-Talatini and her team.

Another possible explanation for the difference in results is the time lag between sample collection and analysis. As already eluded to, there was a steep learning curve for the team on how to obtain accurate results from the MS at the beginning of the project. However, due to the finite amount of time available to collect data, it was agreed that samples be collected from patients and stored at -80°C awaiting analysis once the MS techniques had been perfected. There were no concerns surrounding the effects of freezing samples since storage had previously been demonstrated to not have any effect on AEA measurement. This delay was unexpectedly extended when the ERG was placed under a detailed review by the hospital's R&D department, and all research was suspended for several months. This ultimately meant that there was a delay of over 12 months from collecting the initial samples to being able to analyse them, whereas the samples collected later in the project were processed in a much more timely fashion. There are data to confirm (or refute) whether or not NAEs remain stable for 12 months in deep freeze conditions and so this could possibly have resulted in the difference between results.

As more results became available it became clear that the absolute values obtained from the MS became far more variable over time (although consistency was maintained within each run) with values generated ranging from 0.004868 to 1.172 pmol/mL. Obviously this was incredibly disappointing, especially as the department had already identified absolute values of AEA that could be used to predict pregnancies that would eventually end in a miscarriage (Taylor et al. 2011). In addition, a lot of time and effort (any money) was spent into trying to rectify this. Unfortunately, a change of supervisor (as well as the loss of the mass spec specialist early on in my project) meant that there was no-one in the department who understood the intricacies of the MS method, and the loss of the supervising Professor meant there was a loss of financial drive to help fix problems identified.

The multitude of issues surrounding measuring plasma AEA concentrations, along with the findings contradicting the work of several other teams, leads one to question the validity of the results generated here. It is still likely that the original hypothesis is still valid, and would warrant further investigation. The approach to this would however be different; ensuring that all members of staff involved fully understood the rationale for the work, and with adequate technical support to use the ULPC-MS/MS to its full potential.

8 Expression of NAEs during fertility treatment

The data presented in this Chapter are divided into two clear sections: a pilot study to examine whether there was any value in using precious resources for a full study and then a full study when it was shown that something interesting was occurring.

8.1 Pilot study

In their pilot study, El-Talatini et al. (2009) restricted their focus to the changes in plasma AEA and the outcome of IVF-ET/ICSI. However, as already discussed in Chapter 7, it is known that OEA and PEA exert an effect on the overall levels of AEA by competing with it for degradation by FAAH. The ERG recently identified a method to extract OEA and PEA as well as AEA simultaneously from plasma. It was therefore decided to test the hypothesis by measuring plasma OEA and PEA concentrations in the blood samples of patients involved in IMPLANT study. Prior to embarking on a full study, the plasma from 11 patients already enrolled in the IMPLANT study were analysed.

8.1.1 Materials and Method

The materials and methods in this Chapter are essentially the same as those described in detail in Chapter 7. All deviations are described in detail.

8.1.1.1 Subjects

Patients were the same as those used for the IMPLANT study (Chapter 7) and so had the same inclusion/exclusion criteria and were approached during the follicle tracking stage of their treatment, and then recruited to the study on the day of oocyte retrieval (OR). They had a blood sample collected at this time and then continued with their treatment as planned (day of embryo transfer (ET) based on the advice of the embryologist) and had a repeat blood test taken on that day. Plasma was isolated from the whole blood (as described in Chapter 7) and then stored at -80°C until analysis (i.e. on a day when the MS was working properly).

8.1.1.2 Extraction and analysis of OEA and PEA

OEA and PEA were extracted and analysed following the same method used for extracting and analysing AEA (described in Chapter 7), with detection parameters of the MS for OEA and PEA and the mass transitions set as follows: OEA (m/z 326.5 > 61.9), OEA-d2 (m/z 328.4 > 61.9), PEA (m/z 300.4 > 61.9) and PEA-d4 (m/z 304.4 > 61.9). All other parameters on the UHPLC-MS/MS were identical to the measurement of AEA and had previously been validated by the ERG (Marczylo et al. 2009).

8.1.2 Results

Of the 11 patients included in this pilot study, 7 had a positive urinary pregnancy test 2 weeks after ET. Of these, 5 went on to deliver a baby at >36 weeks gestation, one had a first trimester miscarriage and one was found to have only a biochemical pregnancy.

ROUT analysis identified one of the PEA concentrations (ET value for a patient with a negative pregnancy test following treatment) as being an outlier, and so this was excluded from analysis.

8.1.2.1 NAE concentration on day of OR and pregnancy outcome

Figure 8-1 shows that there was no statistically significant difference in the concentration of OEA (p=0.513) or PEA (p=1888) in the plasma of pregnant or non-pregnant patients, when comparing levels taken on day of OR and ET.

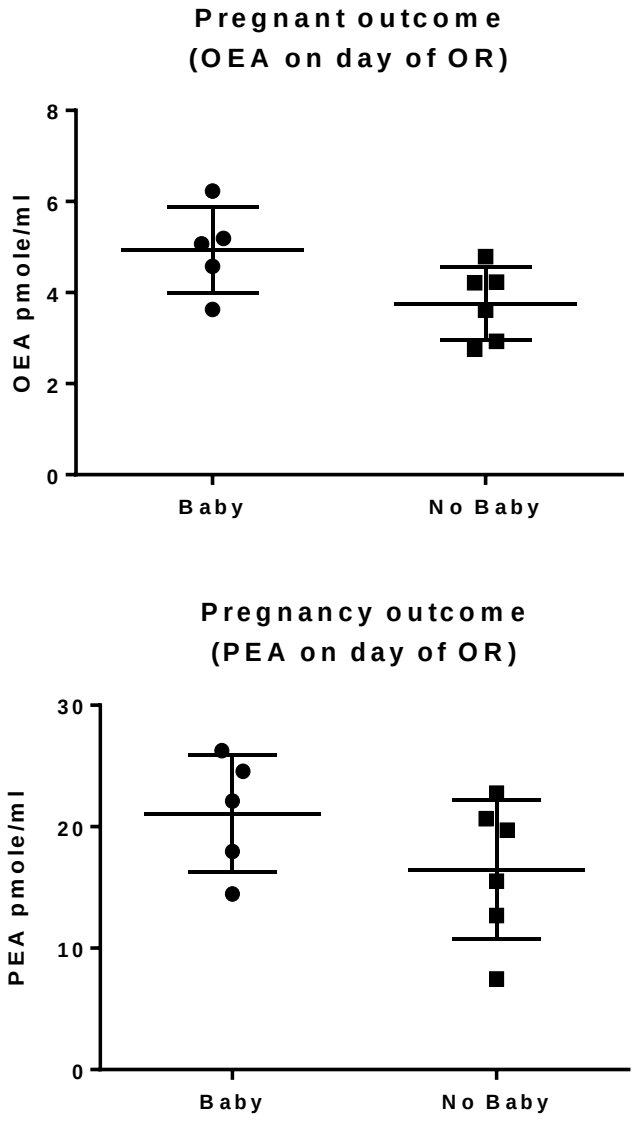

Figure 8-1 The plasma OEA and PEA concentrations of women undergoing IVF or ICSI in the IMPLANT study on the day of OR and their overall pregnancy outcome

The data show either the plasma OEA concentrations (upper panel) or PEA concentrations (lower panel) of women who subsequently delivered a baby at term (Baby) or did not become pregnant, miscarried or only had a biochemical pregnancy (No Baby).

The individual data points are plotted together with the mean (OEA: 4.94 baby, 3.75 No Baby; PEA: 21.07 Baby, 16.47 No Baby) and SD (OEA: 0.94 Baby, 0.80 No Baby; PEA 4.83 Baby, 5.723 No Baby). The data are not significantly different; Student's unpaired t-test (OEA p=0.513, PEA p=0.1888).

8.1.2.2 NAE concentration on day of ET and pregnancy outcome

Figure 8-2 shows that patients who went on to deliver a baby at term had a statistically significantly higher level of OEA in their plasma on the day of ET when compared with those who did not become pregnant, miscarried or only had a biochemical pregnancy ($p= 0.0011$), whereas the plasma levels of PEA on the day of ET were not significantly different ($p= 0.4947$) in the pregnant and non-pregnant women.

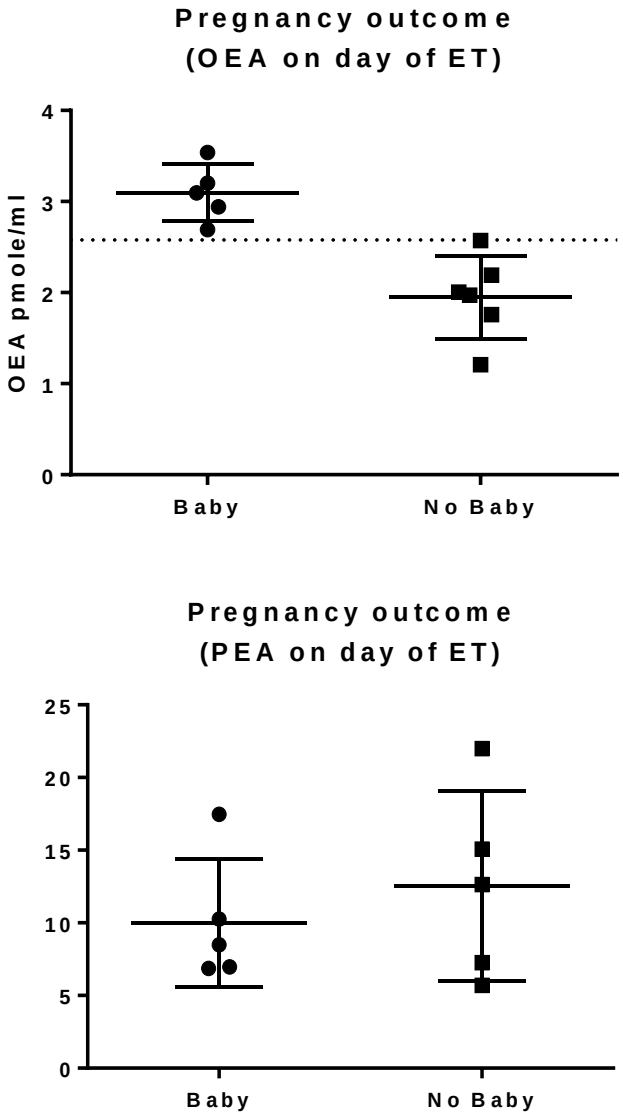

Figure 8-2 The plasma OEA and PEA concentrations of women undergoing IVF or ICSI in the IMPLANT study on the day of ET and their overall pregnancy outcome

The data show either the plasma OEA concentrations (upper panel) or PEA concentrations (lower panel) of women who subsequently delivered a baby at term (Baby) or did not become pregnant, miscarried or only had a biochemical pregnancy (No Baby).

The individual data points are plotted together with the mean (OEA: 3.09 Baby, 1.95 No baby; PEA: 10.01 Baby, 12.53 No Baby) and SD (OEA: 0.31 Baby, 0.45 No Baby; PEA: 4.39 Baby 6.52 No Baby). While the data generated for PEA concentrations were not statistically significant, Student's unpaired t-test ($p= 0.4947$), the difference in OEA concentrations between the 2 groups was statistically significant ($p= 0.0011$).

Of note, all patients with a plasma OEA concentration of ≥2.58 pmol/ml subsequently went on to deliver a live baby at ≥36 weeks gestation.

