**human reproduction update**

# Endocannabinoids as biomarkers of human reproduction

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**BACKGROUND:** Infertility is a condition of the reproductive system that affects ∼10–15% of couples attempting to conceive a baby. More than half of all cases of infertility are a result of female conditions, while the remaining cases can be attributed to male factors, or to a combination of both. The search for suitable biomarkers of pregnancy outcome is a challenging issue in human reproduction, aimed at identifying molecules with predictive significance of the reproductive potential of male and female gametes. Among the various candidates, endocannabinoids (eCBs), and in particular anandamide (AEA), represent potential biomarkers of human fertility disturbances. Any perturbation of the balance between synthesis and degradation of eCBs will result in local changes of their tone in human female and male reproductive tracts, which in turn regulates various pathophysiological processes, oocyte and sperm maturation included.

**METHODS:** PubMed and Web of Science databases were searched for papers using relevant keywords like 'biomarker', 'endocannabinoid', 'infertility', 'pregnancy' and 'reproduction'.

**RESULTS:** In this review, we discuss different studies on the measurements of AEA and related eCBs in human reproductive cells, tissues and fluids, where the local contribution of these bioactive lipids could be critical in ensuring normal sperm fertilizing ability and pregnancy.

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**CONCLUSION:** Based on the available data, we suggest that the AEA tone has the potential to be exploited as a novel diagnostic biomarker of infertility, to be used in association with assays of conventional hormones (e.g. progesterone, β-chorionic gonadotrophin) and semen analysis. However further quantitative research of its predictive capacity is required.

**Key words:** biomarkers / endocannabinoids / human reproduction / infertility / pregnancy

# Introduction

The reproductive events needed for pregnancy establishment consist of multiple steps that must successfully occur at exactly the right time and in the right place. To become fully competent for fertilization, oocytes and sperm cells must undergo a series of differentiation and maturation events (Voronina and Wessel, 2003; Toshimori, 2009). On the female side, aberrations in the correct embryo placement along the uterus, decidualization, placentation and intrauterine embryonic growth can result in the onset of pre-eclampsia, miscarriage and/or preterm birth (Cha et al., 2012). On the male side, sperm cells should undergo capacitation and acrosome exocytosis in order to fertilize oocytes correctly (Buffone et al., 2012; Aitken, 2013). Any derangement in this complex sequence of events may affect the competence of sperm cells and their ability to commit to the final steps of fertilization (Barash et al., 2003; Das and Holzer, 2012). Therefore, the discovery of biomarkers that track the correct progression of pregnancy process, or reveal any possible complication including anomalies in female and male gametes, is a clinical imperative to provide an opportunity for timely and appropriate intervention (Achache and Revel, 2006; Polsani et al., 2013; Volk et al., 2013). Endocannabinoids (eCBs) are a new groups of bioactive lipids that act as critical signals in various aspects of male and female human reproduction. The aim of this review is to outline and discuss the way forward in using eCBs as novel biomarkers of reproductive events, which have the potential to become predictive and diagnostic tools of fertility defects, to be exploited for improving pregnancy outcomes and ameliorating the management of fertility problems in humans.

## Current and potential biomarkers in clinical practice

Nowadays, there is an ongoing research to identify and develop novel molecular and/or genetic biomarkers which might improve diagnostic accuracy or help in detecting different diseases (Achache and Revel, 2006; Lédée et al., 2011). Indeed, the current biomarkers used in clinical practice are limited, since most of the available assays do not have the required sensitivity and specificity (Palmer and Barnhart, 2013). Therefore, it is often difficult to translate results obtained at the bench to the bed site, and research efforts frequently end at the initial phases of biomarker discovery, without any validation of preclinically promising targets (Barnhart et al., 2010). In addition, the phenotyping of samples, collection and storage of biomaterials, use of novel assays, and over-interpretation of confounding/chance findings all represent serious limitations for a biomarker to reach the final step of validation (Pepe et al., 2008; Altman et al., 2012; Hardarson et al., 2012). Since clinicians need reliable biomarkers for the diagnosis of reproductive defects, the use of robust assays is required to qualify new molecules upon their identification. Nevertheless, in view of their clinical relevance, the National Institutes of Health defined a serum biomarker as 'a characteristic that

is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention' (Woodcock, 2010). A biomarker in clinical reproductive medicine may be useful to better understand and predict ovarian reserve, gamete quality, embryo viability and euploidy, as well as endometrial receptivity and pregnancy outcome, including miscarriage, ectopic pregnancy and obstetric complications (e.g. pre-eclampsia or preterm labor) (Palmer and Barnhart, 2013). Also in the management of in vitro fertilization (IVF) protocols, specific biomarkers could be used to discriminate between suitable and unsuitable embryos to be transferred. At present, the only biomarker used routinely in clinical practice is human chorionic gonadotrophin (hCG) (Rausch and Barnhart, 2012; Seeber, 2012). Indeed, once a pregnancy is established, the rise of hCG produced by the trophoblasts indicates the viability of the embryo. Additional biomarkers in serum, including progesterone (P), estrogen ($E_2$), alpha-fetoprotein, fetal fibronectin and inhibin A, have been studied and used in clinical practice to track the progress of a normal pregnancy, or to determine complications related to this process (Stovall et al., 1992; Grosskinsky et al., 1993; Seifer et al., 1996; Ness et al., 1998; Krause et al., 2001; Lambert-Messerlian and Canick, 2004; Abdelazim, 2013). The chemical structures of the lipid hormones P and $E_2$ are shown in Fig. 1A. At an early stage and before placental production, P is secreted by the corpus luteum and becomes a critical signal for the establishment of normal pregnancy. Indeed serum P appears to be the single most specific biomarker for distinguishing viable from nonviable pregnancies in early gestation (Cowan et al., 1992) and, in combination with transvaginal ultrasound hCG, it is useful for diagnosis of ectopic pregnancy (Arck et al., 2007). Additionally, $E_2$ levels provide important information on ovarian function and diseases that affect testes, ovaries or adrenal glands (Domingues et al., 2010). Blood $E_2$ levels can also allow clinicians to monitor the progression of pregnancy, efficacy of fertility treatments, or evaluate menopause symptoms. Furthermore, inhibin A and beta-core hCG, the major metabolite of hCG in maternal urine, have been studied as potential biomarkers for determining ectopic versus normal pregnancies (Cole et al., 1994; Seifer et al., 1996). The biochemical profiles of these biomarkers and their involvement in clinical female reproductive practice are reported in Table I. Incidentally, it should be noted that for pre-eclampsia, pre-symptomatic predictive blood-borne potential biomarkers include activin-A (Diesch et al., 2006), C-reactive protein (Mihu et al., 2008), placenta growth factor and its receptor soluble fms-like tyrosine kinase (s-1FLT) (Shokry et al., 2010), leptin (Sucak et al., 2010), transforming growth factor-α1, and plasminogen activator inhibitor (Belo et al., 2002). Yet, these biomarkers have limited clinical exploitation, due to poor sensitivity and specificity of the available methods for their detection. So far, there is no single marker that allows early prediction of pre-eclampsia in women. Moreover, ovarian reserve tests comprise hormonal markers [basal follicle stimulating hormone (FSH), $E_2$, inhibin-B, antimullerian hormone] and ultrasonographic markers (ovarian volume, antral follicle counts), yet they all have
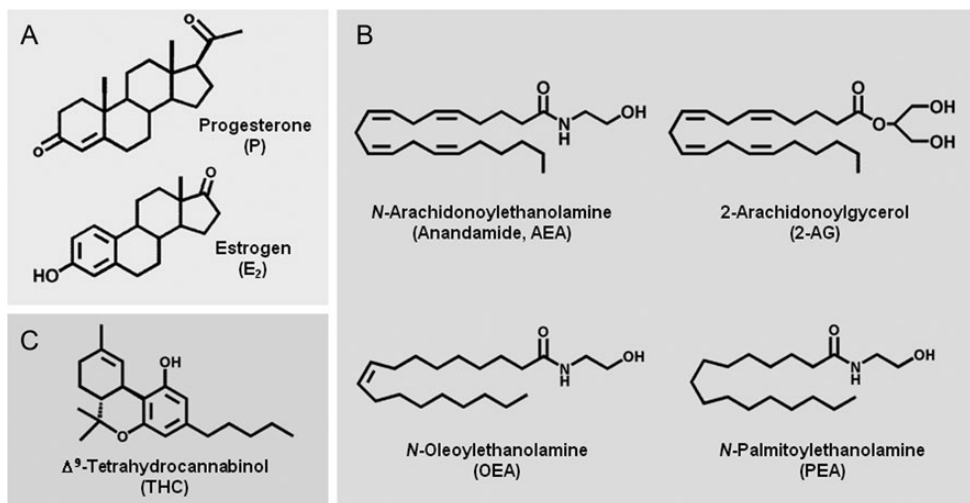
**Figure 1** Chemical structures of current and potential lipid biomarkers of reproduction. (**A**) Steroid hormones; (**B**) eCBs and eCB-like compounds; (**C**) THC. eCB, endocannabinoid.

