Localisation and Function of the Endocannabinoid System in the Human Ovary

Mona R. El-Talatini1, Anthony H. Taylor1, Janine C. Elson2, Laurence Brown3, Allan C. Davidson2, Justin C. Konje1*

1Endocannabinoid Research Group, Reproductive Sciences Section, Department of Cancer Studies & Molecular Medicine, University of Leicester, Leicester, United Kingdom, 2Assisted Conception Unit, Leicester Royal Infirmary, University Hospitals of Leicester NHS Trust, Leicester, United Kingdom, 3Department of Pathology, Leicester Royal Infirmary, University Hospitals of Leicester NHS Trust, Leicester, United Kingdom

Abstract

**Background:** Although anandamide (AEA) had been measured in human follicular fluid and is suggested to play a role in ovarian follicle and oocyte maturity, its exact source and role in the human ovary remains unclear.

**Methods and Findings:** Immunohistochemical examination of normal human ovaries indicated that the endocannabinoid system was present and widely expressed in the ovarian medulla and cortex with more intense cannabinoid receptor 2 (CB2) than CB1 immunoreactivity in the granulosa cells of primordial, primary, secondary, tertiary follicles, corpus luteum and corpus albicans. The enzymes, fatty acid amide hydrolase (FAAH) and N-acylphosphatidylethanolamine-phospholipase D (NAPE-PLD), were only found in growing secondary and tertiary follicles and corpora lutea and albicantes. The follicular fluid (FF) AEA concentrations of 260 FF samples, taken from 37 infertile women undergoing controlled ovarian hyperstimulation for in vitro fertilisation and intracytoplasmic sperm injection with embryo transfer, were correlated with ovarian follicle size (P = 0.03). Significantly higher FF AEA concentrations were also observed in mature follicles (1.43 ± 0.04 nM; mean ± SEM) compared to immature follicles (1.26 ± 0.06 nM), P = 0.0142 and from follicles containing morphologically assessed mature oocytes (1.56 ± 0.11 nM) compared to that containing immature oocytes (0.99 ± 0.09 nM), P = 0.0011. ROC analysis indicated that a FF AEA level of 1.09 nM could discriminate between mature and immature oocytes with 72.2% sensitivity and 77.14% specificity, whilst plasma AEA levels and FF AEA levels on oocyte retrieval day were not significantly different (P = 0.23).

**Conclusions:** These data suggest that AEA is produced in the ovary, is under hormonal control and plays a role in folliculogenesis, preovulatory follicle maturation, oocyte maturity and ovulation.

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* E-mail: jck4@le.ac.uk

**Introduction**

Endocannabinoids (endogenous cannabinoids) are unsaturated fatty acid derivatives widely distributed in the human body [1]. Anandamide (AEA), the most studied endocannabinoid, mimics some of the central and peripheral effects of Δ9- tetrahydrocannabinol (Δ9-THC), the psychoactive component of marijuana [2]. AEA, an ethanolamine amide of arachidonic acid, the precursor of prostaglandins and leukotrienes [3] is synthesised on demand through the sequential actions of N-acetyltransferase (NAT) and a molecule-specific enzyme N-acylphosphatidylethanolamine-phospholipase D (NAPE-PLD) [4]. AEA binds to and activates cannabinoid receptors of which there are two main isoforms, cannabinoid receptor 1 (CB1) and CB2 [5]. In the human, CB1 is considered to be the main receptor in the central nervous system [6] and is expressed in many other peripheral tissues such as the adrenal gland, ovaries, uterus, testis, prostate [5], and placenta [7]. In contrast, CB2, which is not expressed in the brain except in conditions of extreme stress, is mainly found in immune-based tissues [8] such as the spleen, tonsils, thymus, bone marrow, B-cells, natural killer cells, monocytes, polymorphic mononuclear cells, neutrophils and T8- and T4-postive cells [5] and more recently in the 1st trimester trophoblast [9]. On binding and internalisation, AEA is degraded to arachidonic acid and ethanolamine by the microsomal enzyme fatty acid amide hydrolase (FAAH) [10]. FAAH and NAPE-PLD are considered to be the main regulators of AEA in target tissues often referred to as ‘anandamide tone’ [11]. The ligands (endocannabinoids), receptors and enzymes as a group constitute the main components of the endocannabinoid system.

