The Role of Anandamide in Human Folliculogenesis, Implantation and Early Pregnancy

A thesis submitted for the degree of

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by Mona R.T. El-Talatini, MBBCH, MRCOG

Reproductive Sciences Section, Department of Cancer Studies and Molecular Medicine, University of Leicester, Leicester, LE2 7LX

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Dedication

This thesis is dedicated to the two most important people in my life:

my parents (Enam Jarada and Ramadan El-Talatini) who supported and encouraged me throughout my life.

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The Role of Anandamide in Human Folliculogenesis, Implantation and Early Pregnancy

Abstract

Anandamide (AEA) is an endogenous cannabinoid that in the mouse has been shown to be important for gametogenesis, embryo development and successful implantation. Such evidence is, however, lacking in humans. The aim of this thesis was therefore to investigate the role of AEA in human reproduction. Plasma AEA levels were measured by ultra-performance liquid chromatography and mass-spectrometry throughout the menstrual cycle in healthy women both cross-sectionally and longitudinally and its relationship to sex steroid hormones and gonadotrophins that regulate the menstrual cycle were determined. Additionally, the endocannabinoid system [the cannabinoid receptors (CB1 and CB2), the enzymes fatty acid amide hydrolase (FAAH) which degrades AEA and *N*-acyl-phosphatidyl ethanolamine selective phospholipase (NAPE-PLD) which synthesizes AEA] was localised in the ovary by immunohistochemistry. Plasma and follicular fluid (FF) AEA levels were also determined in women undergoing *in-vitro* fertilisation and embryo transfer (IVF-ET) and correlations between FF AEA and follicle size and oocyte maturity were undertaken. Plasma AEA levels were then measured in these women on the day of oocyte retrieval (OR), embryo transfer (ET) and at pregnancy test (PT) and comparisons made between pregnant and non-pregnant women and between those with viable pregnancies and those who had miscarried at 6 weeks. The relationship between plasma AEA and sex steroids in the stimulated cycles was also examined. AEA was found to be hormonally regulated, mainly by gonadotrophins and estradiol, and its highest levels were at ovulation and low at the implantation window in natural and stimulated cycles. The endocannabinoid system was localised in human ovary, FF AEA was associated with folliculogenesis, ovulation and oocyte maturity. ROC analysis indicated that a FF AEA concentration of 1.09 nM discriminated between mature and immature oocytes with area under the curve of 0.77 (P=0.001) with a sensitivity of 72.2% and a specificity of 77.1%. In successful pregnancies a significant decline in plasma AEA levels from ovulation to the day of ET then a significant rise from the day of ET to the day of PT appeared to be important. Plasma AEA levels were high at 4 and 5 weeks gestation and then significantly declined at 6 weeks gestation. These findings therefore suggest that AEA plays a role in human folliculogenesis, ovulation, and oocyte maturity and low levels are important for implantation and early pregnancy success. These data may show the potential for using AEA as a biomarker test for oocyte assessment and to improve pregnancy outcomes in stimulated IVF/ICSI cycles.

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Declaration

This study was approved by Leicestershire and Rutland Local Research Ethics Committee. The ethical approval number is Q6 /Q 2501/49.

I declare that the work presented in this thesis is my own work and has not been submitted for any other degree.

Signed **Date**

Abbreviations

2-AG	2-Arachidonylglycerol
ACN	Acetonitrile
AEA	Anandamide
AMH	Anti-Mullerian hormone
BMI	Body mass index
BMP-15	Bone morphogeneic protein
CB	Cannabinoid receptor
COX	Cyclooxygenase enzyme
d_8–AEA	Octa-deuterated anandamide
DAB	3, 3-diaminobenzamidine
DI	Donor insemination
E2	Estradiol
EDTA	Ethylene diamine tetra-acetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immuno-sorbent assay
ET	Embryo transfer
FAAH	Fatty acid amide hydrolase
FSH	Follicle stimulating hormone
GDF-9	Growth differentiation factor-9
GH	Growth hormone
GIFT	Gamete intrafallopian transfer
GnRH	Gonadotrophin releasing hormone
hCG	Human chorionic gonadotrophin
HFEA	Human Fertilisation and Embryology Authority

hMG	Human menopausal gonadotrophins
HPLC	High performance liquid spectrometry
ICSI	Intracytoplasmic sperm injection
IGF	Insulin like growth factor
IGFBP	Insulin like growth factor binding protein
IS	Internal standard
IUI	Intrauterine insemination
IVF-ET	In-vitro fertilisation and embryo transfer
IVM	In-vitro maturation
LH	Luteinising hormone
LIF	Leukaemia inhibitory factor
LMP	Last menstrual period
mRNA	Messenger ribonucleic acid
NAPE-PLD	N-acyl-phosphatidyl ethanolamine selective phospholipase D
NAT	N-acyltransferase
nM	Nano-molar
MRM	Multiple reaction monitoring
OD	Oocyte donation
OR	Oocyte retrieval
P4	Progesterone
PCOD	Polycystic ovarian disease
PEA	Palmitoylethanolamide
PGE2	Prostaglandin E2
PID	Pelvic inflammatory disease
PMW	Post-menopausal women

PN	Pronuclear
PT	Pregnancy test
r-FSH	Recombinant follicle stimulating hormone
ROC	Receiver-operator characteristic
SD	Standard deviation
SEM	Standard error of the mean
SO	Superovulation
T	Testosterone
TBS	Tris-buffered saline
TGF-β	Transforming growth factor-β
TNF	Tumour necrosis factor
ULPC-MS/MS	Ultra performance liquid chromatography-tandem mass spectrometry
USS	Ultrasound Scan
VEGF	Vascular endothelial growth factor
Δ^9- THC	Δ^9- Tetrahydrocannabinol

Publications arising from this thesis

Papers

1. Lam PC, Marczylo TH, **El-Talatini MR,** Finney M, Nallendran V, Taylor AH, Konje JC. Ultra performance liquid chromatography tandem mass spectrometry method for the measurement of anandamide in human plasma. Analytical Biochemistry 2008; 380: 195-201.
2. **El-Talatini MR,** Taylor AH, Konje JC. The relationship between plasma levels of endocannabinoid, anandamide, sex steroids and gonadotrophins during the menstrual cycle. Fertility and Sterility diol:10.1016/j.fertnstert.2008.12.033.
3. **El-Talatini MR,** Taylor AH, Elson JR, Brown L, Davidson AC and Konje JC. Localisation and function of the endocannabinoid system in the human ovary. PloS One 2009; 4(2): e4579.
4. **El-Talatini MR**, Taylor AH, and Konje JC. Fluctuation in anandamide levels from ovulation to early pregnancy in IVF-ET women and its hormonal regulation. Human Reproduction 2009; 24: 1989-1998.

Abstracts

1. **El-Talatini, MR**, Taylor AH, Elson JR and Konje JC. The endocannabinoid, anandamide, is involved in human folliculogenesis and oocyte maturation during IVF Treatment. Book of Abstracts, the 2nd SGI International Summit in Reproductive Medicine, Valencia, Spain, November 2007, pp59.
2. **El-Talatini MR**, Taylor AH, Spurling SM and Konje JC. Variation of plasma anandamide levels and fatty acid amide hydrolase expression throughout the menstrual

cycle. 55th Annual Meeting of SGI, March 26-29, 2008 - San Diego, USA Reproductive Science 2008; 15(Suppl. 2): 167A-167A.

3. Lam PMW, Marczylo TH, **El-Talatini MR**, Finney M, Nallendran V, Taylor AH and Konje JC. The description of a fast, sensitive and accurate UPLC-MS/MS method for the measurement of anandamide. 55th Annual Meeting of SGI, March 26-29, 2008 - San Diego, USA. Reproductive Sciences 2008; 15(Suppl. 2): 169A-169A.
4. **El-Talatini MR,** Taylor AH and Konje JC. Longitudinal study showing the relationship between anandamide and sex steroids and gonadotrophin hormones in women. Oral presentation at the 18th Annual Symposium of the International Cannabinoid Research Society, June 25-29, 2008 - Aviemore, Scotland, UK, Abstract Book pp 45.
5. **El-Talatini MR,** Taylor AH, Elson JR and. Konje JC. The endocannabinoid ystem in the human ovary. Blair Bell Meeting December 15-16, 2008 - Manchester, UK.
6. **El-Talatini MR**, Taylor AH, Elson JR and Konje JC. Changes in the plasma anandamide levels from ovulation to early pregnancy in women undergoing IVF-ET. Blair Bell Meeting, December 15-16, 2008 - Manchester, UK.
7. **El-Talatini MR**, Taylor AH, and Konje JC. Plasma AEA in Caucasians and non-Caucasians in natural cycles and IVF stimulated cycles. Blair Bell Meeting, December 15-16, 2008 - Manchester, UK.
8. **El-Talatini MR**, Taylor AH, Girish T, Spurling SM, Abbas MS and Konje JC. Localisation of the endocannabinoid system in the human ovary and the role of anandamide in ovarian follicle and oocyte maturation. SGI 56th Annual Meeting, March 17-21, 2009 - Glasgow, UK.
9. **El-Talatini MR,** Taylor AH, S Rana, and J C Konje. A longitudinal study of plasma anandamide and sex steroid hormone levels in pregnant and non-pregnant women

undergoing IVF-ET treatment. SGI 56th Annual Meeting, March 17-21, 2009 - Glasgow, UK.

10. Taylor AH, **El-Talatini MR**, Abbas MS, Spurling SM, Bell SC, Taylor DJ and Konje JC. Localisation of the endocannabinoid system in the human uterus during the menstrual cycle. SGI 56th Annual Meeting, March 17-21, 2009 - Glasgow, UK.

CHAPTER 1

INTRODUCTION

1.1. Infertility

1.1.1. Infertility incidence, definition and types

Infertility affects one in seven couples in the UK (Templeton et al., 1991; Gunnell and Ewings, 1994) and can cause considerable psychological distress (Wright et al., 1989; Whiteforda and Gonzalez, 1995). **Table 1.1** shows the number of couples experiencing infertility in the UK by region and HFEA approved treatment centres (HFEA, 2007). The United Nations stated that reproductive health is "a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity in all matters relating to the reproductive system and to its functions and processes" (United Nations, 1996). Infertility should therefore be considered a disease process worthy of investigation and treatment (Royal College of Obstetrician and Gynaecologists, 1992).

Couples are considered to be infertile if they fail to conceive after 12 to 24 months of unprotected intercourse (Evers, 2002; Gnoth et al., 2005). Data from population-based studies suggest that approximately 10-15% of couples in the Western World experience difficulties in conceiving (Hull et al., 1985; Evers, 2002; Oakley et al., 2008) and half (or approximately 8%) of these will be sub-fertile rather than infertile and will eventually conceive spontaneously or with the help of simple advice; whereas the remaining half (8%) will remain infertile and will require sophisticated treatment from fertility clinics. Of the 8% infertile group about one half (4%) comprise couples with **primary infertility** (couples who have never conceived) while the other half (4%) have **secondary infertility** (couples who have had a pregnancy, although not necessarily a successful one) **(Table 1.2)** (Templeton et al., 2000; Bhattacharya, 2007).

Table 1.1. Couples experiencing infertility and HFEA approved treatment centres by region in the UK. (around 1 in 7 couples are likely to experience problems conceiving).

	TOTAL UK population aged 16-45	Estimated people experiencing fertility problems	Estimated couples with difficulty conceiving 1 in 7	HFEA licensed treatment clinics (DI and IVF) 2006	Treatment clinics (DI, IVF, GIFT, IUI) 2008
East Midlands	1,693,1005	250,000	125,000	6	6
East of England	2,166,977	300,000	150,000	6	5
London	3,487,035	500,000	250,000	21	25
North East	1,018,655	150,000	75,000	7	9
North West	2,722,999	400,000	200,000	5	11
Northern Ireland	728,647	100,000	50,000	2	3
Scotland	2,096,719	300,000	150,000	7	9
South East	3,259,664	500,000	250,000	8	14
South West	1,838,416	250,000	125,000	8	13
Wales	1,125,021	150,000	75,000	2	5
West Midlands	2,125,068	300,000	150,000	7	10
Yorkshire & the Humber	2,026,872	300,000	150,000	6	6
UK Total	**24,289,178**	**3,350,000**	**1,750,000**	**85**	**116**

Source: Human Fertilisation Embryology Authority (HFEA) Fact & Figures 2006.

Key: DI= Donor insemination; IVF= *In-vitro* fertilisation; GIFT= Gamete intrafallopian transfer; IUI= Intrauterine insemination.

Table 1.2. Diagnostic categories and distribution of couples with primary and secondary infertility.

Diagnostic category	Primary (%)	Secondary (%)
Male	25	20
Ovulation	20	15
Tubal	15	40
Endometriosis	10	5
Unexplained	30	20

The proportion of couples in each group varies from population to population depending on environmental factors and referral pattern. Adapted from: Templeton *et al*. Management of infertility for the MRCOG and beyond, 2000.

1.1.2. Infertility causes

These are:

1. Male factor: this results from a lack of sufficient number of competent sperms to fertilize the normal ovum. There are many causes of male infertility such as, varicocele, idiopathic oligozospermia, obstructive azoospermia, ejaculatory inadequacy, immotile cilia syndrome, karyotype abnormalities, pituitary lesions, gonadotrophin deficiency and congenital abnormalities (Rowe et al., 1993).
2. Ovulatory disorders: anovulation (absence of ovulation) or oligo-ovulation (infrequent ovulation) are responsible causes of infertility in about one fifth of all cases (Templeton et al., 2000). Ovulatory disorders can be classified on the basis of the anatomical site in the hypothalamic- pituitary- gonadal axis into:

   Ovarian failure caused by genetic problem such as, Turner syndrome (45XO), autoimmunity, radiotherapy, and premature ovarian failure.

a. Hypothalamus disorders, where abnormalities in gonadotrophins releasing hormone (GnRH) secretion from the hypothalamus affect ovarian function. For example, hyperprolactinemia, Kallman syndrome and excessive exercise or stress which may alter hypothalamic GnRH secretion.
b. Pituitary disorders resulting in deficiency of gonadotrophins secretion. These include pituitary tumours, necrosis, or thrombosis.
c. Hypothalamic-Pituitary dysfunction, such as polycystic ovary syndrome (PCOS) the best known example where the dysfunction is secondary to other endocrine disturbances associated with the syndrome.

3. Tubal factor: this accounts for 15-20% of cases of primary infertility and 40% of secondary infertility. Tubal disease mainly results from either pelvic inflammatory disease (PID), or lower abdominal surgery. PID may occur either spontaneously or as a complication of miscarriage, intrauterine instrumentation and pelvic surgery.

4. Endometriosis: this is defined as the presence of functional endometrial tissue outside the uterine cavity. The common sites are the pelvic peritoneum, ovaries and the rectovaginal septum. The prevalence of pelvic endometriosis in women with infertility has been shown to be 21% (Strathy et al., 1982; Mahmood and Templeton, 1991; Meuleman et al., 2008). The mechanisms of how endometriosis can influence fertility remain unclear, however, it has been shown that the peritoneal fluid from these women contains high levels of cytokines, and activated growth factors which have been shown to be toxic to sperm function and embryo survival (Guidice and Kao, 2004). Additionally, advanced endometriosis is associated with excessive pelvic adhesions which may lead to anatomical distortion.
5. Unexplained infertility: this affects 25-30% of couples and the diagnosis is made when routine investigations including semen analysis, tubal evaluation and ovulation tests all appear normal (Taylor and Collins, 1992).

1.1.3. Infertility treatment

Treatment options for female and male infertility are summarised in **Table 1.3**.

Assisted conception is a term used to describe techniques used to facilitate fertility.

These techniques often referred to as assisted reproductive techniques (ART) are summarised in **Table 1.4.** Assisted conception is mainly used for couples with intractable pathology, failed primary treatment; severe male infertility or unexplained infertility. The most advanced treatment option for UK couples and indeed in Leicester is *in-vitro* fertilisation, intracytoplasmic sperm injection and embryo transfer (IVF/ICSI-ET).

The outcome of treatment of infertility using ART is often determined by the woman's age (Chuang et al., 2003), the presence of previous surgery, the woman's initial FSH levels (Ebrahim et al., 1993), her duration of infertility, and the number of good oocytes (Khalili et al., 2005) and embryos available for replacement at the end of treatment (Testart et al., 1983; Templeton et al., 2000). IVF-ET and ICSI–ET will be discussed below in details.

Table 1.3. Treatment of infertility.

Infertility factor	Treatment options
1. Anovulation	
a. Hypogandotrophic Hypogonadism (WHO I)	Pulstile GnRH Agonists and Gonadotrphins
b. Hypothalamic pituitary dysfunction PCOD (WHO II)	Clomifene citrate or Tamoxifen Metformin, Gonadotrophin, ovarian drilling
c. Ovarian failure (WHO III)	Oocyte donation
d. Hyperprolactinaemia	Bromocriptine or Cabergoline
e. Failure of the above	IVF (*In-vitro* fertilisation)
2. Tubal	
a. Mild tubal disease	Tubal surgery
b. Proximal tubal obstruction	Tubal cannulation
c. Moderate to severe	IVF
3. Endometriosis a. Minimal	Surgical ablation Superovulation (SO) / Intrauterine insemination (IUI).
b. Moderate to severe	IVF
4. Unexplained	Empirical treatment Clomiphene IUI SO/IUI If above failed IVF
5. Male factor	
a. Mild male factor	IUI (Intrauterine insemination)
b. Obstructive azoospermia	Surgery
c. Non-obstructive azoospermia	Surgical sperm retrieval in selected cases
d. Ejaculatory failure	Drugs, sperm recovery
e. Hypothalamic Hypogonadism	Gonadotrophins
f. Severe male factor	Intracytoplasmic sperm injection (ICSI)

Adapted from: Bhattacharya, S. Infertility in Dewhurst's Textbook of Obstetrics and Gynaecology, 2007.

Table 1.4. Techniques used in assisted reproduction and their outcomes.

Technique	Main indications	Outcome
IVF	1.Tubal disease 2.Intractable pathology 3.Failed primary treatment	Pregnancy rate per oocyte recovery 26.1%* Live-birth rate per oocyte recovery 34%$^{\Delta}$
ICSI	1.Sever oligozospermia 2.Failed Fertilization with IVF	Pregnancy rate per oocyte recovery 26.5%* Live-birth rate per oocyte recovery 32%$^{\Delta}$
IUI-H	1.Unexplained infertility 2.Mild male factor infertility	IUI: 12.2% pregnancy rate per cycle*
OD	1.Absent or non-functioning ovaries 2.carrier of serious of genetic disorder 3.Repeated poor response with IVF	Live birth per transfer 50% $^{\Delta}$
DI	1.Azoospermia 2.Infectious disease in the male partner 3.Prevent transmission of genetic condition	Pregnancy rate per cycle 16%* Live birth per cycle11%$^{+}$

Key: IVF= *In-vitro* fertilisation; ICSI= Intracytoplasmic sperm injection; GIFT= Gamete intrafallopian transfer; IUI-H= Intra uterine insemination with husband semen; DI= Donor insemination; OD= Oocyte donation.

* Anderson et al. 2007.

Δ US Department of health and Human services, Centre for Disease Control and prevention, 2004.

+ Human Fertiltilization and Embryology Authority, 2005.

Adapted from: Bhattacharya, S. Infertility in Dewhurst's Textbook of Obstetrics and Gynaecology, 2007.

A. In vitro fertilisation and embryo transfer (IVF-ET)

IVF-ET is used for infertility secondary to tubal disease, ovulatory disorders, endometriosis, unexplained infertility and oligoasthenozoospermia. The treatment involves the following steps:

1. *Ovarian stimulation*

The aim of controlled ovarian stimulation is to produce multiple large follicles from which multiple mature eggs can be obtained (Macklon et al., 2006). This is achieved by pituitary desensitisation using a GnRH analogue often employed prior to and during ovarian stimulation. The stimulation part is achieved by using either human menopausal gonadotrophin (hMG) containing follicle stimulating hormone and luteinising hormone (FSH and LH) (van Wely et al., 2003) extracted from the urine of menopausal women, or purified urinary FSH. More recently recombinant gonadotrophins (r-FSH and r-LH) have become available (Kelly, 2003, Balen et al., 1999) and increasingly being preferred. The above hormones may be used either alone or in combination hence there are many different protocols for ovarian superovulation. Regular monitoring of the follicles by ultrasound scan and serial serum estradiol measurements is essential during ovarian stimulation (Lass, 2003).

2. *Oocyte retrieval*

Oocytes are retrieved from ovaries under ultrasound guidance and this procedure is usually undertaken under light sedation.

3. *Sperm-oocyte mixture*

After the oocytes have been retrieved, sperms are washed and prepared. Insemination is then performed one to six hours after oocyte retrieval with approximately 50,000-200,000 motile spermatozoa placed with each oocyte in a special culture media. After about 16-18 hours the oocytes are examined for fertilization, which is defined by the presence of two pronuclei.

4. *Embryo Transfer*

The embryo (at the four–to eight cell stage) is transferred into the uterine cavity usually 48 or 72 hours after oocyte collection. In the UK a maximum of two embryos may be transferred during a single cycle of infertility treatment in women younger than 40 years of age, and no more than three in women over 40 years in order to reduce the risk of multiple pregnancies (Mayor, 2004).

B. Intracytoplasmic sperm injection (ICSI)

ICSI involves the injection of a single sperm through the outer membrane of the oocyte to reach the cytoplasm (Van Steirteghem et al., 1993). Therefore, the main indication for ICSI is male factor infertility.

A closer look at available data indicates that the number of women requiring fertility treatment in the UK has risen steadily over the years (**Figure 1.1**) (Meikle, 2005), and this problem is not restricted to the UK, as a similar picture is also seen throughout Europe. This could be due to a combination of different factors such as women delaying having babies to later in life (Speroff, 1994; Porter et al., 2006; Laurance, 2007), the increase in sexually transmitted infections (Adler and Meheus, 2000), huge increases in childhood obesity or the recognised decline in male fertility (Jensen et al., 2008). Infertility is therefore an important health problem that requires more effective treatments. To obtain a better understanding of female fertility, it is essential to understand the physiology of folliculogenesis, fertilisation, implantation and the factors regulating these processes.

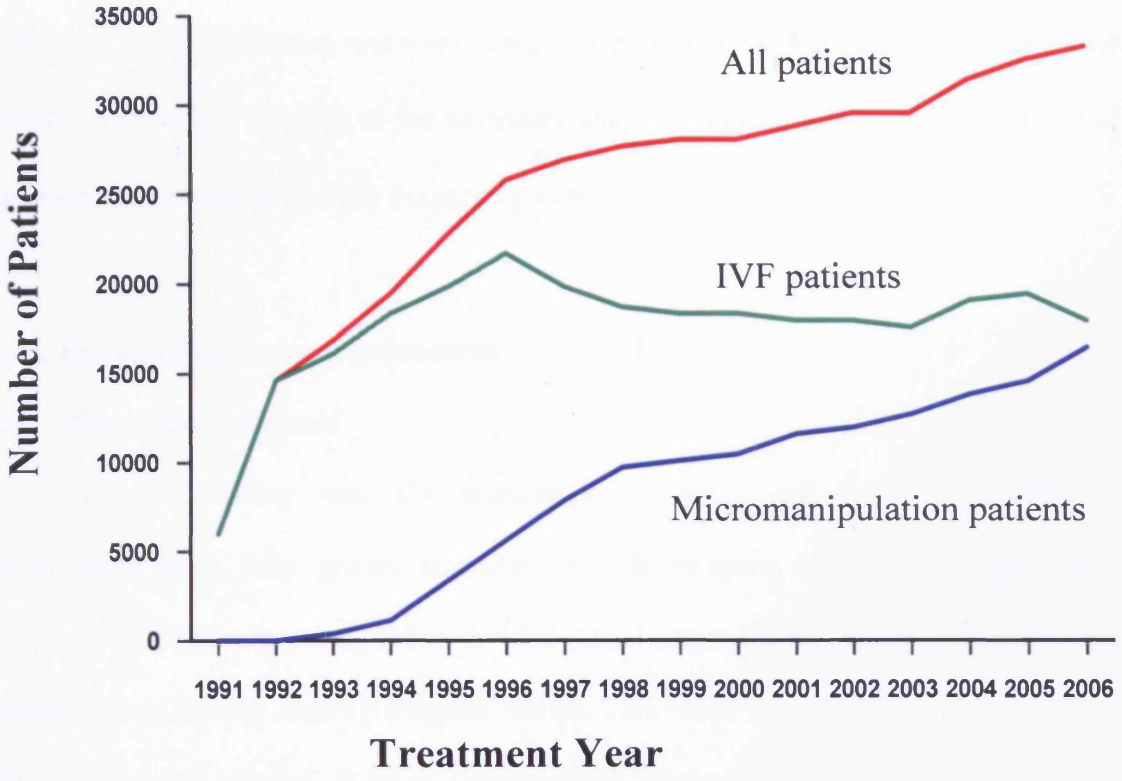


Figure 1.1. Infertility treatment (IVF/ICSI) trends in the UK from 1991-2006. The above graphs are showing an increase in the number of women having IVF/ICSI treatment over the years. Source: HFEA report 2007.

1.2. Folliculogenesis

1.2.1. Definition

Folliculogenesis is a dynamic process that starts with the recruitment of primordial follicles into a pool of growing follicles and ends with either ovulation or death by atresia (Erickson, 2003). In women it is a very long process requiring about a year for a primordial follicle to grow and develop to the ovulatory stage (Gougeon, 1986). It is divided into 2 phases: (i) the preantral and (ii) the antral phase.

1.2.2. Phases of follicular development

i. The preantral phase

This phase is starting with the recruitment of primordial follicles through their development to a fully grown secondary follicle **(Figure 1.2)**. It is gonadotrophin-independent, begins *in-utero*, and is divided into 3 major stages; the primordial, primary and secondary follicle stages (Gougeon, 1986). This phase is characterised by growth and differentiation of oocytes. It takes approximately around 290 days or about 10 regular menstrual cycles for this phase to be completed (Gougeon, 1986).

ii. The antral phase

The phase is starting with the development of early antral follicles to the formation and development of the preovulatory follicle. It is gonadotrophin-dependent, and begins only after the menarche. It is further divided into 4 major stages based on follicle size; small (class 2, 3, 4, 5), medium (class 6), large (class 7) and preovulatory follicles (class 8) (Gougeon, 1986) **(Figure 1.2)**. This phase is characterised by a tremendous increase in the size of the follicle itself (up to approximately 25 mm). After antrum formation occurs at the class 3 stage (0.4 mm in diameter), the rate of follicular growth accelerates. The time

interval between antrum formation and the development of a 20 mm preovulatory follicle (class 8) is about 60 days or about 2 menstrual cycles (Gougeon, 1986).

A dominant follicle is selected from a cohort of class 5 (2-5 mm) follicles **(Figure 1.2)** at the end of the luteal phase of the cycle to grow to the ovulatory stage (Gougeon, 1986). Atresia can occur at class1 (secondary follicle) stage, with the highest incidence occurring in the pool of small and medium (class 5, 6, 7) Graafian follicles (Gougeon, 1986).

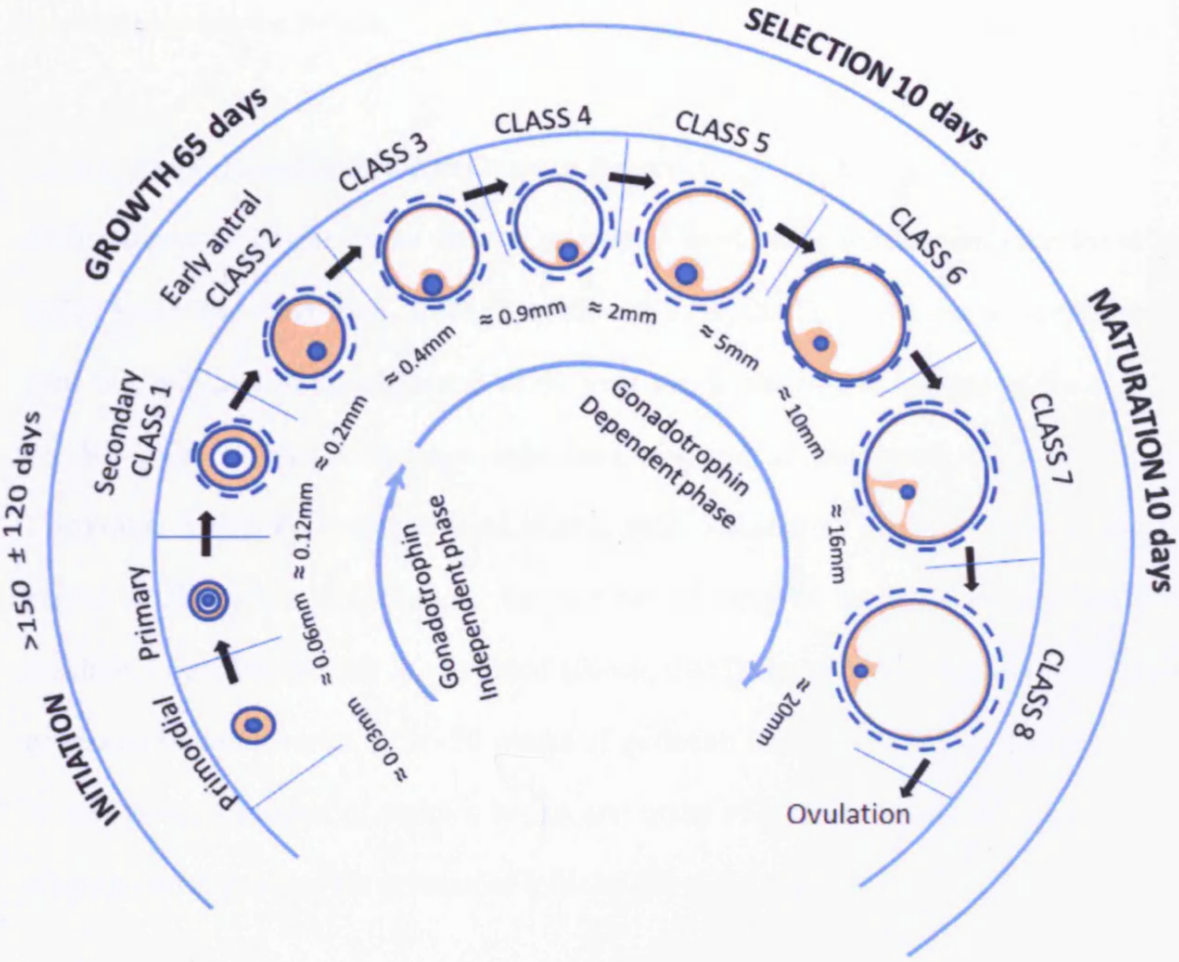


Figure 1.2. Phases of folliculogenesis. Folliculogenesis is divided into two phases: (1) The preantral phase is the gonadotrophin-independent phase of folliculogenesis and is sub-divided into 3 distinct development stages; the primordial, primary and secondary follicles. The secondary follicle is often classed as the class 1 follicle and (2) the antral phase is the gonadotrophin-dependent phase and is classically divided further into 4 developmental stages based on the size of the follicle; small (classes 2, 3, 4 and 5), medium (class 6), large (class 7) and preovulatory (class 8). This classification is adapted from Gougan, A: Dynamics of follicular growth in the human: A Model from preliminary results, 1986.

1.2.3. Morphological and development aspect of folliculogenesis

The study of folliculogenesis is primarily based on the morphological changes **(Figure 1.3)** that occur within the follicle.

1.2.3.1. The Primordial Follicle (Ovarian Reserve)

Folliculogenesis begins in the embryo at about 3 weeks after fertilization (Gondos et al., 1971, Smitz and Cortvrindt, 2002, Speroff and Fritz, 2005c), with the primordial germ cells that originate in the endoderm of the yolk sac, allantois, and hindgut of the embryo. By 5-6 weeks of gestation, these cells have migrated to the genital ridge (Smitz and Cortvrindt, 2002). Following a rapid mitotic multiplication of germ cells (oogonia) that begins at this point of gestation, the number of oocytes rapidly increases until the maximum possible number is produced (Block, 1953). In total, 6-7 million oocytes are generated in both ovaries by 16-20 weeks of gestation (Baker, 1963, Sforza et al., 1993). At this point, a process of meiosis begins and many of the oocytes are destroyed, whilst others go on to produce the primordial follicles (Byskov, 1986).

Between 16-18 weeks of fetal life (Smitz and Cortvrindt, 2002), primordial follicle formation begins in the inner part of ovary, near the rete ovarii (Smitz and Cortvrindt, 2002) and continues until just after birth (Speroff and Fritz, 2005c). The primordial follicle consists of a single oocyte; arrested in the diplotene stage of the meiotic prophase, surrounded by a single layer of spindle-shaped granulosa cells (Erickson, 1995), which are further enveloped by a thin membranous basal lamina (Smitz and Cortvrindt, 2002). It is approximately 0.03 to 0.05 mm in diameter **(Figure 1.3)**. Primordial follicles therefore exist in a micro-environment in which direct contact with other cells does not occur and thus does not have its own independent blood supply (Reynolds, 1950).

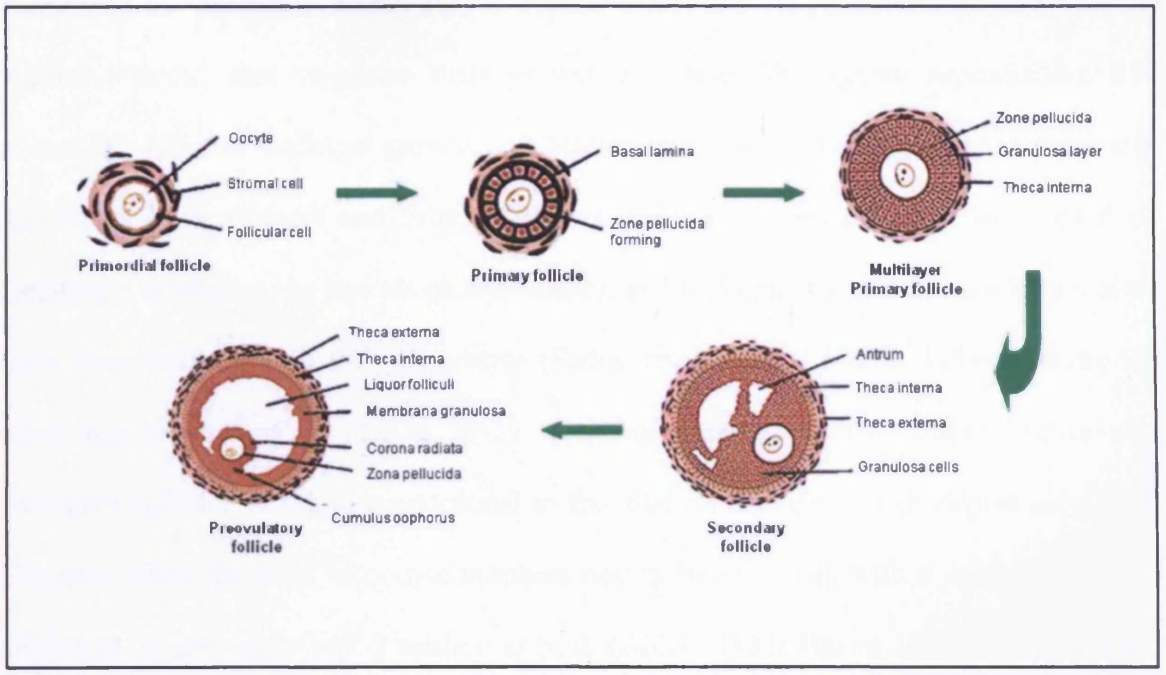


Figure 1.3. A schematic diagram of the major follicle structures present in the adult ovary. The primordial follicle consists of oocyte surrounded by single layer of spindle shaped granulosa layer. One is epithelial in origin the other mesenchymal in origin. The former converts into the cuboidal granulosa cell of the primary follicle and the stromal cell converts into the vestigial thecal layer and starts to produce a basal lamina that separates the two cell types. The granulosa cells proliferate and expand, whilst the stromal cells differentiate into the inner and outer thecal cell layers that define the secondary follicle, which starts to show the presence of a gel-like fluid filled antrum. At the same time the ovum polarises to one side of the follicle under the actions of locally produced growth factors. Further development of a dominant follicle continues under gonadotrophin, growth factor and sex steroid influences to aid the maturation of the follicle ready for ovulation. The presence of multiple granulosa cell types that either surround the ovum, are intimate with the folliclur fluid (liquor folliculi) or are associated with the basal lamina (membrane granulosa) suggest different roles for these different cells at this stage of folliculogenesis. Adapted from: Berne & Levy Principles of Physiology, 2006.

These cells are therefore independent of outside forces and the primordial follicles have an intrinsic 'clock' that regulates their growth and fate. Throughout reproductive life, primordial follicles undergo growth and atresia until their numbers become exhausted (Erickson, 2003; Speroff and Fritz, 2005c). Growth and atresia are not interrupted by pregnancy, ovulation, or periods of anovulation and this dynamic process continues at all ages, from infancy until the menopause (Faddy and Gosden, 1995). After attaining the maximum number of oocytes at 16-20 weeks of pregnancy, this number irretrievably decreases at a rate which is proportional to the total number present (Faddy et al., 1992). The most rapid decrease in oocyte numbers occurs before birth, with a decline from 6-7 million at 16-20 weeks to 1-2 million at birth (Block, 1953; Baker, 1963; Gougan et al., 1994). Although the rate of loss of oocytes declines to a slower rate during early extrauterine life and adolescence, the number of oocytes remaining at puberty for most women is between 300,000 to 500,000 (Baker, 1971) **(Figure 1.4)**. About 400-500 follicles from this reservoir will eventually develop into mature follicles and result in ovulation during the reproductive years (Baker, 1963; Baerwald et al., 2003; Speroff and Fritz, 2005c). The number of follicles that develop from the primordial follicles formed during *in-utero* development and the rate of loss of oocytes during early childhood and adolescence constitute the ovarian reserve (Erickson, 2003).

It is thought that oocytes that are not surrounded by granulosa cells to form primordial follicles are lost, probably via apoptosis (Gougan, 1996; DePol et al., 1997). Initial recruitment (McGee and Hsueh, 2000), the process by which some primordial follicles 'wake up' and leave the resting pool is mediated by a counter-balance of various stimulatory and inhibitory hormones and locally produced growth factors (Gougan, 1996).

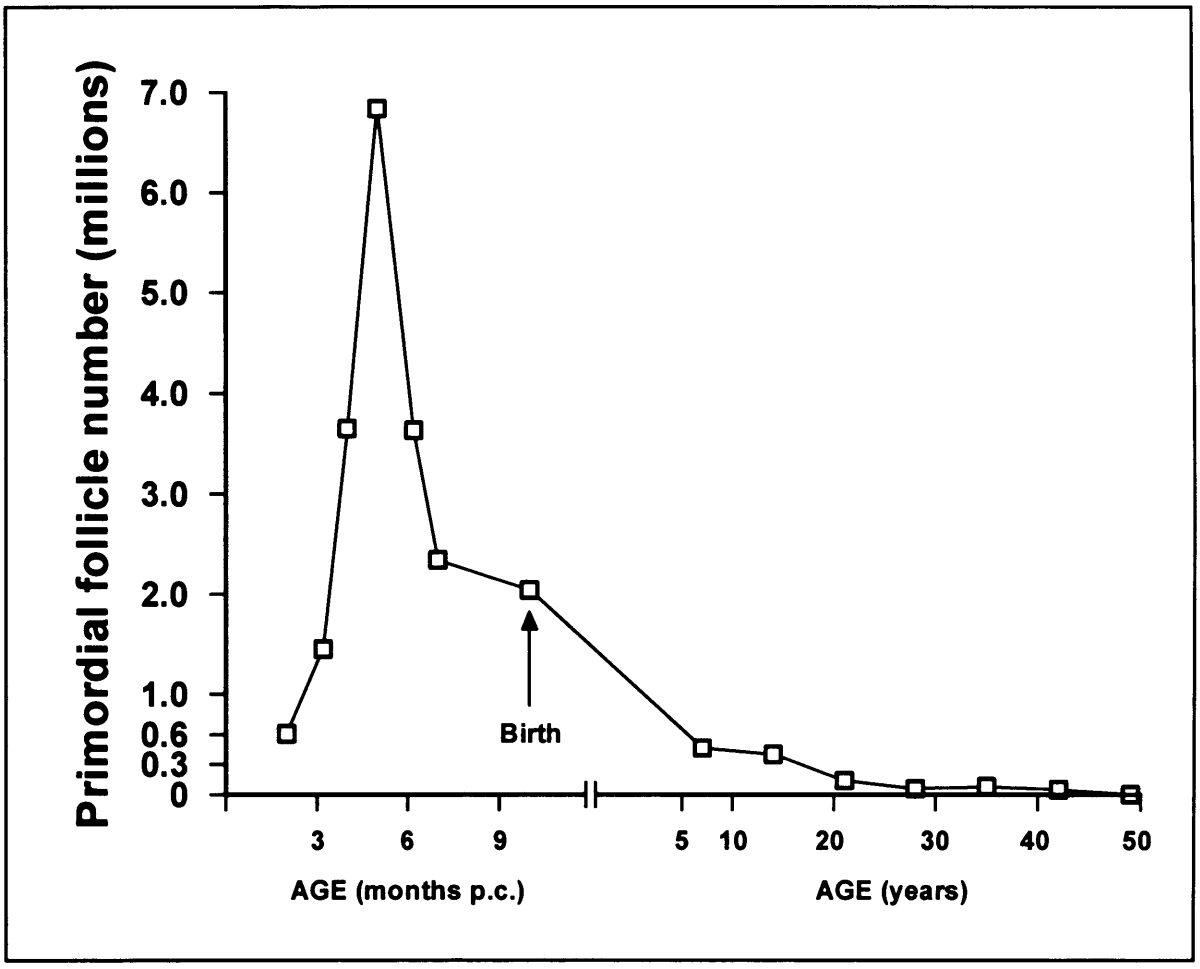


Figure 1.4. Reproductive ageing. All primordial follicles are formed in the fetus between 6-9 month gestation.The number of primordial follicles decrease progressively with age. Redrawn from Baker TG: Radiosensitivity of mammalian oocytes in particular reference to the human female. 1971 and Erickson et al.: Morphology and physiology of the ovary, 2003.

1.2.3.2. The Primary Follicle

The transition from a primordial follicle to a primary follicle (0.1 mm in diameter) **(Figure 1.3)** (Fortune et al., 2000) marks the first sign of 'true' follicular development (Erickson, 2003). This transition is characterised by an increase in the size of the oocyte and a change in the shape of granulosa cells from squamous to cuboidal epithelium **(Figure 1.3)** (Smitz and Cortvrindt, 2002) and the acquisition of a mitotic potential in the granulosa cells. At the same time, small gap junctions, which are intracellular channels of connexin proteins, develop between granulosa cells and oocytes (Wiesen and Midgley, 1994). These gap junctions are essential for growth and multiplication of granulosa cells and for the nutrition and development of the oocyte (Cecconi et al., 2004), in a simple paracrine interaction (Amsterdam and Rotmensch, 1987; Kumar and Gilula, 1996; Erickson, 2003). With multiplication of the cuboidal granulosa cells, the primordial follicle becomes a primary follicle with an inner and an outer granulosa cell layer; the outer granulosa cell layer being separated from the stromal cells by a basement membrane called the basal lamina (Speroff and Fritz, 2005c). During primary follicle development, the surrounding stromal cells differentiate into concentric layers of thecal cells; the theca interna (closest to the basal lamina) and the theca externa (the outer layer) (Speroff and Fritz, 2005c). The theca layers appear when granulosa proliferation produces between 3 and 6 layers of granulosa cells. This process occurs in the absence of gonadotrophins and is supported by the persistence of this initial growth in anencephalic fetuses (Levy et al., 2006).

1.2.3.3. The Secondary Follicle

Towards the end of the preantral stage, the oocyte enlarges and becomes surrounded by a membrane called the zona pellucida **(Figure 1. 3)** (Speroff and Fritz, 2005c). At this point, the granulosa cells undergo multilayer proliferation as the theca layer continues to organize from the surrounding stroma (Speroff and Fritz, 2005c). At the same time one or two

arterioles terminating just outside the basal lamina appear exposing these follicles to factors circulating in blood. Simultaneous with this is the appearance of specific receptors for FSH on the surface of granulosa cells (Oktay et al., 1997; Erickson, 2003). Granulosa-oocyte communication increases and it is thought that this change becomes important for normal preantral follicle development. In the pre-pubertal ovary, the development of the secondary follicle (0.2 mm in diameter) represents the maximal degree of follicular development (Levy et al., 2006) and the actual time taken for early growing follicles to attain the secondary follicle stage is unknown but is thought to take several months (Gougan, 1996).

1.2.3.4. The Antral (Early Tertiary) Follicle

This stage which begins only after the menarche, takes 65-85 days, and thus spans 3 menstrual cycles for completion (Levy et al., 2006). During the late luteal phase of each cycle a small number of antral follicles (2-5 mm in diameter) are recruited for further development (Gougan, 1996).

Under the synergistic influence of estrogen and FSH there is an increase in the production of follicular fluid that accumulates in the intracellular spaces between granulosa cells (Erickson, 1995; Erickson, 2000b; Erickson, 2003). This fluid eventually coalesces to form a cavity **(Figure 1.3)** and as this forms the follicle makes it gradual transition into the antral stage. The role of the follicular fluid is not clearly understood (Speroff and Fritz, 2005c), but it has been suggested that the most probable role is to provide a means whereby the oocyte and surrounding granulosa cells can be nurtured in a specific endocrine environment (Edwards, 1974). Follicular fluid contains a complex mixture of substances (Levy et al., 2006); some secreted by the granulosa and theca cells and some transferred from the surrounding plasma (Edwards, 1974). Many substances have been found in the

follicular fluid and these include mucopolysaccharides, plasma proteins, electrolytes, enzymes for steroid synthesis, steroid hormones (Lobo et al., 1985), FSH, LH, inhibin, activin, follistatin, oxytocin, arginine, vasopressin, coritoctrophic releasing hormone (CRH), growth factors (IGF-1, IGF-2), cytokines (interlukin-1 and tumour necrosis factor), and the components of the renin-angiotensin system (Klein et al., 2000; Erickson, 2003). All of these presumably play some role in the development of the follicle and ovulation. Of classical importance is the presence of steroid hormones, which reach the antrum via granulosa cells secretion and diffusion from theca cells. At this point in folliculogenesis, the granulosa cells surrounding the oocyte are called the cumulus oophorus (Eppig et al., 1997; Speroff and Fritz, 2005c).

The differentiation of cumulus cells is believed to be a response to signals originating in the oocyte. In the presence of FSH, estrogen becomes the dominant substance in the follicular fluid. Conversely, in the absence of FSH, androgen predominates. LH is not normally present in follicular fluid until the mid cycle. If LH is prematurely elevated, mitotic activity in granulosa cells decrease, degenerative changes ensue, and intra-follicular androgen levels rise. Therefore, the dominance of estrogen and FSH is essential for sustained accumulation of granulosa cells and continued follicular growth (Ie Nestoure et al., 1993). Antral follicles with the greatest rates of granulosa proliferation contain the highest estrogen concentrations and lowest androgen/estrogen ratios, and are the most likely to house a healthy, mature oocyte.

1.2.3.5. The Preovulatory (Late Tertiary) Follicle

At this point of the normal ovarian cycle, most of the follicles that started growing 360 days ago would have already died (Hirshfield, 1991). Those that have not died now respond to the increasing FSH concentrations by growing. This phase coincides with the follicular phase of the menstrual cycle and the follicle is ~20 mm in diameter **(Figure 1.3)** (Gougan, 1993).

A rise in pituitary FSH caused by the disintegration of the corpus luteum at the late luteal phase participates in the selection of a cohort of 3-11 (class 5, 2-5 mm diameter) follicles (Chabbert-Buffet and Bouchard, 2002; Zeleznik, 2004). The selected follicles compete with each other for growth-inducing FSH and the dominant follicle starts to secrete estradiol and somewhat later inhibin B, which begins to suppress pituitary FSH production and secretion. Follicles that have lower FSH receptors show retardation of their growth and become atretic. On day 5-7 of the menstrual cycle, a single follicle is selected, called the dominant follicle (Schipper et al., 1998), which grows quickly and dramatically up to 20 mm in diameter. At this stage the follicle is known as the preovulatory follicle. Granulosa cells in the pre-ovulatory follicle enlarge and acquire lipid inclusions, while the theca cells become vacuolated and the thecal layer becomes richly vascular, giving the preovulatory follicle a hyperaemic appearance (Speroff and Fritz, 2005c). Meanwhile, the oocyte proceeds in meiosis, approaching completion of its reduction division of the chromosomes.

As the preovulatory follicle approaches maturity, it produces an increasing quantity of estrogen with estrogen concentrations rising slowly at first during the late follicular phase and then rapidly reaching a peak approximately 24-36 hours prior to ovulation (Pauerstein et al., 1978). When the estradiol level peaks, there is a large quantity of LH release (the LH surge) (Fritz et al., 1992), which stimulates the dominant follicle further and seals the fate

of any remaining follicles with lower estrogen and FSH content, by further increasing androgen superiority (Erickson, 2000a; Vegetti and Alagna, 2006).

1.2.4. Major Stages of Folliculogenesis

Folliculogenesis occurs within the cortex of the ovary and includes four major developmental stages (Erickson, 2003) **(Figure 1.5)**:

i. Follicle Recruitment

ii. Preantral Follicle Development

iii. Selection and Growth of the Preovulatory Follicle

iv. Follicular Atresia

i. Follicle Recruitment

This process starts soon after the formation of primordial follicles in the fetus and continues until the pool of primordial follicles becomes exhausted at the menopause (Erickson, 2003). Recruitment occurs at a relatively constant rate during the first three decades of life, however, when the ovarian reserve reaches a critical number of ~25,000 at around 37 years (Faddy et al., 1992), the rate of loss accelerates by about 2-fold and a decrease in fecundity accompanies the accelerated loss (O'Connor et al., 1998; Piette et al., 1990). The age-related rise in plasma FSH that occurs in women after 36 years of age is believed to be involved in the accelerated recruitment of primordial follicles and reduced fertility (Erickson, 2000a). Presumably, the high concentration of plasma FSH acts to accelerate the transition from primary to preantral follicles (Erickson, 2003).

Recruitment has been divided into 2 distinct types; (a) initial recruitment that occurs before puberty and (b) cyclic recruitment that occurs after puberty has been initiated (McGee and Hsueh, 2000). These two separate events are discussed in detailed below.

a. Initial Recruitment

Initial recruitment is a continuous process that starts just after the primordial follicle formation and long before the onset of puberty. After initial recruitment, oocyte growth is a prominent feature of the growing follicles **(Figure 1.5),** but these oocytes remain arrested in the prophase of meiosis. For those follicles not recruited, the default pathway is to remain dormant (McGee and Hsueh, 2000). Although initial follicle recruitment is thought to be due to a release from the inhibitory stimuli that maintains the resting primordial follicles in stasis, the precise mechanisms controlling the initiation of follicular growth have been difficult to investigate because initial follicular recruitment represents a protracted process characterized by the slow growth of a substantial number of small follicles over a prolonged period of time (Hirshfield 1989). To overcome some of these difficulties, many investigators have monitored the changes in the number of primordial and/or primary follicles that remain in the ovary at any given time, the supposition being that the decline in follicle numbers in this category is due to follicles leaving the resting pool to enter the growing pool. Because of difficulties in distinguishing between non-growing and growing follicles, primordial and primary follicles have been considered a contiguous group (Edwards et al., 1977; Gougan, 1996) despite evidence that primary follicles are growing at this stage of folliculogenesis (Hirshfield 1989).

Resting primordial and primary follicles are likely to be under constant inhibitory influences of systemic and/or local origin (Wandji et al., 1996). A decrease in inhibitory influences and/or an increase in stimulatory factors that allows the initiation of follicle growth, such as elevated serum FSH levels, may be essential for initial follicle recruitment. For example, changes in FSH levels have been shown to be associated with accelerated initial recruitment found in both the early and late stages of reproductive life (McGee and Hsueh, 2000). There is evidence that high tonic LH levels are associated with a reduced

number of resting follicles (Flaws et al., 1997). However, FSH and LH are unlikely to exert any direct action on primordial follicles due to the lack of LH and FSH receptors in the follicle at this time (Oktay et al., 1997). There is also a suggestion that the oocyte may also be involved in the initial recruitment of such follicles (Tsafriri, 1997; Eppig et al., 1997; Erickson and Shimasaki, 2000). It was proposed by Edwards et al., that the order in which follicles are recruited is related to the order in which oocytes originally entered meiosis during development (Edwards et al., 1977). This production line hypothesis predicts that the first oocytes entering meiosis are the first ones to mature and one of them will eventually become the dominant oocyte that is released during ovulation. In addition, Hirshfield (Hirshfield, 1991) has demonstrated that rapidly progressing oocytes located near the cortico-medullary junction of the ovary, begin their growth earlier and are enclosed within follicles that initiate growth during the neonatal and infantile period, a time of accelerated follicle loss. In contrast, oocytes that undergo slower meiotic progression are located closer to the cortex and are enclosed in follicles that grow later in life. Meiotic competence of human follicles declines with age (Volarcik et al., 1998) and this finding has been used to support the production line hypothesis. However, any reduction in oocyte quality could be due to poorer conditions present during folliculogenesis after the age of 35 years (Faddy et al., 1992; McGee and Hsueh, 2000). Because the exact nature of the factors involved in controlling oocyte quality is still unclear, confirmation of the production line hypothesis will not be known for some time. Factors like c-kit, Growth Differentiation Factor-9 (GDF-9) and Bone Morphogeneic Protein-15 (BMP 15) are thought to be involved in oocyte-granulosa cell communication (Nilsson and Skinner, 2001; Cecconi et al., 2004) in early follicles and have been proposed to have a role in initial follicle recruitment (Dong et al., 1996; Aaltonen et al., 1999; Wu and Matzuk, 2002; Skinner, 2005). Until all the factors involved in initial recruitment are

known, the exact mechanisms stimulating primordial follicles to leave the resting pool will remain elusive.

b. Cyclic Recruitment

In contrast to initial recruitment, cyclic follicle recruitment which starts after the onset of puberty, and is the result of an increase in circulating FSH levels during each menstrual cycle that rescues a cohort of antral follicles from atresia **(Figure 1. 5)** (McGee and Hsueh, 2000). During cyclic recruitment, only a limited number of follicles survive because the default pathway is to undergo cell death by atresia (McGee and Hsueh, 2000). Oocytes in these follicles have already completed their growth, acquired a zona pellucida, and are competent to resume meiosis (Trounson et al., 1998).

ii. Preantral Follicle Development

This stage of follicle development is not dependent upon FSH stimulation for growth as there is evidence of an early antral stage, where either hypophysectomy or hypothalamic/pituitary failure has been described in women with absent gonadotrophin secretion (Macklon and Fauser, 2001). It is therefore reasonable to conclude that as gonadotrophins are not necessarily required for stimulating preantral follicle growth, but locally produced growth factors are likely to be critically involved in controlling preantral follicle development during this period.

