universidade de aveiro Departamento de Química
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Luís Filipe da Silva Midão

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Doutora Georgina Correia da Silva, Professora Auxiliar da Faculdade de Farmácia da Universidade do Porto, do Doutor Bruno Fonseca, Investigador Pós Doutoramento, da Faculdade de Farmácia da Universidade do Porto, e do Doutor Francisco Amado, Professor Associado com Agregação, do Departamento de Química da Universidade de Aveiro.

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“Põe tudo quanto és
No mínimo que fazes.”

Ricardo Reis

o júri

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Como diz o provérbio africano, *"Se quer ir rápido, vá sozinho. Se quer ir longe, vá em grupo"*. E que sorte tive eu em ter o grupo que tenho!

palavras-chave

Canabinóides, sistema endocanabinóide, gravidez, hormonas, Δ^9-tetrahidrocanabinol.

Resumo

Com a descoberta do sistema endocanabinóide (ECS), a sinalização canabinóide tem sido identificada em vários processos fisiológicos e patológicos. Este sistema é formado pelos recetores canabinóides do tipo 1 e 2 (CB1 e CB2), pelos endocanabinóides anandamida (AEA) e 2-araquidonoilglicerol (2-AG), e pelas enzimas de síntese e degradação destes endocanabinóides. A sinalização endocanabinóide é essencial para a fertilidade, implantação e decidualização, e poderá também estar envolvida no desenvolvimento da placenta, sendo necessários mais estudos nesta área. O desenvolvimento da placenta é um processo altamente regulado e dinâmico, que envolve a proliferação, diferenciação e apoptose dos trofoblastos, as células especializadas da placenta. Este orgão, além de ser responsável pela proteção imune do feto, e pela troca de gases/nutrientes, desempenha um papel endócrino, produzindo hormonas essenciais para o estabelecimento e manutenção de uma gravidez saudável. Os endocanabinóides poderão ser um dos muitos fatores intervenientes na complexa regulação da produção de hormonas pelos trofoblastos. A cannabis é a droga mais utilizada pelas grávidas e o seu consumo durante a gravidez está associado a várias complicações nomeadamente atraso do crescimento intra-uterino e parto prematuro. Contudo, os mecanismos bioquímicos responsáveis por estas complicações estão, ainda, por desvendar. O principal fitocanabinóide, o Δ^9- tetrahidrocanabinol (THC) poderá levar a um desequilíbrio da homeostasia do sistema endocanabinóide e da função endócrina da placenta. Com este trabalho pretendemos analisar qual o efeito do THC, nos trofoblastos, em especial na sua função endócrina. Para isso foram utilizados explantes de placenta e uma linha celular de trofoblastos, as células BeWo. Os resultados obtidos por qRT-PCR demonstram um aumento da transcrição de genes que codificam para a leptina, proteína placentária 13, progesterona e estradiol, hormonas essenciais para o desenvolvimento placentário e crescimento fetal. Foi também observado um aumento da secreção de gonadotrofina coriónica e estradiol. Esta desregulação hormonal poderá originar alterações nas vias de sinalização que regulam a proliferação, diferenciação e apoptose dos trofoblastos dando origem a uma deficiente placentação associada a complicações na gravidez.

Keywords

Cannabinoids, endocannabinoid system, pregnancy, hormones, Δ^9-tetrahydrocannabinol.

Abstract

With the discovery of the endocannabinoid system (ECS), the relevance of cannabinoid signaling has been recognized in several physiological and pathological processes including reproduction. This system consists of cannabinoid receptors type 1 and 2 (CB1 and CB2), the endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) and the enzymes for degradation and synthesis of these endocannabinoids. Cannabinoid signaling is essential for fertility, implantation and decidualization and possibly also for placental development, which is a research area that needs future studies. The formation and development of the placenta is a highly regulated and dynamic process that involves proliferation, differentiation and apoptosis of trophoblasts, the specialized cells of placenta. Besides being responsible for the immune protection of the fetus, and for the exchange of gases and nutrients, the placenta plays an endocrine role, producing hormones essential for proper establishment and maintenance of a healthy pregnancy. The endocannabinoids may be involved in the complex network that regulates hormone production by trophoblast cells. Cannabis is the drug most commonly used by pregnant women and its consumption during pregnancy is associated with complications, such as, intrauterine growth restriction and preterm delivery. Nevertheless, the biochemical mechanisms that lead to these complications are yet to be unveiled. The major phytocannabinoid, Δ^9-tetrahydrocannabinol (THC) may have an impact in the endocannabinoid system homeostasis and in placenta endocrine function. With this work it was intended to analyze the effect of THC on trophoblasts, especially in its endocrine function. For this it was used placental explants and a trophoblast cell line, BeWo cells. Quantitative PCR results demonstrated an increase in the transcription of genes that encode hormones such as leptin, placental protein 13, progesterone and estradiol fundamental for placental development and fetal growth. It was also observed an increase in the secretion of beta-chorionic gonadotropin and estradiol This hormonal deregulation can have an impact on the signaling pathways for trophoblasts proliferation, differentiation and apoptosis, resulting in an incorrect placentation, which is associated with poor pregnancy outcomes.

Author's Publications/ communications

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Abbreviations List

3β-HSD	3β-hydroxysteroid dehydrogenase
2-AG	2-arachidonoylglycerol
AA	Arachidonic acid
AC	Adenyl cyclase
AEA	Anandamide
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
CBD	Cannabidiol
CNS	Central nervous system
DAG	Diacylglycerol
DAGL	Diacylglycerol lipase
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone sulfate
eCBs	Endocannabinoids
ECS	Endocannabinoid system
EMT	Endocannabinoid membrane transporter
enEVT	Endovascular extravillous trophoblasts
eNOS	Endothelial nitric oxide synthase
E1	Estrone
E2	17β-estradiol
E3	Estriol
ERK	Extracellular signal-regulated kinase
ESCs	Endometrial stromal cells
EVT	Extravillous trophoblasts
FAAH	Fatty acid amide hydrolase
FAK	Focal adhesion kinase
GPCR	G protein-coupled receptors
hCG	Human chorionic gonadotropin

iEVT	Interstitial extravillous trophoblasts
JNK	C-jun N-terminal kinase
LepR	Leptin receptor
LIF	Leukemia inhibitory factor
LH	Luteinizing hormone
MAGL	Monoacylglycerol lipase
MAPK	Mitogen-activated protein kinase
NAPE	N-acyl-phosphatidylethanolamine
NAPE-PLD	N-acyl-phosphatidylethanolamine-specific phospholipase D
NAT	N-acyltransferase
NO	Nitric oxide
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PKA	Protein kinase A
PLC	Phospholipase C
StAR	Steroidogenic acute regulatory protein
THC	Δ^9-Tetrahydrocanabinol
TRPV1	Transient receptor potential vanilloid 1

I. Introduction

1. Cannabinoids: an overview

Cannabis sativa was one of the first plants to be cultivated in human history. The use of cannabis in Chinese medicine is described in Pen ts*'ao* ching, the oldest pharmacopoeia in the world, which refers to the use of cannabis in the treatment of intestinal problems, rheumatic pain, malaria and disorders of the female reproductive organs. In ancient India, cannabis was also used for medical purposes in the treatment of insomnia, fevers and cough (1,2).

Nowadays, the medical use of cannabis gained its clinical relevance in the 1990s, with the discovery of the endocannabinoid system (ECS) (3). The number of studies on cannabinoids has increased since then, reflecting the interest on these substances by the scientific community (4). Cannabinoids are ligands for cannabinoid receptors (cannabinoid receptor 1 – CB1 – and cannabinoid receptor 2 – CB2), though they can also activate other types of receptors to exert their actions (5).

According to their origin, cannabinoids can be divided into 3 groups (**Figure 1**): phytocannabinoids, a group of cannabinoids found in *Cannabis sativa*, like Cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (THC); synthetic cannabinoids, the laboratory synthesized compounds, which are classified in different classes like the cyclohexylphenols (CP-55,940 and CP-47,497) and the aminoalkylindoles (WIN-55,212-2, JWH-018 and JHW-073) and; endocannabinoids (eCBs) that are endogenous compounds, being the most representative the N-arachidonoylethanolamine (anandamide - AEA) and 2-arachidonoyglycerol (2-AG) (6,7).

The interest of cannabis and cannabinoid derivatives in the medical community is growing because of their therapeutic potential. This interest is motivated both by changes in the legal status of cannabis in many countries, by intense research on the role of cannabinoids and by evidence that cannabinoids may be useful for the treatment of various medical conditions (8).

With the increased use of these compounds by the pharmaceutical industry, there is a need to expand cannabis plantations. This year, Portugal was the country chosen by Canadian and Israelite companies to cultivate and export cannabis for medicinal purposes. Nevertheless, in Portugal, the medical use of *Cannabis sativa* is still illegal. However, there are some approved drugs that have cannabinoids in their composition. In 2012, the Portuguese Authority for Medicines and Health Products - INFARMED, approved Sativex®, a combination of THC and CBD indicated to treat muscle spasticity in multiple sclerosis (9).

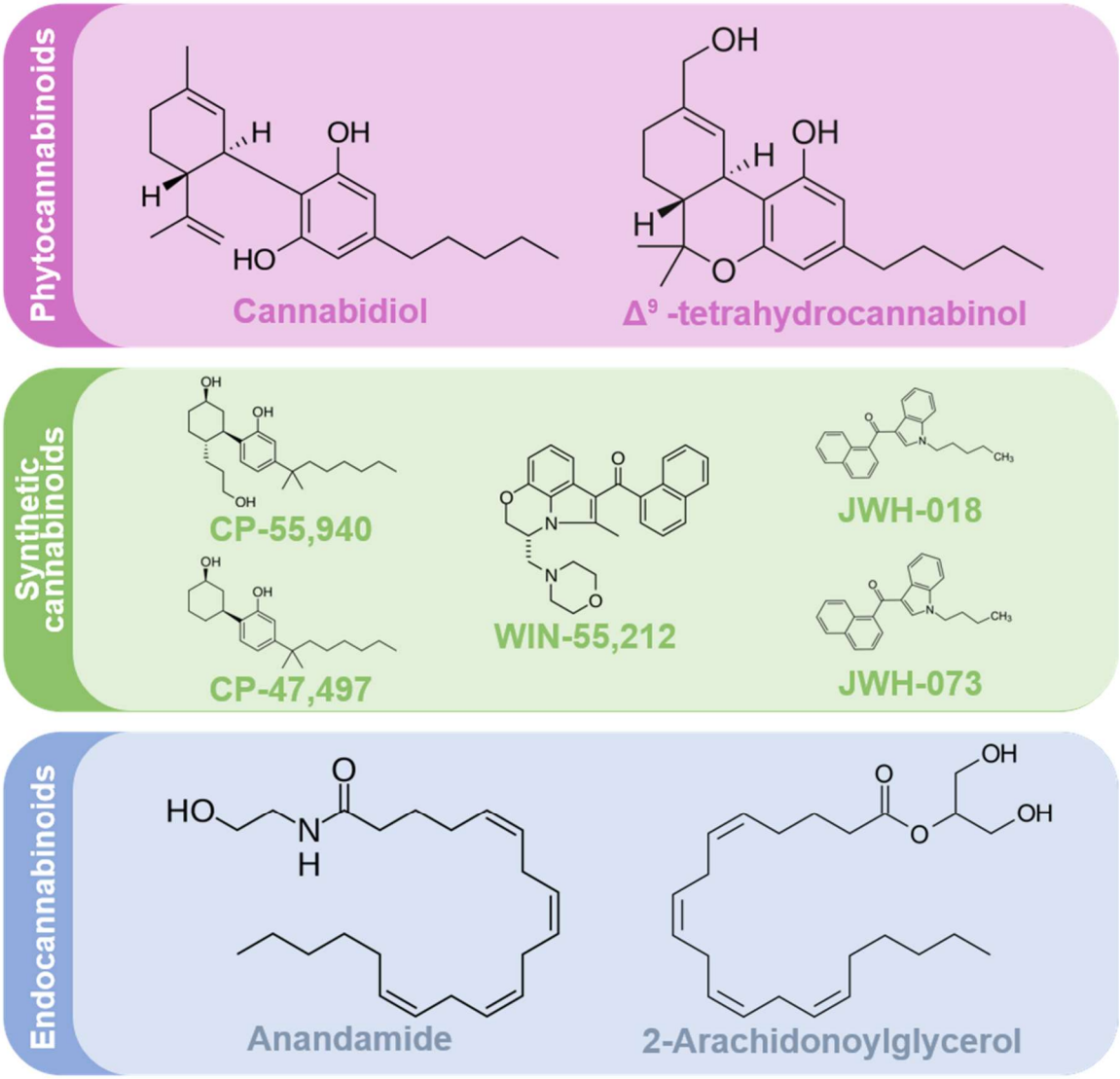

Figure 1 - Chemical structure of some cannabinoids from different groups. Phytocannabinoids are a class of cannabinoids found in *Cannabis sativa*, like CBD and THC; synthetic cannabinoids are produced in laboratory such as CP-55,940, CP-47,497, WIN-55,212, JWH-018 and JWH-073; endocannabinoids are endogenous ligands, like AEA and 2-AG.

1.1. Phytocannabinoids

The term phytocannabinoids represents a group of C_{21} terpenophenolic compounds found in cannabis (10). Among more than 400 compounds, 60 belong to the cannabinoid class, being THC, which has psychoactive effects, and CBD, without any psychoactive effects, the most studied (7,11,12).

Even though it was known for years that the use of cannabis stimulates appetite, reduces nausea, stops seizures and relieves pain, only after the discovery of the structure of THC, scientists understood the clinical, biochemical and pharmacological effects of cannabis (13). The isolation and synthesis of THC came from the Israeli chemist Raphael Mechoulam in 1964 (14). Initially, the properties of THC were thought to be due to its lipophilic nature, so its psychotropic effects would result from interference in membrane fluidity rather than binding to a specific receptor (15). However, in the mid-1980s, several groups showed that cannabinoid activity was highly stereoselective, which led to the search for specific receptors and its endogenous ligands (7,13).

Due to its sedative, analgesic and anti-inflammatory properties, medical cannabis can be used to treat nausea and vomiting-induced by chemotherapy, to stimulate appetite in cancer and AIDS patients, in chronic pain relief, in some types of epilepsy and in the control of spasticity in multiple sclerosis (16–18).

Δ^9-tetrahydrocannabinol can be extracted from the resin that is secreted by the plant or chemically synthesized and used for medicinal purposes. The concentration of THC varies according to its location in the plant: flowers, dried leaves or small stems have a concentration between 1% and 5%; the resin or hashish produced by the glands has a concentration between 5% and 10% and; the oil resulting from resin extraction contains a THC concentration of 50% or more (19,20).

THC has the ability to stimulate dopamine release by neurons, which explains the sensation of euphoria (21). Its consumption may induce the onset of hallucinations and affect memory as it alters the way information is processed by the hippocampus (22,23). The use of this drug by young people can lead to long-term problems, such as decreased memory and cognition (7). On average, its

effects last about two hours and start between 10 to 30 min after consumption. However, due to its lipophilic characteristics, accumulation of THC can occur in adipose tissue, extending its effects (24,25).

1.2. Synthetic Cannabinoids

Synthetic cannabinoids are a family of cannabinoids designed to reproduce specific psychotropic/therapeutic properties of cannabis. These cannabinoids represent the latest breakthrough in designer drugs and are synthesized through small variations of the THC molecule, such as esterification of the phenolic hydroxyl group, extending and branching of the pentyl side chain, or substituting nitrogen for oxygen in the benzopyran ring. These cannabinoids are sold mixed with herbs, in products called K2, Spice or herbal incense, without any prescription or legal restrictions (13,26).

Some of the major synthetic cannabinoids found in Spice, such as JWH-018 and CP-47,497 (Figure 1), were created by pharmaceutical companies such as Pfizer® for the development of analgesics. These drugs have even more potent effects than THC because of their higher affinity for the cannabinoid receptors. For this very same fact, they have been used as drugs of abuse (27–29). There are innumerous cases of overdose with this type of cannabinoids, reason why many countries are taking measures to prevent the use of these substances by adding them to the list of dangerous drugs (27,30).

