

The fundamental role of the endocannabinoid system in endometrium and placenta: implications in pathophysiological aspects of uterine and pregnancy disorders

J. Maia, B.M. Fonseca, N. Teixeira, and G. Correia-da-Silva *

UCIBIO, REQUIMTE, Departamento de Ciências Biológicas, Laboratório de Bioquímica, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal

*Correspondence address. Faculty of Pharmacy, Porto University Biochemistry Lab., Biological Sciences Department Rua Jorge de Viterbo Ferreira 228, 4050-313 Porto, Portugal. E-mail: george@ff.up.pt  <https://orcid.org/0000-0001-5787-9018>

Submitted on October 9, 2019; resubmitted on January 3, 2020; editorial decision on January 28, 2020

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BACKGROUND: The endocannabinoid system (ECS) consists of the cannabinoid receptors CB1 and CB2, the main endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) and their metabolic enzymes *N*-acylphosphatidylethanolamine-specific phospholipase D, fatty acid amide hydrolase, diacylglycerol lipase and monoacylglycerol lipase. This system is involved in the modulation of essential physiological processes. Its role in the reproductive system has become significantly important in recent years, given its major role in events such as gametogenesis, decidualisation, implantation and placentation.

OBJECTIVE AND RATIONALE: In this paper, we review the literature and summarize the role of the ECS elements in reproduction and their potential as early markers for diagnosis of reproductive disorders or as pharmacological targets for treatment.

SEARCH METHODS: Original research and review papers published from 1964 to June 2019 were selected in terms of relevance, reliability and quality by searching PubMed, MEDLINE and Web of Science, using the following search terms: endocannabinoid system and endometriosis; endocannabinoid system and ectopic pregnancy; endocannabinoid system and miscarriage; endocannabinoid system and

pre-eclampsia; endocannabinoid system and endometrial cancer; endocannabinoid system and reproduction; endocannabinoid, endometrium; placenta; *N*-acylethanolamines; anandamide; 2-arachidonoylglycerol; and cannabinoids.

OUTCOMES: This review demonstrates relevant information concerning ECS alterations in endometriosis, ectopic pregnancy, miscarriage, pre-eclampsia and endometrial cancer. We highlight the importance of the endocannabinoids in endometrial and placental physiology and pathophysiology, from studies *in vitro* and *in vivo* and in clinical observations. The most studied of the endogenous cannabinoids is AEA. The levels of AEA were increased in plasma of patients with endometriosis and miscarriage, as well as in the fallopian tube of women with ectopic pregnancy and in endometrial biopsies of endometrial cancer. Changes in the pattern of expression of the cannabinoid receptor CB1 were also observed in endometrial biopsies of endometriosis, fallopian tube and decidua of patients with ectopic pregnancy and pre-eclamptic placenta. Moreover, alterations in CB2 expression have been reported in association with endometrial cancer. In general, studies on the cannabinoid signalling through CB2 and on the biological activities of the other major endocannabinoid, namely 2-AG, as well as its metabolic enzymes are scarce and avidly required.

WIDER IMPLICATIONS: The pathophysiological mechanisms involved in the described endometrial and placental pathologies are still unclear and lack the means for an early diagnosis. Based on current evidence, though alterations in ECS are demonstrated at tissue level, it is difficult to associate plasmatic changes in AEA with specific endometrial and placental diseases. Thus, pairing alterations in AEA levels with 2-AG and/or other endocannabinoid-like molecules may provide more accurate and early diagnoses. In addition, patients may benefit from new therapies that target the ECS and endocannabinoid signalling.

Key words: endocannabinoid system / endocannabinoids / endometrium / placenta / pregnancy / endometriosis / ectopic pregnancy / miscarriage / pre-eclampsia / endometrial cancer

Introduction

Cannabis sativa has been used for centuries for a plethora of purposes. Nowadays, its consumption is growing due to the increasing legalisation for therapeutic applications. Despite its popularity, it was only in 1964 that the main psychoactive compound of *C. sativa*, the cannabinoid Δ^9 -tetrahydrocannabinol (THC), was isolated and characterized (Gaoni and Mechoulam, 1964). Many years later, its molecular targets were discovered. The cannabinoid receptor 1 (CB1) was identified in 1990 (Matsuda *et al.*, 1990) followed by the cannabinoid receptor 2 (CB2) (Munro *et al.*, 1993). Their crystallographic structure has, however, only recently been described (Hua *et al.*, 2016; Shao *et al.*, 2016; Li *et al.*, 2019). Both receptors belong to the seven-transmembrane G protein-coupled receptor superfamily and are expressed throughout the central nervous system (Zou and Kumar, 2018) and in peripheral organ systems such as the immune system (Pandey *et al.*, 2009), the gastrointestinal tract (DiPatrizio, 2016), skin (Rio *et al.*, 2018) and reproductive tissues (Rossato *et al.*, 2008; Walker *et al.*, 2019). The identification of the cannabinoid receptors led to the discovery of the endogenous ligands, termed endocannabinoids (eCBs). The first endocannabinoid to be isolated was the ethanolamide of arachidonic acid, arachidonylethanolamine, also named anandamide (AEA) after 'ananda', which means bliss in Sanskrit (Devane *et al.*, 1992). Shortly after, another derivative of arachidonic acid, 2-arachidonoylglycerol (2-AG), was described (Mechoulam *et al.*, 1995).

The Endocannabinoid System

The endocannabinoid system (ECS) is a lipid signalling system composed of the cannabinoid receptors, the eCBs and their main metabolic enzymes (Fig. 1). AEA is produced from membrane phospholipids by the action of *N*-acetyltransferase followed by *N*-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) (Di Marzo *et al.*, 1994), while 2-AG is synthesized by the enzyme 1,2-diacylglycerol lipase α/β (DAGL) (Sugiura *et al.*, 1995). However,

alternative routes of 2-AG synthesis may exist since it was reported that DAGL^{-/-} mice presented only an 80% reduction of 2-AG levels (Alger and Kim, 2011). Early studies on the eCBs suggested that these were synthesized on demand, meaning that they are not stored but synthesized locally upon need; however, in 2014, it was suggested that the eCBs are stored in intracellular lipid droplets referred to as adiposomes, allowing intracellular accumulation (Maccarrone, 2009; Fezza *et al.*, 2014). The actions of eCBs on their receptors are terminated by degradation through their respective hydrolyzing enzymes. AEA is inactivated by fatty acid amide hydrolase FAAH (Cravatt *et al.*, 1996) mainly located in the intracellular membranes, while the main catabolic route for 2-AG is through monoacylglycerol lipase (MAGL), an enzyme associated with the inner leaflet of plasma membranes (Dinh *et al.*, 2002), and, to a lesser extent, by the recently discovered α/β -hydrolase-6 and -12 (ABHD6 and ABHD12) (Blankman *et al.*, 2007; Chicca *et al.*, 2012). FAAH also hydrolyzes 2-AG although this degradation seems to be minor *in vivo*. Additionally, cyclooxygenase 2 (COX-2) has the ability to metabolize both AEA and 2-AG, generating arachidonic acid (Fowler, 2007). The transport of the eCBs through the plasma membrane is not fully understood. However, being lipophilic, they have the ability to cross the phospholipid bilayer by simple diffusion as well as through the action of the putative endocannabinoid membrane transporter (EMT) (Kaczocha *et al.*, 2009). Furthermore, eCBs may enter the cell by endocytosis (Chicca *et al.*, 2012). However, there remains considerable debate, as the EMT is yet to be characterized (Nicolussi and Gertsch, 2015). In addition to AEA, other ethanolamides including *N*-palmitoylethanolamine (PEA) (Di Marzo *et al.*, 1996) and *N*-oleoylethanolamine (OEA) (Rodriguez de Fonseca *et al.*, 2001) are collectively termed as *N*-acylethanolamines. More recently, other endocannabinoid-related lipids have been discovered, such as virodhamine (Porter *et al.*, 2002) and 2-arachidonoylglycerol ether (Hanus *et al.*, 2001), but their physiological relevance is not fully understood.