8.1.2.3 Change in NAE concentration and overall pregnancy outcome

An examination of the percentage change in plasma OEA and PEA levels taken on the day of ET when compared to those taken on the day of OR showed that there was a fall in both (Figure 8-3). When considering the % change in OEA concentrations, in the pregnant patients OEA fell by a mean of 36% whereas it fell by a mean of 47% in the non-pregnant patients, but this change was not found to be statistically significant (p= 0.1068). However, when considering the %change in PEA concentrations, in the pregnant patients PEA fell by a mean of 53% whereas it only fell by a mean of 26% in the non-pregnant patients, and this change was found to be statistically significant (p= 0.0168).

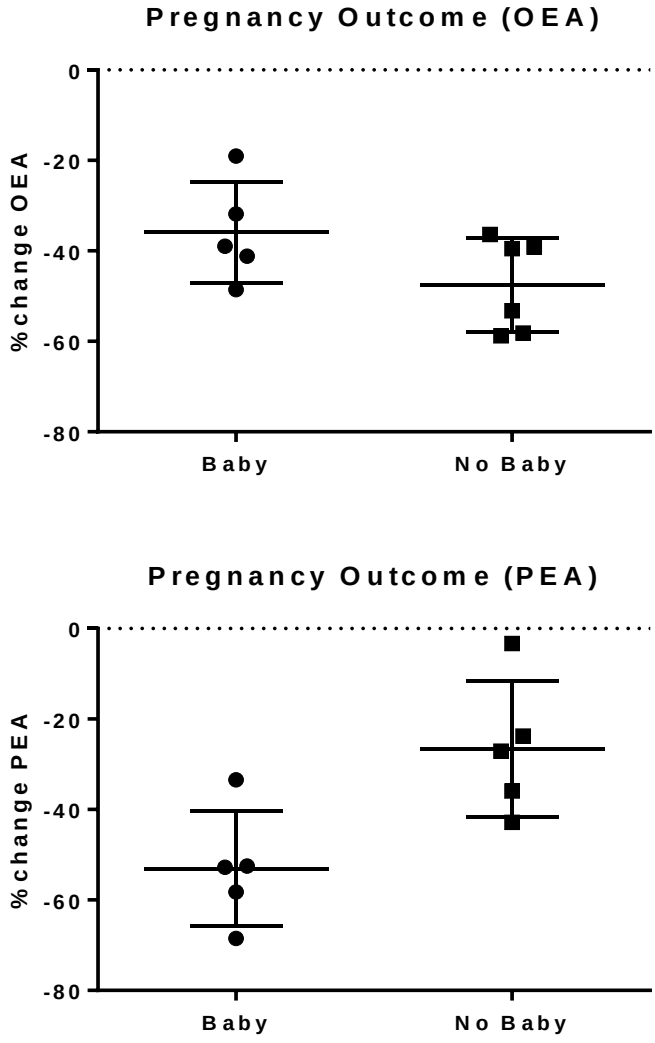

Figure 8-3 The % change in plasma OEA and PEA concentrations of women undergoing IVF or ICSI in the IMPLANT study and their overall pregnancy outcome

The data show the % change in either plasma OEA concentrations (upper panel) or PEA concentrations (lower panel) of women who subsequently delivered a baby at term (Baby) or did not become pregnant, miscarried or only had a biochemical pregnancy (No Baby).

The individual data points are plotted together with the mean (OEA: -35.93 Baby, -47.54 No Baby; PEA: -53.09 Baby, -26.57 No Baby) and SD (OEA: 11.17 Baby, 10.32 No Baby; PEA: 12.74 Baby, 15.00 No Baby). The change in OEA concentrations was not statistically significant; Student's unpaired t-test (p= 0.1068). However, the change in PEA concentrations was statistically significant (p= 0.0168).

8.1.3 Discussion about the pilot study

This pilot study showed that plasma OEA and PEA could be measured in many of the women. There were some problems with incomplete sampling, which could still be related to problems within the MS. However, the concentrations of plasma OEA and PEA were ~10 times and ~40 times higher than that of plasma AEA from the same women, suggesting that this method is more robust than that for AEA alone, and thus these data are more convincing. This point was made in the original paper describing this technique (Marczylo et al. 2010).

This pilot study identified a trend in change in plasma OEA concentrations and pregnancy outcome. While the data do not allow one to make a bold statement such as "IVF/ICSI will be successful if plasma PEA concentrations fall by "x"% between OR and ET", but does provide evidence to warrant exploring the phenomenon further by expanding the study.

When considering OEA, as with the plasma AEA study (Chapter 7), plasma OEA levels did not significantly alter between the day of OR and ET. However, a statistically significant difference was noted when comparing plasma OEA concentrations on day of ET against pregnancy outcome. Indeed, a plasma OEA concentration of 2.58 pmol/mL or more was predictive of patients who would give birth at >36 weeks gestation, i.e. give birth to a healthy baby at close to term; the primary goal of ART. On this basis, it became clear that this observation required more samples in a bigger study to determine whether plasma OEA on the day of ET is indeed a good predictor of pregnancy outcomes. The main limitation of this pilot study was the small number and the lack of consistency in some of the measurements. Therefore a decision to attempt this test on the entire cohort (full study) was made.

8.2 Full study

Following the promising results from the pilot study, the decision was made to extend the pilot study into a full study. As blood samples from patients involved in the IMPLANT study had already been made, the decision was made to analyse OEA and PEA as well as AEA in all the samples taken.

8.2.1 Materials and Method

The materials and methods for the full study are as described in the Pilot study section of this Chapter. Because of the larger dataset, some additional statistical tests were performed on the data. This included a ROUT test (Motulsky and Brown 2006) for the identification and removal of outliers from the data. All subsequent data were examined for significance using Student's t-test once tests for normality had been completed. These tests were performed using GraphPad prism version 6.

8.2.2 Results

As this study was run in conjunction with the study described in Chapter 7, the same 63 patients were recruited into this study, and as a result had the same pregnancy outcomes as already described. As a reminder, treatment outcomes on 2 of the patients were unobtainable and so they have been excluded from the study. Of the remaining 61 patients**,** 31 had a positive pregnancy test 2 weeks after ET. Of these 31 patients, 6 patients were diagnosed with a biological pregnancy, 6 miscarried before 12 weeks gestation and the remaining 19 went on to deliver a live baby at 36 or more weeks gestation.

Again, as with the study described in Chapter 7, the inability to reliably reproduce results from the UPLC-MS/MS from one day to the next (despite being able to obtain reproducible results within each run) meant that rather than comparing absolute values, it was only possible to compare the changes in OEA and PEA from the day of OR to the day of ET. Unfortunately, 6 of the samples collected did not generated meaningful data for OEA (3 patients who had a negative pregnancy test, 2 patients who delivered healthy babies and 1 who miscarried before 12 weeks), and 7 did not generate meaningful data for PEA (4 patients who had a negative pregnancy test and 3 patients who delivered a healthy baby) because either the OR or ET values were absent. In addition, after performing ROUT analysis, 3 results were excluded from OEA measurements (2 patients who had a negative pregnancy test and 1 patient who miscarried) and 3 results from PEA measurements (3 patients who had a negative pregnancy test), because they were identified as "outliers".

8.2.2.1 OEA level on the day of ET and pregnancy outcome

The pilot study suggested that a plasma concentration of OEA of ≥2.58pmol/mL on the day of ET could be used to predict pregnancy outcome. Unfortunately, increasing the number of patients in the study (Figure 8-4) did not support this original hypothesis that OEA levels ≥2.58pmol/mL on day of ET could be used to predict pregnancy outcome ($p = 0.7654$).

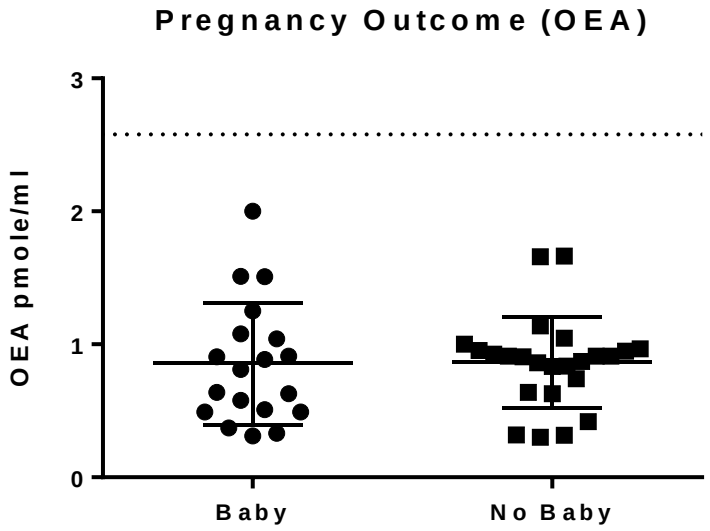

Figure 8-4 Plasma OEA concentration of women undergoing IVF or ICSI in the IMPLANT study on the day of ET and their overall pregnancy outcome

The data show the plasma OEA concentrations of women who subsequently delivered a baby at term (Baby) or did not become pregnant, miscarried or only had a biochemical pregnancy (No Baby).

The individual data points are plotted together with the mean (0.85 Baby, 0.86 No Baby) and SD (0.45 Baby, 0.34 No Baby). While the data generated were not statistically significant, Student's unpaired t-test (p= 0.7654).

8.2.2.2 NAEs and overall outcome of IVF/ICSI treatment

As with the IMPLANT study, the ultimate outcome to measure when considering ARTs is "did the patient take a baby home at the end of their treatment?". In relation to this work, that translated to the question "can a change in OEA or PEA levels between OR and ET be used to predict which patients will take a baby home?".

Contrary to the results in the pilot study, the graph shown in Figure 8-5a suggests that a greater decrease in plasma OEA levels is associated with an increased likelihood of achieving a successful pregnancy following IVF-ET/ICSI, however, analysis did not show any statistical significance between the differences ($p=0.9874$; Student's t-test). Again contrary to these results in the pilot study, the graph in Figure 8-5b shows that there was no change in plasma PEA in the group that did not have a take home baby and that there was a small overall increase in the group that had a successful pregnancy following IVF/ICSI, however, analysis did not show any statistical significance between the differences ($p=0.3791$; Student's t-test).

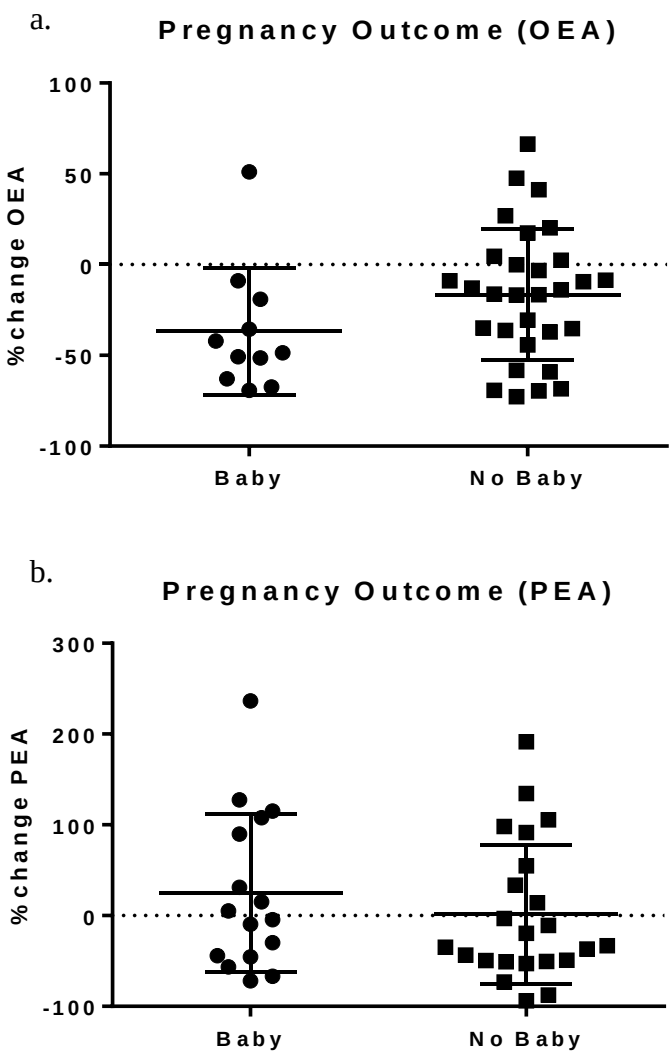

Figure 8-5 The % change in plasma OEA and PEA concentrations of women undergoing IVF or ICSI in the IMPLANT study and their overall pregnancy outcome

The data show the % change in either plasma (a) OEA concentrations or (b) PEA concentrations of women who subsequently delivered a baby at term (Baby) or did not become pregnant, miscarried or only had a biochemical pregnancy (No Baby).