1.3. Endocannabinoids

The search for endogenous ligands for the cannabinoid receptors led to the discovery of a family of polyunsaturated compounds derived from arachidonic acid that are called endocannabinoids (6). Endocannabinoids are lipid mediators that modulate various physiological processes by activating the cannabinoid receptors. The first endogenous ligand was isolated from pig brain in 1992 and named anandamide (31). Three years later, in 1995, a second ligand was isolated from rat

brain and canine gut and called 2-arachidonoyglycerol (32,33). Although other endogenous compounds have also been described, such as O-arachidonoyl ethanolamine (virodhamine) and 2-arachidonoyl glycerol ether (noladin ether), AEA and 2-AG are the most studied and well known eCBs (34).

These lipid mediators may act in an autocrine or paracrine manner, mediating several biological processes, such as cell proliferation, differentiation, and apoptosis (35). Once eCBs are involved in various physiological processes, their levels must be tightly regulated to maintain homeostasis. These compounds are synthesized, *on demand*, through multiple biosynthesis pathways (36). Once synthesized, the endocannabinoids are transported in both directions through the cell membrane by simple diffusion due to their lipophilic nature or by a putative selective endocannabinoid membrane transporter (EMT) (37). Endocannabinoids may target the cannabinoid receptors or may act on intracellular sites of ion channels such as transient receptor potential vanilloid 1 (TRPV1), peroxisome proliferator-activated receptors (PPARs) family and T-type calcium channels (36). The cannabinoid signaling ends with two mechanisms that cooperate: reuptake of the eCBs and their enzymatic degradation. Both steps are important in the modulation of eCBs levels in tissues (38). There is a machinery of metabolic enzymes responsible for biosynthesis and degradation of eCBs, which act in a very effective way (**Figure 2**). In pathological processes, if the endocannabinoid signaling is altered, the metabolic enzymes are recruited to re-establish homeostasis (36).

1.3.1. Anandamide

As previously referred, anandamide was the first endogenous cannabinoid to be discovered in 1992 in the pig brain (31). Its name derives from the Sanskrit "ananda", which means "bliss, pleasure" and amide is due to its chemical structure (39).

AEA is biosynthesized from precursors of membrane phospholipids, N-arachidonoyl-phosphatidylethanolamines (NAPEs), resulting from the transfer of an arachidonoyl group from phosphatidylcholine (PC) to phosphatidylethanolamine (PE) by the action of N-acyl enzyme-transferase (NAT). The generated NAPE is

subsequently cleaved by a phospholipase D (NAPE-PLD), resulting in anandamide and phosphatidic acid, the latter serving as the cellular metabolic intermediate in the synthesis of other phosphoglycerols (34,38). After being used, AEA is mainly degraded by the enzyme fatty acid amide hydrolase (FAAH) that cleaves AEA into arachidonic acid and ethanolamine (40).

Anandamide is a partial agonist for the CB1 receptor, whereas, as THC, it has a lower affinity for CB2. AEA appears as a modulator of several physiological functions not only in the central nervous system (CNS) but also in endocrine and immune system, gastrointestinal tract and reproductive organs (41,42). For

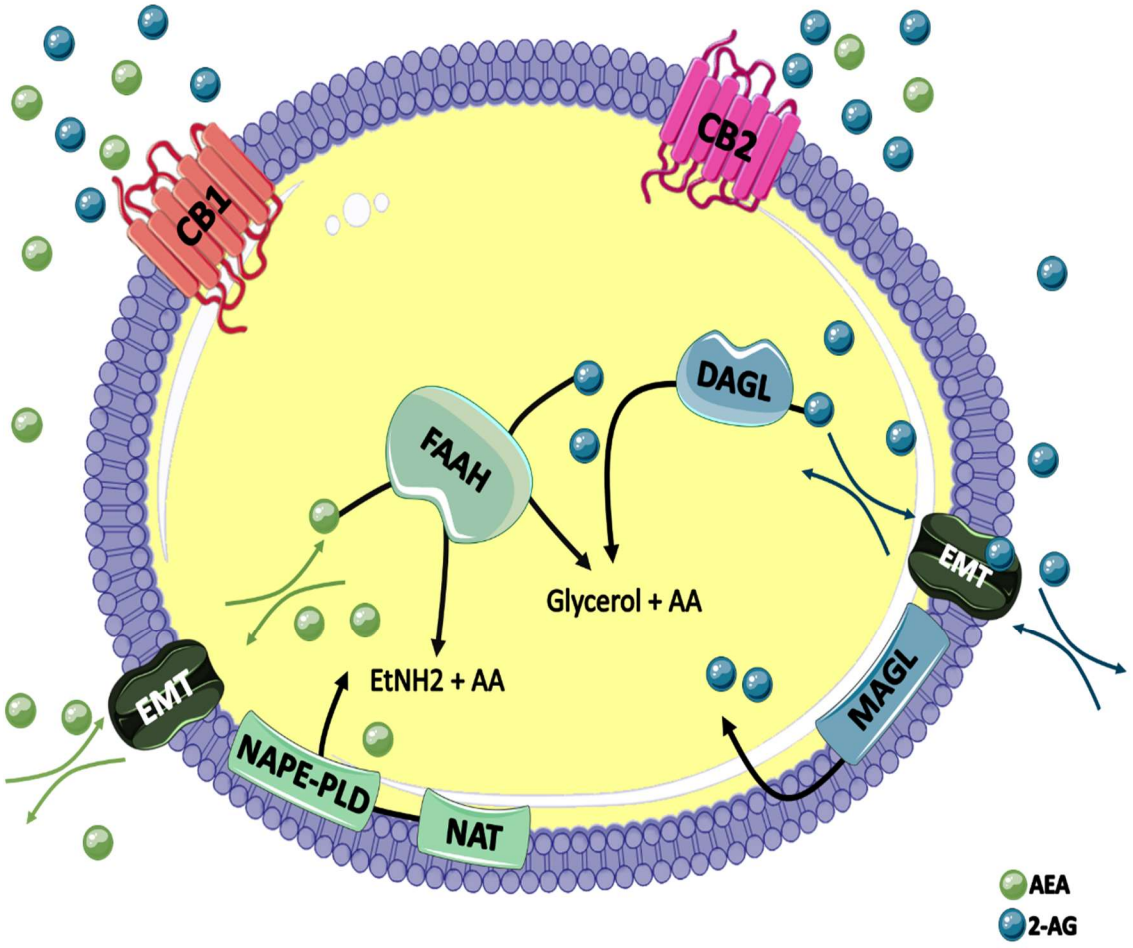

Figure 2 - Components of the endocannabinoid system. Anandamide (AEA; green circles), biosynthesis from membrane precursors is catalyzed by the N-acyltransferase (NAT) followed by N-acyl-phosphatidylethanolamines-specific phospholipase D (NAPE-PLD). 2-arachidonoylglycerol (2-AG; blue circles) synthesis occurs also from membrane precursors but through diacylglycerol lipase (DAGL). AEA is hydrolyzed by fatty acid amide hydrolase (FAAH) into ethanolamine (EtNH2) and arachidonic acid (AA). 2-AG is hydrolyzed through monoacylglycerol lipase (MAGL) or FAAH into glycerol and arachidonic acid.
EMT - endocannabinoid membrane transporter; CB - cannabinoid receptor

example, in the cardiovascular system, AEA induces hypotension and bradycardia and, in the gastrointestinal system, inhibits smooth muscle relaxation. There also appears to exist a modulatory role of AEA and CB1 receptors in uterine-embryo interactions (43,44).

1.3.2. 2-Arachidonoylglycerol

2-Arachidonoylglycerol is the other major endocannabinoid that along with AEA influences cannabinoid signaling in the CNS and peripheral tissues. Unlike AEA, which has higher affinity for CB1, 2-AG is a full agonist at both CB receptors (45).

The principal synthetic pathway for 2-AG is the hydrolysis of membrane phospholipids through a phospholipase C (PLC) producing 1,2-diacylglycerol (DAG) which is subsequently converted to 2-AG by diacylglycerol lipase (DAGL) (32). Once 2-AG can trigger negative effects on certain cells and tissues, its rapid degradation after synthesis is essential and is ensured by monoacylglycerol lipase (MAGL), an enzyme that decomposes 2-AG to arachidonic acid and glycerol (46). This endocannabinoid seems to be important in several physiological processes, such as nociception, inflammation, neuroprotection, cell differentiation and apoptosis, and in reproductive events (36,41,47).

2. Cannabinoid receptors

By using a radioactive THC analog, the CP55,940, it was possible in 1988 to identify specific binding sites of this compound in the rat brain, demonstrating the presence of specific receptors (48). Two years later, in 1990, Matsuda *et al*. cloned cannabinoid receptor type 1, CB1 from rat brain (3) and CB2 was cloned in 1993 from rat macrophages (49), but it was only by the end of 2016 that CB1 crystal structure was reported (50) providing to the pharmaceutical industry new opportunities for the design of cannabinoid receptor modulators. Cannabinoid receptors belong to the superfamily of G protein-coupled receptors (GPCR), which have a seven-domain transmembrane structure. The ligands bind to the extracellular domain, as these receptors are found in cell membranes, and once activated, they signal through different transduction pathways (36).

Generally, cannabinoids exert their effects through the stimulation of $G_{i/0}$ protein-coupled receptor signaling inhibition of adenylyl cyclase (AC), which mediates the conversion of adenosine triphosphate (ATP) to cyclic AMP (cAMP), a second messenger that stimulates protein kinase A (PKA) activity (**Figure 3**). CB1 receptor also modulates ion channels, including inhibition of N- and P/Q-type voltage-sensitive Ca^{2+} channels and activation of rectifying K^+ channels. Additionally, activation of the CB1 receptor stimulates various intracellular kinases, such as extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), focal adhesion kinase (FAK), and p38 MAPK (p38). Yet, CB1 activation leads to sphingomyelin hydrolysis and acute ceramide production and accumulation. Although CB2 receptors also inhibit AC and produce cellular inhibition of PKA, unlike CB1, there is no blockade of Ca^{2+} channels neither activation of K^+ channels. There are however evidences that CB2 activation involves MAPK signaling pathways and ceramide synthesis (51). This multiplicity of responses caused by cannabinoid signalling regulates critical cellular events, such as differentiation, migration, survival, cell death, and intracellular trafficking (36).

CB1 are mainly located in the CNS, and in certain peripheral tissues. They are expressed in the hippocampus, amygdala, cerebellum, substantia nigra, basal ganglia and in the cerebral cortex. At the peripheral level, they are localized in the

lung, liver, heart, bone marrow, adrenal gland, adipose tissue, thymus, tonsils, prostate, uterus, ovaries and testis (52–54). CB2 is mainly found in tissues responsible for the production and regulation of imunne system cells, such as spleen, thymus and tonsils, though being also expressed in other tissues namely in the reproductive tract (36,52,54) .

The knowledge of the involvement of the endocannabinoid system in various physiological and pathological processes, may lead to the development of new therapeutic approaches whether using cannabinoid receptor agonists or antagonists or inhibitors of endocannabinoid-related metabolic enzymes (55).

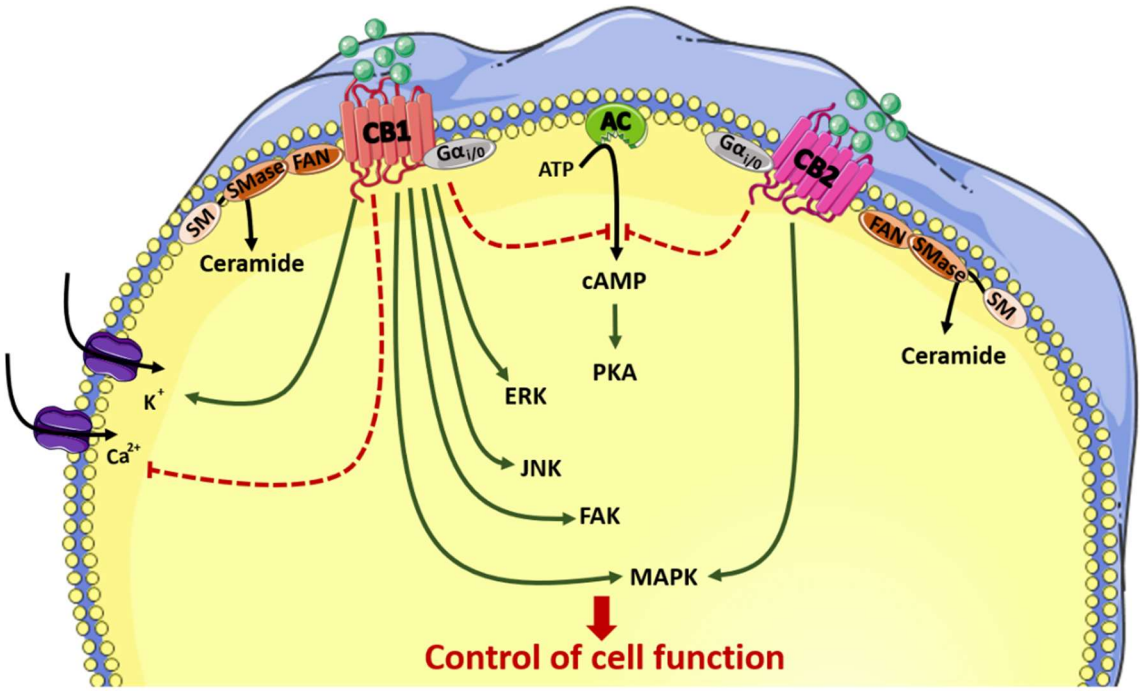

Figure 3 - Cannabinoid receptor-coupled signaling pathways. Cannabinoids exert their effects through the stimulation of $G_{i/0}$ protein-coupled receptor signaling inhibition of adenylyl cyclase (AC), which mediates the conversion of adenosine triphosphate (ATP) to cyclic AMP (cAMP). cAMP binds to the regulatory subunits of protein kinase A (PKA). CB1 and CB2 also mediate ceramide accumulation involving sphingomyelin (SM) hydrolysis via sphingomyelinase (SMase) activation through the adaptor protein FAN (factor associated with neutral sphingomyelinase activation). Contrary to CB2, CB1 modulates ion channels, including inhibition of N- and P/Q-type voltage-sensitive Ca2+ channels and activation of rectifying K+ channels. Activation of CB1 can also stimulate various intracellular kinases, such as extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), focal adhesion kinase (FAK), and p38 MAPK (p38), being this last also activated by CB2. All these events participate in the control of cell function.

3. Cannabinoids and pregnancy

Cannabinoid signaling is also involved in critical events in pregnancy such as gametogenesis, fertilization, oviductal transport, implantation and maintenance of gestation (53,56) and members of the endocannabinoid system are expressed in the myometrium and decidua (57,58). Moreover, in spontaneous abortions, it was reported an increase in CB1 receptor expression and very low levels of FAAH, as well as, an increase in plasmatic levels of AEA (59,60).

Altogether it suggests that an unbalanced cannabinoid signalling may be involved in the pathophysiology of pregnancy related complications (**Figure 4**). In fact, there is a tight control of AEA levels both during the menstrual cycle and pregnancy. During follicular phase AEA levels are higher, whereas in luteal phase or during implantation, they are lower. These low levels of AEA are needed to promote uterine receptivity and maintenance of pregnancy, with a dramatic increase in childbirth (**Figure 5**) (59).