In general, activation of both cannabinoid receptors leads to inhibition of adenylyl cyclase, resulting in a decrease in intracellular cAMP (Matsuda *et al.*, 1990; Slipetz *et al.*, 1995). In addition, downstream

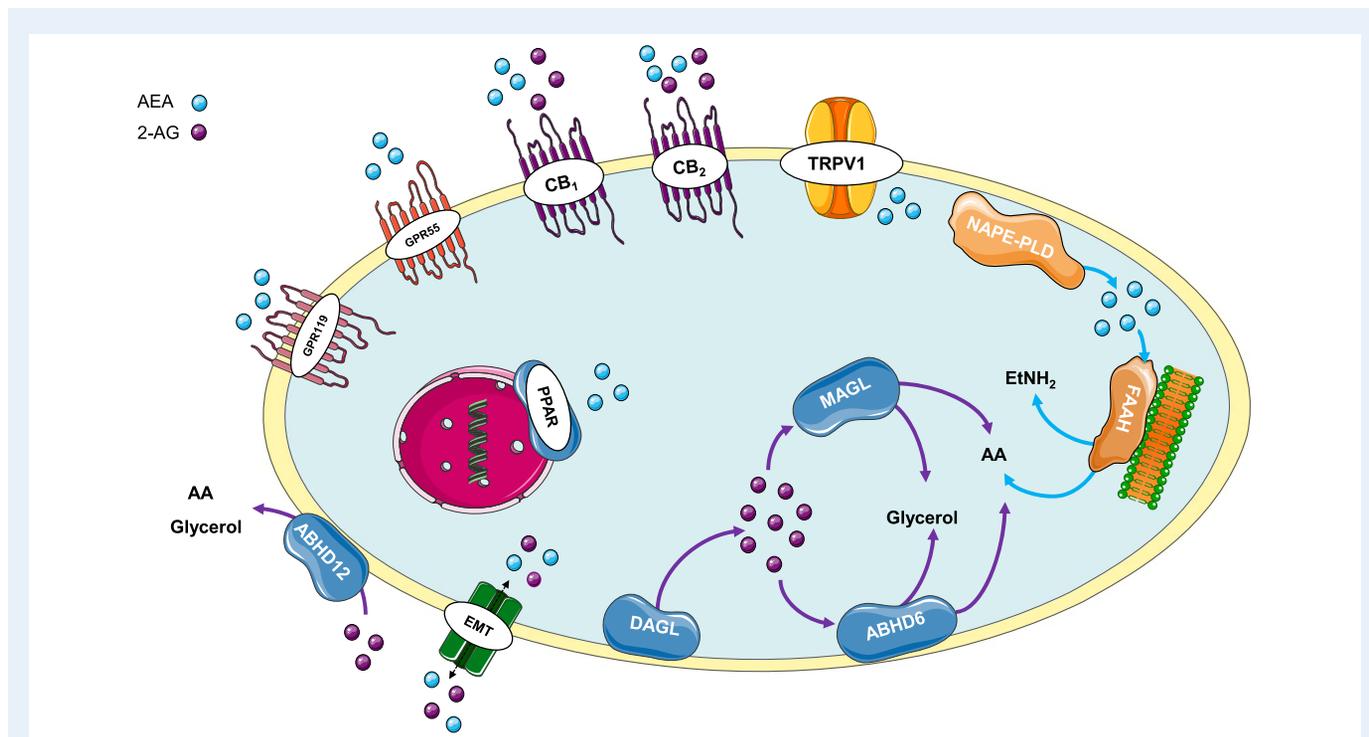


Figure 1 The elements of the endocannabinoid system and related receptors. The endocannabinoid system (ECS) is composed of its two main endocannabinoids AEA and 2-AG, which activate their primary targets CB1 and CB2 as well as their secondary targets GPR55, PPAR and TRPV1, among others. AEA is synthesised from cell membrane lipids by NAPE-PLD and is degraded primarily by FAAH. 2-AG is synthesised also from membrane lipid precursors by the DAGL enzyme and is degraded by MAGL and, also, to a minor extent, by ABHD6 and ABHD12. Besides their proposed ability to move to and from the cell by diffusion, the existence of an endocannabinoid specific transporter, the EMT, has been proposed that eases this bidirectional flow. 2-AG, 2-arachidonoylglycerol; ABHD6, α/β -hydrolase-6; ABHD12, α/β -hydrolase-12, AEA, anandamide; CB1, cannabinoid receptor 1; CB2, cannabinoid receptor 2; DAGL, diacylglycerol lipase; EMT, endocannabinoid membrane transporter; FAAH, fatty acid amide hydrolase; GPR55, G-protein-coupled receptor 55; GPR119, G-protein-coupled receptor 119; MAGL, monoacylglycerol lipase; NAPE-PLD, *N*-acylphosphatidylethanolamine-specific phospholipase D; PPAR, peroxisome proliferator-activated receptors; TRPV1, transient receptor potential vanilloid 1. This image contains some elements adapted from the Servier Medical Art Image Bank (Servier Laboratories (Aust) Pty Ltd) licensed under a Creative Commons Attribution 3.0 Unported Licence.

signalling cascades such as mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinases 1/2 are activated (Bouaboula et al., 1995; Ibsen et al., 2017). Moreover, β -arrestins, Ca^{2+} channels, inward rectifying K^{+} currents and phospholipase C are modulated by cannabinoid receptor activation (Zoratti et al., 2003). Different cannabinoid ligands may activate diverse pathways, leading to diverse responses, a concept termed 'biased signalling' (Ibsen et al., 2017). Furthermore, it has been demonstrated that cannabinoids, the natural or synthetic molecules able to bind the cannabinoid receptors, have the ability to modulate the sphingolipid-metabolizing pathways, by increasing ceramidase activity and leading to higher levels of ceramide, a second messenger that is able to control cell fate (Velasco et al., 2005) (Fig. 2). Besides the classic CB1 and CB2, other receptors can be activated by cannabinoids. Among them are the GPR55 (Ryberg et al., 2007), the transient receptor potential vanilloid 1 (TRPV1) (Huang et al., 2002), the peroxisome proliferator-activated receptors (PPAR) (Sun et al., 2006) and the GPR119 (Syed et al., 2012).

The ECS is able to regulate cell proliferation, differentiation, migration and death. The outcome of these events is dictated by the molecular targets and cell type involved, affecting several physiological systems

and their functions. In the central nervous system, the ECS modulates pain perception, motor functions and cognitive functions (Zou and Kumar, 2018). The ECS is also involved in the control of inflammation (Barrie and Manolios, 2017), energy balance through glucose homeostasis (Tibiriça, 2010), blood pressure and heart rate (Pacher et al., 2005), as well as a variety of functions in the reproductive system (Rossato et al., 2008; Walker et al., 2019). However, it was noticed that alterations in the cannabinoid receptors and eCBs metabolic enzyme expression, as well as in eCBs levels, were associated with diverse pathologies (Di Marzo, 2018). Taking these observations into account, therapies centred on the elements of the ECS started to be developed, mainly agonists/antagonists of CB1 and CB2 or inhibitors of the ECS metabolic enzymes (Di Marzo, 2008). However, eCBs interact with multiple receptors and many chemically related mediators also have the ability to activate these receptors. Therefore, the pharmacological manipulation of the ECS remains a challenge.