**Table I** Main serum biomarkers used in clinical female reproductive practice.

| Type | Description | Role |
|------|-------------|------|
| β-Human chorionic gonadotrophin (hCG) | Glycoprotein produced by trophoblastic cells of the placenta | Pregnancy viability |
| Progesterone (P) | Steroid hormone secreted by corpus luteum and placenta for the preparation of endometrium for a possible pregnancy | Pregnancy viability in early gestation Ectopic pregnancy |
| Estrogen (E$_2$) | Sex hormone produced primarily in ovary and testes by aromatization of testosterone. Small amounts are produced by adrenal glands | Pregnancy dynamics Testes, ovaries or adrenal glands diseases Menopause |
| α-Fetoprotein | Oncofetal glycoprotein produced in the yolk sac during the first trimester, and in the fetal liver and gastrointestinal tract during late pregnancy | Abnormal placentation (placenta previa and placenta accreta) Pre-eclampsia Fetal loss and preterm delivery |
| Fetal fibronectin | Glycoprotein produced by fetal cells, found in amniotic fluid and placenta | Spontaneous preterm birth |
| Inhibin A | Peptide produced by corpus luteum and regulated by hCG | Down's syndrome Ectopic pregnancy |

studied in reproductive medicine, or those already in use, are aimed at diagnosing diseases, like infertility, polycystic ovary syndrome, endometriosis or determining the viability of early pregnancy, just to mention a few (Seeber, 2012; Fassbender et al., 2013; Iliodromiti et al., 2013). In this context, the goal has been to identify individual markers of each disease, whereas only a few examples of multivariate diagnostic markers have been reported until now (Nolen and Lokshin, 2013; Palmer and Barnhart, 2013). However, it should be pointed out that the use of two or more biomarkers may indeed provide a diagnostic test with better sensitivity and specificity (Ridker et al., 2003). Combinations of biomarkers have also been shown to support rapid diagnosis of early or ectopic pregnancies, suggesting that a multiple biomarker strategy might help to distinguish viable from non-viable pregnancies (Kuc et al., 2011; Kagan et al., 2012; Daponte et al., 2013; Nicolaides et al., 2013).

On the male side, conventional semen analysis provides important information about sperm concentration, viability, motility and morphology, but is considered a poor indicator of reproductive potential (Guzick et al., 2001; Jequier, 2010). Although fertile men have higher mean sperm parameters (concentration, motility and morphology) than infertile men, there is a large overlap between fertile and infertile subjects (Guzick et al., 2001). Sperm analysis provides useful clues for the initial evaluation of male infertility, but it does not measure fertility itself (Guzick et al., 1998; Jequier, 2010) nor does it take into account functional aspects of sperm cells like the ability to fertilize the oocyte. In addition, since mammalian fertilization and subsequent embryo development depend partially on the inherent integrity of sperm DNA (Ahmadi and Ng, 1999; Agarwal and Said, 2003), different assays have been developed and used in research laboratories to assess sperm DNA damage, which is more clinically informative and relevant (Vasan, 2011). In any case, the results of sperm DNA integrity testing alone do not predict pregnancy rates achieved through natural or artificial conception, as warned by the American Society for Reproductive Medicine (Practice Committee of the American Society for Reproductive Medicine, 2013).

major limitations in accuracy, invasiveness and cost parameters that need to be considered carefully to make reliable predictions (Domingues et al., 2010). It is important to point out that most of the potential biomarkers

Here we will support the view, based on data from the literature from human studies, that bioactive lipids like eCBs might represent a novel class of biomarkers to be exploited in the clinical practice of reproductive medicine.

## Overview of the eCB system in reproductive events

eCBs are non-classical neurotransmitters, released from membrane phospholipids and produced 'on demand' by the cell (Pacher and Kunos, 2013). The best characterized eCBs are *N*-arachidonoylethanolamine, also known as anandamide (AEA), and 2-arachidonoylglycerol (2-AG), both shown in Fig. 1B. These bioactive lipids act by binding to type-1 and type-2 cannabinoid receptors (CB$_1$ and CB$_2$) (Pertwee *et al.*, 2010), to GPR55, a recently discovered putative 'CB$_3$' (Ross, 2009; Gasperi *et al.*, 2013), and to nuclear receptors like the peroxisome proliferator-activated receptors (PPARs) (Pistis and Melis, 2010). AEA behaves also as an endovanilloid, by binding to transient receptor potential vanilloid type 1 (TRPV1) channels (Di Marzo and De Petrocellis, 2010). The 'canonical pathway' for AEA biosynthesis occurs in two steps, of which the first is catalyzed by the *N*-arachidonoylphosphatidyl ethanolamine-specific phospholipase D (NAPE-PLD), a member of the metallo-β-lactamase family that is molecularly distinguished from the known PLD isoforms that hydrolyze common glycerophospholipids (Okamoto *et al.*, 2009). Alternative metabolic routes for AEA biosynthesis include: (i) members of the PLA/acyltransferase family like Ca$^{2+}$-independent *N*-acyltransferases and (ii) multistep pathways via *N*-acylated lysophospholipids (for a recent review see Ueda *et al.*, 2013, and references therein). On the other hand, the PLC−diacylglycerol lipase (DAGL) pathway is the most important route for the biosynthesis of 2-AG (Bisogno *et al.*, 2003; Ueda *et al.*, 2011). Both AEA and 2-AG signaling pathways are terminated by enzymatic hydrolysis, mediated primarily by the serine hydrolases fatty acid amide hydrolase (FAAH, also called FAAH-1) (McKinney and Cravatt, 2005; Fezza *et al.*, 2008) and monoacylglycerol lipase (MAGL) (Dinh *et al.*, 2002), respectively. However, other oxidative enzymes such as cyclooxygenase-2 (COX-2), different lipoxygenase (LOX) isozymes and cytochrome P$_{450}$ add molecular oxygen to AEA or 2-AG, producing prostaglandin-ethanolamides (Kozak *et al.*, 2002) and glyceryl esters (Kozak *et al.*, 2001), or hydroxy-anandamides and hydroxyleicosatetraenoyl-glycerols (van der Stelt *et al.*, 2002), respectively. In addition, despite controversies on the different routes by which eCBs can cross plasma membranes and be shuttled to their intracellular targets or catabolic enzymes (Fowler, 2013), eCB transport across membranes appears to be a protein-mediated process, which occurs through an endocannabinoid membrane transporter (EMT) (Chicca *et al.*, 2012). Fatty acid binding proteins, heat shock protein 70 and albumin (Maccarone *et al.*, 2010, and references therein) might also contribute to AEA uptake. In addition, a partly truncated FAAH-1, termed FAAH-1 like anandamide transporter (FLAT) (Fu *et al.*, 2011), was proposed as an intracellular transporter of AEA, although a recent paper, published during the preparation of this manuscript, demonstrated that this protein might better mediate AEA inactivation (Leung *et al.*, 2013). Taken together eCBs, their receptors, and biosynthetic and catabolic enzymes, as well as putative transporter(s), form the endocannabinoid system (ECS), schematically depicted in Fig. 2. In this context, it should be noted that *in vivo* levels of AEA including those in reproductive organs are regulated by a tight balance between
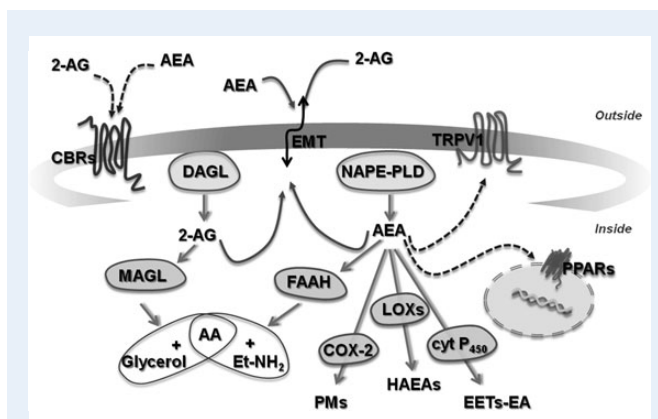