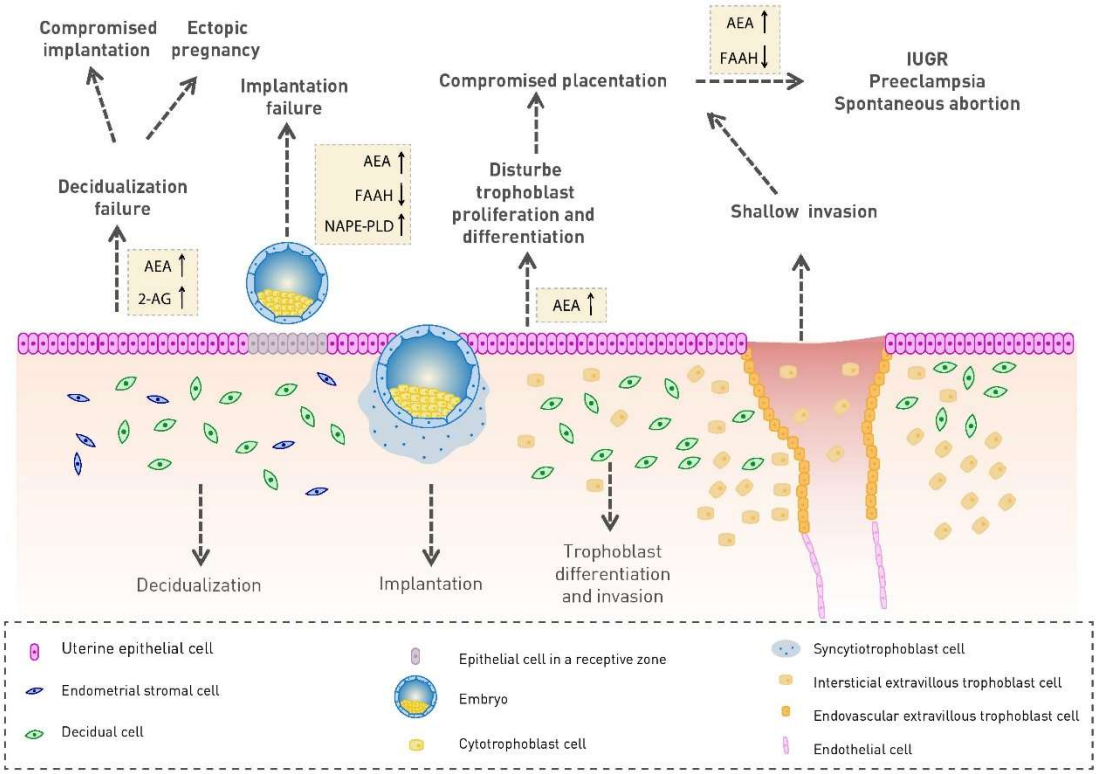

Figure 4 - Deregulated endocannabinoid signaling can lead to adverse pregnancy outcome. Decidualization and placentation are two highly organized processes where the endocannabinoid system acts. Alterations in endocannabinoids levels or the expression of the enzymes involved in its metabolism can compromise pregnancy. Adapted from (66).

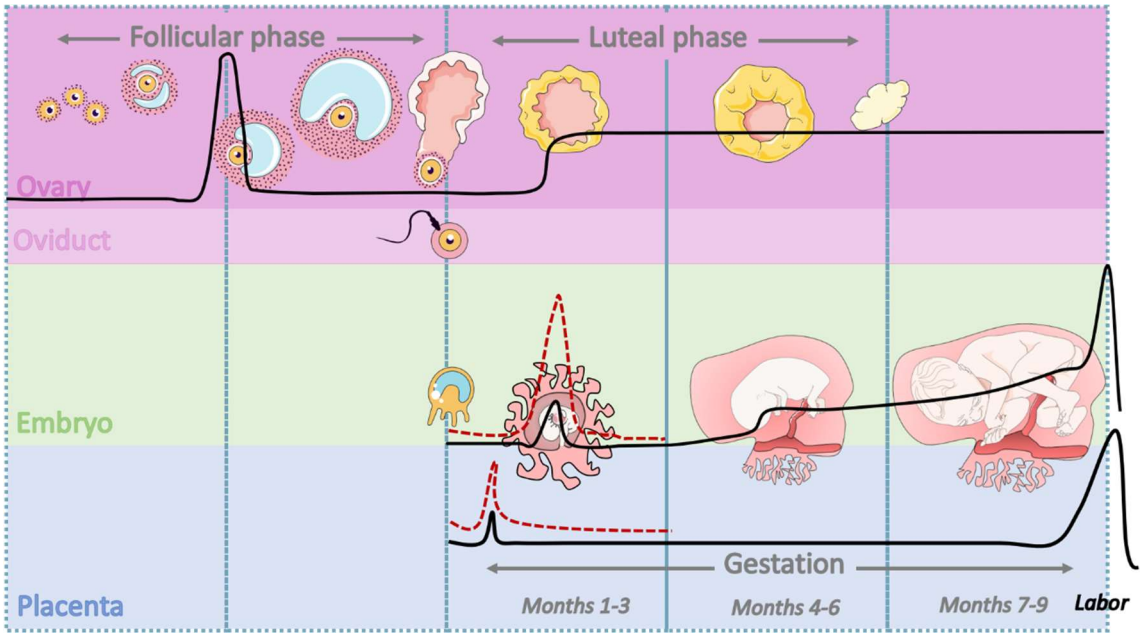

Figure 5 - Fluctuations in AEA levels through the menstrual cycle and gestation. Under physiological conditions (solid line), higher AEA plasma levels in the follicular phase and lower concentrations in the luteal phase of the menstrual cycle, as well as at the beginning of implantation, are required. 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. 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.

Cannabinoid receptors are expressed in human oocytes and the metabolic enzymes of AEA, NAPE-PLD and FAAH, are localized in the follicles and in the corpora lutea (61) suggesting a role for eCBs in the modulation of ovarian function. In addition, the level of AEA in follicular fluid is correlated with oocyte maturation and the expression of NAPE-PLD, FAAH and cannabinoid receptors also vary during the menstrual cycle (53,62).

After fertilization, CB1 is expressed in preimplantation embryos since the 4-cell stage until the blastocyst stage while CB2 receptor is expressed from the 1-cell stage to the blastocyst stage. Also, FAAH and NAPE-PLD are detected in early stages, showing the involvement of AEA during embryo development. In fact, it is

known that AEA is the key link between the development of the embryo and the implantation during the period of the “implantation window” (53,63).

The endocannabinoid system also plays a role in oviductal transport. It was proposed that cannabinoid receptors modulate the correct transport along the oviduct, since in CB1 knockout animals it was observed the retention of embryos (63). It seems that this mechanism is only dependent on CB1, since in CB2 knockout animals this retention does not occur indicating that deficiencies in the CB1 receptor in some women may play a role in ectopic pregnancies or female infertility (**Figure 6**) (64). The implantation of the blastocyst occurs 5 to 7 days after fertilization. For this, it is necessary that the endometrium becomes receptive after the differentiation of endometrial stromal cells (ESCs) into decidual cells (53). There is an uneven distribution of the AEA levels with a lower concentration in the implantation sites compared to the non-implantation sites. In what concerns FAAH, the pattern reverses as there is a greater expression of this enzyme at the sites of implantation compared to the sites of non-implantation (65).

The decidua is a tissue that supports embryonic growth and protects the embryo from the mother's immune system. Decidual formation is highly regulated, and the endocannabinoid system members play a major role during the decidualization process. Pregnancy complications such as miscarriage and foetal growth restriction are associated with alterations in the decidualization process (66). In decidual cell cultures, endocannabinoids induce apoptosis, by a mechanism that is dependent on CB1 receptor, showing the role of ECS in the decidua regression (67,68).

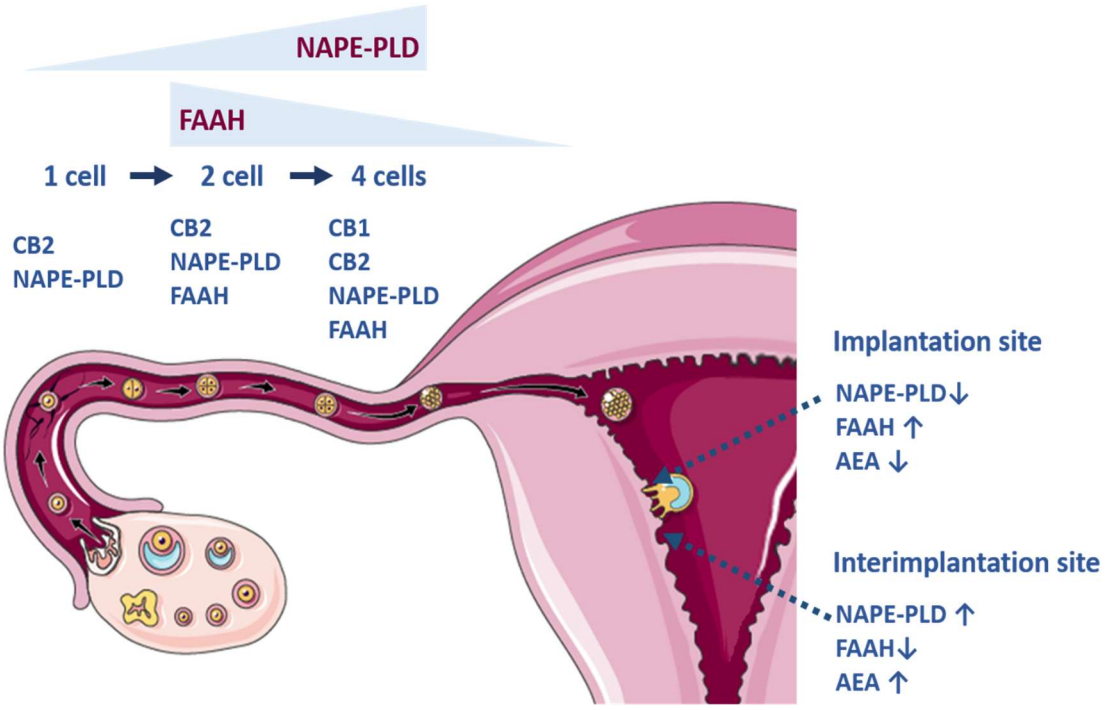

Figure 6 - Regulation of endocannabinoid system throughout oviduct transport and uterus. Since early stages, embryo expresses CB2 and can metabolize AEA by expressing NAPE-PLD and FAAH. During implantation, there is a reduction of NAPE-PLD and an increase in FAHH resulting in reduced AEA levels. In contrast, in the inter implantation sites the AEA-metabolic enzymes are differentially regulated resulting in higher AEA levels.

3.1. Human placenta and trophoblasts

The placenta, from the Greek plakuos, which means "flat cake", is a fetomaternal organ that separates the fetus from the endometrium (69). There are several studies implicating the endocannabinoid system in placental development. In fact, in 1999 the expression of CB receptors in term placenta was described for the first time (70). CB1 was found in all tissues, whereas FAAH was highly expressed in the decidua and amnion (71). FAAH, and both CB1 and CB2 receptors are also expressed in first trimester placenta (65). Moreover, CB1 knockout mice present an abnormal placentation (72). In addition, FAAH regulation of AEA levels on the placenta may play a role in fetal protection against high AEA maternal plasma levels (73). Concerning the other major endocannabinoid, 2-AG, it was reported the expression of the biosynthetic and degradative enzymes in placental cytotrophoblasts (74).

During placental development the specialized epithelial-type trophoblast cells demonstrate invasive properties and undergo proliferation, differentiation and apoptosis, all processes that are essential for pregnancy maintenance (75) (**Figure 7**). Abnormal placental development, either due to alterations in invasion, cell proliferation and differentiation or in apoptosis, can result in pregnancy complications such as abortion, preterm delivery, and preeclampsia, among others (76). Endocannabinoids were shown to intervene in cytotrophoblast apoptosis and, concomitantly, may have a role in placentation (43,74).

There are two main pathways by which the stem cell cytotrophoblasts-differentiation can occur: the extravillous and the villous pathways. From the extravillous pathway, there is a population of extravillous trophoblasts (EVTs) that acquire an invasive phenotype and differentiate into two different types: interstitial (iEVTs) and endovascular (enEVTs). The iEVTs invade the decidua, being involved in immune tolerance, and placental anchorage to the uterus, whereas enEVTs are involved in the remodeling of the maternal uterine spiral artery, ensuring proper blood flow to the placenta (77). From the villous pathway, the cytotrophoblasts (CTs) proliferate, differentiate and fuse to form the syncytiotrophoblasts (STs), the outer epithelial layer of the chorionic villi that directly contact with maternal blood. Syncytiotrophoblasts are involved in transport and metabolism and also produce hormones that are released into fetal and maternal circulation and affect fetal growth, metabolism and parturition (69,77,78).

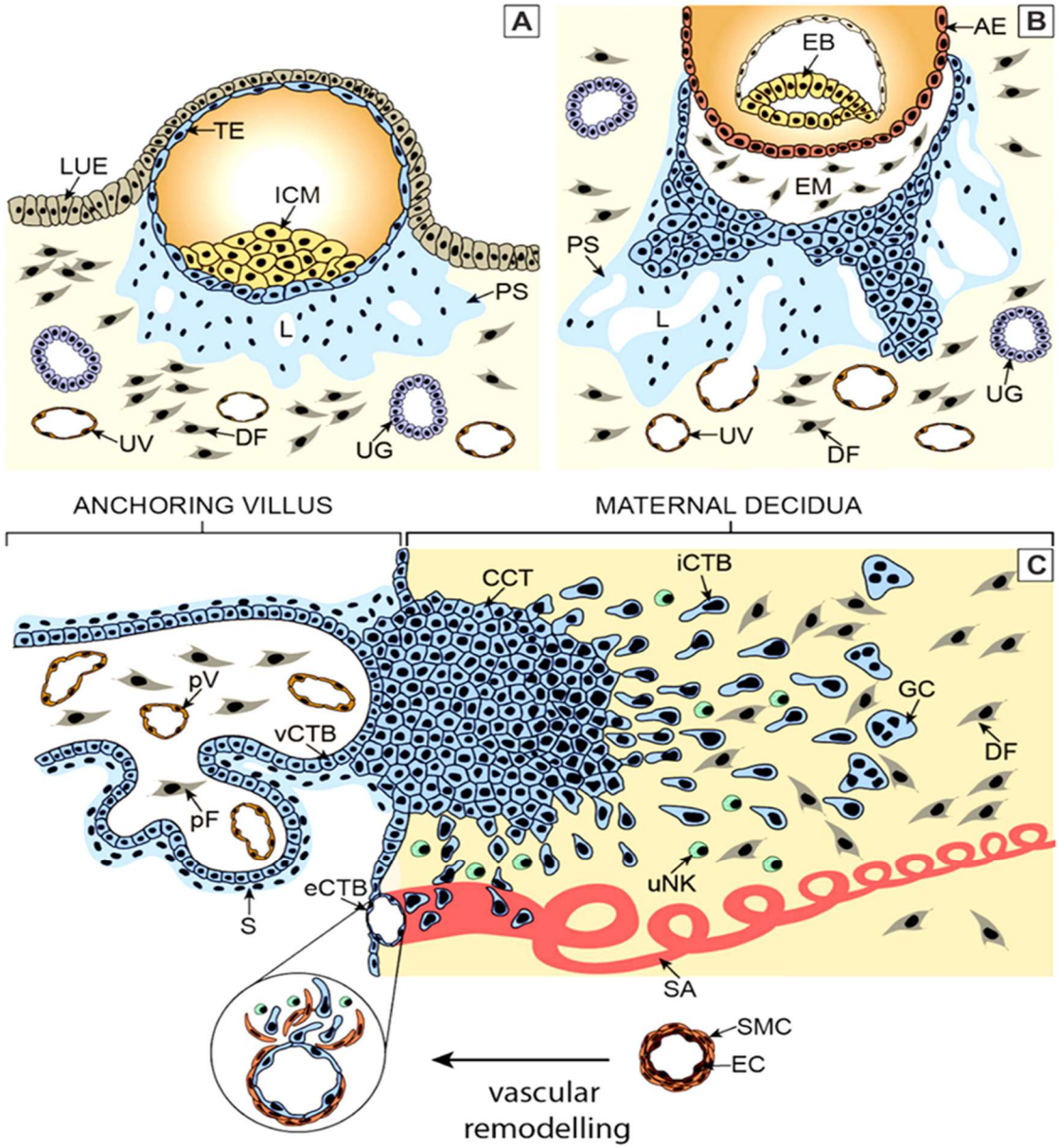

Figure 7 - Critical steps of human placental development. (A) After implantation stems cells of the trophectoderm give rise to the primitive syncytium by cell fusion. Lacunae that originate the intervillous space, are formed. Some of the lacunae erode uterine vessels. (B) At a subsequent stage, proliferative cytotrophoblasts (CTBs) emanate from the trophectoderm, break through the primitive syncytium and contact the basal plate thereby forming primary villi. (C) Tertiary villi are built upon migration of extraembryonic mesodermal cells. At distal sites, proliferative cell columns are formed which give rise to invasive extravillous trophoblast subtypes. iCTBs migrate into decidual stroma, approach vessels from outside and eventually form giant cells as the end stage of the invasive differentiation pathway. Endovascular trophoblasts migrate into spiral arteries and contribute to uNK cell-initiated remodeling within the decidua. AE, amniotic epithelium; CCT, cell column trophoblast; DF, decidual fibroblast; EB, embryoblast; EM, extraembryonic mesoderm; eCTB, endovascular cytotrophoblast; GC, giant cell; ICM, inner cell mass, iCTB, interstitial cytotrophoblast; LUE, luminal uterine epithelium; FALRL, lacunae, pF, placental fibroblast; PS, primitive syncytium; pV, placental vessel; SA, spiral artery; S, syncytium; TE, trophectoderm; UG, uterine gland; uNK, uterine NK cell; UV, uterine vessel; vCTB, villous cytotrophoblast. Adapted from (126).