Several breakthroughs have been made recently on the association between lifestyle factors, such as dietary habits, light exposure and physical activity, and alterations of the ECS and endocannabinoidome (eCBome), which includes endocannabinoid-related medi-

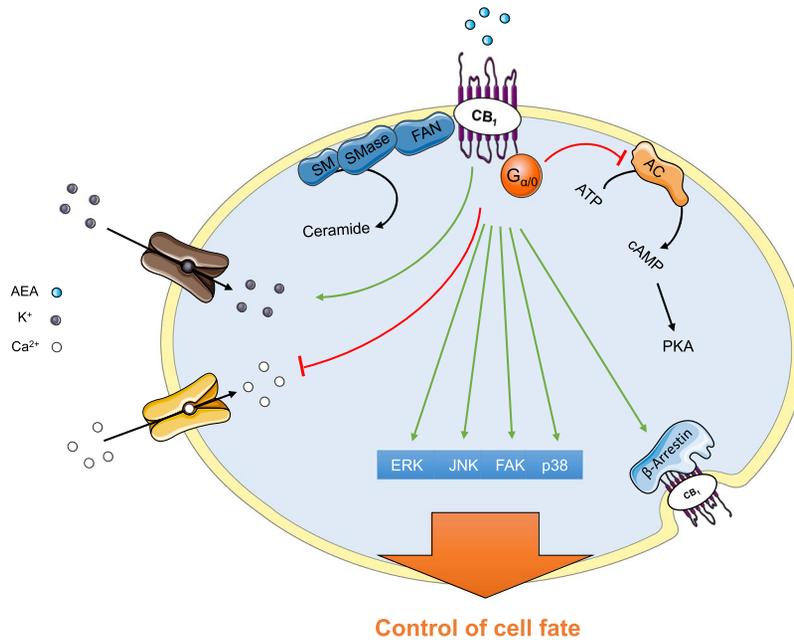


Figure 2 Cannabinoid receptor signalling. Cannabinoids exert their effects by binding to specific G-protein-coupled receptors. The major pathways and channels activated by CB1 receptor are depicted. These include inhibition of the adenylyl cyclase (AC)–cyclic AMP–protein kinase A (PKA) pathway; inhibition of voltage-operated Ca^{2+} channels and activation of K^{+} inwardly rectifying channels; activation of mitogen-activated protein kinase cascades (ERK; JNK, p38 and FAK); ceramide generation and β -arrestin recruitment. This image contains some elements adapted from the Servier Medical Art Image Bank (Servier Laboratories (Aust) Pty Ltd) licensed under a Creative Commons Attribution 3.0 Unported Licence.

ators, their targets and metabolic enzymes. Increasing evidence is now linking the eCBome and the gut microbiome (Di Marzo and Silvestri, 2019). In the gut, it was found that the microbiota and the ECS have a bidirectional relationship, in which an increase in CB1 signalling, paired with obesity and related disorders, may be associated with impaired metabolism through dysbiosis (Di Marzo, 2018). Moreover, CB1 activation was also associated with intestinal dysbiosis caused by altered intestinal permeability (Karwad et al., 2017). In addition, it has been reported that the probiotic *Lactobacillus acidophilus* exerts antinociceptive effects against visceral pain through the increase of CB2 expression in the intestinal epithelial cells (Rousseaux et al., 2007).

In addition to the crosstalk between gut microbiota and ECS elements, dietary habits can also affect homeostasis of the ECS. Obesogenic diets, characterized by high fat content, were shown to lead to increased AEA and or 2-AG levels (Piscitelli et al., 2011). Moreover, this type of maternal diet has an impact on the infant eCBome (Miranda et al., 2018) and induces an increase in adiposity as well as a preference for high fat diets (Dias-Rocha et al., 2018). Furthermore, the eCBome was found to have a role as mediator of the positive effects of spicy food in the decrease of overall mortality by ischemic heart diseases, as well as in the reduction of diabetes. These effects appear to be mediated by the activation of TRPV1 and PPAR α by the active compound of chili peppers, capsaicin (Kang et al., 2010). Sunlight also has an influence on the eCBome, since ultraviolet-B radiation (UVB) has the ability to upregulate CB1 expression and AEA, PEA and 2-AG levels in keratinocytes (Magina et al., 2011). In addition, physical exercise modulates eCBs levels, as AEA and or 2-AG are increased in

active men when compared to sedentary ones (Raichlen et al., 2012; Raichlen et al., 2013). These are examples of the fast-growing roles of the endocannabinoids in several areas of human physiology and how they seem to modulate lipid metabolism by establishing a crosstalk with other lipid mediators.

As addressed previously, eCBs are also oxidized by COX-2 and the resulting endoperoxides are converted by prostaglandin synthases to prostaglandin ethanolamides (prostamides) and prostaglandin glycerol esters (Fig. 3), highlighting the crosstalk between endocannabinoids and prostaglandin metabolic pathways (Rouzer and Marnett, 2011). Both of these families of lipid mediators are very important during several reproductive processes, such as development of the placenta, as well as in endometrium remodelling to receive the implanting blastocyst (Mitchell et al., 2016). It is now clear that the ECS is involved in important reproductive events from oocyte production to parturition. Its relevance has been demonstrated by the tight regulation of eCBs (Maccarrone, 2009) in order to fulfil their functions in the uterus (Guo et al., 2005; Wang et al., 2007; Fonseca et al., 2010a; Fonseca et al., 2010b; Scotchie et al., 2015) and placenta (Kenney et al., 1999; Helliwell et al., 2004; Park et al., 2003; Trabucco et al., 2009), where the presence of the ECS has been reported.

Given the involvement of these cannabinoids in fertility, reproduction and endocrine function (Correa et al., 2016; Walker et al., 2019), in this review we explore alterations of the ECS elements in the reproductive system and associated pathologies, their potential or lack thereof for pharmacological manipulation and how the altered levels of eCBs in these pathologies can be used to monitor disease severity or progression.