Our knowledge about the effect of cannabinoids on the ovary comes from studies in animals [12] and marijuana users [13]. Studies in the in-vitro rat ovary model demonstrated that Δ9-THC exerts a direct inhibitory effect on folliculogenesis [12] and...
ovulation [14] whereas it causes anovulation in rats [15], rabbits and rhesus monkeys [16] as a result of LH surge disruption [17]. The effects of cannabis and THC on the human ovary have been studied and shown to be inconsistent [18] mainly due to the development of tolerance among chronic users [18] and the timing of administration of cannabis in relation to the phases of the menstrual cycle [13]. While in some studies, chronic cannabis smokers appeared to have normal menses after intensive smoking

Figure 1. Immunohistochemical staining for CB1, CB2, FAAH and NAPE-PLD on control tissues. Images on the left side of the panel represent the negative control (IgG/serum) and images on the right side of the panel are sections incubated with specific antibodies/antisera. The tissues used in a–f are human fetal membranes whereas g and h are human endometrium. CB1, CB2 and FAAH Immunoreactivity was observed in the amnion (Am), chorion (Ch) and decidua (Dec) of term fetal membranes, whilst NAPE-PLD immunoreactivity was observed mainly in the endometrial glands (Gl) with sparse staining in endometrial stroma (S). The images are representatives from at least six samples and were taken at 200× magnification. Bar = 50 μm.

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other studies showed increased anovulatory cycles and a short luteal phase. Nevertheless, a direct adverse effect on the ovary were clearly observed as cannabis users were at a higher risk of primary infertility due to anovulation [19], and even when these women had IVF treatment, they produced poor quality oocytes and lower pregnancy rates compared to non-users [20].

AEA has been demonstrated in ovarian follicular fluids at the time of oocyte retrieval in IVF cycles suggesting that it may play a role in ovarian follicle or oocyte maturity [21,22]. However, the source of AEA in the follicular fluid and its possible role within the ovary remains poorly understood. Therefore, our study aimed to localise the endocannabinoid system in the ovary and to investigate whether follicular fluid or plasma AEA levels are related to physiologically important ovarian events such as folliculogenesis, the size and maturity of preovulatory follicle, oocyte maturity, and ovulation.

Materials and Methods

Each volunteer signed an informed written consent prior to entry in to the study which was approved by the Leicestershire and Rutland Research Ethics Committee.

Our study was in 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.

Subjects

For the immunohistochemical studies, 12 ovarian tissue blocks were collected prospectively from women with regular (cycle length 28–32 days) menstrual cycles having a hysterectomy and bilateral salpingo-oophorectomy for benign pathology such as: heavy periods, benign ovarian cyst or prophylactic oophorectomy for family history of ovarian cancer. The woman who had a family history of ovarian cancer was not a carrier of the BRCA1 gene. None of the volunteers had been on any medication for at least one month prior to surgery. The ovaries were confirmed by a gynaecological pathologist 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 [9] and hysterectomy for benign conditions such as myoma or dysfunctional uterine bleeding [23], respectively. All 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 women were healthy and had no other medical disorders, had not used cannabis in the last 10 years and had a basal FSH of ≤10 IU/L in the period prior to starting IVF/ICSI-ET. Eight percent of the volunteers smoked the remainder did not.

Controlled ovarian stimulation protocol, follicular fluid sampling and oocyte retrieval

Ovarian stimulation was performed using a long protocol, with pituitary down-regulation with the gonadotrophin releasing hormone (GnRH) agonist Supercure (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 [24]. 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 [25] once there was no sonographic evidence of 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 [26]. Follicular maturation was assessed by serial (every 2–3 days) transvaginal ultrasound scan and serum estradiol measurements [27], 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 combined with an endometrial thickness of 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). The follicular fluid from each follicle was thereafter collected into Kimble Scintillation vials for AEA measurements.

Measurement of Plasma and follicular fluid AEA concentrations

Blood and follicular fluid samples were processed within two hours of collection. Plasma and follicular fluid AEA concentrations were quantified using the Ultra Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS/MS) method which we previously described [28]. AEA measurements were only made form samples that were at least 1 ml in volume as our extraction method was not valid for lower volumes.

Assessment of ovarian follicle size, oocyte maturity and embryo quality

The follicle size was determined by measuring the volume of the fluid aspirated [29] obtained from individual follicles with Falcon
embryos. Granulosa cells (G), theca cell layers (Th), the oocyte (O) and follicular fluid (ff) demonstrated FAAH immunoreactivity as did lutein
granulosa cells of the corpus luteum (CL) and corpus albicanus (CA), and septa (S). There was a gradation of staining in the granulosa cells of
the tertiary follicle with greatest intensity in the mural cells (panel j). The images are representatives from at least two structures and taken at 50×, 200×
or 400× magnification. Bar = 50 μm.