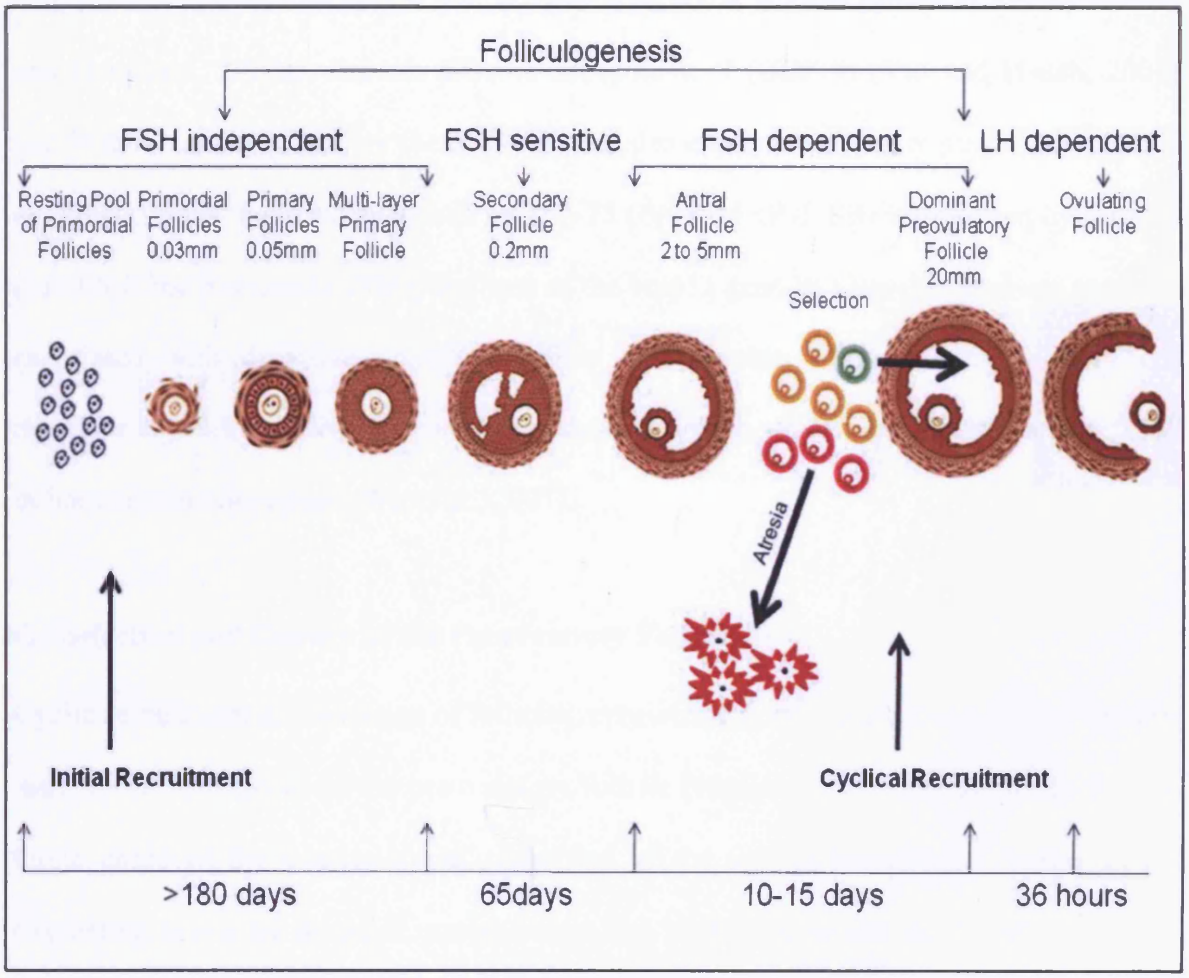


Figure 1.5. Schematic representation of the major stages of folliculogenesis. The follicle drawing was adapted from: Berne & Levy Principles of Physiology, 2006. The information was taken from: Gougan A: Dynamics of follicular growth in the human: A model from preliminary results, 1986. McGee et al. Initial and cyclic recruitment of ovarian follicles, 2000, and Chabbert-Buffet et al. The normal human menstrual cycle, 2002.

Several growth factors including epidermal growth factor (EGF), transforming growth factor (TGF-β), insulin-like growth factors 1 and 2 (IGF1 and IGF II) (Udoff and Adashi, 1996) are all thought to play a role in human preantral follicle growth (Chabbert-Buffet and Bouchard, 2002a). Growth differentiating factor-9 (GDF-9) (Vitt and Hsueh, 2001) and Wilms tumour inhibitory factor (WTI) are also expressed and may play a role (Suh et al., 2002), whilst bone morphogenic protein-15 (BMP-15/GDF-9B) is produced by oocytes (Erickson and Shimasaki, 2000) and loss of the bmp15 gene in a knockout mouse model is associated with defective preantral follicle development (Galloway et al., 2000). It therefore appears that BMP15 may be an essential protein for efficient oocyte development in human folliculogenesis (Wu et al., 2007).

iii. Selection and Growth of the Preovulatory Follicle

Cyclic recruitment and selection of follicles represents a continuous process that eventually leads to the emergence of the preovulatory follicle (McGee and Hsueh, 2000). In the late luteal phase of the ovarian cycle, regression of the corpus luteum and decline in the circulating levels of estradiol, progesterone and inhibin A results in the release of the negative feedback suppression on pituitary FSH production and secretion **(Figure 1.6)**. This allows an increase in circulating FSH levels (Ross et al., 1970) and once a critical threshold is reached (Brown, 1978, Schipper et al., 1998), the growth of a cohort of antral follicles measuring 2-5 mm in diameter is stimulated. One of the selected follicles grows faster than the others and in the mid to late follicular phase of the cycle it is selected to become the dominant follicle (Zelenik, 2001). The exact reasons why one of the follicles emerges as the dominant one are unclear (Zelenik, 2004); however, the feedback inhibition of FSH secretion is suggested as the principle mechanism by which the maturing dominant follicle suppresses the development of less mature follicles (Krasch et al., 1973; Zeleznik, 2004).

During the selection process, FSH induces aromatase activity in the granulosa cells of the dominant follicle resulting in a rise in systemic levels of estradiol (Ie Nestoure et al., 1993). This results in the suppression of pituitary FSH secretion, thus preventing the maturation of less mature follicles (Zelenik et al., 1987). In spite of declining mid follicular FSH levels, the dominant follicle continues to develop and produce more estrogen in the presence of a concentration of FSH that is unable to maintain less mature follicles since as the dominant follicle matures it becomes more sensitive, and less dependent upon FSH for its development (Zeleznik, 2004; Vegetti and Alagna, 2006). The concentration of FSH required to initiate preovulatory follicle development is much greater than that necessary to maintain preovulatory growth. In response to FSH stimulation (Zelenik and Kubik, 1986), granulosa cells of the dominant follicle acquire LH receptors and the concurrent induction of LH receptors may provide the maturing follicle with an additional source of gonadotrophin support which enables it to mature (Filicori et al., 2002).

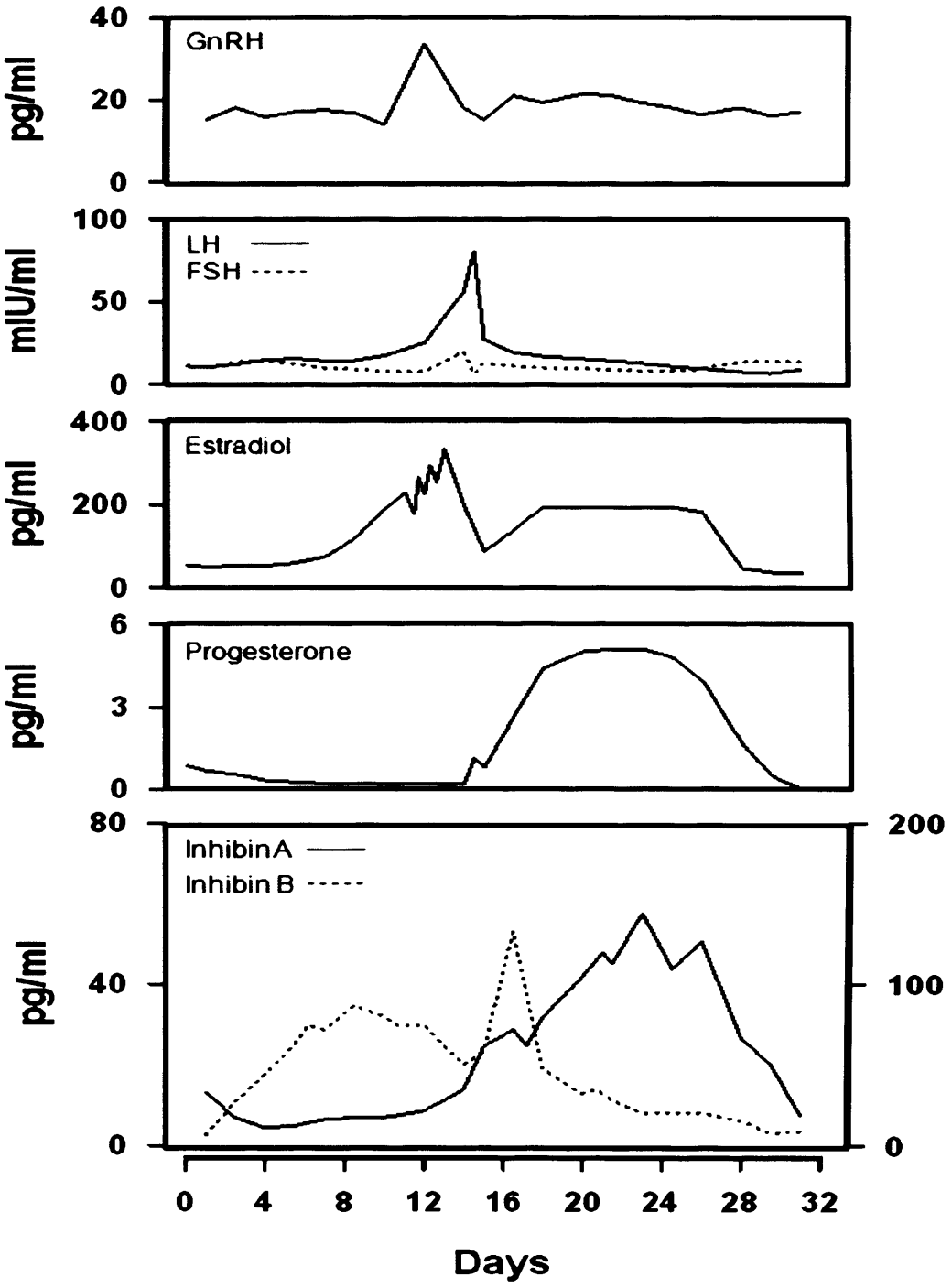


Figure 1.6. The hormones that regulate the menstrual cycle. Gonadatrophin-releasing hormone (GnRH) and preovulatory increases in estradiol occur during the latter stages of the follicular phase and immediately preceding the preovulatory surges of LH and FSH. After ovulation, the broad peak of progesterone and estradiol in the luteal phase are a consequence of secretion by the corpus luteum. The earlier inhibin B peak observed in the follicular phase is an indication of follicular function, whereas the latter inhibin A peak is a consequence of secretion by the corpus luteum. Adapted from: Berne & Levy Principles of Physiology, 2006.

The two-cell-two-gonadotropin theory has been modified (**Figure 1.7**) (Kobayashi et al., 1990; Yamato et al., 1992; Hillier et al., 1994) to indicate a key role for LH not only in thecal cell steriodogenesis stimulation, but also in follicle growth and maturation (Filicori, 1999). In LH and FSH deficient anovulatory women, treatment with FSH alone appears to be sufficient to induce normal follicular development but fails to induce ovulation and luteinisation (Zeleznik, 2004). Studies conducted in women by Sullivan *et al.* (Sullivan et al., 1999) using recombinant FSH and LH, support the hypothesis that the acquisition of LH receptors by granulosa cells protects the dominant follicle from the decline in FSH concentrations during the mid- to late-follicular phase. The accumulation of a greater mass of granulosa cells is accompanied by the advanced development of thecal vasculature and by day 9 of the menstrual cycle thecal vascularity in the dominant follicle is twice that in the other antral follicles. This allows for a preferential delivery of gonadotrophin to the follicle. This could be an additional mechanism by which the dominant follicle retains FSH responsiveness (Zelenik, 2001), sustained development and continued function despite waning gonadotropin levels. Furthermore, inhibin B (Roseff et al., 1989) produced by granulosa cells in the dominant follicle exerts a negative feedback suppression of FSH release which participates further in atresia of the non-dominant follicles (Roseff et al., 1989) and the notion that locally derived factors (Baird, 1983; McGee and Hsueh, 2000) such as insulin like- growth factors (IGFs) are involved in the amplification of FSH action demonstrate the importance of such local factors in controlling the production of the dominant follicle. Although yet to be identified, it has been postulated that teratogenic factors produced by the dominant follicle inhibit the development of subordinate follicles and cause death of such follicles through follicular atresia (McGee and Hsueh, 2000).

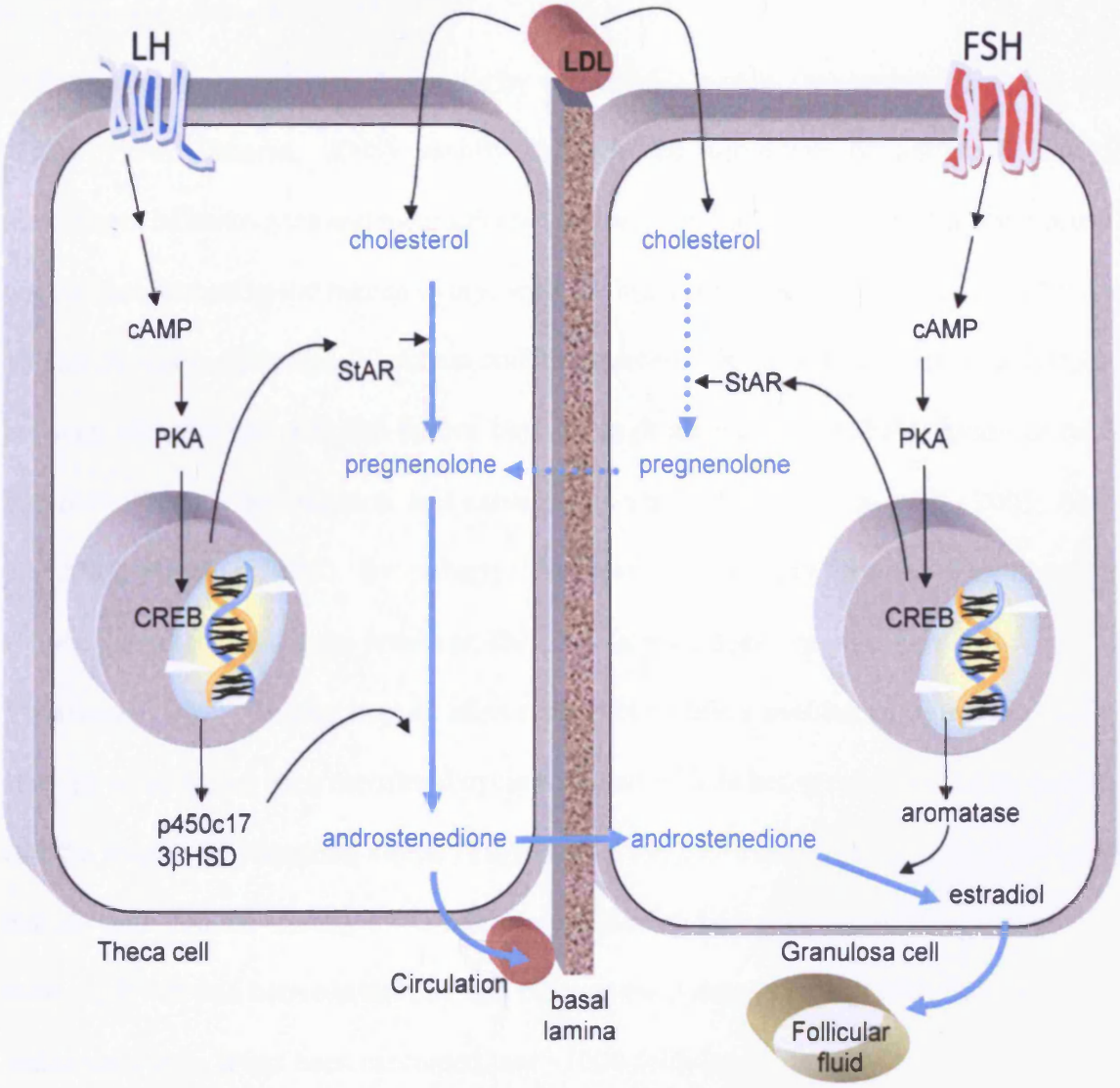


Figure 1.7. The two gonadotrophin two cell model. Theca and granulosa cells cooperate in the synthesis of estradiol. The theca cells produce androgens in response to luteinising hormone (LH). Granulosa cells respond to follicle-stimulating hormone (FSH) by aromatising the androgens to estrogens. Low density lipoproteins provide the cholesterol required and cyclic adenosine monophosphate (cAMP) is produced through the actions of adenylate cyclase. The production of the nuclear transcription factor cyclic AMP response element binding protein (CREB) occurs through the actions of protein kinase A. The binding of CREB to nuclear DNA promotes the production of steroid acute regulatory protein (StAR) and 17α-dehydrogenase/lyase (3βHSD) which collectively convert cholesterol from cholesterol through to and androgen, androstendione, which diffuses across the basal lamina and enters the granulosa cell. A similar stimulation cascade occurs in the granulosa cell that results in the generation of aromatase that converts the androstendione to estradiol. The dashed arrows indicate minor or disputed effects. Adapted from: Goodman, 2003.

iv. Follicular Atresia

Follicular atresia is the normal process by which follicle cells are removed from the ovary (Tilly, 1996; Hussein, 2005) usually through the activation of apoptosis and the recruitment of leukocytes and macrophages. In early fetal life as many as 7 million primary oocytes are formed in the human ovary, with the highest number usually produced between 16 and 20 weeks gestation (Vaskivuo and Tapanainen, 2003). This number falls sharply to between 600,000 and 800,000 before birth through apoptosis and the decrease in oocyte number continues beyond birth and extends into early life (Vaskivuo et al., 2001; Abir et al., 2002; Hussein, 2005). By puberty, the ovary has already lost the majority of the follicles generated *in-utero,* however, follicular atresia does not stop here (Vaskivuo and Tapanainen, 2003). During normal adult reproductive life, a number of primordial follicles start growing during each menstrual cycle with one follicle being released during ovulation and the remaining becoming atretic (Tilly et al., 1991). Ultimately, only ~450 follicles will mature and ovulate during a woman's reproductive life with ~250,000 follicles being removed by atresia between puberty and menopause (Gougan et al., 1994). During a single menstrual cycle, it has been estimated that ~1000 follicles begin growing (Hussein, 2005), with only one or two being released during ovulation (Mortia and Tilly, 1999).

The role of apoptosis in follicular atresia has been extensively investigated *in-vivo* and *in-vitro* in many non-human species, including the rat, pig, chicken, and cow. However, several recent studies (Yuan and Giudice, 1997; Markström et al., 2002; Depalo et al., 2003; Hussein, 2005; Amsterdam et al., 2003) have also demonstrated a strong association between follicular atresia and apoptosis in the human ovary. As such, apoptosis is considered a crucial event in maintaining ovarian follicular dynamics, and ensuring that in every menstrual cycle only one or very few follicles reach the stage of a preovulatory follicle and will ovulate (Amsterdam et al., 2003). This is essential in preventing multiple

embryos and reduces the chances of twins and higher order pregnancies (Hurwitz and Adashi, 1992; Amsterdam et al., 1998; Amsterdam et al., 1999). After ovulation, the dominant follicle collapses to form the corpus luteum and apoptosis is responsible for corpus luteum regression (luteolysis) which is essential for preserving the cyclicity of the menstrual cycle (Tilly et al., 1991; Amsterdam et al., 2003).

With regard to ovarian apoptosis, two general mechanisms are operative **(Figure 1.8)**; one which is triggered by binding of death molecules to cell surface receptors (cell surface receptor-mediated events) (Hussein, 2005), while the other is generated by signals arising within the cell (mitochondria–mediated events) (Hussein, 2005; Markström et al., 2002).

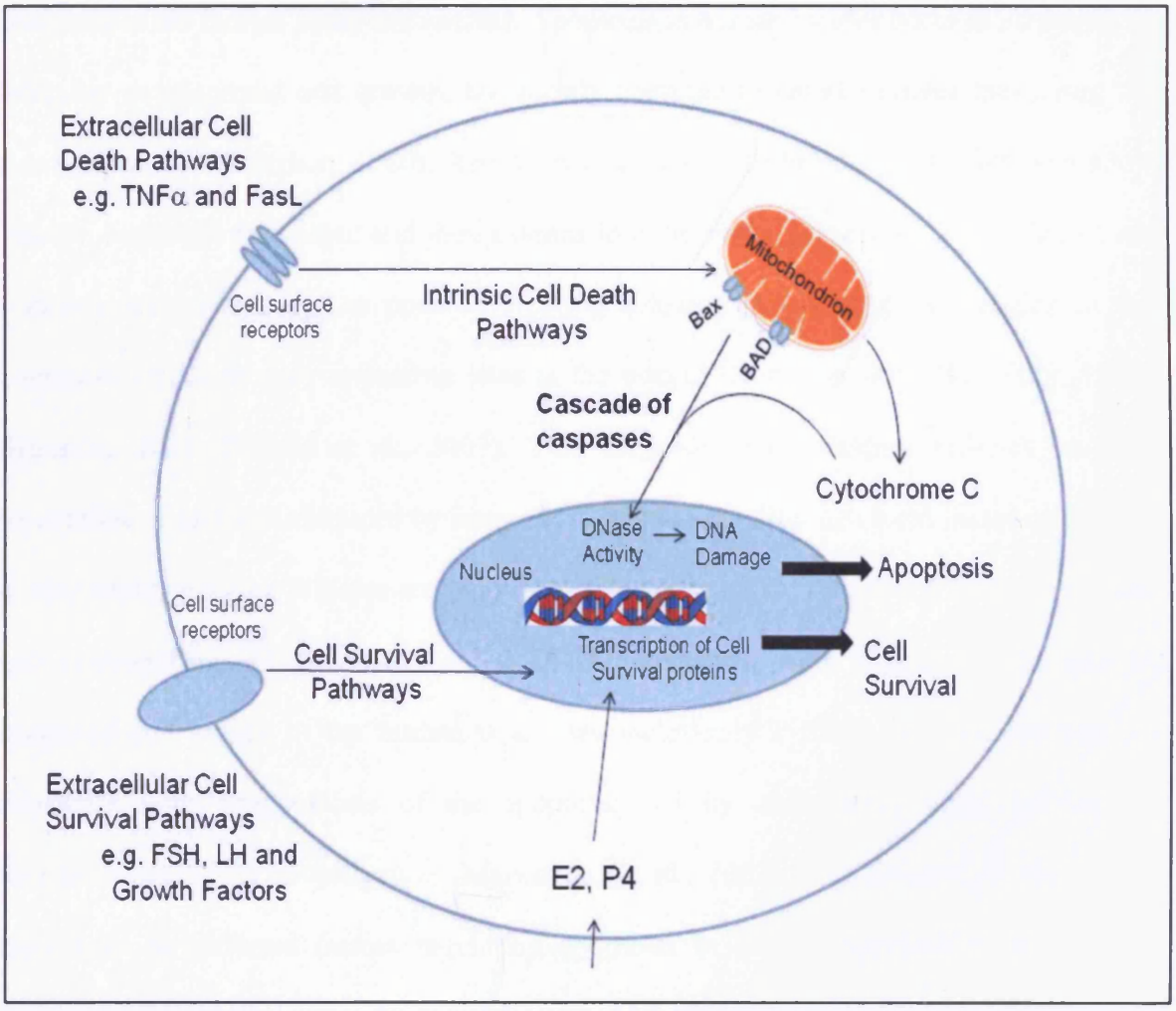


Figure 1.8. The general mechanism of cell survival depends upon a balance between pro- and anti-apoptotic pathways. Pro-apoptotic pathways are divisible into extrinsic (tumour necrosis factor alpha (TNFα) and Fas ligand (FasL) binding to cell surface receptors that activate intracellular cell death pathways and intrinsic pathways (mitochondria-mediated events) that lead to the production of a cascade of chemical mediators (caspases) that act with cytochrome C released from the damaged mitochondria to induce DNases that damage DNA and ultimately to apoptosis. On the other hand, cell survival pathways mediated through the actions of gonodatrophins, growth factors and estrogen and progesterone, leads to the transcription of cell survival proteins that prevent or repair DNA damage and lead to cell survival. Bcl-2= B cell/lymphoma-2 family; Bclx= long form family member; E2= estradiol; P4= progesterone. Adapted from: Markstrom, E. et al. Survival factors regulating ovarian apoptosis-dependence on follicle differentiation, 2002, Hussein, MR. Apoptosis in the ovary: molecular mechanisms, 2005. And from: Hsu, SY et al. Tissue specific BC1-2 protein partners in apoptosis: an ovarian paradigm, 2000.

The exact signals, mechanisms, receptors and intracellular signalling pathways leading to apoptosis in the human ovary are unclear. Apoptosis in human ovaries occur at all stages of follicular development and growth, but mostly confined to antral follicles measuring >5 mm in diameter (Gougeon, 1986). Apoptosis in quiescent follicles, in both fetal and adult ovaries, begins in the oocyte and then extends into the surrounding granulosa cells, which contrasts with atresia in the pool of growing follicles, where apoptosis begins in the granulosa cell layer only appearing later in the oocyte (Kondo et al., 1996, Tilly, 1996) (Hussein, 2005; Depalo et al., 2003). This suggests that immature follicles may be susceptible to apoptosis induced by intra-oocyte signalling, although local factors may play a role, whilst growing follicles are more sensitive to the signals produced by stromal cells and extrinsic signalling (Depalo et al., 2003). However, such studies on the role of apoptosis and atresia in the human ovary are technically difficult because the process concludes with phagocytosis of the apoptotic cell by surrounding cells, leaving no detectable traces for investigation (Markström et al., 2002). Furthermore, in the ovary, there may be different factors regulating apoptosis in follicle populations of different developmental stages, necessitating models in which a homogeneous group of follicles can be isolated at specific stages. To overcome some of these obstacles, many turned to animal models for studying follicular atresia taking advantage of the fact that isolated follicles or granulosa cells undergo spontaneous apoptosis when cultured in the absence of serum (Tilly et al., 1992). The addition of survival or pro-apoptotic factors to incubation medium can affect the rate of spontaneous apoptosis. It therefore seems that the decision of "life or death" in the ovary is dependent upon a delicate balance between pro-survival factors and pro–apoptotic factors (Gougeon, 1986; Chun and Hsueh, 1998; Erickson, 2003; Markström et al., 2002; Hussein, 2005). **Table 1.5** summarises the current list of pro- and anti-apoptotic factors known to affect the ovary.

From many animal studies and from a limited number of human studies it can be concluded that only follicles reaching a critical stage of their development that coincides with appropriate changes in serum gonadotrophin and/or autocrine/paracrine steroids, cytokines, or growth factors will be spared from atresia and go on to reach ovulation (Gougeon, 1986; Chun and Hsueh, 1998; Amsterdam et al., 2003). Further studies into the precise factors involved in the human would be of benefit as it would improve the low rate of conception in IVF by allowing an improvement in the culture method for oocytes (Hussein, 2005). Furthermore, such knowledge might be used in the prevention of or in the delay of premature ovarian failure (Hussein, 2005), and its use will enable further insight into the therapeutic control of malignant ovarian tumour progression through apoptotic induction (Hussein, 2005). This knowledge would, however, only be of value if the other factors involved in the regulation of human folliculogenesis are also known.

Table 1.5. Ovarian cell pro- survival and pro-apoptotic factors.

Survival factors	References	Death factors	References
FSH	(Chun, et al., 1996)	Free radicals	(Tilly and Tilly, 1995)
Estrogen	(Billig et al., 1993)	TNF-α	(Kapia, 1996 et al.,)
IGF-1	(Chun et al., 1994)	BAX	(Felici et al., 1999)
EGF and bFGF	(Tilly et al., 1992)	Androgen	(Billig et al., 1993)
Activin	(Chun et al., 1995)	GnRH	(Billig, et al.,1994)
IL-1β	(Chun et al., 1995)	IL-6	(Gorospe and Spangelo, 1993)
Bcl-2	(Felici et al., 1999)	Caspases	(Fenwick and Hurst, 2002)
BCl-XL	(Felici et al., 1999)	Fas	(Hu, 2001 et al.,)
C-Kit	(McGee et al., 2000)	p53	(Hussein, 2003)

The table lists the different survival and death factors of ovarian follicles in animal models. This was adopted from: Chun, S.Y. et al. Paracrine mechanisms of ovarian follicle apoptosis, 1998 and from: Hussein, M.R. Apoptosis in the ovary: molecular mechanisms, 2005.

1.2.4. Factors regulating folliculogenesis

The factors involved in regulating human folliculogenesis can be divided into 2 broad categories (**Figure 1.9**):

(a) Those whose role is clearly established and

(b) Those whose role is only just emerging.

It is already clear that the early stages of follicular development appear to occur in the absence of gonadotrophin (Macklon and Fauser, 2001) although FSH may be involved in initial recruitment. Follicle growth to the early antral stage has been described in women with absent gonadotrophin secretion, due either to hypophysectomy or hypothalamic/pituitary failure (Speroff and Fritz, 2005c). Also, *in-vitro* studies have demonstrated that factors other than gonadotrophin (Erickson and Shimasaki, 2001) are probably involved in follicular development and apoptosis.

Nevertheless, it took almost 60 years (Brown, 1978) for the concept that FSH is an obligatory factor for dominant follicle selection and development to be accepted and represents the cornerstone of our understanding of ovarian physiology, as the absence of FSH means no dominant follicle, and hence no ovulation (Zelenik, 1993). It is now clear that although FSH is the primary regulator of the final stage of follicular development up to preovulatory stage (Gougan, 1996; Erickson and Shimasaki, 2001) there are other factors produced by the granulosa cell or the oocyte (Ostuka et al., 2005) that may act in an autocrine or paracrine manner to modulate, amplify or attenuate FSH action during follicular development (Findlay, 1993; Macklon and Fauser, 1998; Monget and Bondy, 2000; McGee and Hsueh, 2000; Erickson and Shimasaki, 2001; Britt and Findlay, 2002).

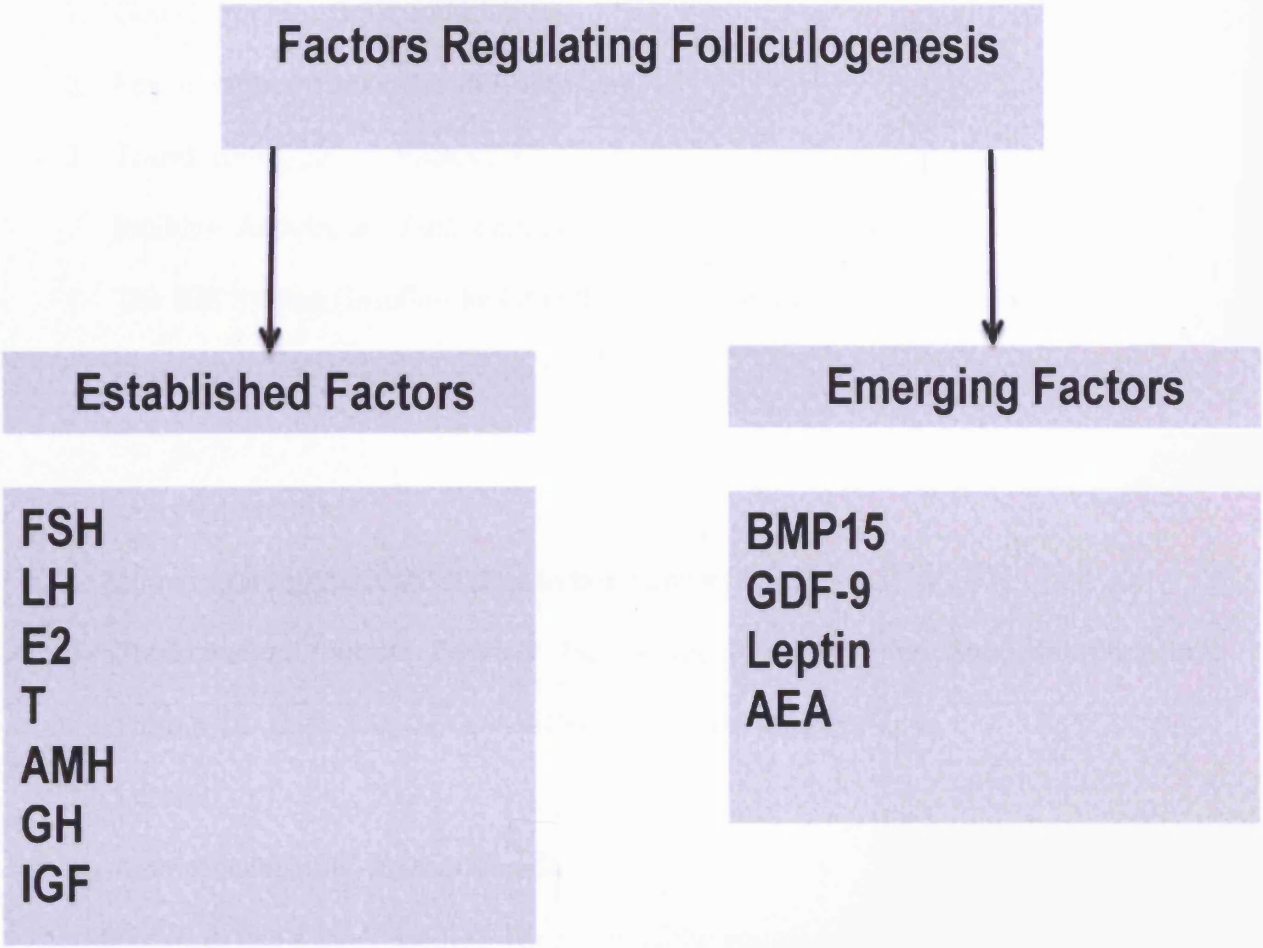


Figure 1.9. Factors known to regulate human folliculogenesis. The established and emerging factors known to play a role in the regulation of human folliculogenesis are listed in the order of their importance. FSH=follicle stimulating hormone; LH=leutinising hormone; E2=estradiol; P4=progesterone; T=testosterone; AMH=Anti-Mullerian hormone; GH=growth hormone; IGF-1=insulin-like growth factor-1; BMP15=bone morphogenic protein 15; GDF-9=growth differentiating factor-9; AEA=anandamide.

a. Established factors:

The established factors include the following:

1. Gonadotrophins (FSH and LH).
2. Sex hormones (Estrogen and Androgen).
3. Transforming growth Factor-β Family members (Anti-Mullerian Hormone (AMH), Inhibins, Activin, and Follistatin).
4. The IGF System (Insulin-like Growth factors, Insulin-like Growth factor Binding Proteins).

b. Emerging factors:

The following are some of the factors in this category:

1. Transforming Growth Factor-β Family members, such as Bone Morphogeneic Protein-15 (BMP-15), Growth Differentiation Factor-9 (GDF-9).
2. Leptin.
3. Endocannabinoids, such as anandamide.

In the next section a more detailed discussion of the gonadotrophins (FSH, LH) and the sex steroid (estrogen) will be provided and the endocannabinoids (anandamide) will be discussed at the end of the Chapter. These factors are relevant to the work described in this thesis.

1. Gonadotrophins

a. Follicle-stimulating hormone (FSH)

FSH is a pituitary glycoprotein hormone composed of two peptide subunits: as α-subunit which is common to LH, β-hCG, and thyroid stimulating hormone (Tryrotrophin; TSH), and a β-subunit that is specific to FSH (Pierce and Parsons, 1981). Circulating FSH acts through binding to specific transmembrane G-protein coupled FSH receptors (Fan and

Hendrickson, 2005) and signals through the actions of intracellular cAMP and cGMP to alter the phenotype of the target cell (Simoni et al., 1997).

The fact that granulosa cells are the only ovarian cell types that express FSH receptors (Findlay and Drummond, 1999) reflects their importance in physiologically mediating FSH action in the ovary. Accumulated data from a large number of *in-vivo* and *in-vitro* human studies have demonstrated that FSH receptors play a fundamental role in the growth and differentiation of the dominant follicle (Messinis and Templeton, 1990; Erickson and Shimasaki, 2001; Adriaens et al., 2004; Vegetti and Alagna, 2006). FSH promotes follicular fluid formation, granulosa cell proliferation, estradiol production (by inducing the P450 aromatase enzyme which converts testosterone and androgen to estradiol and estrone), and LH receptor expression (Macklon and Fauser, 1998). The latter is required for LH to induce ovulation and luteinisation. A high level of LH receptor expression is not induced in the granulosa cells until the dominant follicle reaches the preovulatory stage. This single fact supports the possibility that when LH enters the follicular fluid during the late follicular phase, it may be an important effectors of granulosa function, perhaps even replacing FSH as the principle regulator of cytodifferentiation (Macklon and Fauser, 2001).

FSH regulates folliculogenesis in women by controlling the recruitment of a cohort of follicles at the luteal-follicular transition phase and the selection of the dominant follicle during mid-follicular phase (Macklon and Fauser, 1998; Zeleznik, 2004). To do this, Brown (Brown, 1978) postulated that FSH concentration must exceed a certain level before follicular development proceeds and that when this critical FSH threshold (Hall et al., 1992) is surpassed the growth of a cohort of antral follicles is stimulated. The duration of this peak is limited in the normal cycle by the decrease in FSH which occurs in early- to

mid-follicular phase (Van Dessel et al., 1996) and is controlled by increased estradiol and inhibin B produced by the dominant follicle (Groome et al., 1996). This has led to the concept of an 'FSH window' (Fauser and Van Heusden, 1997; Zeleznik, 2004), which stresses the significance of the duration of FSH elevation above the threshold level (Macklon and Fauser, 2001), rather than the level of elevation of FSH for single dominant selection. This concept has been recently proven by demonstration that elevating FSH concentrations for a short period of time in the early-follicular phase does not increase the number of dominant follicles (Schipper et al., 1998). Conversely, when the physiological decrease of FSH in a normal cycle is prevented by the administration of FSH in the late-follicular phase (Zelenik, 2001), the augmented sensitivity to FSH allows several follicles to gain dominance (Schipper et al., 1998).

The observation that the dominant follicle requires less FSH for continuous development (Macklon and Fauser, 1998) suggests that FSH signals may be modified within the ovary, either at the level of FSH binding to the receptor or by interference with post receptor signal transduction (Macklon and Fauser, 1998). Given the variation that occurs in maximum FSH concentration in the early follicular phase, and the observation that follicles can fully mature without concomitant rise in estradiol (Schoot et al., 1992), it appears that intraovarian factors (Findlay and Drummond, 1999) may determine the magnitude of the response to stimulation by FSH. *In-vitro* studies have shown that growth factors such as IGF, TGF-β, fibroblast growth factor (FGF) and activin all enhance FSH action (Findlay, 1994), while others such as inhibin (Miro and Hillier, 1992), EGF and some IGFBPs have been shown to have an inhibitory effect on the response of cultured granulosa cells to FSH (van Dessel et al., 1996). While FSH is a key factor involved in folliculogenesis, it is also becoming clear that its prominent role is subject to acute modifications.

b. Luteinizing Hormone (LH)

LH is also a pituitary glycoprotein composed of two peptide subunits: an α-subunit which is common to FSH, β-hCG, and TSH, and a β-subunit that is specific to LH. It acts in a similar way to FSH by binding to specific trans-membrane G-protein coupled receptors (Pierce and Parsons, 1981) and by signalling through the actions of intracellular cAMP and cGMP to alter the phenotype of the target cell. Although FSH is considered the central regulator of dominant follicle survival and development, LH signalling also plays a fundamental physiological role (Erickson and Shimasaki, 2001). LH has traditionally been considered to be the main stimulus of the theca interstitial cell and to elicit changes in gene expression that are critical to estradiol production (Erickson et al., 1985). Specifically, activation of the LH receptors in theca cells leads directly to the stimulation of androstendione production, which readily diffuses across membranes into the granulosa cell (Yamato et al., 1992). This is the basis of the classical two gonadotrophin-two-cell model for estradiol biosynthesis (Hillier et al., 1994).

The physiological significance of this LH response is to provide substrate to the granulosa cells where it is metabolised by the enzyme P450 aromatase into estradiol (Hillier et al., 1994). Because estradiol production is unique to dominant follicles, the level of plasma estradiol is a useful marker for monitoring the physiological responses to endogenous or exogenous gonadotrophin (such as hCG) in women (Erickson and Shimasaki, 2001). There are two additional physiologically important functions of LH in the dominant follicle and corpus luteum. Firstly, the surge of plasma LH is responsible for ovulation (Sullivan et al., 1999) and corpus luteum formation (Erickson and Shimasaki, 2001). Secondly, LH is essential for progesterone and estradiol production by the corpus luteum during the early- and mid-luteal phases of the menstrual cycle (Zelenik, 1998; Erickson and Shimasaki, 2001).

2. Estrogen

Estrogen is an obligatory hormone required for normal folliculogenesis beyond the antral stage and for the maintenance of the female phenotype of the somatic cells within the ovaries (Britt and Findlay, 2002). The ability of follicles to make estrogen is first apparent in the late preantral stage when the follicles possess all the components of the two cell-two gonadotrophin model (Drummond, 2006). Although, aromatase activity is present in small antral follicles, estradiol (E2) production at this stage is limited by an inability to produce an androgen substrate for aromatization (Carson et al., 1981). Growth beyond the small antral stage is characterised by an increase in aromatase activity and androgen synthesis, which culminates in follicular E2 production (Hillier, 1981; Drummond, 2006). The preovulatory follicle has the highest intrafollicular level of E2 (Drummond, 2006), primarily due to the size of its granulosa cell population and its capacity for androgen aromatisation (Hillier, 1981; McNatty, 1982).

Despite many studies demonstrating that E2 is the principal estrogen produced by follicles (Britt and Findlay, 2002), the specific role of E2 and its receptors in ovarian function remains unclear (Richards, 2001). What is known is that the action of E2 is related to the action of FSH in folliculogenesis (Kumar et al., 1997; Abel et al., 2000). Although FSH is essential for the final growth of antral follicles, and preantral follicles are responsive but not dependent on FSH for growth (Britt and Findlay, 2002), FSH, IGF-1 and E2 all stimulate the proliferation and differentiation of granulosa cells (Zhou et al., 1997). The specific genes induced in granulosa cells by E2 have not been identified, but it is clear that E2 exerts a supporting role for FSH action (Britt and Findlay, 2002). There are several genes suspected of being regulated in granulosa cells by E2 including cyclin D2 (Richards, 2001), inhibin α and inhibin βB (Charpentier et al., 2000), which are essential for follicle growth and differentiation (Charpentier et al., 2000).

1.3. Fertilisation

1.3.1. Definition and description of the process

Fertilisation is the process whereby a capacitated sperm and ovum unite to form a zygote (**Figure 1.10**), and usually occurs in the ampulla of the fallopian tube (Evans and Florman, 2002). The fusion of the oocyte with the sperm is followed by the fusion of their genetic materials (Johnson and Everitt, 2000). The fertilisable lifespan of a human oocyte and sperm are unknown, but most estimates for the oocyte range between 12 and 24 hours, and for sperm the range is between 24 and 48 hours. Thus, fertilisation occurs in the fallopian tube 12-24 hours after ovulation. The details of the various components of this process are shown in **Figure 1.11** (Speroff and Fritz, 2005e).

1.3.2. Factors regulating fertilisation

Fertilisation is regulated by two factors; sperm capacitation and egg transport.

Capacitation is defined as the cellular changes that spermatozoa must undergo to fertilize. This is regulated by the ability of spermatozoa to undergo the acrosome reaction, bind to the zona pellucid and to acquire hypermotility. The transport of the egg depends on smooth muscle contractions of the fallopian tube that brings the fimbriae in contact with the ovarian surface, synchronised unidirectional cilia movements and alteration in the levels of estrogen and progesterone.

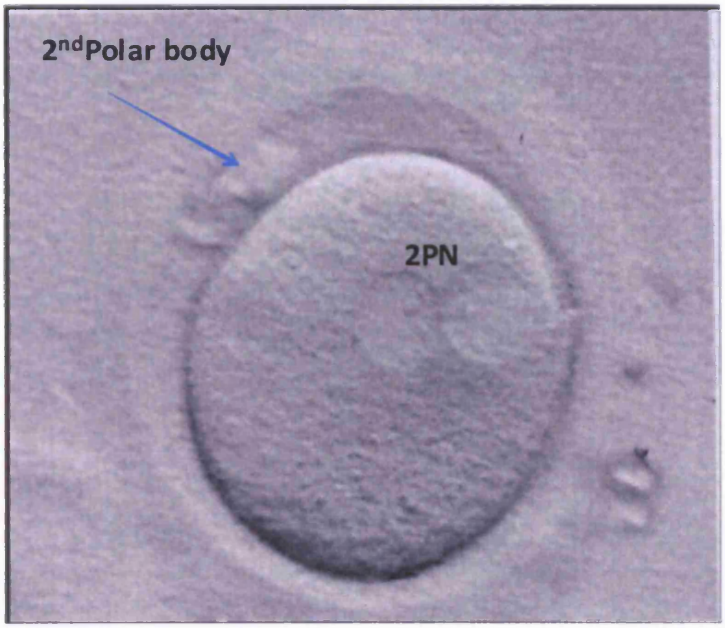


Figure 1.10. Human embryo at the 2 pro-nuclear (PN) stage (day 1 of normal fertilisation). This photo has been taken with permission from one of the study volunteers.

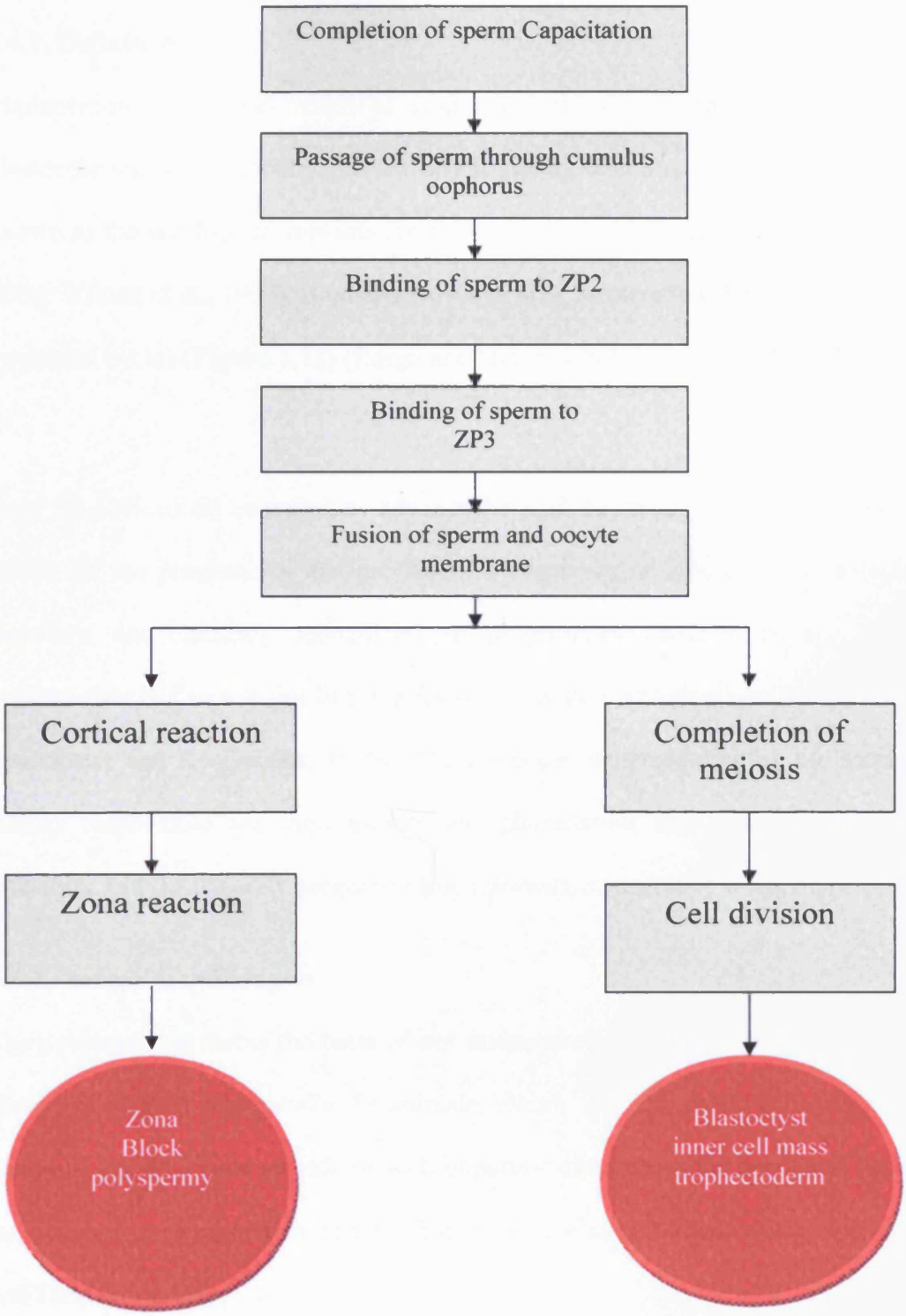


Figure 1.11. Graphical description of the fertilisation process. Adapted from: Speroff et al. Clinical Gynaecologic Endocrinology and Infertility, 2005.

1.4. Implantation and early pregnancy

1.4.1. Definition

Implantation is the end result of complex molecular interactions between a mature blastocyst and a hormonally primed uterus during a limited time in the cycle, which is known as the window of implantation (Norwitz et al., 2001; Diedrich et al., 2007; Lessey, 2000; Wilcox et al., 1999). It occurs 5-7 days after fertilization (Days 20 to 24 of a 28 day menstrual cycle) **(Figure 1.12)** (Bergh and Navot, 1992; Norwitz et al., 2001).

Only 50-60% of all conceptions advance beyond 20 weeks of gestation (Wilcox et al., 1988). Of the pregnancies that are lost, 75% represent a failure of implantation and are therefore not clinically recognized as pregnancies (Wilcox et al., 1988). Failed implantation is also a major limiting factor in assisted reproduction (Diedrich et al., 2007; Spandorfer and Rosenwaks, 1999). Thus, a better understanding of the mechanism and factors responsible for implantation and placentation may improve the outcome of infertility and reduce early pregnancy loss (Norwitz et al., 2001; Wang and Dey, 2006).

1.4.2. Normal implantation

The evidence that forms the basis of our understanding of the implantation process in the human is derived from studies in animals, mainly rodents, sheep and primates (Lee and DeMayo, 2004). These provide us with important clues about the hormonal, molecular and morphological changes that might occur in women prior to and during implantation (Lee and DeMayo, 2004).

Following fertilisation, the initial development of the pregnancy occurs in the fallopian tube with the fertilised ovum (zygote) developing into a mass of 12 to 16 cells (morula) by a process of mitosis (Croxatto et al., 1978; Norwitz et al., 2001). Two to three days after

fertilisation, the morula enters the uterine cavity. Next, the morula develops into the blastocyst by the establishment of a fluid filled cavity within the mass of cells **(Figure 1.12).** This is accompanied by a process of cellular differentiation with the outer cell mass (towards the surface) becoming the trophoblast that ultimately develops into the extra-embryonic structures such as the placenta, while the inner cells mass develops into the embryo. Up to this stage, the developing embryo is surrounded by the zona pellucida, a non-adhesive protective coating layer. Before the process of implantation can occur, the embryo needs to hatch from the zona pellucida. This process occurs some 5-6 days after fertilisation (conception) (Norwitz et al., 2001) and the blastocyst is now able to implant into the wall of the uterus.