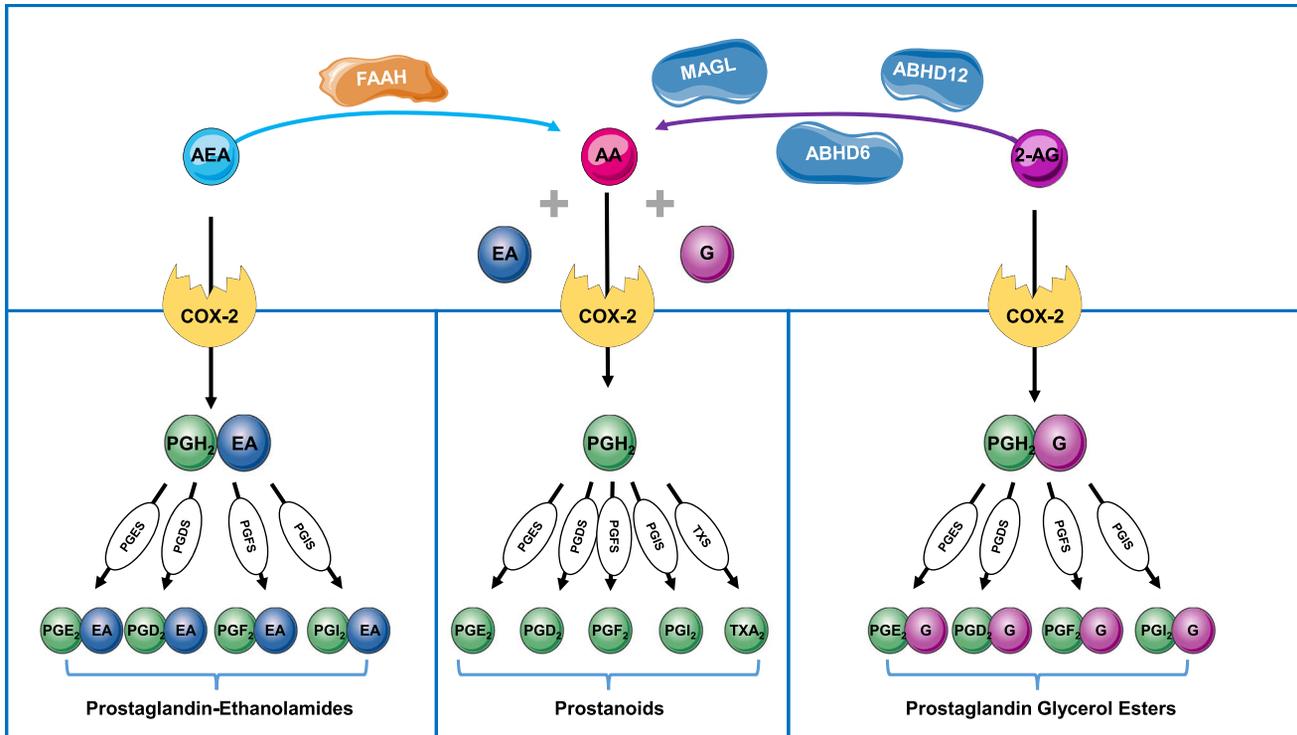


Figure 3 Crosstalk between the endocannabinoid and eicosanoid system pathways. FAAH is the main enzyme responsible for the hydrolysis of AEA into arachidonic acid (AA) and ethanolamine (EA). 2-AG is mainly degraded by MAGL and, to a smaller extent, by ABHD6 and ABHD12 into AA and glycerol (G). AA is metabolised by cyclooxygenase 2 (COX-2) leading to the formation of the endoperoxide PGH₂. Tissue specific metabolism of PGH₂ by PG synthases (PGS) yields the different prostanoids: prostaglandins (PGD₂, PGE₂ and PGF_{2α}), prostacyclin (PGI₂) and thromboxane A₂ (TXA₂). AEA and 2-AG are also metabolised by COX-2 to produce prostaglandins–ethanolamides (or prostamides) and prostaglandin glycerol esters, respectively. However, as yet the molecular targets of these lipid mediators have not been totally elucidated. This image contains some elements adapted from the Servier Medical Art Image Bank (Servier Laboratories (Aust) Pty Ltd) licensed under a Creative Commons Attribution 3.0 Unported Licence.

ECS in the Reproductive Tissues

The majority of ECS elements are expressed in human ovaries (El-Talatini *et al.*, 2009), oviduct (Wang *et al.*, 2006) and uterus (Das *et al.*, 1995; Paria *et al.*, 2001; Scotchie *et al.*, 2015). In fact, the endometrium has the highest levels of *N*-acylethanolamines found in reproductive tissues (Das *et al.*, 1995).

The cannabinoid receptors, alongside AEA and 2-AG metabolic enzymes, have also been characterized in uterine tissues throughout the menstrual cycle (Taylor *et al.*, 2010; Scotchie *et al.*, 2015). The expression of the metabolic enzymes and of the cannabinoid receptors was assessed by western blot, immunohistochemistry and real-time PCR, showing that these elements undergo changes according to the fluctuations in steroid hormones (Ribeiro *et al.*, 2009; Scotchie *et al.*, 2015; Maia *et al.*, 2017). These variations consequently lead to changes in the eCB levels throughout the menstrual cycle, with an increase in AEA levels in the ovary upon ovulation (El-Talatini *et al.*, 2009) and higher plasma levels in the follicular phase when compared to those in the luteal phase (Habayeb *et al.*, 2004). Curiously, while in the ovarium high intrafollicular levels of AEA must be achieved, uterine and plasma levels must be low for the successful implantation of a fertilized oocyte (Maccarrone, 2009). Furthermore, CBI expression is higher in the

glandular epithelium than in stroma and is under the regulation of progesterone (Resuehr *et al.*, 2012).

ECS in the endometrium

During the menstrual cycle, the endometrial stromal cells proliferate and differentiate giving rise to decidual cells in a process called decidualisation (Okada *et al.*, 2018). This specialized tissue possesses the two main cannabinoid receptors and AEA metabolic enzymes (Habayeb *et al.*, 2004; Almada *et al.*, 2016a). The process of decidualisation can be disrupted by high levels of AEA, which inhibit endometrial proliferation through deregulation of the cell cycle, as well as by impaired differentiation by activation of CBI receptor (Almada *et al.*, 2016a). In addition, our group recently demonstrated that the negative impact of AEA in decidualisation seems to be caused by a disruption of oestrogen signalling due to the inhibition of aromatase, an enzyme responsible for a key step in the biosynthesis of oestrogens, and to a decrease in aromatase and oestrogen receptor transcripts (Almada *et al.*, 2019). Furthermore, it was shown that WIN 55212-2, a CBI agonist, decreases the transcription of specific decidualisation markers in human decidual cells, while a CBI antagonist, AM251, induces the opposite effect (Moghadam *et al.*, 2005). Moreover, AEA is important

during decidualisation since AEA levels are lower in decidual cells when compared to non-differentiated cells (Almada *et al.*, 2016b). In addition, miscarriage decidua and uterine natural killer cell-conditioned medium from miscarriage samples exhibit high AEA levels, which, in turn, interfere with the decidualisation process (Fonseca *et al.*, 2019).

Rat models have provided evidence of the importance of CBI in this process, due to its increased expression during the peak of decidua development (Fonseca *et al.*, 2009). Additionally, AEA induced apoptosis of rat decidual cells through a mechanism that involves ceramide synthesis and p38 MAPK activation, compromising the decidualisation process (Fonseca *et al.*, 2013). Moreover, in primary endometrial stromal cells isolated from pregnant rat uterus and stimulated for decidualisation, AEA treatment markedly diminished the differentiation programme and the administration of AEA in the uterine lumen of pseudopregnant rats prevented the decidualisation process (Fonseca *et al.*, 2015). Several studies in rats have shown that throughout pregnancy there is an AEA gradient in the uterus. This gradient seems to be directly involved in the implantation process and embryo development (Paria *et al.*, 1995; Guo *et al.*, 2005), while promoting trophoblast differentiation and outgrowth (Das *et al.*, 1995). Once the maximum development of decidua is reached, it undergoes regression by apoptosis (Gu *et al.*, 1994; Dai *et al.*, 2000). This phase is associated with the development of the placenta, which requires the genesis of different trophoblast types.

Globally, these studies and observations show the importance of a tight control of eCB levels by the enzymes of synthesis and degradation in order to achieve a successful decidualisation process and blastocyst implantation.

ECS in the placenta

The importance of AEA levels in endometrial turnover and the implications for the success of implantation is well described, but the role of the ECS during gestation, namely in placentation, is still obscure. Throughout pregnancy, plasma AEA levels decrease from the first to the second and third trimester, with no changes between the second and third trimesters. At term, plasma AEA levels dramatically increase during labour (Habayeb *et al.*, 2004). In addition to these evident fluctuations in plasma AEA, local endocannabinoid signalling may also modulate critical processes of cellular turnover during placental development.

Proliferation and differentiation of the trophoblasts is required for normal placentation and physiological functions. Among the most important trophoblast cell types are cytotrophoblast (CT) and syncytiotrophoblast (ST). The CT proliferates and differentiates into extravillous trophoblasts and ST. ST are responsible for the endocrine function and constitute the physical barrier between maternal and foetal blood, where nutrient and gas exchange take place (Kidima, 2015). Therefore, there is a need for tight regulation between proliferation, differentiation and apoptosis among these cells for normal placental development to occur (Hemberger *et al.*, 2019; Knöfler *et al.*, 2019). The cannabinoid receptors, the main eCBs and their metabolic enzymes have been reported in the CT and ST of the first trimester and term placenta and in the trophoblastic type BeWo cell line (Kenney *et al.*, 1999; Park *et al.*, 2003; Helliwell *et al.*, 2004; Habayeb *et al.*, 2008; Trabucco *et al.*, 2009; Marczylo *et al.*, 2010). However, 2-AG levels were only measured in baboon placenta (Brocato *et al.*, 2013) (Table I).