The individual data points are plotted together with the mean (OEA: -36.80 Baby, -16.52 No Baby; PEA: 25.01 Baby, 1.52 No Baby) and SD (OEA: 34.75 Baby, 36.02 No Baby; PEA: 87.27 Baby, 76.52 No Baby). The change in OEA or PEA concentrations was not statistically significant; Student's unpaired t-test (OEA p= 0.9874, PEA p= 0.3791).

8.2.2.3 NAEs and urinary pregnancy test result

Having shown that neither OEA nor PEA could be used to predict overall pregnancy outcome, the next question to answer was whether OEA or PEA could be used to predict early pregnancy outcomes. Patients undergoing IVF/ICSI return for a pregnancy test (both urinary and plasma HCG) two weeks after their embryo transfer and the question then was "can a change in OEA or PEA level between OR and ET be used to predict which patients will have a positive pregnancy test 2 weeks after embryo transfer?".

The graph shown in Figure 8-6a supports the hypothesis that lower levels of OEA appear to be linked to successful embryo implantation, however, analysis did not show a statistically significant different between the pregnant and non-pregnant groups ($p=0.6634$; Student's t-test). However, the graph in Figure 8-6b reached a different conclusion regarding the role of PEA. In the pilot study it was found that PEA fell in all patients, but concentrations fell less in the patients who went on to give birth to a live infant, whereas in the full study it was shown that an increase in PEA levels from day of OR to the day of ET may be linked to successful embryo implantation. While the trend identified in the pilot study was not supported by statistical analysis, the full study data is supported by statistical significance ($p=0.0287$; Student's t-test).

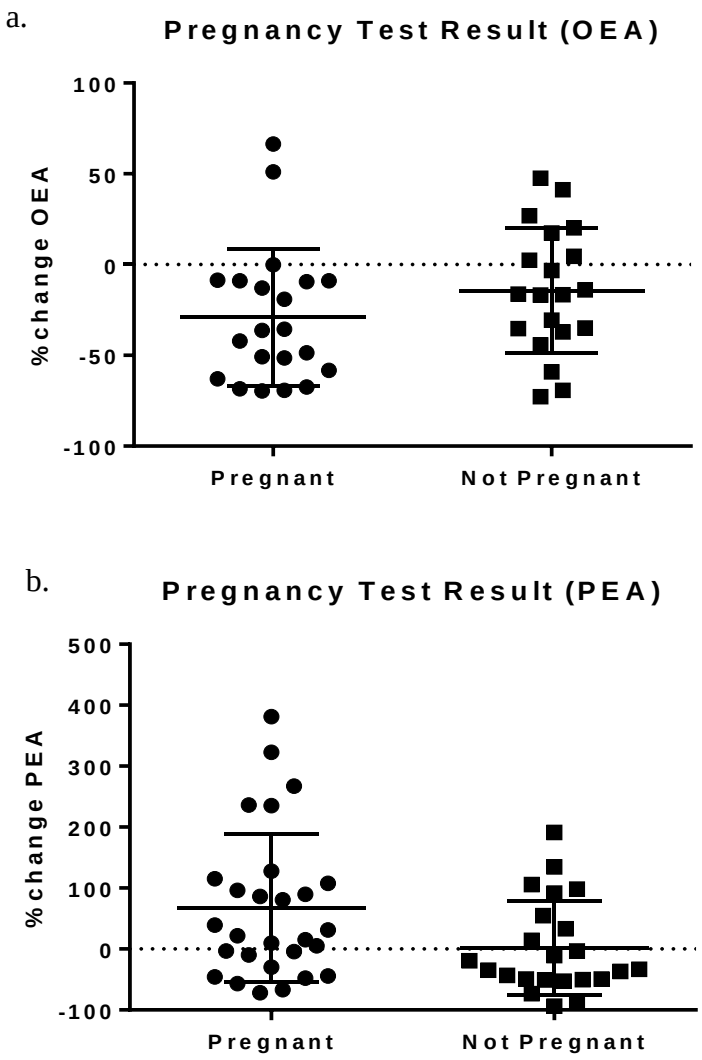

Figure 8-6 The % change in plasma OEA and PEA concentrations of women undergoing IVF or ICSI in the IMPLANT study and their pregnancy test result

The data show the % change in either plasma (a) OEA concentrations or (b) PEA concentrations and their pregnancy test results (urinary hCG)

The individual data points are plotted together with the mean (OEA: -29.10 Pregnant, -14.49 Not Pregnant; PEA: 67.49 Pregnant, 1.52 Not Pregnant) and SD (OEA: 37.59 Pregnant, 34,46 Not Pregnant; PEA: 121.8 Pregnant, 76.52 Not pregnant). The change in OEA concentrations was not statistically significant; Student's unpaired t-test (p= 0.6634). However, the change in PEA concentrations was statistically significant (p= 0.0287).

8.2.2.4 NAEs and pregnancy outcome in patients with a "positive" pregnancy test

In order to explore the role of OEA and PEA in embryo implantation, the levels of these NAEs in patients who had a positive urinary pregnancy test 2 weeks after ET were measured. Following a positive pregnancy test, patients in this study had one of 3 outcomes:

1) Biochemical Pregnancy
2) Spontaneous miscarriage
3) Ongoing pregnancy resulting in a baby being delivered >36 weeks

The change in OEA and PEA levels from OR to ET in these 2 groups, as shown in Figure 8-7a and 8-7b respectively were therefore examined**.**

Figure 8-7a shows a fall in plasma OEA with all outcomes following a positive pregnancy test. One way ANOVA analysis did not show any statistically significant difference between the three groups. Figure 8-7b shows that an increase in plasma PEA happened in all outcomes after a positive pregnancy test. One way ANOVA analysis did show a statistically significant difference between the three groups.

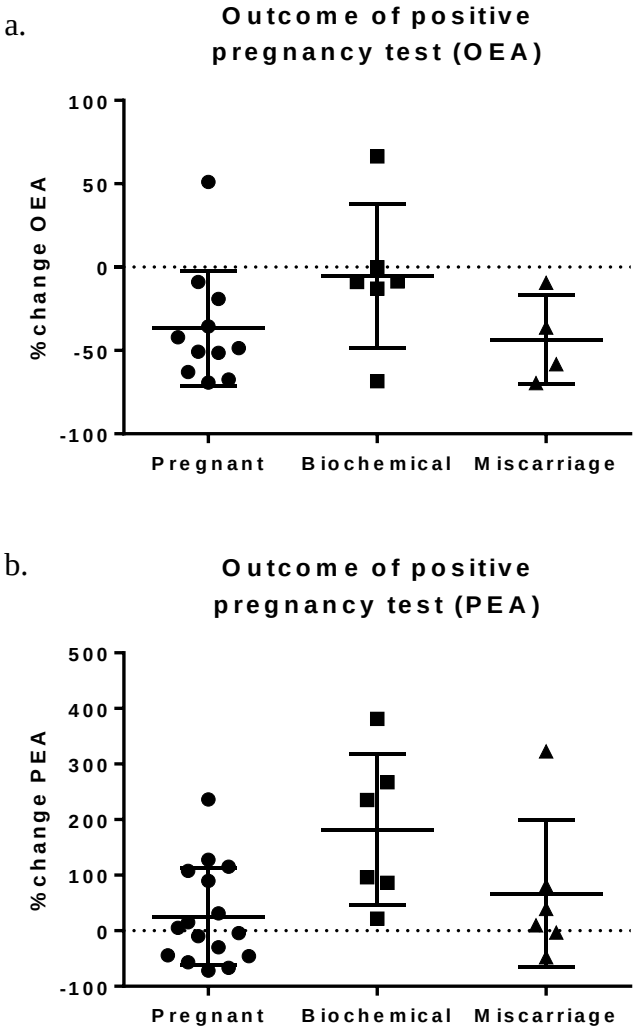

Figure 8-7 The % change in plasma OEA and PEA concentrations of women undergoing IVF or ICSI in the IMPLANT study and the outcome of a positive pregnancy test

The data show the % change in either plasma OEA concentrations (a) or PEA concentrations (b) and the outcome of their positive pregnancy test.

The individual data points are plotted together with the mean (OEA: -36.80 Pregnant, -5.44 Biochemical, -43.41 Miscarriage; PEA: 25.01 Pregnant, 181.40 Biochemical, 66.85 Miscarriage) and SD (OEA: 34.75 Pregnant, 43.00 Biochemical, 26.50 Miscarriage; PEA: 87.27 Pregnant, 135.60 Biochemical, 132.6 Miscarriage). One way ANOVA did not show any statistically significant differences between the three groups for either NAE. The change in OEA concentrations was not statistically significant; Student's unpaired t-test (p= 0.6634). However, the change in PEA concentrations was statistically significant (p= 0.0287).

8.2.2.5 The entourage effect

The results from the pilot study and full study gave contrasting outcomes. While in the pilot study plasma PEA concentrations fell in both groups (more so in the pregnant group than the non-pregnant group) the opposite was seen in the full study where plasma PEA concentrations actually rose. OEA concentrations fell in both groups, but again there was a difference between the pilot and full study when considering which group had the greatest reduction of plasma OEA levels.

One suggestion for this could be the proposed "entourage effect" whereby OEA and PEA are thought to compete with AEA for degradation by FAAH, thereby potentiating AEA activity (Bambang et al. 2010). To investigate this potential, the average change in NAE concentrations was examined, combining the results generated from AEA, OEA and PEA and comparing them against the pregnancy test result taken 2 weeks after embryo transfer. This time point was chosen because it was the only aspect of treatment that generated statistically significant results in the full study. Figure 8-8 clearly shows that there was no significant difference in the average change in NAE concentration and pregnancy test result (Student's unpaired t-test; $p= 0.9914$).

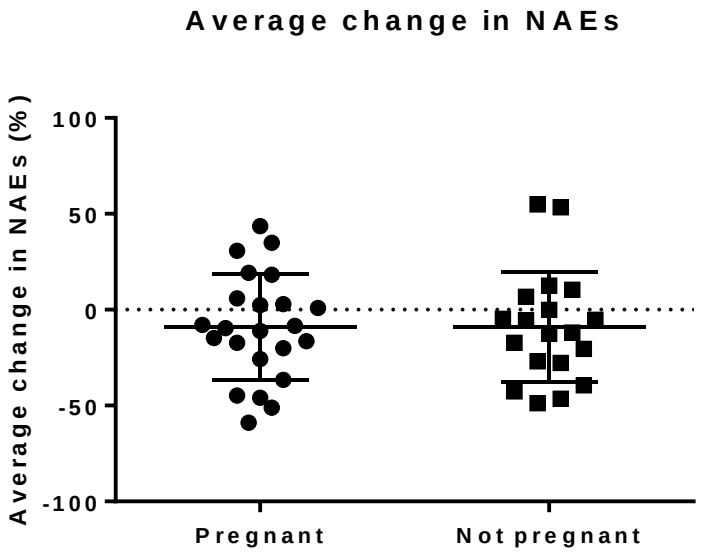

Figure 8-8 The average change in plasma NAE concentrations of women undergoing IVF or ICSI in the IMPLANT study and their urinary pregnancy test result

The data show the average % change in e plasma NAE concentrations of women and their pregnancy test (urinary hCG) result taken 2 weeks after embryo transfer.