**Figure 2** Biosynthesis, degradation and target receptors of AEA and 2-AG. AEA is mainly synthesized by NAPE-PLD, whereas DAGL is the most important enzyme for the biosynthesis of 2-AG. AEA and 2-AG signaling pathways are terminated by enzymatic hydrolysis, mediated primarily by the serine hydrolases FAAH and MAGL, respectively. The transport of eCBs across the plasma membrane is due to a putative EMT. eCBs exert their biological activity by binding to CBRs, whereas TRPV1 and PPARs are the main intracellular targets for AEA. The latter compound can also undergo oxidation to HAEAs by LOXs, or to PMs by COX-2. Additionally, cyt P$_{450}$ can also oxygenate AEA to generate EETs-EA. Abbreviations: AEA, anandamide; 2-AG, 2-arachidonoylglycerol; NAPE-PLD, *N*-arachidonoylphosphatidylethanolamine-specific phospholipase D; DAGL, diacylglycerol lipase; FAAH; fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; eCB, endocannabinoid; EMT, endocannabinoids membrane transporter; CBRs, cannabinoid receptors; TRPV1, transient receptor potential vanilloid type 1; PPARs, peroxisome proliferator-activated receptors; AA, arachidonic acid; Et-NH$_2$, ethanolamine; HAEAs, hydroperoxy-anandamides; LOXs, lipoxygenases; PMs, prostamides; COX-2, cyclooxygenase-2; cyt P$_{450}$, cytochrome P$_{450}$; EETs-EA, epoxygenated fatty acids-ethanolamide.

biosynthetic and degradative enzymes (Wang *et al.*, 2006a, and references therein). Changes in the activity and/or expression of these enzymes determine significant fluctuations in AEA levels, which in turn can lead to success or failure of pregnancy (Schuel, 2006; Karasu *et al.*, 2011).

The ECS system regulates diverse physiologic processes, and has attracted considerable attention in reproductive pathophysiology for the potential use of its distinct elements for the diagnosis and/or treatment of human infertility (Maccarone, 2009). Indeed, there is accumulating evidence that alterations of some ECS components in the various stages of reproductive events may negatively affect the final outcome (Di Blasio *et al.*, 2013). Previous data have highlighted that AEA metabolizing enzymes, especially FAAH, are fundamental to ensure proper AEA levels, and hence to avoid impairments of fertility signals networks, both on the female (Maccarone *et al.*, 2000; Sun *et al.*, 2009; Trabucco *et al.*, 2009) and male side (Francavilla *et al.*, 2009; Aquila *et al.*, 2010a; Lewis *et al.*, 2012; Amoako *et al.*, 2013). Studies performed on *Cb$_1$* and *Cb$_2$* knock-out mice demonstrate that they suffer from pregnancy loss (Wang *et al.*, 2004; Sun and Dey, 2008), and that CB$_1$ deficiency leads to embryo retention in the oviduct for an extended period, and hence to ectopic pregnancy and reduced fertility (Wang *et al.*, 2004). Complete sequestration of CB$_1$-mediated signaling causes abnormal embryo development (Wang *et al.*, 2004), whereas female mice

lacking $CB_2$ do not present embryos correctly hatched to the implantation sites (Wang et al., 2004). Embryo retention, due to failure in the oviductal-uterine embryo transport, might be ascribed to deficient $CB_1$ expression in maternal oviducts (Wang et al., 2004; Guo et al., 2005; Sun and Dey, 2012), or to high oviductal AEA levels, as reported in *Faah* knock-out mice (Wang et al., 2006b). Similarly, both high and low eCB signaling compromise the placentation process (Sun et al., 2010), as well as parturition (Wang et al., 2008). On the male side, binding of eCBs to cannabinoid and vanilloid receptors plays a key role in controlling spermatogenesis, the acquisition of sperm fertilizing ability (i.e. sperm viability and motility), capacitation and sperm-oocyte fusion (Francavilla et al., 2009). Very recently, hot spots in the regulation of sperm quality by some ECS members have been discussed (Maccarrone, 2013), and involve plasma membrane dynamics, epigenetic control and chromatin remodeling (Chioccarelli et al., 2010; Battista et al., 2012). Additional information in humans reported in the following sections complement comprehensive reviews that have appeared recently in the literature (Bambang et al., 2012; Battista et al., 2012; Chan et al., 2013; Di Blasio et al., 2013).

## Methodologies for detection of eCBs in human reproductive matrices

eCBs have been detected in several human matrices, demonstrating that alterations in their levels might be associated with pathological dysfunctions (Pertwee, 2013). A variety of analytical techniques have been developed for the qualitative detection and quantitative determination of these compounds, as detailed in a recent review (Battista et al., 2014, and references therein). It should be stressed that AEA and 2-AG do not have chromophores or fluorescent moieties in their structures, which could make these molecules easily detectable by gas chromatography (GC) separation and direct UV absorbance detection. In addition, the low eCB content in some non-conventional matrices (i.e. follicular fluids and seminal plasma) requires sensitive, accurate and reproducible methods for proper detection in clinical samples. The search for novel methodological approaches is constantly evolving to overcome the pitfalls due to the matrix effect, extraction yield and low chemical stability of eCBs (i.e. acyl transmigration from 2-AG to 1-AG), which still affect available analytical procedures (Buczynski and Parsons, 2010). Moreover, sample storage and extraction (through solid phase extraction or liquid–liquid extraction methods) represent crucial steps, which might interfere with the analysis and affect the final results. Liquid chromatography (LC) coupled to one mass spectrometry (MS) detector (LC–MS) or two (LC–MS/MS) is largely used for the determination of eCBs in biological samples (Zoerner et al., 2011). Lately, ultra high performance LC (UPLC) has been also used in the form of UPLC–MS/MS, in order to increase the analytical performance (Lam et al., 2008, 2010; Amoako et al., 2010; Fanelli et al., 2012). Taking into account that studies on humans generate data with wide confidence intervals, the use of largely standardized methodologies with improved sensitivity may markedly reduce the variability of results obtained so far by independent investigators.

The quantitative detection of eCB content in human reproductive cells, tissues and fluids through different methodologies is summarized in Table II, and represents an emerging issue because of its potential exploitation for the diagnosis and/or therapy of male and female infertility, as detailed in the next sections.