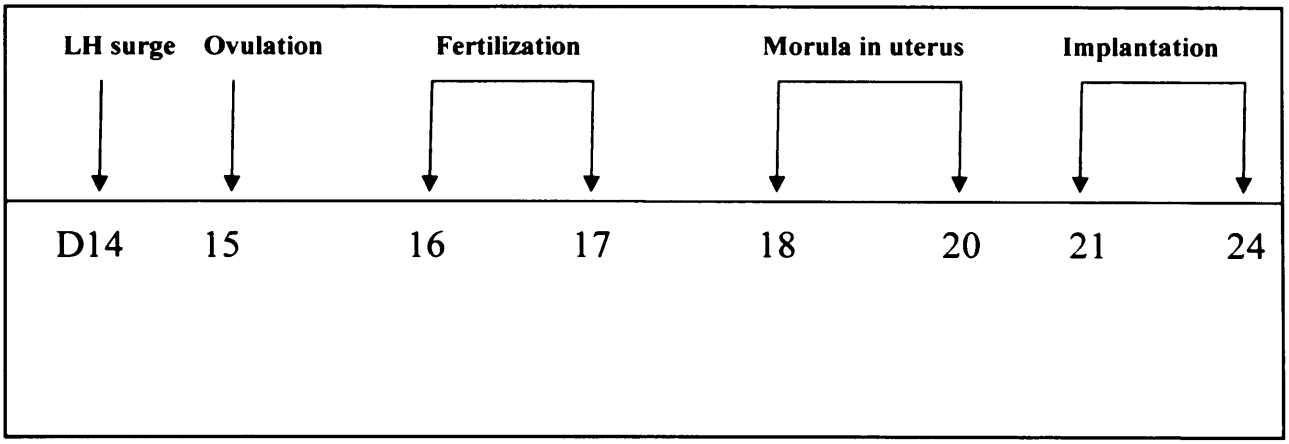


Figure 1.12. Sequence of events from ovulation, fertilisation, to the stage of implantation in the human. D= days of a normal menstrual cycle.

The process of implantation in humans commences 5-7 days after conception and is divided into three stages (Diedrich et al., 2007; Norwitz et al., 2001). During the first stage, called "apposition", the blastocyst aligns itself with microvilli on the apical surface of the syncytiotrophoblast inter-digitating with micro-protrusions from the apical surface of the uterine epithelium (pinopodes) (Nikas, 1999). The nature of this process is necessarily unstable to enable the blastocyst to find the most appropriate adhesion point for implantation. In the second stage, known as "stable adhesion", there is increased physical interaction between the blastocyst and the uterine epithelium through a series of molecular interactions that are poorly understood (Aplin and Kimber, 2004; Norwitz et al., 2001). In the third stage, known as "trophoblast invasion", the syncytiotrophoblasts produce digestive enzymes that allow the trophoblasts to penetrate through the uterine epithelium with the embryonic pole orientated towards the uterine epithelium. During the process of invasion the uterine epithelium is destroyed.

By the 10^{th} day post conception, the blastocyst is completely embedded in the stromal tissue of the uterus, the uterine epithelium has re-grown to cover the site of implantation and the cytotrophoblasts stream out of the trophoblast layer and begin the process of endometrial invasion, which extends into the inner third of the myometrium (interstitial invasion) and the uterine vasculature (endovascular invasion). These processes enable the trophoblasts to come into direct contact with maternal blood (Norwitz et al., 2001). Several chemical mediators are involved in these processes including prostaglandins, leukaemia inhibitory factor (LIF) and epidermal growth factors. Successful implantation is the end result of the complex molecular interactions that occur between the blastocyst and the hormone-primed uterus. Failure to synchronise the component processes involved in these interactions with a receptive uterus results in failed implantation and early pregnancy loss (Paria et al., 2002a; Norwitz et al., 2001).

1.4.3. Uterine receptivity

Uterine receptivity is defined as the state during the period of endometrial maturation during which the blastocyst can achieve implantation (Diedrich et al., 2007). From assisted reproductive experiments, the optimal time (window of implantation) has been found to be between days 20 and 24 in a regular 28 day menstrual cycle (Lessey, 2000; Sharkey and Smith, 2003).

The features of uterine receptivity include histological changes with the endometrium becoming more vascular and oedematous and the endometrial glands displaying enhanced secretory activity with the development of pinopodes on the luminal surface of the epithelium (Nikas, 1999; Sharkey and Smith, 2003).

Multiple signals synchronise the development of the blastocyst and the preparation of the uterus (Daftary and Taylor, 2001; Dimitriadis et al., 2005). Among these factors are sex steroid hormones. Implantation requires (a) a pre-ovulatory surge in 17β-estradiol, that stimulates the proliferation and differentiation of uterine epithelial cells and (b) progesterone produced from the corpus luteum after ovulation, which stimulates the proliferation and differentiation of stromal cells (Kodaman and Taylor, 2004). Several factors have been identified as potential markers of endometrial receptivity (Cavagna and Mantese, 2003; Dimitriadis et al., 2005). For example, the levels of LIF in both the luminal and glandular epithelium of the uterus rise dramatically in the mid-secretory phase of the menstrual cycle (Cavagna and Mantese, 2003) and decreased secretion of LIF has been found to be associated with recurrent pregnancy loss. Other molecules that are involved in the regulation of endometrial receptivity include adhesion molecules (Achache and Revel, 2006) and mucins which are proteins that have high sugar content and cause an increase in the expression of oligosaccharide receptors on the surface of the uterine epithelial cells.

The current evidence suggests that the blastocyst actively participates in the process of implantation (Paria et al., 2002a). Among the factors that enable the blastocyst to initiate implantation through a process known as "activation", are catecholestrogens, which are a class of estrogen metabolites. Evidence for signalling between the blastocyst and the uterus is derived from animal studies in the murine pregnancy (Wang and Dey, 2006). In the mouse, implantation can be delayed indefinitely by the manipulation of the hormonal milieu. During this delay, it was found that the expression of endometrial heparin-binding epidermal growth factor genes does not increase despite the blastocyst being positioned next to the uterine lining. However, when the estrogen is replaced by injection, the implantation process resumes with activation of the blastocyst associated with a rapid increase in the expression of endometrial heparin-binding epidermal growth factor genes at the site of apposition of the blastocyst (Das et al., 1994). To complete this process, embryos at or near the implantation site, express epidermal growth factor receptors and heparin sulphate proteoglycans, both of which interact with the endometrial heparin-binding epidermal growth factor-like ligands. Indeed, addition of heparin-binding epidermal growth factors to cultured embryos stimulates their proliferation and maturation (Das et al., 1994). It is considered that these findings are directly applicable to human pregnancy, as the addition of heparin-binding epidermal growth factor has similar effects on the human embryo *in-vitro* (Martin et al., 1998). Thus, the interaction between an activated blastocyst and a receptive uterus is part of a complex process that leads to successful implantation (Norwitz et al., 2001).

1.4.4. Factors associated with the regulation of the implantation and the maintenance of early pregnancy

The factors and molecular mechanisms that regulate uterine receptivity and embryo development in human are not well elucidated, and the current understanding of these processes comes primarily from research in rodents. The role of steroid hormones in implantation and early pregnancy maintenance are better understood than other molecules. However, there are several growth factors and cytokines that have been identified and appear to control multiple steps in the process of implantation **(Table 1.6).** For example, LIF appears to be important for both decidualisation and implantation (Dey et al., 2004). LIF is produced by both uterine glandular epithelial cells before implantation and stromal cells surrounding the active blastocyst at the time of implantation (Cavagna and Mantese, 2003).

The synthesis of prostaglandins is also essential for successful of implantation. Cyclooxygenase, the rate limiting enzyme in the synthesis of prostaglandin E_2, has two isoforms: a constitutive (COX-1) and an inducible (COX-2) isoform. Estrogen and progesterone reduce the production of COX-1 in the endometrium; hence COX-1 level is decreased in the mid-luteal phase of the menstrual cycle (coinciding with the period of increased uterine receptivity) in anticipation of implantation (Norwitz et al., 2001). On the other hand, COX-2 production, which is not affected by sex steroid hormones, is restricted to the site of implantation and depends on the presence of a blastocyst that is ready for implantation for its induction (Lim et al., 1999). In addition, interleukin-1, detected in the medium in which human embryos have been cultured (Sheth et al., 1991), induces the expression of COX-2 in stromal endometrial cells in culture (Huang et al., 1998). Prostaglandin I_2 (prostacyclin), produced by the actions of COX-2 is a ligand for the nuclear receptor peroxisome proliferator activated receptor δ (PPAR δ) (Lim et al., 1999),

which appears to be critical for implantation, as fetal mice lacking a related receptor (PPARγ) die in the middle of the gestational period because of defective placentation (Barak et al., 1999).

The exact molecular mechanisms involved in the regulation of the trophoblast of the endometrium are poorly understood (Tranguch et al., 2005; Diedrich et al., 2007). The temporal and spatial expression of several growth factors and cytokines within the uterus including LIF, interleukin-1 and its receptors, insulin-like growth factors I and II and their binding proteins, colony stimulating factor 1 and transforming growth factors α and β (Dimitriadis et al., 2005) suggests that they may all have important functional roles in the regulation of cytotrophoblast invasion. Physiological factors may also be important, for example oxygen tension promotes some aspects of trophoblast differentiation including the production of integrin $\alpha_1\beta_1$ (Genbaceva et al., 2001). The exact roles of these various factors are yet to be elucidated. However, endocannabinoids could be one the molecules that could be important for implantation and early pregnancy success (Maccarrone and Finazzi-Agro, 2004; Turco et al., 2008; Habayeb et al., 2008b).

Table 1.6. Factors associated with implantation and the maintenance of early pregnancy.

Factor	Example	Suggested Roles
Hormones	Estradiol, Progesterone	Promote proliferation and differentiation of endometrial stromal and epithelial cells
	Human chorionic gonadotrophin	Maintain progesterone release from corpus luteum
Cytokines and growth factors	Leukemia inhibiting factor; heparin-binding epidermal growth factor; hepatocyte growth factor, interleukin; vascular endothelial growth factor	Facilitate signalling between blastocyst and uterus; regulate endometrial prostaglandin production; promote endometrial invasion, proliferation, regulate vascular permeability and remodelling.
Changes in endometrial luminal epithelium	Pinopodes; alteration in adhesion-molecules and mucin expression	Facilitate blastocyst attachment ; promote trophoblast invasion
Immunological factors	Interleukin-10 HLA-G	Immunosupression Prevents immune recognition and rejection of semi-allograft
	Indoleamine 2,3-dioxygenase	Degrades tryptophan, which is essential for macrophage action
Trophoblast proteinases, inhibitors, and adhesion molecules	Matrix metalloproteinases-tissue inhibitor of metalloproteinases; cathepsin B and L; integrins	Regulate trophoblast invasion
Other factors	Cyclooxygenase-2	Regulate prostaglandin production
	Oxygen tension	Regulate the balance between trophoblast proliferation and differentiation

Source: Norwitz *et al.*, Implantation and the Survival of Early Pregnancy, 2001.

1.5. Endocannabinoids

1.5.1. Introduction

Endocannabinoids (endogenous cannabinoids) are unsaturated fatty acids derivatives widely distributed in the human body (Devane et al., 1992; Mechoulam and Hanus, 2000). Anandamide (*N*-arachidonylethanolamine; AEA), the most studied endocannabinoid, and other members of the family such as 2-arachidonylglycerol (2-AG), palmitoylethanolamine (PEA) are based on arachidonic acid (Di Marzo, 1998; Di Marzo et al., 1999) **(Figure 1.13)** the precursor of prostaglandins and luekotrienes (Habayeb et al., 2002).

Anandamide synthesis occurs on demand through the sequential actions of *N*-acyltransferase (NAT) and *N*-acyclphosphatidylethanolamine selective Phospholipase D (NAPE-PLD) (Piomelli et al., 2000), with the activities of NAPE-PLD being the rate-limiting step. On release into the interstitial space, AEA either diffuses into the lymph or blood or acts locally to affect cellular and tissue phenotypes (Beltramo et al., 1997). AEA binds to and activates cannabinoid receptors to produce its cellular effects (Habayeb et al., 2002) with the main cannabinoid receptors being CB1 and CB2 (Galiègue et al., 1995). Anandamide mimics some of the central and the peripheral effects of Δ^9-tetrahydrocannabinol (THC), the psychoactive component of marijuana (Childers and Breivogel, 1998).

In the human, the CB1 receptor is the main receptor in the central nervous system (Gonzalez et al., 1999; Wenger et al., 1999a) and is expressed in other peripheral tissues such as the adrenal gland, (Liu et al., 2002) spleen, retina (Porcella et al., 2000), heart, lungs, bone marrow, thymus, tonsils, uterus, placenta (Kenney et al., 1999), testis (Gerard et al., 1991), prostate and ovaries (Galiègue et al., 1995). In contrast, the CB2 receptor, which is seldom expressed in the brain, is abundant in immune tissues (Parolaro et al.,

2002) such as the spleen, tonsils, thymus, bone marrow, B-cells, natural killer cells, monocytes, polymorphic nuclear cells, neurtrophils and T8- and T4-positive T-cells (Galiègue et al., 1995). AEA is degraded to arachidonic acid and ethanolamine by a specific fatty acid amide hydrolase (FAAH) enzyme (Cravatt et al., 1996; Giang and Cravatt, 1997). Together AEA, cannabinoid receptors, FAAH, NAT and NAPE-PLD form the endocannabinoid system (Sugiura et al., 2002).

1.5.2. The role of Anandamide in human folliculogenesis

Most of our knowledge about the role of cannabinoids in human ovaries comes from studies that have examined the effect of the psychoactive component of marijuana, Δ^9-THC on the ovary. Clearly animals studies and studies done in marijuana users have demonstrated that Δ^9- THC affects ovulation through a direct damaging effect on the ovary (Adashi et al., 1983) and indirectly by altering the hypothalamic pituitary axis. Δ^9- THC also suppresses the preovulatory surge of LH in female rats leading to anovulation (Field and Tyrey, 1984). This suppression is reversed by GnRH administration, suggesting that this effect may be primarily due to hypothalamic inhibition of GnRH secretion (Ayalon et al., 1977). The effect of endocannabinoids on the hypothalamic-pituitary-ovarian axis is thought to be similar to that of Δ^9- THC (Reich et al., 1982). However, AEA has only been quantified in the follicular fluid during egg collection in IVF women (Schuel et al., 2002) with the suggestion that AEA may play a role in oocyte maturity. A definitive role for AEA in human ovaries has, however, never been examined.

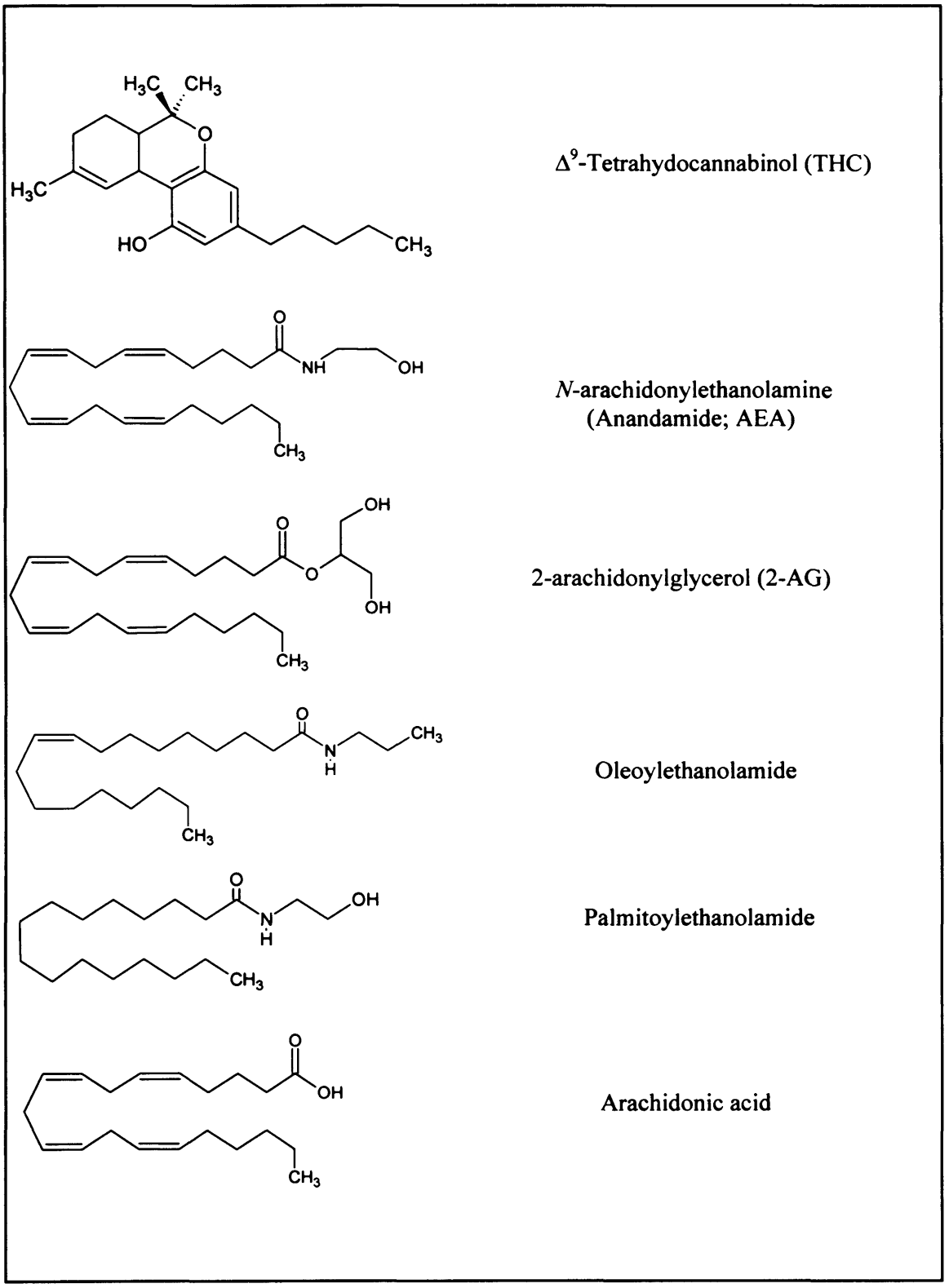


Figure 1.13. The chemical structures of the cannabinoids; Δ^9-THC, AEA , 2-AG, PEA, OEA. All are derivatives of arachidonic acid (structure shown).

1.5.3. The possible role of AEA in fertilisation

Our knowledge of the role of AEA in fertilisation comes mainly from the sea urchin model. In this model, fertilisation is inhibited because AEA blocks the acrosome reaction which is an essential step in egg and sperm fusion (Schuel et al., 1994; Schuel and Burkman, 2005). In the human, CB1 receptors have been detected in the sperm head and midpiece (Rossato et al., 2005). This distribution of receptors probably reflects the role of AEA in the regulation of sperm motility and the acrosomal reaction (Schuel and Burkman, 2005). The presence of AEA in human seminal plasma and oviductal fluids (Schuel et al., 2002) suggest that AEA signalling may regulate sperm capacitation and the fertilisation potential in human sperms (Schuel and Burkman, 2006). Also, AEA has been implicated in oviductal motility (Schuel, 2006) and embryo transport in mice, as CB1 deficient mice retained large numbers of embryos in their oviducts (Wang et al., 2004).

1.5.4. AEA and implantation, and early pregnancy

The role of cannabinoids and the endocannabinoid system in implantation and early pregnancy is better understood than that in folliculogenesis and fertilisation (Battista et al., 2008), mainly because there have been more studies on these aspects. Previous studies have shown that smoking marijuana adversely affects implantation, fetal growth and development (Fergusson et al., 2002; Park et al., 2004). CB1 and CB2 receptors have been detected in the human uterus (Galiègue et al., 1995). CB1 receptors have shown high levels of expression in the uterus where the highest levels of AEA in the mouse have also been measured (Das et al., 1995). It has also been demonstrated that FAAH and NAPE-PLD (the enzymes that regulate AEA concentrations) are expressed in the mouse uterus and peri-implantation embryos (Paria et al., 1996; Klinger et al., 2006; Wang et al., 2006).

Regional differences in the levels of AEA in the uterus determine potential implantation sites; low levels of AEA are associated with uterine receptivity (Paria et al., 2002b), while high levels are correlated with failed implantation (Schmid et al., 1997). Also, low levels of AEA are essential for embryonic development (Piomelli, 2004); for example, in the murine *in-vitro* embryo culture model, levels of AEA above 28 nM resulted in embryonic death, while embryo exposed to 7 nM survived and progressed to the blastocyst stage (Schmid et al., 1997; Maccarrone and Finazzi-Agro, 2004). Since Maccarrone *et al.* also found that high levels of AEA and low levels of FAAH in women having IVF and ET are associated with increased risk of miscarriage (Maccarrone et al., 2002b), it seems logical to conclude that in the human low levels of AEA may be important for early pregnancy maintenance as recently demonstrated by Habyeb et al., (Habayeb et al., 2008b).

1.6. Conclusions

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

1.7. Hypotheses

From both animal and human data, it is hypothesized that there is an association between endocannabinoids such as anandamide and reproduction. The aim of the studies in this thesis was to investigate the roles of AEA in human folliculogenesis, oocyte maturity, in human implantation and early pregnancy.

The key hypotheses tested are that:

1. AEA concentration in follicular fluid would be predictive of follicle and oocyte maturity.
2. plasma AEA levels are modulated by sex steroid and gonadotrophin hormones in natural and stimulated cycles.
3. the endocannabinoid system is present and active in the ovary.
4. the plasma AEA concentration in women undergoing IVF/ICSI-ET differs between those with successful and ongoing pregnancies and those who miscarry.

CHAPTER 2

Measurement of Anandamide

2.1. Introduction

The aim of this part of the thesis was to develop a robust and reproducible assay technique for the extraction and measurement of anandamide (AEA) from human biological fluids (plasma, follicular fluid, urine, and saliva) and cell culture media.

A review of the literature indicated that anandamide had previously been measured in human plasma and in follicular fluid but not in urine or saliva (Schuel et al., 2002; Habayeb et al., 2004; Vogeser et al., 2006). One of the difficulties anticipated prior to starting this research was that obtaining 3 or 4 samples of blood from each volunteer might reduce recruitment and increase drop-out rates, because taking blood is an invasive and relatively painful procedure when compared to the provision of repeated urine or saliva samples. Therefore, the idea of quantifying AEA from urine and saliva was very appealing and worth investigating.

Although, AEA had been measured in human plasma, follicular fluid, seminal fluid, breast milk, and oviductal fluid using high liquid chromatography mass spectrometry (HPLC-MS) technology (Schuel et al., 2002; Habayeb et al., 2004), those studies had not been repeated and showed sample analysis times of $\geq$ 30 minutes. The recent demonstration that ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) could be used in quantifying AEA in rat brain and human plasma with reduced analysis times to 7-15 minutes, suggested that this newer technology could be applied to the studies in this thesis (Fernandez-Rodriguez et al., 2004; Vogeser et al., 2006). The advantages of this method over the others are that it is fast, extremely sensitive and precise in measuring AEA, which should therefore allow for the accurate analysis of a large number of samples in a relatively short time. Therefore, modifications were made to the existing AEA

quantification method using an ultra performance liquid chromatography tandem mass spectrometry system (ULPC-MS/MS) and the method evaluated.

2.2. Methods

2.2.1. Preparation of calibration curves

Anandamide (AEA) and octa-deuterated anandamide (d_8-AEA) were obtained from Cayman Chemicals (Ann, Arbor, MI, USA). The purity of d_8-AEA is $\geq$ 95% (Cayman Chemical product datasheet, 2008).

AEA and d_8-AEA as supplied were dried down under nitrogen gas and reconstituted in acetonitrile (ACN) as stock solutions at 5 mg/ml and 100 µg/mL, respectively. The stock solutions were then stored as 100µM aliquots for single use at -20°C. Further ice-cold dilutions were made in acetonitrile on the day of analysis.

1. Standard calibration curves consisting of 7 points were created with each standard point (S1-S7) represented by variable concentrations of AEA and fixed amount of d8-AEA (**Table 2.1**). For calculating the concentrations of AEA from samples the standard calibration curve was used.
2. S1-S7 were made by adding 190 µl of each diluted AEA standard to 10 µL of 0.25 pmol/µL of d_8-AEA.
3. The resulting 200 µL solution was mixed on a vortexer for 10 seconds and transferred to glass vials which were placed in the ULPC machine.
4. To increase assay accuracy, three calibration curves were prepared on the day of the sample analysis, and the mean result of three injections of the three curves used to calculate the concentrations of AEA in the unknowns.
5. Not more than 10% difference in calibration curve results between the days was accepted.

Table 2.1. Preparation of AEA standards S1 to S7.

	Final AEA Concentration (nM)	Amount of AEA (µL)	Amount of ACN (µL)
S1	20	200 µl of 100 nM	800 µL
S2	10	500 µl of 20 nM	500 µL
S3	5	500 µl of 10 nM	500 µL
S4	2	400 µl of 5 nM	600 µL
S5	1	500 µl of 2 nM	500 µL
S6	0.5	500 µl of 1 nM	500 µL
S7	0.25	500 µl of 0.5 nM	500 µL

2.2.2. ULPC-MS/MS

The UPLC-MS/MS system (**Figure 2.1**) comprised of an Acquity UPLC system connected inline to a Waters Quattro Premier Tandem mass spectrometer (Waters Ltd., Elstree, UK). The column utilised was an Acquity UPLC BEH (Bridged Ethyl Hybrid) C_{18} (2.1 x 50 mm, 1.7μm) maintained at 40°C in a heated compartment. The mobile phases used were Buffer A (2 mM ammonium acetate containing 0.1% formic acid) and buffer B (acetonitrile containing 0.1% formic acid). The liquid chromatography gradient conditions were as follows: 80% A for 0.5 min changing to 0% A for the next 1.0 min, returning to 80% A over the next min. The column was then re-equilibrated at 80% A until 3.5 min had elapsed, at which point the next sample was injected. Samples were maintained at 4°C throughout.

The analytes were quantified using tandem electrospray mass spectrometry in positive ion mode (ES^+) with the capillary voltage set at 0.6 kV, the cone voltage at 18 V, a source temperature of 120°C, a desolvation temperature of 440°C, a cone gas flow of 49 L/h, and desolvation gas flow of 800 L/h. The MS/MS conditions for monitoring each precursor $[M+H]^+$ ion comprised entry, collision and exit energies of -2, 17 and -17eV, respectively.

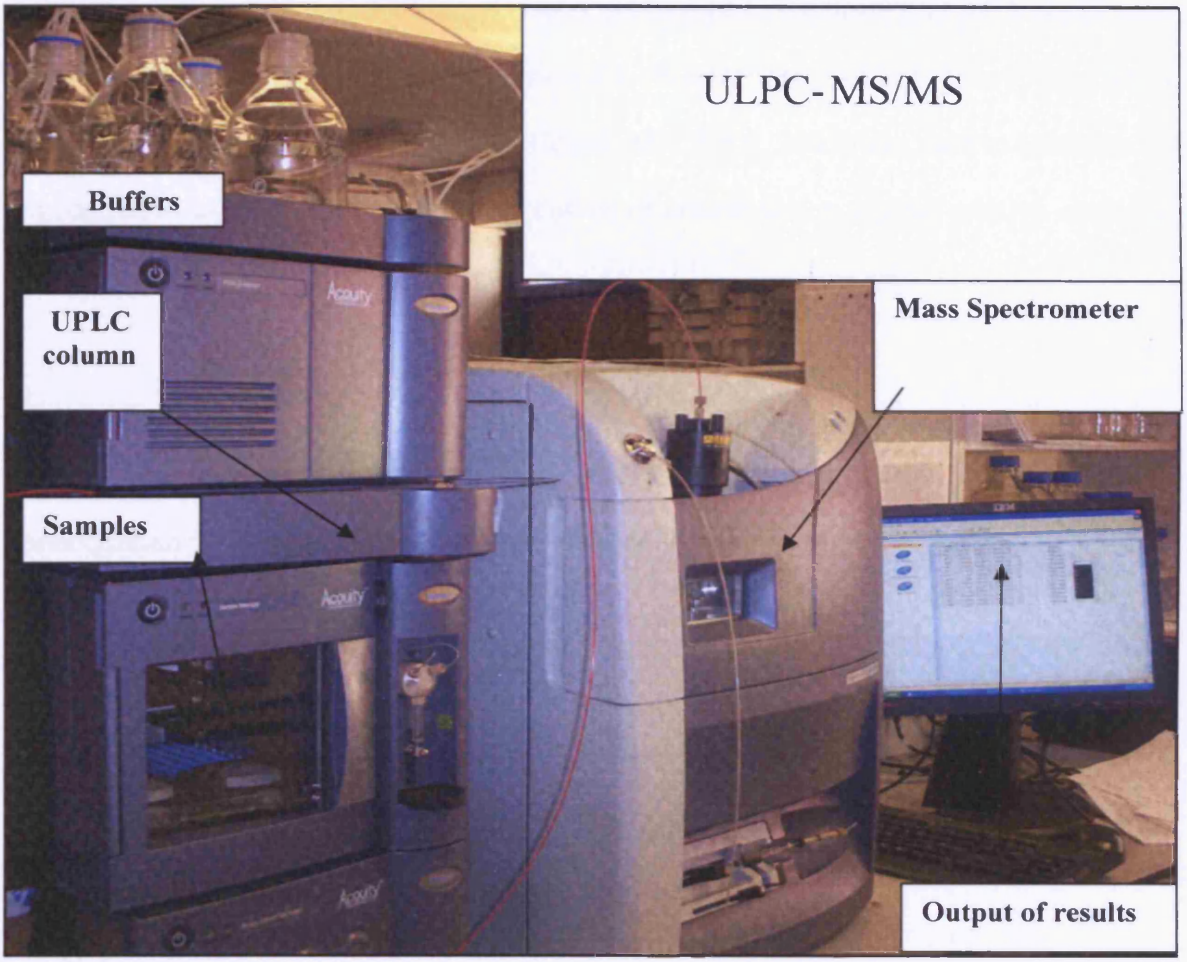


Figure 2.1. ULPC-MS/MS. The system comprises of buffers, the Acuity Liquid Chromatography system (dark grey boxes on left side of the image), a heated UPLC column, a chilled sample chamber, the double chambered mass spectrometers and a computerised system for analysis and output of results.

Product ions were monitored in multiple reactions monitoring (MRM) mode and the injection volumes for samples and standards were 7 μL with needle overfill. The calibration curves and samples analyses were performed in triplicate and AEA peaks were integrated using Masslynx software version 4.1 (Waters Inc., Milford, MA, USA, 2005), whilst Quanlynx software (Waters Inc. Milford, MA. USA, 2005) was used to calculate the concentration of AEA using calibration curves of concentration against relative response, calculated as follows:

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

Where *IS area* = the peak area of the AEA-d_8 internal standard, [*AEA-d8*] = the concentration of the internal standard, and *Peak area* = the peak area of AEA.

The strongest MRM transitions found for AEA and AEA- d_8 were m/z 348.3 > 62.3 and m/z 563.3 > 63.3, respectively. Represenatative spectra of the parent ion and daughter ion scans for AEA and AEA-d_8 are presented in **Figure 2.2.**

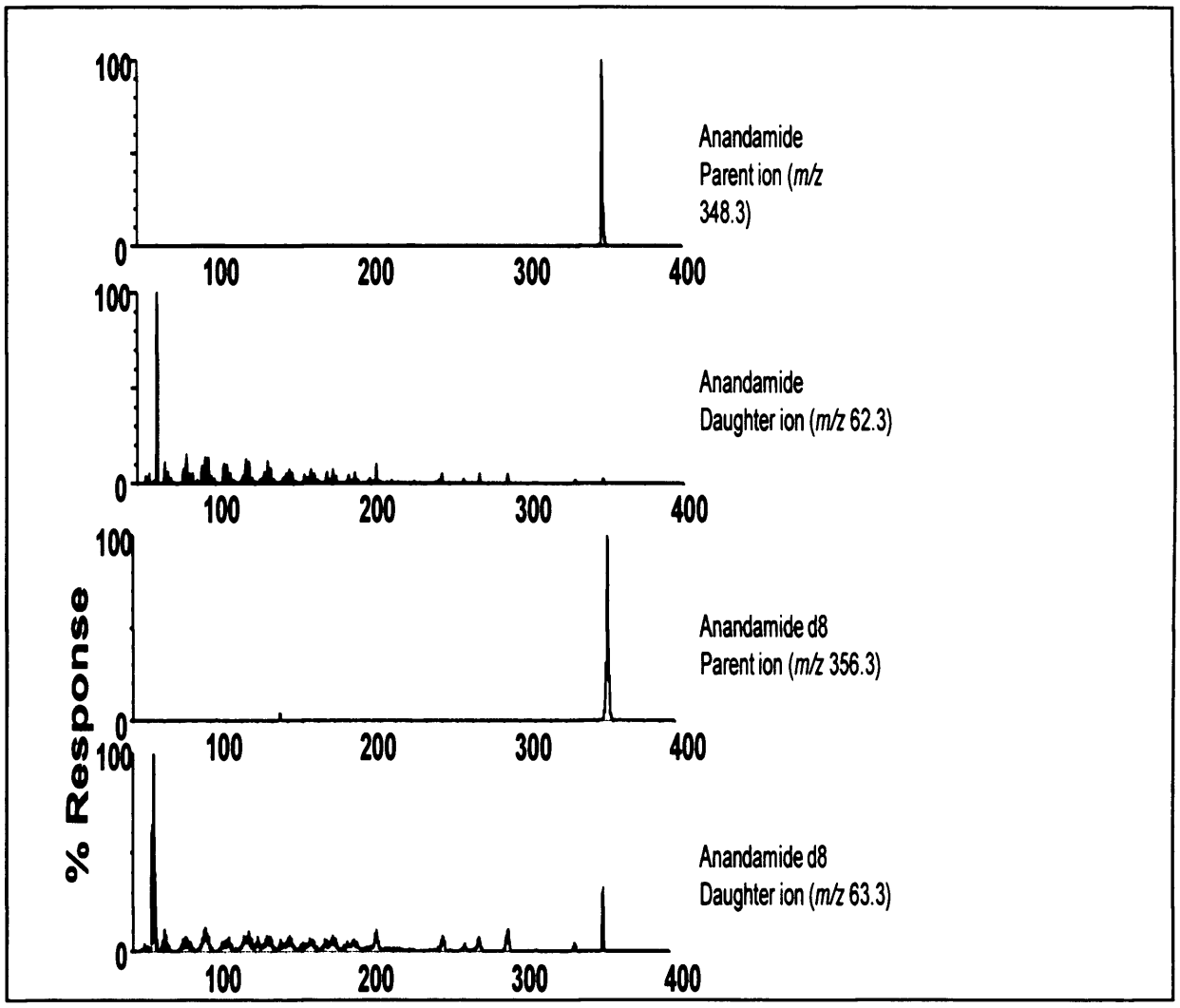


Figure 2.2. Parent and daughter ion mass spectra for AEA and AEA-d_8.

2.2.3. Validation of UPLC-MS/MS method

To determine the robustness and accuracy of AEA measurements, several UPLC analytical parameters such as the consistency of the retention time, the accuracy (the deviation of observed concentration from expected) of reported concentrations and precision (repeated injection) were investigated with 20 injections of 19 nM, 0.95 nM and 0.237 nM AEA standards in acetonitrile (133 fmol, 6.65 fmol and 1.66 fmol AEA on the column).

Linearity for the assay was determined on 7 seven-point standard curves (1.66 to 133 fmol on column; 0.237 nM to 19 nM AEA) using linear regression analysis. The limits of quantification and detection were defined as AEA responses which yielded a signal to noise ratio, without smoothing, of >10 and >3, respectively and were calculated for extraction of AEA-d_8 from both plasma and from saline. The limits of quantification for AEA were determined only from saline because of the presence of endogenous AEA in plasma samples.

2.2.4. Blood, follicular fluid, culture medium, urine and saliva collection

2.2.4.1. Blood

Venous human blood was collected from veins in the antecubital fossa after the application of an appropriate tourniquet into an evacuated EDTA containing collection tube (Sarstedt Ltd, Leicester, UK). A total volume of 4 mL was collected and the blood placed on ice for transport to the analytical laboratory where the blood was transferred into 15 mL polypropylene conical tubes (Becton Dickenson Labware Europe, France) and immediately centrifuged at 1200 x g for 30 minutes at 22°C to separate blood cells from plasma.

2.2.4.2. Follicular fluid and culture medium

Follicular fluid was obtained from women undergoing oocyte collection for IVF/ICSI-ET in the Assisted Conception Unit. Oocyte retrieval was performed, after the administration of light sedation with Fresenius Propoven 1% (Fresenius Kabi Ltd, Cheshire, UK), Alfentanil Hydrochloride (Janssen-Cilag Ltd, Buckinghamshire, UK), and Midazolam (Roche, Hertfordshire, UK). Aspiration of the follicular fluid was done under transvaginal ultrasound guidance using a Siemens USS (Siemens, Surrey, UK), needle and suction system, as seen in **(Figure 2.3)**. After visualisation of the target follicle, the aspiration needle (17 Gauge x 900mm; Rocket Medical PLC, Washington, UK) was inserted into the follicle and follicular fluid aspirated into 14 mL polystyrene tube. The follicular fluid was immediately examined by the embryologist for presence of oocytes, which was then removed with a Pasteur pipette (Poulten & Graf Ltd, Barking, UK), and the remaining follicular fluid transferred to 7 mL Kimble scintillation vials on ice.

To determine the concentrations of AEA in Sydney IVF Fertilization Medium (Cook Ireland Ltd., Ireland) and Sydney IVF embryo cleavage culture medium (Cook Medical, Limerick, Ireland) samples obtained prior to and after 16 hours incubation with sperm and egg from ICSI patients were assayed. Samples of embryo cleavage culture medium, before embryo incubation and on the day of embryo transfer (48 hours after oocyte retrieval) were also assayed. Samples were placed in Kimble scintillation vials and centrifuged at 1200 x g at 22°C for 30 minutes

2.2.4.3. Saliva

Saliva was collected by using the Salivette system (Sarsted Ltd, Leicester, UK), which consists of a cotton piece and plastic collecting tube. Volunteers were asked to insert the cotton piece in their buccal cavity for 5 minutes to thoroughly wet the cotton piece with

saliva. After returning the cotton piece to the plastic tube, the saliva was collected at the bottom of plastic tube by centrifugation at 1200 x g at 22°C for 30 minutes **(Figure 2.4)**.

2.2.4.4. Urine

Mid-stream urine samples were collected directly into 25 mL polycarbonate tubes in the absence of preservatives **(Figure 2.4)**. Any cells or debris were collected by immediate centrifugation at 1200 x g at 22°C for 30 minutes.

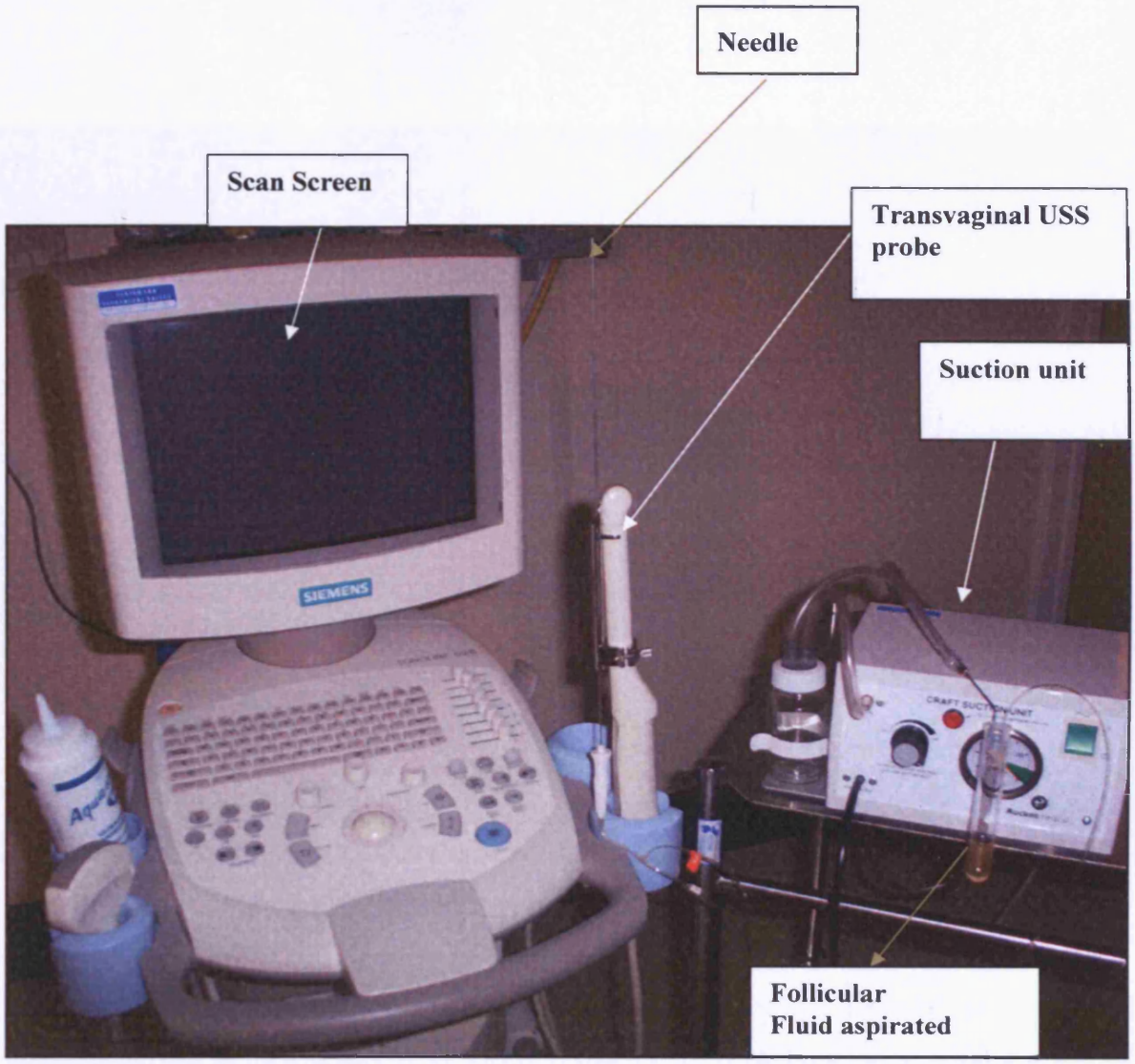


Figure 2.3. Transvaginal needle system used in ovarian follicles aspiration. The system consists of an ultrasound machine (seen to the left), transvaginal USS probe to which a needle guide is attached. This is in turn connected by sterile tubing to a vacuum unit (suction unit) that is used to retrieve the contents of the follicle that is visualised on the scan screen.

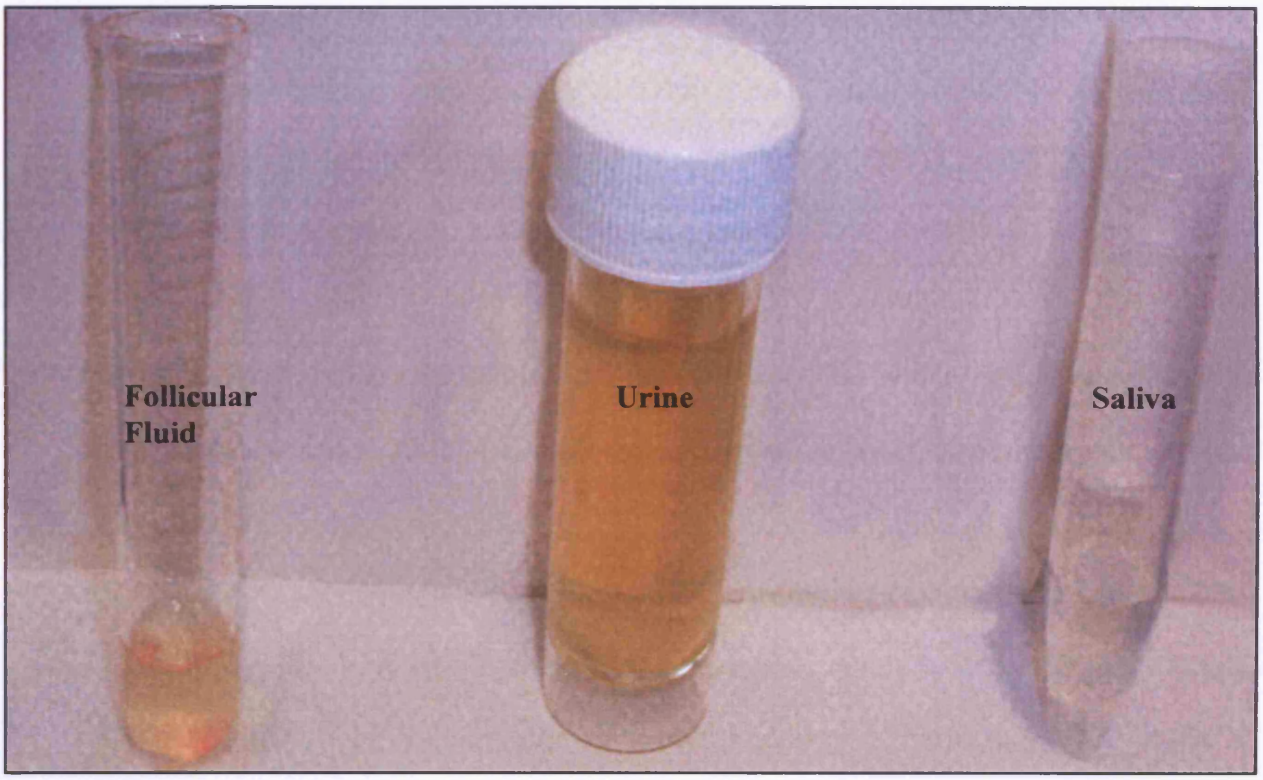


Figure 2.4. Biological fluids at the time of samples collection. Each of the biological fluids is shown in their respective collection tubes after centrifugation at 1200 x g for 30 min to sediment cells and particulate material. Cells are seldom seen in the urine sample and insoluble material is retained on the cotton swab inside the salivette tube.

2.2.4.5. Extraction of AEA from biological fluids using the liquid phase extraction method

AEA was extracted from plasma, follicular fluid, saliva, urine and culture medium used for fertilisation and embryo cleavage using a modification of a liquid phase extraction method as described by Habayeb *et al.*, and Giuffrida *et al.*, (Giuffrida et al., 2000, Habayeb et al., 2004, Lam et al., 2008).

All biological fluids were transported on ice to the analytical laboratory and centrifuged at 1200 x *g* for 30 min at 22°C to remove cells and debris. Plasma, follicular fluid, urine or saliva (2 mL) was transferred to a fresh 7 mL Kimble scintillation vial (Kinesis, St Neots, and Cambridge, UK). For the purpose of determining extraction efficiency, 2.5 pmol AEA-d_8 internal standard were added prior to thorough mixing with a desktop vortexer. Protein was precipitated by the addition of 2 mL ice-cold acetone followed by centrifugation at 1200 x *g* for 10 min at 4°C and the supernatant transferred to a fresh Kimble scintillation vial and the acetone evaporated under a gentle stream of nitrogen gas. Lipid extraction was then performed on the remaining mixture by the addition of 2 mL methanol: chloroform (1:2 v/v) followed by gentle mixing by repeated inversion. The samples were centrifuged at 1200x g for 10 minutes at 4°C and the lower chloroform layer was recovered into a fresh Kimble vial and dried under a gentle stream of nitrogen before reconstitution in acetonitrile (80 µL).

2.2.4.6. Extraction of AEA from biological fluids using the solid phase extraction method

The liquid phase extraction procedure suffers from two limitations: time taken and the amount of sample required, i.e. 3.5 hours to process a minimum of 2 mL of sample. To improve the extraction procedure a solid phase extraction method which uses the same analytical column material was utilised since it is reported to be easier, faster and requires a smaller volume of fluid (0.5 mL) compared to the liquid phase extraction method (2 mL), which also requires a steady supply of Kimble vials that are a special manufacture. Therefore, the solid phase extraction method was compared with the liquid phase extraction procedure for follicular fluid and plasma from a small number of women (2% of all samples).

The solid phase extraction method steps were as follow:

1. Plasma or follicular fluid (0.5 mL) was mixed with 20 μL of d_8-AEA internal standard (0.0627 pmol/μL) and 0.5 mL of de-ionised water in a microfuge tube.
2. The solution was vortexed for 10 seconds and centrifuged for 5 minutes at 13,000 rpm to pellet insoluble material and then placed on ice.
3. Meanwhile an Oasis HLB 1 cc cartridge (Waters Corporation) placed in the vacuum manifold and pre-conditioned by the addition of 1ml of methanol at rate of 1ml/minute by applying a vacuum (5 mm of Hg).
4. The column was conditioned for samples by passing 1 mL of de-ionised water through the column at 1 mL/minute.
5. The dilute plasma/ AEA-d_8 mix was added to the cartridge at a rate of 1 mL/min and the non-adsorbent material washed off the column with 1 mL of 40% methanol into a collection tube.
6. The waste fluid was then discarded and replaced with clean collection tubes.

7. The AEA was eluted from the cartridges by adding 1mL of acetonitrile at a rate of 1 mL/min.
8. The solution was dried under nitrogen gas and reconstituted in 80 µL of acetonitrile, vortexed for 30 seconds and injected into the UPLC-MS/MS.

2.2.4.7. Effect of sample volume on anandamide measurement

The standard AEA extraction with liquid phase method was based on a 2 mL plasma volume. Occasionally, it was not possible to obtain 2 mL of biological fluid for analysis; i.e. taking 4 mL of blood was sometimes difficult and the volume of aspirated follicular fluid could be as small as 300 µL, so the standard extraction method needed modification for smaller volumes. Since that needed validation, a series of experiments were designed to examine the effect of sample volume on the final AEA measurement. The groups taken were:

a. Small and normal volumes of plasma:

   Two and 4 mL of blood collected in EDTA tubes, centrifuged for 30 minutes at 1200 x g and 1 mL or 2mL of plasma reserved on ice. AEA was extracted using the liquid phase extraction method with additions reduced by 50% for the 1 mL plasma samples.

b. Small and normal volumes of follicular fluids:

   The follicular fluid from large follicles were centrifuged for 30 minutes at 1200 x g and 2 mL, 1 mL, 0.75 mL, 0.5 mL and 0.25 mL volumes transferred to Kimble vials on ice. For volumes <1 mL, physiological saline or de-ionised was added to increase the total volume to 2 mL. AEA was then extracted using the liquid phase extraction method.

c. Sydney IVF Fertilisation and embryo cleavage culture media:

These media which are used to fertilise eggs and also during embryo cleavage contain human serum albumin in addition to glucose, proteins, and various essential salts (Cook Medical, Limerick, Ireland). For the measurement of AEA, 2 mL of the culture medium prior to incubation with sperm and egg or prior to incubation with embryos was transferred to Kimble vials on ice. The Assisted Conception Unit's usual practice is to keep each sperm and egg in a 20 μL volume of culture medium, for example in a woman with 6 eggs having ICSI treatment in total the volume of culture medium used will be 120 μL. Therefore, the amount of media was measured and adjusted to 1 mL with deionised water and half the amount of AEA-d_8 added. All samples of $\leq$ 2 mL volume were extracted using the liquid phase extraction method.

2.3. Statistical analysis

All the data were normally distributed (Shapiro-Wilk test; Prism version 5 software, GraphPad Inc., San Diego, California, USA, www.graphpad.com) therefore, parametric tests were used. Student's unpaired *t*-test was used to compare the variances between 2 groups. A P-value of <0.05 was considered to be statistically significant.

2.4. Results

2.4.1. UPLC-MS/MS and calibration curves

Both d_0-AEA and d_8-AEA were eluted from the UPLC and detected by MS/MS with a mean retention time of 1.67 ± 0.0009 min after injection. Retention time was thus consistent over the lifetime of the column which represented in excess of 9000 injections. Regression analysis of seven calibration curves was described by the equation: Response (y) = (2.44 ± 0.19) [d_0-AEA] nM + (0.06 ± 0.23*)* with a mean r^2 value of 0.999 (**Table 2.2**). The measurement of anandamide using the seven standards S1-S7, indicated that the calibration curves were linear from below and above the levels reported previously for plasma and follicular fluid (**Figure 2.5**) (Schuel et al., 2002, Habayeb et al., 2004) and showed inter-assay linearity (**Figure 2.6**). There was no statistical difference between the calibration curve parameters (**Table 2.2**) over the duration of the project with the % coefficient of variation of the slope mean being only 8.07%. The inter-assay coefficient of variation for 20 injections at the 0.24 nM (1.66 fmol) level was 3.9 % and at the 0.95 nM (6.65 fmol) level was 4.8%. The intra-assay coefficient of variation at the 0.24 nM level was 3.7% and at the 0.95 nM level was 2.1%. The limit of quantification (LOQ; a signal to noise ratio >10) of AEA was 0.22 fmol on the column (0.092 nM) and the limit of detection (LOD; a signal to noise ratio=3) was 0.055 fmol on the column (0.0079 nM). The recovery from plasma was efficient (19%) with an interday and intraday variability of 2.9% and 12.6%, respectively. The LOD from and LOQ from five plasma samples were 18.75 pM and 25.0 pM, respectively (Marczylo et al., 2009). Furthermore, this method represents marked improvements in LOQ, LOD, precision, and accuracy when compared with previously published methods **(Table 2.3).**

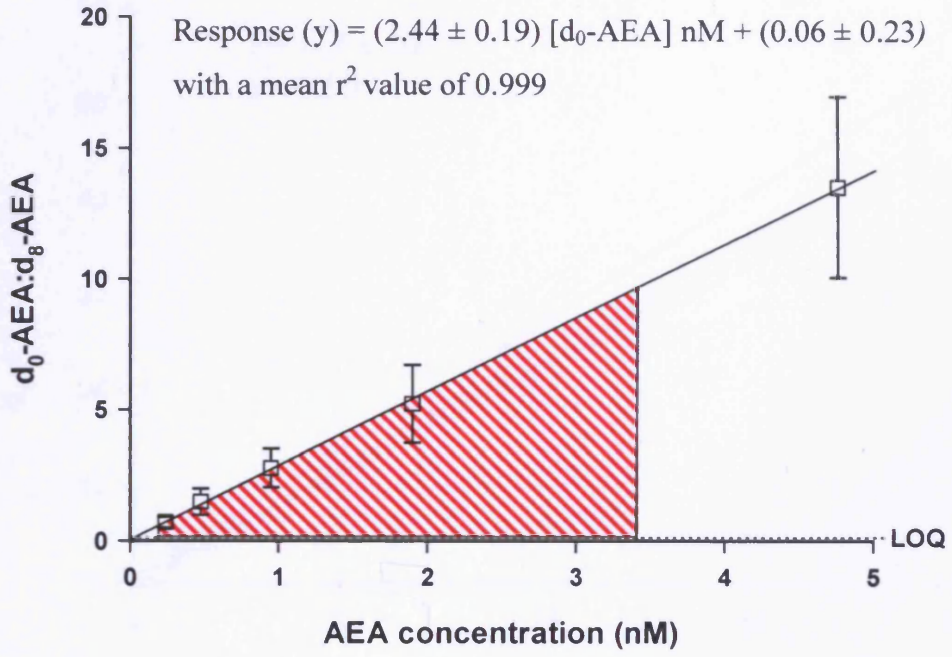


Figure 2.5. The standard curve for the measurement of AEA. The shaded area represents the AEA concentrations of tested plasma and follicular fluid samples. The x-axis represents the concentration of AEA in nM on the column while the y-axis is the ratio between AEA peak and d_8-AEA peak.