In rat placentation, the ECS has been found as early as the 10th day of gestation in the trophoblastic cells of the ectoplacental cone and on the 14th day on the spongiotrophoblast cells (Sun *et al.*, 2010). Not only is the ECS present in placental tissues, but also its importance in placental development has been demonstrated in several studies with knockout mouse models. In CBI^{-/-} mice, trophoblast cells present reduced proliferation and the placenta has a lower weight when compared with wild-type mice (Sun *et al.*, 2010). A lower trophoblast proliferation was also observed in FAAH^{-/-} animals, which suggests the involvement of ECS in trophoblast proliferation and differentiation (Sun and Dey, 2012).

In cell line models, it has been demonstrated that AEA prevents BeWo cell proliferation (Habayeb *et al.*, 2008) and induces apoptosis (Costa *et al.*, 2015a) and that 2-AG also causes cell death by a CB2-dependent mechanism (Costa *et al.*, 2014). In addition, high levels of both AEA and 2-AG impair the synthesis of ST-related proteins as well as the endocrine function of primary ST (Costa *et al.*, 2015a; Costa *et al.*, 2015b). Once again, this highlights the role of the eCBs, this time in trophoblast turnover, reinforcing the importance of cannabinoid signalling in the fundamental cellular processes for placentation. Over the past few years, growing evidence proposes the ECS as a part of the relevant mechanisms that regulate the complexity of events that occur in the placenta. Nevertheless, further studies are needed to investigate the role of the ECS in the occurrence of placental-mediated complications in pregnancy.

ECS in Placental and Endometrial Disorders

The presence of the ECS in the female reproductive tissues and its strict regulation suggest that changes in the homeostasis of its key components may be associated with several pathologies such as endometriosis, miscarriage, ectopic pregnancy, pre-eclampsia and endometrial cancer (Table II) (Fig. 4).

Endometriosis and adenomyosis

Endometriosis is defined by the presence of endometrial glands or stroma at ectopic sites, such as the peritoneum and ovary, causing a chronic inflammatory reaction (Farquhar, 2007). Among the subtypes of endometriosis, there is one characterized by the migration of endometrial cells into the myometrium, named adenomyosis.

Endometriosis is one of the most common gynaecological disorders, with a prevalence of 2–10% among women worldwide (Horton *et al.*, 2019). However, the prevalence may be higher in women of reproductive age, causing infertility (Meuleman *et al.*, 2009). The great majority of patients present with pain, which is one of the major clinical features and is often associated with psychological distress and fatigue (Gibbons, 2004). In addition, in more than 95% of the cases, patients with deep infiltrating endometriosis are prone to severe pain (Bouaziz *et al.*, 2017). There is currently no cure, and treatments are mainly intended to reduce endometriosis-associated pain and improve fertility (Dunselman *et al.*, 2014).

Alterations in the ECS have been reported in endometriosis; however, little is known about its potential role in disease establishment and progression. Nevertheless, this is a topic that deserves further

Table 1 Endocannabinoid system elements described in the uterus and placenta.

ECS element	Model	Study
AEA	Term placenta	(Marczylo et al., 2010)
2-AG	Baboon placenta	(Brocato et al., 2013)
CBI	Uterus	(Taylor et al., 2010)
	Term placenta	(Kenney et al., 1999)
	BeWo cells	(Kenney et al., 1999)
	Ist Trimester placenta	(Helliwell et al., 2004)
CB2	Amniotic epithelial cells	(Park et al., 2003)
	Uterus	(Taylor et al., 2010)
	Term placenta	(Kenney et al., 1999)
	BeWo cells	(Kenney et al., 1999)
NAPE-PLD	Ist Trimester placenta	(Helliwell et al., 2004)
	Uterus	(Taylor et al., 2010)
	Term placenta	(Aban et al., 2013)
FAAH	Ist Trimester placenta	(Trabucco et al., 2009)
	Uterus	(Taylor et al., 2010)
	Term placenta	(Park et al., 2003)
	Ist Trimester placenta	(Helliwell et al., 2004)
DAGLα	Amniotic cells	(Park et al., 2003)
	Decidua	(Park et al., 2003)
MAGL	BeWo cells	(Costa et al., 2014)
	Term placenta	(Costa et al., 2014)

2-AG, 2-arachidonoylglycerol; AEA, anandamide; CBI, cannabinoid receptor 1; CB2, cannabinoid receptor 2; DAGL, diacylglycerol lipase; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; NAPE-PLD, N-acylphosphatidylethanolamine-specific phospholipase D.

investigation since endocannabinoids modulate inflammation, cell proliferation and cell survival, as well as cell migration, all of which are processes that are critical to endometriosis (Sanchez et al., 2012). When it comes to cell migration, this condition is characterized by a higher endometrial expression of matrix metalloproteinase-2 (MMP-2) and lower expression of tissue inhibitor of MMP (TIMP)-2 and TIMP-3, compared with the endometrium of women without endometriosis (Chung et al., 2002). It is known that in cancer cells the cannabinoids have the ability to reduce their migratory effects, as shown by the inhibition of metastatic nodule formation in a Lewis lung carcinoma model (Portella et al., 2003), and block the migration of human colon carcinoma cells SW480 through a CBI receptor-dependent mechanism (Joseph et al., 2004; Laezza et al., 2008). Moreover, the migration of endometrial cells is dependent on CBI receptor activation (Gentilini et al., 2010).

Inflammation is one of the hallmarks of endometriosis. In experiments performed in mouse models of endometriosis, a specific subset of macrophages in the peritoneal fluid and macrophages in the ectopic lesions are involved in the angiogenic process (Capobianco et al., 2011). Moreover, several cytokines and growth factors are increased in the

peripheral blood and peritoneal fluid of patients with endometriosis (Pizzo et al., 2002). It is well described that the CBI and CB2 receptors are expressed in human and mouse immune cells, CB2 being present at higher levels (Parolaro, 1999; Lee et al., 2001), though the cannabinoids have the ability to modulate the immune response by mechanisms that are dependent or independent of CB signalling. In fact, the phytocannabinoid THC has the ability to reduce the chemotactic response of peritoneal macrophages to early inflammatory response (Raborn et al., 2008), while Rimobant, a CBI receptor inverse agonist, also inhibits the inflammatory response in human umbilical vein endothelial cells (Huang et al., 2010).

Given the importance of the ECS in the cellular processes that characterize endometriosis, we reviewed the recent findings regarding an association between this condition and alterations in the ECS elements. Using a rat model of endometriosis, Dmitrieva et al. (2010) demonstrated that CBI receptor is expressed in the somata and fibres of the sensory and sympathetic neurons that innervate endometriotic lesions. This same study showed that CBI agonists decrease endometriosis associated hyperalgesia, whereas antagonists increase it (Dmitrieva et al., 2010). Moreover, in women with endometriosis, there occurs

Table II Summary of main findings regarding the alterations in the endocannabinoid system elements in endometrial and placental diseases.