**Table II** Different methods for eCB detection in human female and male reproductive matrices.

| Matrix | Methodology | References |
|---|---|---|
| Plasma | LC-MS | Maccarrone et al. (2002a) and Habayeb et al. (2004, 2008a) |
| | UPLC-MS/MS | Lam et al. (2008), El-Talatini et al. (2009a, b, 2010), Nallendran et al. (2010), Taylor et al. (2011), Tong et al. (2012) and Gebeh et al. (2013a, b) |
| Fallopian tubes | UPLC-MS/MS | Gebeh et al. (2012) |
| Follicular fluid | LC-MS | Schuel et al. (2002a) |
| | UPLC-MS/MS | El-Talatini et al. (2009b) |
| Amniotic fluid, placenta, fetal membranes | UPLC-MS/MS | Marczylo et al. (2010) |
| Milk | GC-MS | Di Marzo et al. (1998) |
| | LC-MS | Schuel et al. (2002a) |
| | UPLC-MS/MS | Lam et al. (2010) |
| Sperm cells | LC-MS | Francavilla et al. (2009) and Lewis et al. (2012) |
| Seminal plasma | LC-MS | Schuel et al. (2002a), Francavilla et al. (2009) |
| | UPLC-MS/MS | Amoako et al. (2010, 2013) |

LC-MS, liquid chromatography-mass spectrometry; UPLC-MS, ultra-high performance liquid chromatography-mass spectrometry; GC-MS, gas chromatography-mass spectrometry.

# Methods

PubMed and Web of Science databases were searched for papers using relevant keywords, like 'biomarker', 'endocannabinoid', 'infertility', 'pregnancy', 'reproduction' and 'sperm'.

We only included English journal articles published since 1973, based on their widely recognized relevance to the topic. Database research was focused on human and mammalian species.

# eCBs in the female reproductive tract

eCBs play an important role in several female reproductive processes, including folliculogenesis, ovulation and oocyte maturation, as well as implantation and early pregnancy (Lazzarin et al., 2004; Taylor et al., 2007; El-Talatini et al., 2009a, b, 2010; Peralta et al., 2011). In the endometrium, high doses of eCBs alter these latter processes; additionally, they play a role in labor and parturition (Wang et al., 2006a; Maccarrone, 2009; Nallendran et al., 2010; Bari et al., 2011; Xie et al., 2012). On this basis, several independent groups have investigated the presence of eCBs in female reproductive fluids such as blood plasma, breast milk and oviductal fluid, by measuring the levels of AEA, 2-AG and related compounds, like OEA (N-oleoylethanolamine) and PEA (N-palmitoyl ethanolamine) (Schuel et al., 2002a; El-talatini et al., 2009a; Fonseca et al., 2010; Lam et al., 2010; Gebeh et al., 2013a). The chemical structures of the latter two compounds are shown in Fig. 1B. It should be noted that studies on human subjects have been performed in women

undergoing IVF–embryo transfer programs or healthy volunteers with uncomplicated pregnancy, which provide the opportunity to perform eCB measurements only at different stages of the menstrual cycle or of pregnancy.

## eCB levels in blood during pregnancy

It is well known that plasma AEA levels vary throughout the menstrual cycle, with highest levels in the follicular phase compared with luteal phase and with early pregnancy (Maccarrone et al., 2002a; Habayeb et al., 2004). A recent study analyzed plasma AEA concentrations obtained from female volunteers at different stages of the menstrual cycle, or from pregnant women, by using UPLC–MS/MS (Lam et al., 2008). This highly specific and reproducible method allowed an accurate analysis in reduced plasma sample volumes, showing an increase of AEA concentration at the follicular phase ($1.45 \pm 0.81$ nM) compared with the luteal phase ($0.77 \pm 0.30$ nM), in keeping with previous studies (Maccarrone et al., 2002a; Habayeb et al., 2004). Higher AEA contents in plasma at the time of ovulation, and lower concentrations in the luteal phase of the menstrual cycle, as well as at the beginning of implantation, are required to ensure a successful pregnancy (El-Talatini et al., 2009a). On the other hand, low FAAH and high AEA levels in blood are associated with spontaneous miscarriage, as reported in two studies where low FAAH activity was found in lymphocytes of women who spontaneously aborted (Maccarrone et al., 2000) compared with healthy controls. In keeping with these findings, a pilot study limited to a small number of participants reported higher plasma AEA level (~3-fold) in women with threatened miscarriage with respect to the birth group (Habayeb et al., 2008a). Similarly, women undergoing successful IVF/intracytoplasmatic sperm injection (ICSI) show low levels of AEA at the time of implantation, compared with women who fail to become pregnant (El-Talatini et al., 2009a). In particular, a lower AEA level has been demonstrated on the day of embryo transfer, along with a higher level at 4 and 5 weeks of gestation (the time of the pregnancy test), and a drop at 6 weeks (time of the first ultrasound scan) to values similar to those at the pregnancy test (Maccarrone et al., 2000; El-Talatini et al., 2009a; Taylor et al., 2011). Consistent with these studies on human plasma, an analysis of ECS in peripheral blood mononuclear cells during human menstrual cycles showed the highest FAAH activity and the lowest AEA concentrations on Day 21 compared with Day 7 and Day 14, a time point corresponding to the luteal phase and the putative window of uterine receptivity for implantation (Lazzarin et al., 2004). Conversely, serum AEA levels measured in asymptomatic women at 6–10 weeks of gestation have been reported to be the same in those who miscarried and those who did not (Tong et al., 2012). It is should be noted that differences in sample processing (e.g. frozen samples versus fresh samples, and serum versus plasma) may have contributed to these divergent results (Tong et al., 2012).