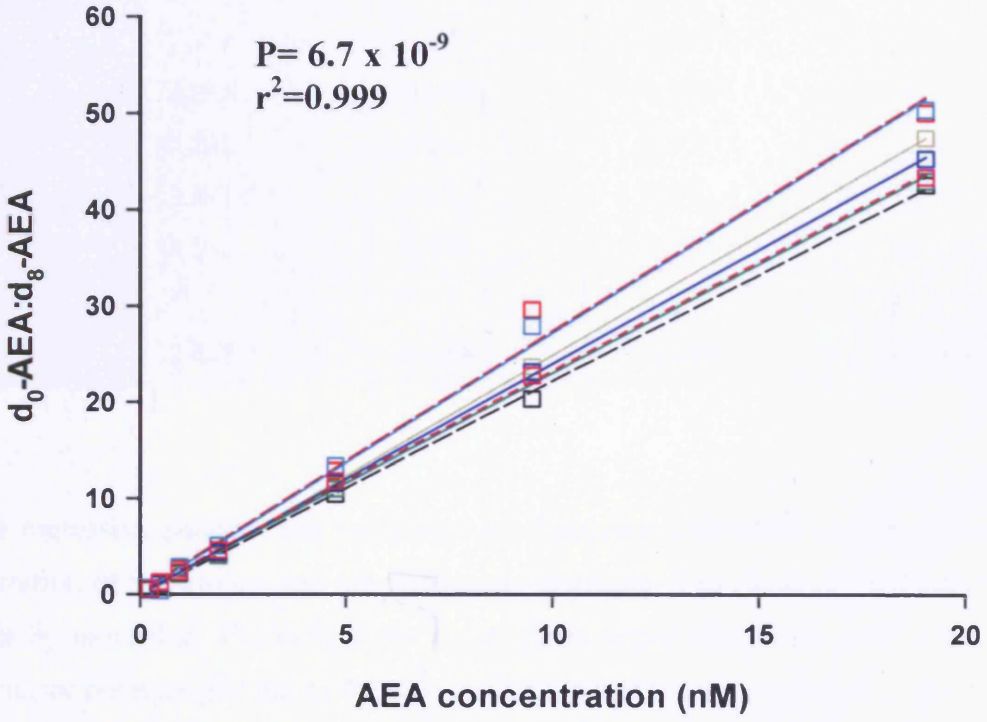


Figure 2.6. Seven random calibration curves collected over the duration of the project shows inter-assay linearity. There was no statistical difference between these calibration curves.

Table 2.2. Summary of AEA calibration curves.

Curve number	Slope	Intercept	R^2	P-value
1	2.229	-0.112	0.999	2.4×10^{-9}
2	2.707	0.303	0.992	1.8×10^{-6}
3	2.517	-0.330	0.999	4.5×10^{-11}
4	2.293	0.090	0.998	2.8×10^{-8}
5	2.702	0.171	0.996	2.2×10^{-7}
6	2.397	0.025	0.999	7.3×10^{-11}
7	2.292	0.327	0.999	5.1×10^{-9}
means	**2.448**	**0.068**	**0.999**	**6.7×10^{-9}**

Linear regression analysis was performed on 7 random calibration curves collected over the duration of the project and the parameters tabulated. The slope of each curve did not deviate by more than 10.6% from the mean. There was a significant correlation between the detector response for the d_0-AEA:d_8-AEA ratio and the amount of AEA standard used to make the calibration curves.

Table 2.3. Comparison of the UPLC-MS/MS AEA measurement method with data obtained from two recently described LC-MS/MS methods.

	Richardson et al	Vogesser et al	UPLC-MS/MS
Range	25 fmol-250 pmol[c]	0.069-4.32 pmol[c]	1.66-133 fmol[c]
Linearity	$R^2 = 0.992$	n.d.	$Y=2.48 \pm 0.14$ $+ (0.44 \pm 0.04)$ $R^2 = 0.999$
Precision (RSD)	13.6% (20 pmol/g)	6.8% (180 fmol [c]) 5.3 (1097 fmol [c])	3.9% (1.66 fmol[c]) 4.8% (6.65 fmol[c]) 3.7% (133 fmol)
LOQ **LOD** **Accuacy**	10 pmol/g [b] 25 fmol[c] 65-155%	n.d.	25.0 fmol/mL plasma 18.75 fmol/mL plasma 97.5 ± 9.5% (3.33 fmol[c]) 98.5 ± 6.1% (6.65 fmol[c]) 104.5 ± 3.2 (133 fmol[c])
Run time (min)	15	7	4

[a] Represents data for extraction from brain tissues. n.d. = not determined.

[b] Rat brain tissue.

[c] Represents values as mass of AEA on Column.

2.4.2. AEA measurements with liquid phase and solid phase methods

There was no statistically significant difference between the two methods of AEA extraction from plasma or follicular fluid using solid and liquid phase extraction; (P= 0.715) (**Tables 2.4 and 2.5**). AEA was extracted from plasma and follicular fluid in significant amounts but it was undetectable in saliva and urine **(Table 2.6)**.

Table 2.4. Comparison of the effect of liquid or solid phase extraction methods on plasma AEA levels.

Plasma AEA levels (nM)			
Volunteers	**Liquid phase**	**Solid phase**	**%Difference**
V1	1.215	1.230	1.23
V2	1.907	1.821	-4.51
V3	1.162	1.184	1.89
V4	0.982	0.813	-17.21
V5	0.782	0.594	-24.04
V6	1.665	1.339	-19.57
V7	0.691	0.643	-6.94
V8	1.782	1.512	-15.15
V9	0.792	0.730	-7.83
V10	0.420	0.406	-3.33
V11	0.661	0.720	8.92
V12	0.828	0.901	8.82
V14	1.082	1.299	20.05
V15	2.069	1.799	-13.05
V16	1.368	1.310	-4.24
V17	0.932	1.243	33.36
V18	1.544	1.522	-1.42
V19	1.670	1.489	-10.84

The AEA levels in 2 mLof plasma taken from 15 female and 3 male volunteers (V17-V19) and extracted by the liquid phase extraction method were compared to levels from 0.5 mL of plasma extracted using the solid phase extraction method. An apparent increase and decrease in plasma AEA levels are depicted with a (+) and (-), respectively. The difference in measurement was not significantly different (P=0.7157; Student's unpaired *t*-test).

Table 2.5. Comparison of the effect of liquid or solid phase extraction methods on follicular fluid AEA levels.

Follicle Number	Follicular fluid AEA Liquid phase	Follicular fluid AEA Solid phase	% difference
1	1.391	1.459	4.88
2	1.438	1.409	-2.02
3	0.930	0.965	3.76
4	0.442	0.518	17.19
5	0.846	0.634	-25.06
6	0.975	0.871	-10.66
7	1.646	1.891	14.88

The AEA levels in two mL of follicular fluid taken from 7 follicles were extracted by the liquid and solid phase extraction methods. The difference in levels were assessed by two-tailed Student's unpaired *t*-test; P= 0.964.

Table 2.6. Plasma, urine, saliva AEA levels measured from 3 male volunteers.

AEA levels (nM)					
Volunteer	**Plasma Liquid phase**	**Plasma Solid phase**	**Urine Liquid phase**	**Urine Solid phase**	**Saliva Liquid phase**
V1	1.544	1.522	0.0020	0.0045	U
V2	0.932	1.243	0.0022	0.0026	U
V3	1.670	1.489	0.0050	0.0029	U

The levels of AEA in urine and saliva samples were below the limit of quantification (LOQ = 0.092 nM) and the limits of detection (LOD = 0.0079 nM). U=undetectable.

2.4.3. AEA measurements in 2 mL and smaller volumes

Plasma and follicular fluid AEA levels quantified from 2 mL and 1 mL volume of sample were similar, P=0.977 (**Table 2.7**). Less than 1 mL results were not robust. Adding physiological saline to increase the volume did not produce reproducible results, and although water showed better results, the number of the samples were small (n=6) and so no meaningful conclusions could be made (**Table 2.8**).

AEA levels were undetectable in culture medium post-fertilisation or in culture medium where the embryo was incubated and underwent cleavage (**Tables 2.9** and **2.10**).

Table 2.7. Comparison between AEA levels in different volumes of plasma and follicular fluid (2 mL *vs*.1 mL).

Volunteers	AEA Levels in nM 2mLof Plasma	AEA Levels in nM 1mL of Plasma	% Difference
V1	0.622	0.673	8.19
V2	0.860	0.851	-1.05
V3	1.134	1.129	-0.44
V4	1.073	1.086	1.21
V5	1.056	1.049	-0.66
V6	0.908	0.886	-2.42
V7	0.894	0.910	1.79
V8	0.347	0.331	-4.61
V9	0.266	0.259	-2.63
V10	0.573	0.599	4.54
Follicular Fluid (nM)			
F1	1.546	1.530	-1.03
F2	2.052	2.048	-0.19
F3	1.289	1.300	0.85
F4	1.708	1.698	-0.58
F5	2.377	2.351	-1.09
F6	0.634	0.610	-3.78

Student's two-tailed unpaired t-test for plasma, P=0.977 and follicular fluid, P=0.974.

Table 2.8. Comparison of AEA levels in 2 mL follicular fluid with and smaller (0.25 mL to 1 mL) volumes.

Follicle	Volume of additive	AEA level in 2mL of sample (nM)	% difference
F1	none	1.537	
F1	1 mL saline	0.542	-64.74
F2	none	0.948	
F2	1 mL saline	0.426	-55.06
F3	none	2.075	
F3	1 mL saline	1.969	-5.10
F4	none	1.468	
F4	1 mL saline	0.716	-51.22
F5	none	0.551	
F5	1 mL dH_2O	0.520	-5.62
F5	1.5 mL dH_2O	0.491	-10.89
F5	1.75 mL dH_2O	0.458	-16.88
F6	none	0.459	
F6	1 mL dH_2O	0.459	0
F6	1.5 mL dH_2O	0.406	-11.54
F6	1.75 mL dH_2O	0.371	-19.17

AEA levels were measured in standard (2 mL) follicular fluid and smaller volumes (1 mL, 0.5 mL and 0.25 mL) of follicular fluids using the liquid phase extraction method and UPLC-MS/MS. The sample volumes were all increased to 2 mL with either saline or de-ionised water (dH_2O) before extraction.

Table 2.9. Comparison in the levels of AEA in fertilisation culture medium before and after fertilisation. The volumes used were 2 mL before and 120-240 μL after fertilisation.

Sample	Before Fertilisation (Volume of sample = 2 mL)	After Fertilisation (Volume of sample = 120-740 μL)
1	0.17	0.093
2	0.27	0.034
3	0.30	0.081

AEA concentrations are given in nM.

Table 2.10. The effect of culture medium volume on the AEA levels (nM) measured before and after adding embryos to the culture medium.

AEA levels in embryo culture medium (nM)		
Sample	**Prior to adding the embryo**	**After embryo cleavage**
1	0.128	0.022
2	0.126	0.025
3	0.128	0.024
4	0.126	0.012
5	0.128	0.015
6	0.129	0.019

2.4. Discussion

Anandamide (AEA) is a highly lipophilic molecule that is soluble in organic solvents (Giuffrida et al., 2000). The synthetic AEA (d_0-AEA) and the internal standard (d_8-AEA) are supplied as standard solutions in ethanol and in methyl acetate (Cayman Chemical), respectively. However, the integrity of these standards is reported to be compromised by evaporation once the sample has been opened (Habayeb et al., 2004). So others (Giuffrida et al., 2000; Habayeb et al., 2004) have dissolved AEA in chloroform/methanol for use. However, chloroform is also extremely volatile and is damaging to the Waters UPLC system.

Consequently, continued stability and improved reproducibility of signal response was only observed if the synthetic AEA and the internal standard were dried under a gentle stream of nitrogen and re-dissolved in acetonitrile, aliquoted for daily use and placed as aliquots at -20°C. The stability of the compounds remained consistent if all further sample handling was conducted on ice. These data are seldom included in papers that describe analytical techniques, but they should be, as misappropriate handling of samples could negate the accurate measurement of the molecule in question. To ensure that the method was working efficiently and likely to provide meaningful data, the precision, sensitivity and reproducibility of the assay was determined.

The assay response was linear over a wide range of AEA concentrations both above and below the levels of biological samples. The limit of detection at 0.0187 nM and the limit of quantification at 0.025 nM were ~100-fold better than those reported by Richardson et al. (Richardson et al., 2007), or Vogeser et al., (Vogeser et al., 2006) thereby providing an improved method. Additionally, the run time for a single sample of 4 minutes is

significantly faster by 7-15 minutes than either of these methods, suggesting that a larger throughput of samples was possible.

On the column, the accuracy for 3.33 fmol of AEA was 97.5 ± 9.5%, 98.5 ± 6.1% for 6.65 fmol and was 104.5 ± 3.2% for 133 fmol. This sensitivity was also a marked improvement over the aforementioned methods (Richardson et al., 2007; Vogeser et al., 2006), implied that smaller sample volumes could be used for analysis and that this method could be employed to analyse biomatrices with significantly lower AEA concentrations.

Although AEA had previously been measured in human plasma and follicular fluid, AEA had not been quantified in urine or saliva. Such measurements had not been undertaken on the present UPLC-MS/MS system. Additionally, since obtaining repeat or sufficient samples from volunteers can be difficult, hence it was investigated whether dilution with saline or water to the required 2 mL volume of clinically relevant samples and then using the standard liquid phase extraction method (Habayeb et al., 2004), (with the exception that the extracted material was re-constituted in acetonitrile instead of chloroform: methanol) would affect the results obtained. The data indicated that AEA could be extracted and measured with precision from 2 mL of plasma or follicular fluid, but not from urine and saliva. The sensible explanation as to why AEA was not detected in urine or saliva could be because AEA is not excreted by kidneys, or is destroyed by salivary gland enzymes, although there are no data to support the presence of fatty acid amide hydrolase (FAAH; the enzyme degrades AEA) in either of these tissues. Alternatively, the relatively similar protein content and pH of plasma and follicular fluid are different to that of urine and saliva and since the liquid phase extraction method was designed for the extraction of AEA from plasma, that method may only be amenable to biological fluids that are similar to

plasma. Therefore, an alternative method may be needed to be developed for urine and saliva.

With regard to smaller sample volumes, dilution of the samples to 2 mL with saline proved to be unworkable with an approximately 50% loss of material. Diluting the sample to 2 mL with de-ionised water was better with a less than 10% loss of material, but both methods were unsuitable and unusable. The alternative method for dealing with smaller volumes of biological sample (1 mL) using the liquid phase extraction method was to reduce the amount of solvents used in the various extraction steps to 50% of those for the 2 mL volumes. Analysis of the data indicated that this modification was not only workable, but was also robust. However, applying the same approach to volumes < 1 mL proved to be inaccurate. Therefore, no results of AEA measurements for sample volumes below 1 mL were included in the results of this project.

Previous reports indicated that a solid phase extraction method had advantages over the liquid phase extraction method because of ease and speed (Richardson et al., 2007). In addition, the solid phase method requires a smaller volume (0.5 mL compared to 2 mL) of fluid. An analysis of the AEA levels measured from plasma and follicular fluid extracts demonstrated that there was no significant difference between the two methods.

All the previously published data quantifying AEA in plasma and follicular fluid were performed using the liquid phase extraction method with external standards (Giuffrida et al., 2000; Schuel et al., 2002; Habayeb et al., 2004; Vogeser et al., 2006; Habayeb et al., 2008b) and isotope dilution, as this methodology provides increased accuracy (Caymen Chemical product datasheet). In order to compare the results in this thesis with those of

previous studies and because only 2% of the samples were extracted using the solid phase method, the liquid phase extraction method was preferred rather than using both methods.

The difficulty with culture medium was the small volumes involved. Such small volumes meant that the AEA levels were below the level of detection. It would have been better if larger volumes could have been used, but it was not possible to change the clinical practice of the Assisted Conception Unit for this purpose, as that may have affected fertilisation of the oocyte and ultimately the pregnancy rate in the Unit. Therefore, this part of the project on the role of AEA in fertilisation and embryo cleavage was abandoned.

2.5. Conclusions

The UPLC with MS/MS method described for the analysis of AEA in plasma and follicular fluid showed that the method is sensitive, reproducible and precise in comparison to previous methods, which made this method suitable for the analysis of clinical samples. The improved sensitivity over previous methods suggested that this method could be used for the analysis of AEA in samples with reduced volumes or in biomatrices with lower AEA levels, especially when coupled to solid phase extraction. The faster analysis time for the method represents a saving in time, potentially allowing for the analysis of larger sample numbers. Nevertheless, the method needed to be further 'validated' with 'real' samples. This will be described in Chapter 3.

CHAPTER 3

Validation of the Anandamide Assay

3.1. Introduction

In Chapter 2 it was demonstrated that the ULPC-MS/MS is a very sensitive and precise method for the measurement of AEA in plasma and follicular fluid. The next logical step was to validate the assay technique by measuring the AEA levels in the plasma on a cohort of volunteers. Since the research group had previously published data on plasma AEA levels during the menstrual cycle, early pregnancy and in post-menopausal women (Habayeb et al., 2004) and Schuel's group had previously measured AEA in follicular fluids (Schuel et al., 2002) the plan was to quantify levels in similar cohorts of women. Two cross-sectional studies were therefore conducted. The first study aimed to measure the AEA plasma levels during (a) the menstrual cycle, (b) the postmenopausal period, and (c) in the first trimester pregnancy. The second study aimed at measuring AEA levels in follicular fluid from a woman undergoing IVF/ICSI treatment.

3.2. Subjects and Methods

3.2.1. Subjects

All volunteers were interviewed, and a detailed medical history obtained. Eligible volunteers gave signed informed consent to take part in the studies, which was approved and conducted according the guidelines of the Leicestershire and Rutland Local Research Ethics Committee. The approval number was Q6/ Q2501/49.

3.2.1.1. Plasma Measurements

Volunteers were recruited and divided in to three groups; pregnant (first trimester), pre-menopausal, and post-menopausal. The inclusion criteria for the first trimester pregnant volunteers were non-obese, body mass index (BMI) less than 27 kg/m^2, no vaginal

bleeding or co-existing medical condition and ultrasound confirmed viable single intrauterine pregnancy at 6-8 week of gestation.

The non-pregnant volunteers were divided into two groups: pre-menopausal, and post-menopausal. The inclusion criteria for the pre-menopausal volunteers were; regular menstrual cycles for the last 6 months, a written record of the date of the start of their last menstrual period, a BMI less than 27 kg/m^2 (i.e. non-obese), not on any hormonal contraception, or any medication for at least 6 months, and not suffering from any medical disorders. Blood samples were collected and divided into two subgroups: follicular phase (days 2-14) and luteal phase (days 20-30) based on the first day of the volunteer's menstrual period.

The inclusion criteria for the post-menopausal volunteers were; a minimum of 2 years post menopause, a BMI less than 27 kg/m^2 (non-obese), not receiving hormonal replacement therapy in the last 6 months prior to the study and not on any medication or suffering from any medical disorders. For AEA quantification a 4 mL blood sample was collected from each volunteer as described in Chapter 2.

3.2.1.2. Follicular Fluid Measurements

A single volunteer was recruited from the Assisted Conception Unit at the Leicester Royal Infirmary for ICSI treatment. She was a 32 year old healthy woman with primary infertility for the preceding 3 years due to male factor, had a BMI of 23 kg/m^2 and was a non-smoker. Her ovaries were stimulated using the long stimulation protocol (see Chapter 5) and on the day of oocyte retrieval she presented with 9 different sized follicles from which follicular fluid was collected (this process will be described in detail in Chapter 5).

3.2.2. AEA Measurements

To be consistent with previous studies, both plasma and follicular fluid were processed within 2 hours of collection, AEA extracted using the liquid phase method and the levels measured using ULPC-MS/MS, as described in Chapter 2.

3.3. Statistical analysis

All the data were normally distributed (Shapiro-Wilk test; Prism version 5 software, GraphPad Inc., San Diego, California, USA, www.graphpad.com) therefore, parametric tests were used. Patient demographics presented as mean ± SD and range, AEA results presented as mean ± SEM. Comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference test and Student's unpaired *t*-test was used to compare the variances between 2 groups. A P-value of <0.05 was considered to be statistically significant.

3.4. Results

3.4.1. Subjects

A total of 51 pre-menopausal and post-menopausal women were recruited into the two studies. Of these, 8 were pregnant, 30 were pre-menopausal (non-pregnant) and 13 were post-menopausal. There were no significant differences in the ages between the pre-menopausal volunteers (**Table 3.1**).

Table 3.1. Demographic Characteristics of the Volunteers (data are presented as means ± SD with ranges in parentheses).

Study group	Age (years)	BMI (kg/m^2)	Gestational age (Weeks)
First trimester (n=8)	31.25 ± 3.41 (26-36)	23.5 ± 2 (20-26)	6.5 ± 0.67 (6-8)
Pre-menopausal (n=30)	30.71 ± 9.03 (20-42)	22.14 ± 2.41 (19-26)	Not applicable
Post-menopausal (n=13)	60.0 ± 5.73 (52-68)	23.0 ± 1.58 (21-25)	Not applicable

Pre-menopausal women: 15 were in the follicular phase and 15 in the luteal phase.

n= the number of volunteers.

3.4.2. Plasma AEA Measurements

Figure 3.1 shows that the mean plasma AEA level in the follicular phase of the menstrual cycle (1.45 ± 0.21 nM) was almost double the level in the luteal phase (0.77 ± 0.08 nM). The difference in plasma AEA levels between the two phases was highly significant (P=0.0054). The levels of plasma AEA in the luteal phase of cycle, however, did not significantly differ (P=0.922) from that in the first trimester of pregnancy (0.78 ± 0.08 nM).

The mean post-menopausal plasma AEA level (0.96 ± 0.05 nM) was significantly lower (P=0.044) than in the follicular phase (1.45 ± 0.21 nM), but there was no statistically significant difference between the post-menopausal and the luteal phase (P=0.066).

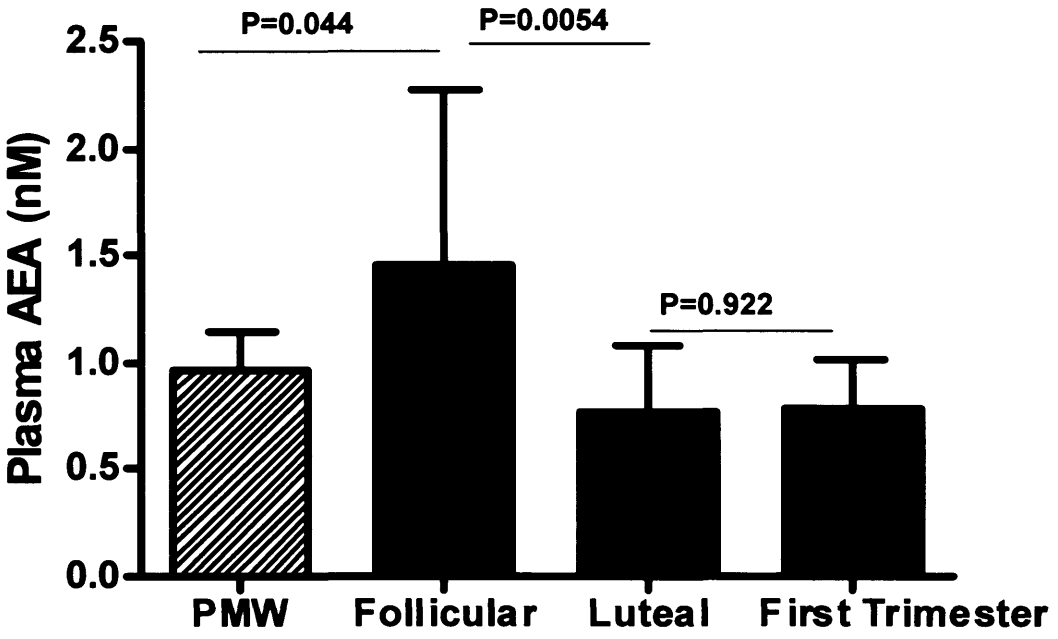


Figure 3.1. Comparison of the plasma AEA levels in adult woman at different stages of the menstrual cycle, post-menopause (PMW), and pregnancy. The number of volunteers are; PMW=13, Follicular phase=15, Luteal=15 and the first trimester of pregnancy=8. Data are expressed as the mean ± SEM. One-way ANOVA with Tukey's honestly significance difference post-test was used to determine the P-values; PMW *vs.* follicular phase, P=0.044; follicular *vs.* luteal phase, P=0.0054; luteal phase *vs.* first trimester of pregnancy, P=0.922.

3.4.3. Follicular fluid AEA measurements

Table 3.2 shows the AEA levels measured in the follicular fluids of 9 follicles. There were differences in the levels from individual follicles; the lowest level was 0.416 nM and the highest 2.272 nM. The data from the follicular fluids and the plasma samples were then compared to the levels from the literature (**Table 3.3**) and found to be similar.

Table 3.2. Follicular fluid AEA levels from 9 aspirated follicles. Mean ± SEM of the follicular fluid AEA measurement from 9 follicles are shown.

Follicle Number	AEA Concentration (nM)
F1	1.980
F2	2.028
F3	1.341
F4	1.741
F5	1.461
F6	2.272
F7	0.416
F8	0.602
F9	1.440
Mean	**1.476**
SEM	**0.21**

Table 3.3. Comparison of plasma and follicular fluid AEA measurements from this study and published data.

Study cohort	AEA concentration (nM)	n-value (Published data)[a,b]	AEA concentration (nM)	P-value
Plasma AEA				
Postmenopausal (n=13)	0.96 ± 0.05	8	0.67 ± 0.01[a]	**0.0001**
Menstrual cycle				
Follicular phase (n=15)	1.45 ± 0.21	9	1.68 ± 0.16[a]	0.393
Luteal phase (n=15)	0.77 ± 0.08	8	0.87 ±0.19[a]	0.639
First trimester of pregnancy (n=8)	0.78 ± 0.08	10	0.89 ± 0.14[a]	0.571
Follicular fluid (n=9)	1.48 ± 0.21	9	2.90 ± 0.90[b]	0.163

Data are expressed in mean ± SEM and are from this study (column 2), from (Habayeb et al., 2004)[a] and (Schuel et al., 2002)[b] (columns 3 and 4). Student's two-tailed unpaired *t*-test with Welch's correction for unequal variances was used to determine the P-values.
n= number of volunteers.

3.5. Discussion

A literature review showed that there were only two published studies that measured AEA in human biomatrices; the first measured plasma AEA in reproductive and post-reproductive women (Habayeb et al., 2004) and the second measured AEA in a number of reproductive fluids including ovarian follicular fluid (Schuel et al., 2002). Both studies used the liquid phase extraction method and a HLPC-MS system, which is considered to be a less sensitive system compared to the UPLC-MS/MS system used in this thesis. MS/MS is thought to be more sensitive than MS because it is more specific; MS determines the parent ion mass and MS/MS measures both the parent and daughter ion mass (Lam et al., 2008). The reason for the increased sensitivity of the UPLC-MS/MS method over the HPLC-MS methods previously used is unclear but could be related to the retention times of the products on the analytical column being much shorter using the BEH columns, so that the entire amount of compound/analyte is released to the detector immediately. Alternatively, the C18 columns used in the standard HPLC method may act as a catalyst for chemical degradation of anandamide (Hardison et al., 2006) leading to reduced concentrations at the detector.

The levels of AEA measured in plasma and follicular fluid using the ULPC-MS/MS were comparable to those obtained with the HPLC-MS (**Table 3.3**). There was a significant reduction in plasma AEA levels during the menstrual cycle from the follicular to the luteal phase as previously reported by Habayeb et al., (Habayeb et al., 2004). However, although the AEA levels in the post-menopausal women were not significantly different from that in the luteal phase, as previously reported (Habayeb et al., 2004), the levels in post-menopausal women were significantly higher than the published results for this age group (Habayeb et al., 2004). The reason for this is unclear but could either be related to the increased sensitivity of the UPLC-MS/MS machine compared to the HPLC-MS/MS

machine, or to the fact that there might have been significant differences in the two cohorts. The fluctuations of plasma AEA concentrations during the menstrual cycle; high level during the follicular phase and lower levels at the luteal phase and equally low levels during early pregnancy, suggest that AEA is a hormonally-regulated molecule.

Further validation of the UPLC-MS/MS values came from the measurement of AEA in follicular fluid. Although there was a significant variation in AEA concentration from follicle to follicle, the mean AEA concentration of 1.476 ± 0.629 nM was not significantly different to the previously published value of 2.9 ± 0.9 nM from follicular fluids (Schuel et al., 2002). The AEA concentrations in the woman was approximately one half of that found in Schuel's study suggesting that follicular fluid AEA concentration vary either from woman to woman, or from follicle to follicle. Indeed, as is shown in **Table 3.2** AEA concentrations varied from one follicle to another and this variability could be related to the fact that follicles are also variable in size. This observation will be further investigated in Chapter 5.

3.6. Conclusions

The results of measuring AEA using ULPC-MS/MS were comparable to the published data using a slightly different machine, providing confidence in the robustness and the precision in the method used to measure AEA in plasma and follicular fluid.

Although the plasma AEA levels in the follicular and luteal phase were similar to those reported by Habyeb et al., (Habayeb et al., 2004) it was difficult to define the precise changes in the levels of AEA during the menstrual cycle. An understanding of these changes is essential to allow for a better appreciation of how AEA may be involved in reproduction. It was therefore, considered extremely important to establish the changes in

plasma AEA levels throughout the menstrual cycle and to determine how these are related to the hormones controlling the normal ovulatory menstrual cycle prior to studying the levels of AEA in relation to fertility and early pregnancy. These studies are described in Chapter 4.

CHAPTER 4

Plasma Anandamide throughout the Menstrual Cycle, and its Relation to Sex Steroids and Gonadotrophin Hormones

4.1. Introduction

Our understanding of the effect of cannabinoids on the menstrual cycle and the regulation of the hypothalamic-pituitary-ovarian axis has come mainly from studies on cannabis smokers and animals. Marijuana smoking is associated with suppression of LH, and a shortened luteal phase in women (Tyrey, 1980; Jones, 1983; Mendelson et al., 1986). The main psychoactive ingredient in marijuana delta-9-tetrahydocannabinol (Δ^9-THC) causes delayed puberty in the female rat (Field and Tyrey, 1984), and suppresses the pre-ovulatory surge of LH leading to anovulation in rats, rabbits and rhesus monkeys (Nir et al., 1973; Smith et al., 1979). The inhibitory effect of Δ^9-THC on ovulation is reversed by the administration of gonadotrophin releasing hormones (Ayalon et al., 1977) suggesting that this effect is mediated via inhibition of the activity of a hypothalamic factor (Reich et al., 1982) which is essential for the release of pituitary LH.

Several studies have suggested that in addition to the central effect of cannabinoids, there is also a direct gonadal effect (Ayalon et al., 1977). Studies in the *in-vitro* rat ovary model demonstrated that Δ^9-THC inhibited follicular steroidogenesis and prostaglandin synthesis and because prostaglandins have an essential role in follicle rupture and ovulation (Reich et al., 1982), prevented ovulation. Other rat studies showed that Δ^9-THC suppresses progesterone secretion from the corpus luteum (Adashi et al., 1983), suggesting a role for Δ^9-THC in preventing early pregnancy success. These direct ill-effects on the ovary were also observed in cannabis users (Griffin et al., 1986; Mendelson et al., 1986) these women had poor oocyte quality and lower pregnancy rates during IVF treatment (Klonoff-Cohen et al., 2006).

The effects of endogenous cannabinoids (endocannabinoids) on the hypothalamic-pituitary–ovarian axis are quite similar to those of Δ^9-THC. Anandamide (AEA) one of the

most potent endocannabinoids decreases serum LH and prolactin levels in both male and female rats, but does not affect serum FSH (Wenger et al., 1999b). Initially, the site of action of anandamide was thought to be mainly in the hypothalamus, i.e. no direct effect on the anterior pituitary, as the cannabinoid receptors were found in the arcuate nucleus and the pre-optic areas (Fernandez-Ruiz et al., 1997). The CB1 receptor has, however, also been identified in the anterior pituitary (Gonzalez et al., 1999; Wenger et al., 1999a) suggesting that anandamide may modulate its action on the anterior pituitary by direct action as well by interfering with hypothalamic regulatory factors. The expression of CB1 receptor protein in the rat anterior pituitary has been shown to be regulated by sex steroid hormones (Gonzalez et al., 2000). The amount of CB1 receptor-mRNA transcripts in the anterior pituitary fluctuates throughout the ovarian cycle in rats with the highest levels found during diestrous and the lowest during estrous (Gonzalez et al., 2000). In contrast, anandamide levels in the anterior pituitary peak in the estrous phase and are lowest in the diestrus phase, whilst AEA concentrations in the hypothalamus showed the reverse pattern, suggesting that anandamide concentrations in both the anterior pituitary and hypothalamus are influenced by sex steroids (Gonzalez et al., 2000).

The actions of the ovarian sex steroids may not only be confined to the hypothalamic-pituitary-gonadal axis, indeed, estrogen has been shown to stimulate, while progesterone has been demonstrated to inhibit the expression and activity of the enzyme fatty acid amide hydrolase (FAAH) responsible for the degradation of anandamide in the rat uterus (Maccarrone et al., 2000a). The significant reduction of FAAH expression in the uterus of ovariectomised rats was reversed with estradiol treatment, suggesting that sex steroids might regulate FAAH (Maccarrone et al., 2000a).

Only a limited number of studies have examined the levels of AEA in the human during the menstrual cycle (Habayeb et al., 2004; Lazzarin et al., 2004). In plasma, AEA levels were found to be highest in the follicular phase in comparison to the levels in the luteal phase, and in post-menopausal women (Habayeb et al., 2004), again suggesting that AEA levels may be related to ovarian sex steroids. Another study (Lazzarin et al., 2004) found that FAAH and AEA levels fluctuated in human lymphocytes during the ovulatory cycle. The lowest levels of FAAH were noted peri-ovulation and the highest were 7 days post ovulation, a time that is characterised by elevated serum progesterone levels and known as the endometrial 'window of implantation' (Sharkey and Smith, 2003). This fluctuation of FAAH levels during the menstrual cycle suggests that FAAH is possibly associated with or regulated by sex steroid hormones in humans. Indeed, in another study, the same research group demonstrated that progesterone up-regulated FAAH enzyme expression and activity in human lymphocytes (Maccarrone et al., 2001) and suggested that low plasma AEA levels are required for successful implantation. In contrast to progesterone, estrogen stimulated AEA release from human endothelial cells (Maccarrone et al., 2002a), which would be consistent with the elevated plasma AEA levels found in the follicular phase of the cycle. The role of AEA in the function of the ovary is unclear. Schuel et al., quantified AEA in follicular fluid during oocyte retrieval in women undergoing *in-vitro* fertilisation (IVF) (Schuel et al., 2002). The presence of AEA in the human follicular fluid suggested that AEA may either be involved in folliculogenesis or ovulation or associated with the factors regulating these processes.

From all of the available literature it is clear that little is known about the interplay between endcannabinoids, especially anandamide, and the hormones that regulate the menstrual cycle. Since AEA levels are considered to be key to the synchronous development of the embryo and the priming of the endometrium for implantation in mice

(Paria et al., 2002b; Liu et al., 2002) and that gonadal steroid hormones may be involved in controlling plasma AEA levels (Gammon et al., 2005), the aim of this study was therefore to investigate the changes in plasma AEA levels throughout the menstrual cycle cross sectionally and longitudinally and how the levels might be related to those of key ovarian steroid hormones and gonadotrophins involved in the regulation of the menstrual cycle.

4.2. Material and Methods

4.2.1. Subjects

This study was undertaken in two parts. The first part was cross-sectional and designed to confirm previous observations (Habayeb et al., 2004; Lam et al., 2008) and furthermore to examine those observations in greater detail (i.e. not only in the early follicular and late luteal phases) plasma changes during the menstrual cycle while the second was longitudinally designed with the volunteers acting as their own controls to allow for further investigation of whether the changes in AEA levels observed cross-sectionally were mirrored in the same individuals. All the volunteers included in the study were healthy, non-obese, and had regular menstrual cycles (defined as occurring every 26-32 days) for six months prior to entry into the study, were not on any medication or hormonal contraception for at least six months prior to recruitment into the studies and were non-smokers or smoked <20 cigarettes per day. The post-menopausal women had all been amenorrhoeic for two years, had not been on hormonal replacement therapy or any medication for six months prior to the study and were recruited as controls for the pre-menopausal studies. All the women (both pre-menopausal and post-menopausal) had an intact uterus and both ovaries were present. The post-menopausal women had all passed through a natural menopause. Each volunteer gave a written signed informed consent prior to recruitment into the study, which had ethics approval from the Leicestershire and Rutland Local Research Ethics Committee (approval number Q6/ Q2501/49).

In both parts of the study, the menstrual cycle was divided into five phases based on the normal physiological changes in the levels of sex steroid hormones and gonadotrophins that occur throughout the ovulatory menstrual cycle (Chabbert-Buffet and Bouchard, 2002): early follicular (days 2-6), late follicular (days 8-12), ovulatory (days 13-16), mid luteal (days 18-23), and late luteal (days 24-30).

Forty-two volunteers (35 pre-menopausal aged 20-40 years, and 7 post-menopausal aged 52-68 years), with a BMI of 19-26 kg/m^2 took part in the cross-sectional part of the study. They were recruited for sample collection at defined periods of their menstrual cycle, based on their self-reported last menstrual period (LMP). For the pre-menopausal group, a single sample of blood was taken from each volunteer to measure the plasma anandamide (AEA) level for the particular period for which they were recruited. A single blood sample was obtained from the post-menopausal controls.

A total of 12 volunteers (7 pre-menopausal aged 21-40 years and 5 post-menopausal aged 54-68 years) with a BMI of 20-24 kg/m^2 were recruited into the longitudinal part of the study. The 7 pre-menopausal volunteers kept a menstrual diary of their cycle length for 3 months prior to entering the study. Blood was collected serially for the measurement of plasma AEA, serum 17β-estradiol (E2), progesterone (P4), follicle stimulating hormone (FSH) and luteinising hormone (LH). The start of menstruation was considered as day 1 of the cycle and the volunteers were asked use a urine LH surge kit (Clear Blue Ovulation Test, Unipath Ltd., Bedford, UK) from day eight of their cycle until the day of a positive urine test (14). Twenty four hours after a positive urine LH surge test, ovulation blood samples were collected (i.e. LH+1) (Speroff and Firtiz, 2005).

Due to variations in the normal lengths of the natural menstrual cycle it was necessary to determine the phase of the cycle in the following manner **(Table 4.1).** Day 2 to day 6 represented the early follicular phase of the cycle, therefore blood samples were taken between days 2 and 5 to ensure accuracy. The mean sampling day was 3.7 ± 1.11 (SD). A blood sample taken from day 8 to 24 hours prior to a LH surge represented the late follicular phase, but since the day of a LH positive urine test was difficult to predict, women with a short cycle had blood taken on day 8, and women with a longer cycle sample on day 10 or 11. The date range for the late follicular phase was therefore day 8 to 11 with a mean of 10.3 ± 1.11 days. The samples that represented ovulation day were taken 24 hours after a positive urinary LH surge test which ranged between days 13 and 16 of the cycle. The mean day of ovulation was therefore 14.7 ± 1.25 days. The mid luteal phase sample was collected 7 days post ovulation and thus the mean sample day was 20.8 ± 2.11 and ranged between days 18 and 23 of the cycle. To ensure that the late luteal phase sample was representative of this phase of the cycle, blood samples were taken from day 11 onwards post ovulation so that, for example, a woman with a 26 day cycle length and who ovulated on day 13 had her late luteal sample taken on day 24. The late luteal samples were therefore taken between day 24 to 30 based on an individual woman's cycle length had a mean of 26.3 ± 2.13 days. The start day of the next menstrual period was noted so that the studied cycle length could be calculated. For this group the mean length was 29.5 ± 1.98 and ranged between 26-32 days. The phases of the cycle were further confirmed using the results of serum sex steroid hormones and gonadotrophin levels, which have defined specific levels for each phase of the cycle (Chabbert-Buffet and Bouchard, 2002). For the post-menopausal women, blood samples were taken weekly over a four week period for the measurement of plasma AEA and serum hormones.

Table 4.1. Absolute cycle length in days and the day of the cycle on which blood samples were taken from the volunteers (V) in the longitudinal study. LH refers to the day of initial LH^+ test using the Clear-Blue Urinary LH testing kit.

Timings	V1	V2	V3	V4	V5	V6	V7	Mean
Cycle length (days)	30	30	31	32	26	30	28	29.5
Early Follicular Sample (day)	3	4	4	3	5	5	2	3.7
Late Follicular Sample (day)	10	11	8	11	10	11	11	10.3
Ovulation (LH+1)	14	14	16	16	13	16	14	14.7
Mid Luteal Sample (day)	18	18	21	23	21	23	22	20.8
Late Luteal Sample (day)	24	24	28	30	26	26	26	26.3

4.2.2. Methods

4.2.2.1. Blood sampling and plasma AEA measurement

All samples were collected between 10:00 and 12:00 am to avoid any diurnal variation. The volunteers in the cross-sectional study had four mL of blood obtained for plasma AEA alone whereas volunteers in the longitudinal study had eight mL of blood collected for both plasma and serum. The blood for plasma AEA was collected into EDTA tubes while that for serum was collected into serum gel tubes. The blood in the serum tubes was left to clot for 15 minutes before being centrifuged at 1200 x g for 30 min and separated serum stored at -20°C for later hormone measurements. Separated plasma was processed within 2 hours of blood collection for AEA measurement as described in detail in Chapter 2.

4.2.2.2. Hormone measurements [Serum Luteinising Hormone (LH), Follicle Stimulating Hormone (FSH), Estradiol (E2), and Progesterone (P4)]

All stored samples were processed at the same time to prevent any inter-assay variation on an automated ADVIA Centaur Assay System (Bayer Diagnostics, Newbury, Berkshire, UK) performed by the Chemical Pathology Department of Leicester Royal Infirmary. The range of detection was 0.07-200 IU/L for LH, 0.3-200 IU/L for FSH, 36.7-3670 pmol/L for estradiol and 0.48-190.8 nmol/L for progesterone and similar to normal reference values (Stricker et al., 2006).

4.3. Statistical Analysis

Power calculations based on previous published plasma AEA data in women (Habayeb et al., 2004, Habayeb et al., 2008b), indicated that a minimum of 6 women in each of the groups would allow a clinically significant difference of at least 40% in plasma AEA concentrations to be observed with 80% power, assuming a 2-sided $\alpha = 0.05$.

Data were normally distributed and are therefore expressed as mean ± SD or mean ± SEM, where appropriate. Parametric tests were used for statistical analysis and comparison between groups was performed with a one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference test or Student's *t*-test. Pearson correlation was used to evaluate the relationship between different variables, with $P<0.05$ considered to be significant.

4.4. Results

4.4.1. Subjects

There were no statistically significant differences in the ages and BMIs of the volunteers in the pre- and post-menopausal cohorts **(Table 4.2)**. As expected, the women in post-menopausal group were significantly older than the pre-menopausal women ($P<0.05$).

Table 4.2. Comparison of the distribution, age, and BMI of the women in the two studies. Values are presented as means ± SD with ranges in parentheses. P values were calculated using Student's unpaired *t*-test. The BMI for all groups of women was not significantly different. n= number of volunteers.

Distribution	Cross-sectional Study (n=42)	Longitudinal Study (n=12)	P-value
Pre-menopausal women	n=35	n=7	-
Post-menopausal women	n=7	n=5	-
Pre-menopausal women age (years)	30 ± 9 (20-42)	28.8 ± 7.4 (21-40)	0.617
Post-menopausal women age (years)	60 ± 5.2 (52-68)	59.4 ± 4.9 (54-68)	0.628
Pre-menopausal women BMI (kg/m^2)	22 ± 2.4 (19-26)	21.3 ± 1.4 (20-23)	0.690
Post-menopausal women BMI (kg/m^2)	23 ±1.6 (21-25)	23.4 ± 0.9 (22-24)	0.635

4.4.2. Plasma AEA levels

Plasma AEA levels in the pre- and post-menopausal women in the cross-sectional and longitudinal arms of the study were comparable **(Table 4.3).** Plasma AEA levels were significantly lower (P=0.038) in the early follicular phase of the cycle in the longitudinal arm when compared to those in the cross-sectional arm **(Figure 4.1)**. There were, however, no significant differences in AEA levels in the other phases of the menstrual cycle or in the postmenopausal women between the two arms of the study.

Table 4.3. A comparison of the plasma AEA levels from pre-menopausal women during each phase of the menstrual cycle and post-menopausal controls in the two studies (cross-sectional and longitudinal). Data are expressed as the mean ± SEM. The P values were calculated using Student's unpaired *t*-test. n= number of volunteers.

Phase of the cycle	Cross-sectional (n=42)	Longitudinal (n=12)	P-value
Early Follicular (d2-d6)	0.89 ± 0.06 nM (n=7)	0.73 ± 0.03 nM (n=7)	0.038
Late Follicular (d8-d11)	0.77 ± 0.09 nM (n=7)	0.63 ± 0.08 nM (n=7)	0.281
Ovulation (d13-16)	1.38 ± 0.14 nM (n=7)	1.33 ± 0.16nM (n=7)	0.815
Mid Luteal (d18-23)	1.09 ± 0.13 nM (n=7)	0.96 ± 0.10 nM (n=7)	0.453
Late Luteal (d24-30)	0.66 ± 0.07 nM (n=7)	0.56 ± 0.06 nM (n=7)	0.308
Post-menopausal	0.98 ± 0.07 nM (n=7)	1.05 ± 0.075 nM (n=5)	0.441

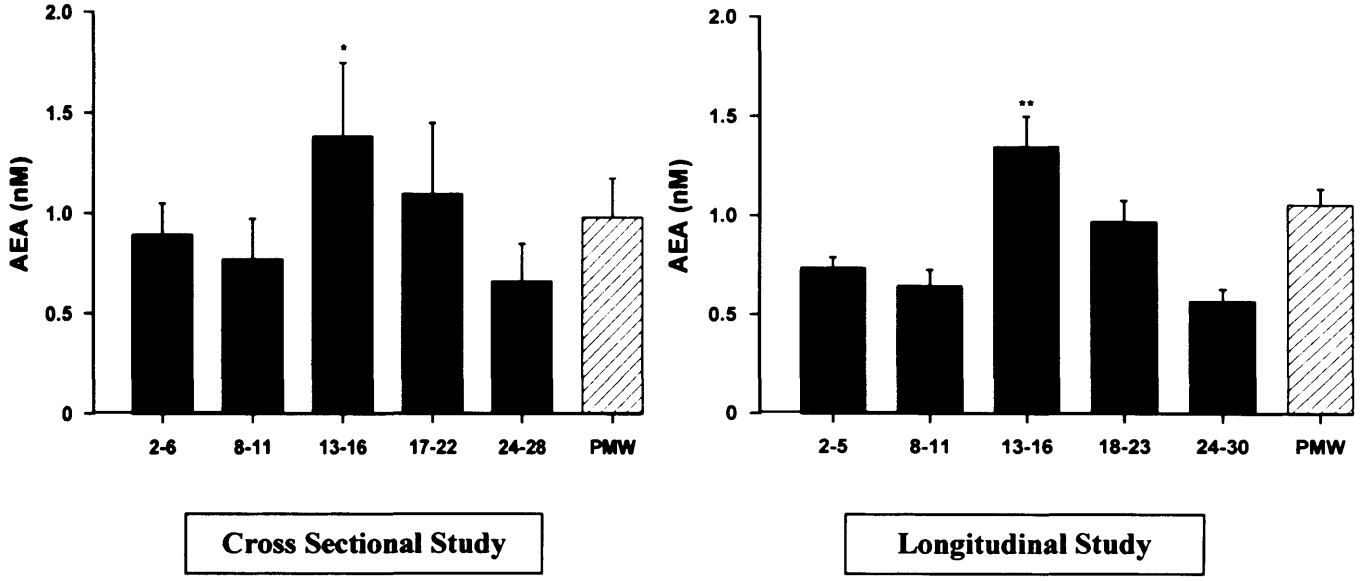


Figure 4.1. Comparison of plasma AEA levels in pre- and post-menopausal women in both cross-sectional (n=42) and longitudinal studies (n=12). The anandamide levels pre-menopausal women are (**black columns**) and post-menopausal women (**hatched columns**). Data are presented as mean ± SEM; *P<0.05, **P<0.01; ***P<0.001; One-way ANOVA with Tukey's honestly significance difference post-test compared to the mean plasma anandamide recorded at the d2-6 of the menstrual cycle with the rest of the cycle measurements. Plasma AEA levels were significantly lower (P=0.038) in the early follicular phase of the cycle in the longitudinal arm when compared to those in the cross-sectional arm. However, there were no significant differences in AEA levels in the other phases of the menstrual cycle or in the postmenopausal women between the two arms of the study. n=number of volunteers.

In the longitudinal arm of the study, the plasma AEA level (0.73 ± 0.03 nM) in the early follicular phase (day 2-5) of the menstrual cycle were higher than those (0.63±0.08 nM) in the late follicular (day 8-11) phase **(Figure 4.2)**, but this was not statistically significant ($P>0.05$). There was a statistically significant ($P<0.0001$) rise in plasma AEA level from 0.63 ± 0.08 nM in the late follicular (day 8-11) phase to 1.33 ± 0.16 nM at the time of ovulation (LH+1) followed by a statistically non significant decrease to 0.96 ± 0.10 nM in the early luteal (day18-23) phase. The lowest plasma AEA level (0.56 ± 0.06 nM) was observed in the late luteal (day 24-30) phase and was significantly lower ($P<0.05$) than that of the early luteal (day 18-23) phase. When the highest plasma AEA level measured at the time of ovulation (LH+1) was compared with the lowest level observed in the late luteal (day 24-30) phase, there was a statistically significant difference ($P<0.0001$).

In the post-menopausal women of the longitudinal arm of the study, plasma AEA levels fluctuated slightly, but there was no statistically significant difference over the four-week sampling period ($P=0.329$; **Figure 4.2**). The mean AEA level was 1.05 ± 0.07 nM, which was comparable to the levels in post-menopausal controls of the cross-sectional arm of the study.

4.4.3. Ovarian steroids and gonadotrophins levels

Figures 4.2 and 4.3 show that the measured serum levels of E2, P4, LH and FSH in pre- and post-menopausal women were in keeping with the classically described patterns for these hormones in pre-menopausal ovulating and post-menopausal women (Nulsen and Peluso, 1992).

Serum E2 levels in the pre-menopausal women were highest around the time of ovulation (1326 ± 193.2 pmol/L). In the post-menopausal women there were statistically insignificant minimal fluctuations in E2 levels over the four-week period (P=0.445); the mean E2 level over the 4 weeks was 36.4 ± 4.55 pmol/L. Serum P4 levels were highest (45.5 ± 10.01 nmol/L) in the mid-luteal phase of the cycle (**Figure 4.2**). By contrast, P4 levels in postmenopausal women remained stable over the four weeks (mean value of 1.920 ± 0.06 nmol/L). The peak serum LH level in the pre-menstrual women at the time of ovulation was 16.13 ± 2.24 IU/L. The mean post-menopausal serum LH level was 35.25 ± 0.42 IU/L, with no significant change over the four-week period (P=0.976; **Figure 4.3**). The highest serum FSH level (6.76 ± 0.96 IU/L) in pre-menopausal women was observed at the time of ovulation. In the post-menopausal women the mean serum FSH level was 74 ± 0.75 IU/L, with no significant fluctuations in the levels over the four-week period (P=0.958). This was as expected, higher than the level in the pre-menopausal women.