Pathology	Altered ECS	Tissue	Study
Endometriosis	AEA	↑Plasma	(Sanchez <i>et al.</i> , 2016)
	2-AG	↑Plasma	(Sanchez <i>et al.</i> , 2016)
	CBI	↓Endometrium	(Sanchez <i>et al.</i> , 2016)
		↑Myometrium	(Shen <i>et al.</i> , 2019)
CB2	↑Myometrium	(Shen <i>et al.</i> , 2019)	
Ectopic pregnancy	AEA	↑Fallopian tubes	(Horne <i>et al.</i> , 2008)
	OEA	↑Plasma	(Gebeh <i>et al.</i> , 2013)
		↑Plasma	(Gebeh <i>et al.</i> , 2013)
	PEA	↑Plasma	(Gebeh <i>et al.</i> , 2013)
	CBI	↓Decidua and Fallopian tubes	(Horne <i>et al.</i> , 2008)
	FAAH	↓Fallopian tubes	(Gebeh <i>et al.</i> , 2012)
↓Plasma		(Gebeh <i>et al.</i> , 2013)	
Miscarriage	AEA	↑Plasma	(Maccarrone <i>et al.</i> , 2002)
	CBI	↑Uterine NK cells	(Fonseca <i>et al.</i> , 2019)
		↑1st Trimester placenta	(Trabucco <i>et al.</i> , 2009)
	NAPE-PLD	↑1st Trimester placenta	(Trabucco <i>et al.</i> , 2009)
	FAAH	↓Peripheral lymphocytes	(Maccarrone <i>et al.</i> , 2000)
		↓1st Trimester placenta	(Trabucco <i>et al.</i> , 2009)
Pre-eclampsia	AEA	↓Plasma	(Molvarec <i>et al.</i> , 2015)
	CBI	↑Placenta	(Fugedi <i>et al.</i> , 2014)
	NAPE-PLD	↑Placenta	(Fugedi <i>et al.</i> , 2014)
		↓Placenta	(Aban <i>et al.</i> , 2013)
	FAAH	↓Placenta	(Fugedi <i>et al.</i> , 2014)
		↑Placenta	(Aban <i>et al.</i> , 2013)
Endometrial cancer	AEA	↑Endometrium	(Ayakannu <i>et al.</i> , 2019)
		↑Endometrium	(Schmid <i>et al.</i> , 2002)
		↑Plasma	(Ayakannu <i>et al.</i> , 2014)
	2-AG	↑Endometrium	(Guida <i>et al.</i> , 2010)
	CBI	↓Endometrium	(Ayakannu <i>et al.</i> , 2014)
	CB2	↑Endometrium	(Guida <i>et al.</i> , 2010)
		↓Endometrium	(Ayakannu <i>et al.</i> , 2014)
MAGL	↓Endometrium	(Guida <i>et al.</i> , 2010)	

NK, natural killer; OEA, *N*-oleoylethanolamine; PEA, *N*-palmitoylethanolamine.

a significant increase in plasmatic AEA and 2-AG levels together with a decrease in CBI expression in endometrial biopsies, when compared with a control group in the secretory phase. This suggests a tissue downregulation of the cannabinoid signalling (Table II) (Sanchez *et al.*, 2016). A similar conclusion regarding the decrease in CBI receptor was also obtained in a study with human endometrial biopsies but this time in all phases of the cycle, not only in the secretory phase (Resuehr *et al.*, 2012). On the other hand, CBI and CB2 mRNA and protein

levels are significantly higher in junctional zone and outer myometrium tissue samples from patients with adenomyosis than from women without adenomyosis. Also, CBI levels are directly correlated with dysmenorrhea severity (Shen *et al.*, 2019). Nonetheless, the involvement of the ECS in this condition may also be a way to relieve pain, since deep-infiltrating endometriotic nodules implanted in nude mice treated with the synthetic cannabinoid WIN 55212-2, a CBI agonist, led to a reduction in nodule size. In addition, WIN 55212-2 also exerted

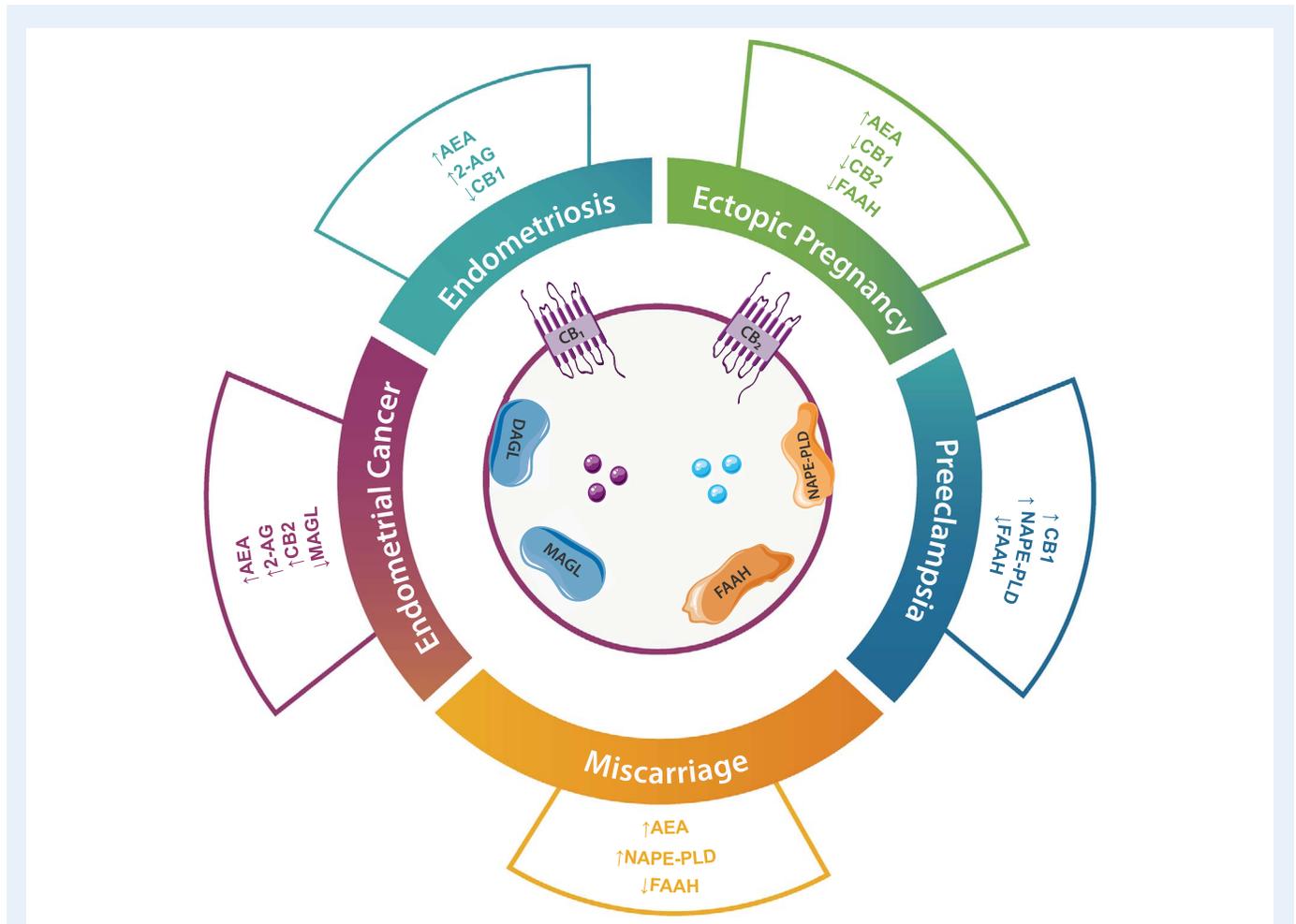


Figure 4 Alterations in the ECS elements in endometrial and placental diseases. Main findings regarding alterations in members of the ECS in endometriosis, miscarriage, ectopic pregnancy and endometrial cancer. This image contains some elements adapted from the Servier Medical Art Image Bank (Servier Laboratories (Aust) Pty Ltd) licensed under a Creative Commons Attribution 3.0 Unported Licence.

in vitro antiproliferative effects on human endometriotic cells, coupled with an inhibition of the AKT (or protein kinase B) pathway (Leconte *et al.*, 2010). In a mouse model of endometriosis, methanandamide increased the lesion volume, as well as the mRNA levels of survivin, *N*-cadherin, integrin β 1 and interleukin-6, molecules that are involved in cell adhesion and inflammation (Sanchez *et al.*, 2017). Moreover, in this study, by using CB₁^{-/-} mice, the authors demonstrated that the lack of CB₁ expression in the recipient peritoneal environment leads to an impairment in lesion volume and lower survivin and *N*-cadherin expression levels (Sanchez *et al.*, 2017). Although the observed changes in ECS indicate the involvement of cannabinoid receptors and of the major eCB, AEA, in endometriosis, little is known about the expression of the metabolic enzymes and their potential contribution to the eCB fluctuation.