Moreover, AEA measurements have also been performed during early pregnancy, showing no difference in the first ($0.91 \pm 0.28$ nM) and second ($0.91 \pm 0.30$ nM) trimesters with respect to the levels measured in the luteal phase of the menstrual cycle; instead, AEA levels are significantly elevated (1.48 nM) in women with non-viable first trimester pregnancies compared with the values (1.21 nM) found in confirmed viable pregnancies (Lam et al., 2008; Taylor et al., 2011). In the viable pregnancies, high AEA levels apparently correlate with low levels of P (Maccarrone et al., 2003a, b), and are possibly associated with implantation failure and aberrant development of the feto–maternal interface, culminating overall in miscarriage. Furthermore, AEA levels in women with non-viable first trimester pregnancies are similar to those found in the follicular phase (Lazzarin et al., 2004; El-Talatini et al., 2010; Taylor et al., 2011). No significant association has been reported between AEA and P levels in plasma at the time of implantation or during early pregnancy, or in normally cycling women (El-Talatini et al., 2009a, b). However, a positive correlation between $E_2$ and AEA was found during the menstrual cycle of healthy women and in non-pregnant women after IVF and embryo transfer (El-Talatini et al., 2009a, b, 2010). In addition, an increase in plasma AEA levels has been observed in women during active labor, suggesting a role for this eCB also in the final phase of pregnancy (Habayeb et al., 2004; Lam et al., 2008). In women spontaneously laboring at term, plasma AEA levels were ~4-fold higher than the levels in non-laboring women at term (Habayeb et al., 2004). This latter observation was confirmed by an independent study (Nallendran et al., 2010), where a ~1.5-fold increase in the levels of laboring versus non-laboring subjects was documented. Incidentally, the smaller increase observed in the latter study might be due to a different mechanism of labor (induced versus spontaneous), and to a different method of plasma analysis (Nallendran et al., 2010). In this context, it should be noted that AEA undergoes spatiotemporal distribution specific changes, in order to support pregnancy onset: during the early stage, at the implantation site, low levels are needed to promote uterine receptivity and maintenance of pregnancy, while at the time of labor, high levels of AEA may be useful for parturition, probably because its hydrolysis releases arachidonic acid, which in turn increases the concentration of prostaglandins (Di Marzo and Petrosino, 2007; Mitchell et al., 2008). These fluctuations of AEA content are schematically depicted in Fig. 3. Moreover, as already shown in mouse models, COX-2 might be involved in the regulation of AEA content in humans, in order to guarantee normal implantation and then preservation of pregnancy (Yu et al., 1997; Kozak et al., 2002). In particular, AEA oxygenation by COX-2 at implantation sites would produce prostamides, which can be essential for processes such as ovulation, fertilization, implantation and decidualization, by analogy with classical prostanoids (Lim et al., 1997). Interestingly, as $CB_1$ is expressed in placental villi of women undergoing elective Cesarean section, it can be suggested that maternal AEA may maintain uterine quiescence through this receptor subtype, by producing myometrial relaxant factors like nitric oxide and gonadotrophin-releasing factor (Acone et al., 2009). eCBs seem to be involved also in early pregnancy complications, since higher levels of AEA have been found in Fallopian tubes of pregnant women compared with non-pregnant luteal phase controls (Gebeh et al., 2012). Furthermore, plasma samples obtained from women with ectopic pregnancies exhibit a decreased FAAH activity compared with normally pregnant controls, which leads to higher AEA levels (Gebeh et al., 2013a). Surprisingly, also the plasma concentrations of other eCBs (i.e. OEA and PEA), measured by UPLC-MS/MS, were found to be elevated in ectopic pregnancy and in vitro studies showed that AEA and OEA, but not PEA, have a negative effect on the Fallopian tube cilia beat frequency (Gebeh et al., 2013a). It is generally accepted that OEA and PEA act as 'entourage' compounds, which potentiate the effects of eCBs at their receptor targets by indirectly inhibiting their degradation (Jonsson et al., 2001; Di Marzo and Petrosino, 2007; García Mdel et al., 2009). These data support a role for AEA and related eCBs in modulating tubal function in ectopic pregnancy (Gebeh et al., 2013a), though it cannot be ruled
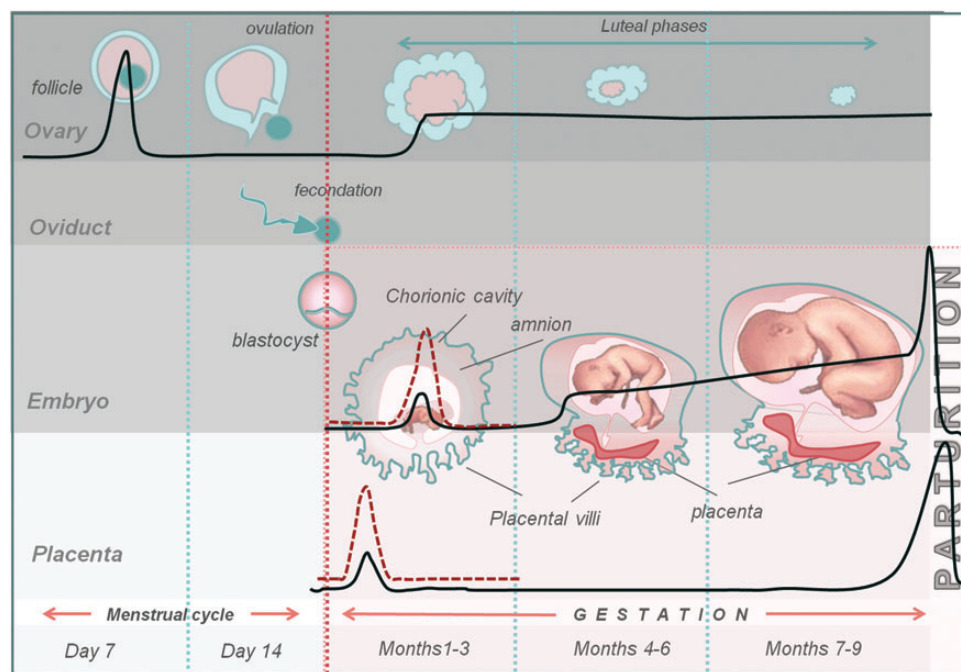
**Figure 3** Fluctuations in AEA levels through the menstrual cycle and gestation. Under physiological conditions (solid line), higher AEA plasma levels in the follicular phase and lower concentrations in the luteal phase of the menstrual cycle, as well as at the beginning of implantation, are required to ensure the successful outcome of pregnancy. During early pregnancy, low levels of AEA, with respect to the levels measured in the luteal phase of the menstrual cycle, are needed to promote uterine receptivity and pregnancy maintenance. Finally, high AEA levels in the placenta at term and at the time of labor may be necessary for parturition. Under pathological conditions (dashed line), AEA levels are increased in the placenta obtained from pre-eclampsia patients, and in ectopic or non-viable pregnancies, as well as in lymphocytes from women who miscarry. AEA, anandamide.

out that high levels of plasma AEA are more generally associated with defects in uterine wall implantation. In this context, it has been clearly documented in mouse models, but not yet in humans, that different ECS elements play a major role in regulating blastocyst development, in order to guarantee successful implantation in the uterus (Paria et al., 1998; Liu et al., 2002; El-Talatini et al., 2009b; Maccarrone, 2009; Sun et al., 2010; Taylor et al., 2010). Indeed, the levels and activity of uterine AEA and FAAH, as well as of blastocyst $CB_1$ receptors and FAAH, are coordinately regulated to synchronize preimplantation development and uterine receptivity (Wang et al., 2006b; Maccarrone, 2009).

## eCB levels in the ovary and follicular fluid

Adverse effects of $\Delta^9$-tetrahydrocannabinol (THC, shown in Fig. 1C), the active principle of cannabis extracts like hashish and marijuana (Izzo et al., 2009), are reported in folliculogenesis and ovulation, where this phytocannabinoid is associated with poor quality oocytes, and eventually anovulation and infertility (Nir et al., 1973; Ayalon et al., 1977). Also AEA, PEA and OEA have been quantified in follicular fluid retained following oocyte aspiration from women undergoing IVF treatment (Schuel et al., 2002a). Recently, AEA measurements have been in the human ovary and follicular fluid, to demonstrate that AEA is produced by the granulosa of growing (i.e. secondary and tertiary) follicles, corpus luteum and corpus albicans, but not by oocytes (El-Talatini et al., 2009b). On this basis, the authors suggested that AEA may play a role during the antral phase of folliculogenesis (El-Talatini et al., 2009b). Interestingly, AEA measured in the follicular fluid obtained

from women undergoing controlled ovarian hyperstimulation for IVF/ ICSI was higher in follicles with mature oocytes than in those with immature oocytes ($1.56 \pm 0.11$ versus $0.99 \pm 0.09$ nM) (El-Talatini et al., 2009b). Yet, receiver operating characteristic analysis, which allows the visualization and analysis of the behaviors of diagnostic systems (Fawcett, 2006), revealed that a concentration of AEA of 1.09 nM in follicular fluid was predictive of mature oocytes in 77% of the cases, supporting the hypothesis that AEA in follicular and oviductal fluids may be involved in oocyte maturity (Schuel et al., 2002a; El-Talatini et al., 2009b). This effect could probably occur through the $CB_2$ receptors, which were indeed localized by immunohistochemical analysis in oocytes of tertiary follicles, but not in those at other stages of development (El-Talatini et al., 2009b). Since other ECS components have been identified in the rat ovarian medulla and cortex, eCB signaling could also be involved in the regulation of follicular maturation and development in humans (Bagavandoss and Grimshaw, 2010).