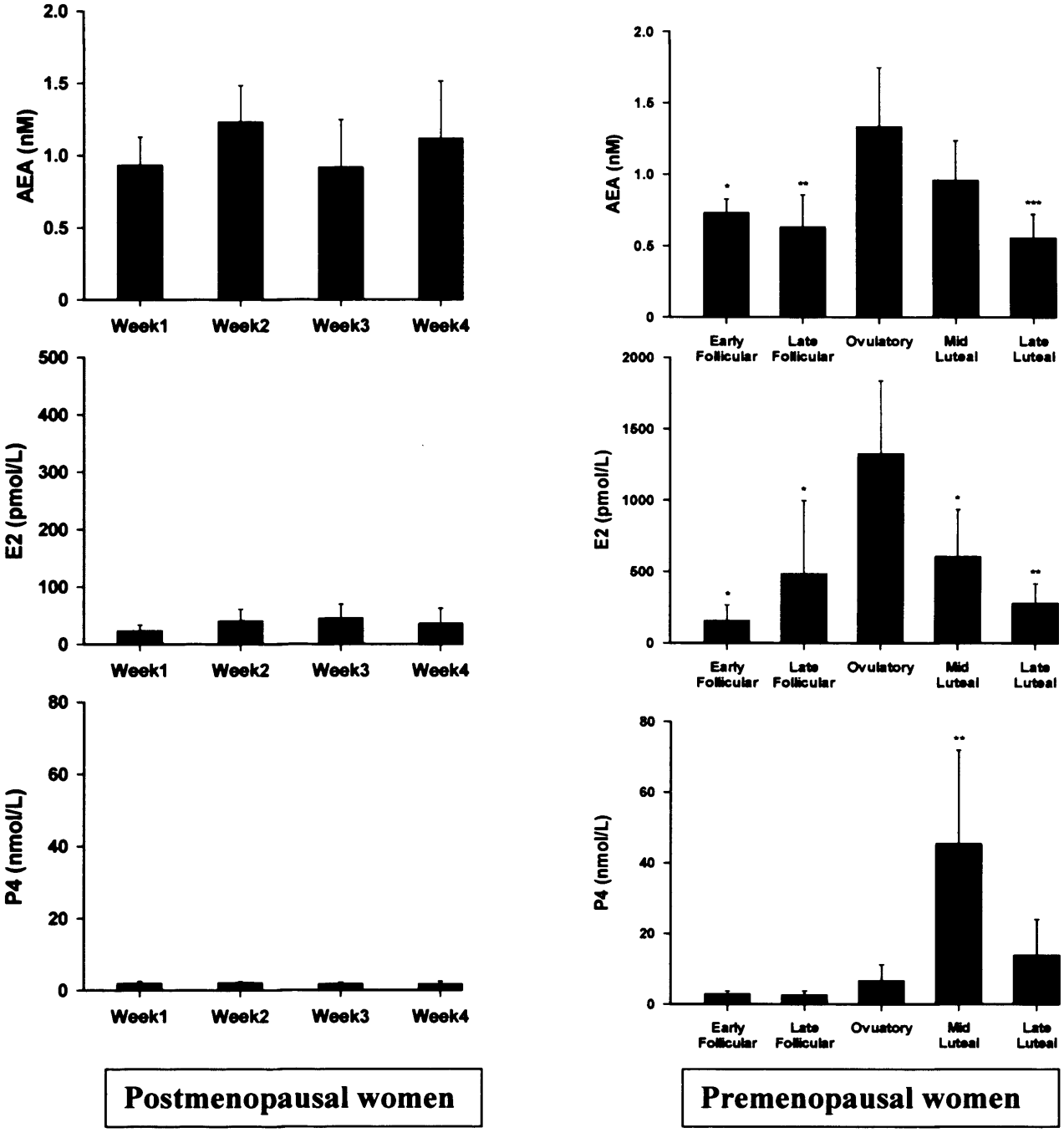


Figure 4.2. Comparison of plasma AEA (nM) and serum estradiol (E2; pmol/L), progesterone (P4; nmol/L) levels through the menstrual cycle in the longitudinal study with levels found in postmenopausal women over the 4 weeks period. Data are presented as mean ± SEM for 7 women in pre-menopausal and 5 women in post-menopausal group. *P<0.01; **P<0.001; One-way ANOVA with Tukey's honestly significance difference test when compared with the measurement in week 1 for the post-menopausal women and with early follicular phase.

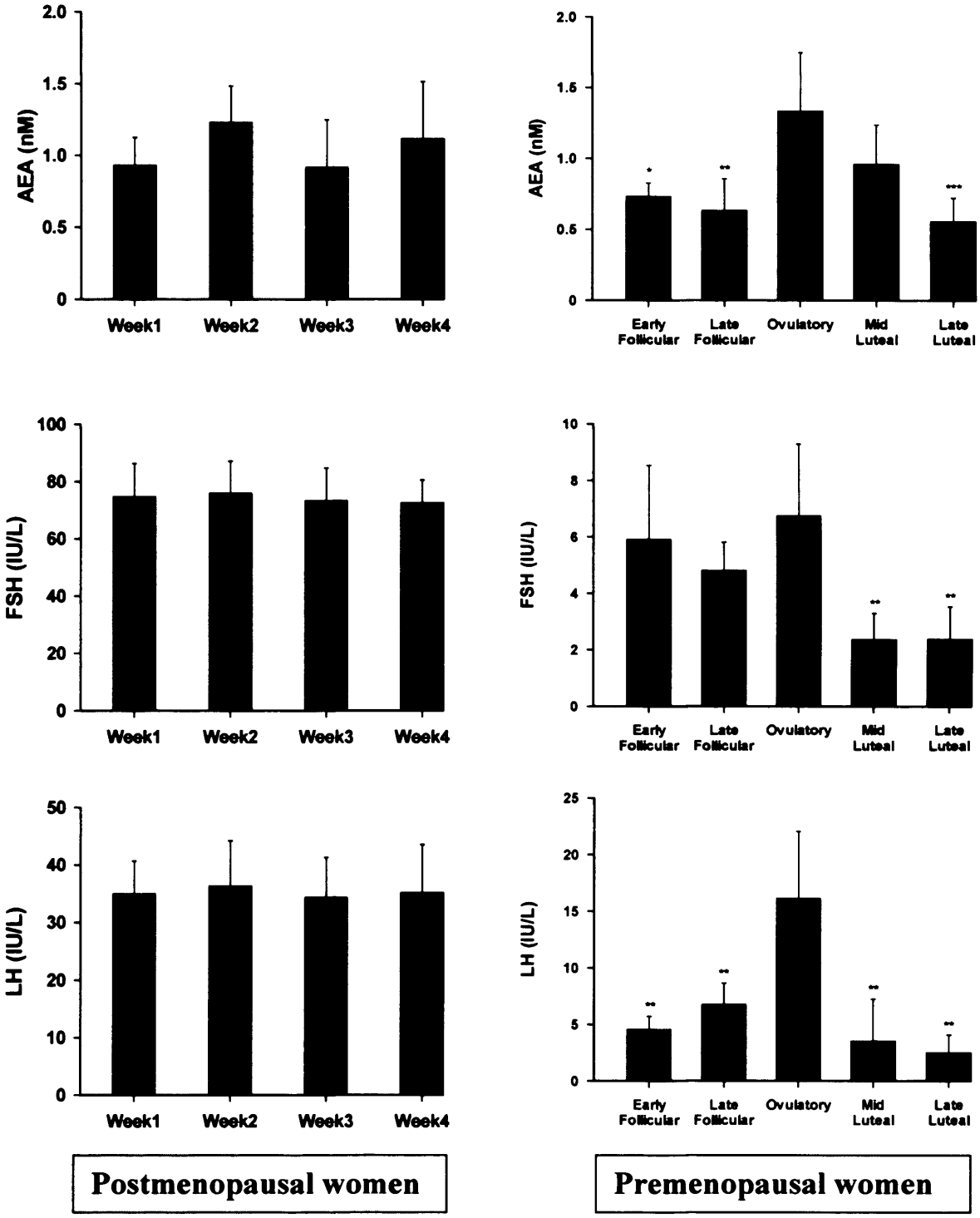


Figure 4.3. Comparison of plasma AEA and serum luteinizing hormone (LH; IU/L) and follicle stimulating levels (FSH; IU/L) levels through the menstrual cycle in the longitudinal study with levels found in post-menopausal women over the same period. Data are presented as mean ± SEM for 7 women in pre-menopausal and 5 women in post-menopausal group. *P<0.01; **P<0.001; One-way ANOVA with Tukey's honestly significance difference test when compared with the measurement in week 1 for the post-menopausal women and with early follicular phase.

4.4.4. Correlations between AEA and LH, FSH, E2, and P4

The correlations between plasma AEA levels and serum LH, FSH, E2 and P4 in pre-menopausal women are shown in **Figure 4.4**, and for the post-menopausal women in **Table 4.4**. There were highly statistically significant positive correlations between plasma AEA levels and serum LH ($P<0.0001$), AEA and FSH levels ($P=0.022$) and AEA and serum estradiol levels ($P=0.0015$), but not with progesterone ($P=0.841$) in the pre-menopausal women. By contrast there were no correlations between AEA and these hormones in the postmenopausal women (**Table 4.4**).

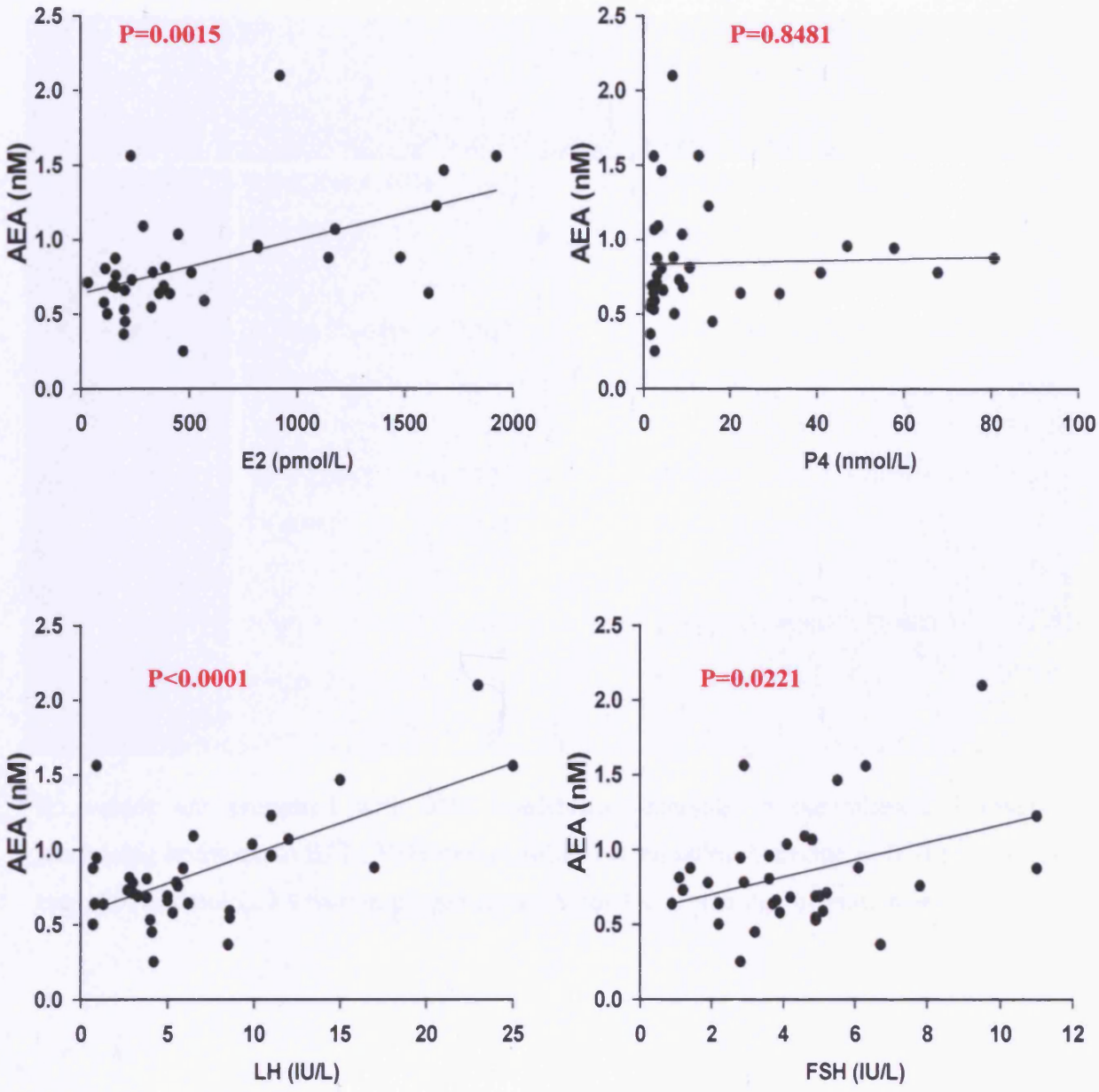


Figure 4.4. Pearson correlations between plasma anandamide (AEA), and serum luteinising hormone (LH), follicle stimulating hormone (FSH), estradiol (E2) and progesterone (P4) for the pre-menopausal women (n=7). n=number of volunteers.

Table 4.4. Correlations between AEA, estradiol, progesterone, luteinising hormone follicle stimulating hormone in pre-and post-menopausal women.

Correlation	Pre-menopausal women (n=7)	Post-menopausal women (n=5)
AEA and LH	R^2=0.399 (0.378 to 0.797)	R^2=0.0002 (-0.454 to 0.430)
	P<**0.0001**	P=0.949
AEA and FSH	R^2=0.148 (0.060 to 0.637)	R^2=0.1439 (-0.703 to 0.076)
	P= **0.022**	P=0.099
AEA and E2	R^2=0.266 (0.220 to 0.724)	R^2=0.017 (-0.329 to 0.543)
	P= **0.001**	P=0.578
AEA and P4	R^2=0.001 (-0.274 to 0.584)	R^2=0.036 (-0.301 to 0.364)
	P=0.842	P=0.419

R^2 values are presented with 95% confidence intervals in parentheses. LH=serum luteinising hormone in IU/L; FSH=serum follicle stimulating hormone in IU/L; E2=serum estradiol in pmol/L; P4=serum progesterone in nmol/L. n=number of volunteers.

4.5. Discussion

The results of the both the cross-sectional and the longitudinal arms were similar and show that plasma AEA fluctuated throughout menstrual cycle (El-Talatini et al., 2009). They confirm the previous observations of Habyeb et al., (Habayeb et al., 2004) that AEA levels were higher in the early follicular phase than the late luteal phase of the menstrual cycle. These data are, however, qualitatively different to those studied before where plasma AEA levels were highest in the early follicular phase (D2-7) of the menstrual cycle (Habayeb et al., 2004). However, that study was limited in its scope because it did not examine the AEA levels throughout the menstrual cycle. The other limitations of the study by Habayeb et al., and the cross sectional arm of this study, was that volunteers were recruited based on menstrual history without proven ovulation and how plasma AEA levels were related to those of ovarian steroids or gonadotrophins was not established.

In both arms of the study, plasma AEA levels varied throughout the menstrual cycle with a similar pattern and both were characterised by peak plasma AEA levels at the time of presumptive ovulation (cross-sectional arm; days 13-16) and validated ovulation (longitudinal arm; LH+1). The peak plasma AEA level observed at the time of ovulation in both arms suggests a possible association between AEA and folliculogenesis and/or ovulation. In a previous report, the AEA levels in follicular fluid obtained at the time of egg collection in stimulated cycles in women undergoing IVF-ET treatment (Schuel et al., 2002) were similar to the levels obtained at the time of ovulation in this study, which is not surprising as the oocyte retrieval stage in IVF cycles corresponds to the time of ovulation in the natural menstrual cycle. This suggested that either AEA may be produced locally in the ovarian follicle, or its production elsewhere in the body maybe intimately related to folliculogenesis or ovulation (El-Talatini et al., 2009).

The observation that plasma AEA levels declined slightly during the mid luteal phase of the menstrual cycle, coinciding with the 'window of implantation', is in keeping with studies in animals which suggest that low AEA levels are beneficial to implantation. Lazzarin et al., demonstrated that at this key point in the menstrual cycle the peripheral mononuclear cells increase production and activity of fatty acid amide hydrolase (FAAH), the principle enzyme involved in the degradation of AEA (Lazzarin et al., 2004). This activity of FAAH is thought to be under the control of progesterone (Maccarrone et al., 2001), which is elevated in the mid-luteal phase of the menstrual cycle, and so it is reasonable to speculate that once ovulation occurs, the increase in progesterone-induced FAAH expression and activity leads to a decrease in plasma AEA levels.

If peripheral FAAH activity was the only determinant of plasma AEA levels, then there should be a strong inverse correlation between plasma AEA and serum progesterone levels; we observed no such correlation and the patterns of AEA and P4 levels did not appear to be related suggesting that in the normal menstrual cycle, P4 is unlikely to be the main controlling factor of plasma AEA levels. The fact that serum E2 levels and plasma AEA levels are significantly correlated suggests a closer association between these two molecules. Maccarrone and co-workers (Maccarrone et al., 2002a) showed that AEA release from endothelial cells was stimulated by E2, supporting the possibility that E2 and AEA levels are closely linked.

Recently a report by Habayeb et al., that plasma AEA levels below 2 nM in the first trimester of pregnancy were predictive of live birth in women with a threatened miscarriage (Habayeb et al., 2008b), suggested that plasma AEA levels above this value were not conducive to successful pregnancy. In this study, the level of plasma AEA in the early luteal phase of the menstrual cycle, which coincides with the implantation window

were all lower than 1 nM. Although, there is evidence that high levels of AEA are embryotoxic and have effects on peri-implantation events in the mouse (Liu et al., 2002; Sun and Dey, 2008) and sheep (Turco et al., 2008), the exact relationship between AEA and human implantation has not yet been studied. Investigating the possible relationship between plasma AEA and serum hormones with implantation in IVF women required further investigation (El-Talatini et al., 2009) (see Chapter 6).

The findings of positive correlations between plasma AEA and serum FSH, LH and E2 levels in pre-menopausal women, suggest that these hormones may be involved in the regulation of AEA or *vice-versa*. The gonadotrophins are, however, unlikely to be the sole regulators of AEA as there was no corresponding increase in the levels of plasma AEA in the post-menopausal women, where serum FSH and LH levels were grossly elevated. However, since reproductive/endocrine physiology in pre-and post-menopausal women is different it may well be that the AEA regulating mechanisms are different in these two periods or there are other factors involved which are yet to be thoroughly understood. Further studies will need to undertaken to explore in detail precisely how this regulation may occur.

In summary, the above findings suggest that AEA may be controlled by either gonadotrophins, or most probably by estradiol, or by both during the menstrual cycle (El-Talatini et al., 2009).

4.6. Conclusions

The observations in this Chapter suggest that the regulation of AEA during the menstrual cycle may be linked or associated with the hormones that regulate the menstrual cycle. It is possible that AEA could be produced by ovarian follicles and that high plasma AEA levels are required at or for successful ovulation. These observations are critical to a further understanding of the role of anandamide in the regulation of human fertility. In the next Chapter, this possibility is further explored by investigating the role of the endocannabinoid system in the human ovary.

CHAPTER 5

Localisation and Function of the Endocannabinoid system in the Human Ovary

5.1. Introduction

In Chapters 3 and 4 it was clearly demonstrated that ovarian follicle fluid contains significant quantities of anandamide and that there were significant fluctuations in anandamide (AEA) levels during the menstrual cycle with the highest concentrations associated with ovulation (El-Talatini et al., 2009), suggesting that AEA may play a role in folliculogenesis, oocyte maturity and ovulation. However, the source of AEA in the follicular fluid and its possible role within the ovary remains uncertain.

Our knowledge about the effect of cannabinoids on the ovary comes from studies in animals (Kostellow et al., 1980; Adashi et al., 1983) and marijuana users (Mendelson et al., 1986). Studies in the *in-vitro* rat ovary model demonstrated that Δ^9-THC exerts a direct inhibitory effect on folliculogenesis (Adashi et al., 1983) and ovulation (Nir et al., 1973) whereas it causes anovulation in rats (Ayalon et al., 1977), rabbits and rhesus monkeys (Smith et al., 1979) as a result of LH surge disruption (Tyrey, 1980). The effects of cannabis and Δ^9-THC on the human ovary have been studied and shown to be inconsistent (Brown and Dobs, 2002) mainly due to the development of tolerance among chronic users (Brown and Dobs, 2002) and the timing of the administration of cannabis in relation to the phases of the menstrual cycle (Mendelson et al., 1986). While in some studies, chronic cannabis smokers appeared to have normal menses after intensive smoking (Mendelson et al., 1986), other studies showed increased anovulatory cycles and a short luteal phase. Nevertheless, a direct adverse effect on the ovary was clearly observed as cannabis users were at a higher risk of primary infertility due to anovulation (Muller et al., 1990), and even when these women had IVF treatment, they produced poor quality oocytes and lower pregnancy rates compared to non-users (Klonoff-Cohen et al., 2006).

AEA has been demonstrated in ovarian follicular fluids at the time of oocyte retrieval in *in-vitro* fertilisation (IVF) cycles (Chapter 3) suggesting that it may play a role in ovarian follicle or oocyte maturity (Schuel et al., 2002). Therefore, this aim of this part of the thesis was to localise the endocannabinoid system in the ovary and to investigate whether follicular fluid and plasma AEA levels are related to physiologically important ovarian events such as folliculogenesis, the size and maturity of the preovulatory follicle, oocyte maturity, and ovulation.

5.2. Materials and Methods

This study consisted of 2 parts; the first was mainly to localise the endocannabinoid system in the ovary using immunohistochemistry, and the second to investigate the role of AEA in ovarian follicles in relation to folliculogenesis, follicle size and oocyte maturity.

5.2.1. Subjects

For the immunohistochemical studies, 12 ovarian tissue blocks were collected prospectively from women having a hysterectomy and bilateral salpingo-oopherectomy for benign pathology such as; heavy periods, benign ovarian cyst or prophylactic oophorectomy for family history of ovarian cancer. The ovaries were confirmed by histopathology to be normal. Control tissues including fetal membranes (for CB1, CB2 and FAAH) and secretory phase endometrium (for NAPE-PLD) were obtained from women undergoing elective Caesarean section at term (Habayeb et al., 2008a) and hysterectomy for benign conditions such as myoma or dysfunctional uterine bleeding (Taylor et al., 2005), respectively. Tissues were fixed in 10% neutral buffered formalin for 4 days before being embedded in paraffin wax.

For the assessment of follicular fluid AEA concentrations and the possible role of AEA in ovarian physiology, a total of 37 women undergoing ovarian stimulation for *in-vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) with embryo transfer (ET) between July 2007 and December 2007 were recruited into the study at the Assisted Conception Unit of the Leicester Royal Infirmary Hospital. All the women were healthy and had no other medical disorders, had not used cannabis in the last 10 years and had a basal FSH of their previous cycle of ≤10 IU/L in the period prior to undergoing IVF/ICSI-ET (Bancsi et al., 2003). Eight percent of them smoked less than 20 cigarettes per day. Each volunteer signed an informed written consent prior to partake in to the study which was approved by the Leicestershire and Rutland Research Ethics Committee (approval number Q6/ Q2501/49).

5.2.2. Controlled ovarian hyperstimulation protocol, follicular fluid sampling and oocyte retrieval

Ovarian stimulation was performed using a long protocol, with pituitary down-regulation with the gonodatrophin releasing hormone (GnRH) agonist Supercur (Aventis Pharma Ltd, Kent, UK) commenced in the mid luteal phase of the previous cycle and continued until ovulatory human chorionic gonadotrophin (hCG) was given (Macklon et al., 2006; Arslan et al., 2005; Speroff and Fritz, 2005b). Stimulation was initiated with either human menopausal gonadotrophin (hMG) Menopur (Ferring, Langley, UK) or recombinant follicle stimulating hormone (rFSH) Puregon (Organon Laboratories Ltd, Cambridge, UK) or a combination of rFSH and hMG (Agrawal et al., 2000; Strehler et al., 2001; Ng et al., 2001) once there was no sonographic evidence of no ovarian follicular activity and serum estradiol levels were below 200 pmol/L. The dosage was based on the patient's age, BMI and early follicular phase serum FSH levels (Cahil et al., 1994). Follicular maturation was assessed by serial (every 2-3 days) transvaginal ultrasound scan and serum estradiol

measurements (Lass, 2003). hCG 10,000 IU (Pregnyl; Organon Laboratories Ltd, Cambridge, UK) was administered subcutaneously to induce final oocyte maturation, when at least 4 follicles measuring at least 17 mm in diameter **(Figure 5.1)** combined with an endometrial thickness of at least 8 mm were observed. Thirty-six hours after hCG administration, oocyte retrieval was performed transvaginally under ultrasound guidance and intravenous sedation with a combination of Propofol (Propoven 1%; Fresenius Kabi Ltd, Cheshire, UK), Alfentanil hydrochloride (Rapifen; Janssen-Cilag Ltd, Buckinghamshire, UK) and Midazolam (Hypnovel; Roche, Hertfordshire, UK). All the identified follicles were aspirated until the follicle wall collapsed. Prior to the administration of sedation, 4 mL of blood was collected into an EDTA tube for AEA assay. The aspirated fluid was then examined under a low power stereomicroscope (Nikon SM200) by an experienced embryologist for the presence of oocytes. The corona-cumulus-oocyte complex was identified, and removed with a Pasteur glass pipette (Poulten & Graf Ltd, Barking, UK) **(Figure 5.2)**. The follicular fluid from each follicle was thereafter collected into Kimble Scintillation vials for AEA measurements. For each aspirated follicle, its diameter (mm) and volume of fluid obtained (mL) and whether an oocyte was present in the aspirated fluid or not were recorded on a data sheet as shown by the example in **Figure 5.3.**

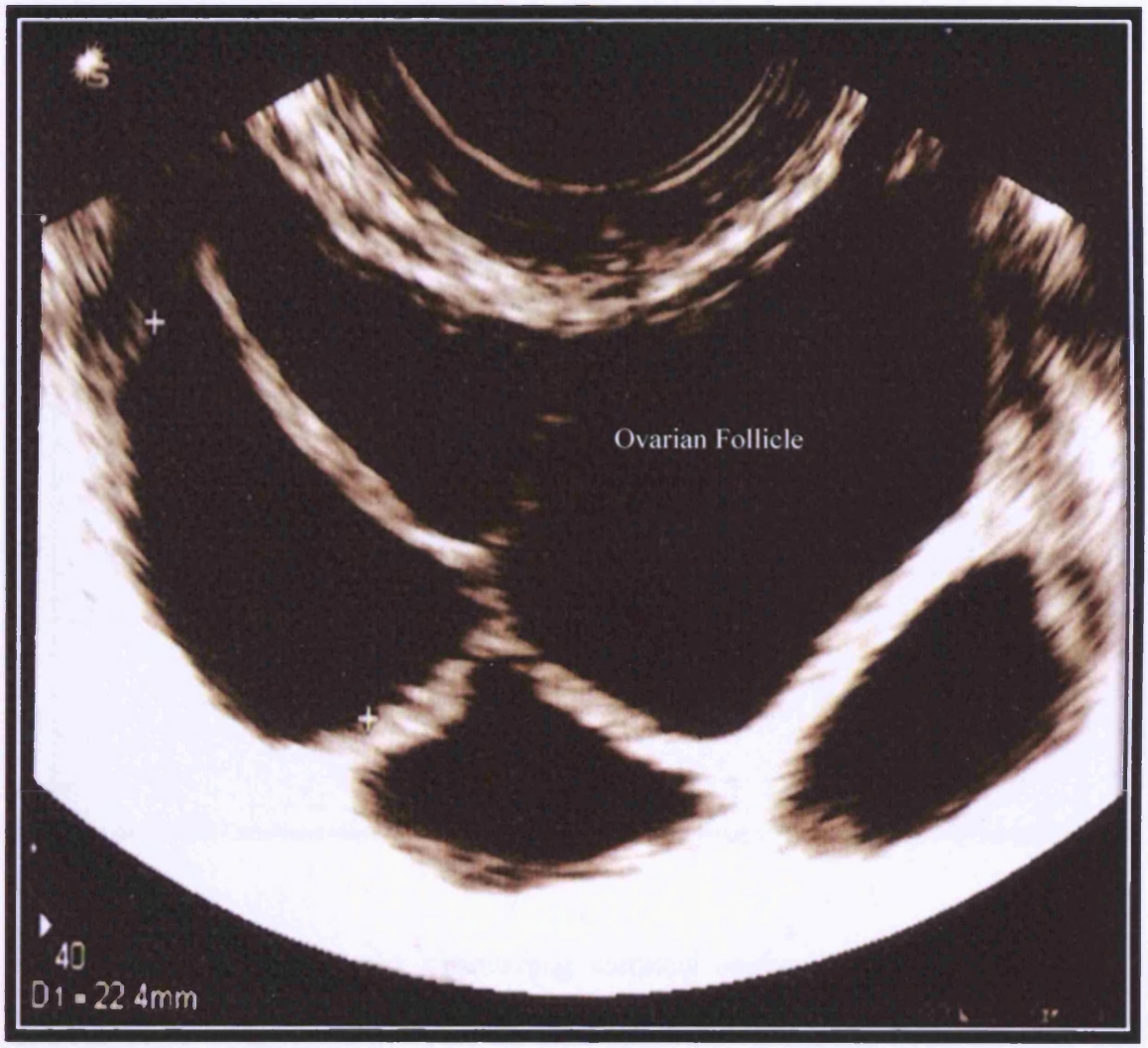


Figure 5.1. A transvaginal scan image of the ovary showing 5 ovarian follicles during oocyte retrieval.

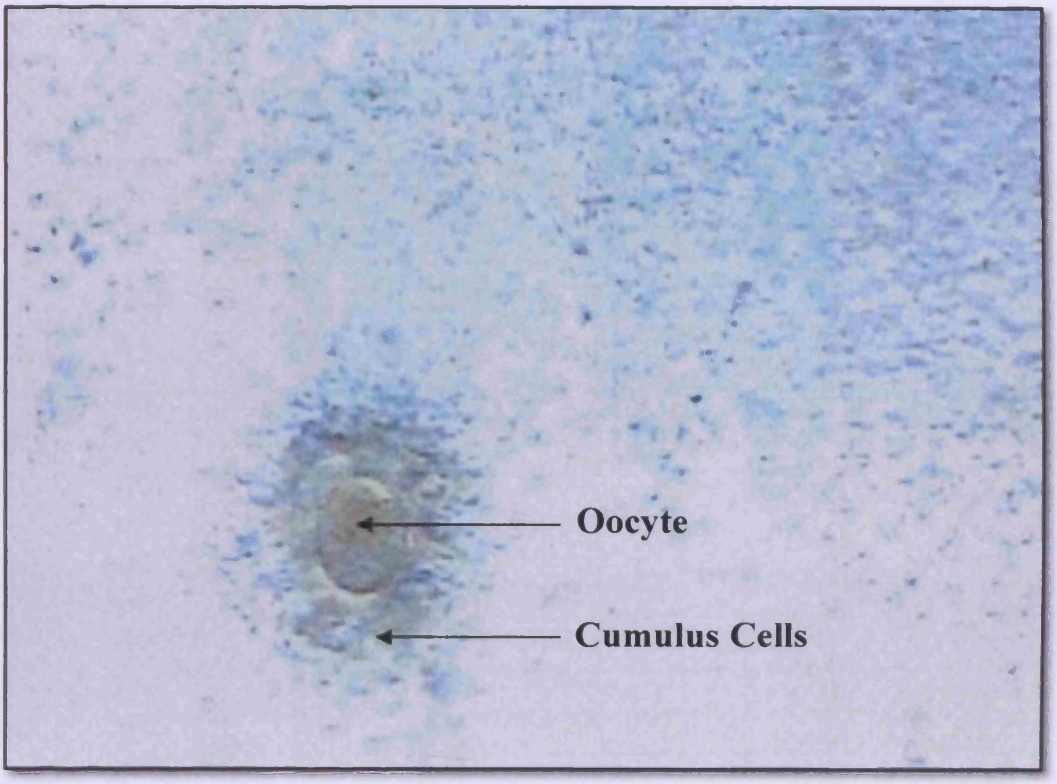
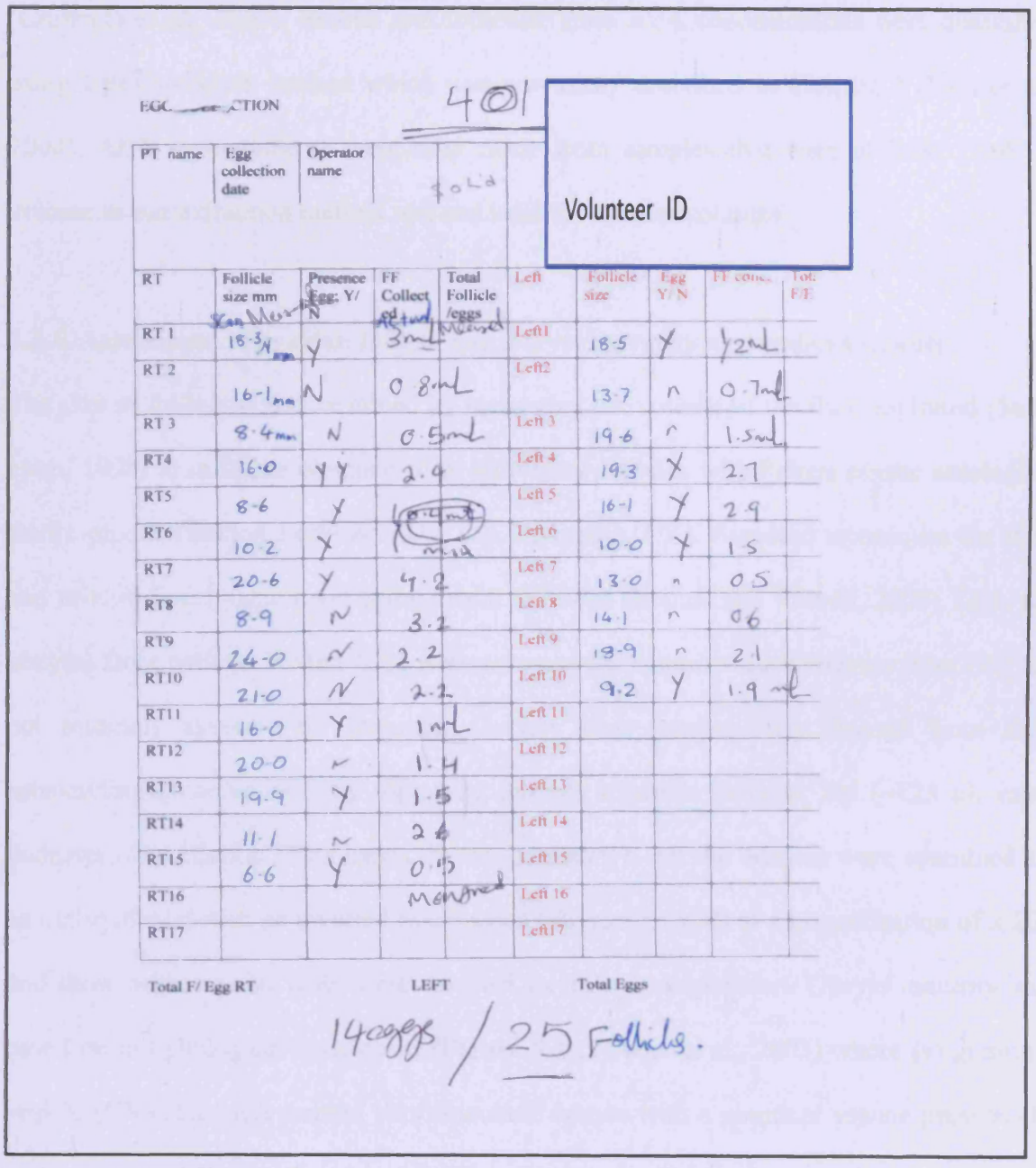


Figure 5.2. Oocyte and the surrounding cumulus oophorus cells as seen under the microscope immediately after retrieval. The photomicrograph has been taken at 100 x magnification.



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PT name	Egg collection date	Operator name			Volunteer ID

RT	Follicle size mm	Presence Egg: Y/N	FF Collected	Total Follicle /eggs	Left	Follicle size	Egg Y/N	FF conc.	Tot. FF
RT 1	18·3	Y	3mL		Left1	18·5	Y	½mL	
RT 2	16·1	N	0·8mL		Left2	13·7	n	0·7mL	
RT 3	8·4	N	0·5mL		Left 3	19·6	n	1·5mL	
RT4	16·0	Y Y	2·4		Left 4	19·3	Y	2·7	
RT5	8·6	Y			Left 5	16·1	Y	2·9	
RT6	10·2	Y			Left 6	10·0	Y	1·5	
RT7	20·6	Y	4·2		Left 7	13·0	n	0·5	
RT8	8·9	N	3·2		Left 8	14·1	n	0·6	
RT9	24·0	N	2·2		Left 9	19·9	n	2·1	
RT10	21·0	N	2·2		Left 10	9·2	Y	1·9mL	
RT11	16·0	Y	1mL		Left 11				
RT12	20·0	N	1·4		Left 12				
RT13	19·9	Y	1·5		Left 13				
RT14	11·1	N	2·6		Left 14				
RT15	6·6	Y	0·5		Left 15				
RT16			Missed		Left 16				
RT17					Left17				

Total F/ Egg RT LEFT Total Eggs

140eggs / 25 Follicles.

Figure 5.3. The data sheet for one of the volunteers at the time of oocyte retrieval. RT refers to the right ovary and the number refers to the follicles and the same applies to the left side. FF= follicular fluid.

5.2.3. Measurement of Plasma and follicular fluid AEA concentrations

Blood and follicular fluid samples were processed within two hours of collection (Giuffrida et al., 2000). Plasma and follicular fluid AEA concentrations were quantified using UPLC-MS/MS method which was previously described in Chapter 2 (Lam et al., 2008). AEA measurements were only made from samples that were at least 1 mL in volume as our extraction method was not valid for smaller volumes.

5.2.4. Assessment of ovarian follicle size, oocyte maturity and embryo quality

The size of follicle was determined by measuring the volume of the fluid aspirated (Salha et al., 1998) in millilitre obtained from individual follicles with Falcon plastic serological sterile pipette (Becton Dickinson UK Ltd, Plymouth, UK). Standard techniques for ICSI and *in-vitro* insemination and culture were followed (Brauda and Rowell, 2003). Only the oocytes from patients having ICSI were assessed for maturity since oocytes from IVF are not routinely assessed for maturity. Oocytes were mechanically cleaned from their surrounding cumulus cells by aspiration through a plastic Stripper Tip (~125 μL inner diameter; MidAtlantic Diagnostics, Berlin, Germany). All the oocytes were examined by an embryologist with an inverted microscope (Olympus I x70) at a magnification of x 200 and those with a polar body were selected for micromanipulation. Oocyte maturity was based on morphological assessment **(Figure 5.4);** (Ebner et al., 2003) where (a) germinal vesicle (GV) stage represented very immature oocyte with a germinal vesicle present, (b) metaphase I (MI) stage oocytes were still immature but the germinal vesicle had disappeared and the first polar body had not been extruded and (c) metaphase II (MII) oocytes were mature and the first polar body was present.

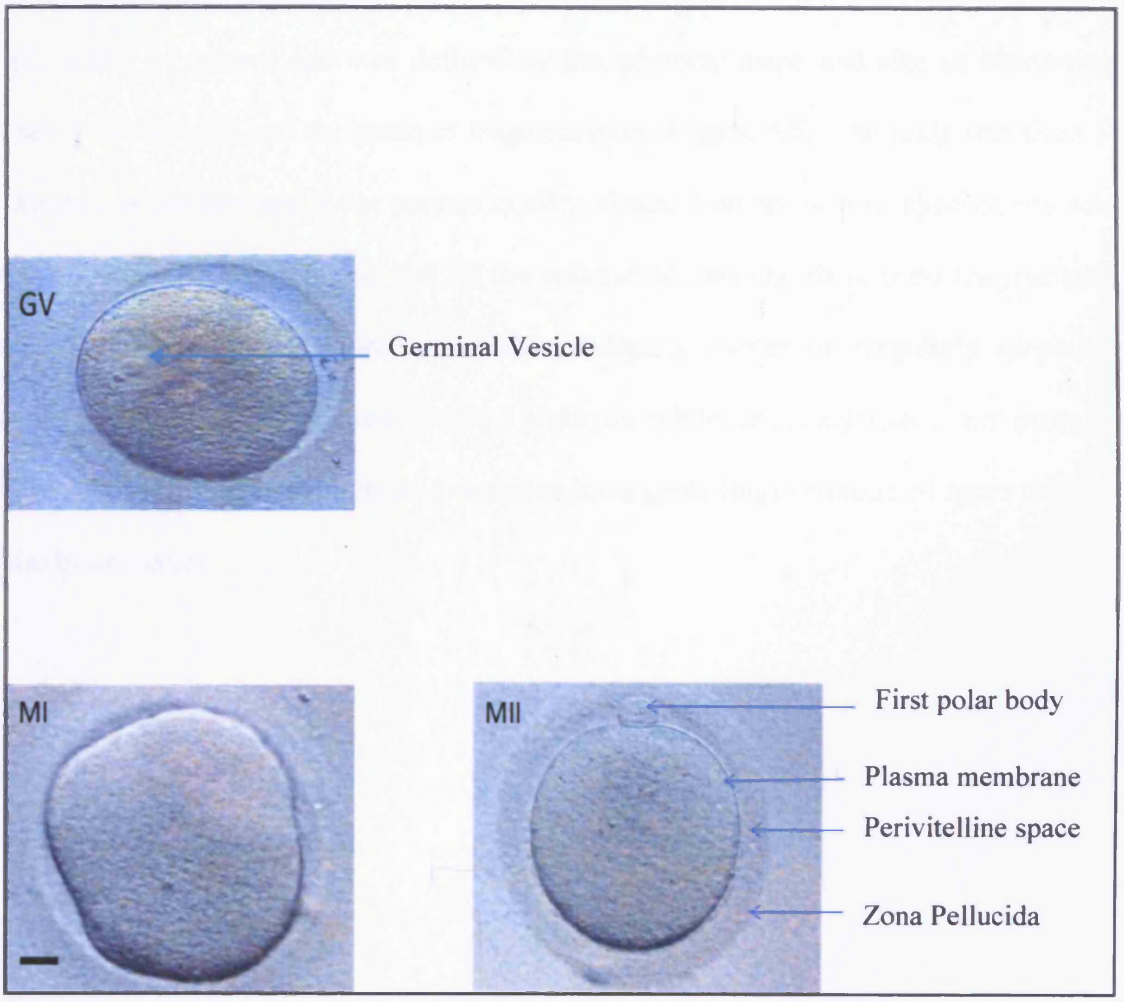


Figure 5.4. Photomicrographs showing immature oocytes: GV (Germinal vesicle), Metaphase (MI) and a mature oocyte - Metaphase (MII). Metaphase (MII) oocytes were used in ICSI treatment. Images were taken at 200x magnification. Bar=100 μm.

Embryo quality assessment

Embryo quality was assessed during the second and third day (Hoover et al., 1995; Cutting et al., 2008) of culture and was defined by the number, shape and size of blastomeres, presence of vacuoles and the grade of fragmentation **(Figure 5.5)**. The scale was from 1 to 4, where 1 is the best and 4 the poorest quality. Grade 1 embryos have blastomeres which are even and spherical in shape and fill the volume of zona and there is no fragmentation; Grade 2 embryos have blastomeres which are slightly uneven or irregularly shaped and there is up to 10% fragmentation; Grade 3 embryos exhibit fragmentation of not more than 50% of the blastomeres, and grade 4 embryos have gross fragmentation of more than 50% of the blastomeres.

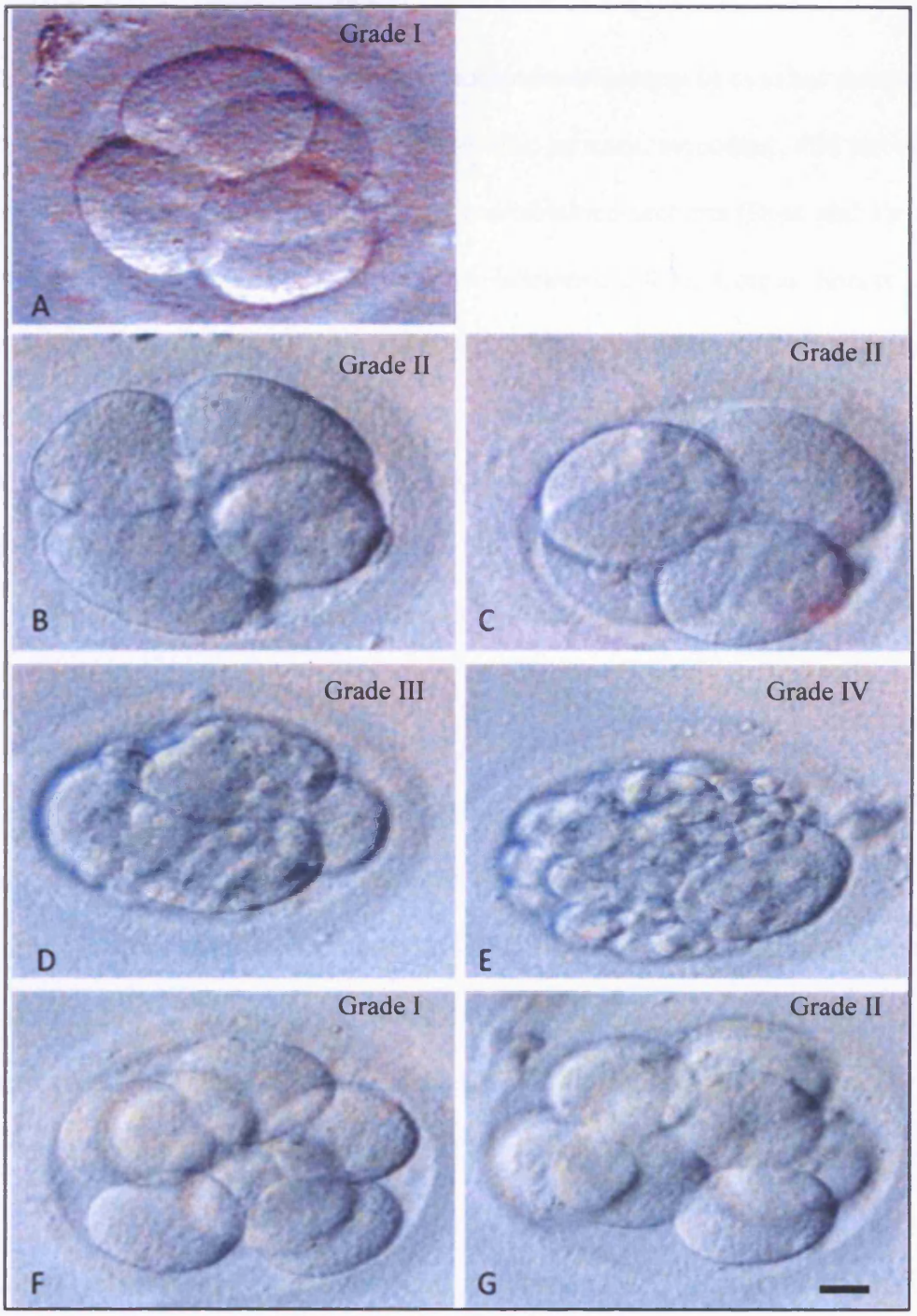


Figure 5.5. The grading system used for the embryos quality assessment.
Photomicrographs; A, B, C, D, E represent 4 cell stage embryos at 48 hours after oocyte retrieval. Grade 1 and II are good quality embryos, while grade III and IV are poor quality embryos. In grade I and II the bastomeres are spherical, regular in shape and with no or minimal fragmentation, in grade III the fragmentation of not >50% of blastomeres, and grade IV embryos have gross fragmentation. Images F and G depict the 7 cell stage embryos at 72 hr after oocyte retrieval of embryos transferred to one of the volunteers that resulted in a successful singleton pregnancy. These photomicrographs were taken with permission from study volunteers and are from different treatment cycles. Bar = 100 μm.

5.2.5. Immunolocalisation of the endocannabinoid system in ovarian tissues

Ovarian follicles were classified as primordial, primary, secondary, and tertiary based on classical histology of haematoxylin and eosin-stained sections (Ross and Pawlina, 2003; Speroff and Fritz, 2005d; Williams and Erickson, 2008). Corpus luteum and corpus albicans were also identified using classical histological criteria. After obtaining positive immunoreactivity on the control tissues with the cannabinoid receptors (CB1 and CB2), fatty acid amide hydrolase (FAAH) and *N*-acyl-phosphatidyl ethanolamine selective phospholipase (NAPE-PLD) antibodies, the same concentrations of these antibodies and IgG/rabbit sera were applied to ovarian sections in duplicate.

Immunolocalisation of CB_1, CB_2 and FAAH

The CB1 and CB2 rabbit polyclonal antibodies (Sigma-Aldrich Ltd., Poole Dorset, UK) were used at 1:500 and 1:250 dilutions, respectively in TBS. Non-immune rabbit IgG (Dako, Glostrup, Denmark) diluted in TBS to the same concentration was used as the negative control. Serum from rabbit immunised with human FAAH protein (Alpha Diagnostics International, San Antonio, TX) was used at a 1:2000 dilution in TBS with normal rabbit serum (DAKO) diluted in TBS to the same concentration as control.

Tissues sections (5 μm) were mounted onto silanized glass microscope slides and dried for 7 days at 37°C prior to use. Slides were de-waxed in xylene three times for 3 min and re-hydrated in graded alcohol for 3 min followed by incubation in distilled water for 3 min. Microwave antigen retrieval was performed for CB1 and CB2 only by incubating the slides in 10mM citric acid buffer (pH 6.0) heated at 700 watts for 10 min (Taylor and Al-Azzawi, 2000). Endogenous peroxidase activity was then blocked by incubation in 6% H_2O_2 in water for 10 min. Blocking of non-specific protein binding sites was performed by incubation in 10% normal goat serum for 10 min at room temperature. Endogenous avidin

and biotin sites were blocked using the Avidin-Biotin Blocking Kit (Vector Laboratories) as recommended by the manufacturer. Primary antibodies diluted in tris-buffered saline (TBS; 0.5 M Trizma, 1.5M NaCl, 2 mM $MgCl_2$, pH 7.6; 100 µl/slide) were added and the slides were incubated in a humid chamber overnight at 4°C. Slides were then washed in TBA [Tris-buffered saline containing 0.1% bovine serum albumin (Fraction V; Sigma-Aldrich Ltd.)] for 30 min. After washing the slides for 30 min in TBS, biotinylated goat anti-rabbit antibody (Vector Laboratories) diluted to 1:400 in TBA was applied for 30 min at room temperature. After an additional wash in TBS, and ABC Elite reagent was applied according to the manufacturer's detailed instructions. After additional washing in TBS for 20 min, DAB was added to each slide (100 µL /slide) for 5 min. Slides were then washed in distilled water for 5 min before counterstaining in Mayer's haematoxylin for 15 seconds. After washing in running tap water for 5 min, slides were dehydrated in graded alcohols, cleared in xylene twice for 6 min before mounting with DPX mounting medium (BDH Poole, Dorset).

Immunolocalisation of NAPE-PLD

NAPE-PLD immunohistochemistry was performed according to the manufacturer's instructions (ABIN110270; Cayman Chemical, Ann Arbor, MI). Briefly after de-waxing and rehydration to water, endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 in ice-cold water for 15 min and non-specific protein binding sites blocked with 5% normal goat serum in TBS (TBS; 0.5M Trizma, 1.5M NaCl, pH 7.4; 100 µl/slide) for 30 min at room temperature. Primary antibodies diluted in TBS (1:200) were added and the slides were incubated in a humid chamber overnight at room temperature. After washing in TBS containing 0.1% Tween 20 (TBS-T; Sigma-Aldrich Ltd.) three times for 5 min with buffer changes, biotinylated goat anti-rabbit antibody (Dako; Glostrup, Denmark) diluted to 1:400 in TBS was applied for 30 min at room temperature. After an additional wash in

TBS-T, ABC Elite reagent (Vector Laboratories) was applied and the slides washed again. Immunoreactivity was visualised with 3,3'-diaminobenzidine for 5 min and lightly counterstained with Mayer's haematoxylin. After washing in running tap water for 5 min, slides were dehydrated in graded alcohols, cleared in xylene twice for 6 min before mounting with DPX mounting medium (BDH Poole, Dorset).

Photomicrography

Photomicrographic images were taken on an Axioplan transmission microscope equipped with a Sony DXC-151P analogue camera connected to a computer running Axiovision image capture and processing software (Axiovison version 4.4, Carl Zeiss Ltd., Welwyn Garden City, Hertfordshire, UK). Images were captured at either 50x, 200x, or 400x magnification in the presence of daylight and medium value neutral density filters with the lamp set at 6400K. Image backgrounds were colour corrected to neutral grey with the use of ColorPilot software (version 4.62; www.colorpilot.com).

5.3. Statistics

Demographic and cycle characteristic data are expressed as mean ± SD (range) or median where appropriate. Follicular fluid and plasma AEA levels are expressed as mean ± SEM. Means were compared using the Student's unpaired *t*-test and $P<0.05$ was considered statistically significant. For correlations between follicle size and follicular fluid AEA concentrations Spearman correlation test was used.

5.4. Results

5.4.1. Immunohistochemistry patients and localisation of endocannabinoid system in ovarian tissues

The mean age of the 12 volunteers from whom ovarian sections were obtained was 41.8 ± 2.8 (range 39-45 years). The indications for removal of the ovaries were menorrhagia (6 women), benign ovarian cyst (5 women) and prophylactic oopherectomy for a family history of ovarian cancer (1 woman).