3.2. Placenta endocrine function

The placenta besides being responsible for gases and nutrients exchange, it provides protective immunity to the fetus and has an important endocrine function, producing hormones that can act at the systemic, paracrine or autocrine level, affecting metabolism, fetal growth and parturition (79). It is not clear the importance of the cannabinoids in the complex network of molecules that orchestrate the production of placental hormones essential for gestational success, although a few studies proposed a regulatory role (43,47,80,81). In this section some of the major placental hormones and their functions will be described.

3.2.1. Human chorionic gonadotropin

Human chorionic gonadotropin (hCG) is a hormone that is critical for establishment and maintenance of pregnancy. This hormone is a glycoprotein that binds to the LH-HCG receptor, which is expressed by cytotrophoblasts, syncytiotrophoblasts, and extravillous trophoblasts (82,83). Human chorionic gonadotropin can be detected in maternal plasma and urine seven days after conception (being urine hCG detection the most commonly used pregnancy test). The plasma concentration increases exponentially up to 9-12 weeks, subsequently decreasing to a plateau that remains stable throughout the rest of pregnancy (78,84).

The syncytiotrophoblasts produces hCG, that during the first eight weeks is essential for the maintenance of the corpus luteum, preserving estradiol and progesterone secretion and, thus, preventing menstruation. When the placenta becomes capable of synthesizing steroids, the secretion of hCG decreases (83). hCG acts on the fetus, stimulating at the adrenal level the synthesis of dehydroepiandrosterone sulfate (DHEA-S) and, at testicular level, the synthesis of testosterone while in the mother inhibits the secretion of the luteinizing hormone (LH) by the pituitary gland. Moreover, hCG ensures that uterus maintains the optimal state for implantation as it acts on the decidual cells of the endometrium, preventing their apoptosis. In addition, promotes angiogenesis in the uterine vasculature,

thereby ensuring a correct blood supply and while the placenta is formed, is involved in the growth and development of the umbilical cord (78). hCG contributes to the continuous growth of the uterus, allowing the development of the fetus as it promotes the proliferation of smooth muscle cells of the myometrium. The contractility of the uterus is depressed allowing maintenance of pregnancy. During the last weeks of pregnancy, hCG levels tend to decrease in preparation for delivery, allowing uterine contractions caused by prostaglandins and oxytocin (85,86).

The secretion of this hormone is regulated by other hormones: there is a stimulation by leptin, GnRH, cortisol, and an inhibition caused by progesterone and inhibin (83).

3.2.2. Progesterone

Progesterone is produced after ovulation and up to the tenth week of pregnancy by the corpus luteum, and then by placenta until delivery. When there is no fertilization, the corpus luteum degenerates, but in case of pregnancy, the secretion of hCG by the syncytiotrophoblasts keeps the corpus luteum, and thus the production of progesterone (78).

Progesterone is produced from cholesterol. The first step requires transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane, facilitated by steroidogenic acute regulatory (StAR)-like domain of a protein called Metastatic Lymph Node Gene 64 Protein (MLN64), constitutive of the placenta (87). The conversion of cholesterol to pregnenolone is performed by the enzyme CYP11A being then the conversion of pregnenolone to progesterone, catalyzed by the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD). After production, progesterone is secreted into either the maternal or fetal compartment where it is converted in fetal adrenal steroids such as cortisol and dehydroepiandrosterone (DHEA) (81,88). Progesterone actions are exerted through binding to its receptors progesterone receptor A (PR-A) and progesterone receptor B (PR-B) (86).

Known as “the pregnancy hormone”, progesterone is a major player in pregnancy. It has an important role in the vasodilation of uterine vessels. It is

involved in the rapid increase in endothelial nitric oxide synthase (eNOS) activity and nitric oxide (NO) production in endothelial cells (73). In early pregnancy, progesterone receptors are expressed in the decidual endothelial cells thereby having an important role during decidualization, formation of blood vessels in the placenta and regulating the invasion of EVTs (89).

Progesterone decreases the contractility of the myometrium and stimulates the release of leukemia inhibitory factor (LIF), allowing a correct implantation and maintenance of pregnancy (81,88,90). Another crucial function of progesterone is the contribution to the development of the mammary glands, since it is associated with ductal proliferation and lobuloalveolar differentiation, allowing the production of milk after childbirth (78). Progesterone may play a role in the regulation of the endocannabinoid system (81,91). It inhibits the release of AEA from endothelial cells, and upregulates the activity and expression of FAAH in human peripheral lymphocytes (90,92).

3.2.3. Estrogens

Estrogens are steroid hormones that can diffuse through the cell membrane, and once inside the cell bind to specific receptors ER-alpha and ER-beta, up-regulating several genes. The main estrogens are estrone (E1), 17β-estradiol (E2) and estriol (E3). Estrone is obtained from progesterone, estradiol from testosterone, and estriol from androsterone, being the enzyme CYP450 aromatase involved in the estrogen biosynthesis pathways (78,83).

In the beginning of pregnancy, the corpus luteum is responsible for the production of estrogens. After five weeks of gestation as it regresses, the placenta becomes the main secreting organ. The levels remain low during the first trimester, and then increase until labor (88).

The placenta requires steroidal precursors formed by the mother and the fetus to synthesize estrogens. While cholesterol, required for progesterone synthesis, is provided by the maternal circulation; the androgens, necessary for estrogen synthesis, are supplied by the fetus, since the placenta is devoid of 17alpha-hydroxylase/17,20-lyase, essential for the synthesis of androgens.

Therefore, placenta uses the dehydroepiandrosterone-sulphate (DHEA-S) produced by maternal and fetal adrenals and synthesizes estradiol and estrone. Estriol is almost all synthesized at the expense of fetal precursors (83).

Estrogenic signaling is involved in the regulation of trophoblast differentiation and in its invasive capacity, suggested by estrogen receptors, ERα and ERβ, expression in placental villous trophoblasts (93). Estrogen can regulate placental angiogenesis increasing the vascularity and blood vessel density in placental tissues (89). Besides this, it stimulates uterine growth and development of mammary glands, in the preparation to parturition (78). 17β-estradiol seems to be involved in the regulation of the endocannabinoid system (94) E2 stimulates NAPE-PLD and inhibits FAAH, with subsequent increase in the levels of AEA (92).

3.2.4. Leptin

Leptin is a peptide hormone, encoded by *LEP*, that is primarily secreted by adipocytes, having functions in weight control, appetite and energetic homeostasis, being also involved in thermogenesis, hematopoiesis and angiogenesis (81,83). This hormone has effects on several reproductive events, such as regulation of ovarian function, oocyte maturation, embryo development, and implantation. It is also produced by the placenta (95).

Leptin has its effect mediated by its receptors (LepR) that exist in long and short isoforms, which couple with different signaling pathways. The long isoform, which mediates most of the effects of leptin, activates the JAK-STAT, ERK 1/2 and PI3K pathways (81). Both leptin and its receptors are located in syncytiotrophoblasts and endometrium (83,96). Considering that the pre-implantation embryo expresses LepR, and the endometrium is a target of leptin actions, there seems to be a role for this hormone in the blastocyst-endometrial dialogue. In fact, there is an increase of leptin secretion by the endometrium in the presence of a blastocyst, showing the importance of leptin in implantation. Leptin also stimulates the proliferation and survival of trophoblasts and the production of hormones thus regulating fetal growth and development (81,96).

The secretion is also regulated by other hormones during pregnancy, such as hCG, insulin and estradiol, which increase the synthesis of leptin, whereas progesterone has an inhibitory effect (83). Given its role during pregnancy, deregulation of the hormone's function and/or metabolism may be implicated in a poor pregnancy outcome, related to conditions such as intrauterine growth restriction, preeclampsia, recurrent miscarriage, and gestational diabetes (96). In relation to the endocannabinoid system, it was shown that leptin reduces the levels of AEA and enhances FAAH activity (97,98).

3.2.5. PP13

Isolated and purified from term placenta by Hans Bohn and colleagues in 1983, placental protein 13 (PP13) is a 32 kDa protein comprised of two identical 16 kDa subunits bounded by disulfide bonds (99). It is a member of the galectin super-family, a family of carbohydrate-binding proteins, which are key proteins of immune-homeostasis and inflammation, being for that, also known as galectin-13 (100). These proteins modulate cell adhesion, signal transduction and molecular recognition, by binding to β-galactoside residues on cell surfaces (101).

LGALS13 is the gene that encodes for PP13, and it is localized on chromosome 19, next to other 5 members of the super-family of galectins, and it is expressed in the maternal-fetal interface of the placenta (102). Although it has been isolated from human placenta,PP13 might be expressed in some other maternal/fetal tissues, during pregnancy (103). In the placenta, it is localized in the syncytiotrophoblast, and in the endothelium of fetal vessels. In fetal membranes, the PP13 gene is expressed in amnio, extravilous trophoblasts and syncytiotrophoblast microvillous membrane, where maternal-fetal immunological interactions occur (104).

The role of this protein produced mainly by the syncytiotrophoblasts during pregnancy is not fully understood . When it binds to proteins of the extracellular matrix on the interface between placenta and endometrium, acts as a lysophospholipase, assisting maternal remodeling and placental implantation (105,106). PP13 leads cytotrophoblasts to release linoleic and arachidonic acids,

increasing prostacyclin and thromboxane levels, both acting as vasoactive prostagladins (103). For this, PP13 may have a role regulating blood pressure of the uterine vasculature from and to the placenta. This protein prepares the utero-placental vessels for the needs of pregnancy, and drives the T-leukocytes to apoptosis, reducing the immune response to the invading trophoblasts (101). In addition, placental down-regulation of this gene, is associated with high risk of preeclampsia (107,108).

3.3. Δ^9-tetrahydrocannabinol and pregnancy

Cannabis is the most commonly used drug by people in general, and also by pregnant women (109,110). As the cannabinoid signaling is tightly regulated during pregnancy, the use of cannabis may lead to its deregulation and, thus, to adverse pregnancy outcome (53).

Although one of the functions of the placenta is to act as a barrier against toxins, the lipophilicity of THC allows it to cross the placental barrier, reaching the bloodstream of the fetus, and thereby it can affect fetal development. In fact, in women that had an acute consumption of cannabis, it was possible to verify the presence of THC in the urine 3 months after stopping its consumption (111). THC also passes into breast milk, thus extending the time it can harm the newborn (112,113). Moreover, THC can compromise fertility, since it affects follicle development, maturation of the oocyte, as well as the production of steroid hormones by the ovaries, potentially leading to deregulated/non-existent menstrual cycles (52,114).

The placenta expresses the two main cannabinoid receptors and thereby THC may exert biological responses in placental cells. As already referred, during placental development stem cytotrophoblast cells undergo proliferation and differentiation into other trophoblast cell populations namely syncytiotrophoblasts and extravilous trophoblasts. *In vitro* studies on THC effects in BeWo cells, a choriocarcinoma cell line, showed a decrease in cell proliferation, although the mechanisms were not disclosed (115). In an extravilous trophoblast cell line, THC impaired trophoblast migration and invasion (116), and in term placenta trophoblast

primary cultures, the differentiation of cytotrophoblasts into syncytiotrophoblasts was also affected (117), thus interfering with a correct placentation.

Cannabis use both before and during pregnancy is associated with several gestational complications. Fetuses that have been exposed to THC during pregnancy had lower levels of insulin, which can explain one of the common complications related with THC consumption during pregnancy, impaired fetal growth (118). Preterm birth, intrauterine growth restriction, abortion and low birth weight, are also associated with THC exposure (118–122). Clinical studies indicate that children born from mothers who consumed cannabis during pregnancy may experience behavioral and neurodevelopmental problems such as tremors, low verbal skills, poor memory, lower intelligence scores. While adults, they would have more propensity to be impulsive and to develop drug addiction (118).

4. Aims

Cannabis sativa has been used for medicinal purposes for hundreds of years, and nowadays THC is being increasingly used in the pharmaceutical industry, in drug design and for treatment of various conditions. It is also known that cannabis, besides being the drug most consumed by the population in general, is also the most consumed drug by pregnant women. Cannabis use during pregnancy is associated with gestacional complications, such as premature delivery, low birth weight, and miscarriage. However, these clinical evidences are not yet perceived at the biochemical level and the role of cannabinoid signaling in placentation is not fully understood.

Therefore, the main objective of this thesis was to study the impact of the main cannabinoid of cannabis plant, Δ^9-tetrahydrocannabinol, in one of the most important functions of the placenta, the endocrine function. For that, the effects of THC on the production of crucial hormones to the establishment and maintenance of pregnancy, such as estradiol, progesterone, leptin and PP13, will be studied in placental explants, an accepted model for endocrine and toxicological studies, and also in BeWo cells, a cell line that provides a good model of placental cytotrophoblasts.

This work may provide new insights into the mechanisms underlying the negative impact of THC during pregnancy.

II. Materials and Methods

1. Materials

Dulbecco's Modified Eagle Medium - F12 (DMEM-F12), fetal bovine serum (FBS), antibiotic/antimicotic solution (100 U/mL penicillin G sodium, 100 µg/mL streptomycin sulphate and 0.25 µg/mL amphotericin B) and trypsin were from Gibco/Invitrogen Corporation, CA, USA. Mouse monoclonal antibodies for aromatase, 3β-HSD and β-actin, and goat anti-mouse antibody were from Santa Cruz Biothecnology, CA, USA. The VIDAS® Progesterone, VIDAS® Estradiol II and VIDAS® hCG kits were from bioMérieux SA , France. RNAlater™ Stabilization Solution was from ThermoFisher Scientific, Waltham, MA, USA. GRS cDNA Synthesis MasterMix was from GRiSP Lda, Porto, Portugal. Δ^9-tetrahydrocannabinol was from Lipomed AG, Switzerland. TriSure was from Bioline Reagents Ltd, UK. Bradford reagent, and PCR plates were from Bio-Rad, Laboratories (Melville, NY, USA). SYBR™ Green was bought from Kapa Biosystems, MA, USA. Ethylenediaminetetraacetic acid (EDTA), diethyl pyrocarbonate (DEPC), isopropanol, chlorophorm, primers, cocktail of protease and phosphatase inhibitors, Triton X-100, activated charcoal, sulforhodamine B (SRB), Mayer's haematoxylin, eosin and tetrazolium salt [3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) were bought from Sigma-Aldrich Co, MO, USA. All plastics used in cell culture techniques were from Falcon™, SD, USA. Nitrocellulose membranes and ECL Prime Western Blotting Detection Reagent were from GE Healthcare, UK.

2. Placental explants culture

Placental explants are accepted as a good model for both toxicological and endocrine placental studies, since they hold both cytotrophoblasts and syncytiotrophoblasts, thus expressing all the enzymatic machinery for the production of hormones involved in processes related to placentation (123). Term placentas (n=7) of normal pregnancies (38-40 weeks gestation) of Caucasian women from the Porto region were collected immediately after spontaneous delivery

or elective cesarean section from the Centro Materno-Infantil do Norte – Centro Hospitalar do Porto. All procedures were conducted according to the Ethics Committee of the Centro Hospitalar do Porto, Porto.

Placental explants were collected as previously described (124,125). Briefly, placentas were washed with saline solution to remove most of the blood. The placental cotyledons were then cut from at least 5 different areas of the placenta, and rinsed in cold DMEM-F12. After removal of the decidua, explants were obtained by dissecting the top layer of the villous trees and removing visible connective tissue, veins, and calcium deposits. All fragments, with about 30 mg of weight, were then washed twice with ammonium chloride, for 5 min, for removal of the red blood cells. After two passages per saline solution, they were allowed to stabilize in 24-well plates (1 explant per well) for 24 h in 600 µL of DMEM-F12 with 5% antibiotic/antimicotic solution (100 U/mL penicillin G, 100 µg/mL streptomycin, 0. 25 µg/mL amphotericin B) and 10% Charcoal Treated Fetal Bovine Serum (FBS-CT) at 37°C, 5% CO_2. After 24 h, the cell culture medium from the explants was removed and replaced with medium at the following THC concentrations: 0, 10, 20 and 40 µM, or vehicle (ethanol 0.01%), in the case of contols. Fetal bovine serum was previously treated with activated charcoal, to remove a variety of endogenous compounds including steroid and pepetide hormones. After treatment, the supernatants were collected and centrifuged at 10.000 g for 15 min, and stored at -80 ºC, until furher hormonal measurements.Moreover, the explants were weighted and also stored at -80 ºC.