## eCBs at the maternal–fetal interface

AEA has also been quantified by advanced methodologies in human cord and maternal blood, amniotic fluid, placenta and fetal membranes collected during Cesarean section (Marczylo et al., 2010). Higher AEA levels were found in freshly processed placenta ($2.72 \pm 1.04$ pmol/g of tissue) than in fetal membranes ($1.19 \pm 0.63$ pmol/g of tissue) (Marczylo et al., 2010), where a consistently higher FAAH expression was demonstrated (Park et al., 2003). In this context, it should be noted that AEA concentrations, in the human brain as well as in human

plasma, may increase due to repeated freeze-thaw cycles or delays in tissue processing (Vogeser et al., 2006; Palkovits et al., 2008). Therefore, it is crucial to strictly control the experimental procedures, in order to avoid large fluctuations in AEA levels. In addition, $CB_2$ but not $CB_1$ expression has been found in human placental macrophages during the first trimester (Helliwell et al., 2004). As placental tissues from spontaneously miscarrying women are characterized by very low FAAH levels and high $CB_1$ expression in trophoblastic cells (Acone et al., 2009; Trabucco et al., 2009), increased FAAH expression (Habayeb et al., 2008b) and consequently reduced AEA levels in the placenta in the early stages of pregnancy may be associated with a better pregnancy maintenance (Habayeb et al., 2008b). Therefore, FAAH may have a protective role for the placental barrier between fetal and maternal blood cells, by decreasing circulating maternal AEA (Helliwell et al., 2004; Trabucco et al., 2009). Consequently, the fetus is exposed to changes in AEA levels during its development within the uterus: these levels are low at the beginning, but then become high in the placenta at term. In addition, higher AEA levels in the umbilical vein ($0.88 \pm 0.33$ nM) compared with the artery ($0.77 \pm 0.30$ nM) suggest that there may be a transport across the placenta and/or a biosynthesis of AEA by the placenta itself (Marczylo et al., 2010). Incidentally, it should be noted that conflicting data have been reported on AEA concentrations in amniotic fluid, varying from $0.02-0.18$ nM (Marczylo et al., 2010) to 8 nM (Schuel et al., 2002a). This is likely due to different gestational ages and sample collection procedures between the two studies. AEA seems to be produced also by human placenta, where higher and lower expression of NAPE-PLD and FAAH, respectively, were observed in pre-eclampsia compared with normotensive women, and might possibly contribute to fetus damage (Abán et al., 2013). Also the newborn is exposed to eCBs, and indeed from the day after delivery, the mother produces human milk with 2-AG concentrations ($\sim 1$ µM) that are $\sim 125$-fold higher than those of AEA ($\sim 8$ nM) (Di Marzo et al., 1998). This pioneering study was performed by GC-MS analysis, and was extended later on through more sophisticated methodologies (Table II). LC-MS and UPLC-MS/MS showed that indeed human milk contains different N-acylethanolamines in the nanomolar range: $0.1-5.0$ nM for AEA, $2.0-67.0$ nM for OEA and $9.0-23.0$ nM for PEA (Schuel et al., 2002a; Lam et al., 2010). Collectively, available data suggest a positive role for eCBs in milk intake by the newborn.

Overall, the positive or negative effects of eCBs depend on the site of action and on the duration of exposure, as well as on the spatiotemporal expression patterns of the key synthetic/hydrolytic enzymes and target receptors of these substances. Available literature data reviewed here suggest that AEA levels in plasma of pregnant women might be considered as a diagnostic biomarker for natural or assisted pregnancy outcome. They also suggest that modulating the ECS could potentially be useful as a therapeutic alternative to prevent preterm labor. By using a specific cutoff of AEA levels, also in combination with other widely accepted biomarkers (e.g. P or hCG), diagnostic tests on whole blood could also deliver more reliable information on the risk of miscarriage.

# eCBs in the male reproductive tract

The involvement of eCBs in human male reproductive events was recognized shortly after the identification of these bioactive lipids (Schuel et al.,

2002a). Since then, eCBs have been quantified in seminal plasma and human sperm cells (Francavilla et al., 2009; Marczylo et al., 2009; Amoako et al., 2010, 2013). Much like boar sperm cells, where a complete ECS was demonstrated for the first time (Maccarrone et al., 2005), human sperm cells have been shown to express $CB_1$ (Rossato et al., 2005; Aquila et al., 2009), $CB_2$ (Agirregoitia et al., 2010) and TRPV1, along with all major components of the ECS needed to synthesize (NAPE-PLD) and degrade (EMT and FAAH) AEA (Francavilla et al., 2009). Interestingly, ECS elements are expressed differently in distinct sperm segments: NAPE-PLD and FAAH are localized in the post-acrosomal region and in the midpiece; $CB_1$ is present in the plasma membrane over the acrosomal region, in the middle region and along the tail; $CB_2$ is found in the plasma membrane at the sperm head; and finally TRPV1 is restricted to the post-acrosomal region (Francavilla et al., 2009; Agirregoitia et al., 2010). The location of different ECS components in the human sperm cell is represented in Fig. 4. These immunocytochemistry data support functional and pharmacological studies, which document a different role for $CB_1$, $CB_2$ and TRPV1 receptors in the complex process of fertilization (Maccarrone, 2009, 2013).

## eCBs in the regulation of sperm functions

The binding of AEA to $CB_1$ and/or $CB_2$ receptors seems relevant for the acquisition of sperm fertilizing ability, both in invertebrates (Schuel et al., 1994), vertebrates (Cobellis et al., 2006, 2010; Cacciola et al., 2008; Sun et al., 2010) and humans (Rossato et al., 2005; Agirregoitia et al., 2010). The control of sperm motility, as well as the induction of the acrosome reaction (AR), has been ascribed to $CB_1$ activation (Schuel et al., 2002b; Rossato et al., 2005; Aquila et al., 2010a; Barbonetti et al., 2010), although more recently also a role for $CB_2$ has been documented (Agirregoitia et al., 2010). Indeed, motility analysis on semen samples incubated with specific agonists or antagonists of $CB_1$ and $CB_2$ demonstrated that the distinct activation of these receptors modulates the proportion of motile sperm cells, with $CB_1$ increasing the percentage of
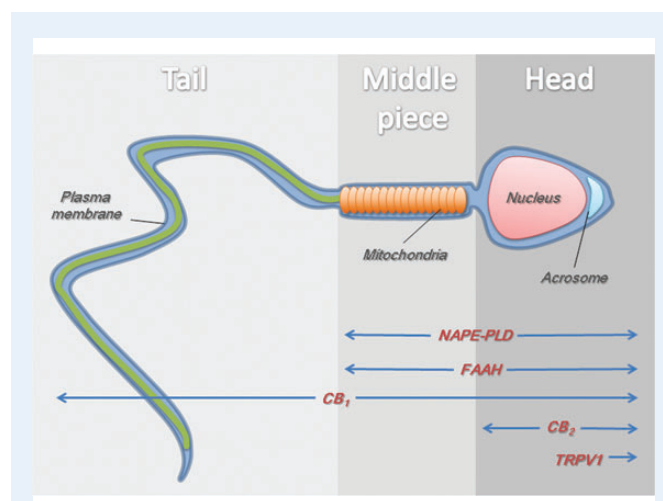


**Figure 4** Schematic drawing of the localization of endocannabinoid system (ECS) elements in the human sperm cell. Abbreviations: NAPE-PLD, N-arachidonoylphosphatidylethanolamine-specific phospholipase D; FAAH; fatty acid amide hydrolase; $CB_{1/2}$, type-1 or 2 cannabinoid receptor; TRPV1, transient receptor potential vanilloid type 1.

immobile cells and reducing the proportion of rapidly progressive sperm cells in favor of slow, sluggish sperm cells (Agirregoitia et al., 2010).

Sperm motility is directly related to mitochondrial function, which in turn is connected to mechanisms of energy depletion, possibly due to deficits in mitochondrial oxidative phosphorylation (Rossato, 2008; Badawy et al., 2009) or to glycolysis blockage (Barbonetti et al., 2010). In this context, it has been suggested that AEA inhibits sperm mitochondrial activity in a dose-dependent manner (Rossato et al., 2005), and that the presence of $CB_1$ in the midpiece of human sperm cells, where mitochondria are localized (Aquila et al., 2010b), affects their motility by decreasing the mitochondrial transmembrane potential in a non-apoptotic manner (Barbonetti et al., 2010). TRPV1 receptors were identified in the testis from rats (Stein et al., 2004), but as yet not from humans. In general, the latter family of channel receptors may be involved in regulating calcium-dependent functions of mammalian sperm cells, including motility, capacitation and AR (Kumar and Shoeb, 2011). They might also be involved in controlling human sperm/oocyte fusion (Francavilla et al., 2009). Indeed, functional studies based on capsazepine, a selective antagonist of TRPV1, demonstrated a marked reduction of the ability of sperm cells to fuse with oocytes in the presence of P, and a reduction of the P-induced AR rate (Francavilla et al., 2009). Interestingly, the responsiveness to P was restored by using a specific EMT inhibitor, which was able at the same time to prevent the effect of capsazepine (Francavilla et al., 2009).