The individual data points are plotted together with the mean (-9.11 Pregnant, -9.02 Not Pregnant) and SD (27.51 Pregnant, 28.98 Not Pregnant). The data are not significantly different; Student's unpaired t-test (p= 0.9914

It was then investigated whether or not the combined effects of NAE concentrations affected the outcome of IVF/ICSI treatment. Again, the data shown in Figure 8-9 did not reveal any significant difference in the total change in NAE concentration and pregnancy test result (Student's unpaired t-test; p=0.3682).

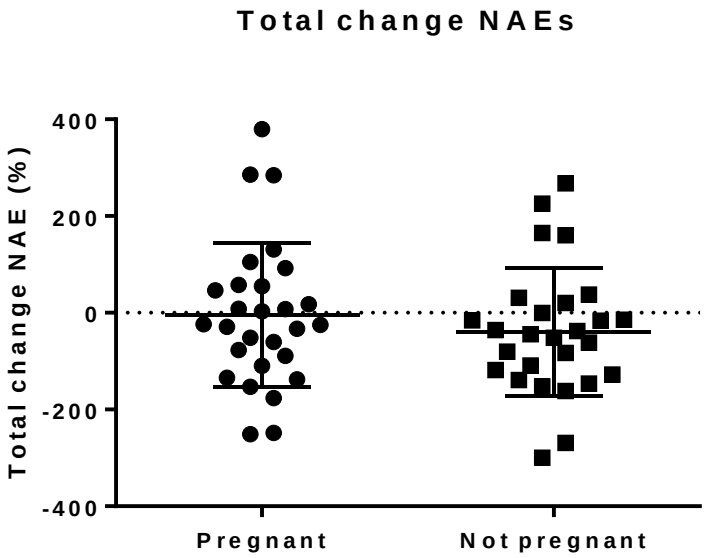

Figure 8-9 The total change in plasma NAE concentrations of women undergoing IVF or ICSI in the IMPLANT study and their urinary pregnancy test result

The data show the total % change in plasma NAE concentrations of women and their pregnancy test (urinary hCG) result taken 2 weeks after embryo transfer.

The individual data points are plotted together with the mean (-4.56 Pregnant, -39.18 Not Pregnant) and SD (149.5 Pregnant, 132.5 Not Pregnant). The data are not significantly different; Student's unpaired t-test (p= 0.3682).

Next, the combined effects of the change in OEA and PEA concentrations against the change in AEA concentrations as the entourage effect suggests OEA and PEA are competing with FAAH's activity and it is that event that affects AEA's effects on implantation success were explored. The results of this are shown in Figure 8-10**,** which again shows there was no significant difference between pregnancy test result and the change of AEA concentration compared to the change in OEA+PEA concentrations.

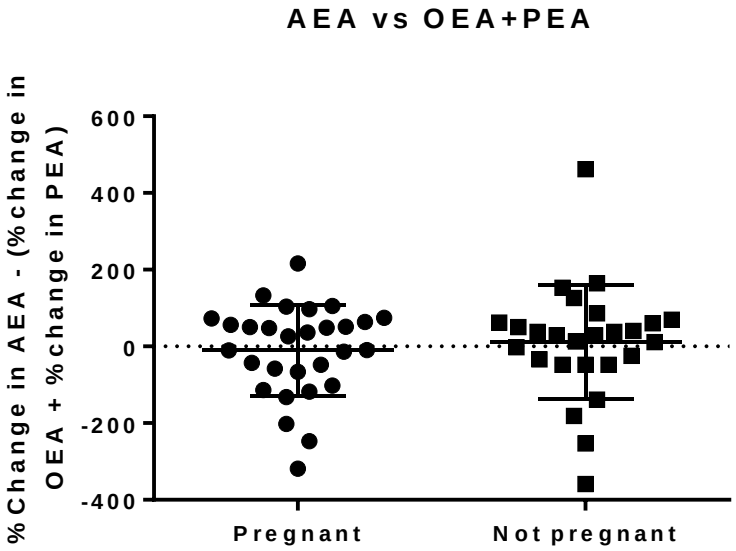

Figure 8-10 The difference between change in plasma AEA concentration and plasma OEA and PEA (combined) concentrations of women undergoing IVF or ICSI in the IMPLANT study and their urinary pregnancy test result

The data show the difference between the % change in plasma AEA and plasma OEA+PEA (combined) concentrations of women and their pregnancy test (urinary hCG) result taken 2 weeks after embryo transfer.

The individual data points are plotted together with the mean (-10.26 Pregnant, 11.41 Not Pregnant) and SD (118.50 Pregnant, 149.40 Not Pregnant). The data are not significantly different; Student's unpaired t-test (p= 0.5517)

Finally, the change in NAEs as individual entities was examined. While the proposed entourage effects suggests that it is the combined effects of OEA and PEA competing with FAAH to maintain AEA concentrations, my *in-vitro* work suggested that PEA plays more of a role than OEA.

Results of the *in-vivo* work showed very similar result to that of *in-vitro* studies. In the non-pregnant population, there was no significant difference in the plasma concentration of the NAEs. However, in the pregnant population, there was a statistically significant change in plasma PEA concentrations, while maintaining fairly stable OEA and AEA concentrations (Figure 8-11).

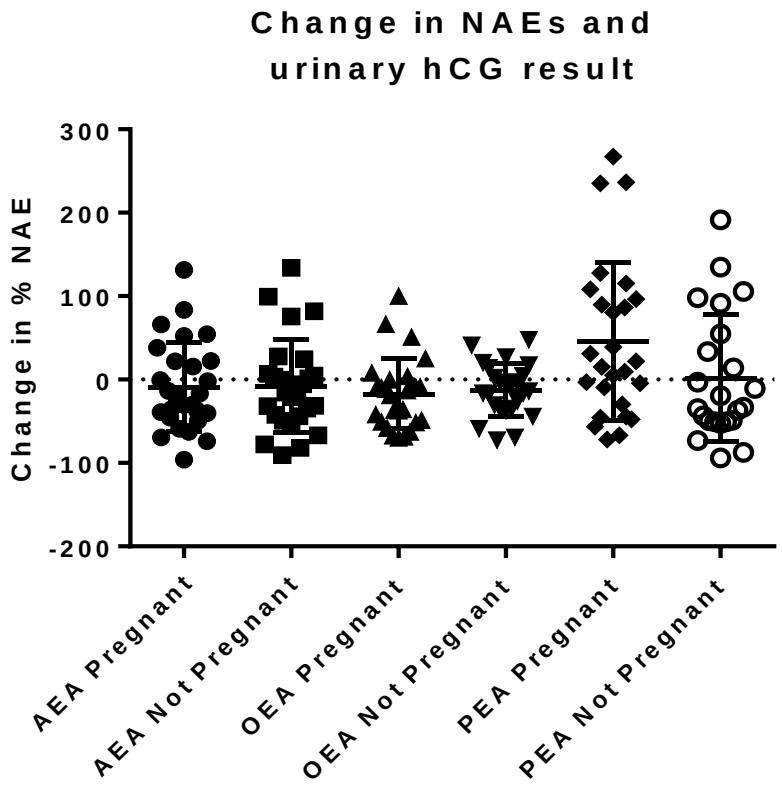

Figure 8-11 Percent (%) change in each NAE and pregnancy test outcome

The data show the difference between the % change in individual plasma NAE concentrations of women and their pregnancy test (urinary hCG) result taken 2 weeks after embryo transfer.

The individual data points are plotted together with the mean and SD (values as in chapters 7.3.1 and 8.1.2.3). The change in plasma PEA concentration in patients with a positive pregnancy test showed statistical significance when comparted to the change in plasma AEA or OEA concentrations in patients with a positive pregnancy test (one way ANOVA, $p<0.05$ AEA and $p<0.01$ OEA).

8.3 Discussion

The rational for extending this study from a pilot to a full study was the early results suggesting that an OEA level of >2.58pmol/mL could be used to identify patients undergoing IVF-ET/ICSI who would then go on to deliver a baby at >36 weeks. Unfortunately this finding was not supported in the full study. Also, as already discussed, as more results became available it became clear that the absolute values obtained from the MS became far more variable over time (although consistency was maintained within each run). The range of NAE measurements generated meant that it quickly became obvious that using an absolute OEA level to predict IVF-ET/ICSI outcome would not be possible. It was therefore decided to abandon plans to pursue absolute NAE values, and instead compare plasma OEA and PEA levels on the day of ET against those on the day of OR (with both samples being processed at the same time on the MS).

However, the issues resulting from the slowly failing MS do not explain why there was such variability in the results in the change in plasma PEA; in the pilot study it fell whereas in the full study it rose. As already discussed, OR and ET samples from each patient were grouped together for MS analysis, and consistency in results within runs was confirmed by regular "quality control" samples being analysed. As already discussed in Chapter 7, there was a time delay of over 12 months between collecting and analysing the samples in the pilot study, whereas in the full study samples were collected and analysed in a much more timely fashion. For that reason, it is surmised that the full study results to be the true results as the samples were processed in a much more timely fashion.

When looking at the full study, this study has sadly generated more negative findings than positive ones. It was not possible to have an absolute value for OEA or PEA that could be used to predict treatment outcome in patients undergoing IVF or ICSI; furthermore the % change in either NAE that could not be used to predict which patients are likely to have successful treatment.

It was however shown that there is a statistically significant difference in the % change in PEA between patients with a negative or positive pregnancy test 2 weeks after embryo transfer. Sadly though, this again could not be used to predict overall pregnancy outcome,

because there was no statistically significant difference between the patients who went on to have a successful pregnancy and those with positive pregnancy test that ended with a biochemical pregnancy or a miscarriage. The change in PEA concentration and early pregnancy investigation results are in keeping with the cell culture work, which suggested that PEA concentrations alter more dramatically in receptive cells (Chapter 3).

While a statistically significant finding would normally suggest that an expansion of the study would be beneficial to explore the result further, in a resources conscious society is it necessary to consider how the expansion of the study will help improve patient care. In this particular study, it was possible to show that there is a difference in the change in plasma PEA concentrations between OR and ET and the outcome of the patient's pregnancy test. This cannot be used to help plan IVF or ICSI treatment, nor can it be used to counsel patients appropriately about their progress as the change in plasma PEA concentrations does not represent to overall pregnancy outcome. It is therefore unlikely that the change in NAE concentrations between OR and ET in patients undergoing IVF/ICSI warrants further investigation. However, quantifying absolute values of NAEs (analysed on a MS that produces consistent results) may have some benefit in pursuing.

9 Reflection on the project

9.1 How does these studies influence our understanding of endometrial receptivity?

This project first ignited my interest when I learnt about the IMPLANT trial and the egotistical part of my personality thought: "Wow! If this works then we might have found a way to dramatically improve IVF/ICSI success rates". Now, with the beauty of hindsight, I can see that it was a pretty naïve attitude, and showed how little I understood about the world of research. However, while my work hasn't "cured" infertility, it has helped add another building brick into the wall of the knowledge concerning uterine receptivity.