Despite the fact that some of these reports were exploratory, their findings on the alteration of ECS elements and cannabinoid signalling in endometriosis provides momentum for further investigation of the pharmacotherapy for this disease (Pertwee, 2009). Nevertheless, several factors need to be taken into account, including the time and tissue-specific availability of mediators involved and their modulation

by endogenous factors (such as hormones), inflammatory factors and environmental stimuli (Bisogno & Maccarrone, 2014). However, two clinical trials in patients with deep endometriosis-induced hyperalgesia that include cannabinoids have been ongoing for some time. The oral formulation of THC/CBD (1:1) (clinical trial NCT03875261) and the combination of PEA and transpolydatin (clinical trial NCT02372903) are being tested, although the results have not yet been published.

Given the relevance of pain in this pathology, the use of phytocannabinoids as in the clinical trial to ease the pain seems to be a good strategy. However, given the existence of cannabinoid receptors throughout the body, this approach is not very specific. Since the ECS is closely related to pain modulation, future strategies could involve the downregulation and antagonism of the cannabinoid receptors and/or TRPV1, or to modulate their actions locally.

Ectopic pregnancy

Ectopic pregnancy occurs as a result of an embryo implanting outside the uterus, most commonly in the Fallopian tube, with a prevalence of 1–2% of all pregnancies (Shaw *et al.*, 2010). The characteristics of

this pathology are abnormal bleeding, pain and rupture of the fallopian tube, which accounts for the leading cause of pregnancy-related first trimester maternal death in Europe and the USA (Farquhar, 2005; Varma & Gupta, 2009).

Given the risk of mortality and morbidity associated with the lack of a reliable method of diagnosis, knowledge about the molecular mechanisms that lead to tubular implantation is needed. Alterations in the ECS elements have been linked with ectopic pregnancy. Studies in mice have shown that pharmacological and genetic silencing of the CBI receptor leads to retention of embryos in the oviduct and, consequently, implantation failure (Wang *et al.*, 2004). A similar effect was reported when methanandamide was administered. In humans, the fallopian tube and decidua of women with ectopic pregnancy express less CBI when compared to healthy women (Horne *et al.*, 2008). In addition to the downregulation of the receptors, it has been reported that the fallopian AEA levels in these women are higher than in healthy non-pregnant women, which seems to be related to a reduced FAAH expression in the fallopian tube epithelium (Table II) (Gebeh *et al.*, 2012). A similar increase in AEA levels, as well as in OEA and PEA, were also reported in plasma, this effect being mainly due to a decrease of FAAH levels in the peripheral blood cells (Gebeh *et al.*, 2013). Therefore, the local and plasma levels seem to be directly correlated with the downregulation or reduced activity of local or peripheral FAAH.

Nonetheless, these studies suggest that the high eCB levels observed in women with an ectopic pregnancy may have a functional impact in modulating fallopian tube function. However, even though local alterations in AEA levels are reflected in the plasma, given the variety of physiological functions in which the AEA is involved, it is not easy to use this eCB as an early predictor of ectopic pregnancy. Therefore, AEA would always have to be associated with other molecules related to the condition. Further studies are required to evaluate the complementary mechanisms and pathways involved in how eCBs modulate cilia movement and/or tubal smooth muscle contractility.

Miscarriage and preterm birth

The correlation between cannabinoids and adverse effects on pregnancy has been demonstrated through several epidemiological studies. Among the described negative effects, there are early foetus loss, preterm birth and low birthweight (Fried, 1995; El Marroun *et al.*, 2009). The importance of a proper AEA tonus for embryo implantation has been described, as deficiencies in FAAH levels lead to impaired implantation and, consequently, infertility (Wang *et al.*, 2006). These observations suggest that possible alterations in AEA levels may cause miscarriage and implantation failure. In fact, a study that compared FAAH with CBI protein expression and NAPE-PLD transcription in first trimester placenta of spontaneous miscarriage and voluntary pregnancy termination reported a decrease in FAAH protein expression and an increase in both NAPE-PLD transcripts and CBI protein in women of the spontaneous miscarriage group when compared to the voluntary pregnancy termination group (Trabucco *et al.*, 2009). Moreover, a decrease in FAAH was found in peripheral lymphocytes of women who miscarried in comparison with pregnant women at 6–11 weeks of gestation (Maccarrone *et al.*, 2000) and in the endometrium of women with unexplained infertility (Cui *et al.*, 2017). A similar study also observed that the same

decrease in FAAH activity and protein level, resulting in high AEA levels, is related to failure in IVF embryo transfer and spontaneous miscarriage (Table II) (Maccarrone *et al.*, 2002). Furthermore, the rise in plasma AEA levels observed in non-viable first-trimester pregnancies seems to be correlated with pregnancy-related hormones such as pregnancy-associated plasma protein A (PAPP-A) (Taylor *et al.*, 2011). Contrary to the decrease in FAAH observed in most of these reports, in the trophoblasts that invaded the decidua from patients who experienced multiple miscarriages, this enzyme is in fact overexpressed and was found in the nucleus of these cells in 67% of the women when compared to those who had early pregnancy termination (Chamley *et al.*, 2008).

These reports undoubtedly indicate that in order to allow pregnancy progression a fine regulation of AEA levels in the placental environment is necessary. In addition, low FAAH and CB2 levels and high CBI and NAPE-PLD expression characterize peripheral blood cells and/or placental tissues from spontaneous miscarriage. Since progesterone and estradiol have the ability to activate the FAAH promoter (Maccarrone *et al.*, 2003; Grimaldi *et al.*, 2012), more research on the molecular events leading to placental FAAH regulation should be carried out. Moreover, given the inhibition of choriocarcinoma growth *in vitro* by AEA via CB2 (Habayeb *et al.*, 2008), additional mechanisms related to CB2 activity have to be taken into account.

Preterm birth is the leading cause of perinatal morbidity and mortality worldwide. As previously referred to, plasma AEA levels increase dramatically during labour (Habayeb *et al.*, 2008). Also as mentioned above, COX-2 has the ability to oxidize the eCBs. In addition, the degradation product of AEA and 2-AG, arachidonic acid, is also a substrate for the synthesis of prostaglandins, showing an important crosstalk between eCBs and prostaglandins (Alhouayek and Muccioli, 2014). Given the implications of AEA and other lipid mediators, such as prostaglandins, in uterine contractions, by using a mouse model of preterm labour, the downregulation of CB2 as well as an upregulation of CBI and NAPE-PLD accompanied by an increase in prostaglandin F2 α has been identified (Bariani *et al.*, 2015).

The first study on a single blood test of AEA levels as a predictor of preterm labour has recently been published (Bachkangi *et al.*, 2019). Both AEA and PEA levels predicted the gestational age of delivery and remaining period of pregnancy with better accuracy than conventional tests. Although further studies need to be performed for both PEA and OEA (since the number of women tested was significantly lower when compared to the group tested for AEA), this approach is remarkable, since it uses eCB for the first time as biomarker for reproductive pathology in a single blood test.