The levels of expression of $CB_1$ and $CB_2$ receptor subtypes and of TRPV1, as well as their distinct compartmentalization in sperm cells, may critically regulate sperm function. Also, the content of eCBs in the male reproductive tract may differentially activate these molecular targets, thus affecting the different steps of fertilization.

## eCBs and other peripheral factors involved in reproduction

Besides the well-known direct effects of eCBs in fertility, ECS elements are also known to interact with sex steroid hormones and cytokines, thus indirectly regulating these complex processes (Karasu et al., 2011; Bambang et al., 2012). Sex steroid hormones (i.e. P, $E_2$, luteinizing hormone, FSH and testosterone) are the main factors involved in fertility; more recently also leptin, an hormone that helps the body to regulate fat accumulation, has been added to the list, although its role remains unclear (Karasu et al., 2011; Ahrens et al., 2013). P up-regulates lymphocyte FAAH activity through the transcription factor, Ikaros, leading to decreased AEA levels (Maccarrone et al., 2001, 2003a, b). An indirect correlation between $E_2$ and eCB tone was also documented in human endothelial cells, where enhanced AEA levels, due to increased NAPE-PLD and decreased FAAH activities, may modulate the cardiovascular and immune systems (Maccarrone et al., 2002b). Crosstalks between the endocrine and immune systems are known to regulate a large number of biological processes, with a basic role in implantation (Sen et al., 2014). Several immune cells (i.e. macrophages and T cells) and signaling/regulatory molecules (i.e. hormones, cytokines and growth factors) play functional roles during the establishment of pregnancy (Karasu et al., 2011). Endometrial and trophoblast cells, as well as peripheral leukocytes and natural killer (NK) cells, represent a source of cytokines that exert a widespread regulation of basic cellular functions, like proliferation and differentiation (Lee et al., 2011).

Type-1 helper (Th1) and type-2 helper (Th2) cytokines derived from peripheral T lymphocytes act with opposite effects on trophoblast growth, depending on the balance and timing of their production. Th1 cytokines are anti-fertility factors that stimulate NK cells and enhance tumor necrosis factor-$\alpha$ (TNF-$\alpha$) secretion from macrophages, whereas Th2 cytokines are pro-fertility factors that act through the suppression of NK cells activity and the stimulation of natural suppressor cells (Piccinni, 2010; Lee et al., 2011; Battista et al., 2012). Leukemia inhibitory factor (LIF), a member of the interleukin (IL)-6 family, plays important roles in the immune and hematopoietic systems, and is also essential for reproduction (Smith et al., 1998; Aghajanova, 2004). A relationship between reduced LIF expression and recurrent miscarriage has been shown in women (Piccinni et al., 1998; Taupin et al., 1999), suggesting that this cytokine is critical for implantation and pregnancy maintenance in humans (Ahima and Flier, 2000; Maccarrone and Wenger, 2005). LIF levels have been investigated as predictive markers of successful embryo implantation, since they decrease in the late luteal phase of the menstrual cycle (Laird et al., 1997; Sharkey et al., 1999), and in secretory phase endometrium of patients with infertility (Wu et al., 2013). Interestingly, eCBs have been demonstrated to increase the production of IL-1, IL-4, IL-6 and IL-10 (Derocq et al., 2000; Kishimoto et al., 2004), and to inhibit the release of TNF-$\alpha$ and interferon-$\gamma$ from human lymphocytes (Klein et al, 2004; Cencioni et al., 2010). Moreover, it is known that high levels of AEA in blood inhibit the release of LIF via $CB_1$, culminating in pregnancy failure (Maccarrone et al., 2001). In this context, it has been demonstrated that IL-4 and IL-10 enhance FAAH activity, which
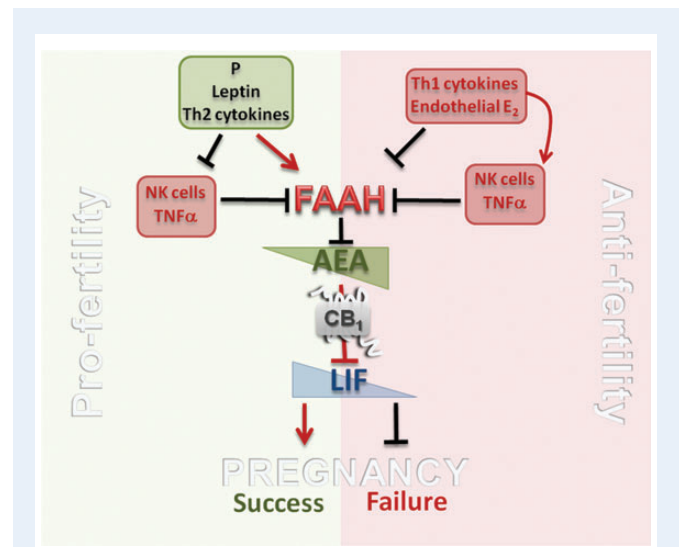


**Figure 5** High levels of LIF and low levels of AEA are essential for successful pregnancy. AEA reduces LIF release via $CB_1$ receptors. P and leptin decrease AEA levels by up-regulating the *FAAH* promoter, and hence FAAH expression and activity. Instead, anti-fertility Th1 cytokines increase AEA levels by inhibiting FAAH, and additionally they potentiate NK cells and TNF$\alpha$ production. Pro-fertility Th2 cytokines potentiate FAAH activation by P, and inhibit NK cells and TNF$\alpha$ secretion. Finally, endothelial $E_2$ increases AEA levels, by reducing FAAH activity. Abbreviations: LIF, leukemia inhibitory factor; AEA, anandamide; $CB_1$, type-1 cannabinoid receptor; P, progesterone; FAAH; fatty acid amide hydrolase; Th, T-helper; NK, natural killer; TNF$\alpha$, tumor necrosis factor $\alpha$; $E_2$, estrogen.

instead is inhibited by IL-2 and IL-12, leading to decreased or increased AEA levels, respectively (Maccarrone and Finazzi-Agrò, 2004). Consistently, defective FAAH in peripheral blood has been proposed as a diagnostic marker of human infertility (Maccarrone et al., 2000). The effects on pregnancy outcome due to cytokines-hormones-AEA networks are summarized in Fig. 5.

In plasma, the leptin content correlates with P levels during the luteal phase of the menstrual cycle, and with hCG concentrations during human pregnancy (Hardie et al., 1997). Recent studies have shown that obese women undergoing IVF treatment have a lower conception rate (Wang et al., 2000; Fedorcsák et al., 2004) and higher risk of miscarriage (Fedorcsák et al., 2000; Wang et al., 2002). In line with this, it has been demonstrated that there is a different regulation of eCBs in maternal fat and placental tissue, depending on maternal obesity. Indeed, in obese mothers increased AEA was observed in the subcutaneous fat, and decreased 2-AG was observed in the placenta, leading to the hypothesis that both eCBs may exert a distinct control during fetal development (Brocato et al., 2013). In addition, AEA levels are also indirectly controlled by leptin, which up-regulates the *FAAH* promoter via signal transducer and activator of transcription 3 (STAT3), and concomitantly

reduces AEA levels in T cells (Maccarrone et al., 2003a). Moreover, alterations of leptin levels have been found in the ovulatory and luteal phases of women of reproductive age (Ajala et al., 2013), overall supporting a link between obesity, eCBs, leptin, sex hormones and fertility (Linne, 2004; Henson and Castracane, 2006; Metwally and Ledger, 2008).