Firstly, I feel I have expanded our understanding of the entourage effect of NAEs. Previously, it was suggested that both OEA and PEA competed with AEA to be degraded by FAAH (and thus maintaining an appropriate anandamide tone). My work has suggested that there is a correlation between PEA and NAPE-PLD, with increasing expression of NAPE-PLD resulting in an increase in concentrations of PEA while concentrations of AEA and OEA remain fairly constant. The importance of PEA was further demonstrated in my *in-vivo* work, which saw an increase in PEA in patients who became pregnant, whereas AEA and OEA concentrations remained stable.

While this work suggests that NAPE-PLD is important in maintaining a necessary "anandamide tone" (*via* an entourage effect with PEA), it is not to say that FAAH does not play an equally important role in endometrial receptivity. The work with SNAP showed that increasing concentration of SNAP resulted in an increasing expression of FAAH, but this phenomenon was restricted to receptive endometrial cells.

When considering other differences between receptive and non-receptive endometrial cell lines, the relative amounts of transcripts for both NAPE-PLD and FAAH were significantly higher in receptive cells than non-receptive cells. Furthermore, the amount of FAAH was 10x higher in Ishikawa cells when compared to HEC-1A cells (whereas NAPE-PLD was only 2x higher).

Treating Ishikawa cells with SNAP showed significant changes in the amounts of transcripts for both NAPE-PLD and FAAH. Again, these changes were restricted to the receptive cell line. What is yet to be confirmed is whether the changes seen were the result of an increase of integrin β3 expression, an increase availability of nitric oxide, or both. I am confident that at least some of the changes are a result of increased integrin β3 expression, as similar changes were seen in cells treated with an alternative chemical that is known to increase the expression of integrin β3 (gal-3), but further work is needed before firm conclusions can be drawn on this point.

As far as I am aware, this is the first time a direct comparison of ECS enzymes has been made between receptive and non-receptive endometrial cell lines or human cells (with past work being carried our using animal studies). In addition, this is the first time the effect of integrin β3 on the ECS enzymes has been explored. Finally, work surrounding the entourage effect has so far been restricted to OEA and PEA's combined effect on AEA *via* FAAH, whereas this is the first evidence detailing a direct correlation between NAPE-PLD transcript levels and PEA concentrations.

9.2 Things that did not go to plan

While having written about the experiments that generated data, this thesis could equally be written about "projects that did not work". The American comedian W.C. Fields is famously quoted as saying "never work with animals or children". In the context of this project, this should be changed to "never work with administration staff or mass spectrometers"(!).

By far the biggest setback encountered was the loss of all 3 of my supervisors (my Professor went on sabbatical, my Clinical supervisor accepted a job at a different hospital and my Scientific supervisor was made redundant), along with the loss of 2 post-doctoral fellows who developed the method for extracting and analysing the concentration of NAEs from various substances. The loss of so many key mentors naturally hindered the process of the project in numerous ways, including the loss of a person of "authority" to help when administration staff placed unnecessary limitations on projects and the loss of

people with the knowledge base to help when experiments were not going to plan (not to mention the loss of laboratory space when my Scientific supervisor left the University).

A large proportion of the frustration here was directed towards the UPLC-MS/MS. As already discussed, immediately prior to starting my work the department lost both post-doctoral fellows with the detailed knowledge of how to maximise the results obtained from the UPLC-MS/MS. A new member of staff was recruited, and while she had the knowledge to maintain the ULPC-MS/MS she did not understand the intricacies of the methods used to analyse NAEs. The ULPC-MS/MS would periodically stop detecting NAEs; sometimes this was resolved with a deep clean and replacing all the solvents, but often it involved alterations to the method (which invariably resulted in a delay of experiments and the generation of data).

Even when the UPLC-MS/MS was detecting NAEs, it soon became apparent that the concentration of NAEs detected varied. She was confident that samples analysed in a single run generated reproducible results (confirmed by steady QC values), but could not be replicated with samples analysed in different runs (due to differing interassay QC values). Again, steps were taken to try to resolve this but ultimately no cause was identified and so eventually it was decided that it would be unwise to compare samples processed in different runs. In addition to a lot of time being lost while trying to 'fix' problems with the UPLC-MS/MS, this decision also dramatically restricted the range of possible analyses.

Because the ULPC-MS/MS was an older machine, it started to develop numerous faults, which (once the service contract expired) needed to be 'fixed' by in-house staff. For a large part of my project, it was only possible to use half the UPLC-MS/MS, and once the only functional motor broke, the project effectively ceased, because repairs would have not been cost-effective. This again meant that a lot of potential data were lost as quantification of NAEs in the culture medium (i.e. most of the cell culture work) could not be performed.

Another significant setback occurred after a review by the Research and Development (R&D) team from within the UHL NHS Trust, who stopped recruitment to all projects within our department while concerns related to previous projects were addressed. While

this was an extremely frustrating time, it enabled me to work closely with the R&D team. By spending several weeks in their office helping to resolve identified issues and I gained much greater insight into the administrative processes necessary to run a successful clinical research project.

In conclusion, the work here has helped to further our understanding of the role endocannabinoids in endometrial receptivity, however, there were also several other avenues that were unsuccessful or unexplored for a variety of reasons, and it would be unfair not to mention them at all. The full details of the projects are included in the Appendices. They included:

a) Comparing the expression of the endocannabinoid system at the implantation zone and inter-implantation zone (appendix 1)
b) Comparing the concentration of NAEs in plasma and endometrial samples (appendix 2)
c) Measuring FAAH activity in culture medium of "good quality" and poor quality embryos

There was a long delay in starting project a) because nobody within the R&D department knew who was responsible for projects undertaken in peripheral hospitals, and although the project eventually gained approval from the Local Research Ethics Committee and recruitment commenced, it ultimately failed because the hysteroscopes available in the theatres were of insufficient quality to take accurate biopsies (and I could not convince other departments to lend me their newer, more accurate, hysteroscopes).

Project b) successfully gained ethics approval, patients were recruited into the study, and tissue samples were obtained. Unfortunately, no data were generated from the samples because the UPLC developed a fault and repairs were considered financially impracticable.

Project c) did not even reach the point of submitting an application to the Local Ethics Research Committee because neither the local R&D team nor the Human Fertilisation and Embryology Association (HFEA) knew who (if anyone) needed to give consent for the culture medium to be collected.

While it is disappointing that it was not possible to complete the above projects, I feel they are worth mentioning because I believe that if they had have been studied they would have helped to expand our understanding of the role of the endocannabinoid system in endometrial receptivity and would encourage any interested readers to pursue these projects further.

10 Discussion

10.1 Principal findings

As stated in the introduction (Chapter 1), the aim of this thesis was to explore the role of the endocannabinoid system in human uterine receptivity. This was explored using both *in-vitro* and *in-vivo* methods.

The most promising results came from my *in-vitro* studies, which ultimately found that increasing the expression of integrin β3 decreased the amount of NAPE-PLD mRNA, and increased the amount of FAAH mRNA in a dose dependant manner. Furthermore, while these changes were demonstrated in a receptive cell line, the same was not shown in a non-receptive cell line.

Sadly, my *in-vivo* studies were not as forthcoming with meaningful results, as already discussed in Chapter 9.

10.2 Strengths and weaknesses of the studies

All studies have strengths and weaknesses, and the results generated in my thesis are no different. An undeniable strength of both the *in-vivo* and *in-vitro* work is that every step of all of the experiments was carried out by the same person. This will obviously have removed any possibility of inter-observer error.

When considering the work conducted using cell lines, it is important to note that certain assumptions have to be made before interpreting the results. First, one has to assume that results generated by cell lines mirror those that would be generated if primary tissue culture had been used and are reflective of what happens *in-vivo*. The benefit of using cell lines is that they are uniform, and results should be reproducible whereas studies utilising primary tissue culture give rise to the inevitably that some degree of inter-sample variation will occur.

The second assumption to be made is that the amount of cDNA amplified and measured in the PCR equates to the amount of functioning enzyme present in the cell. The initial assumption to be made is that the amount of cDNA created is identical to the amount of mRNA in the cell. Even after accepting this assumption, we know that relative amounts of mRNA produced do not necessarily equate to relative amounts of protein produced. Furthermore, the amount of protein produced does not necessarily equate to the production of a functioning enzyme. Therefore, the results from the cell culture must be interpreted as a "suggestion" rather than a "proven fact". Potential methods for exploring further whether the amount of cDNA correlates to enzyme expression and activity are discussed in Section 10.5.

Unfortunately, it was not possible to explore the effects of increasing concentrations of integrin β3 on NAE concentrations due to problems with the ULPC-MS/MS. Also, due to the methods employed to increase integrin β3 activity, it can only be suggested that increasing concentrations of integrin β3 decreased NAPE-PLD expression while increasing FAAH transcript levels. Further investigation (e.g. removing the effects of nitric oxide) is necessary before one can conclusively report on the effects of integrin β3 on the expression of NAPE-PLD and FAAH. Again, this is discussed further in section 10.5.

One questions that clearly needs addressing at this point is whether or not analysis using a ULPC-MS/MS was the most appropriate choice of method for measuring samples used in clinical practice. The simple answer would be "yes, because UPLC-MS/MS is the only technique currently used for measuring NAEs". However, when considering the suitability of this piece of equipment in clinical samples the question needs to be considered at a deeper level. Vogeser et al. (2016) talk at length about the many limitations of the mass spectrometry measurement systems, including the inconsistency of signal nature (which can be affected by something as simple as a change in atmospheric pressure), effects of cleaning on the fine-tuning of instruments and the extent of training necessary for successful and safe operation to name but a few. In the context of clinical samples, samples are much harder to reproduce than commercially available samples and so the effects of equipment failure are more problematic. With all this in mind, it must be concluded that while UPLC-MS/MS is not the best choice of method of analysing NAEs, it is the most suitable as it is the only method currently available.

Although it seems that most promising results came from the *in-vitro* studies, there is also value in the *in-vivo* studies. It was to be expected that the results from *in-vivo* studies would generate less clear-cut results, because no two patients are identical. When working with cell cultures it is possible to maintain strictly reproducible conditions, whilst the same is impossible with human subjects. Furthermore, the causes of infertility in the patients were many and varied, which again potentially affected outcome. Finally, the quality of embryo replaced in each patient varies, which again could have influence the overall outcome. That said, despite the numerous limitation to the *in-vivo* studies, and the fact that this portion of the project was seriously hindered by numerous problems, it has provided some data to help further our understanding of the role of the ECS in human fertility.

10.3 Strengths and weaknesses in relation to other studies

While the studies presented in this thesis may have some weaknesses, the data generated have also helped support pre-existing work. For example, in Chapter 2 the effects of gal-3 on the enzymes and receptors of the ECS were considered and gal-3 was found to have no effect on the expression of the CB1 and CB2 receptors, whereas it had a statistically significant effect on the expression of NAPE-PLD. These findings were in keeping with past work showing that while the expression of CB1 and CB2 remained constant throughout the menstrual cycle, the expression of NAPE-PLD varied (Taylor, Abbas et al. 2010). While the study by Taylor et al. explored the expression of the ECS in endometrial biopsy samples, this is the first study to demonstrate that Ishikawa cells produce CB1 and CB2 cDNA. In addition, the results from Chapters 2-5 are the first to document that Ishikawa and HEC-1A cells both produce NAPE-PLD and FAAH transcripts.