Immunohistochemistry

Specificities for each of the antibodies used in these studies using control tissues are shown in **Figure 5.6** using term fetal membranes for CB1, CB2 and FAAH and secretory phase endometrium for NAPE-PLD. Immunohistochemical analysis of the ovarian tissues for immunoreactive CB1, CB2, FAAH and NAPE-PLD revealed a widespread pattern of immunostaining in the ovarian cortex and medulla **(Figures 5.7, 5.8, 5.9 and Table 5.1)**. The immunoreactivity for CB1 was observed in the medulla and the cortex. With regard to the follicles CB1 was localised in primordial, primary, secondary and tertiary follicles but mainly in the granulosa and theca cells of the secondary and tertiary follicles. No immunostaining was observed in the oocytes of these follicles although immunopositive staining was observed in the corpora lutea and corpora albicantes **(Figure 5.7)**. Similarly, CB2 was expressed in a similar manner as CB1 except the CB2 staining was more intense in the ovarian follicles in comparison to CB1 and more intense in the oocytes and granulosa cells of tertiary follicles in comparison to the theca cells **(Figure 5.7)**. However, FAAH immunostaining was not observed in the granulosa cells or the oocytes of primordial, primary, secondary or tertiary follicles but was expressed in the theca cells of secondary and tertiary follicles, corpus luteum and corpus albicans **(Figure 5.8)**. NAPE-PLD immunostaining was observed in the oocytes of primordial follicles, but not in the

granulosa cells of primordial or primary follicles **(Figure 5.9)**. NAPE-PLD immunostaining was also absent from the oocytes of other types of follicles. However, NAPE-PLD was clearly expressed in the granulosa and theca cells of the secondary and tertiary follicles and corpus luteum, corpus albicans, and medulla **(Figure 5.9)**. The intensity of immunostaining of NAPE-PLD in the corpus albicans appeared to be of a lower intensity when compared to that of FAAH staining in corpus albicans **(Figure 5.8)**.

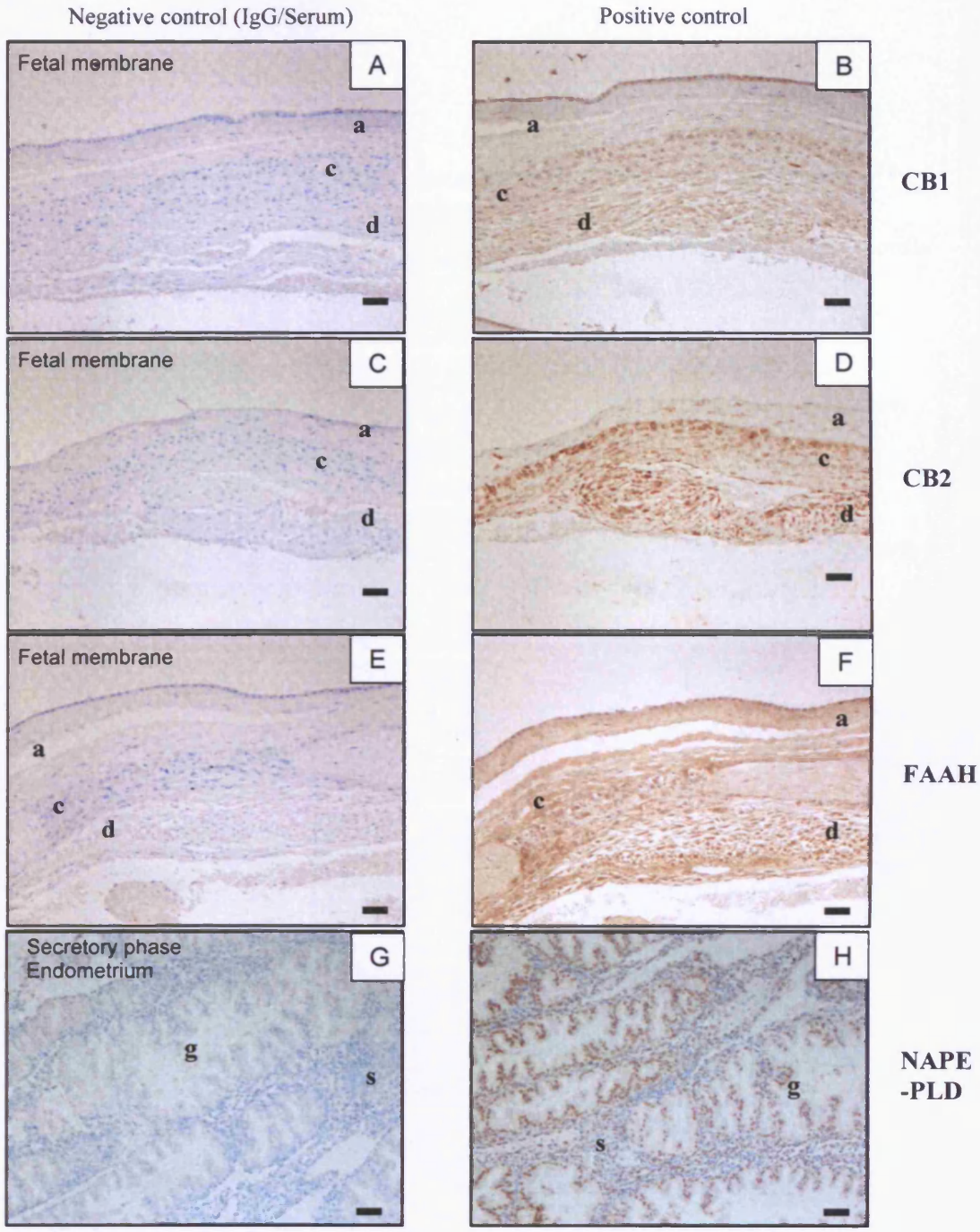


Figure 5.6. Immunohistochemical staining for the cannabinoid receptors (CB1 and CB2); fatty acid amide hydrolase (FAAH) and *N*-acyl-phosphatidyl ethanolamine selective phospholipase D (NAPE-PLD) on control tissues. Images on the left represent the negative control (IgG/serum) and images on the right are sections incubated with specific antibodies/antisera. The tissues used in A-F are human fetal membranes (**a** = amnion; **c** = chorion and **d** = decidua) whereas G and H are human endometrium (**g** = glands and **s** = stroma). The images are representatives from at least six samples and were taken at 100x magnification. Bar = 50μm.

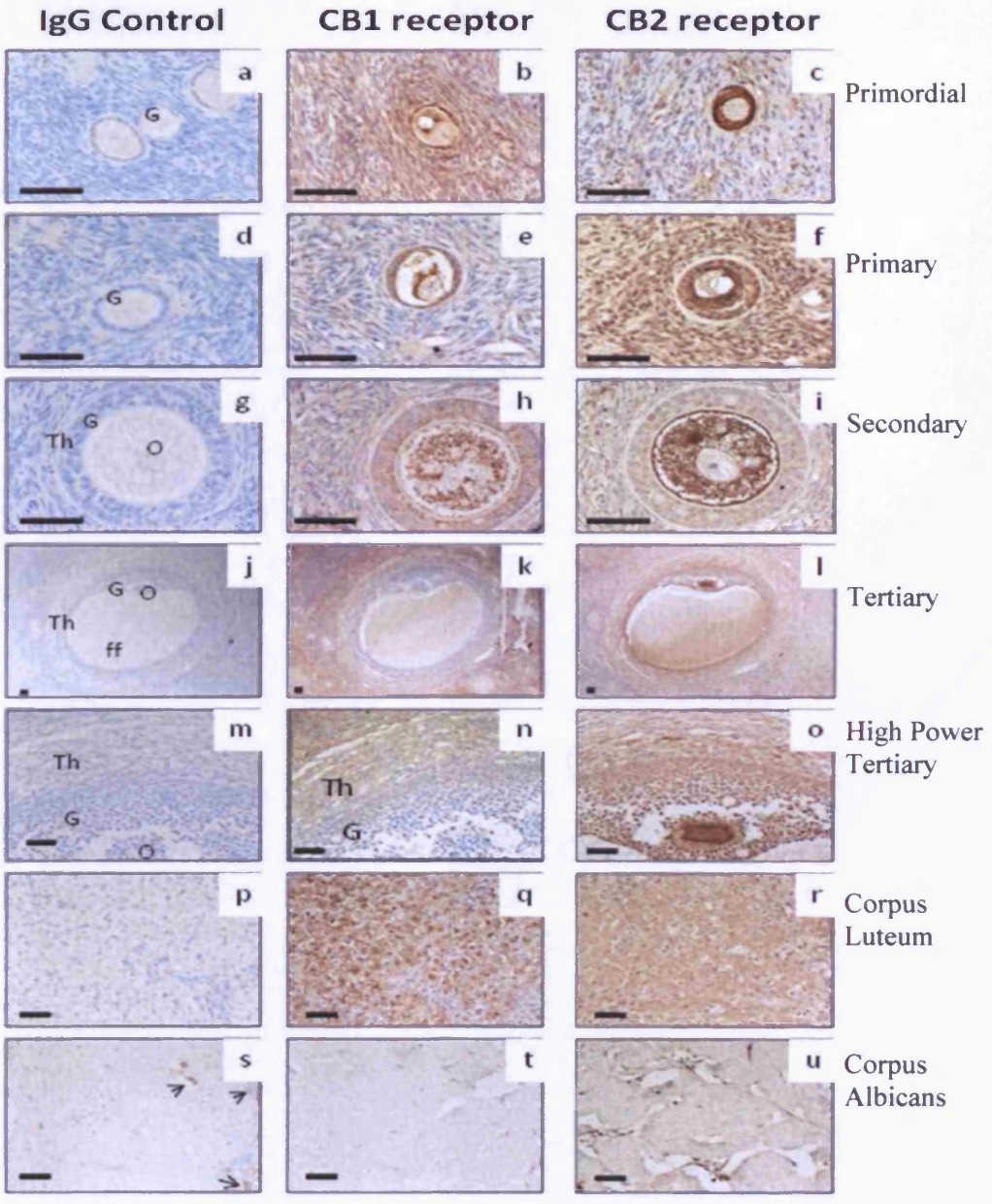


Figure 5.7. Immunohistochemical staining for CB1 and CB2 receptors. Images on the left panel represent the negative control (IgG) and images in the middle are for CB1 on the right are for CB2. Images a, b and c are primordial follicle; d, e and f are primary follicles; g, h and i are secondary follicles; j, k and l are low power images of tertiary follicles; m, n an o are high power images of tertiary follicles, p, q and r are corpus luteum, r, s and t are images of the corpus albicans. The images are representatives from at least two structures and taken at 50x, 200x or 400x magnification. Bar = 50μm.

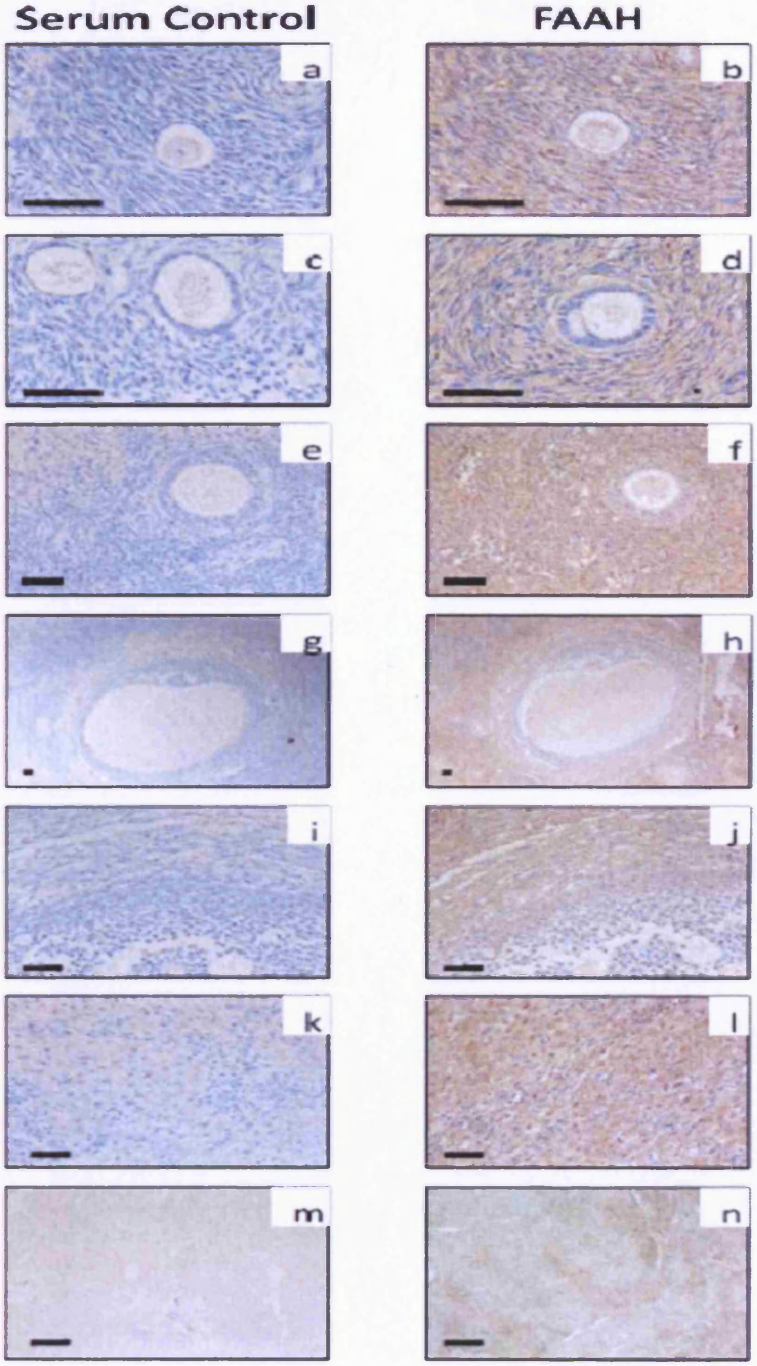


Figure 5.8. Immunohistochemical staining for FAAH. Images on the left panel represent the negative control (non-immune rabbit serum and images on the right are for FAAH. Images a and b are primordial follicle; c and d are primary follicles; e and f are secondary follicles; g and h are low power images of tertiary follicles; i and j are high power images of tertiary follicles, k and l are corpus luteum, and m and n are images of the corpus albicans. The images are representatives from at least two structures and taken at 50x, 200x or 400x magnification. Bar = 50μm.

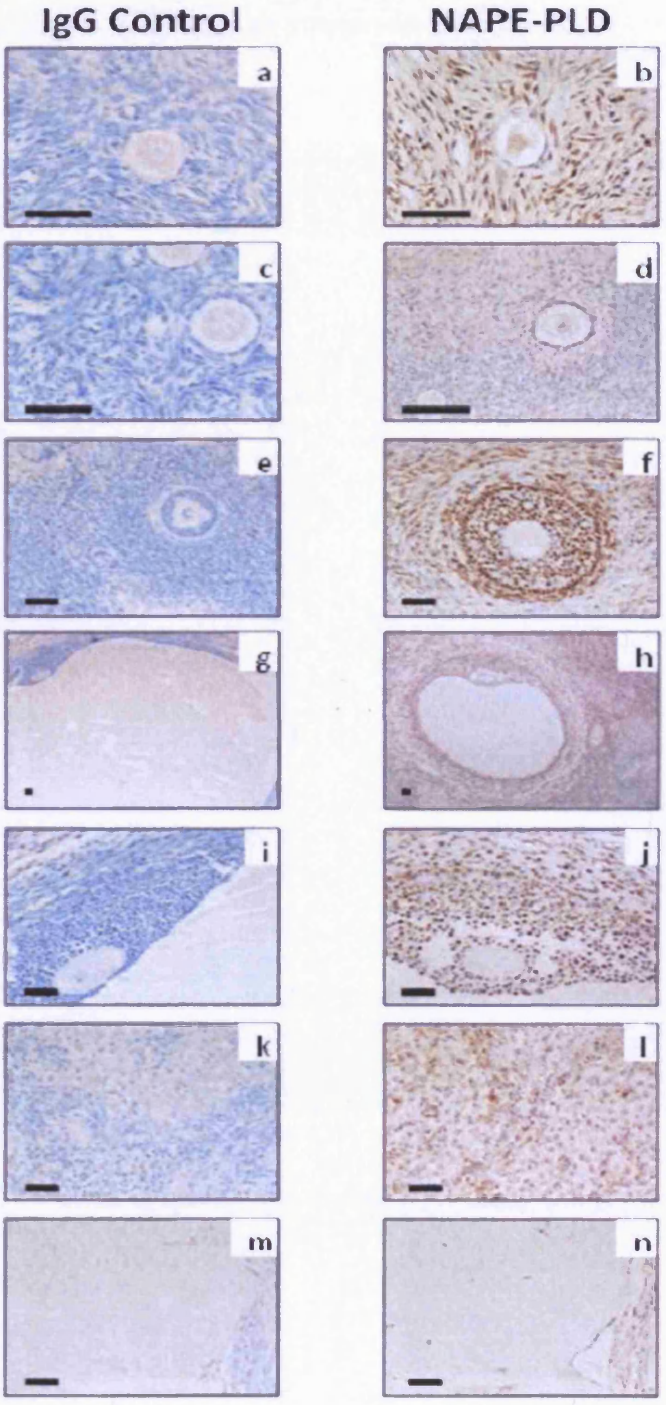


Figure 5.9. Immunohistochemical staining for NAPE-PLD. Images in the panel represent the negative control (rabbit IgG) and images on the right are for NAPE-PLD. Images a, and b are primordial follicles, c and d are primary follicles, e and f are secondary follicles; g and h are low power images of tertiary follicles, i and j are high power images of tertiary follicles, k and i are images of corpus luteum, and m and n are images of corpus albicans. The images are representative from at least two structures and taken at 50x, 200x, or 400x magnification. Bar= 50 μm.

Table 5.1. Immunohistochemistry localisation of the various components of the endocannabinoid system in the human ovarian cortex.

Structure	Cell type	CB1	CB2	FAAH	NAPE-PLD
Primordial	oocyte granulosa	- +	- ++	- -	- -
Primary Follicle	oocyte granulosa	- +	- +	- -	- -
Secondary Follicle	oocyte granulosa theca	+ + +	- ++ +	- - +	- + +
Tertiary Follicle	oocyte granulosa theca	- + +	++ ++ +	- - +	- + +
Corpus luteum		++	+	+	++
Corpus albicans		+	+	+	+

Scoring system (-) = staining absent; (+) = staining visible; (++) = strong staining

5.4.2. IVF/ICSI-ET patients and follicular AEA measurements

A total of 37 women undergoing IVF/ICSI-ET were recruited in to this part of the study, their mean age and BMI were 33.28 ± 5.08 years, 24.25 ± 2.68 kg/m^2, respectively (Chuang et al., 2003; Matalliotakis et al., 2008). **Tables 5.2 and 5.3** show the demographic characteristics and medications used for controlled ovarian hyperstimulation in these women.

A total of 260 follicles from these women were studied, of which 193 contained at least 1 mL of follicular fluid; their volume having been accurately measured. There was a significant (P=0.035) positive correlation between follicular size and follicular fluid AEA concentration **(Figure 5.10)**. The measured AEA concentration (1.43 ± 0.04 nM) in the follicular fluid of the 172 follicles from which eggs were retrieved was significantly higher than that (1.26 ± 0.06 nM) in the follicular fluid of 88 follicles where oocytes were not retrieved (P=0.014).

Table 5.2. Characteristics of the 37 volunteers undergoing IVF/ICSI-ET.

	Mean/ number	SD	Range
Age (years)	33.28	5.08	23-42
BMI (kg/m^2)	24.25	2.68	19.5-30
Infertility period (years)	3.86	2.45	1-14
Type of infertility: Primary infertility	26		
Secondary infertility	11		
Infertility cause: Male	16		
Unexplained	11		
Tubal	7		
Endometriosis	1		
PCOD + male	1		
tubal + male	1		
Treatment: ICSI	20		
IVF	15		
ICSI + IVF	2		
Basal hormones:			
Basal FSH (IU/L)	5.69	1.68	2.5-9.9
Basal LH (IU/L)	4.81	1.72	2-10

SD = standard deviation; Range = minimum to maximum values; BMI = body mass index; PCOD = polycystic ovary syndrome; IVF = *in-vitro* fertilization; ICSI = intracytoplasmic sperm injection; FSH = follicle stimulating hormone; LH = luteinizing hormone.

Table 5.3. Details of the ovulation stimulation regimen, number of oocytes collected per cycle and number of embryos on the day of embryo transfer.

	Mean/ number	SD	Median	Range
Duration of stimulation (days)	11.55	1.38	12	9-15
Dose of Puregon (rFSH) IU	2251.08	1478.99	2025	675-6450
Dose of Menopur (hMG) IU	3967.50	1895.86	3600	1950-7650
Dose of Puregon IU + Menopur IU	3425 + 1850	1242.22 + 482.18	3300 + 1650	2250-5725 + 1500-2400
Number of women stimulated by Puregon (rFSH)	25			
Number of women stimulated by Menopur (hMG)	9			
Number of women stimulated by hMG + rFSH	3			
Number of follicles aspirated per woman	16.41	6.89	15	2-29
Number of oocytes collected per woman	11.37	5.07	11	1-23
Total number of embryos per woman on the day of embryo transfer	5.30	3.09	5.50	1-14
Number of good quality embryos on the day of transfer	4.08	3.24	4	1-14

hMG= human menopausal gonadotrophins; rFSH= recombinant follicle stimulating hormone.

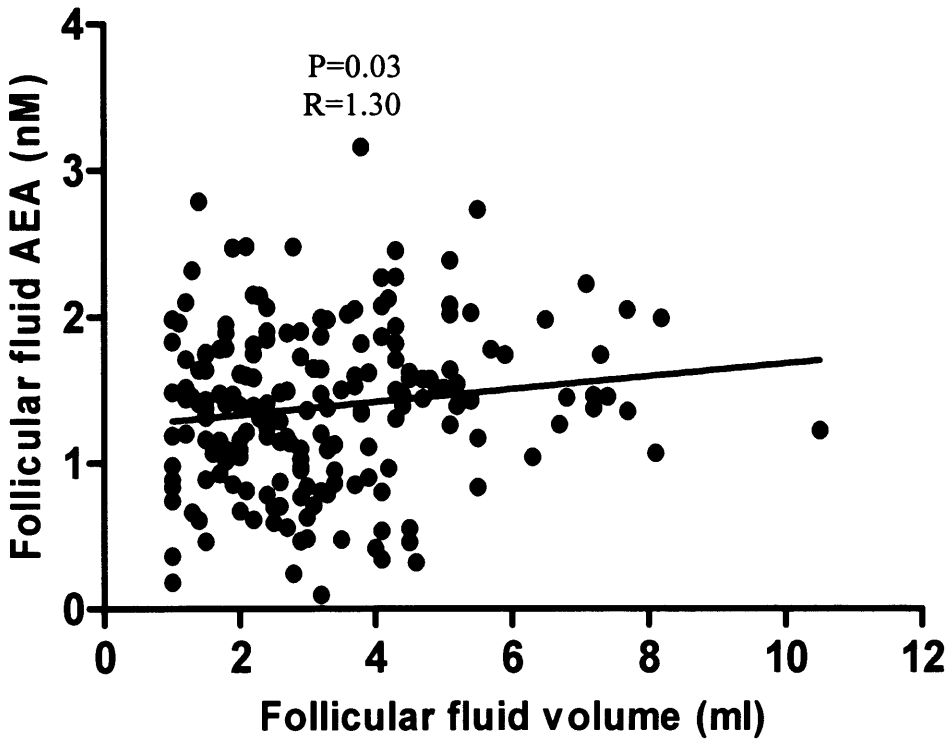


Figure 5.10. Spearman correlation between follicular fluid AEA concentration and follicle size. A positive correlation between the size of preovulatory follicle (in mL) and the concentrations of follicular fluids AEA from IVF/ICSI patients is shown. Follicular fluids from 193 follicles were analysed R = 0.1304; P = 0.03.

5.4.3. Prediction of oocyte maturity from follicular fluid AEA measurements

A total of 53 oocytes were retrieved from those undergoing ICSI and 35 of these were mature and 18 immature. The mean follicular fluid AEA concentrations (1.56 ± 0.11 nM) in follicles with mature oocytes was significantly higher (P=0.001) than that (0.99 ± 0.09 nM) of the immature oocytes **(Figure 5.11)**. Furthermore, ROC curve analysis revealed that a cut-off level for follicular AEA concentrations that identified a mature oocyte, was 1.09 nM with an area under the curve of 0.76 ± 0.07 (P=0.001) with a sensitivity of 72.2% and a specificity of 77.14% **(Figure 5.12)**. After sperm injection and incubation, of the 35 mature oocytes 21(60%) were fertilised; and resulted in 13 (61.9%) good and in 8 poor quality embryos on the day of embryo transfer (48 to 72 hours later). The mean AEA concentrations in follicles that produced good (1.49 ± 0.21 nM), and in those producing poor (1.48 ± 0.19 nM) quality embryos were similar (P=0.99).

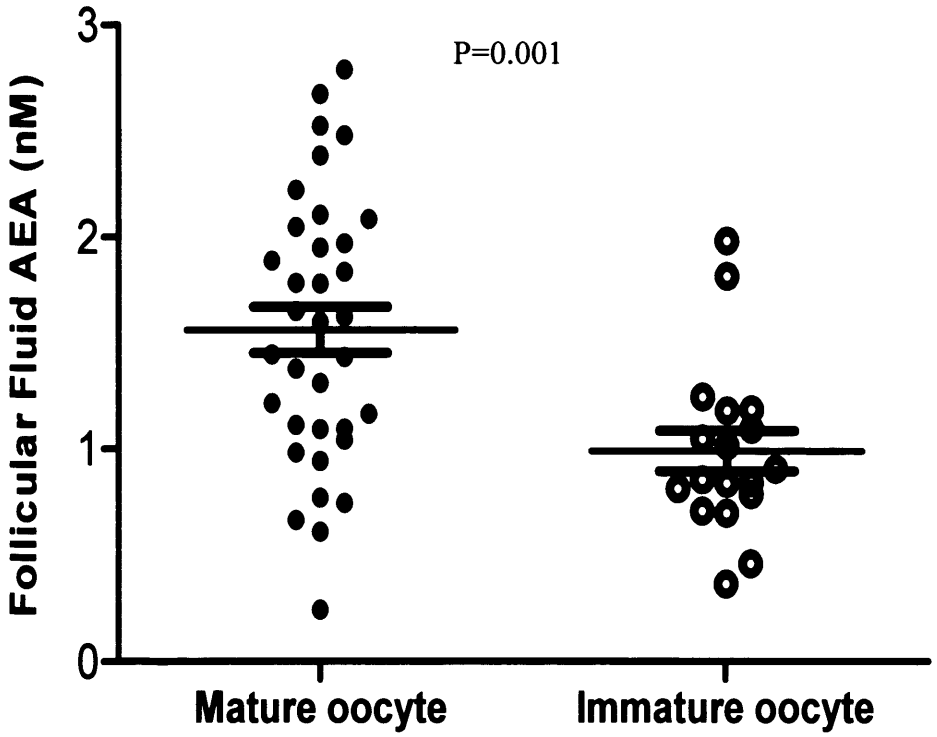


Figure 5.11. Follicular fluid AEA concentrations from follicles with mature and immature oocytes. Follicular fluid AEA concentrations from individual follicles containing mature MII oocytes (n=35) and follicles containing immature oocytes (n=18) from volunteers had ICSI cycles are shown. The long horizontal bar represents the mean and the shorter bars the SEM of follicular fluid AEA concentration. The P=0.001 value was calculated using the Student's unpaired *t*-test. n=number of volunteers.

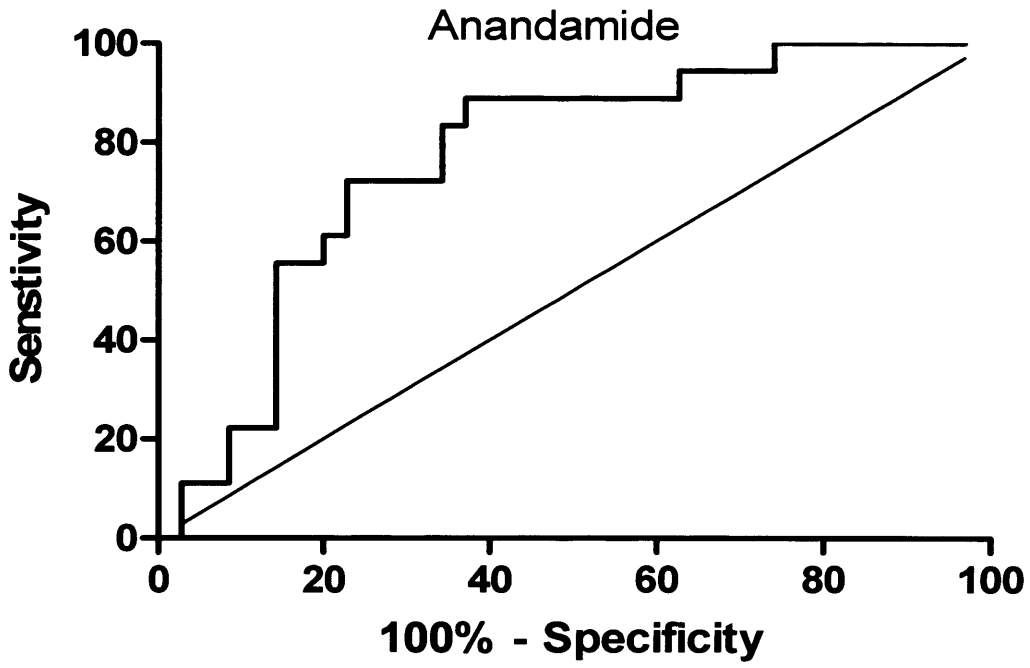
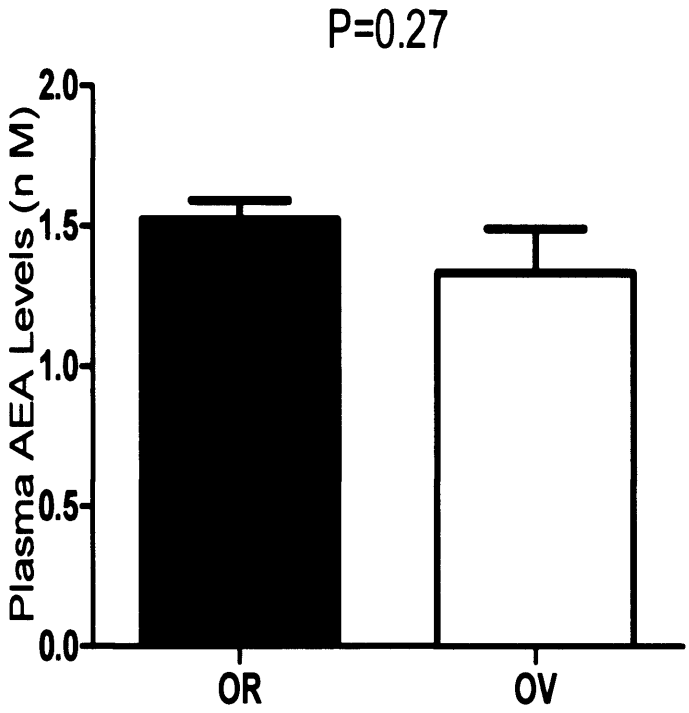


Figure 5.12. A receiver-operating characteristic curve (ROC) analysis for the prediction of the production of mature oocytes from follicular AEA concentration. The sensitivity and specificity relationship for measurements of anandamide in follicular fluid is plotted. The optimum cut off point for the identification of mature oocytes at 1.09 nM, provided a sensitivity of 72.2%, (95CI=46.52% to 90.31%) and a specificity of 77.14% (95CI=59.86 to 89.58%) and likelihood ratio of 3.16. The area under the curve was 0.768 ± 0.067, (Mean ± SEM; 95%CI=0.63-0.89, P=0.001).

5.4.4. Plasma AEA measurements

There was no significant difference between the mean plasma AEA concentrations (1.52 ± 0.07 nM) of the 37 women on the day of oocyte retrieval and the mean follicular fluid concentrations (1.42 ± 0.05 nM) from all the follicles for each woman (P=0.23). In addition, the mean plasma AEA concentrations on the day of oocyte retrieval were similar (P=0.27) to the values obtained in plasma (1.33 ± 0.16 nM) at the time of ovulation in natural cycles (see Chapter 4) **(Figure 5.13).**



undergoing IVF/ICSI-ET (n=37) and on the day of ovulation (OV) in naturally cycling women (n=7). The P-value of 0.27 was calculated using Student's unpaired *t*-test. n=number of volunteers.

5.5. Discussion

The endocannabinoid system in the human ovary has very rarely been investigated, and the observations in Chapter 4 that plasma anandamide concentrations increase at the time of ovulation in the natural menstrual cycle suggested that AEA may be produced in the ovary. Although Schuel *et al.* (Schuel et al., 2002) quantified anandamide and some of its congeners in follicular fluid, there was sparse evidence in the literature that either confirmed this observation or explained the exact function of AEA in the human ovary. Therefore, the current study was designed to localise the endocannabinoid system in the human ovary and to examine if there was a role for AEA in relation to the physiologically vital processes that occur in the ovary, such as folliculogenesis, preovulatory follicle maturation or oocyte maturity.

To the best of my knowledge, this is the first time that the entire endocannabinoid system; i.e. the ligand, the receptors and the enzymes responsible for AEA regulation have been localised in the human ovary (El-Talatini et al., 2009). The immunostaining showed widespread expression of CB1 and CB2 receptors in the medulla and cortex of the ovary. In the cortex the receptors were expressed in the granulosa cells of primordial, primary, secondary and tertiary follicles and in the theca cells of secondary and tertiary follicles. Immunostaining for both receptors was also observed in the corpus luteum and corpus albicans. In general, CB2 immunostaining was more intense than CB1 in the ovary, but interestingly, oocytes of follicles at all stages of development did not show positive expression of CB1 or CB2 except the oocytes of tertiary follicles, which expressed the CB2 receptor. These data suggest that the follicles and corpora can all respond to AEA, but the oocyte cannot respond to AEA until the last stage of its development. These observations suggest that AEA is involved in oocyte maturity, through the actions of the CB2 receptor.

FAAH, the enzyme responsible for the degradation of AEA, was expressed only in theca cells of secondary and tertiary follicles, the corpus luteum and corpus albicans. These data suggest that AEA is acting in an autocrine manner on the granulosa cell to stimulate unknown phenotypic changes, and that an alternative degradation pathway that does not involve FAAH may be present in the granulosa cell. Indeed, recent evidence suggests that AEA can be converted to a prostaglandin E2-ethanolamine through the actions of cyclooxygenase 2, an enzyme that is expressed in the ovarian granulosa cell (Fowler, 2007) and which is under leptin control.

NAPE-PLD on the other hand was expressed in the granulosa and theca cells of secondary, and tertiary follicles, the corpus luteum and corpus albicans suggesting that AEA is mainly produced from the granulosa of growing (secondary, tertiary) follicles but not from oocytes. Since NAPE-PLD was immunolocalised to the corpus luteum, and to a lesser extent the corpus albicans, this also suggests that AEA is synthesised by these from the two sources during the latter stages of the menstrual cycle.

These observations suggest that AEA probably acts mostly on CB2 receptors to produce its physiological actions in the ovary and that it is degraded in the theca cells and corpora lutea and albicantes. They also provide evidence that AEA is produced in the human ovary and that this process is probably under hormonal control as it is produced by growing follicles, the corpus luteum and corpus albicans. The evidence thus suggests that AEA affects the antral phase of folliculogenesis (Erickson, 2000a) **(Figure 5.14)**. Nevertheless, recent evidence suggests that in the mouse, the main actions of endocannabinoids are mediated through the actions of the CB1 receptor causing an imbalance between E2 and P4 signalling (Wang et al., 2008). The data presented herein are not at odds with this observation as Wang et al., did not examine the presence of the CB2 receptors, although

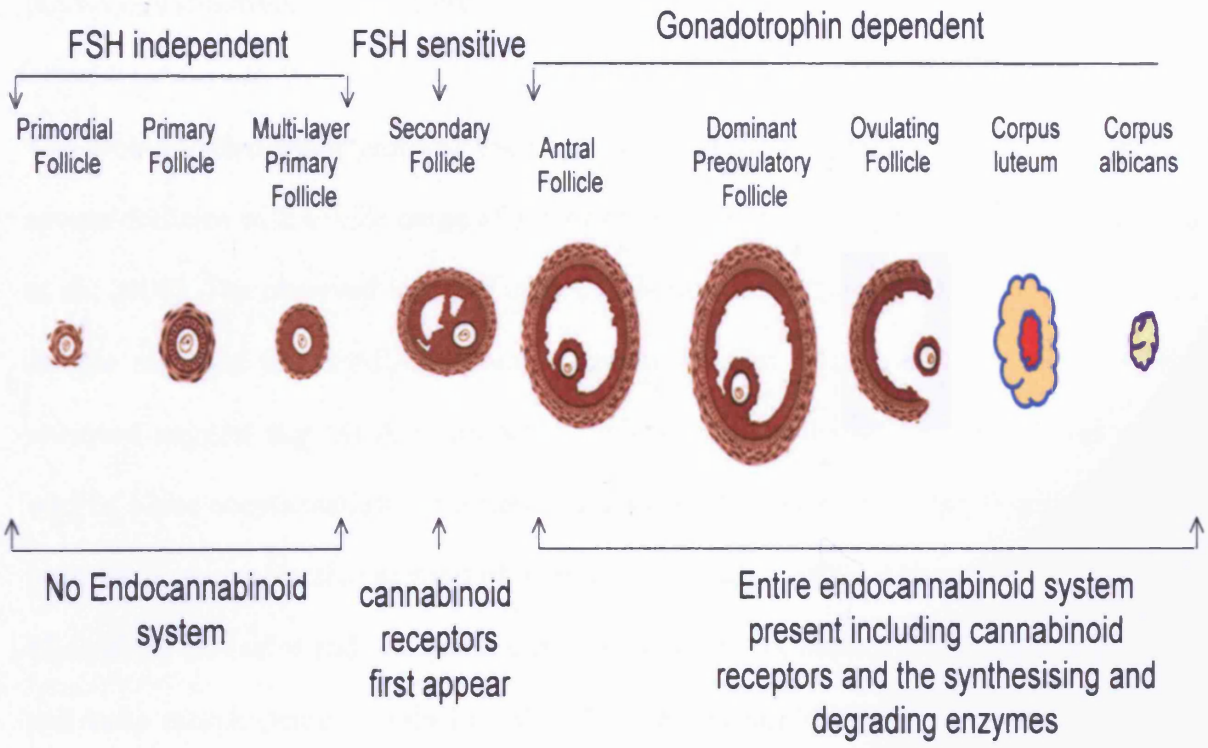


Figure 5.14. Diagrammatic representation of the role of the endocannabinoid system in the human ovary and its regulation.

they demonstrated that CB2 was not involved through the use of a specific CB2 antagonist. These observations therefore highlight a potential difference between murine and human ovarian physiology, as has been previously observed with other markers of ovarian physiology (Shimasaki et al., 2004).

The ovarian stimulation protocol used for IVF/ICSI women causes the development of several follicles with a wide range of sizes and at different developmental stages (Macklon et al., 2006). The observed increase in follicular fluid AEA concentrations with increased follicle size and lower AEA concentrations in follicles from which oocytes were not retrieved suggest that AEA is probably involved in the maturation of follicles or the oocyte. Since oocyte maturity is currently assessed subjectively by embryologists there is a need for a more objective method of assessment (Veeck, 1988). Although many potential biomarkers (estradiol and testosterone (Costa et al., 2004), inhibin B (Chang et al., 2002), and bone morphogenic protein-15 (BMP-15) (Wu et al., 2007)) in follicular fluid have been suggested to discriminate between mature and immature oocytes, none of these are in current clinical practice. In this regard, the ROC analysis demonstrating that a 1.09 nM follicular fluid AEA level was predictive of mature oocytes in 77.14% of the cases is especially encouraging (El-Talatini et al., 2009). Although this study clearly indicates that follicular fluid AEA levels are associated with both follicle and oocyte maturity, there are likely to be a number of other factors that influence oocyte maturity and quality (Tarin and Pellicer, 1992; Greenblatt et al., 1995; Van Blerkom et al., 1997). Follicular fluid AEA levels should be further investigated in larger studies as a possible biomarker for the assessment of oocyte maturity in advanced reproductive technology procedures. This needs to be done in conjunction with other potential biomarkers.

With regards to embryo quality, there was no significant association between follicular fluid AEA concentrations and embryo quality. This suggests that several other factors including sperm quality may be involved in determining embryo quality (van Golde et al., 2001).

The fact that there was no significant difference between plasma and follicular fluid AEA concentrations at the time of oocyte collection suggests that systemic AEA concentrations reflect ovarian AEA levels. Also, plasma AEA concentrations in women undergoing IVF/ICSI at the time of oocyte collection were similar to those found in women at ovulation in the natural cycles (El-Talatini et al., 2009) which is evidence that AEA may be involved in the ovulation process, whether that ovulation occurred naturally or was stimulated.

5.6. Conclusions

The studies in this Chapter provide significant evidence which will improve our understanding of the mechanisms of action of AEA in the human ovary which is crucial for fertility treatment and its improvement. Nevertheless, these interesting observations have posed more questions such as, (a) how does AEA affect oocytes maturity, i.e. does it affect nuclear or cytoplasmic maturation? or (b) can AEA be used in *in-vitro* maturation of human oocytes (IVM) as an emerging infertility treatment?

Chapter 6

The Role of Anandamide from ovulation to Early Pregnancy

6.1. Introduction

In the preceding Chapters it was shown that anandamide (AEA) is most likely involved in ovulation and has association with some of the sex steroid hormones and gonadotrophins and its levels in the follicular fluid could be used as a marker for oocyte maturity (El-Talatini et al., 2009). The significant relationship between anandamide and sex steroids and the changes described throughout the menstrual cycle suggest that it is likely that AEA is involved in the success of implantation.

It is thought that the endocannabinoid system could be one of the key modulators of human reproduction (Maccarrone and Finazzi-Agro, 2004; Battista et al., 2008). This suggestion is derived from animal studies (Das et al., 1995; Turco et al., 2008) especially, the mouse model which has provided us with a wealth of knowledge suggesting that AEA signalling is involved in reproduction (Park et al., 2004; Taylor et al., 2007). Endocannabinoid signalling via CB1 is considered to be the main regulator for the cleavage of mouse oocytes, oviductal transport of preimplantation embryos (Wang et al., 2004; Schuel and Burkman, 2006), blastocyst hatching from the zona pellucida, and implantation of the blastocyst in the uterine mucosa (Schmid et al., 1997; Paria et al., 2002b; Piomelli, 2004), and recently the role of the CB2 in reproduction has also been considered (Maccarrone, 2008).

Unlike the mouse, the evidence for a role of anandamide (AEA) in human reproduction is poor and include data that show that AEA (a) may be associated with human folliculogenesis and oocyte maturity, since it has been quantified in follicular fluid (Schuel,

et al., 2002; El-Talatini et al., 2009) (Chapter 5), (b) may be associated with ovulation, since higher levels of AEA have been reported at midcycle (El-Talatini et al., 2009) and (c) may be associated with implantation, since lower levels have been observed at the implantation window (Lazzarin et al., 2004; El-Talatini et al., 2009). In males, AEA has been quantified in seminal plasma and has also been shown to influence sperm function (Schuel et al., 1994; Schuel and Burkman, 2005). In addition to all of these, low plasma levels of AEA have been reported to be associated with viable pregnancies; the earliest gestation where AEA had been studied was 6 weeks (Maccarrone et al., 2000b; Habayeb et al., 2002; Maccarrone et al., 2002b; Habayeb et al., 2008b). The endocannabinoid system has been also shown to be expressed in first trimester placenta (7-12 weeks gestation) (Habayeb et al., 2008a). However, the role AEA in the human from the time of ovulation to 6 weeks gestation is not well understood, due to the fact that it is not possible to measure the local levels of anandamide in the human, and because most women in natural cycles report their pregnancy when they have already missed one to two weeks of their excepted menstrual period (i.e. 5-6 weeks of gestation) (Fossum et al., 1988). Nevertheless, Assisted Reproductive Techniques such as IVF and ICSI-ET provide the opportunity to study plasma AEA at specific time periods during these procedures, accurately.

Aims of the study:

The aims of the studies presented in this Chapter were therefore to investigate:

(a) whether plasma AEA levels in women undergoing IVF-ET at different treatment stages [ovulation (oocyte retrieval), implantation window (just prior to transferring embryos to the uterus)] and at pregnancy test were similar to those at ovulation and at the implantation window during natural cycles.

(b) whether plasma AEA levels during these IVF-ET treatment stages are different in pregnant (positive pregnancy test) and non pregnant (negative pregnancy test) women at 2 weeks post embryo transfer.

(c) the plasma AEA levels at first viability ultrasound scan in pregnant women with viable pregnancies (positive implantation) are comparable to those women who had miscarried by 6 weeks gestation (failed implantation).

(d) the changes in AEA levels in women with viable from 2 to 7 weeks gestation, and

(e) the effect of ethnicity on AEA in this cohort of women. Since the effect could be related to the treatment received (IVF/ICSI) or genuinely related to the ethnic differences between the two groups, the results of plasma AEA of the two groups in natural and stimulated cycles were also compared.

6.2. Materials and methods

6.2.1. Subjects

A total of 111 infertile women undergoing controlled ovarian hyperstimulation for IVF/ICSI-ET treatment between July 2007 and August 2008 were recruited into the study at the Assisted Conception Unit of the Leicester Royal Infirmary Hospital. All the volunteers were invited to take part in the study at their first visit to the unit after having been given a detailed explanation of the study protocol. Those who agreed to partake were interviewed personally and a detailed medical history and information about their ethnic

origins recorded. Each volunteer then signed the consent form prior to starting their IVF/ICSI-ET treatment. Ethical approval for the study had been obtained from the Leicestershire and Rutland Research Ethics Committee (number Q6/ Q2501/49). Only women aged ≤ 42 years (Chuang et al., 2003), who had no chronic diseases, were not on any medication, had a body mass index (BMI) ≤ 30 kg/m^2 (Matalliotakis et al., 2008), a basal FSH (day 2-4) in the previous cycle of <10 IU/L (Ebrahim et al., 1993) or smoked <20 cigarettes a day, were included in the study.

6.2.2. Treatment protocol

Ovarian stimulation was as described in Chapter 5. Oocyte retrieval was performed by transvaginal ultrasound guided aspiration of ovarian follicles and the retrieved oocytes processed as detailed in Chapter 5. Only oocytes from ICSI cycles were graded for maturity, as described in Chapter 5. The recovered oocytes were incubated with spermatozoa and fertilisation confirmed 16 hours later. Resulting embryo quality was assessed morphologically by an embryologist as described in Chapter 5. For women <40 years old, either one or two embryos were transferred into the uterus depending on their choice (Mayor, 2004). Those >40 years old, had the option of having up to 3 embryos transferred. Embryo transfer (Monday to Friday) was performed 48 or 72 hours post oocyte retrieval. Thereafter, twice daily 400 mg of vaginal progesterone pessaries (Cyclogest, Shire) were given for luteal phase support until the day of pregnancy test for non-pregnant women and up to 12 weeks for the pregnant group (Nosarka et al., 2005). A urinary pregnancy test and serum β-hCG were performed 2 weeks after embryo transfer. Women with a serum β-hCG level <5 IU/L were considered not pregnant and no more blood samples were collected from them. Those with a serum β-hCG of >5IU/L were considered pregnant. All pregnant woman had a second serum β-hCG a week later (i.e. at 5 weeks of pregnancy) and if the second serum β-hCG value was >1500 IU/L a transvaginal

ultrasound scan was performed 4 weeks after embryo transfer (i.e. equivalent to 6 weeks pregnancy) to confirm that their pregnancy was intrauterine and viable by presence of gestational sac and the fetal heart movements **(Figure 6.1)**. These women were considered as the viable pregnancy group and this was considered to indicate positive implantation.

Since serum β-HCG levels are expected to double approximately every 48 to 72 hours in normal pregnancy (Check et al., 1992) women whose second β-HCG at 5 weeks was not doubling normally and were below 1500 IU/L were followed up with serial serum β-HCG measurements until these levels declined to less than 5IU/L. Women in the miscarriage group included those in whom at two consecutive transvaginal ultrasound scans the pregnancy sac was smaller than the expected for dates and no fetal heart movements were seen or the sac was empty. This was in addition to women who initially had a normal pregnant serum β-HCG but then their levels started dropping at 5 and 6 weeks gestation. These women were considered to have a failed implantation.

All women were asked to report any vaginal bleeding or significant abdominal pain. The non-pregnant women reported vaginal bleeding which started one or two days before the urinary β-hCG pregnancy test.

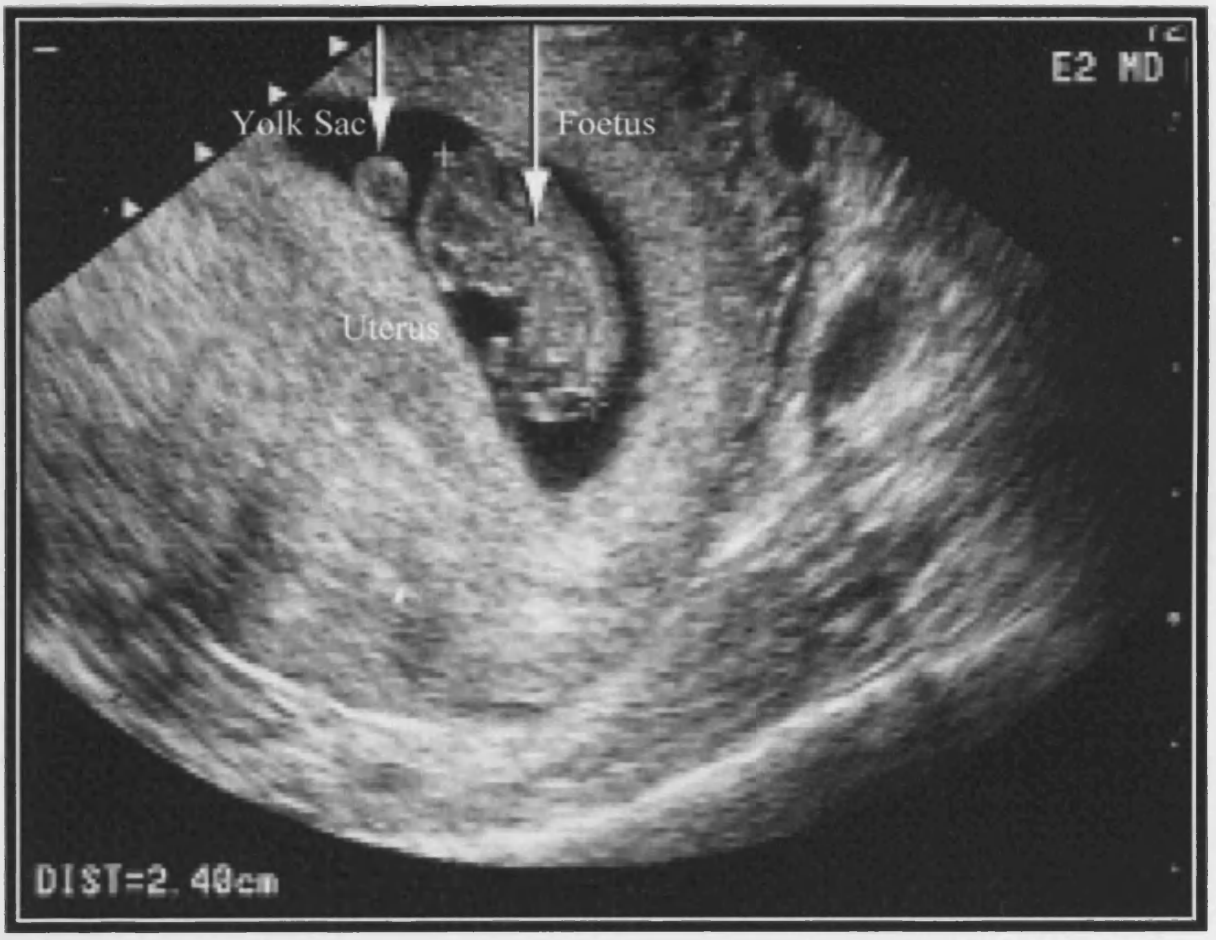


Figure 6.1. A transvaginal ultrasound image of an intrauterine viable pregnancy at six weeks gestation.

6.2.3. Timing of blood samples

From each of the volunteers recruited, the following blood samples were taken:

(a) For anandamide (AEA) measurements 4 mL of bloods were collected:

(i) 30 minutes before oocytes retrieval.

(ii) 30 minutes prior to embryo transfer (48 or 72 hours after oocyte retrieval).

(iii) On the day of the first pregnancy test (2 weeks after embryo transfer i.e. equivalent to 4 weeks gestation), on the day of the first ultrasound scan (6 weeks) and from women with abnormal doubling of serum β-hCG at 6 weeks (i.e. 4 weeks after embryo transfer). In a subset of 13 pregnant women with normal doubling of serum β-hCG extra samples for the measurement of plasma AEA were collected at 5 weeks of pregnancy (3 weeks after embryo transfer) and in a further 9 women, plasma AEA levels were also determined at 7 weeks in order to investigate longitudinally the changes in levels of AEA over these weeks of pregnancy.

(b) For β-hCG measurements, 4 mL of blood was collected

(i) on the day of the first pregnancy test and

(ii) at 3 weeks after embryo transfer or at 5 weeks gestation (from women with a positive pregnancy test)

(c) 4 mL of blood for serum estradiol and progesterone measurements in a minimum of selected cases and controls with viable pregnancies and non-pregnant (pregnancy test was negative) women were collected;

(i) 30 minutes before oocytes retrieval,

(ii) 30 minutes prior to embryo transfer (48 hours after oocyte retrieval)

(iii) on the day of pregnancy test (2 weeks after embryo transfer), and

(iii) on the day of ultrasound scan in women with a viable pregnancy.

6.2.4. Plasma anandamide (AEA) measurements

Plasma AEA concentrations were quantified using the Ultra Performance Liquid Chromatography-tandem Mass Spectrometry (UPLC-MS/MS) liquid phase method as described in Chapter 2 (Lam et al., 2008).