3. Histological tissue preparation and HE staining

For the histological studies, explants were cultured in 24-well plates and treated with THC for 24 and 72 h. After these treatments, the cell culture medium was removed, and the explants were fixed in 4% paraformaldehyde for 24 h, at room temperature, and then dehydrated, through increasing concentrations of ethanol (70, 85, 96 and 100%, 2 times 15 min), followed by 1 h on xylol, and then included in paraffin. Blocks were then sectioned by microtomy, and 4 µm sections were

collected onto glass slides. For morphological analysis it was used hematoxylin-eosin staining. Hematoxylin is a basic dye that reacts with anionic components of cells and tissues, which include phosphate groups, nucleic acids, sulfate groups of glycosaminoglycans, and carboxyl groups of proteins. Eosin, being acidic, predominantly stains the cytoplasm, collagen fibers and other structures with a basic character. For haematoxylin and eosin staining, the slides were deparaffinized, hydrated, stained with Mayer's hematoxylin solution for 4 min, washed with tap water, stained with 1% Eosin for 7 min, again washed with tap water, dehydrated through increasing concentrations of ethanol, finishing in xylene, mounted with DPX (Merck, MA, USA), and observed under a bright field microscope (Eclipse E400 Nikon, Japan) equipped with image analysis NIS-Elements Documentation software (Nikon, Japan).

4. BeWo cell culture

BeWo cell line is a human choriocarcinma cell line purchased from ATCC (Manassas, USA). BeWo cells are widely used as a model of cytotrophoblasts, since these cells have the main characteristics of human cytotrophoblasts (126).

Cells were cultured in DMEM-F12, supplemented with 10% FBS-CT, and 1% antibiotic-antimycotic solution, at 37°C in 95% air/5% CO_2 humidified atmosphere. During the culture of these cells, the medium was changed every 3 days. After reaching 80% confluency, cells were subcultured into new culture flasks. For this, cells were treated with 2 mL of 0.25% trypsin/ 1 mM EDTA solution for 3 min at 37°C, to which 2 mL of 10% FBS medium was added to inactivate trispsin. They were then washed with PBS, and transferred to 15 mL centrifuge tubes for further centrifugation, at 600 g, at 4°C for 5 min. The supernatant was then discarded, and the cells resuspended in culture medium, counted in a Neubauer chamber, and cultured at the following densities: the cells plated at 96-well plates, at $1,5x10^4$ cells/well (final volume 200 µL), and at 6-well plates, at 6,0x $x10^5$ cells/well (final volume 2000 µL). After adherence (24 h), the cell culture medium was removed and replaced with medium with THC at the same concentrations used for explants experiments or vehicle (ethanol 0.01%), in the case of controls.

5. Cell viability assays

In order to evaluate the effects of THC on BeWo cells viability MTT and Sulforhodamine B (SRB) assays were performed. For this, BeWo cells were plated in 96-well plates. After the adhesion for 24 h, the medium was removed, and cells were treated with THC (0,1, 10, 20 and 40 μM) or vehicle (ethanol 0.01%), in the case of contols, for 24, 48 and 72 h.

After treatment, cells were incubated with MTT (0.5 mg/mL final concentration) for 3 h at 37ºC in 95% air 5% CO_2. After removal of the medium, DMSO:isopropanol mixture (3:1) was added to stop the reaction and cells were left for 15 min under agitation, to dissolve the formazan crystals. After this, the purple final solution was spectrophotometrically quantified at 540 nm, using a PowerWave™ microplate spectrophotometer (BioTek, WA, USA).This assay is an indirect measure of cell viability, once it relies on the mitochondrial metabolism to convert MTT (yellow) to formazan (purple).

The SRB assay is used for cell density determination based on the measurement of cellular protein content. After the treatment periods, cells were fixed with 40% (vol/vol) trichloroacetic acid and incubated with a solution of 0.4% SRB (in 1% acetic acid) for 20 min, after which the excess dye was removed by washing with 1% (vol/vol) acetic acid. Then, the protein-bound dye was dissolved in 10 mM Tris-base solution for O.D. determination at 492 nm using a a PowerWave™ microplate spectrophotometer (BioTek, WA, USA).

6. RNA extraction

All materials used, such as microcentrifuge tubes, tips, and water, were previously treated with diethyl pyrocarbonate (DEPC) to inactivate all RNAses, preventing RNA loss. To study the effect of THC on the transcription levels of the genes related to the hormones of our interest, placental explants were harvested after 24, 48 and 72 h of THC treatment and stored in 600 μL of RNAlater at 4°C until further RNA extraction. At 24 h, culture medium of the BeWo cells was removed, cells were washed with PBS, and 500 μL of TriSure was added to the 6-well plates,

and stored at -80ºC. For both explants and cells, RNA extraction was done according to the manufacturer's instructions.

For RNA extraction, the explants were moved to new tubes, 500 µL of TriSure was added, and the tissue was homogenized using a small rotor. For BeWo RNA extraction, after adding 500 µL of TriSure, the cells were scraped from the bottom of the wells, and transferred to tubes. Hereafter, the extraction steps were the same. After, 100 µL of chloroform was added to the samples, the tubes were vortexed vigorously for approximately 15 sec, and left at room temperature for 15 min. Then, the samples were centrifuged at 12000 g for 15 min at 4°C. After this, the samples presented 3 phases: a superior, aqueous and transparent, containing the RNA; a whitish and viscous interface, which contains the DNA and an organic phase of greenish color, containing the proteins. Carefully and gently, the aqueous phase was transferred to a new tube, to which 250 µL of isopropranol was added, to precipitate the RNA. The tube was inverted 30 times, and stood for 15 min at room temperature. Once again the samples were centrifuged under the same conditions, and the supernatant was then discarded, leaving a whitish-colored pellet where the RNA was found. Then, for washing the pellet, 500 µL of 75% ethanol (in DEPC treated water) were added to the samples, which were vigorously vortexed, and centrifuged at 7500 g for 5 min at 4°C. Most of the ethanol was removed with a pipette, and the remnant was allowed to air dry. Thereafter, 20 µL of DEPC-treated water was added, and the RNA dissolved by pipetting the solution. After complete dissolution, RNA was quantified in the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) using 1 µL of the samples, and RNA quality was assessed through the 260/280 and 260/230nm ratios. A 260/280 ratio of approximately 2, and a 260/230 ratio in the range of 2.0-2.2, indicates that samples had no protein, phenol or other contaminants. Samples that went beyond these limits were discarded.

7. cDNA Synthesis

After extraction, RNA was converted to cDNA by reverse transcription using the GRS cDNA Synthesis Kit (GRiSP Lda, Porto, Portugal). For each reaction, 10 µL of GRS RT Mastermix were added, along with 1 µL of Oligo(dT)$_{20}$ primer, 1 µg of RNA, and DEPC-H_2O up to 20 µL. The samples were then placed in a thermocycler (BioRad, CA, USA) , as the following: 10 min at 25ºC, allowing primers to bind to the RNA strand, 30 min at 45 ºC, to the reverse transcriptase conversion of RNA into cDNA, and 5 min at 85 ºC, for the inactivation of the enzyme. The samples were then stored at -20 ºC until use.

8. Quantitative Polymerase Chain Reaction (qPCR)

For the preparation of the qPCR reactions, the KAPA SYBR FAST qPCR kit was used. To each reaction, 10 µL of KAPA SYBR FAST qPCR Mastermix, 1 µL of primer forward and 1 µL of primer reverse (both at 4 µM) specifically designed for the desired genes, 7 µL of water and 1 µL of cDNA, were added. The samples were then placed in the thermocycler and subjected to the following cycles of temperatures: 1 cycle of 3 min at 95ºC, for the activation of DNA polymerase; 40 cycles of 3 sec at 95 °C for denaturation followed by 30 sec at the annealing temperature, allowing the primers to bind and the DNA polymerase to create a new copy of DNA. After the amplification reaction, the samples were subjected to an extra cycle of 5 sec, from 65 to 95ºC, with increments of 0.5ºC, to determine the dissociation temperature of the primers, creating the so-called melting curve. This curve is essential to understand if a single product has formed or if non-specific products have also been formed. Although all primers used were designed specifically for the target genes, the annealing temperatures were previously tested, so that only one peak was present in the melting curve, ensuring their specificity. The characteristics of the primers used are shown in **Table 1**, and GAPDH was used

as housekeeping gene. Relative changes in gene expression from qCR were analyzed trough the $2^{-\Delta\Delta C_T}$ method.

Table 1 - Primer sequences and qPCR conditions used to assess the expression of genes encoding leptin, PP13, aromatase and 3β-HSD. AT - annealing temperatura; MT - melting temperature; AL - amplicon lengh.

Gene	GenBank	Primer sequence (5'-3')	AT (ºC)	MT (ºC)	AL (bp)
GAPDH	NM_001289745.1	F: CGCGAAGCTTGTGATCAATGG R: GGCAGTGATGGCATGGACTG	55.0	83.0	358
LEP	NM_000230.2	F: TGCGGATTCTTGTGGCTTTG R: CTGACTGCGTGTGTGAAATGT	60.0	85.0	133
LGALS13	NM_013268.2	F: GATGGCAAACAATTTGAGC R: GGGATTCGATGGACAAAGCC	54.0	88.0	98
HSD3B1	NM_000862.2	F: TCCACTCTTCTGTCCAGCTTTT R: TTCTCCTTCACCAAGAGGCG	59.0	81.5	203
CYP19	NM_000103.3	F: GACGTCGCGACTCTAAATTG R: ACCCGGTTGTAGTAGTTGCAG	57.3	84.5	285

9. Protein extraction and Western Blot analysis

After 24, 48 and 72 h of treatment, explants were transferred to microcentrifuge tubes and stored at -80°C. To prepare protein samples it was used 200 µL of lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) and a cocktail mix of protease and phosphatase inhibitors (both at 1:100 v/v). The explants were homogenized using a mini-rotor, centrifuged at 14000 g for 10 min at 4ºC, and the supernatant was transferred to new tubes. The protein concentrations were further quantified by the Bradford assay.

Protein samples (50 µg) were prepared in electrophoresis sample buffer (0.125 M Tris-HCl pH 6.8; 4% SDS; 20% glycerol; 10% of 2-mercaptoethanol). After boiling, in order to denature proteins, the samples were loaded into a SDS-polyacrylamide (10%) gel, to perform SDS-PAGE. Proteins were then transferred from the gel to a nitrocellulose membrane, using Trans-Blot semi dry transfer cell, (BioRad, California, USA), for 50 min at 2000W. Prior to incubation with the antibody

of interest, the membrane was blocked with blocking buffer (5% dry milk in TBS with 0.1% Tween 20), for 1 h at room temperature, in order to block non-specific binding sites. After that, membranes were incubated with primary mouse monoclonal antibodies CYP19 and 3β-HSD (1:200), overnight, at 4ºC. Then, the membranes were washed with TBS/Tween 20 and incubated for 1h at room temperature with an appropriate peroxidase-conjugated secondary antibody (goat, anti-mouse, IgG-HRP, 1:2000). After 2 times 10 min washes with TBS/Tween 20, the membranes were exposed to a chemiluminescence detection reagent and analyzed using ChemiDoc™ MP Imaging system (Bio-Rad, California, USA). Densitometry analysis was performed through ImageLab software (Bio-Rad, California, USA). The membranes were next stripped and reincubated with anti-β-actin (mouse, monoclonal, 1:500) for a loading control, using a goat anti-mouse IgG-HRP 1:2000, as secondary antibody. As a positive control it was used a placenta microsomal fraction extract.

10. Hormonal quantification by ELFA

β-hCG, estradiol and progesterone were measured by the enzyme-linked fluorescence assay (ELFA), using MINI VIDAS® (bioMérieux SA , France), with the VIDAS® hCG, VIDAS® Progesterone and VIDAS® Estradiol II kits (bioMérieux SA , France). The principle of this technique associates the same immunoenzymatic method of known ELISA assays, with a detection by fluorescence, thereby increasing the sensitivity of the method. The hormones present in the medium, and the derivatives of hCG, estradiol and progesterone in the conjugate compete for antibody sites that line the interior of the solid phase receptacle. The boundless constituents are removed with the washing steps. During the final stage of detection, the substrate (4-methyl-umbelliferyl phosphate) is cycled in and out of the receptacle and the conjugate enzyme catalyses the hydrolysis of this substrate in a fluorescent product (4-methyl-umbelliderone), being then the fluorescence measured at 450 nm. The intensity of the fluorescence is inversely proportional to the concentration of antigen present in the sample. Hormone levels were normalized to explant weight.

11. Statistical analysis

All data presented are the result of at least three independent experiments, carried out in triplicate, and are expressed as mean ±SEM. Statistical analysis was carried out by ANOVA, followed by the Turkey post-hoc test for multiple comparisons (GraphPad PRISM v.6.0, GraphPad Software, Inc., CA, USA). Values of $p<0.05$ were considered as statistically significant.

III. Results

1. THC effects on placental explants

1.1. Analysis of explants viability

In long-term cultures of placental explants, tissue viability should be controlled to ensure the feasibility of functional studies. The evaluation of the structural integrity of the explants by microscopic analysis of the morphology is one of the most important tests for *in vitro* tissue viability (127). The syncytiotrophoblasts are extremely susceptible to environmental conditions and easily degenerate under conditions commonly used in mammalian cell culture systems. For this, the first step in the study of the viability of the explants was to analyse paraffin sections of the explants stained with HE (**Figure 8**). Alterations such as syncytial sloughing and detachment, and necrosis can be easily assessed through this technique (128). After 72 h of culture, the explants remained viable, wihout significant morphological alterations, presenting a syncytiotrophoblast layer well defined.

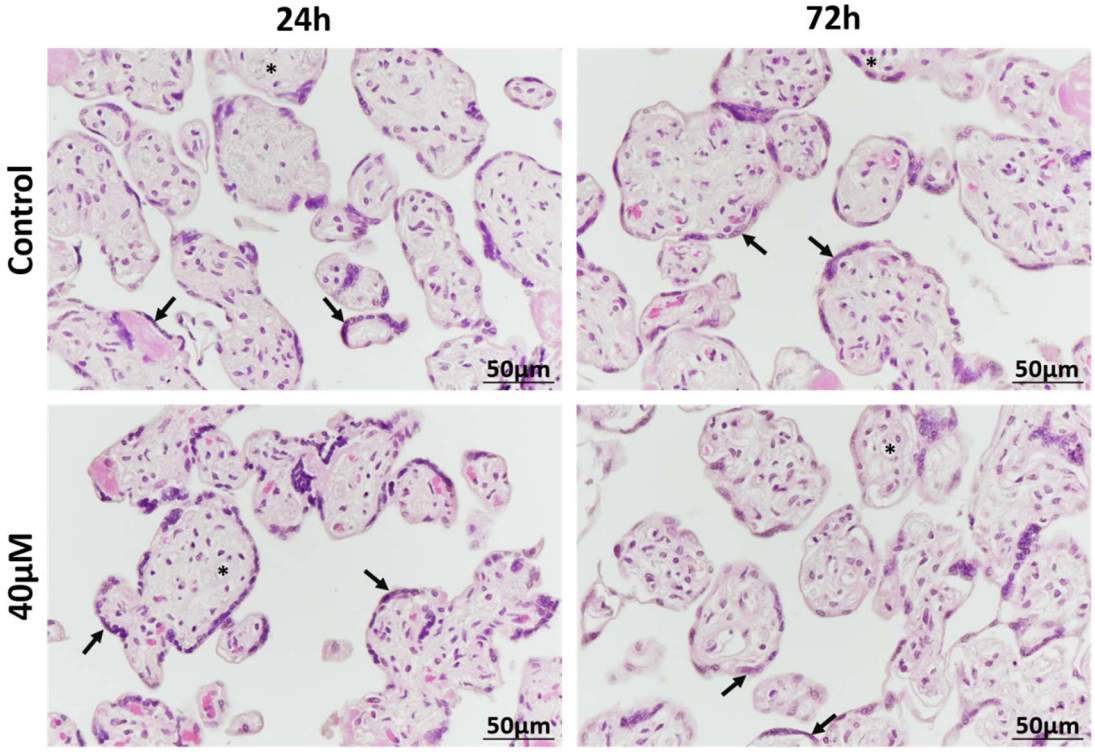

Figure 8 - Effects of time of incubation and THC treatment on placental explants morphology. Placental explants morphology was analyzed in the absense and presence of THC (40 µM) after 24 and 72 h of treatment. No morphological alterations were observed with time of incubation of THC treatment. Arrow- syncytiotrophoblast; *- Fetal blood vessel.