As well as being the first study to identify the expression of components of the ECS in Ishikawa and HEC-1A cells, this is also the first study to consider the effects of integrin β3 on the ECS. When considering how this study supports others' work, the most exciting area is the differences identified between the receptive and non-receptive cell lines and the effects of integrin β3 on the amounts of NAPE-PLD and FAAH transcript produced.

As already discussed in the introduction (Chapter 1), Guo et al. (2005) demonstrated that higher expression of NAPE-PLD was noted in a "non-receptive" endometrium when compared to an endometrium that was "receptive" to implantation. Interestingly, they showed that NAPE-PLD expression was significantly lower at the implantation site when compared to the peri-implantation site. The natural conclusion from these data suggest that these differences are the result of integrin β3 activity in the endometrium.

While much of the data in this thesis supports others' work, not all of the previous work could be corroborated. It was not possible to replicate the promising results of El-Talatini et al. (2009) with regards to the change in plasma AEA concentrations and IVF/ICSI success. Furthermore, I was not able to show any correlation between the change in plasma AEA concentrations and any stage of early pregnancy investigations. As already discussed, while the patients in the study all had some similarities (see the "inclusion criteria"), their causes for infertility were many and varied, as were the quality of embryos replaced and did not correlate exactly with those used previously. This means that to corroborate and expand the previous studies, a close attention to the inclusion and exclusion criteria must be employed. Furthermore, the UPLC-MS/MS developed numerous faults and detected AEA less consistently than OEA and PEA, which resulted in less data with which to perform statistical analysis. This was a serious problem, and it can only be conjectural to suggest that if the equipment and its attendant resources had been available, then similar data to those produced previously would have been generated here.

The preliminary study examining plasma OEA and PEA concentrations was more promising, and during the pilot study it appeared that plasma OEA concentrations on the day of ET could be used to predict pregnancy outcome. Although these results were not confirmed in a larger study, the reason for this discrepancy may be because the full study was performed when the UPLC-MS/MS was not working reliably. Therefore, I have to be reticent in making any firm conclusion into the role of OEA in IVF/ICSI success.

It would be very easy to become disheartened about the lack of conclusive results generated by the IMPLANT study. However, it is not uncommon for large clinical studies and trials to fail to replicate the promising results from their pilot studies. One notable example of this is a study looking into the roles of vitamins C and E in reducing the risk

of pre-eclampsia, where a pilot study suggested that treating patients identified as "at high risk" of developing pre-eclampsia with vitamins C and E reduced their risk of developing pre-eclampsia (Zhang et al. 2002). This led to the development of a large, multi-centre trial to explore this effect further. Unfortunately, the full study was terminated prematurely as it was found that patients treated with vitamins C and E had an increased rate of adverse effects, including higher risk of acidified arterial umbilical cord (pH<7), an increased need for intravenous antihypertensive therapy, and a higher rate of gestational hypertension. The vitamin taking group also had a greater rate of stillbirth. The study authors concluded that concomitant supplementation of vitamins C and E did not prevent pre-eclampsia in women "at risk" and in fact may be detrimental in pregnancy (Poston, Briley et al. 2006). Considering examples helps to not only put the lack of data from my *in-vivo* study into context, but also remind the reader that the challenges I encountered are not uncommon in clinical studies.

10.4 Meaning of the study

The purpose behind this study was to explore the role of the ECS in endometrial receptivity, and I feel that both the *in-vitro* and *in-vivo* work has helped do this.

Regardless of whether the demonstrated changes in NAPE-PLD and FAAH transcript levels were the result of increasing integrin β3 or nitric oxide (or both), there were significant differences noted when comparing the effect of SNAP treatment on receptive (Ishikawa) and non-receptive cell (HEC-1A) lines. Of key note, treating Ishikawa cells with increasing concentrations of SNAP resulted in up to a 10x increase in relative expression of FAAH, and also resulted in a decrease in NAPE-PLD expression (both of which were statistically significant) whereas treating HEC-1A cells did not have any effect on the amount of NAPE-PLD or FAAH transcripts.

The preliminary work also explored effects of treatment of Ishikawa cells with increasing concentrations of gal-3 and the resulting concentrations of AEA, OEA and PEA, as well as the concept of the "entourage effect" exerted by OEA and PEA on NAPE-PLD and FAAH activity. While increasing concentrations of gal-3 did not cause any statistically significant difference in AEA concentrations (although the concentrations did not remain stable either, with some concentrations of gal-3 causing an increase in AEA whereas other

concentrations caused a decrease in AEA), there were statistically significant changes in both OEA and PEA concentrations, and this led to an exploration of any potential link between enzyme expression and NAE production. While it was not possible to show any link between FAAH and any of the NAEs, it was however demonstrated that a statistically significant correlation exists between the amount of NAPE-PLD transcripts and the resulting concentration of PEA.

Further data highlighting a potential role of PEA in endometrial receptivity came from the *in-vivo* studies. Here interesting data were generated with regards to the change in PEA concentrations during IVF/ICSI treatment and early pregnancy events, but there was no value in using changes in PEA concentrations and overall pregnancy outcome. My data suggest that immediately prior to embryo transfer there is a statistically significant rise in plasma PEA concentrations in patients whose initial tests suggest they are pregnant (a positive urinary hCG). However, the size of this increase in plasma PEA is also important as it was statistically significantly lower in patients who went on to have a successful pregnancy when compared to those who had a miscarriage or biochemical pregnancy.

To date, the majority of studies exploring the role of the ECS in early pregnancy events have focussed on AEA. However, based on the data presented in this thesis, there is now evidence to suggest that PEA may also play an important role and certainly warrants further investigation.

10.5 Unanswered questions and future research

While the studies presented in this thesis has increased our understanding of the role of endocannabinoids in endometrial receptivity, I feel it has also created more questions than answers, with the role of PEA in early pregnancy events needing extensive exploration. When considering my project, I feel there are some important questions that need answers before firm conclusions can be reached. In addition to the studies listed in Appendices 1 and 2, areas that I feel need further investigation include the following.

10.5.1 Can plasma concentrations of OEA on the day of embryo transfer be used to predict IVF/ICIS outcome?

The work in the pilot study suggested that a plasma OEA concentration of ≥2.58 pmol/mL on the day of ET could be used to predict IVF/ICSI success. Unfortunately, the UPLC-MS/MS was not working reliably enough to explore this fully within this project. There is thus a need to further explore the potential of OEA in this context.

10.5.2 With a working UPLC-MS/MS (that reliably detects AEA), can the studies of El-Talatini et al. be reproduced?

My findings were disparate to those of El Talatini et al., and because the UPLC-MS/MS was so unreliable I find it hard to fully discount their work. I, therefore, feel it is important to repeat the work of El Talatini et al., and if their work is reproducible to explore the IMPLANT study further. However, for such a study to work, I feel it would need to be a multi-centre study as a single unit did not have a high enough throughput of patients to generate results in a timely fashion.

10.5.3 Does an increased expression of integrin β3 just affect the amount of NAPE-PLD and FAAH mRNA, or does it also affect enzyme expression? Furthermore, does this translate to enzyme activity?

My work suggested that increased expression of integrin β3 affects the amount of transcripts for both NAPE-PLD and FAAH. An obvious next step would be to explore whether this change results in a change of enzyme expression, using immunohistochemistry, immunofluorescence, western blotting or all three techniques. Following on from this, cellular enzyme activity could be assessed using an enzyme assay (Fonseca et al. 2014).

10.5.4 Is it an increased expression of integrin β3 or an increased availability of nitric oxide, or both, that affects the expression of NAPE-PLD and FAAH in receptive endometrial cells?

My work suggested that increased expression of integrin β3 affects the amounts of both NAPE-PLD and FAAH. However, as already discussed, it is unclear if these findings are a result of integrin β3, nitric oxide or both and further investigation is necessary to reach a conclusion.

10.5.5 Does the expression of integrin β3 vary between the implantation zone and inter-implantation zone?

It is already known that the expression of NAPE-PLD and FAAH differ between the implantation and inter-implantation zones in animal studies. As I have shown that integrin β3 expression affects the amounts both of NAPE-PLD and FAAH in receptive cells (but not in non-receptive cells), a logical step would be to identify whether the expression of integrin β3 also varies between the implantation and inter-implantation zones in the human endometrium.

11 Appendices

Here I have included the protocols and patient information leaflets for the 2 projects that I gained ethical approval to start, but unfortunately was not able to generate any data from. I have also included publications and presentations arising from my thesis.

11.1 Appendix 1 - Comparing the expression of the endocannabinoid system at the implantation zone and inter-implantation zone

11.1.1 Protocol

OBJECTIVES

Primary Objective
The aim of this project is to define the expression of the endocannabinoid system at the implantation zone in the human endometrium.

Secondary Objectives
To identify whether there is a correlation between the expression of endocannabinoids in the plasma and at the implantation zone.

STUDY DESIGN

Summary of Study Design
This is a pilot study, where volunteers will be recruited from a cohort undergoing a surgical termination of pregnancy. They will be identified during their visit of Leicester Fertility Control Clinic, and at that time given written and verbal information (along with the contact details for the research team). On the day of their operation, those that express an interest will be consented to join the study.

Following signing a consent form, recruited patients will donated a blood sample immediately prior to their operation, and two endometrial will be taken during the operation. Their termination will then proceed as planned. No further patient input is necessary.

Primary and Secondary Endpoints/Outcome Measures
The endpoint will be when 10 complete measurements of the ECS components have been made.

Study Participants
Participants will be healthy females with proven fertility requesting surgical termination of pregnancy.

Inclusion Criteria

- Participant is willing and able to give informed consent for participation in the study.
- Currently pregnant with a single pregnancy which shows no signs of abnormality.
- Requesting a surgical termination.
- Willing to allow her Consultant to be notified of participation in the study.

Exclusion Criteria

- The participant may not enter the study if ANY of the following apply:
- Decision to keep the pregnancy.
- Surgical termination offered after a failed medical termination.
- History of cannabis use in the last 6 months.
- History of hormonal contraception use in the last 2 months.
- Any significant disease or disorder which, in the opinion of the Investigator, may either put the participants at risk because of participation in the study, or may influence the result of the study, or the participant's ability to participate in the study.
- Participants who have participated in another research study involving an investigational product in the previous 12 weeks.

Study Procedures

Patients enrolled in the study will donate both a blood and two endometrial biopsies. The blood sample will be obtained before the patients' operation begins, and the endometrial biopsies will be taken during their operation.

Plasma samples will be obtained after centrifugation of whole blood samples (mixed with EDTA), collected using standard venepuncture techniques.

All samples will then be analysed as described by Marczylo et al. (69). Briefly, tissue spiked with deuterated AEA internal standard will be introduced unto preconditioned cartridges, washed with aqueous methanol and then eluted in acetonitrile. The eluate will be dried under constant stream of nitrogen, reconstituted in 80µl of acetonitrile and transferred to an HPLC sample vial for UPLC-MS/MS analysis.

Screening and Eligibility Assessment

Patients will be identified as those listed for a surgical termination of pregnancy at University Hospital of Leicester. They will be approached by a member of the research team after they have discussed the termination with their consultant, and given written and verbal information about the study. This information will explain the exact nature of the study; the implications and constraints of the protocol and any risks involved in taking part. It will be clearly stated that the participant is free to withdraw from the study at any time for any reason without prejudice to future care, and with no obligation to give the reason for withdrawal.

The participant will then meet with the Investigator on the day of their operation to confirm they meet the inclusion criteria (and do not meet any of the exclusion criteria).

At this point, the patient information leaflet will be discussed, with ample opportunity to question the Investigator.

Informed Consent

The participant will personally sign and date the latest approved version of the informed consent form before any study specific procedures are performed.