6.2.5. Hormonal measurements

Serum estradiol (E2), progesterone (P4) and β human chorionic gonadotrophin (β-hCG) measurements were undertaken using an automated ADVIA Centaur Assay System (Bayer Diagnostics, Newbury, and Berkshire, UK) performed by the Chemical Pathology Department of Leicester Royal Infirmary. The inter-assay coefficients of variation for E2, P4, β-hCG were 5.6, 3.6, and 2.9%, respectively, whilst the intra-assay coefficients of variation were 8.4, 5.2 and 2.7%, respectively. β-hCG was measured on the day blood samples were collected whereas serum for E2 and P4 measurements were stored at -20°C for later quantification.

6.3. Statistical Analyses

Power calculation based on previous published plasma AEA data in women (Habayeb et al., 2004, Habayeb et al., 2008b), indicated that a minimum of 6 women in each of the groups would allow a clinically significant difference of at least 40% in plasma AEA concentration to be observed with 80% power, assuming a 2-sided α=0.05.

The analysis was performed in defined groups as shown below:

(a) Stimulated cycle versus natural cycle (Chapter 4)

(b) Pregnant versus non-pregnant IVF women

(c) White versus non-White IVF and natural cycles (Chapter 4)

(d) White British versus Asian Indian IVF cycles

(e) Viable pregnancy versus miscarriage

(f) Viable pregnant versus non-pregnant women

Shapiro-Wilk analysis of plasma AEA levels for all the above groups indicated that plasma AEA measurements were normally distributed and so parametric tests were used to analyse the data. Plasma AEA and hormonal measurements are presented as mean ± SEM.

One-way ANOVA with Tukey's multiple comparison tests was used to compare plasma AEA levels in each group separately. Patient demographics data are presented as means ± SD (range) and cycle characteristics data presented as means ± SD (range). Comparison between two groups was performed using Student's unpaired *t*-test or Mann-Whitney U-test, when it was appropriate. In all cases, a $P<0.05$ was considered significant. Linear regression analysis was used to calculate relationships between plasma AEA and serum E2, P4 and β-hCG. Data were analyzed using InStat Version 3.01 (GraphPad Software Inc, San Diego, California).

6.4. Results

6.4.1.1. Subjects

Out of a total of 111 women recruited, 4 (3.6%) withdrew after entry into the study and after giving the first blood sample. The main reason for withdrawal was the frequency of blood sampling required for the remainder of the study. These women were excluded from

the analysis. In 12/111 (10.8%) women, the treatment cycle was cancelled after initiation of ovarian stimulation. The cancellation in 9 of the 12 (75%) was either after the 3rd scan (6 to 7 days post gonadotrophin stimulation), or after the final scan (48 hours before oocyte retrieval) due to poor ovarian response. In these 9 women, the expected response at the 3rd scan was at least 3 follicles ≥ 11 mm in diameter and estradiol (E2) levels doubling every 48 or 72 hours. In the remaining 3 (25%), cancellation was due to an over response as they produced multiple follicles and their final E2 levels exceeded 25,000 pmol/L.

One out of the 111 (0.9%) woman had an ectopic pregnancy while waiting for treatment and 16/111 (14.4%) were lost to follow up after consenting to participate in the study. Therefore, a total of 78 stimulated cycles were studied but 5 women had no embryos transferred, hence 73/78 (93%) of the women in total completed all treatment stages. The volunteers mean age and BMI were 33.50 ± 4.24 (23-42) years and 24.07 ± 3 .02 (18-30) kg/m^2 respectively. Seventy (95%) women never smoked cigarettes and none of them admitted using marijuana in the last 10 years. The patient demographics, duration of infertility and type of infertility (whether primary or secondary), cause of infertility, and assisted reproductive techniques (whether IVF or ICSI offered), are shown in **Tables 6.1a, 6.1b and 6.2.**

Eighty percent of the women recruited into this study were funded by the NHS for one treatment cycle, which is counted only when oocytes are retrieved. If these women did not have at least 4 follicles ≥ 17 mm at their last scan, the cycles were cancelled and they were offered another free treatment cycle.

Table 6.1a. Demographic characteristics, duration of infertility of the study group.

	Mean ± SD (n=78)	Range (Min-Max)
Age (years)	33.50 ± 4.24	23-42
BMI (kg/m^2)	24.07 ± 3.02	18-30
Duration of Infertility (years)	4.48 ± 2.72	1-16

Table 6.1b. Shows the ethnicity, smoking habits and types of infertility of the study group.

	Number (%)
Ethnic group: **White** **non-White**	 57 (73%) 21 (27%)
Smoking: **Yes** **No**	 4 (5%) 74 (95%)
Type of infertility: **Primary infertility** **Secondary infertility**	 56 (72%) 22 (28%)
Women had a previous live birth	5 (6%)

Table 6.2. Infertility causes and infertility treatments offered to the study group.

Data are expressed in mean ± SD, percentages, and the range shows the minimum to maximum values.

	Mean/number (%)	Range (Min-Max)
Cause of infertility:		
Male	28 (35.9%)	
Tubal	14 (17.9%)	
Endometriosis	4 (5.1%)	
Unexplained	26 (33.3%)	
Combined causes:	5(6.4%)	
(Male + ovulatory)	3	
(Male + tubal)	2	
Others: (advanced age)	1 (1.3%)	
Treatments:		
IVF	32 (41%)	
ICSI	43 (55%)	
ICSI+IVF	3 (4%)	
Basal hormone levels:		
FSH (IU/L)	5.9 ± 1.71	2.5-10
LH (IU/L)	4.93 ± 1.97	2-12

BMI = body mass index; IVF = in-vitro fertilization; ICSI= intracytoplasmic sperm injection; FSH = follicle stimulating hormone; LH = luteinizing hormone; PCOS = polycystic ovary syndrome.

6.4.1.2. Cycle characteristics

The mean number of days of ovarian stimulation was 11.45 (range 7-15). Forty one (53%) women were stimulated with recombinant follicle stimulating hormone (rFSH), 33 (42%) with human menopausal gonadotrophins (hMG) and 4 (5%) with both rFSH and hMG **(Table 6.3)**. The average number of oocytes retrieved per woman on the day of oocyte retrieval was 10.9 ± 6.06 (range 1-38) (**Table 6.4**). Seventy four (94%) of the 78 women produced oocytes that were fertilised, but the mean fertilisation rate was only 61.5% (range 13.3-100%). The average number of embryos per woman on the day of embryo transfer was 5.76 ± 3.59 (range 1-18) but the mean number of good quality embryos on the day of embryo transfer was only 3.84 ± 3.01 (range 0-14). In total, 73 out of the 78 women had embryo transfer as 4 had no fertilisation and 1 woman had a thin endometrium and therefore the embryos were not transferred. Forty five/73 (61.6%) of the women had their embryos transferred 48 hours after oocyte retrieval and 28/73 (38.8%) had embryo transfer 72 hours later. Sixty five (89%) of the 73 women had 2 embryos transferred and 8/73 (11%) had only one embryo transferred.

Table 6.4 shows the number of oocytes retrieved per woman, and the number of good quality embryos on the day of transfer. **Table 6.5** shows the number and quality and stage of the embryos transferred to the uterus. The mean number of cells in the embryos transferred were 4.75 ± 2.09 (range 2-8) and 4.83 ± 1.98 (range 2-8) for embryo 1 and embryo 2 respectively.

Table 6.3. The regimen of gonadotrophins including dose used for ovarian stimulation.

	Mean ± SD	Range
Days of stimulation (days)	11.45 ± 1.41	7-15
Dose of Puregon (rFSH) IU	2234 ± 1356	675-6450
Dose of Menopur (hMG) IU	4220 ± 1939	1650-9150
Dose of Puregon+ Menopur IU	2906 ± 1451 + 1838 ± 394.5	1350- 4725 + 1500-2400

rFSH = recombinant follicle stimulating hormone; hMG = human menopausal gonadotrophins.

Table 6.4. Total number of oocytes retrieved, fertilisation rate, number of embryos on the day of transfer of the study group.

	Mean ± SD	Range
Total oocytes retrieved per woman	10.92 ± 6.06	1-38
Number of mature oocytes (ICSI cycles) (n=47)	8.51 ± 4.38	0-18
Mean percentage of oocytes fertilisation (n =74)	61.52 ± 23.67	13.30-100
Total number of good of embryos per woman available on the embryo transfer day (n=73)	3.85 ± 3.01	(0-14)

(n) = indicates the number of women studied from a total cohort of 78 recruits.

Table 6.5. Number and quality of embryos transferred.

	Number (%)
Total number of women had embryo transfer	73 (93.6%)
Number of women had 2 embryos transferred	65 (89%)
Number of women had 1 embryo transferred	8 (11%)
Quality of embryos transferred **Embryo 1**	**(Grades):** G1 = 11 (15.6%) G1-2 = 7 (50.6%) G2 = 20 (27.4) G3 = 2 (2.7%) G2-3 = 3 (4.1%)
Embryo 2	G1 = 4 (6.1%) G2 = 31 (47.7%) G1-2 = 23 (35.4%) G2-3 = 5 (7.7%) G3 = 2 (3.1%)

Grades 1, 2, 1-2 are good quality embryos and grades 2-3, 3, 4 are poor embryos.

6.4.1.3. Plasma AEA levels in natural and stimulated IVF/ICSI-ET cycles

An assumption was made that the day of oocyte retrieval in stimulated cycles was equivalent to the day of ovulation in natural ovulatory cycles, and that the day of embryo transfer corresponded to the mid luteal phase (implantation window). Plasma AEA levels on the ovulation day (Chapter 4) in natural ovulatory cycles (n=7; 1.33 ± 0.16 nM) were therefore compared to those on the day of oocyte retrieval (n=78; 1.27 ± 0.05 nM), and were found not to be statistically different (P=0.74) **(Figure 6.2)**. Similarly, there was no significant difference between plasma AEA levels (0.96 ± 0.10 nM) at the mid luteal phase and those (n=73; 0.89 ± 0.06 nM) on the day of embryo transfer (P=0.71). In stimulated cycles, the decline in AEA levels from the day of oocyte retrieval to the day of embryo transfer was statistically significant (P<0.0001). In the natural cycle there was a decline in AEA levels from the day of ovulation to the mid luteal phase levels but this decline was not statistically significant (n=7; P=0.076).

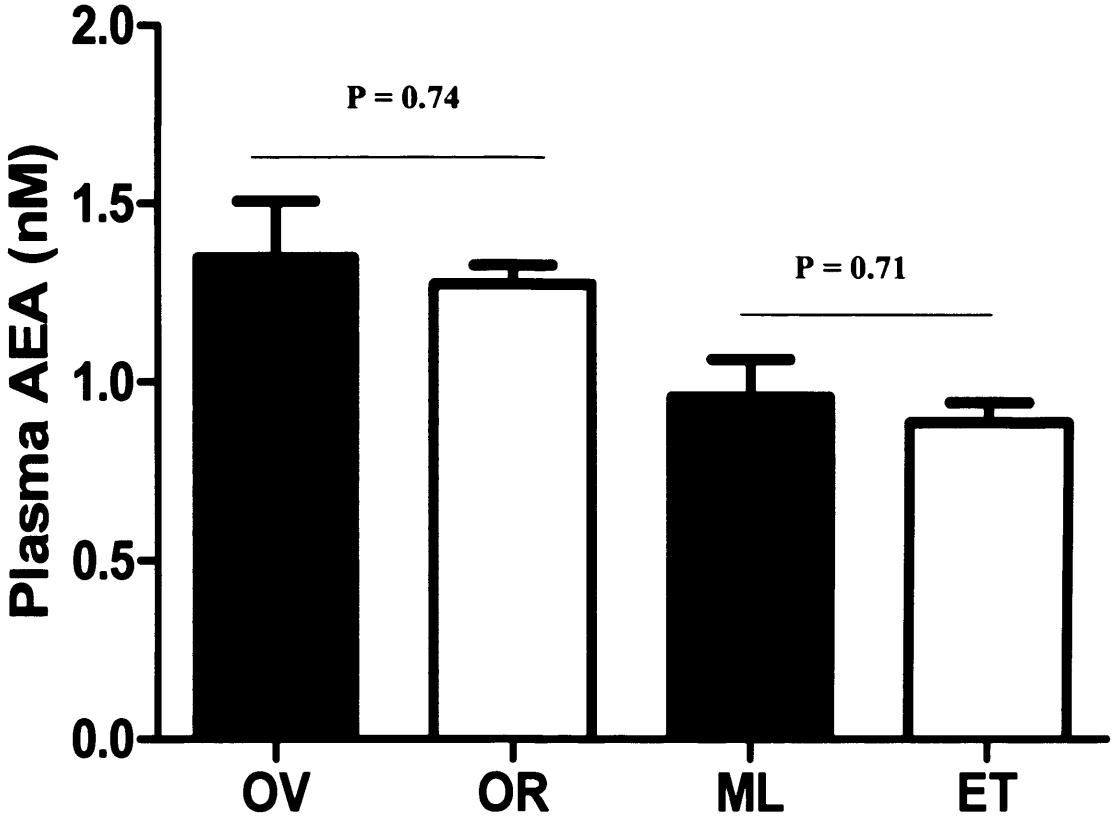


Figure 6.2. Plasma AEA levels in natural ovulating cycles at ovulation (n=7) and in the mid luteal phase (n=7) and in stimulated cycles at oocyte retrieval (n=78) and at embryo transfer (n=73). Data are presented by mean ± SEM. There was no significant difference in plasma AEA levels between natural and stimulated cycles at the time of ovulation (P=0.74) and implantation window (P=0.71). In both cycles, AEA levels were high at ovulation and low at the implantation window. OV= ovulation in natural cycles; OR= oocyte retrieval; ML= mid luteal in natural cycles; ET= embryo transfer. n=number of volunteers.

6.4.1.4. Plasma AEA in pregnant and non-pregnant women

There were 30 pregnancies (serum βhCG >5 IU/L at 2 weeks post embryo transfer or at 4 weeks gestation) that resulted from the 73 women who had embryo transfer. The mean plasma AEA levels in these 30 women decreased steeply from 1.29 ± 0.08 nM on the day of oocyte retrieval to 0.79 ± 0.05 nM on the day of embryo transfer ($P<0.0001$) **(Figure 6.3)**. The levels increased from the day of embryo transfer to the day of the first pregnancy test (1.17 ± 0.07 nM; $P<0.0001$). In the non-pregnant women (n=43), the change in AEA levels from the day of oocyte retrieval to that of the pregnancy test was similar in trend but much less than in the pregnant group. In the non-pregnant women the levels decreased from 1.26 ± 0.07 nM on the day of oocyte retrieval to 0.95 ± 0.08 nM on the day embryo transfer ($P<0.05$) and then increased to 1.23 ± 0.08 nM on the day of pregnancy test. However, the percentage decline in AEA levels between the day of oocyte retrieval and the day of embryo transfer in the pregnant women was 39% compared to 24% in the non-pregnant group, and the percentage increase in AEA levels from the day of embryo transfer to the day of pregnancy test was 49% in the pregnant group compared to only 28% in the non-pregnant women.

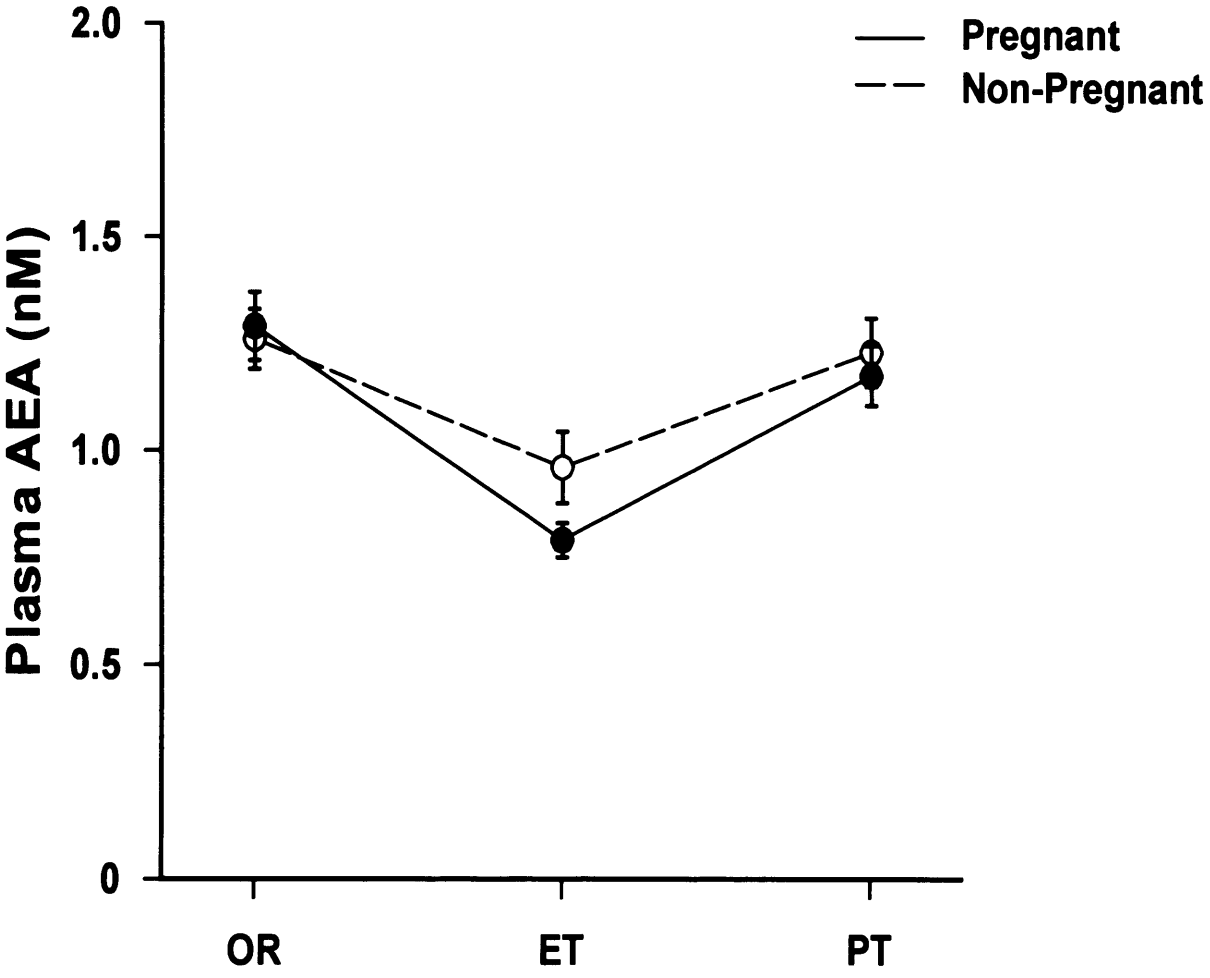


Figure 6.3. Plasma AEA levels in pregnant and non-pregnant women. There was no statistically significant difference in AEA levels between the pregnant (n=30) and non-pregnant (n=43) women at the three studied periods; oocyte retrieval (OR), embryo transfer (ET), pregnancy test (PT) (P=0.79; P=0.14; P=0.64, respectively). There was however, a significant difference in the rate of change in the AEA between OR and ET and between ET and PT in pregnant women compared to the non-pregnant women. The data are presented as the mean ± SEM. P-values were calculated using Student's unpaired *t*-test. n=number of volunteers.

6.4.2. AEA and ethnicity

6.4.2.1. Subjects and cycle characteristics

Since the success rate from Assisted Reproductive Techniques (ART) such as IVF/ICSI reported in a few studies is influenced by ethnicity, a further sub-analysis of the data was undertaken to examine whether ethnicity was an important determinant of the differences in plasma AEA levels in women undergoing IVF/ICSI-ET. Fifty seven (73%) of the 73 women were White (British) and 21(26.9%) were non-White. These percentages reflect the ethnic composition of Leicester **(Table 6.6).** The groups were similar in age, BMI, duration of infertility and basal FSH. There was, however, a difference in the cause of infertility, which influenced the type of assisted reproductive technique, offered to the volunteers (**Table 6.7).** There was no statistically significant difference in both groups with respect to the number of ovarian stimulation days, fertilisation rate, number of oocytes retrieved, and the number of resulting good quality embryo on the day of transfer as shown in **Tables 6.8 and 6.9**.

Table 6.6. A comparison of the ethnic groups in the study with the published data of Leicester ethnic groups in Census 2001.

Ethnic groups / Sub–groups in the study (n=78)			**Leicester population (n=279,921)**
White			**178,739 (63.8%)**
	British	57 (73%)	169,456 (60.5%)
	Irish	0	3,602 (1.29%)
	Other White	0	5,681 (2.03)
Asian Or Asian British			**83,751 (29.9%)**
	Indian	16 (20.5%)	72,033 (25.7%)
	Pakistani	0	4,276 (1.53%)
	Bangladeshi	0	1,926 (0.69%)
	Other Asian	0	5,516 (1.97%)
Black Or Black British			**8,595 (3.07%)**
	Caribbean	2 (2.6%)	4,610 (1.64%)
	African	1 (1.28%)	3,432 (1.22%)
	Other Black	0	553 (0.20%)
Mixed	White and Asian	0	1,908 (0.68%)
	Other Mixed	0	1,218 (0.44%)
Chinese		1 (1.3%)	1,426 (0.5%)
Other ethnic groups		1 (1.3%)	904 (0.32%)

Source of information: Leicestershire County Council, Research and Information, Department of Planning & Transportation. www.Leicester.gov.uk. National values are highlighted.

Table 6.7. Demographic characteristics, duration, types and causes of infertility and treatment offered to White and non-White volunteers. Data are presented as mean ± SD, (range), percentage (%), P values were calculated by using Student's unpaired *t*-test.

	White (n=57)	non-White (n=21)	P Value
Age (years)	33.7 ± 4.53 (23-42)	32.9 ± 3.32 (27-37)	0.49
BMI (kg/gm^2)	24.1 ± 2.96(19-30)	23.9 ± 3.22 (18-28.50)	0.83
Duration of infertility (years)	4.2 ± 2.44(1-16)	5.2 ± 3.31 (2-14)	0.14
Type of infertility:			
Primary infertility	37 (64.9%)	18 (85.7%)	
Secondary infertility	20 (36.1%)	3 (14.3%)	
Previous child birth	3 (5.3%)	2 (9.5%)	
Cause of infertility:			
Male	20 (35.1%)	8(38.1%)	
Tubal	9 (15.8%)	5 (23.8%)	
Unexplained	20 (35.1%	6 (28 %)	
Endometriosis	3 (5.3%)	1(4.7%	
Old age	1(1.7%)	0	
Combined infertility causes:	Total 4 (7%)	1 (4.7%)	
Male+ ovulatory	3		
Male + Tubal	1	1	
Treatment:			
IVF	20 (35.1%)	12 (57.1%)	
ICSI	35 (61.4%)	8 (38.1%)	
IVF + ICSI	2 (3.5%)	1 (4.7%)	

Table 6.8. Basal FSH, duration of stimulation, total number of oocytes retrieved, fertilisation rate and the number of mature oocytes in White and non-White.
Data are presented as mean ± SD, range (minimum-maximum).

	White (n=57)	Non-White (n=21)	P Value
Basal FSH	6.1 ± 1.7 (2.5-10)	5.7 ± 1.5 (3.9-10)	0.45
Days of stimulation	11.4 ± 1.5 (7-14)	11.6 ± 1.1 (9-15)	0.67
Total oocyte number retrieved per woman	11.1 ± 6.2 (1-38)	10.3 ± 5.6 (3-24)	0.60
Number (%) of women who had fertilisation	55 (96.5%)	19 (90.5%)	
Mean percentage of oocytes fertilisation per woman	63.1 ± 23.3 (13-100)	56.9 ± 24.8 (25-100)	0.33
Number of mature oocyte (ICSI cycles)	8.3 ± 4.3 (0-18)	9.3 ± 4.9 (3-18)	0.54

Table 6.9. Total number of embryos, number of good embryos per woman on the day of transfer and the percentage of those who had embryo transfer (White versus non-White). Data are presented as mean ± SD, range (minimum-maximum), percentage (%).

	White (n=55)	Non-White (n=19)	P Value
Total number of embryos per woman available on the day of transfer (both 48hrs and 72hrs).	6 ± 3.7 (1-18)	5 ± 3.3 (1-12)	0.34
Total number of good of embryos per woman available on the day of transfer.	3.9 ± 3 (0-14)	3.5 ± 2.9 (1-9)	0.46
Number (%) of women who had embryos transfer.	54 (94.7%)	19 (90.5%)	
Number (%) of women had 1 embryo transferred.	6 (11.1%)	2 (10.5%)	
Number (%) of women had 2 embryos transferred.	48 (88.9%)	17(89.5%)	

6.4.2.2. Plasma AEA levels in IVF/ICSI-ET cycles

Plasma AEA levels in White women (n=57) on the day of oocyte retrieval (1.34 ± 0.06 nM) were significantly higher than that in non-White volunteers (n=21) (1.10 ± 0.08 nM) (P=0.04) **(Figure 6.4)**. There was no significant difference in plasma AEA levels between the two groups on the day of embryo transfer (P=0.74), and on the day of pregnancy test (P=0.63). Only 54 out of 57 White women had embryo transfer (2 had no fertilisation and in 1 the endometrium was < 8 mm, i.e. not appropriately thickened for embryo transfer) and 19 non-White out of the 21 non-White women had embryo transfer (2 had no fertilisation).

The mean plasma AEA levels in the White women on the day of oocyte retrieval decreased significantly from 1.34 ± 0.06 nM to 0.88 ± 0.05 nM on the day of embryo transfer (P<0.0001) and then increased significantly to 1.19 ± 0.06 nM on the day of pregnancy test (P<0.001). There was, however, no significant difference in plasma AEA levels on the day of oocyte retrieval and pregnancy test. In the non-White group, the mean plasma AEA levels on the day of oocyte retrieval was 1.10 ± 0.08 nM and then decreased to 0.92 ± 0.16 nM on the day of embryo transfer but, this change was not statistically significant (P=0.30). Plasma AEA levels then increased to 1.25 ± 0.11 nM on the day of pregnancy test, but this was also not statistically significant (P=0.09).

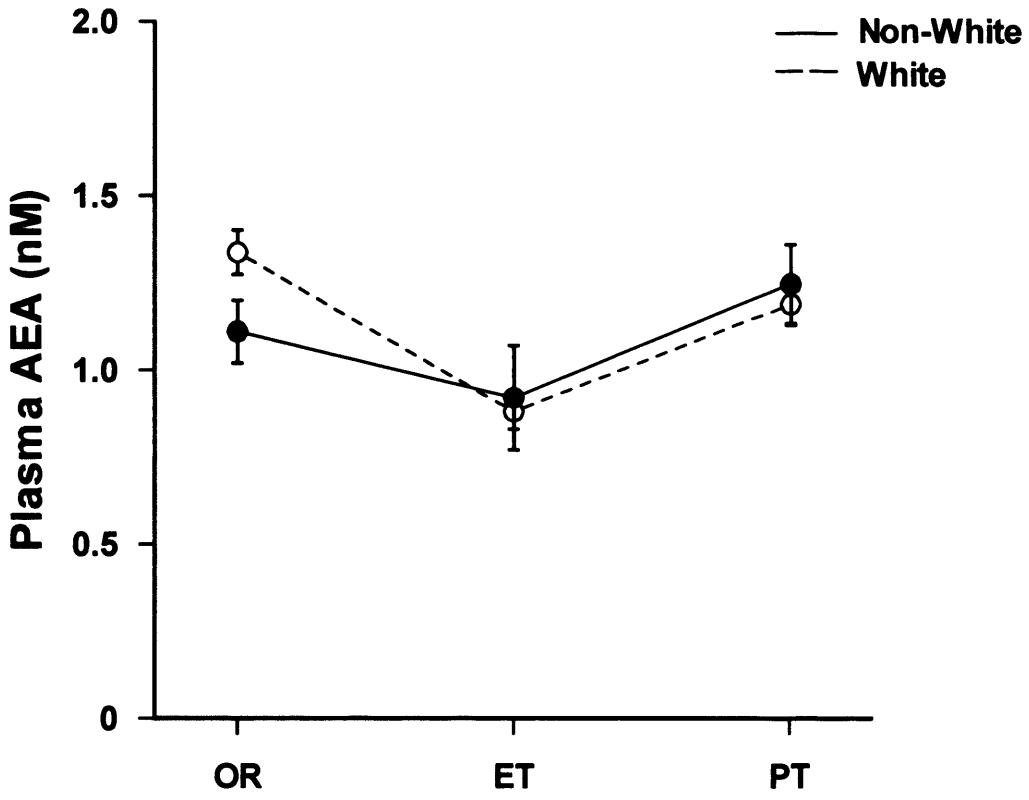


Figure 6.4. Changes in plasma AEA levels from the day of oocyte retrieval to the day of pregnancy test in White (n=57) and non-White women (n=21). There was a significant difference the levels between the two groups on the day of oocyte retrieval (P=0.04), but not on the day of embryo transfer (P=0.74) and pregnancy test (P=0.63). AEA levels in the White group changed significantly throughout the studied periods (P<0.0001). In the non-White group AEA levels did not, however, change significantly throughout the three studied periods (P=0.17).

6.4.2.3. Plasma AEA levels in natural and stimulated cycles

A further analysis was undertaken to determine the differences in plasma AEA levels between White and non-White women observed on the day of oocyte retrieval but not on the day of embryo transfer were not limited solely to the stimulated cycles. Plasma AEA levels in White and non-White women at ovulation and mid luteal phase in natural cycles were therefore compared. In addition, natural and stimulated cycles in the two groups were also compared.

In the White group, the mean plasma AEA levels on day of ovulation (1.49 ± 0.17 nM) were similar to the levels on the day oocyte retrieval (1.33 ± 0.06 nM) (P=0.45) **(Table 6.10).** In non-White group, there were also no significant differences between the plasma AEA levels on day of ovulation (0.98 ± 0.12 nM) and on the day of oocyte retrieval (1.10 ± 0.08 nM) (P=0.45). There was, however, a significant difference between the White and non-White groups at ovulation in the natural cycle (P=0.03), and on the day of oocyte retrieval in the stimulated cycle (P=0.04) **(Figure 6.5).** By contrast, in White and non-White women there were no significant differences in plasma AEA levels between the two groups at the mid luteal phase (0.98 ± 0.12 nM versus1.11 ± 0.32 nM; P=0.68) and on the day of embryo transfer (0.88 ± 0.05 nM versus 0.92 ± 0.16 nM ; P=0.56).

In White and non-White women there were no statistically significant differences in plasma AEA levels in the natural and stimulated cycles, however, there was a significant difference in plasma AEA levels between the two groups on the day of ovulation in the natural and the stimulated cycles, but not at the implantation window.

Table 6.10. Plasma AEA levels in White and non-White women at ovulation, oocyte retrieval, midluteal phase and embryo transfer.

	Ovulation AEA (nM)	Oocyte Retrieval AEA (nM)	P Value	Mid luteal Phase AEA (nM)	Embryo Transfer AEA (nM)	P Value
White	(n=7) 1.49 ± 0.17	(n=57) 1.33 ± 0.06	0.41	(n=9) 0.98 ± 0.12	(n=54) 0.88 ± 0.05	0.44
Non-White	(n=7) 0.98 ± 0.11	(n=21) 1.10 ± 0.08	0.45	(n=7) 1.11 ± 0.32	(n=19) 0.92 ± 0.16	0.56

Data are presented as mean ± SEM. The values of volunteers in the natural cycles are derived from Chapter 4. n=the number of volunteers.

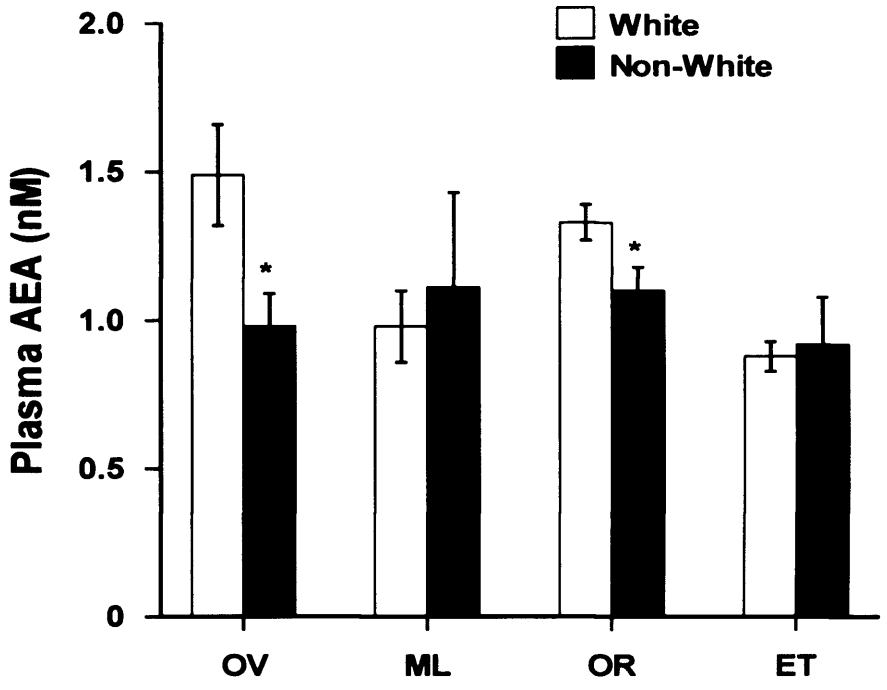


Figure 6.5. Plasma AEA levels in White and non-White women during natural and stimulated cycles. Samples were taken at ovulation (OV), and mid-luteal (ML) phase of the natural cycle, at oocyte retrieval (OR) and at embryo transfer (ET). The data are presented as the mean ± SEM. *P-values: Student's unpaired *t*-test. There was a significant difference between the two groups at ovulation in the natural cycle (P=0.03), and in the stimulated cycle (P=0.04), but not at the implantation window.

6.4.2.4. Treatment outcomes in stimulated cycles

Forty three of the 73 women studied had a negative urine pregnancy test and a serum β-hCG <5 IU/L 2weeks after embryo transfer i.e. equivalent to 4weeks gestation; 31 of these were White and 12 were non-White women. Thirty women, however, had a positive urine pregnancy test and a serum β-hCG > 5 IU/L; 23 of these were White and 7 were non-White women. The percentage of pregnant White women (42.6%) was slightly higher than that of non-White women (36.8%) at 4 weeks **(Table 6.11)**.

At 6 weeks, 21 women of 30 the pregnant women had a viable pregnancy confirmed by ultrasound scan, and 9 women had a spontaneous miscarriage. Seventeen of these 21 were White and 4 were non-White. Of those who miscarried 6 were White and 3 were non-White. The viable pregnancy rate in this study was therefore 31.48% and 21.05% for the White and non-White groups, respectively. These rates were not significantly different ($P>0.05$; Fisher's exact test).

Table 6.11. Outcome of ART in White and non-White women.

	Total women studied (n=73)	White (n=54) 73.9%	Non-White (n=19) 26.2%
Non –pregnant	43 (58.9%)	31 (57.4%)	12 (63.1%)
Pregnant women (positive pregnancy test at 4 weeks)	30 (41.1%)	23 (42.6%)	7 (36.9%)
Viable pregnancy (positive fetal heart at 6 weeks)	21 (28.8%)	17 (73.9%)	4 (57.1%)
Miscarriage	9 (12.3%)	6 (26.1%)	3 (42.9%)

6.4.2.5. Plasma AEA levels in pregnant and non-pregnant women at two weeks post embryo transfer.

AEA levels were similar in pregnant White and non-White women on the day of oocyte retrieval, on the day of embryo transfer and on the day of pregnancy test (P values were 0.41, 0.87 and 0.41 respectively). There were also no statistically significant differences in AEA levels in the non-pregnant White and non-White women (P=0.08, 0.68, 0.32).

In the pregnant White (n=23) and non-White (n=7) groups, plasma AEA levels increased significantly from the day of oocyte retrieval to the day of embryo transfer (P<0.0001, P=0.008), respectively. AEA levels thereafter increased significantly from the day of embryo transfer to the day pregnancy test (P values were P=0.007, P=0.002 respectively for Whitw and non-White women), but there was no statistically significant difference in AEA levels on the day of oocyte retrieval and on the day of pregnancy test in both White and non-White groups **(Figure 6.6)**. Nevertheless, in the non-pregnant group, plasma AEA pattern were different in White women in comparison to non-White women.

In White non-pregnant women (n=31), the mean plasma AEA levels dropped significantly from1.34 ± 0.08 nM on the day of oocyte retrieval to 0.93 ± 0.07 nM on the day of embryo transfer (P<0.001) and there was an increase in AEA levels from the day of embryo transfer to the day of pregnancy test (1.18 ± 0.08 nM) but this increase was not statistically significant (**Figure 6.6).** In the non-White group, however, there was no statistically significant change in plasma AEA levels from the day of oocyte retrieval to the day of pregnancy test (P=0.38).

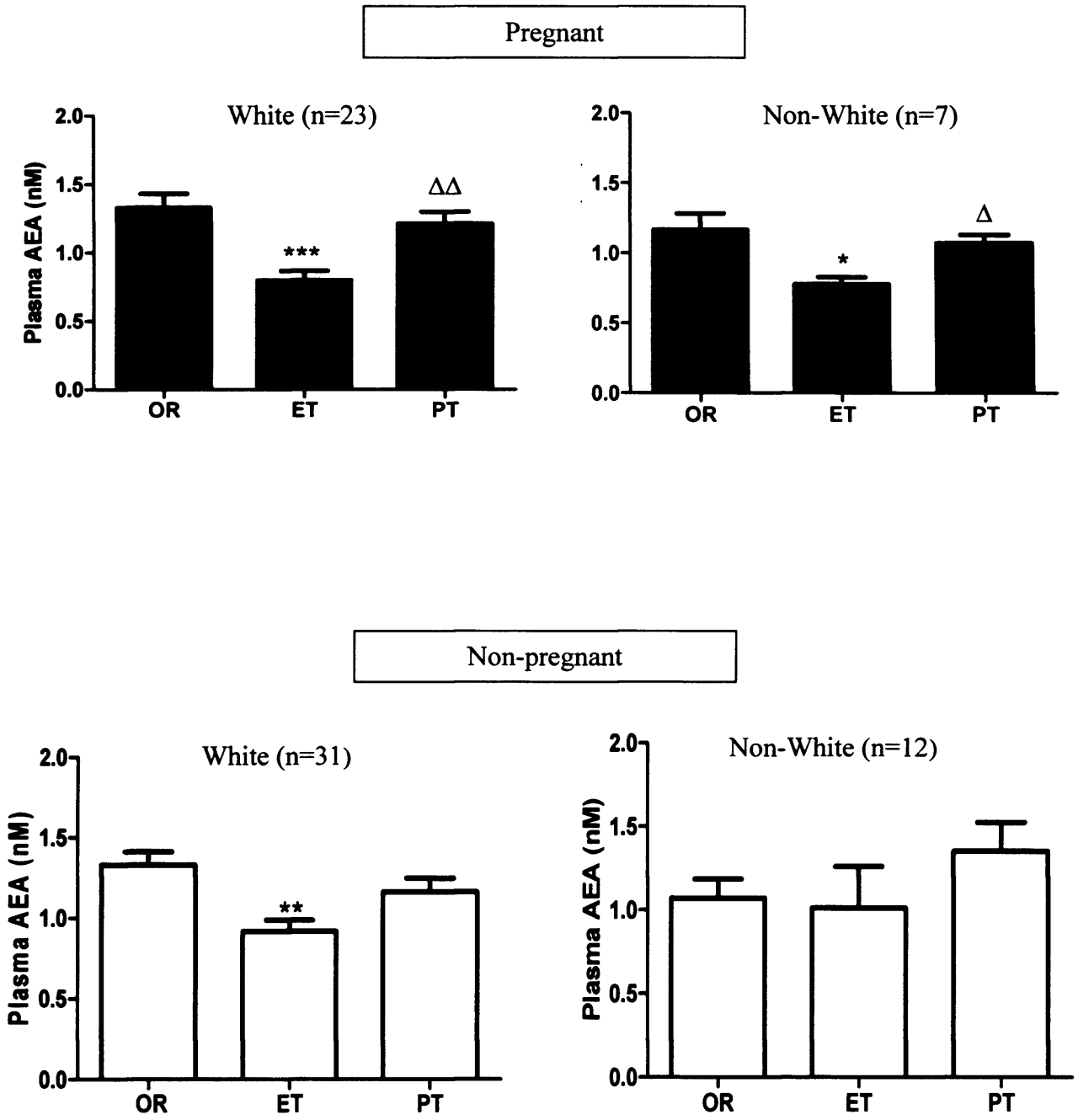


Figure 6.6. Plasma AEA levels in pregnant and non-pregnant White and non-White on the day of oocyte retrieval (OR), embryo transfer (ET) and pregnancy test (PT) day.Data are presented as mean ± SEM; *P<0.05; **P<0.001. ***P<0.0001 compared to OR; $^{\Delta}$P <0.05, $^{\Delta\Delta}$P<0.001 compared to ET. n=number of volunteers.

The mean plasma AEA levels were not significantly different (P=0.23) in women with a viable pregnancy on the day of a positive ultrasound scan in the White (n=17; 0.75 ± 0.06 nM) and in non-White groups (n=4; 0.61 ± 0.05 nM). However, AEA levels at 6 weeks were much higher in women who had miscarriages whether they were White (n=6; 1.21 ± 0.35nM), or non-White (n=3; 1.97 ± 0.66 nM).

6.4.3. AEA and ethnicity – White British and Asian Indians

Since most of the non-White women in this study were Asian Indians (20.5%), a comparison between White British (n=57) and Asian Indians (n=16) was performed.

From **Table 6.12** the White British and Asian Indians were similar in age, BMI, duration of infertility and their basal FSH. However, there were differences between the two groups in the type of infertility (primary or secondary) and the infertility causes. Asian Indians had a higher percentage of (32.2%) tubal factors in comparison to the White British (15.8%). This was reflected on the type of assisted conception treatment offered to the volunteers **(Table 6.12).** There were no statistically significant differences between the groups with regard to the cycle characteristics, ovarian stimulation treatment and outcome as shown in **Tables 6.13 and 6.14.** Fifty four of the 57 White British women and 14 of the 16 Asian Indians had embryo transfer.

Table 6.12. Demographic characteristics, duration, type and cause of infertility and treatment offered to White British and Asian Indians. Data are presented as mean ± SD, (range), percentage (%).

	White British (n=57)	Asian Indians (n=16)	P Value
Age (years)	33.7 ± 4.53 (23-42)	32.8 ± 3.26(27-37)	0.52
BMI (kg/m^2)	24.1 ± 2.96(19-30)	23.4 ± 3.31(18-28)	0.38
Duration of infertility (years)	4.2 ± 2.44(1-16)	5.4 ± 3.68(2-14)	0.14
Type of infertility:			
Primary infertility	37 (64.9%)	14(87.5%)	
Secondary infertility	20 (36.1%)	2(12.5%)	
Previous child birth	3(5.3%)	2(12.5%)	
Cause of infertility:			
Male	20 (35.1%)	5 (31.25%)	
Tubal	9 (15.8%)	5 (31.25%)	
Unexplained	20 (35.1%	4 (25%)	
Endometriosis	3 (5.3%)	1 (6.25%)	
Old age	1 (1.7%)		
Combined infertility causes:	4 (7%)	1 (6.25%)	
Male + ovulatory	3		
Male + Tubal	1	1	
Treatment			
IVF	20 (35.1%)	10 (62.5%)	
ICSI	35 (61.4%)	5 (31.2%)	
IVF + ICSI	2 (3.5%)	1 (6.25%)	

Table 6.13. Basal FSH, duration of stimulation, total number of oocytes retrieved, fertilisation rate and the number of mature oocytes in White British and Asian Indians. Data are presented as mean ± SD.

	White British (n=57)	Asian Indians (n=16)	P Value
Basal FSH	6.1 ± 1.7 (2.5-10)	5.6 ± 1.5 (3-9.10)	0.32
Days of stimulation	11.4 ± 1.5 (7-14)	11.7 ± 1.1 (10-15)	0.81
Total oocyte number retrieved per woman	11.1 ± 6.2 (1-38)	10.5 ± 5.5 (3-24)	0.80
Number (%) of women who had fertilisation	55 (96.5%)	14 (87.5%)	
Mean percentage of oocytes fertilisation per woman	63.1 ± 23.3 (13-100)	52.7 ± 21.6 (25-100)	0.17
Number of mature oocyte (ICSI cycles)	8.3 ± 4.3 (0-18)	10.8 ± 5.3 (3-18)	0.20

Table 6.14. Total number of embryos, number of good embryos per woman on the day of transfer and the percentage of those who had embryo transfer (White British versus Asian Indians). Data are presented as mean ± SD, range (minimum-maximum), or (percentage).

	White British	Asian Indians	P Value
Total number of embryos per woman available on the day of transfer (both 48hrs and 72hrs)	6 ± 3.7 (1-18)	5.3 ± 3.67 (1-12)	0.54
Total number of good of embryos per woman available on the of transfer	3.9 ± 3.1 (0-14)	4 ± 3.3 (1-9)	0.85
Number (%) of women who had embryos transferred	54 (94.7%)	14 (87.5%)	
Number (%) of women had 1 embryo transferred	6 (11.1%)	2(14.3%)	
Number (%)of women had 2 embryos transferred	48 (88.9%)	12 (85.7%)	

Table 6.15 shows that there was no significant difference in the pregnancy rates at 4 weeks in White British (n=23/54; 42.6%) and Asian Indians (n= 5/14; 35.7%).

At 6 weeks gestation, 17 of the 23 (73.9%) White British women had a viable pregnancy and 6 women (26.1%) had a miscarriage. The Asian Indian women (n=5) had a similar viable pregnancy rate (60%) at 6 weeks gestation, (40%) miscarriage rate. This rate was not statistically different from that observed in the White British women.

The successful pregnancy rate at 6 weeks gestation was 31.5% for the White British group and 21.4% for the Asian Indian group (P>0.05).

Plasma AEA levels in pregnant White British women (n=23) and Asian Indian women (n=5) on the day of oocyte retrieval, on the day of embryo transfer and pregnancy test day (2weeks after embryo transfer) were similar (P values were 0.49, 0.68, 0.59 respectively) **(Figure 6.7)**. Similarly, there were no statistically significant differences in AEA levels in the non-pregnant White British (n=31) and Asian Indian (n=9) women (P=0.24, 0.33, 0.21).

Table 6.15. Pregnancy outcomes in the White British and Asian Indian groups.

	White British (n=54)	Asian Indian (n=14)
Non-pregnant	31 (57.4%)	9 (64.3%)
Pregnant (positive pregnancy test)	23 (42.6%)	5 (35.7%)
Viable pregnancy (Scan evidence at 6 weeks)	17 (73.9%)	3 (60%)
Miscarriage at 6 weeks gestation	6 (26.1%)	2 (40%)

There was no significant difference in the proportions of White British and Asain Indian women who were pregnant versus non-pregnant (Fisher's Exact test, **P=0.76).** Also, there was no significant difference in the proportions of White British and Asian Indian women with a viable pregnancy versus non-viable pregnancy, (Fisher's Exact test, **P=0.61**).

The mean plasma AEA levels (1.17 ± 0.16 nM) in the pregnant Asian Indian women were similar to that of pregnant White British women (1.34 ± 0.08 nM) and the levels decreased significantly ($P<0.05$) from the day of oocyte retrieval to embryo transfer (0.74 ± 0.05 nM). The changes in Asian Indian group were however, different from that in the White British in one respect, although the plasma AEA levels increased from 0.74 ± 0.05 nM on the day of embryo transfer to 1.10 ± 0.08 nM on the day of pregnancy test, the increase was not statistically significant ($P>0.05$). By contrast, in the Asian Indian non-pregnant women (n=9), however, there was no statistically significant change in plasma AEA levels from the day of oocyte retrieval to the day of pregnancy test (P=0.62).

The mean plasma AEA levels were not significantly different (P=0.38) in women with a viable pregnancy on the day of a positive ultrasound scan in The White British group (n=17; 0.75 ± 0.06 nM) and in Asian Indian group (n=3; 0.61 ± 0.08nM). However, AEA levels were similar in women who had miscarried whether they were White British (n=6; 1.21 ± 0.35 nM), or Asian Indian (n=2; 1.26 ± 0.06 nM, P=0.94).

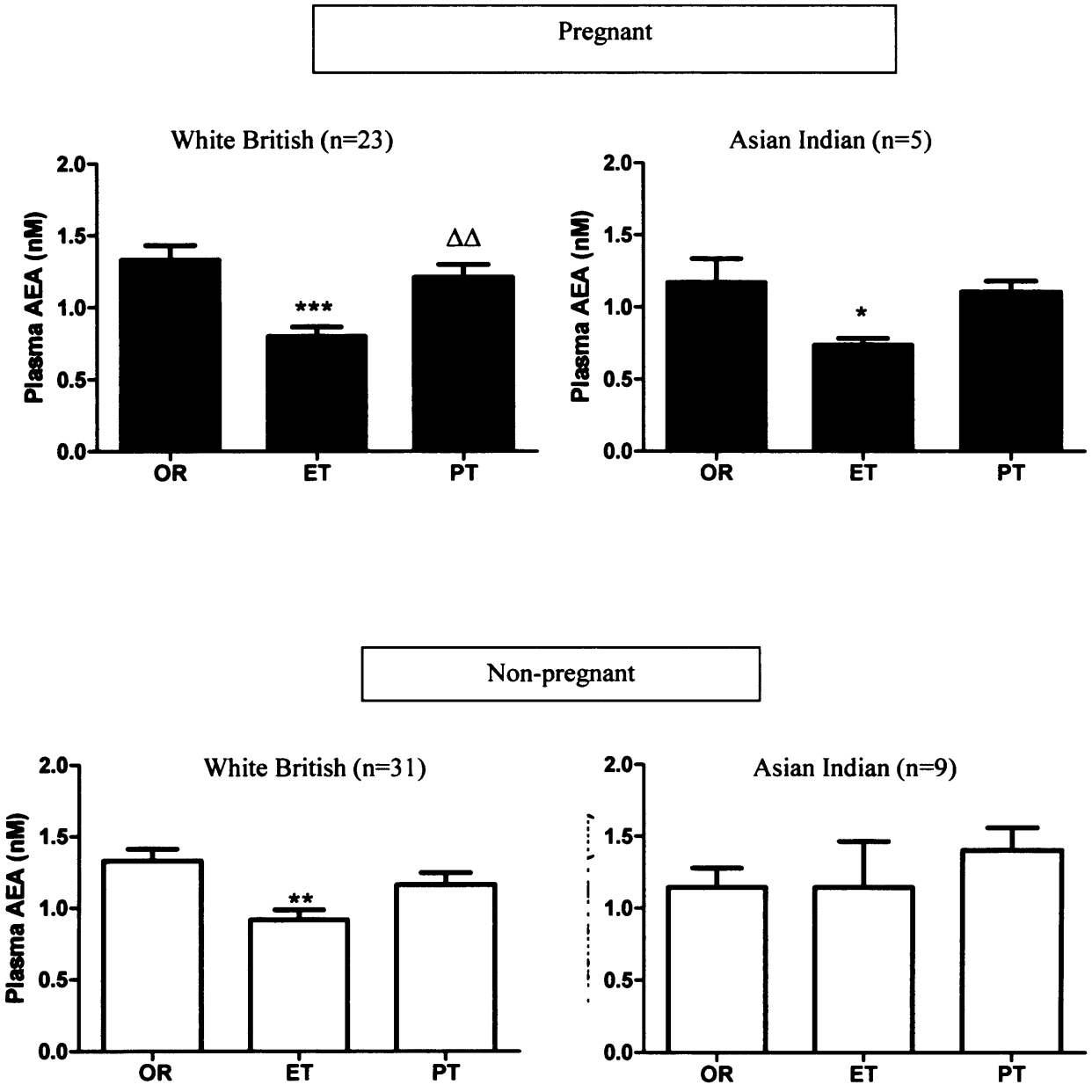


Figure 6.7. Plasma AEA levels in pregnant (n=23) and non-pregnant (n=31) White British and pregnant (n=5), non–pregnant (n=9) Asian Indians on the day of oocyte retrieval (OR), embryo transfer (ET) and pregnancy test (PT) day. Data are presented as mean ± SEM; *P<0.05; **P<0.001. ***P<0.0001 compared to OR. $^{\Delta}$P <0.05, $^{\Delta\Delta}$P<0.001 compared to ET. n=number of volunteers.

6.4.4. Plasma AEA in viable pregnancy and Miscarriage groups at 6 weeks

The changes in plasma AEA levels in women with a viable pregnancy (positive implantation; n=21) and in those who had a spontaneous miscarriage (failed implantation; n=9) are shown in **Figure 6.8**. The mean plasma AEA levels in the viable pregnancy group were 1.22 ± 0.09 nM on the day of oocyte retrieval and then declined sharply to 0.73 ± 0.05 nM on the day of embryo transfer (P<0.0001). The levels then increased significantly to 1.17 ± 0.07 nM on the day of pregnancy test (P<0.0001); there was also, a statistically significant decline in the AEA levels to 0.72 ± 0.05 nM on the day of a positive ultrasound scan (P<0.0001). In the miscarriage group, there was no significant difference in the AEA levels at the different time periods studied. Plasma AEA levels were 1.47 ± 0.14 nM on the day of oocyte retrieval, 0.94 ± 0.12 nM on the day of embryo transfer, on the day of pregnancy test and 1.36 ± 0.27 nM at 6 weeks. There was no significant difference in AEA levels in the two groups on the day of oocyte retrieval (P=0.16), 1.18 ± 0.17 nM on the day of embryo transfer (P= 0.07), and on the day of pregnancy test (P=0.99). However, there were significant differences on the day of ultrasound scan at 6 weeks (P=0.002).

An ROC analysis performed on AEA levels at 6 weeks to determine if it was possible to use AEA levels to discriminate between women with a viable pregnancy and those who miscarried is shown **in Figure 6.9 and Table 6.16.** An AEA level of 1.25 nM had a100% specificity, and 55% sensitivity for the prediction of miscarriage.