# Clinical relevance of eCBs in female and male fertility

eCBs levels may be altered not only in pregnancy disorders, but also under some pathological conditions that affect the uterus, such as endometritis (Iuvone et al., 2008), and in some types of female reproductive cancers (Guida et al., 2010). Indeed, it has been reported that the levels of 2-AG, but not of AEA, are elevated in biopsies from women affected by endometrial carcinoma, as a result of decreased expression of MAGL (Guida et al., 2010). In parallel, increased 2-AG and up-regulated $CB_2$ receptors counteract endometrial carcinoma growth in human endometrioma (Guida et al., 2010). However plasma eCB levels, measured by

**Table III** ECS elements and AEA as biomarkers of human reproduction.

| Reproductive cells, tissues and fluids | AEA levels | Other ECS elements | Outcome |
|---|---|---|---|
| **Blood** | | | |
| Lymphocytes | | ↓ FAAH | Miscarriage |
| **Plasma** | | | |
| Menstrual cycle | ↑ follicular phase ↓ luteal phase | ↓ FAAH | Uterine receptivity |
| Early pregnancy | ↓ | | Progression of pregnancy and/or embryo transfer |
| Labor | ↓ | | Parturition |
| Early pregnancy complications | ↑ | ↑ OEA, ↑ PEA ↓ FAAH | Ectopic pregnancy |
| **Placenta** | | | |
| Early pregnancy | ↓ | ↑ FAAH ↓ $CB_1$ | Maintenance of pregnancy |
| 'At term' pregnancy | ↑ | ↑ NAPE-PLD, ↓ FAAH ↑ $CB_1$ | Parturition Pre-eclampsia Placental development |
| Follicles (secondary and tertiary) | ↑ | ↑ $CB_2$ | Folliculogenesis Oocytes maturation |
| Follicular fluid | ↑ | ? OEA, ? PEA | Follicles/oocytes maturation |
| Amniotic fluid | ↑ | ? OEA, ? PEA | Supply of oxygenated and nutrient-rich blood to the fetus through the umbilical vein |
| Sperm cells | ↑ | ↓ $CB_1$, ↑ $CB_2$ ↑ TRPV1 ↓ TRPV1 | Acquisition of fertilizing ability Sperm-oocyte fusion Sperm infertility |
| **Seminal plasma** | | | |
| Normozoospermia | ↑ | ? OEA, ? PEA | Regulation of sperm reproductive functions |
| Asthenozoospermia and oligoasthenoteratozoospermia | ↓ | ↓ $CB_1$ | Alteration of physiological sperm cells kinematic parameters |
| Idiopathic infertility | ↓ | | Reduction of sperm ability to penetrate an oocyte |

FAAH, fatty acid amide hydrolase; GEA, N-oleoyethanolamide; PEA, N-palmitoyl ethanolamine; CB1/2, type-1 or 2 cannabinoid receptor; NAPE-PLD, N-arachidonylphosphatidylethanolamine-specific phospholipase D; TRPV1, transient receptor potential vallinoid type 1.

UPLC-MS/MS, were found to be the same in women with hyperemesis gravidarum (HG), a condition whose aetiopathogenesis remains unknown, and normally pregnant subjects (Gebeh *et al.*, 2013b). Although eCBs were reported to modulate emesis (Mechoulam and Parker, 2013), both in humans and in animal models, these findings seem to rule out their involvement in HG.

On the male side, the evaluation of eCB content in human sperm cells and/or in seminal plasma could be a novel diagnostic tool for reproductive medicine. The concentrations of AEA in seminal plasma of normozoospermic men range from ~0.20 nM (Amoako *et al.*, 2010, 2013) to 13–26 nM (Schuel *et al.*, 2002a; Lewis and Maccarrone, 2009; Lewis *et al.*, 2012). In line with this, we have recently reported a marked reduction of AEA (26.4 ± 3.6 versus 7.3 ± 1.2 nM) and 2-AG (218.8 ± 55.4 versus 56.7 ± 14.1 nM) content in infertile seminal plasma, paralleled by increased degradation in infertile versus fertile semen samples (Lewis *et al.*, 2012). However, no significant alterations were found in infertile versus fertile sperm cells (0.9 ± 0.3 versus 0.8 ± 0.1 pmol/mg of protein for AEA; 37.9 ± 9.2 versus 31.3 ± 6.8 pmol/mg of protein for 2-AG). Instead, we observed a marked decrease in TRPV1 binding in infertile versus fertile sperm cells (Lewis *et al.*, 2012), further supporting a major role for this ion channel in sperm function (Maccarrone, 2013). On this basis, we might speculate that the reduction of AEA causes infertile sperm cells to lose their quiescent state, and with that the ability to prevent premature capacitation. This condition could then precipitate a premature AR, reducing the ability of the sperm cell to penetrate an oocyte *in vivo*, as well as in IVF. These findings have been lately strengthened by a clinical study performed on men affected by asthenozoospermia and oligoasthenoteratozoospermia (Amoako *et al.*, 2013). Here, AEA levels in seminal plasma, measured by UPLC-MS, were found to be almost halved in patients with respect to normal subjects (~0.08 nM versus ~0.20 nM). These differences in AEA content in men with different pathological semen subtypes were associated with poor semen quality, such as decreased sperm count and abnormal sperm motility, and also with alterations of $CB_1$ transcription (Amoako *et al.*, 2013). Besides AEA, also PEA might play a role in male infertility. The presence of this compound in male reproductive tract might modulate plasma membrane polarity, with an effect on $Ca^{2+}$ influx during the capacitation process (Ambrosini *et al.*, 2003). Additionally, PEA might affect some kinematic parameters of sperm cells (i.e. their motility), and might act on the development of hyperactivation during capacitation, leading to idiopathic infertility (Ambrosini *et al.*, 2005). Collectively, these data pinpoint eCBs (and in particular AEA) as potential new biomarkers that may be used to evaluate male reproductive defects, thus opening new avenues for the treatment of infertility in humans.

# Conclusions

In this review, we have discussed human studies on the assay of eCB content in female and male reproductive tracts (cells, tissues and fluids), focusing our attention on how endogenous AEA tone might exert its function in regulating different reproductive events, from oocyte and follicle maturation, and normal and pathological pregnancy, to sperm capacitation, motility and fusion with the oocyte. In females, the levels of eCBs have been reported from different stages of follicular development to the gestational period. In males, ECS components were identified in human sperm cells and seminal plasma, but the

endogenous tone of eCBs remains to be addressed at different stages of gametogenesis.

Keeping in mind that fertilization fails in up to 10% of IVF cycles (Lewis *et al.*, 2012), available data might point to AEA and other ECS elements as new candidate biomarkers for infertility, and for devising strategies for fertility preservation under normal and pathological conditions (Table III).

The identification of AEA as a potential biomarker to be exploited to determine pregnancy outcome and sperm fertilizing ability appears to hold some potential for reproductive health. Overall, assessing the content of eCBs as biomarkers in female and male reproductive tracts may be an addition to the assays of conventional hormones (e.g. P and hCG) and to semen analyses. On a final note, assays of FAAH activity/expression and of AEA content in peripheral blood offer the advantage of being routinely performed in easily accessible cells, by means of high throughput techniques like radiochromatography/enzyme-linked immunosorbent assays, and LC-MS/UPLC-MS/MS. Therefore, these analyses have the potential to be useful in predicting impaired sperm fertilizing ability as well as the outcome of natural and/or assisted reproduction in pregnant women and in pregnancy complications. However further quantitative research of their sensitivity and specificity for predicting the various fertility defects is required.

# Authors' roles

All authors conceived the topic of this study, and were involved in the drafting of the manuscript and figures. M.M. revised and approved the final version.

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# Conflict of interest

All authors have no conflicts of interest to declare.

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