Written Informed Consent will then be obtained by means of participant dated signature and dated signature of the person who presented and obtained the informed consent. The person who obtained the consent will be suitably qualified and experienced, and have been authorised to do so by the Chief/Principal Investigator. A copy of the signed Informed Consent will be given to the participants. The original signed form will be retained at the study site.

Definition of End of Study

The end point of the study will be when 10 complete measurements have been obtained.

Discontinuation/ Withdrawal of Participants from Study Treatment

Participants have the right to withdraw study within four weeks of their operation. In addition, the investigator may discontinue a participant from the study at any time if the investigator considers it necessary for any reason including:

- Pregnancy
- Ineligibility (either arising during the study or retrospective having been overlooked at screening)
- Consent withdrawn

At the time of signing the consent form, participants will be provided with the contact details for the Principle Investigator, and advised to contact the PI if at any point they wish to withdraw from the study.

Withdrawal from the study will result in exclusion of the data for that participant from analysis. The future of tissue samples already donated, along with any demographic data help will be discussed at the time of withdrawal from the study, and will be destroyed if requested by the patient. The reason for withdrawal will be recorded in the case report form.

If the participant is withdrawn due to an adverse event, the investigator will arrange for follow-up visits or telephone calls until the adverse event has resolved or stabilised.

Source Data
All documents will be stored safely under confidential conditions. On all study-specific documents, other than the signed consent, the participant will be referred to by the study participant number/code, not by name.

11.1.2 Patient Information Leaflet

Title of Study: ***The expression of the endocannabinoid system at the Implantation Zone***
Principal Investigator: Dr Sarah Melford

Introduction

We invite you to take part in a research study investigating the role of naturally occurring chemicals called endocannabinoids in the human body. Our team are interested in how these chemicals affect pregnancies, and in addition to the further knowledge gained, the data collected from this study will be used by Dr Melford to gain a PhD. Before you decide we would like you to understand why the research is being done and what it would involve for you. One of our team will go through the information sheet with you and answer any questions you have. We believe that this should take about 30 minutes.

Talk to others about the study if you wish and ask us if there is anything that is not clear.

What is the purpose of the study?
Endocannabinoids are naturally occurring substances in the body that act through the same pathways that the active ingredients found in cannabis do. Studies have shown that these substances are vital for pregnancy to succeed. Low levels are required for successful pregnancy but much higher levels are associated with miscarriages. The precise way in which these substances influence human reproduction is not known and this is because very little research has been undertaken in women. The main reason for this has been the inability of researchers to accurately measure these substances in blood and tissues. 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 support 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. In this study, we are looking at the role endocannabinoids play at the "Implantation Zone"; a specific point in the lining of the womb (the endometrium) that has the properties needed for embryo to implant successfully. Research in animals has already shown the endometrium expresses different levels of components of the endocannabinoid systems at the implantation zone when compared with the rest of the endometrium. What we want to investigate if the same thing happens in humans.

We believe that by learning more about the role the endocannabinoid system plays in embryo implantation we will be able to help improve the outcome of pregnancies especially in women who undergo repeated miscarriages. However, before we can investigate the "abnormal", it is important we establish the "normal" role of endocannabinoids.

Why have I been chosen?
You have been chosen because you have requested a surgical termination of pregnancy. Prior to your inclusion in the study, a member of the research team will ask you a few to

ensure you are suitable to participate. We want to know the "normal" role of endocannabinoids and so unfortunately cannot include anyone who has a chronic medical condition, or has used cannabis in the last 6 weeks. If you would rather you were not asked such questions, simply inform the research team that you do not want to take part in the study.

Do I have to take part?
No. We will explain the study and go through this information sheet with you, but is up to you to decide whether to join the study or not. If you agree to take part, we will then ask you to sign a consent form. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive.

What will happen to me if I take part?
Immediately prior to your operation, one of the research team will take a blood sample. Then, during your operation, a hysteroscope (a thin metal tube with a camera attached to the end) will be inserted through your cervix into your womb and used to visualise the implantation zone. A biopsy of the implantation zone, as well as one other area of endometrium will be taken. Your termination will then continue as already explained by your surgeon.

What will I have to do?
If you would like to take part in the study, then we will go through a form (consent form) which you will need to sign to allow you to take part in the study and to allow us to take the blood sample and endometrial biopsies as described above.

The blood sample and endometrial biopsies will be taken as described above, and then your termination will proceed as planned. The samples taken will be analysed for endocannabinoids, and then destroyed.

Participation in the study will not change your recovery after the procedure, and you will be allowed home as soon as you feel well enough to do so. There will be no further requirements from you after this.

Will I receive payment for the tissue that I donate for this research study?
No, you will not receive any payment for the tissue because the samples are considered to be a gift - neither yourself nor your relatives will benefit from any inventions that result from the use of the tissue.

What are the possible disadvantages and risks of taking part?
Taking part in this study will mean that you need an extra blood test (in addition to any tests taken in preparation for your operation). It also means you will be under anaesthetic for an additional 20 minutes. However we do not anticipate this will cause any significant side effects.

Taking the biopsies has not been shown to cause any risks that have not already associated with a surgical termination of pregnancy (which will have been discussed with you by your surgeon).

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

You may experience some discomfort of having the blood test.
You may experience some mild period pains following the endometrial biopsy (although you are also likely to experience these following your operation).

What are the possible benefits of taking part?
While the results generated from this research will not benefit you directly, by increasing our knowledge about endocannabinoids, we hope they will eventually help patients who struggle to conceive or suffer early pregnancy loss.

What will happen if I change my mind about being involved in the study?
You are free to withdraw your consent to participate in the study at any time. The decision is yours as to whether you would like the tissue samples, the demographic data held, or both to be destroyed.

If within 4 weeks of your operation you decide you want to withdraw from the study, please contact Dr Sarah Melford (details below) who will discuss with you what you would like to happen to the data already collected and any tissue samples donated.

What if there is a problem?
If you have a concern about any aspect of this study, you should ask to speak to the researchers (through the number given at the end of this information sheet) who will do their best to answer your questions. If you remain unhappy and wish to complain formally, you can do this through the University of Hospitals of Leicester complaints department [following NHS Complaints Procedure or Private Institutional arrangements]. Details can be obtained through the University of Hospitals of Leicester's Patient Information and Liaison Services who can be contacted on:

Free phone line: 08081 788337 (Opening times are Monday to Friday 10am to 4pm)
Email: pils@uhl-tr.nhs.uk

Patient Information and Liaison Service
The Firs
C/O Glenfield Hospital
Groby Road
Leicester LE3 9QP
If you are harmed by taking part in this research project there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

Will my taking part in this study be kept confidential?
Yes. We will follow ethical and legal practice and all information about you will be handled in confidence.

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.

In addition, sections of any of your medical notes may be looked at by responsible individuals from the study team, the sponsor, NHS Trust or from regulatory authorities for audit and monitoring purposes.

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, will have your name and address removed so that you cannot be recognised from it.

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

Who is organising and funding the research?
This research project is both organised and funded by the Endocannabinoid Research Group, which is attached to the University of Leicester.

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 (REC) before it goes ahead. This particular study has been reviewed by the Northampton REC and given a favourable opinion. Approval does not guarantee that you will not come to any harm if you take part. However, approval means that the committee is satisfied that your rights will be respected, that any risks have been reduced to a minimum and balanced against possible benefits and that you have been given sufficient information on which to make an informed decision.

For more information, whom do I contact?

Dr Sarah Melford, MBChB
Reproductive Sciences Section
Department of Cancer Studies and Molecular Medicine
University of Leicester
Robert Kilpatrick Clinical Sciences Building
Leicester Royal Infirmary
Leicester LE2 7LX

Email: sm662@le.ac.uk
Telephone: 07817715574.

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

Thank you for reading this.

11.2 Appendix 2 - Comparing the concentration of NAEs in plasma and endometrial samples

11.2.1 Protocol

OBJECTIVES

The aim of this project is to clarify whether or not levels components of the Endocannabinoid system in blood correlate with the levels found within the womb.

STUDY DESIGN

Summary of Study Design

This is a pilot study, where volunteers will be recruited from a cohort undergoing elective gynaecological surgery. They will be identified from the theatre waiting list, and sent an invitation letter and patient information leaflet in the post at least 2 weeks before their operation. On the day of their operation, they will be verbally invited to join the study, and the information leaflet discussed, if they are interested.

Following signing a consent form, recruited patients will donated a blood sample immediately prior to their operation, and endometrial samples (aspirate, lavage and biopsy) will be taken during the operation. No further patient input is necessary.

Primary and Secondary Endpoints/Outcome Measures

The endpoint will be when 20 complete measurements of the ECS components have been made

Study Participants
Participants will be healthy females with proven fertility undergoing elective gynaecological surgery (under general anaesthetic).

Inclusion Criteria

- Participant is willing and able to give informed consent for participation in the study.
- Female, aged 25-45
- Healthy patient with proven fertility (successfully carrying at least one baby to term).
- Scheduled for elective gynaecological surgery (under general anaesthetic)
- Willing to allow her Consultant to be notified of participation in the study.

Exclusion Criteria
The participant may not enter the study if ANY of the following apply:

- Aged less than 25 or more than 45
- History of hormonal contraception use in the past 2 months.
- History of cannabis use in the past 6 months.

- Any other significant disease or disorder which, in the opinion of the Investigator, may either put the participants at risk because of participation in the study, or may influence the result of the study, or the participant's ability to participate in the study.
- Participants who have participated in another research study involving an investigational product in the previous 12 weeks.
- Unable or unwilling to give consent

Study Procedures

Patients enrolled in the study will donate both a blood and endometrial sample. The blood sample will be obtained before the patients' operation begins, and the endometrial sample will be taken during their operation.

Plasma samples will be obtained after centrifugation of whole blood samples (mixed with EDTA), collected using standard venepuncture techniques.

Three main methods of assessing the uterine environment have been identified; aspiration of endometrial secretions, uterine lavage and endometrial biopsy. The different methods have been shown to collect different components expressed by the endometrium (Hannan et al. 2012) and while biopsy looks at the endometrium itself, aspiration and lavage give information about uterine secretions (and thus the environment the embryo first encounters). For this reason, the plan is to investigate the uterine environment using a combination of uterine aspirate and lavage and endometrial biopsy.

Uterine aspirate: This will be performed as described by Hannan et al. (2012). Briefly, an embryo transfer catheter will be inserted into the uterine cavity, and an aspirate (5-10 µL) will be collected. The outside of the catheter containing the sample will be wiped to remove any cervical mucus and then the tip will be cut off, dropped into an Eppendorf tube, and snap frozen.

Uterine lavage: This will be performed as described by Li et al.(1993). Briefly, a size 8 (paediatric) Foleys catheter will be inserted into the uterine cavity and the balloon of the catheter inflated with 1 ml of normal saline. Following this, 2 ml of normal saline solution will be gradually flushed into the uterine cavity via the opening connected to the inner lumen; followed by gentle suction via the same opening to recover the fluid. The flushing will be carried out slowly (over 15 s) and repeated five times, each time using a fresh sample of 2 ml of physiological saline solution (and so a total of 10ml of saline solution will be used).

Endometrial biopsy: An endometrial curette will be taken using a standard curetting technique and immediately snap frozen.

All samples will then be analysed as described by Marczylo et al. (69). Briefly, tissue spiked with deuterated AEA internal standard will be introduced unto preconditioned cartridges, washed with aqueous methanol and then eluted in acetonitrile. The eluate will be dried under constant stream of nitrogen, reconstituted in 80µl of acetonitrile and transferred to an HPLC sample vial for UPLC-MS/MS analysis.