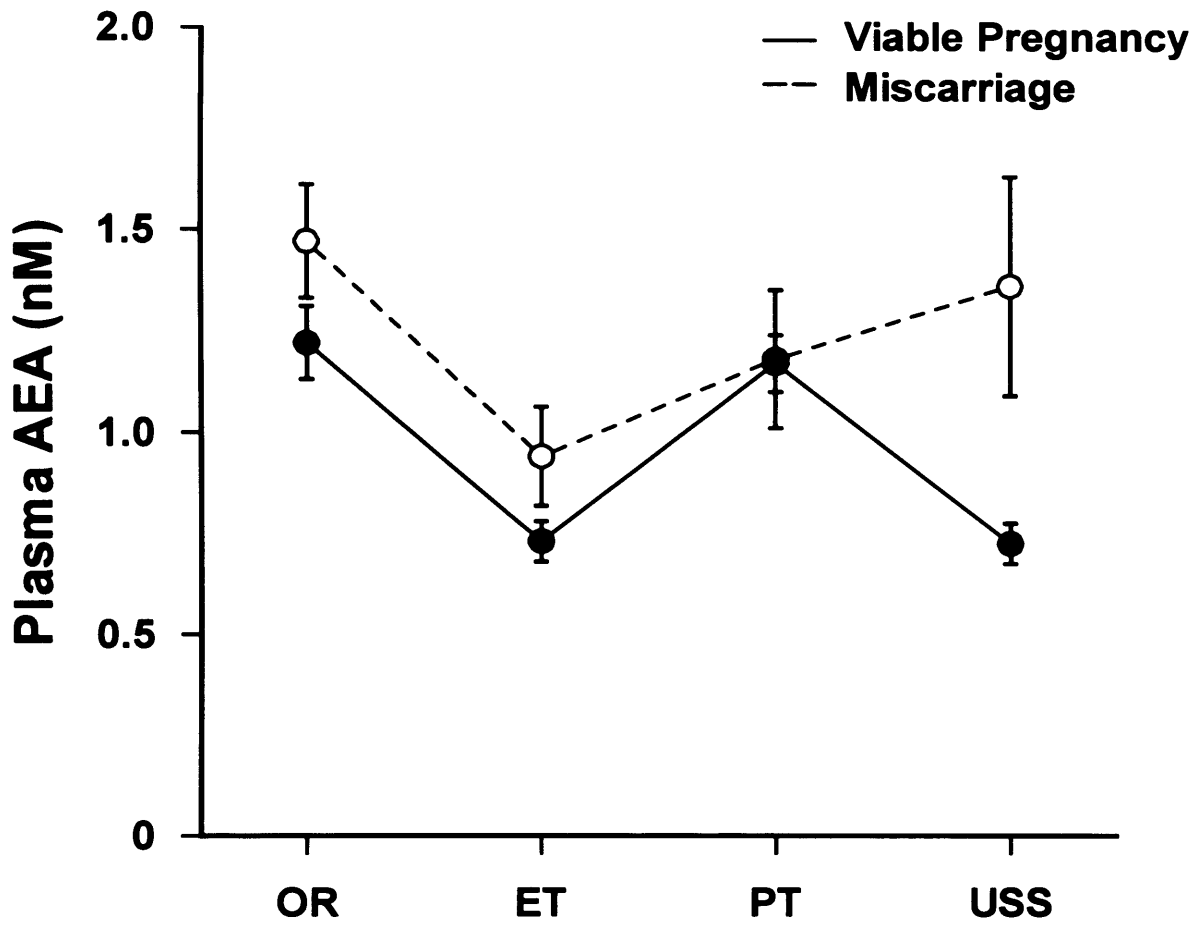


Figure 6.8. Plasma AEA levels in women with viable pregnancy (n=21) and women had miscarriage (n=9) at 6 weeks gestation. There was significant difference between the 2 groups at 6 weeks (P=0.002). The data are presented as the mean ± SEM. P-values were calculated using Student's unpaired *t*-test. OR= oocyte retrieval; ET= embryo transfer; PT= pregnancy test; USS= ultrasound scan.

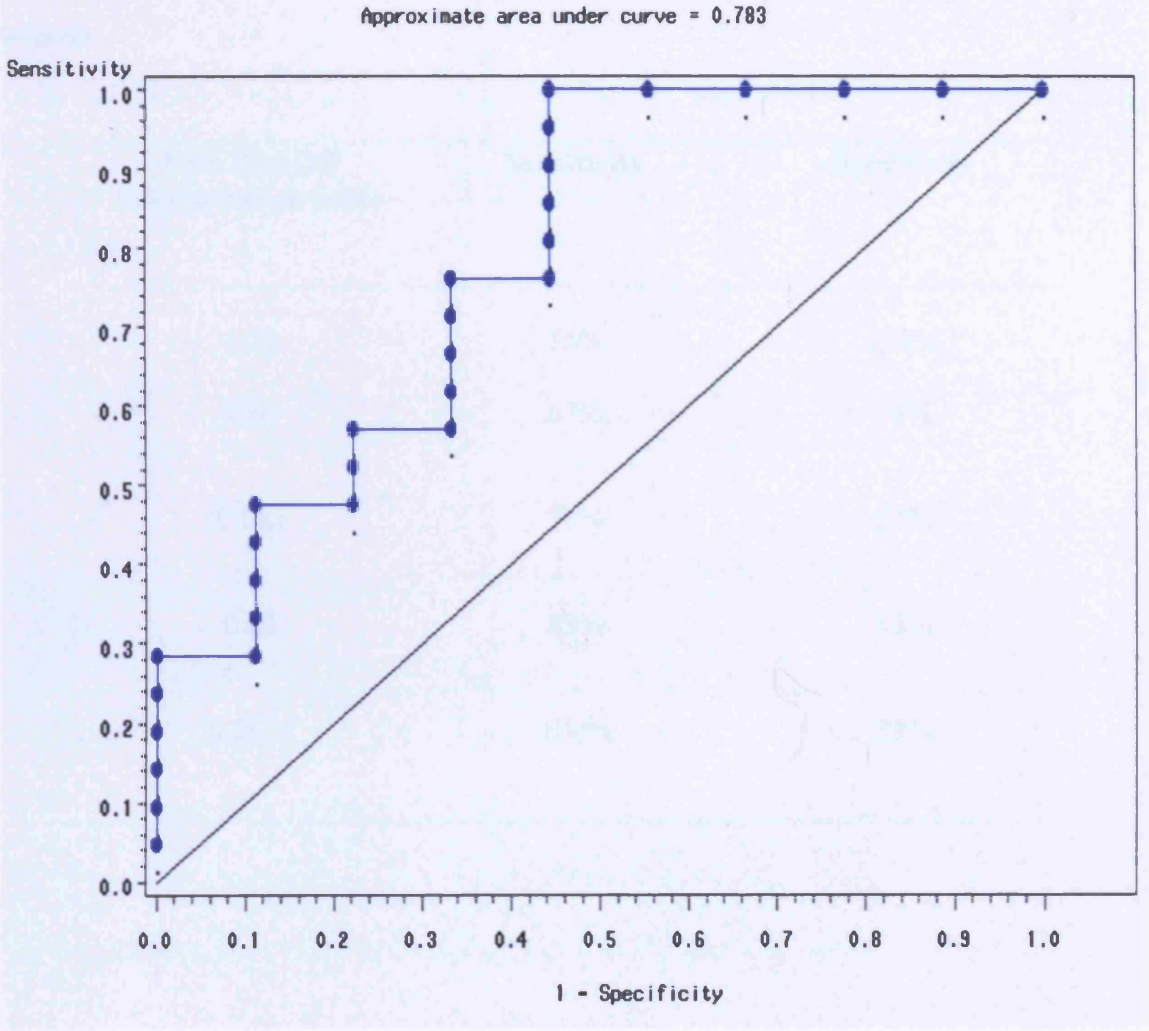


Figure 6.9. ROC Curve for plasma anandamide concentrations at 6 weeks gestation. Logistic Regression: AEA level at 6 weeks identifies those who had miscarried: Odds Ratio (Miscarry) = 19.01 (95% CI=1.24, 291.9), P=**0.0346**. AUC = **0.783**.

Table 6.16. Cut-off points, sensitivity and specificity measurements for plasma AEA concentrations in the predication of miscarriage at 6 weeks gestation in IVF/ICSI-ET women.

AEA Cut Off concentration (nM)	Sensitivity	Specificity
1.25	55%	100%
0.80	67%	76%
0.682	78%	57%
0.66	89%	52%
0.5625	100%	29%

6.4.4.1. Plasma AEA levels in a viable pregnancy from 2 to 7 weeks gestation

On a small cohort of women with a viable pregnancy (positive implantation), extra measurements of plasma AEA levels were undertaken at week 7 of gestation. AEA values from the day of embryo transfer (i.e. week 2 of gestation) to week 7 of pregnancy are shown in **Table 6.17 and Figure 6.10.** The mean plasma AEA levels of these women fluctuated from 0.72 ± 0.05 nM on the day of embryo transfer (n=20) to 1.17 ± 0.07 nM on the day of pregnancy test (i.e. 4 weeks gestation) (n=20,), and then to 1.18 ± 0.11 nM at week 5 (n=13), 0.72 ± 0.05 nM at week 6 (n=21) and to 0.78 ± 0.11 nM at week 7 of gestation (n=13). Plasma AEA levels were significantly lower on the day of embryo transfer at 6 and 7 weeks gestation but significantly higher at weeks 4 and 5 of pregnancy.

There was a significant difference in plasma AEA levels between week 2 and week 4 ($P<0.0001$), but not significantly different from weeks 4 and 5 ($P=0.94$). There was however, a significant difference between weeks 5 and 6 ($P=0.0002$), and between weeks 4 and 6 of pregnancy ($P<0.0001$).

6.4.4.2. The relationship between serum β-hCG and plasma AEA levels in women with viable pregnancies (positive implantation) and those who miscarried (failed implantation).

An analysis of the relationship between plasma AEA and serum β-hCG in those with viable (n=21) and non-viable pregnancies (n=9) at 4 and 5 weeks gestation indicated that there was no statistically significant correlations at either time point (for the viable pregnancy group $r^2=0.06$; $P=0.34$ at 4 weeks; $r^2=0.01$; $P=0.78$ at 5 weeks and for the non-viable pregnancies $r^2=0.05$; $P=0.56$ at 4 weeks; $r^2=0.05$; $P=0.61$ at 5 weeks).

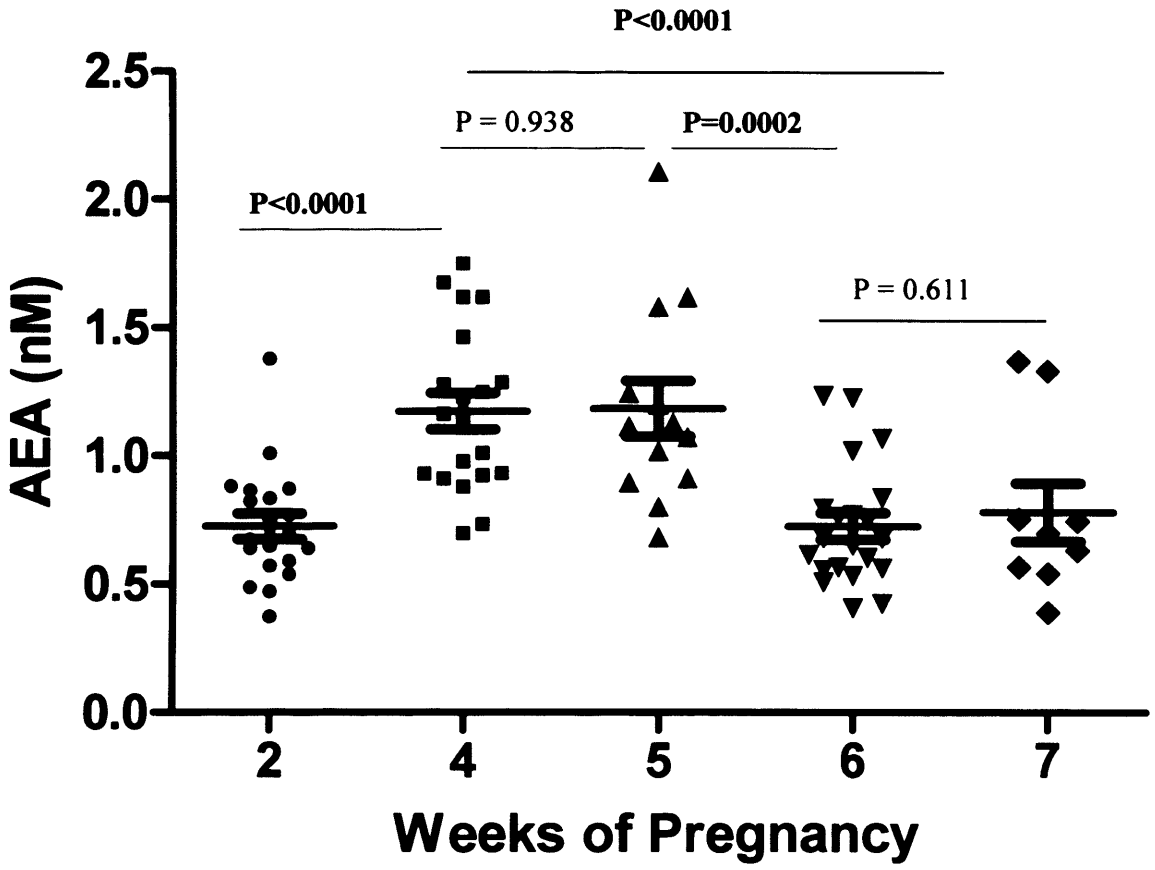


Figure 6.10. Plasma AEA levels in women with a viable pregnancy (positive implantation) from the day of embryo transfer (n=20), week 4 (n=20), week 5 (n=13) and week 7 (n=9) of gestation. The long horizontal bar indicates the mean, the shorter horizontal bars the SEM. P-values were calculated using Student's unpaired *t*-test.

Table 6.17. Plasma AEA concentrations in the pregnant women from the day of embryo transfer to week 7 of gestation.

Patient	AEA_{ET} (nM)	β-hCG (IU/L)	AEA_1 (nM)	β-hCG2 (IU/L)	AEA_2 (nM)	USS Wk 6	AEA_{USS} (nM)	USS Wk 7	AEA (nM)
1	-	168	1.249	5320	1.20	$6+^1$	0.562	$7+^1$	0.698
2	0.65	227	0.930	2200	-	6	0.747	7	0.755
3	1.01	60	1.620	2610	1.131	6	1.068	$7+^6$	0.746
4	0.77	197	1.164	2430	2.11	$6+^5$	0.679	$7+^5$	0.632
5	1.379	65	1.011	4250	1.020	$6+^1$	1.019	-	
6	0.835	136	1.750	2662	0.803	6	1.226	-	
7	0.751	99	0.910	3000	1.583	6	0.604	7	0.568
8	0.882	144	0.924	3690	0.897	6	0.565	-	
9	0.571	132	0.979	2310	-	$6+^1$	0.424	-	
10	0.871	299	1.279	4800	0.684	6	0.764	$7+^1$	1.330
11	0.64	71	1.151	4141	1.115	$6+^5$	0.533	-	
12	0.472	189	0.734	2938	-	6	0.507	7	1.370
13	0.694	150	1.619	4805	1.61	$6+^5$	0.613	-	
14	0.865	392	1.464	4590	-	$6+^5$	0.652	$7+^5$	0.542
15	0.640	303	1.231	6654	-	$6+^3$	0.794	$7+^3$	0.390
16	0.373	256	1.286	8968	-	$6+^6$	0.556		-
17	0.539	983	0.881	8353	1.245	$6+^5$	0.408		-
18	0.489	296	0.70	4430	1.074	$6+^4$	0.77	-	-
19	0.673	161	0.933	5978	-	6	1.234		-
20	0.591	-	-	2489	-	$6+^1$	0.681		-
21	0.823	179	1.676	2618	0.914	$6+^4$	0.837		-

The table shows plasma AEA measurements on the day of embryo transfer (AEA_{ET}), at 4 weeks (AEA_1) at 5 weeks (AEA_2) and at 6 and 7 weeks (AEA_{USS}). Serum β-hCG at 4 and 5 weeks gestation are shown. USS = the date of transvaginal scan ($weeks^{+days}$). AEA = AEA measurement; β-hCG = serum β-hCG levels; (-) = not determined

6.4.5. The relationship between plasma AEA and serum hormones

Further analysis was undertaken in a subgroup of 24 women in whom various hormones (E2, P4) were also measured in 12 with a viable pregnancy and 12 non-pregnant controls. They were matched for age, BMI, duration of infertility, type of infertility (primary, secondary), cause of infertility, basal FSH and LH levels as shown in **Table 6.18**.

These women were from the cohort of 78 studied. Once a pregnant case was identified the next non-pregnant case matched for age, BMI, duration, type and cause of infertility, was selected as control. The treatment protocols in the two groups were very similar **(Table 6.19)**. There was, however, a significantly higher number of ovarian stimulation days for the non-pregnant controls when compared to the pregnant group (P=0.01). Despite the fertilisation rates not being significantly different in both groups (P=0.11), the women with viable pregnancy produced almost double the number of embryos avilable for transfer compared to the non-pregnant women (P=0.01). In addition, the total number of good quality embryos available on the day of embryo transfer was significantly higher in the pregnant group (P=0.003).

Table 6.18. Demographic characteristics, duration, type and cause of infertility and treatment offered for the study group. Data are expressed in mean ± SD, the range in parentheses shows minimum to maximum values.

	Women with a viable pregnancy (n=12)	Non- Pregnant women (n=12)	P Value
Age (years)	32.17 ± 3.27 (28-38)	32.75 ± 3.72 (27-38)	0.69
BMI (kg/gm^2)	23.42 ± 3.60 (19-30)	23.83 ± 3.12 (19-29.50)	0.76
Duration of infertility (years)	4.66 ± 1.72 (2-7)	4.17 ± 1.80 (2-7)	0.49
Type of infertility:			
Primary infertility	7	7	
Secondary infertility	5	5	
Cause of infertility:			
Male	4	3	
Tubal	3	3	
Unexplained	5	6	
Treatment:			
IVF-ET	5	6	
ICSI-ET	7	6	
Basal hormones levels:			
Basal FSH (IU/l)	5.79 ± 1.89 (3.3-10)	6.19 ± 1.74 (4.0-9.6)	0.59
Basal LH (IU/l)	4.55 ± 1.78 (3.0-8.4)	4.31 ± 1.77 (2.3-7.7)	0.75

BMI = body mass index; IVF = in-vitro fertilization; ICSI = intracytoplasmic sperm injection; FSH= follicle stimulating hormone; LH = luteinizing hormone.

Table 6.19. Shows the regimen used for the ovarian stimulation, fertilisation rate and number of embryos per woman on the day of embryo transfer.

	Viable pregnancy (n=12)		Non Pregnant (n=12)		
	Mean ± SD	Range	Mean ± SD	Range	P
Days of stimulation	10.75 ± 1.76	7-12	12.17 ± 0.71	11-14	**0.01**
Dose of Puregon (rFSH) IU	1639 ± 496.6	1000-2475	2463 ± 1546	1350-5400	0.21
Dose of Menopur (hMG) IU	4275 ± 2679	1650-8250	4004 ± 2551	1800-9150	0.86
Mean percentage of oocytes fertilisation per woman	59.86 ± 20.72	13.3-100	45.06 ± 22.67	22.20-100	0.11
Total number of embryos available per woman on the day of embryo transfer	6.82 ± 3.99	2-15	3.42 ± 1.8	2-8	**0.01**
Total number of good embryos available per woman on the day of embryo transfer	5.67 ± 3.312	2-11	2.33 ± 1.07	1-4	**0.003**

rFSH = recombinant follicle stimulating hormone; hMG = human menopausal gonadotrophins.

6.4.5.1. Plasma AEA levels in women with viable pregnancies and in non-pregnant women

In women with a viable pregnancy (positive implantation), the highest plasma AEA levels (mean ± SEM) were observed on the day of oocyte retrieval (1.16 ± 0.15 nM) and on the day of pregnancy test (2 weeks post embryo transfer) (1.18 ± 0.09 nM). The lowest measurable plasma AEA levels were from samples taken just prior to embryo transfer and on the day of a positive ultrasound scan (0.66 ± 0.044 nM and 0.67 ± 0.06 nM respectively). In this pregnant group, plasma AEA levels decreased significantly (P=<0.001) from the day of oocyte retrieval to the day of embryo transfer with a mean difference in the fall of 43.2%. However, AEA levels rose significantly (P=<0.001) from the day of embryo transfer to that of pregnancy test followed by another significant (P<0.001) decrease from the time of pregnancy test to the first ultrasound scan (**Figure 6.11).**

By contrast, in the non-pregnant group the highest plasma AEA levels (1.31 ± 0.14 nM) were seen on the day of the pregnancy test, and the lowest levels were on the day of oocyte retrieval (0.89 ± 0.07 nM) and on the day of embryo transfer (0.71 ± 0.08 nM). Unlike the significant changes seen in plasma levels of the pregnant women from the day of oocyte retrieval to the day of embryo transfer, there was no statistically significant difference in plasma AEA levels from the day of oocyte retrieval to the day of embryo transfer in the non-pregnant group. The mean difference in the decline in plasma AEA levels between the two periods was only (20.2%). There was a significant increase in plasma AEA levels from the day of oocyte retrieval to the day of pregnancy test (P<0.05) and a significant increase from the day of embryo transfer to the day of pregnancy test (P<0.0001).

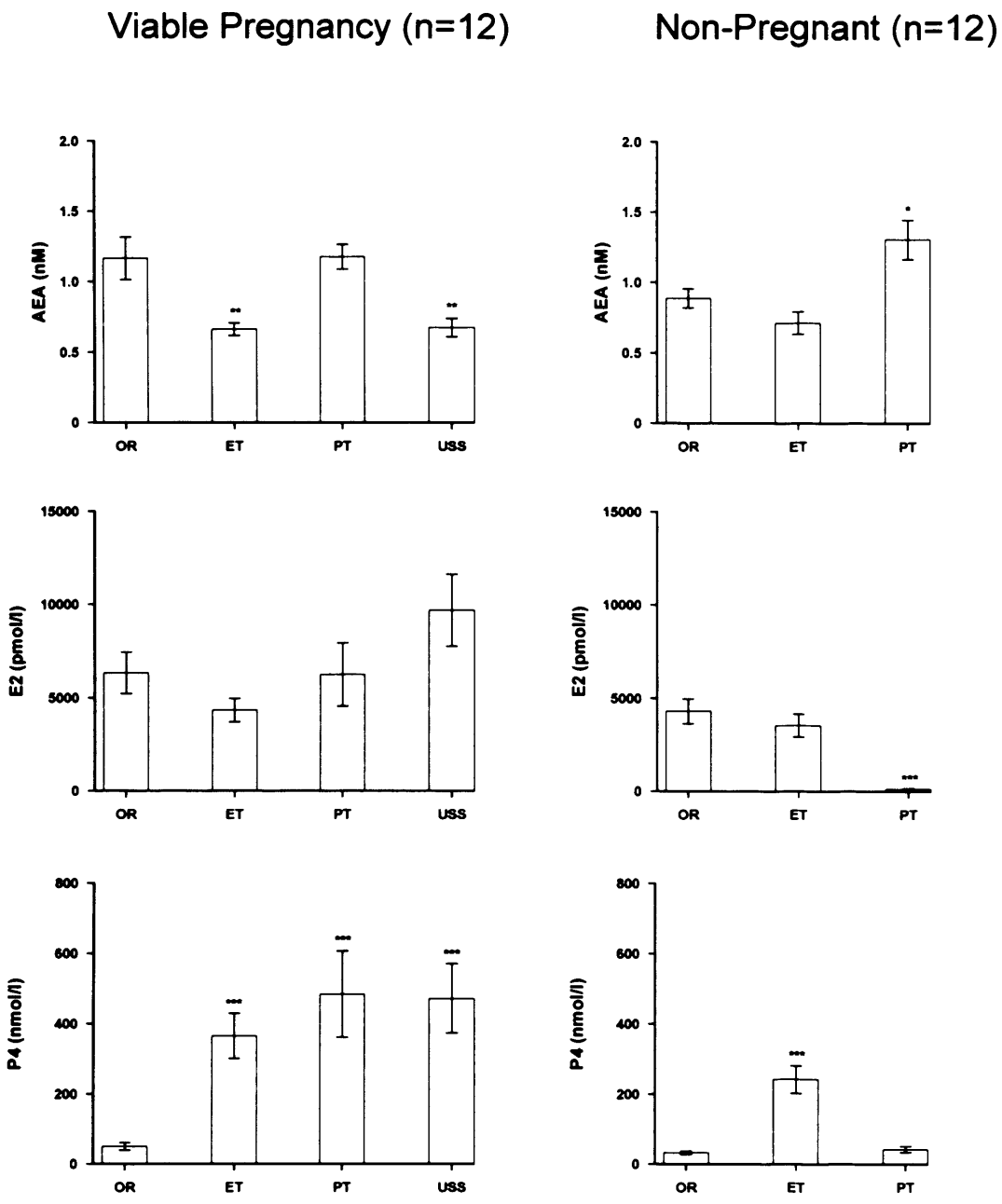


Figure 6.11. Plasma AEA, serum E2 and P4 in the women with a viable pregnancy (n=12) and non pregnant women (n=12) during the different stages of IVF/ICSI-ET. The stages are; OR = Oocyte retrieval; ET= embryo transfer; PT = pregnancy test; USS= first positive ultrasound scan. E2 = estradiol, P4 = progesterone; AEA = anandamide. Data are presented as mean ± SEM; *P<0.05; **P<0.0001.

An analysis of plasma AEA levels in the women with a viable pregnancy and non-pregnant women at the 3 studied time periods showed that there was no statistically significant difference between the two groups.

6.4.5.2. Serum E2, P4, and β-hCG levels in women with viable pregnancies and non-pregnant women

The highest level of serum E2 (9689 ± 1935 pmol/L) in the pregnant women was on the day of ultrasound scan and the lowest (4324 ± 635 pmol/L) on the day of embryo transfer (**Figure 6.10**). Although there appeared to be a subtle fluctuation in serum E2 concentrations over the course of treatment, these fluctuations were not significantly different. For the non-pregnant group, the highest level of serum E2 (4295 ± 622.8 pmol/L) was noted on the day of oocyte retrieval and the lowest level (129.6 ± 21.7 pmol/L) on the day of the negative pregnancy test (**Figure 6.11**). The decrease in serum E2 concentrations observed from the day of oocyte retrieval to the day of pregnancy test in the non-pregnant group was significantly different ($P<0.0001$). A similar decrease in serum E2 concentrations from 3534 ± 616.5 pmol/L on the day of embryo transfer to 129.6 ± 21.7 pmol/L on the day of pregnancy test was also significantly different ($P<0.0001$). When serum E2 levels in the pregnant and non-pregnant groups were compared (**Table 6.20**) only the levels on the day of pregnancy test were significantly different ($P=0.002$).

Serum P4 levels in the pregnant group were highest (483.7 ± 123 nmol/L) on the day of positive pregnancy test and on the day of ultrasound scan (471.7 ± 99.3 nmol/L) (**Figure 6.10**). The P4 level was lowest (49.6 ± 11.2nmol/L) on the day of oocyte retrieval and increased significantly ($P<0.0001$) on the day of embryo transfer (364.9 ± 64.08 nmol/L). The serum P4 levels on the day of embryo transfer, pregnancy test and ultrasound scan were not significantly different (**Figure 6.11**). In the non-pregnant group, however, the

highest P4 level (241.8 ± 39.4 nmol/L) was on the day of embryo transfer and the lowest level (32. 2 ±4.8 nmol/l) was on the day of oocyte retrieval. There was a significant ($P<0.0001$) increase in serum P4 levels from that on the day of oocyte retrieval to that on the day of embryo transfer, and a significant decrease to that (42.1 ± 8.5 nmol/L) on the day of negative pregnancy test ($P<0.0001$).

A comparison between serum P4 levels in the pregnant and non-pregnant groups (**Table 6.20**) showed that only the levels found on the day of pregnancy test were significantly different ($P=0.002$).

Serum β-hCG levels measured exactly two weeks after embryo transfer were significantly higher in the pregnant group (202.5 ± 29.59 IU/L; range 70-392 IU/L) than in the non-pregnant group (all were <2 IU/L).

Table 6.20. Serum progesterone (P4), estradiol (E2) and plasma anandamide (AEA) levels in women with a viable pregnancy and non pregnant women during IVF/ICSI treatment stages. Data are expressed with mean ± SEM. P-values were calculated by Student's unpaired *t*-test and significant values are shown in bold.

	AEA Viable Pregnancy	AEA Non Pregnant	P	P4 Viable Pregnancy	P4 Non Pregnant	P	E2 Viable Pregnant	E2 Non Pregnant	P
OR	1.16 ± 0.15	0.89 ± 0.07	0.11	49.63 ± 11.24	32.25 ± 4.76	0.17	6317 ± 1111	4295 ± 662.8	0.132
ET	0.66 ± 0.04	0.71 ± 0.08	0.62	364.9 ± 4.08	241.8 ± 39.36	0.11	4324 ± 635.4	3534 ± 616.5	0.382
PT	1.18 ± 0.09	1.31 ± 0.14	0.44	483.7 ± 123	42.08 ± 8.50	**0.002**	6238 ± 1695	129.6 ± 21.71	**0.002**
USS	0.67 ± 0.06			471.7 ± 99.27			9689 ± 1935		

OR=oocyte retrieval; ET= embryo transfer; PT= pregnancy test; USS= ultrasound scan.

6.4.5.3. Hormonal associations with plasma AEA

The relationship between plasma AEA levels and serum levels of the sex steroids (E2, P4) in the pregnant and non-pregnant groups at oocyte retrieval, embryo transfer and pregnancy test are show in **Table 6.21**. There were no statistically significant correlations in the pregnant and non-pregnant groups between plasma AEA and serum P4 (P=0.95 and P=0.15 respectively). While there was no statistically significant correlation between plasma AEA levels and E2 in the pregnant group (P=0.95) the relationship in the non-pregnant group was statistically significant (P=0.022). Analyzing the data at positive ultrasound scan (6 weeks) separately showed that there were no statistically significant correlations between plasma AEA and serum E2 (P=0.88), plasma AEA and serum P4 (P=0.75), and plasma AEA and serum β-hCG (P=0.58) at 4 weeks gestation in the pregnant group.

Table 6.21. The relationship between plasma AEA, estradiol (E2), progesterone (P4) and β-hCG in women with a viable pregnancy and non-pregnant IVF-ET women. Data were obtained using linear regression analysis. r = linear regression co-efficient.

Parameters	Women with a viable Pregnancy	Non-Pregnant Women
Plasma AEA and E2	$r^2 = 0.000$	$r^2 = 0.145$
	P= 0.949	**P = 0.022**
Plasma AEA and P4	$r^2 = 0.013$	$r^2 = 0.058$
	P = 0.508	P = 0.155
Plasma AEA and β-hCG	$r^2 = 0.031$	
	P = 0.581	

The significant correlation is shown in bold.

6.5. Discussion

Advanced assisted reproductive techniques such as IVF and ICSI-ET provided an excellent opportunity to accurately determine the plasma AEA levels at the time of ovulation, at the implantation window and during early pregnancy. Additionally, it allowed for comparison of plasma AEA levels in a cohort of women who had a successful implantation, as evidenced by a viable pregnancy (positive implantation) at 6 weeks gestation, and another cohort who failed to implant. Moreover, when AEA levels in IVF stimulated cycles were compared with those of natural cycles, the values at ovulation and oocyte retrieval and those at the mid luteal phase (implantation window) and at the time of embryo transfer were similar, suggesting that higher AEA levels are associated with ovulation and lower levels are associated with the window of implantation. These observations are in keeping with those of Lazzarin et al., who showed that AEA levels fluctuated during the ovulatory menstrual cycle with higher levels around the time of ovulation and significantly lower levels at the implantation window (Lazzarin et al., 2004).

A distinctive pattern of plasma AEA changes were observed in both pregnant and non-pregnant IVF-ET women. In both groups there was an initial reduction in AEA levels from the day of oocyte retrieval (OR) to the day of embryo transfer (ET), followed by a significant increase on the day of pregnancy test (2 weeks after embryo transfer). However, an important finding in this study was a statistically significant (43%) reduction in plasma AEA levels (from OR to ET) in the successful pregnant group, but not in the non-pregnant group. The plasma AEA levels were ascertained prior to placing the embryo in the uterus, so they were not influenced by the presence of the embryo. These observations are in agreement with those of Guo et al., (Guo et al., 2005) who suggested that the levels of AEA in the uterine cavity need to fall for implantation to be successful.

Since ethnicity has been shown in some studies to influence outcome in ART programmes (Mahmud et al., 1995; Lashen et al., 1991; James et al., 2002), but not in other studies, a contentious state has arisen with the consensus view is that there are no significant difference in IVF outcomes among different ethnic groups (Lashen et al., 1999; Bendikson et al., 2005). A sub-analysis of the data along ethnic groups was therefore undertaken. The distribution within the study population was similar to the demographics of Leicester as a whole (Research & Information. The Department of Planning and Transportation of Leicestershire County Council, National Census 2001). There was no difference between White and non-White women with regard to ovarian stimulation results and pregnancy outcomes, since the majority of non-White group were Asian Indians and all of the White group were British, a detailed analysis of IVF outcomes of Asian Indians compared to White British was undertaken. There was no difference in IVF outcomes between the White British and Asian Indian women which is in keeping with published studies that reported that ethnicity has no effect on ART outcomes (Lashen et al., 1999).

There were interesting observations with regard to differences in plasma AEA levels between White and non-White women and this was not exclusive to ART cycles, as similar differences were observed in natural cycles. There was a significant difference between the two groups on the day of ovulation, whether this occurred naturally or was stimulated. There was however, no difference in the levels of AEA at the implantation window in both groups suggesting that AEA may be influenced by ethnicity, an effect which has not been reported previously. Plasma AEA levels in all pregnant women fell significantly from the day of oocyte retrieval to the day of embryo transfer. There was also a significant increase in plasma AEA levels between the day of embryo transfer and pregnancy test. The rise in plasma AEA levels in the Indian Asians was however, not statistically significant. This could be related to the fact that there were only 5 pregnant women in this group.

In the non-pregnant women, where the number of volunteers was considered adequate, there was a clear difference in AEA levels between the White British and Asian Indian groups. In the non-pregnant White British group there was a significant decline in the AEA levels from the day of oocyte retrieval to the day of embryo transfer, an observation similar to that in pregnant women. This was not unexpected since plasma AEA levels were measured in everyone on the day of oocyte retrieval and also on the day of embryo transfer prior to placing embryos in the uterus. However, in non-pregnant White British women there was not a statistically significant rise in plasma AEA levels from the levels on the day of embryo transfer to the levels on the day of pregnancy test. In addition, the non-pregnant Asian Indian women exhibited no fluctuations in their plasma AEA levels during this period.

This part of study has suggested that AEA is affected by ethnicity and helped to clarify that a distinctive pattern in the changes of plasma AEA levels in pregnant women occurs. In the pregnant group, regardless to the ethnic background, a higher level of AEA at ovulation and significant reduction in AEA level on the day of embryo transfer (Lazzarin et al., 2004), followed by an increase in AEA level on the day of pregnancy test is required. These observations suggest that changes in plasma AEA levels may be essential for successful implantation (Schmid et al., 1997); a low level is probably required to modulate uterine receptivity prior to the actual implantation process. Additionally, when the interaction between the embryo and endometrium occurs, a higher AEA level is probably then required since the plasma AEA levels then rose. Alternatively, the rise may reflect production of AEA by the trophoblast. This will require further investigation to confirm or refute as evidence for this is currently lacking. Additionally, there is no evidence that the plasma AEA levels are proxy to the uterine levels.

The changes in plasma AEA levels from the day of embryo transfer up to 7 weeks gestation revealed that in a viable pregnancy, AEA levels were low on the day of embryo transfer and higher at 4 and 5 weeks gestation and then significantly fell from week 6 onwards. The plasma AEA levels in women with a viable pregnancy at 6 weeks gestation were low and similar to that previously reported (Maccarrone et al., 2000b; Habayeb et al., 2008b). An ROC analysis indicated that plasma AEA levels of 1.25 nM could discriminate between women with viable pregnancies and those who had miscarried. In addition, all of the women with viable pregnancies had plasma AEA values <2 nM at 6 weeks and none had miscarried by 12 weeks in keeping with the previous observation that plasma AEA levels >2 nM were associated with spontaneous miscarriage (Habayeb et al., 2008b).

With regards to the regulation of plasma AEA, the data presented here and the well recognised physiological levels of the hormones controlling the periods studied (Dodson et al., 1975) provide clues to possible mechanisms regulating AEA levels. Investigating the relationship between plasma AEA and P4 at implantation and during early pregnancy generated results which suggest that there was no significant association between plasma AEA and P4. Previous studies have suggested that P4 down-regulates AEA levels during the implantation window (Maccarrone et al., 2003; Lazzarin et al., 2004). Since both the pregnant and non-pregnant groups in this study had similar levels of P4 on the day of embryo transfer, prior to starting progesterone supplementation, a similar low plasma AEA level in both groups would have been expected. The data, however, showed that the pregnant group had high P4 and high AEA levels, and the non-pregnant group had low P4 levels and high plasma AEA levels suggesting that P4 may not be the modulating factor for AEA. Furthermore, the lack of a correlation between plasma AEA levels and serum P4 at 6 weeks gestation may also supported this conclusion. These data are qualitatively similar to those reported in normal cycling women (Chapter 4) where no association between AEA

and P4 was found. It can thus be concluded that plasma AEA levels in women are most probably not regulated by P4.

When the well-documented physiological changes in the levels of gonadotrophins in early pregnancy (Duncan et al., 1996) are compared to AEA levels in these women, it is evident that AEA is associated with gonadotrophin and E2 production. Since oocyte retrieval was assumed to correspond to the ovulation stage in the natural cycle, embryo transfer equivalent to the implantation window or mid luteal phase of the natural cycle and a negative pregnancy test equivalent to the early follicular phase of the next cycle (these women started bleeding due to the withdrawal of their progesterone levels), then it is reasonable to conclude that the changes in plasma AEA levels correlate with changes in gonadotrophin levels and that there is an inverse relationship with E2. However, for women with an intact hypothalamic-pituitary-ovarian axis it is not possible to separate the intimate relationship between gonadotrophins and E2 and study them separately. It is well known that hCG can mimic the action of LH (Speroff and Fritz, 2005a) in the corpus luteum. Indeed, hCG injection was given to all these women 36 hours before oocyte retrieval to induce ovulation (Fischer et al., 1993), mimicking the LH surge. However, in pregnant women, at the time of a positive pregnancy test, physiologically, LH levels and hCG levels are also elevated (Duncan et al., 1996).

The hCG secreted from the syncytiotrophoblast after implantation and the LH secreted from maternal anterior pituitary both support the corpus luteum in its role as a source of steroids which are important for pregnancy maintenance (Speroff and Fritz, 2005a). The corpus luteum is totally dependent on hCG levels until the seventh week of pregnancy (Baird et al., 2003) and from then until the 10^{th} week of pregnancy, its function is gradually taken over by the placenta (Speroff and Fritz, 2005a). Since it was concluded that AEA

may be regulated by gonadotrophins in non-pregnant women (Chapter 4), the elevated plasma AEA levels in pregnant women at 4 and 5 weeks gestation may also be related to the elevated LH levels. Since these were not measured in this study further investigation would be essential. By the 6^{th} week of normal pregnancy, LH receptors in the corpus luteum which starts to regress and gradually decline (even though hCG levels remain high) as the placenta gradually become the main source of steroid to support the pregnancy (Speroff and Fritz, 2005a). This is accompanied by a fall in LH levels from this stage of pregnancy. It is therefore logical to extrapolate from this a defined relationship between plasma AEA levels and LH in the non-pregnant women such that plasma AEA levels show a decline at 6 weeks gestation. In fact this was the crucial finding in this study.

The fact that no relationship was found between plasma AEA levels and serum β-hCG, concentrations, a hormone exclusively produced from trophoblast, is in keeping with those of Maccarone et al., suggesting that AEA during the early weeks of pregnancy may be regulated mainly by LH (Maccarrone et al., 2002b). These data are consistent with the proposition that during the follicular phase of the menstrual cycle FSH is the main controlling factor regulating plasma AEA levels, whereas during ovulation, implantation and early pregnancy, AEA is regulated by LH and E2.

6.6. Conclusions

These studies suggest that in a viable pregnancy AEA levels fluctuate from the time of ovulation to early pregnancy, the highest levels being at the time of ovulation and the lowest at 6 weeks gestation. However, the significant decline in the plasma AEA levels (around 40%) at the implantation window prior to implantation appears to be important. Additionally, the changes in plasma AEA levels from oocyte retrieval to embryo replacement (implantation window) could be used to predict the cases in which implantation would be successful in women undergoing IVF/ICSI-ET. Finally plasma AEA is mainly regulated by gonadotrophins and estradiol. Larger studies are, however, essential to confirm these potentially important observations.

Chapter 7

Discussion and future directions

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This thesis was designed to test the hypothesis that the endogenous cannabinoid, anandamide (AEA) is important in human folliculogenesis, ovulation, oocyte maturity and also, in implantation and early pregnancy in women undergoing IVF/ICSI-ET.

It was hypothesised that any effect would be mediated by an alteration in either the systemic or local levels of anandamide, but since local AEA levels could not be measured, systemic levels were used as proxy. Therefore, the first important step of this thesis involved the development and evaluation of an accurate method for AEA measurement in different biometrics using the modern ULPC-MS/MS technology. As multiple blood sampling was required from each volunteer, and repeated sampling was considered to be a problem, potentially limiting the recruitment process, effort was expended into quantifying AEA in urine and saliva in order to replace blood sampling. The data were disappointing in that AEA was undetectable in saliva and urine, which was a surprise because the exocannabinoid Δ^9-THC is readily measured in these fluids (Menkes et al., 1990; Niedbala et al., 2001). The reason for the inability to measure AEA in saliva and urine is unknown, but could be related to the methodology used and as such alternatives may need to be developed. Of course, the reason could also be that AEA is destroyed in the salivary glands and kidney, although there are no data to support the presence of FAAH in either of these tissues. Because AEA was undetectable in saliva and urine, the remaining experiments were performed on plasma and follicular fluid samples. In the initial experiments with follicle fluids there were some follicles that produced less than 2 mL of fluid. Since the normal procedure required 2 mL of fluid for AEA extraction, experiments were performed to investigate the minimum volume of plasma and follicular fluid required to produce a

precise AEA measurement. The data indicated that the minimum amount of fluid that could be processed with accuracy was 1 mL. That meant that the measurement of AEA in cell culture media to determine the precise role of AEA in fertilisation and early embryo development could not be followed.

However, once a reproducible assay for measuring plasma and follicular fluid AEA was established, validation of the assay was achieved by comparing the results of measuring AEA in plasma during the menstrual cycle, in post menopausal women, the first trimester of pregnancy and in the follicular fluid with the published human data. The results of plasma AEA during the menstrual cycle and first trimester of pregnancy were comparable to the published data by Habayeb *et al.*, (Habayeb et al., 2004) and AEA levels in the follicular fluid were comparable to those reported by Schuel *et al.*, (Schuel et al., 2002), suggesting that an appropriate assay was in use. In both menstrual cycle studies, plasma AEA levels were high in the follicular phase and low in the late luteal phase of the menstrual cycle; similarly low levels of AEA were obtained in the first trimester of pregnancy. Data produced by other members of the research group using the same methodology showed that other phases of pregnancy produced comparable results to the published data (Lam et al., 2008). However, the results of plasma AEA levels from post-menopausal women in this study were slightly higher than the published data. The reason for this is unclear, but could be related to the type of women chosen. For example, the women in this study were all White British, and it was clear that ethnicity does play a role in regulating plasma AEA levels (Chapter 6). If the women in the previous study were from different ethnic groups that have lower plasma AEA levels this could explain this discrepancy. The reason was not due to sampling or processing error, because the levels were remarkably similar over a 4 week period (Chapter 4).

A good understanding of the levels of AEA throughout the menstrual cycle and its relationship to the hormones controlling the menstrual cycle were considered crucial for understanding the role of AEA in human fertility and, as there were no published data AEA and these hormones were measured throughout the menstrual cycle. Although Habayeb *et al.*, (Habayeb et al., 2004) had investigated AEA levels at the beginning (early follicular phase) and at the end (late luteal phase) of the cycle, they had not considered the variation in hormones occurring throughout the cycle. It was therefore important to in addition to confirming these pilot findings to do so throughout the phases of the cycle cross-sectionally. It was the basis for dividing the cycle into 5 distinct phases and using postmenopausal women as a negative control. Once this was achieved a longitudinal study was undertaken to further explore the changes and the relationship between AEA and these hormones. To ensure that the study was thoroughly controlled, post menopausal women were examined over the same 4 week period. The important observation from these studies was that there was a fluctuation in plasma AEA levels throughout the cycle, and the highest level was observed at ovulation and lowest at the end of the cycle. These observations suggested that AEA is a hormonally-regulated molecule, a finding in keeping with that of Lazzarin *et al.*, (Lazzarin et al., 2004). The longitudinal part of the study confirmed the results of the cross-sectional study, indicating that the results of previous studies were not due to inter patient variability, but was a real effect possibly due to hormonal influences. Examining the relationship between plasma AEA and the measured hormones indicated that AEA may be hormonally-regulated and this regulation was most likely by either estradiol and gonadotrophins or both. Indeed, Maccarrone and co-worker's had suggested that AEA released from endothelial cells is regulated by estradiol (Maccarrone et al., 2002a) which supports the above results.

An important observation in this part of the thesis was the fact that plasma AEA levels during the menstrual cycle did not correlate with serum progesterone levels. This was contrary to the previous published speculation (Maccarrone et al., 2001; Maccarrone et al., 2003; Lazzarin et al., 2004) in which the activity of fatty acid amide hyrolase (FAAH), the principle enzyme involved in the degradation of AEA, was thought to be under the control of progesterone. None of these reports provided any evidence to support their speculation. The observation here thus questions this speculation and pointed to the need for further studies before definite conclusions can be made.

The fluctuation in plasma AEA levels during the menstrual cycle study, highest at the time of ovulation and the demonstration that follicular fluid AEA was related to size of follicle suggest that AEA may be produced locally in the ovary and could be related to physiological functions of the ovary such as folliculogenesis and ovulation. Previously, Schuel *et al.*, (Schuel et al., 2002) quantified AEA in the follicular fluid in a limited number of follicles and suggested that AEA could be involved in oocyte maturity (Schuel et al., 2002). However, that was the only evidence that AEA is present in the human ovary and there was no evidence that AEA was truly associated with folliculogenesis, ovulation and oocyte maturity. The only other evidence that the endocannabinoid system exists in the human ovary came from a PCR study in which transcripts for CB1 were detected (Galiègue et al., 1995). Therefore, the localisation of the endocannabinoid system in the normal human ovary was undertaken and AEA measured in the plasma and follicular fluid of women undergoing IVF/ICSI-ET. From these studies a series of important observations were made. Firstly, that the endocannabinoid system was localised to the ovary and mainly in the growing follicles and in the corpus luteum and corpus albicans, which suggested that AEA may not only be associated with human folliculogenesis but may also be hormonally regulated. This system was localised in follicles during folliculogenesis which are under

the control of FSH and in the corpus luteum and corpus albicans which are both under control of LH. The additional findings of a positive correlation between follicular fluid AEA levels and oocyte maturity could be clinically relevant. An analysis of the data indicated that a follicular AEA level of 1.09 nM could be used to reasonably discriminate between mature and immature oocytes. The fact that the plasma AEA levels of these IVF/ICSI volunteers, which was taken at least 30 minutes prior to oocyte collection, were similar to the follicular fluid AEA levels measured at the oocyte retrieval and were similar to plasma AEA levels taken on the day of ovulation in the natural cycle, suggested that AEA may be intimately involved in the process of ovulation.

Previous studies had measured plasma AEA levels in the first trimester of pregnancy (Maccarrone et al., 2000b; Maccarrone et al., 2002b; Habayeb et al., 2002) and indicated that a level >2 nM in the first trimester was associated with spontaneous miscarriage (Habayeb et al., 2008b), but there have been no reports on changing plasma AEA levels from ovulation until the end of the first trimester of pregnancy. The only data came from studies that started at 6 weeks of gestation (Maccarrone et al., 2000b; Maccarrone et al., 2002b; Habayeb et al., 2002), as most women would take their first pregnancy test when they have already missed at least 1 week of their expected period (i.e. the earliest reported pregnancy would be 5 to 6 weeks gestation). In natural cycles, the study of the role of AEA from ovulation to the 6^{th} week of pregnancy would be difficult to investigate, hence women undergoing IVF/ICSI-ET were considered ideal to address this question and importantly to explore differences, if any, between those who achieved a pregnancy and those who did not.

The important observation in this part of the study was that during the early stage of pregnancy a significant decline in plasma AEA levels from the day of oocyte retrieval to

the day of embryo transfer (prior transferring embryos) occurred. These data were in keeping with the previous reports that a lower AEA level is required at the implantation window in human (Lazzarin et al., 2004) and that high AEA levels could be embryotoxic as reported in the mouse (Liu et al., 2002) and in sheep (Turco et al., 2008). This study also showed that in viable pregnancies plasma AEA levels significantly increase at 4 and 5 week gestation, and that these levels start to drop significantly from 6 weeks. The women who miscarried had a significantly higher plasma AEA levels than those with viable pregnancies, a finding similar to that of Maccarrone et al., (Maccarrone et al., 2002b) in an IVF cohort. These data suggest that significant changes in female physiology occur during pregnancy that is reflected in plasma AEA levels. The increase at 4 and 5 weeks gestation is probably linked to embryo/trophoblast implantation and outgrowth, since low concentrations of AEA are known to affect trophoblast development (Piomelli, 2004) but also high levels are known to prevent trophoblast growth (Habayeb et al., 2008a). Since the absolute site of AEA production during pregnancy is unknown, it is not possible to explain why some women had much higher levels of plasma AEA and miscarried, but this could be related to a dysfunction in any part of the hypothalamic-pituitary-ovarian-endometrial axis. Nevertheless, by measuring plasma AEA and those of serum sex steroid hormones from the time of ovulation to the viability scan in pregnant women and then comparing those levels in non-pregnant women, a better understanding of the regulation of AEA was made. The data suggested that, AEA is most probably regulated by estradiol and gonadotrophins, and that progesterone may not be associated with the regulation of plasma AEA levels. These data confirmed the menstrual cycle observations and make a compelling argument for AEA not being regulated mainly, if at all, by progesterone.

The effect of ethnicity on plasma AEA concentrations had never been previously reported. Since our population was heterogeneous and there are conflicting reports about the effect of ethnicity on outcomes in ART programs, it was felt important to investigate the effect of ethnicity on plasma AEA levels in these volunteers. Although, the numbers are small, IVF outcomes were not affected by ethnicity as reported (Lashen et al., 1999; James et al., 2002; Bendikson et al., 2005). Interestingly ethnicity affected plasma AEA levels in women from both stimulated and natural cycles. Just how this can be explained is unclear, but it could be related to several factors; some of which may be genetic. This interesting observation needs further investigation.

The novel results of studies presented in this thesis suggest that AEA may be associated with several aspects of female reproduction such as, folliculogenesis, ovulation, oocyte maturity, implantation and early pregnancy success. The potential clinical implications of these findings could be summarised as follows:

1. since plasma AEA levels on the day of ovulation in women in natural and stimulated cycles were similar to those of the follicular AEA levels from ovulatory follicles on the day of oocyte retrieval, plasma AEA levels could be a biomarker for ovulation in women in natural cycles.
2. the fact that AEA is localised to growing follicles and the positive correlation between AEA levels and follicle size indicates that plasma AEA levels could be used in monitoring (tracking) follicular growth in stimulated IVF/ICSI–ET cycles. In addition, follicular fluid AEA levels could be used as a biomarker to assess oocyte maturity, replacing the current non-standardised method used by embryologists, which it is based on subjective morphological assessment of oocytes.
3. in stimulated cycles, measuring plasma AEA levels on the day of embryo transfer prior to transferring the embryos could be used to predict pregnancy outcome at an early

stage. Such measurements would also enable clinicians to decide the best day for transferring the embryos, which subsequently would increase the success rate per treatment cycle.

4. since the plasma levels in women at ovulation and at the implantation window were similar in stimulated and natural cycles and the higher level of plasma AEA at ovulation was followed by a decline on average of at about 40% in the plasma AEA levels at the implantation window for successful implantation, measuring for this change in plasma AEA levels could be used in planning pregnancy.

Future directions

The results of the studies within this thesis have raised many important questions and the following are some suggestions for future research projects:

1. An extension of the study measuring follicular fluid AEA levels to determine oocyte maturity, with the aim of producing an objective biochemical test. A highly specific test such as this to assess oocyte maturity would be useful for embryologists in the assessment of oocytes, as currently there is no objective test available for the assessment of oocytes in the ART practice.
2. Understanding the mechanism of how AEA is affecting oocyte maturity. It would be important to know whether AEA affects nuclear or cytoplasmic maturation. It is anticipated that the results of such studies could be used to improve *in-vitro* maturation of human oocytes, which is an emerging infertility treatment.
3. Since successful pregnancy is also dependent upon a properly receptive endometrium, the localisation of the endocannabinoid system in the human uterus and applying the methodology presented herein to measure AEA in the uterus during the implantation window and similarly in the human embryo (as embryo biopsy is performed for pre-

implantation genetic testing), might help our understanding of how AEA is involved in human implantation.

4. A larger study to confirm and determine the exact levels of plasma AEA levels required during the implantation window, which would allow for an accurate prediction of pregnancy outcomes at an early stage in both natural and stimulated cycles.

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