Pre-eclampsia

Pre-eclampsia is a pregnancy-specific disorder characterized by the development of hypertension and proteinuria after Week 20 of gestation in previously normotensive women. This condition has a prevalence of 3–8% worldwide (Abalos *et al.*, 2013) and is among the leading causes of maternal and perinatal mortality and morbidity. The main causes of this condition seem to be an excess of maternal systemic inflammatory response to pregnancy and a systemic oxidative stress, as well as an impairment of circulating angiogenic and antiangiogenic factors (Redman and Sargent, 2004; Aouache *et al.*, 2018; Helmo *et al.*, 2018). The diagnosis of this condition is still very ineffective. Some

women with pre-eclampsia report an absence of hypertension and proteinuria, indicating that adverse events occur in the mother even when the standard clinical definition of pre-eclampsia is yet to be met (Kent, 2008).

Unrecognized foetal compromise contributes to the rate of foetal demise, and 1 in 20 stillbirths without congenital abnormality is complicated by, or attributable to pre-eclampsia (Heslehurst et al., 2010). Recent works have demonstrated that changes in the ECS expression pattern are associated with this condition. Firstly, women with pre-eclampsia exhibit reduced levels of AEA (Molvarec et al., 2015). However, this reduction was not correlated with the currently used standard pre-eclamptic markers, soluble Fms-like tyrosine kinase-1 and phosphatidylinositol-glycan biosynthesis class F protein. When looking into other ECS members, CB1 receptor levels are higher in pre-eclamptic placental tissues, though no changes have been reported at the CB2 and FAAH levels (Fugedi et al., 2014). These impairments are not consensual, since a previous study verified no changes in CB1 protein levels, but instead an increase in NAPE-PLD and a decrease in FAAH in pre-eclamptic placentas when compared to placentas of normotensive subjects (Aban et al., 2013) (Table II). Moreover, a study regarding the association between polymorphisms in the CB1 gene (CNRI) and pre-eclampsia in the population of central Europe found that the single nucleotide polymorphism rs806368 in this gene is correlated with this condition. This suggests a potential role of this polymorphism as a susceptibility marker for pre-eclampsia (Bienertova-Vasku et al., 2011).

The described reports have shown that serum AEA concentrations are lower in women with pre-eclampsia. However, it is also possible that changes in the ECS elements are a consequence rather than a cause of pre-eclampsia; nevertheless, there is an association between placental ECS expression levels and pre-eclampsia risk factors or pathophysiological signals. The study of this pathology in the early stages is still a challenge. Moreover, given the lack of correlation of lower concentrations of AEA with other known markers of pre-eclampsia, other lipids such as 2-AG, OEA or PEA should be evaluated in order to potentially provide a better association with this pathology.

Endometrial cancer

Endometrial cancer is the most prevalent gynaecological cancer in developed countries (Torre et al., 2015). The number of cases is increasing mainly due to a variety of environmental and lifestyle factors, life span and obesity being among them (Amant et al., 2005), as well as genetic mutations (Colombo et al., 2013). This cancer is classified as either type I oestrogen-dependent or type II oestrogen-independent. Type I tumours are mainly low grade, oestrogen-dependent, hormone-receptor-positive adenocarcinomas with endometrioid morphology and are often referred to as endometrioid endometrial cancers (Buhtoiarova et al., 2016; Morice et al., 2016). Type II ECs are generally high grade, hormone-receptor negative and have poor survival rates (Morice et al., 2016). Therefore, the latter requires a more aggressive treatment (Colombo et al., 2013).

Cannabinoids have the ability to regulate or inhibit certain cellular processes essential for cancer progression and development. It has been shown that AEA (Laezza et al., 2006) and THC (Caffarel et al., 2006) arrest the cell cycle progression in breast cancer cells, being THC

effects dependent on CB2 activation. In addition, cannabinoids can either inhibit cancer cell proliferation or induce apoptosis, as reviewed by Ayakannu et al. (2015). Moreover, the capacity of cannabinoids to inhibit cancer angiogenesis was shown by a decrease in vascular endothelial growth factor (VEGF) and VEGF receptor-1 (R-1) in thyroid cancer (Portella et al., 2003), as well as VEGF and VEGFR-2 expression in patients with glioblastoma (Blazquez et al., 2004).

In this endometrial disease, it has been reported that AEA has the ability to induce the migration of endometrial cancer cell line HEC-1B by a mechanism independent of CB1 (McHugh et al., 2012). This observation remains controversial, as it has been described as dependent on this same receptor for migration following Met-AEA treatment (Gentilini et al., 2010). Moreover, in human endometrial carcinoma biopsies, CB2 expression and 2-AG levels were higher and MAGL expression was lower, when compared to normal endometrium (Guida et al., 2010). This same increase in AEA was verified in endometrial carcinoma biopsies (Schmid et al., 2002). Again, these results are not consensual, since another study using biopsies of patients with both types of endometrial cancer reported a decrease in CB2 and CB1, accompanied by an increase in plasma AEA (Ayakannu et al., 2014; Ayakannu et al., 2019). These data show that although changes in AEA levels as well as in CB1 and CB2 expression seem to occur, there is no definitive correlation between these alterations and endometrial cancer (Table II).

Overall, the literature addressing this issue suggests that downregulation of CB1 and CB2 receptor transcript levels and higher tissue and plasma AEA concentrations are correlated with endometrial cancer. This allows speculation about the role of ECS in the aetiology of this cancer, although further research is warranted. In addition, the correlation between plasma and tissue AEA concentrations suggests that AEA could be used as a predictor for some forms of endometrial malignant disease, especially type I endometrial adenocarcinoma. Another strategy may be the search in endometrial biopsies for alterations in CB2 expression since these studies have found a downregulation of this receptor in endometrial cancer. Thus, the ECS presents an attractive target for pharmacological intervention in some endometrial malignancies.

Discussion

The study of the ECS in human reproduction started nearly 20 years ago. Nowadays, the importance of a tight regulation of eCB levels throughout the menstrual cycle, decidualisation and placenta development is well known. Alterations in ECS homeostasis can lead to abnormal modulation of fundamental cellular processes involved in reproductive pathologies, such as pre-eclampsia, ectopic pregnancy and endometriosis. However, despite an apparent link between alterations in AEA levels and most of these pathologies, it is difficult to use AEA as a biomarker since it is highly unspecific, an exception being made for its successful use as a predictor of preterm birth. Yet, even in this case, the combination of AEA with other lipid mediators, such as OEA or PEA, could improve even further the accuracy of this test. As in this case, in other pathologies, the use of AEA as a biomarker for diagnosis would also benefit from the association with other molecules. Moreover, alterations in the expression of CB1 and CB2 in biopsies could be used to predict alterations that seem to be characteristic of some pathologies such as endometrial cancer.

The great challenge of these observations remains whether or not the alterations in the elements of the ECS are the cause or the consequence of the genesis of some of these pathologies. Still, much has yet to be unveiled and concluded about the role of the ECS since 2-AG and its metabolic enzymes, as well as other promising endocannabinoid-related lipids such as PEA or OEA, were not included in these studies.

Patients could potentially benefit from therapies developed to correct alterations in the ECS and the ongoing clinical studies will contribute to the understanding of how this complex system could be pharmacologically manipulated in these pathologies.

Acknowledgements

Some parts of the images used in this article were adapted from the Servier Medical Art Image Bank (Servier Laboratories (Aust) Pty Ltd). The authors also thank Joana Macedo for helping with image design.

Authors' roles

J.M. collected the information, designed the figures and wrote the manuscript. B.M.F, N.T. and G.C.-S. critically revised the manuscript. All the authors have seen and approved the final version.

Funding

FEDER Funds through the Operational Competitiveness Factors Program (COMPETE); National Funds through FCT (Foundation for Science and Technology) within the scope of the project 'PTDC/DTP-FTO/5651/2014—POCI-01-0145-FEDER-016562'; FCT (PhD grant BD/136105/2018 to J.M.).

Conflict of interest

The authors declare no conflict of interest.

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