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Immunolocalisation of NAPE-PLD

NAPE-PLD immunohistochemistry was performed according to the manufacturer's instructions (ABIN110270; Cayman Chemicals, Ann Arbor, MI). Briefly after de-waxing and rehydration to water, endogenous peroxidase activity was blocked by incubation in 3% 
H2O2 in ice-cold water for 15 min and non-specific protein binding sites blocked with 5% normal goat serum in TBS (TBS; 0.5 M Trizma, 1.5 M 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, 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).

Photomicrographs 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 (Axiovision version 4.4, Carl Zeiss Ltd., Welwyn Garden City, Hertfordshire, UK). Images were captured at 50×, 200× or 400× 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). Immunohistochemical staining was subjectively scored as absent (−), present (+), or intense staining (+++).

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 unpaired Student’s 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.
Figure 4. Immunohistochemical staining for NAPE-PLD. Images in the left side of 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 l are images of corpus luteum, and m and n are images of corpus albicans. Granulosa cells (G), theca cell layers (Th), the oocyte (O) and follicular fluid (ff) demonstrated NAPE-PLD immunoreactivity as did lutein-granulosa cells of the corpus luteum (CL) and corpus albicans (CL) and septa (S). The images are representative from at least two structures and taken at 50×, 200×, or 400× magnification. Bar = 50 μm.

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Results

The mean age of the 12 volunteers from whom ovarian sections were obtained was 41.8±2.8 years (range 39–45 years). The indications for removal of the ovaries were meno-haemorrhage (6 women), benign ovarian cyst (5 women) and prophylactic oophorectomy 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 1, 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 2, 3, 4, and Table 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. Immunostaining was observed in the oocytes of these follicles and increased in the granulosa cells of the tertiary follicle and was observed in the corpora lutea and corpora albicantes (Figure 2). Likewise, CB2 (Figure 2o) was expressed in a similar manner as CB1 (Figure 2n) except the CB2 staining was more intense in the ovaries follicles in comparison to CB1 and more intense in the oocytes and granulosa cells in comparison to the theca cells (Figure 2c, 2f, 2i and 2o). However, FAAH immunostaining was not observed in the granulosa cells of the ovary or the oocytes of primordial, primary or secondary follicles but was expressed in the theca cells of secondary and tertiary follicles, and luteinized granulosa cells of the corpus luteum and corpus albicans (Figure 3l and 3n). NAPE-PLD immunostaining was occasionally observed in the oocytes of primordial follicles, but not in the granulosa cells of primordial or primary follicles (Figure 4). 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 4). The intensity of immunostaining of NAPE-PLD in the corpus albicans (Figure 4n) appeared to be of a lower intensity when compared to that of FAAH staining in corpus albicans (Figure 3n).

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/m², respectively. Tables 2 and 3 show the demographic characteristics of these women and the medications used for controlled ovarian hyperstimulation.

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). The measured AEA concentration

Table 1. Immunohistochemistry localisation of the various components of the endocannabinoid system in the human ovary.

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

Table 2. Characteristics of the 37 volunteers undergoing IVF/ICSI-ET.

| Category          | Sub-Category | Mean/ number SD | Range |
|-------------------|--------------|-----------------|-------|
| Age (years)       |              | 33.28 ± 5.08    | 23–42 |
| BMI (Kg/m²)       |              | 24.25 ± 2.68    | 19.5–30|
| Infertility period (years) |       | 3.86 ± 2.45     | 1–14  |
| Type of infertility | Primary       | 26              |       |
| Infertility cause  | Secondary     | 11              |       |
| Treatment         | ICSI         | 20              |       |
| Basal hormones    | ICSI+IVF     | 2               |       |
| Basal FSH (IU/l)  |              | 5.69 ± 1.88     | 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 ovarian syndrome; IVF = in-vitro fertilization; ICSI = intracytoplasmic sperm injection; FSH = follicle stimulating hormone; LH = luteinizing hormone.
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(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).

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 (P = 0.001) higher than that (0.99±0.09 nM) of the immature oocytes (Figure 6a). 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 6b).

After sperm injection and incubation, of the 35 mature oocytes, 21(60%) were fertilised; and 13 of these (61.9%) were good and 8 were 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).

There was no significant difference (P = 0.23) 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. In addition, the mean plasma AEA concentrations on the day of oocyte retrieval were similar (P = 0.27) to the values we previously reported in plasma AEA (1.33±0.16 nM) at the time of ovulation in natural cycles [34].

**Discussion**

The endocannabinoid system in the human ovary has very rarely been investigated, and our recent observations that plasma anandamide concentrations increase at the time of ovulation in the natural menstrual cycle prompted us to look at the ovary in more detail [34]. Although Scheul et al. [21] 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, our 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 our 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. 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 are all likely to respond to AEA, but oocytes may not respond to AEA.