β-hCG hormone secretion analysis is another very powerful and widely used tool (129). As a biochemical marker of placental explants viability was assessed by the measurement of β-hCG secreted by the explants into cell culture medium, by the ELFA technique (**Figure 9**).

After the incubation of the explants with THC, we found an increase in the levels of hormone produced after 24 h and 72 h of treatment, compared with controls, demonstrating that over time, explants remained viable (**Figure 9**). Hormone measurements were also performed after treatment with THC 40 μM, the highest concentration of THC that was used in experiments, to evaluate if this concentration would interfere with cell viability. Comparing the results at 24 and 72 h, there was an increase in the production of this hormone with treatment, being this increase statistically significant for 72 h ($p<0.05$).

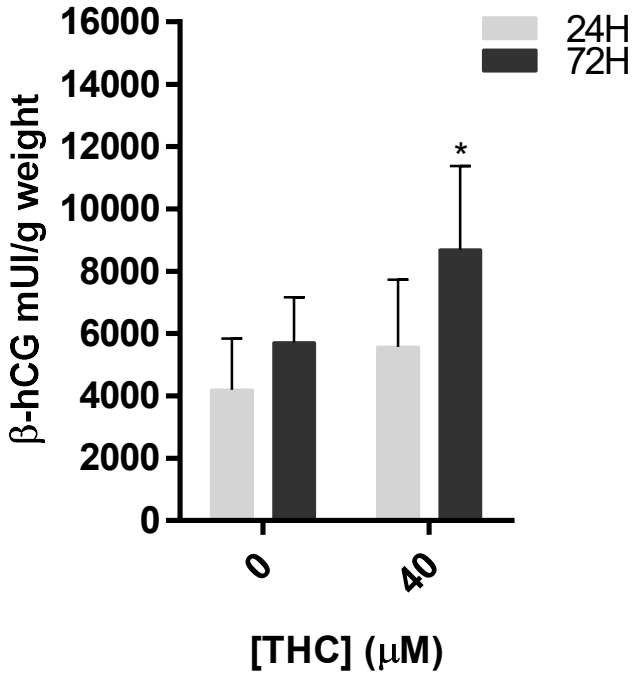

Figure 9 - Effects of THC on β-hCG placental explants production at 24 and 72 h of treatment. The explants remained viable throughout the time of study, once they kept producing β-hCG. At 72 h, there was an significant increase on hormone production at 40 μM of THC, in comparison to control. Significant differences between the treatment and control are denoted by * ($p<0.05$).

1.2. THC effects on endocrine function

It was investigated the impact of THC in placental explants endocrine function concerning some of the hormones that have major influence on placental development by evaluating time of treatment and dose-responses. It were selected short and long periods of treatment and THC concentrations ranging from 10 to 40 μM. In some cases due to lack of placental explant samples the time of 48 h incubation and the concentration of 20 μM were not included.

1.2.1. Leptin

To investigate whether THC induces alterations in the transcription levels of the gene encoding leptin, *LEP*, qPCR was performed using cDNA samples from explants treated with 0, 10 and 40 μM of THC, during 24 and 72 h (**Figure 10**).

The evaluation of mRNA levels revealed that placental explants treated with THC (10 μM) for 24 h presented a 2.4 fold increase in *LEP* gene transcription.

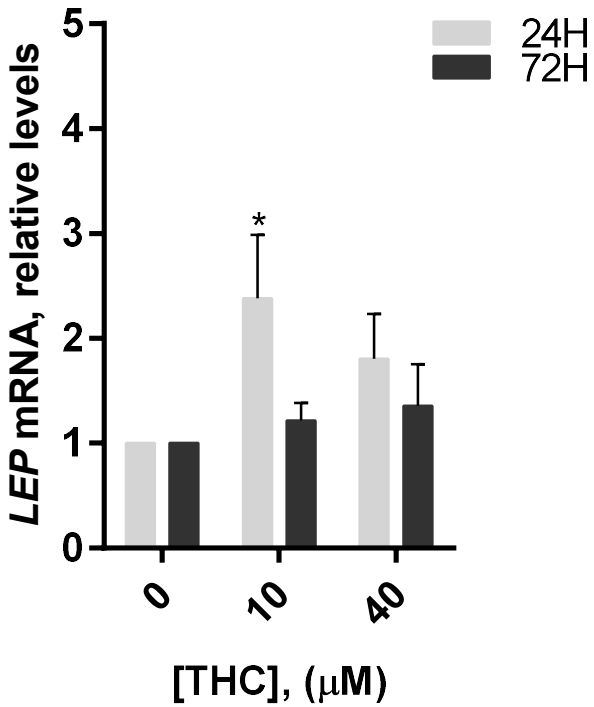

Figure 10 - Effects of THC on the transcription levels of *LEP* at 24 and 72 h of treatment, on placental explants. THC increased gene transcription after 24 h of treatment, with THC 10 μM, in comparison to control. Significant differences between the treatment and control are denoted by * ($p<0.05$).

1.2.2. Placental Protein 13

In order to explore if THC induces alterations on *LGALS13* transcription, the gene encoding PP13, explants were treated with 0, 10 and 40 μM of THC, at 24 and 72 h of incubation and qPCR experiments were performed (**Figure 11**).

The mRNA levels of PP13 were upregulated with the highest THC concentration, in comparison with the control, being this increase statistically significant at 40 μM, after 24 h of treatment.

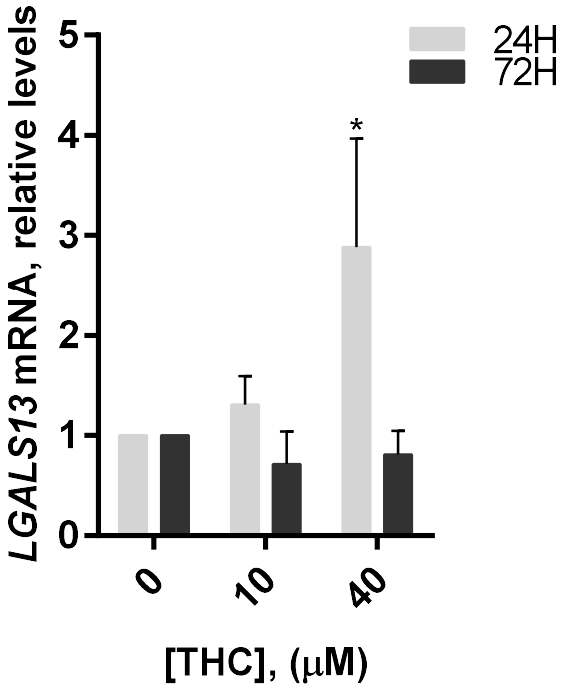

Figure 11 - Effects of THC on the transcription levels of *LGALS13* at 24 and 72 h of treatment, on placental explants. Although no significant changes were observed at 72 h, THC led to an inscrease in gene transcription after 24 h of placental explants treatment, for 40 μM THC, in comparison to control. Significant differences between the treatment and control are denoted by * ($p<0.05$).

1.2.3. Progesterone

To understand the impact of THC exposure on the transcription levels of *HSD3B1*, the gene that encodes for the enzyme involved on the final step of progesterone biosynthesis, it was performed qPCR with specific primers for the enzyme using explant samples treated with THC in a concentration range between 0 and 40 μM, and treatment times varying between 24 and 72 h (**Figure 12**).

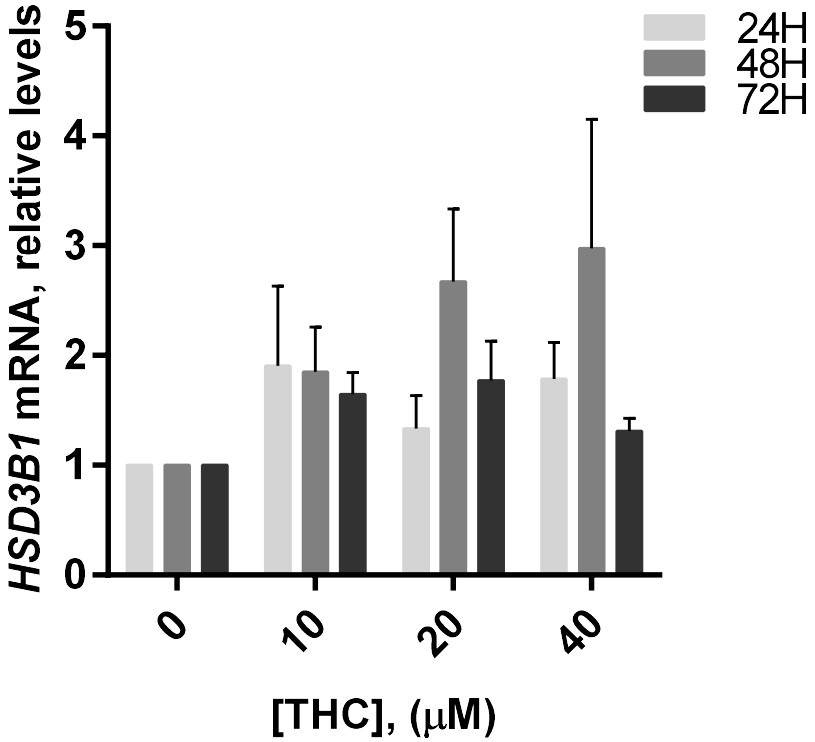

Figure 12 - Effects of THC on the transcription levels of *HSD3B1* at 24, 48 and 72 h of treatment, on placental explants. Although no significant results were obtained, THC seems to increase the transcription levels, at 48 h, in a concentration dependent-manner.

Although no significant effect was found, with no major variations at either 24 or 72 h, there is a tendency for an increase in gene expression with increasing concentrations at 48 h.

Furthermore, the expression of 3β-HSD at protein level in placental explants was evaluated by Western Blot (**Figure 13**). Preliminary results suggest an increase in 3β-HSD expression in placental explants treated with 10 and 40 μM of THC, after 48 h.

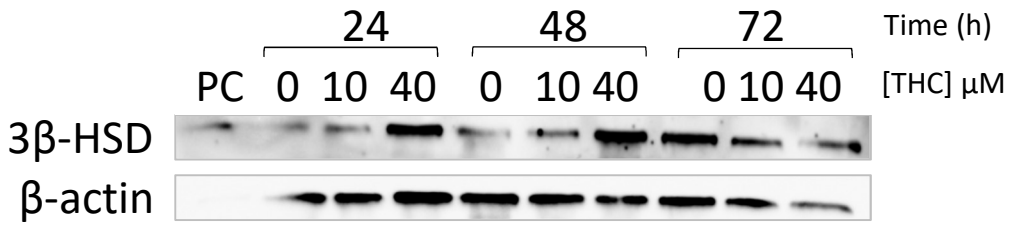

Figure 13 - Effects of THC on the expression levels of 3β-HSD at 24, 48 and 72 h of treatment, on placental explants, analyzed by western blot. Although these are preliminar results, expression of this enzyme seems to increase with THC treatment after 48 h of treatment, in accordance with the tendency found in qPCR. PC- placenta microsomal fraction extract.

Moreover, the secretion of progesterone by placental explants into cell culture medium was also evaluated through the ELFA technique (**Figure 14**). Although no significant effect on hormonal production was found, at 72 h of incubation there appears to be a tendency for increased progesterone synthesis.

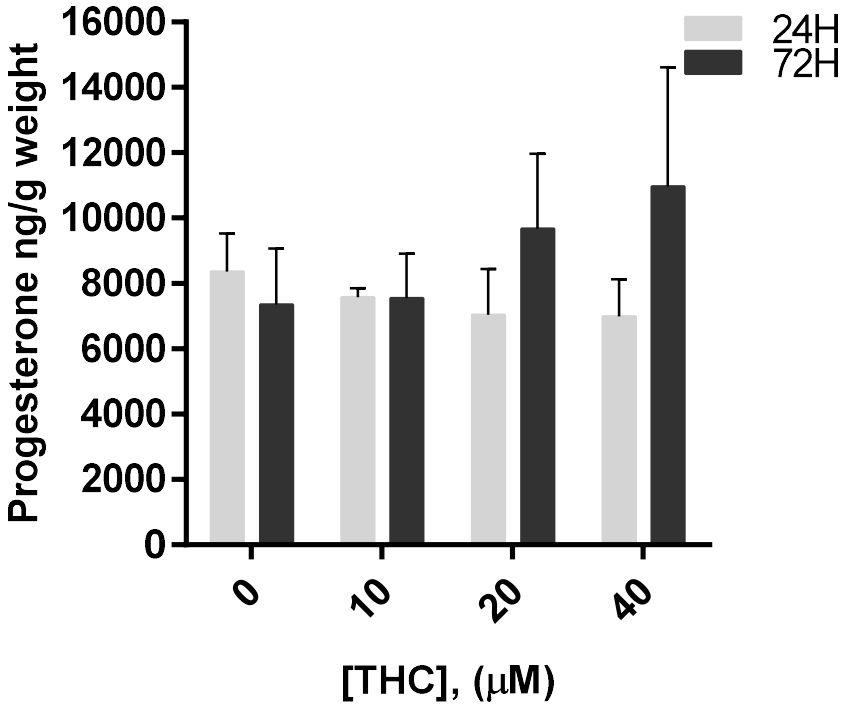

Figure 14 - Effects of THC in the secretion of progesterone by placental explants, at 24 and 72 h of treatment. No significant changes were observed with time and THC concentrations.

1.2.4. Estradiol

The impact of THC on the transcription levels of *CYP19* encoding gene for aromatase, the enzyme involved on the final step of estradiol biosynthesis, was also evaluated. For that, qPCR with specific *CYP19* primers was performed, using THC-treated samples with concentrations between 0 and 40 μM for 24, 48 and 72 h (**Figure 15**).

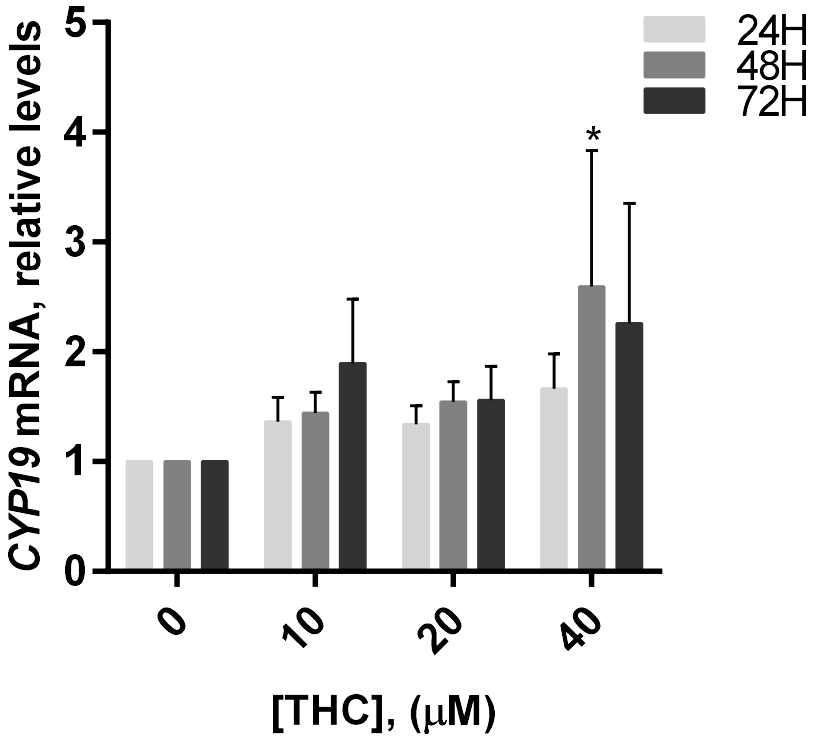

Figure 15 - Effects of THC on the transcription levels of *CYP19* at 24, 48 and 72 h of treatment, on placental explants. THC, at 40 μM, after 48 h, led to a significant increase in the transcription of the gene that encodes for aromatase. Significant differences between the treatment and control are denoted by * ($p<0.05$).