Screening and Eligibility Assessment

Patients will be identified as those listed for elective gynaecological surgery (under general anaesthetic) who have proven fertility.

Written versions of the participant information (along with a letter of information) will be posted to the participants at least two weeks before their operation, detailing the exact nature of the study; the implications and constraints of the protocol; the known side effects and any risks involved in taking part. It will be clearly stated that the participant is free to withdraw from the study at any time for any reason without prejudice to future care, and with no obligation to give the reason for withdrawal.

The participant will then meet with the Investigator on the day of their operation to confirm they meet the inclusion criteria (and do not meet any of the exclusion criteria). At this point, the patient information leaflet will be discussed, with ample opportunity to question the Investigator.

Informed Consent
The participant will personally sign and date the latest approved version of the informed consent form before any study specific procedures are performed.

Written Informed Consent will then be obtained by means of participant dated signature and dated signature of the person who presented and obtained the informed consent. The person who obtained the consent will be suitably qualified and experienced, and have been authorised to do so by the Chief/Principal Investigator. A copy of the signed Informed Consent will be given to the participants. The original signed form will be retained at the study site.

Definition of End of Study
The end point of the study will be when 20 complete measurements have been obtained.

Discontinuation/ Withdrawal of Participants from Study Treatment
Each participant has the right to withdraw study at any time. In addition, the investigator may discontinue a participant from the study at any time if the investigator considers it necessary for any reason including:

- Pregnancy
- Ineligibility (either arising during the study or retrospective having been overlooked at screening)
- Consent withdrawn

At the time of signing the consent form, participants will be provided with the contact details for the Principle Investigator, and advised to contact the PI if at any point they wish to withdraw from the study.

Withdrawal from the study will result in exclusion of the data for that participant from analysis. The future of tissue samples already donated, along with any demographic data help will be discussed at the time of withdrawal from the study, and will be destroyed if requested by the patient. The reason for withdrawal will be recorded in the case report form.

If the participant is withdrawn due to an adverse event, the investigator will arrange for follow-up visits or telephone calls until the adverse event has resolved or stabilised.

11.2.2 Patient Information Leaflet

University Hospitals of Leicester NHS
NHS Trust

Investigating the link between plasma and endometrial endocannabinoid levels
Principal Investigator: Dr Sarah Melford

Introduction

We invite you to take part in a research study investigating the role of naturally occurring chemicals called endocannabinoids in the human body. Before you decide we would like you to understand why the research is being done and what it would involve for you. One of our team will go through the information sheet with you and answer any questions you have. We believe that this should take about 30 minutes.

Talk to others about the study if you wish and ask us if there is anything that is not clear.

1. What is the purpose of the study?

Endocannabinoids are naturally occurring substances in the body that have been shown to be vital for pregnancy to succeed. Low levels are required for successful pregnancy, but much higher levels are associated with miscarriages. The precise way in which these substances influence human reproduction is not known and this is because very little research has been undertaken in women. The main reason for this has been the inability of researchers to accurately measure these substances in blood and tissues. We very recently developed a means of measuring these endocannabinoids and successfully used the method in a small group of pregnant women and non-pregnant women. Our results were very encouraging and support 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 answering these questions, we will improve the outcome of pregnancies especially in women who undergo repeated miscarriages. However, before we can investigate the "abnormal", it is important we establish the "normal" role of endocannabinoids.

2. Why have I been chosen?

You have been chosen because you have proven fertility (that is to say, you have successfully carried a baby to term), and are planning to undergo elective surgery.

3. Do I have to take part?

No. We will explain the study and go through this information sheet with you, but is up to you to decide whether to join the study or not. If you agree to take part, we will then ask you to sign a consent form. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive.

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

Immediately prior to your operation, one of the research team will take a blood sample. Then, during your operation (while you are under a general anaesthetic), the same member of the research team will take 3 different samples from the lining of your womb:

1) Endometrial Aspirate
A small flexible tube will be passed through your cervix into your womb and gentle suction applied to collect any fluid present in the cavity.

2) Endometrial Lavage
A small volume (10ml) of warm saline solution will be flushed into your womb, again through a flexible tube passed through your cervix into your womb) and the flushed liquid collected in the same way as the endometrial aspirate. The small flexible tube will then be removed.

3) Endometrial Biopsy
A endometrial curette will be passed through your cervix into your womb and a biopsy (measuring a few millimetres) will be collected.

In addition to the procedures listed above, your operation will continue as already explained by your surgeon.

5. What will I have to do?

If you would like to take part in the study, then we will go through a form (consent form) which you will need to sign to allow you to take part in the study and to allow us to take the blood and endometrial samples as described above.

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

No, you will not receive any payment for the tissue because the samples are considered to be a gift - neither yourself nor your relatives will benefit from any inventions that result from the use of the tissue.

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

Taking part in this study will mean that you need an extra blood test (in addition to any tests taken in preparation for your operation).

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

You may experience some discomfort of having the blood test.

9. What are the possible benefits of taking part?

While the results generated from this research will not benefit you directly, by increasing our knowledge about endocannabinoids, we hope they will eventually help patients who struggle to conceive or suffer early pregnancy loss.

10. What will happen if I change my mind about being involved in the study?

You are free to withdraw your consent to participate in the study at any time. The decision is yours as to whether you would like either the tissue samples, the demographic data held, or both destroyed.

If at any point you decide you want to withdraw from the study, please contact Dr Sarah Melford (details below) who will discuss with you what you what you would like to happen to the data already collected and any tissue samples donated.

11. What happens when the research study stops?

This will not affect you, as you are not receiving any medication for this study.

12. What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers (through the number given at the end of this information sheet) who will do their best to answer your questions. If you remain unhappy and wish to complain formally, you can do this through the University of Hospitals of Leicester complaints department [following NHS Complaints Procedure or Private Institutional arrangements]. Details can be obtained through the University of Hospitals of Leicester Patient Information and Liaison Services who can be contacted on:

Free phone line: 08081 788337 (Opening times are Monday to Friday 10am to 4pm)
Email: pils@uhl-tr.nhs.uk

Patient Information and Liaison Service
The Firs
C/O Glenfield Hospital
Groby Road
Leicester LE3 9QP

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?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence.

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, will have your name and address removed so that you cannot be recognised from it.

Your GP will be informed of your involvement in the study.

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 a year 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.

In addition, sections of any of your medical notes may be looked at by responsible individuals from the study team, the sponsor, NHS Trust or from regulatory authorities for audit and monitoring purposes.

15. Who is organising and funding the research?

This research project is both organised and funded by the Endocannabinoid Research Group, which is attached to the University of Leicester.

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. This study has been reviewed by the Northampton Research Ethics Committee. 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, whom do I contact?

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

Appendix 3 – Publications and presentations arising from my thesis

11.2.3 Publications

Of mice and (wo)men: factors influencing successful implantation including endocannabinoids.
Melford SE[1], Taylor AH, Konje JC. Human Reproduction Update. 2014 May-Jun;20(3):415-28.

11.2.4 Formal presentations

The role of the endocannabinoid system an embryo implantation - Invited plenary speaker at "Fertility 2015" (Birmingham, January 2015).

Abstract: A question that keeps scientists, clinicians, embryologists and infertile patients awake at night is, "What is it that controls whether an embryo implants into the endometrium, or prevents it?" Despite extensive research, this question seems to be further from an answer than it was a decade ago; this is probably because the mechanisms involved in embryo implantation are multi-factorial. A historical treatise on some of these mechanisms will be presented before the endocannabinoid system is introduced. There is ever increasing evidence that the endocannabinoid system may play a critical part in both rodent and human implantation and both systems will be presented, thus introducing the audience to the history of the endocannabinoid system, before detailing the role it plays in embryo implantation, both in the laboratory and in clinical practice. After discussing what is already known about the endocannabinoid system and embryo implantation, this lecture hopes to 'whet the appetite' of the inquisitive researcher by highlighting a possible future for this promising system in answering the question posed above. The evidence presented may stimulate further research into the mechanisms involved in the precise role of the endometrium in accepting the blastocyst, allowing implantation and maintenance of early pregnancy.

11.2.5 Poster presentations

The effects of Integrin B3 on endocannabinoid system of a receptive endometrium - Presented at ESHRE 2016 (Helsinki, July 2016)

Study question

What effects does integrin β3 have on the enzymes of the endocannabinoid system (NAPE-PLD and FAAH) and are any differences linked to endometrial receptivity.

Summary answer

Receptive endometrium cells showed increased FAAH and decreased NAPE-PLD expression in response to an increase in Integrin β3 activity. Non-receptive endometrium cells showed no change.

What is known already

Endogenous cannabinoids (endocannabinoids) concentrations need to be carefully titrated to ensure uterine receptivity, and this "Anandamide tone" is vital for successful embryo implantation. Anandamide tone is achieved by a careful balance between the activity NAPE-PLD and FAAH. Furthermore, the expression ratio of NAPE-PLD:FAAH differs at the implantation zone and the interimplation zone.

Integrin B3 expression coincides with the window of implantation, and aberrant expression of integrin B3 has been linked to infertility. Integrin β3 expression has also been linked to pinopode expression, suggesting that integrin B3 might be involved in producing the preferred site of embryo-endometrial interaction.

Study design, size, duration

Ishikawa cells were used as a receptive endometrium model. HEC1A cells were used as a non-receptive endometrium model. S-nitroso-N-acetylpenicillamine (SNAP) was used to up-regulate the expression of the integrin β3 (in a dose dependent manner). 5 increasing concentrations of SNAP were used (in addition to a control of 0 μM), and each concentration was added to 6 wells of cells.

Materials, settings, methods

Ishikawa and HEC1A cells (5×10^5) were seeded into a 6-well plate and cultured in 2ml of culture medium (37°C, 5% CO_2) for 24 hours. Following this, culture medium was replaced with medium augmented with S-nitroso-N-acetylpenicillamine (SNAP, 0-2000 μM) and the cells were cultured for a further 4 8hours. Following this, the total cellular RNA extracted and quantified (Taqman PCR) and the relative levels of NAPE-PLD and FAAH transcripts measured.

Main results, role of chance

The work with Ishikawa cells (receptive endometrium) showed there was a clear inverse and statistically significant correlation between the concentration of SNAP and the expression of NAPE-PLD when compared against the control ($p<0.01$ for 500 and 1000μM, $p<0.05$ for 2000μM; Tukey's multiple comparison test). It also showed positive correlation between the concentration of SNAP and the relative expression of FAAH expression. Again this was statistically significant when compared against the highest concentration (2000μM) of SNAP ($p<0.001$ for 0 and 50μM, $p<0.01$ for 100 and 500μM SNAP; Tukey's multiple comparison test).

In contrast, HEC1A cells (non-receptive endometrium) treated with increasing concentrations of SNAP did not show any significant variation in their expression of NAPE-PLD or FAAH (One way ANOVA; NAPE-PLD $p= 0.1862$, FAAH $p=0.2170$).

Furthermore, the relative expression of NAPE-PLD in Ishikawa cells was twice that of HEC1A cells, and the relative expression of FAAH in Ishikawa cells was 100 times that of HEC1A cells.

Limitations, reason for caution

The small sample size (n=6 for each concentration) means further work is necessary before decisive conclusions can be met. However, the strong statistical significance is promising. Also, we have only shown relative expression of NAPE-PLD rather than exploring their functionality.

Wider implications of finings

This study supports existing literature, as there is already work looking individually at integrin β3 and the endocannabinoid system and their role in endometrial receptivity. However, this is the first time a link has been made between the two groups.

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