**Table 3.** Details of the ovulation stimulation regimen, number of oocytes collected per cycle and number of embryos on the day of embryo transfer.

| Duration of stimulation (days) | Mean/ number | SD | Median | Range |
|--------------------------------|--------------|----|--------|-------|
|                                | 11.55        | 1.38 | 12     | 9–15  |
| Dose of Puregon (rFSH) IU/L    | 2251.08      | 1478.99 | 2025  | 675–6450 |
| Dose of Menopur (hMG) IU/L     | 3967.50      | 1895.86 | 3600  | 1950–7650 |
| Dose of Puregon IU/L+Menopur IU/L | 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 | 5.09 | 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. 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. doi:10.1371/journal.pone.0004579.g005](https://www.plosone.org/)
studies, if feasible, would be more comprehensive. and that studies with other techniques, e.g., in vitro functional
acknowledged that immunohistochemistry as the only approach
through the actions of the CB2 receptor. However, it is
us to suggest that AEA may be involved in oocyte maturity,
until the last stage of its development. These observations have led
us to suggest that AEA may be involved in oocyte maturity,
through the actions of the CB2 receptor. However, it is
acknowledged that immunohistochemistry as the only approach
investigating the endocannabinoid system in the ovary is limited,
and that studies with other techniques, e.g., in vitro functional
studies, if feasible, would be more comprehensive.

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 [33] and which is under leptin control.

Figure 6. Prediction of oocyte maturity from follicular fluid
AEA measurements. Panel a shows the follicular fluid AEA
concentrations from follicles that gave rise to mature and immature
oocytes during ICSI cycles. The long horizontal bar represents the mean
and the shorter bars the sem. Panel b shows 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).
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N-APE-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 N-APE-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
albicans. 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 [36].

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 [37]. The data presented herein are not at odds
with this observation as Wang et al., did not examine the
presence of the CB2 receptor, although they demonstrated that
CB2 was not involved through the use of a specific CB2
antagonist. These observations therefore highlight a differential
between murine and human ovarian physiology, as has been
previously observed with other markers of ovarian physiology
[38] and to our mind suggest that to really understand human
ovarian function the human not murine ovary needs to be
studied in more detail. Our volunteers from whom ovarian tissue
was obtained were approaching the end of their reproductive age
and therefore it would be interesting to replicate these studies in
younger women, to ensure that the effect of age on ovarian
physiology did not influence our observations.

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. The observed
increase in follicular fluid AEA concentrations with increased
follicle size and lower AEA concentrations in follicles from which
oocytes were not retrieved indicate that AEA is probably
involved in the maturation of follicles or the oocyte. Since
oocyte maturity is currently assessed subjectively by embryolo-
gists there is a need for a more objective method of assessment.

Although many potential biomarkers (estradiol and testosterone
[39], inhibin B [40], BMP-15 [41]) 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. 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 in the local hormonal environment that we do not
know about [42]. It would be ideal to relate the follicular fluid
and plasma AEA levels with oocyte/embryo quality in women
with different causes of infertility; however, the numbers in this
study were too small for such sub-analyses. We believe that
follicular fluid AEA levels should be further investigated as a
possible biomarker for the assessment of oocyte maturity in
advanced reproductive technology procedures. This needs to be
done in conjunction with other biomarkers.

With regards to the embryo quality we did not find a significant
association between follicular fluid AEA concentrations and

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embryo quality. This suggests that several other factors including sperm quality are involved in determining embryo quality. The fact that there was no significant difference between plasma and follicular liquid AEA concentrations at the time of oocyte collection suggests that systemic AEA concentrations reflect those found in women at ovulation in natural cycles [34] which suggest that plasma AEA is involved in the ovulation process whether it occurred naturally or was stimulated.

Conclusion
This study is a step forward in helping with the understanding of the mechanisms of action of AEA in the human ovary. A better understanding of the mechanism whereby AEA is produced in the human ovary and how it affects oocyte maturity, whether nuclear or cytoplasmic maturation, may help in the development of strategies which may improve the outcome of ART. Furthermore, there is a need to undertake larger studies to investigate the potential use of follicular liquid AEA measurement as a predictor of oocyte maturity.

Author Contributions
Conceived and designed the experiments: MRET AHT JCE LB ACD. Wrote the paper: MRET AHT LB JCK. Performed the experiments: MRET AHT JCE LB. Analyzed the data: MRET AHT JCE LB. Contributed reagents/materials/analysis tools: AHT JCE LB ACD. Contributed reagents/materials/analysis tools: MRET AHT JCE LB. Conceived and designed the experiments: MRET AHT JCE LB ACD. Wrote the paper: MRET AHT LB JCK.

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