After 24 h of THC treatment, no relevant effects were observed. At higher concentrations and times, *CYP19* transcription was increased by THC, being this statiscally significant for 40 μM, at 48 h (**Figure 15**).

The expression of aromatase at the protein level was also evaluated through Western blot (**Figure 16**).

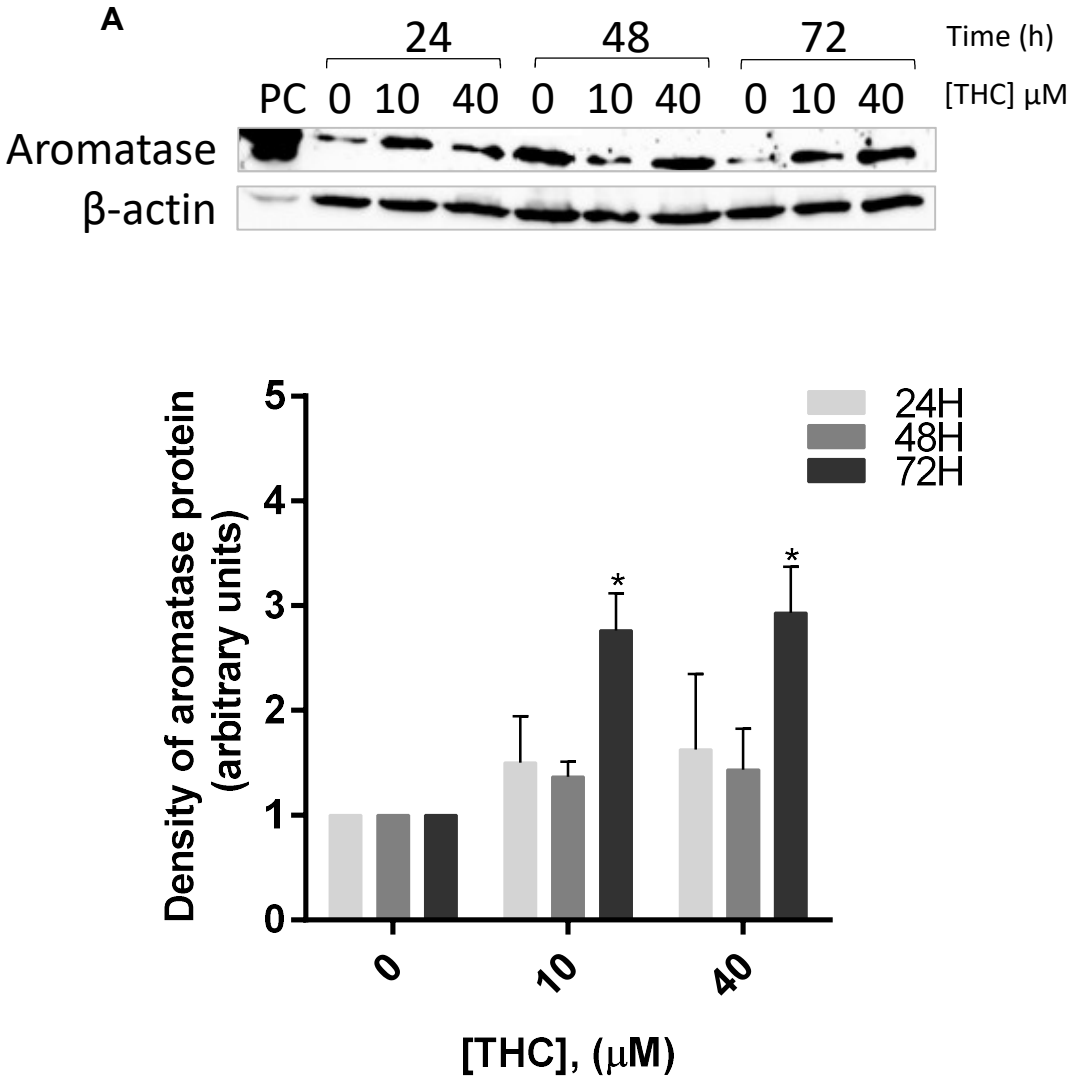

Figure 16 - Effects of THC on the expression levels of aromatase assessed by western blot at 24, 48 and 72 h of treatment, on placental explants. A - Representative western blot. B – Densitometry analysis considering 3 different experiments. At 72 h, at 10 and 40 µM of THC led to an increase of the aromatase expression. Significant differences between the treatment and control are denoted by * ($p<0.05$). PC- placenta microsomal fraction extract.

THC treatments did not lead to major alterations in aromatase expression at 24 and 48 h. However, at 72 h, the expression of this enzyme had a significant increase, either at 10 and 40 µM of THC.

Furthermore, the endocrine function of placental explants, regarding to estradiol synthesis was studied. For this, released estradiol into the culture medium, was quantified through ELFA (**Figure 17**).

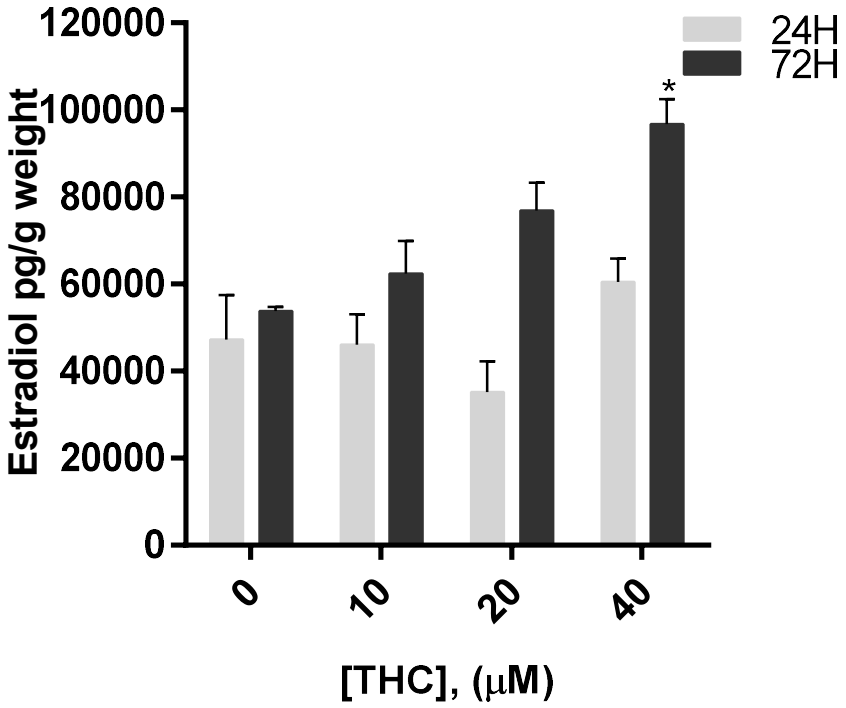

Figure 17 - Effects of THC on the secretion of estradiol by placental explants, at 24 and 72 h of treatment. After 72 h, at concentration of 40 μM, THC enhances estradiol. Significant differences between the treatment and control are denoted by * ($p<0.05$)

Although at 24 h there is no significant changes in estradiol production, at 72 h, THC appears to stimulate the production of estradiol in a concentration-dependent manner, being this increase statistically significant.

2. THC effects on BeWo cells

In addition to the studies on placental explants that hold both cytotrophoblasts and syncytiotrophoblasts, it was also investigated the impact of THC in BeWo cells, a widely used model of cytotrophoblast cells. It was first analysed the effects of THC in cell viability by the MTT and SRB assays, followed by the evaluation of the effects on the endocrine function at the selected conditions.

2.1. Analysis of BeWo cells viability

To evaluate the effects of THC on BeWo cells viability, cells were plated in 96-well plates with THC concentrations ranging between 0 and 40 μM for 24, 48 and 72 h, and MTT and SRB assays were performed (**Figure 18**).

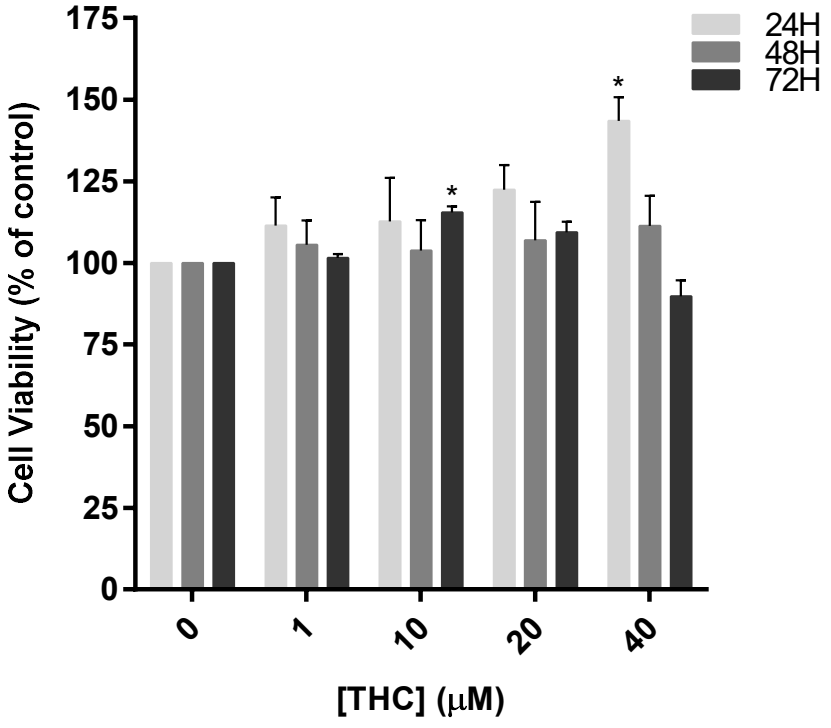

Figure 18 - Effects of THC on BeWo cells viability by MTT assay. Significant enhanced MTT metabolism was observed for THC at 40 μM, after 24 h treatment, and at 10 μM, after 72 h of incubation. Significant differences between the treatment and control are denoted by * ($p<0.05$).

At lower concentrations THC had no impact on cell viability. However, for the concentration of 40 μM at 24 h and 10 μM at 72 h, a significant increase in MTT

metabolism was observed. Since the MTT assay is based on mitochondrial enzyme activity, thus, an indirect method to measure cell viability, another method was used, the sulforhodamine B (SRB) assay, which measures the protein content of the cells (**Figure 19**).

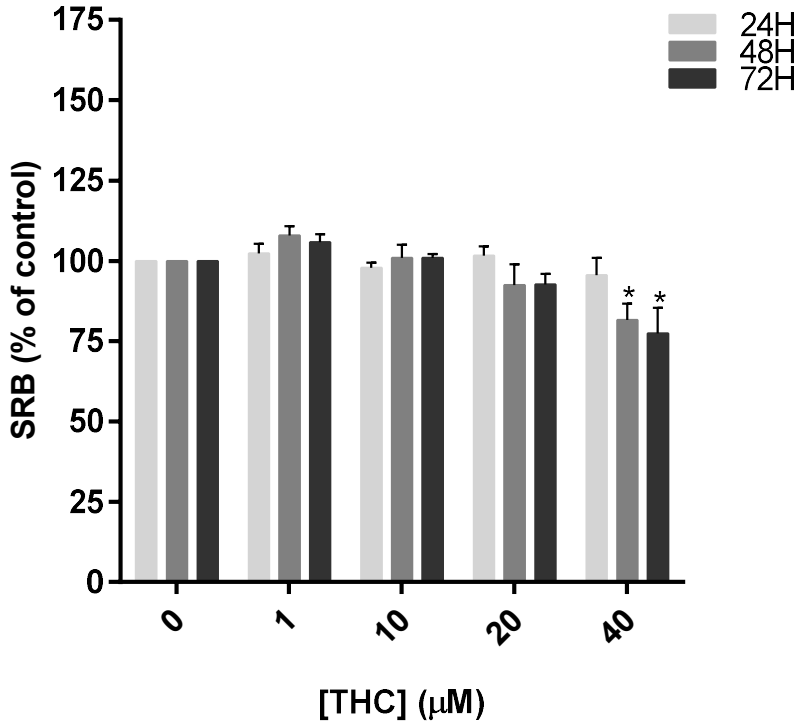

Figure 19 - Effects of THC on BeWo cells viability by SRB assay. A significant decrease in protein content was observed at 48 and 72 h of THC treatment, both for 40 μM concentration. Significant differences between the treatment and control are denoted by * ($p<0.05$).

The effect observed on MTT assay was different from the SRB assay results. Distintly, no differences were observed for 40 μM at 24 h and 10 μM at 72 h in SRB assay, suggesting that THC may affect mitochondrial activity, without inducing cell proliferation. By the SRB assay, it was possible to detect, for 40 μM, at 48 and 72 h, a decrease on SRB, suggesting a decreased cell number (**Figure 19**). Therefore the time of 24 h treatment was selected for the following experiments on THC effects on BeWo cells endocrine function.

2.2. THC effects on endocrine function

2.2.1. Placental Protein 13

BeWo cells express *LGALS13* gene, reason why it was also studied the effect of THC in PP13 expression. For this, qPCR was performed using cDNA samples of BeWo cells treated with 0, 10, 20 and 40 μM of THC, for 24 h (**Figure 20**).

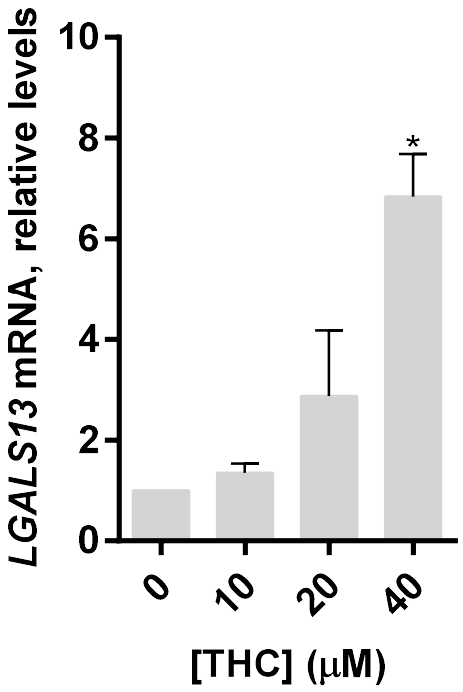

Figure 20 - Effects of THC on the transcription levels of *LGALS13* at 24 h of treatment on BeWo cells. THC causes an upregulation of the PP13 gene, being this increase statistically significant at 40 μM. Significant increase in the transcription between the treatment and control are denoted by * ($p<0.05$).

After 24 h of treatment, *LGALS13* transcription appears to increase along with THC concentration, in comparison with the control, being this increase statistically significant at 40 μM (**Figure 20**).

2.2.2. Progesterone

Similarly, the gene that encodes for the enzyme of the final step of progesterone biosynthesis, 3β-HSD, was studied in response to THC. For this, qPCR with *HSD3B1* specific primers was performed for cDNA samples of BeWo cells treated with 0, 10, 20 and 40 μM of THC, for 24 h (**Figure 21**).

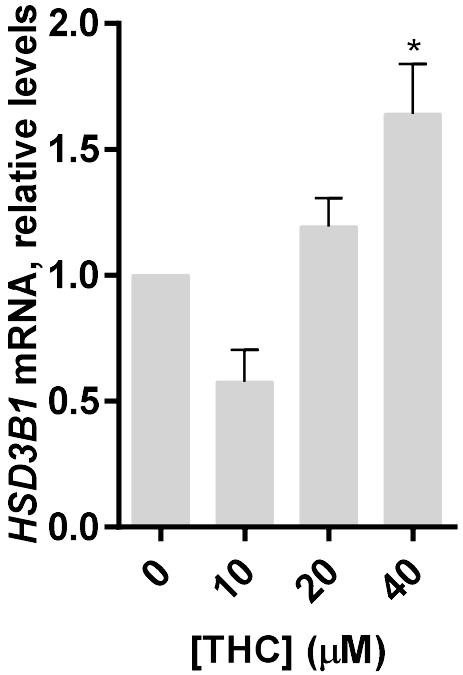

Figure 21 - Effects of THC on the transcription levels of *HSD3B1* at 24 h of treatment on BeWo cells. Higher treatment doses of THC increases the transcription levels of this gene, being this increase statistically significant at 40 μM. Significant differences between the treatment and control are denoted by * ($p<0.05$).

Also *HSD3B1* transcription increases with THC after 24 h of treatment, being statistically significant at 40 μM.

2.2.3. Estradiol

THC impact on the expression of *CYP19,* the gene that encodes aromatase, was also assessed by qPCR (**Figure 22**).

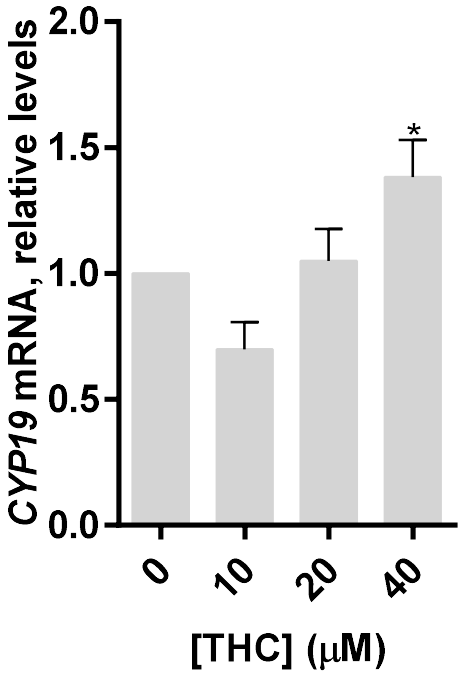

Figure 22 - Effects of THC on the transcription levels of *CYP19* at 24 h of treatment on BeWo cells. A statistically significant increase at 40 μM ($p<0.05$), was also assessed for THC treatment. Significant differences between the treatment and control are denoted by * ($p<0.05$).

Also, in this case it was observed an increase in *CYP19* gene transcription with THC treatment, being statistically significant at 40 μM.

IV. Discussion

The placenta besides being responsible for exchange of nutrients and gases and fetal protection, has also an important endocrine function as it produces a wide number of hormones that play essential roles in the establishment and maintenance of pregnancy (130). The specialized epithelial-type trophoblast cells of the placenta, are divided in various subtypes namely cytotrophoblasts, syncytiotrophoblasts and extravilous trophoblasts (131,132). In this work it was emplyed term placental villous explants, widely used as a model to study transport, metabolism and hormone production at the maternal-fetal interface. Explants contain several cell types besides trophoblasts, like mesenchymal stroma cells, endothelial cells, and placental immune cells. The main advantage of the explants model is that the trophoblast is conditioned by co-culture with other cells thus allowing experimental conditions that more closely mimic placental environment. In contrast, the choriocarcinoma cell lines such as BeWo cell line is well-accepted as a cytrophoblast cell model and represents a suitable and simple system for studying trophoblast cells turnover.

During placental development, trophoblast cells undergo tightly regulated processes of proliferation, differentiation and apoptosis (133). Cytotrophoblasts behave as stem cells for the other types. Proliferation of cytotrophoblasts is followed by differentiation and fusion into syncytiotrophoblasts being these processes regulated by lipids, fusogenic proteins (syncitin-1, syncitin-2), growth factors and hormones (134). Regulation of cytotrophoblast apoptosis is also involved in syncytium formation. The balance between proliferation differentiation and apoptosis is the key to normal placental development and alterations in this delicate turnover may lead to pregnancy complications. These processes are under control of various cytokines and growth factors, and endocannabinoids may play a role in this complex network of modulators. In fact, cannabinoid signaling is involved in cytotrophoblast cell apoptosis (43,74) and differentiation (47,80) and the endocannabinoid system members are expressed in placental tissues from early gestation to labor (66).The intake of exogenous cannabinoids can cause disturbances in both cannabinoid signaling and in the endocannabinoid system homeostasis, affecting placentation and placental functions. *Cannabis sativa* is the most consumed illicit drug by pregnant women (109) but few studies have examined

the effects of cannabinoid consumption on placental developmentl. Clinical evidences such as decreased gestational length, preterm birth and poor pregnancy outcome have been associated with cannabis use (135). However, the biochemical mechanisms underlying these effects are unknown. *In vitro* studies indicate that Δ^9-THC interferes with trophoblast invasion (116), proliferation and turnover (117), which may negatively impact placentation. Besides THC interferes with nutrient transport across the placenta affecting folic acid uptake in chronic exposure to THC (136). However, the impact of cannabis use in one of the most important placental functions, the endocrine function is poorly understood.

In the current study, it is demonstrated that THC affects placental hormone production. THC treatment led to an increase in hCG levels in explants culture medium with time of treatment. This hormone stimulates adenylyl cyclase, and increase cAMP levels thus promoting cytotrophoblast differentiation (131). Interestingly, this phytocannabinoid appears to have an opposite effect to either 2-AG or AEA (47,80). Primary syncytiotrophoblast cells exposure to AEA, results in decreased secretion of hCG, diminished expression of aromatase, leptin and PP13, having no effect on 3β-HSD gene expression. In contrast, 2-AG reduces hCG secretion and transcription of genes encoding leptin, aromatase, and PP13, enhancing 3β-HSD transcription. Moreover, the results of this thesis show that THC leads to increased PP13 and leptin gene transcription. PP13 is expressed predominantly by the syncytiotrophoblast, from where it is released into the maternal blood. PP13 plays a role in the regulation of vascular remodeling and in the promotion of a tolerogenic environment that facilitates trophoblast invasion mostly as it has a pro-apoptotic effect on activated decidual T cells, critical in the down-regulation of maternal immune responses (102). Moreover, the pro-inflammatory actions of this hormone may also play a role in early placentation events. In fact, decidua is infiltrated by natural killer cells, and macrophages that produce cytokines, matrix metalloproteinases and angiogenic factors that regulate trophoblast invasion and uterine spiral artery remodeling. So, these immune cells besides being involved in the establishment of the immune tolerance promote local pro-inflammatory responses that facilitate implantation, trophoblast invasion and placentation (137). In fact, in the second and third trimesters, maternal serum PP13 concentrations rise

paralleling the escalation in the physiological maternal systemic inflammation. In preeclampsia, the abnormal ischemic placental stress and pro-inflammatory changes at the maternal-fetal interface are also reflected by the increased maternal blood PP13 concentrations compared to normal pregnancy. As a consequence, PP13 has a good diagnostic value for the diagnosis of preeclampsia (138). In addition, PP13 is involved in the differentiation and syncialization of trophoblasts. *In vitro* assays with BeWo cells demonstrated that *LGALS13* expression is related to trophoblast fusion and syncytium formation induced by the cAMP-analogue Forskolin and that a PKA inhibitor could inhibit *LGALS13* expression (126). Another study in primary trophoblast cells confirmed these findings and demonstrated a relation between syncialization and differentiation and *LGALS13* expression (139). The results of this thesis demonstrate an increase in *LGALS13* expression that may reflect alterations in cytotrophoblast differenciation occurring in both explants and BeWo cells under THC action, especially when used in high concentrations.

Dysregulation of leptin metabolism and/or function in the placenta may be implicated in the pathogenesis of various disorders during pregnancy, such as recurrent miscarriage, intrauterine growth retardation, and preeclampsia. In fact, leptin gene expression in the placenta is increased in preeclampsia, and maternal plasma leptin levels are higher than those in normotensive pregnant women. Furthermore, leptin may play a role in implantation due to its stimulatory effect on matrix metalloproteinase expression (140).

The results show an increase in LEP mRNA levels. This hormone may stimulate the proliferation and survival of trophoblasts (81,86). In addition, it has pleotropic effects in trophoblast cells, once enhances hCG production, and decreases progesterone and estradiol biosynthesis (141,142). Leptin effects are due to the activation of JAK-STAT, ERK 1/ 2 and PI3K pathways (143). On the other hand, the complex regulation of leptin production in the placenta is still poorly understood. Previous works demonstrated that hCG and cAMP enhances leptin expression through a crosstalk between PKA and MAPK signaling pathways (144,145) and that 17β-estradiol up-regulates placental leptin expression (146). Moreover, the results of this thesis indicate an increase in estradiol levels with THC treatment that can contribute to leptin mRNA increased levels observed in explant

cultures. In fact, the synthesis of leptin may also depend on estrogen regulation as we observed a decrease in LEP gene transcription and β-hCG secretion after THC treatment in primary trophoblast cultures a simple model lacking the trophoblast turnover involving proliferation, differentiation and apoptosis (117).

Human placenta is known to synthesize estrogens during pregnancy in association with the cytotrophoblast invasion. There is evidence to suggest that placental derived estrogen may play an autocrine role in trophoblast differentiation (147,148). This steroid hormone enhances cytotrophoblast syncialization via ERα (149). Estradiol biosynthesis is enhanced by cAMP, hCG, p38 phosphorylation and ERK 1/ 2 inhibition (47,150,151). In our case, the studied phytocannabinoid demonstrated a regulatory effect on estradiol secretion and induced aromatase expression by the cultured explants and the transcription of aromatase encoding gene in either explants and BeWo cells. Supporting these results, the increase in estradiol production was also observed in animal studies that showed that high doses of Δ^9-THC-like drugs increased LH and estrogen in pregnant rats (152). Moreover, Rhesus monkeys who received Δ^9-THC had significantly elevated estrogen levels compared with vehicle controls (153). Just as the endocannabinoid AEA, THC seems to have no significant effect on the expression of the gene encoding for 3β-HSD, nor at protein expression level, neither in progesterone secretion in the explants contrary to the observed increase in BeWo cells indicating that it is important to increase the number of placental samples for statistical power. Progesterone production by the placenta is regulated by numerous factors. While leptin inhibits the production of progesterone by syncytiotrophoblasts, PKA and estradiol stimulate progesterone synthesis (154,155). Also, p38 and ERK 1/ 2 phosphorylation increase the transcription of the gene encoding 3β-HSD (47).

A previous study from our group showed that THC has a dual effect on primary cytotrophoblast cultures. At lower concentrations, THC increased MTT metabolism and revealed an antioxidant capability, while at higher concentrations, THC decreased cell viability (117). In our study, the metabolism of MTT by BeWo cells was significantly increased at 24 h for 40 μM treatment and at 72 h for 10 μM concentration. Under the same conditions, the whole-cell protein content as reflected by the SRB test was not significantly altered, suggesting that THC may

prevent cell death and enhance mitochondrial activity. In what concerns endocrine function, at 24 h treatment, THC induced an upregulation of all genes studied in BeWo cells, whereas this effect was only found for explants at later times.

With this work, and in general, we have demonstrated that THC affects placental endocrine function. All hormones studied, except for the case of progesterone, suffered significant variations at either mRNA, protein or hormone secretion levels. It was observed an increase in progesterone secretion, though not statistically significant. A higher number of studied placentas would be necessary to comprehend these results. This hormonal deregulation caused by the consumption of THC may interfere with cellular processes of proliferation, differentiation and apoptosis. The hormones produced by the placenta regulate implantation, placentation, vascular remodeling and childbirth. However, the direct involvement of each of the hormones produced by the placenta during pregnancy is not demonstrated, since they are highly regulated by several factors, including by other hormones in a complex network of interactions (**Figure 23**). Nevertheless, deregulation in the balance of these hormones can have a negative impact on any of these steps, leading to pregnancy complications, such as miscarriage, preterm labor, intra-uterine growth restriction, all conditions associated with cannabis consumption during pregnancy. In addition, THC may also affect the endocannabinoid system homeostasis and thus indirectly deregulate placental hormone secretion adding complexity to the signaling pathways involved in THC actions.

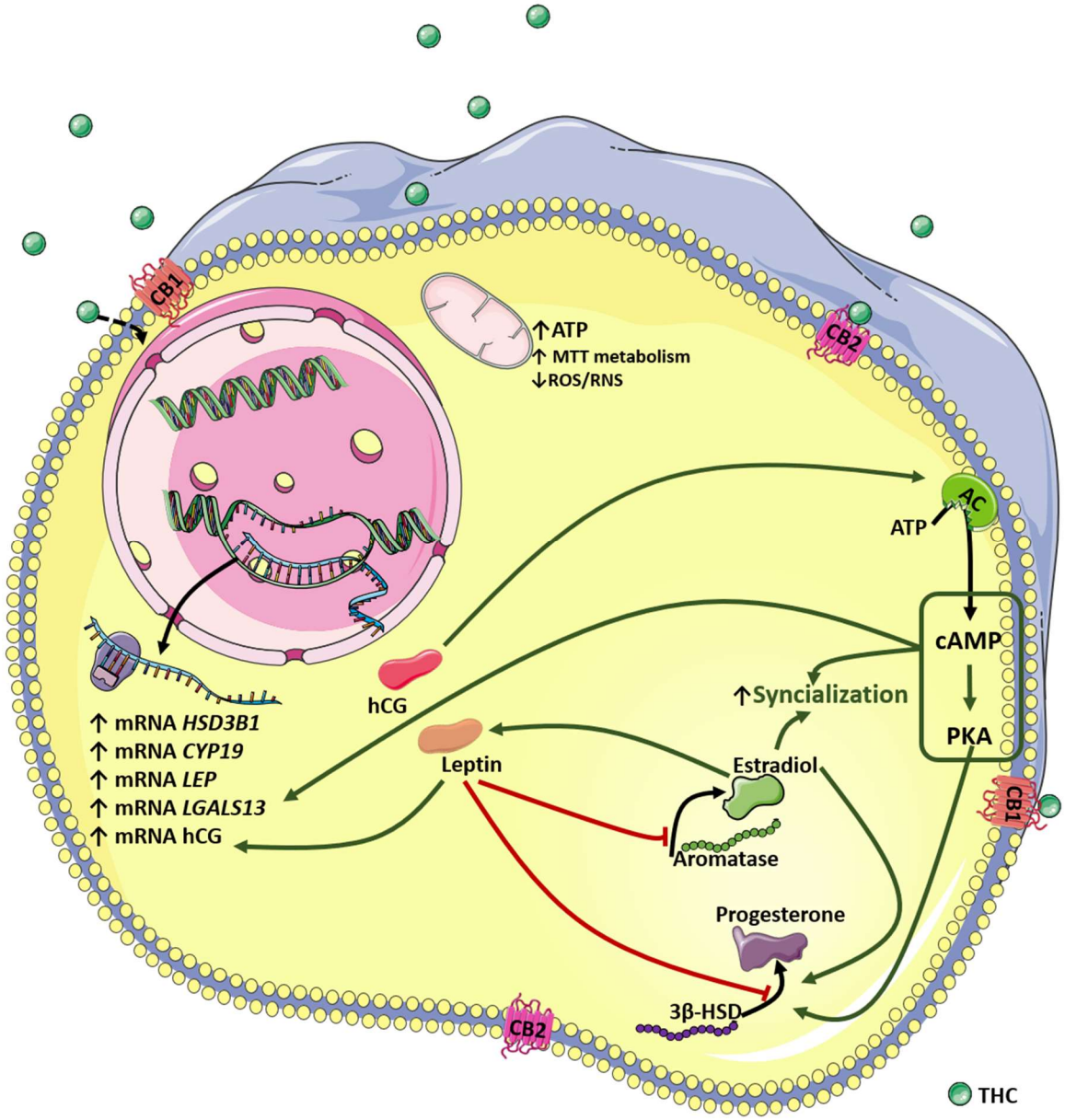

Figure 23 - A model for illustrating THC effects on trophoblast endocrine function. THC causes upregulation of genes responsible for encoding hormones such as PP13, leptin, progesterone and estradiol. The regulation of these hormones affect the synthesis of others, and activate signalling pathways, responsible for cell proliferation, differentiation and apoptosis. Also THC enhances MTT metabolism, increases ATP production and diminishes ROS/RNS generation.

Final conclusions

The study developed during this master's thesis describes the impact of the main psychoactive compound of *Cannabis sativa*, THC, on endocrine placental function. Previous studies suggest that THC may alter trophoblast cell proliferation, differentiation and invasion. This work reveals that THC can affect placental hormone secretion by increasing transcription of the genes encoding PP13, leptin, aromatase and 3β-HSD. Moreover, THC stimulates estradiol and β-hCG secretion. This hormonal dysregulation can have an impact on the signaling pathways for trophoblasts proliferation, differentiation and apoptosis, resulting in an incorrect placentation, which is associated with poor pregnancy outcomes, conditions associated with cannabis consumption during gestation.

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