



# **“EFFECTS OF SYNTHETIC CANNABINOIDS IN ENDOMETRIAL STROMAL CELLS”**

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Master degree in Analytical, Clinical and Forensic Toxicology

Dissertation thesis for the Master Degree in Analytical, Clinical and Forensic Toxicology  
submitted to the Faculty of Pharmacy

Dissertação de candidatura ao Grau de Mestre em Toxicologia Analítica, Clínica e  
Forense apresentada à Faculdade de Farmácia

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August 2016



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## Agradecimentos

Alguns agradecimentos são devidos a todos os que me ajudaram a chegar ao final desta etapa da minha vida:

Ao Doutor Bruno Fonseca, agradeço pela sua orientação e por me ter acompanhado durante quase dois anos. Obrigada pelo conhecimento transmitido, pela confiança e apoio, pela disponibilidade e paciência, e por me ter ensinado a trabalhar em Ciência. À Doutora Natércia Teixeira, por me ter recebido na família que é o laboratório de Bioquímica e pela sua orientação, quero agradecer por ter estado sempre presente e sempre com uma palavra de apoio, especialmente quando nem tudo correu bem, pois “não ter resultados já é um resultado”. À Doutora Georgina Correia-da-Silva, obrigada por todo o apoio, pelos conselhos e pelo interesse que sempre mostrou pelo meu trabalho.

À Doutora Cristina Amaral e à Doutora Marta Almada, quero agradecer por todas as dúvidas esclarecidas, pelas ideias e por todas as palavras de encorajamento que tanto me ajudaram.

À Doutora Susana Rocha e à Doutora Sandra Ribeiro, obrigada por toda a ajuda.

Aos rapazes, João Maia e Tiago Augusto, agradeço por me terem ajudado mesmo quando não queriam e por me terem feito rir todos os dias durante este ano. Às minhas meninas, Fernanda Bernardo, Maria Miguel Castro, Sónia Rocha, e Mestres Lia Costa e Carina Brito, obrigada por todas as conversas de rapariga, pelos desabafos e apoio, pela ajuda que me deram, e por toda a paciência que tiveram para aturar a complicada que eu sou. Foram todos vocês que me ajudaram a manter a pouca sanidade que ainda me resta e que fizeram com que todos os dias fossem uma nova aventura.

À Ana Paula e à Dona Casimira, obrigada por toda a ajuda e por alegrarem o laboratório mesmo em dias de chuva.

Aos restantes professores e alunos do laboratório de Bioquímica, obrigada por me terem recebido e feito com que me sentisse em “casa”.

Às minhas colegas de mestrado e amigas, Maria João Martins e Mariana Soares, obrigada por estarem sempre presentes e prontas a estudar, e além disso, sempre com uma palavra amiga.

Às melhores biólogas de sempre, Sara Vieira, Francisca Diniz, Marta Teixeira e Ana João Mascarenhas, quero agradecer por existirem. Vocês são as melhores amigas e companheiras de aventuras que alguém poderia ter e conhecer-vos tornou a minha vida muito mais colorida. Ao Tiago Moreira, quero agradecer por todas as histórias e todos os risos em momentos inoportunos. Obrigada por todos os momentos partilhados nestes 5 anos e por todos os que ainda virão.

A toda a minha família, quero agradecer por sempre me apoiarem, pelos conselhos e sabedoria que me deram durante toda a minha vida e por estarem sempre interessados nos últimos capítulos da minha jornada. Obrigada por saber que sempre que precisar posso contar convosco.

Por fim, quero agradecer aos meus pais. Não há palavras para descrever o quanto estou agradecida por vocês serem como são, por me apoiarem sempre e sem hesitação, por terem sempre as palavras certas na hora certa, por me encorajarem a ser a melhor pessoa que posso ser, por me deixarem seguir os meus sonhos mesmo quando são irrealistas e, acima de tudo, por estarem ao meu lado mesmo quando não estou no meu melhor. Quero dedicar-vos este trabalho, pois é por vossa causa que cheguei aqui e é por vossa causa que não vou ficar por aqui.

## Abstract

The cannabinoids compounds interact with cannabinoid receptors (CB<sub>1</sub> and CB<sub>2</sub>), and can be divided into three categories: the phytocannabinoids, endocannabinoids and synthetic cannabinoids. Phytocannabinoids are present in the *Cannabis* plant, and the most studied compound is  $\Delta^9$ -tetrahydrocannabinol ( $\Delta^9$ -THC), though more than 60 natural cannabinoids have been described. Endocannabinoids were discovered *in vivo* and are involved in several physiological processes. Synthetic cannabinoids are molecules synthesized in laboratory with cannabimimetic effects, primarily used for research purposes, but also as abuse drugs. Nowadays, the consumption of cannabis has increased worldwide, particularly between young adults, even though its possession and use is prohibited. These prohibitions lead to the emergence and increase in abuse of synthetic cannabinoids, known as “legal highs”, due to lack of legislation. Besides the fact that these substances have higher affinity to cannabinoid receptors than  $\Delta^9$ -THC, not much is known about them. A successful pregnancy is the combination of a series of well-timed events and it was already shown that a tightly controlled endocannabinoid signalling is necessary. So, the consumption of exogenous substances may affect negatively these reproductive events. In this work, it was studied the influence of synthetic cannabinoids in endometrial stromal cells. These cells were treated for 24 and 48 hours with the synthetic cannabinoids THJ-2201, JWH-122, AB-FUBINACA, UR-144, 5F-PB-22 and WIN 55,212-2 (0.01-50  $\mu$ M). Treatments with JWH-122, AB-FUBINACA, UR-144 and 5F-PB-22 caused an increase in mitochondrial activity, although no changes were seen in proliferation studies or in the ratio between mitochondrial DNA (mtDNA) and nuclear DNA (nDNA). Interestingly, WIN 55,212-2 caused a decrease in cell viability, which was accompanied by an increase in caspase -3/-7 activities, suggesting the involvement of apoptosis. Furthermore, WIN 55,212-2 induced changes in cell morphology, like chromatin condensation as observed by Giemsa and H $\ddot{o}$ chst staining. The effects of these compounds in endometrial stromal cells function were also investigated by assessing the activity of matrix metalloproteinases and concentration of prostaglandin E<sub>2</sub>, although no significant alterations were observed. This work showed the impact that synthetic cannabinoids may have in the endometrium cycle and in pregnancy establishment, by influencing cell proliferation and/or death. With the increasing consumption of these substances, especially by women in fertile age, it is important to fully understand their impact in reproduction.

Keywords: cannabinoids, synthetic cannabinoids, endometrium, pregnancy

## Resumo

Os compostos canabinóides interagem com os recetores canabinóides (CB<sub>1</sub> e CB<sub>2</sub>), e podem ser divididos em três categorias: os fitocannabinóides, endocannabinóides e canabinóides sintéticos. Os fitocannabinóides encontram-se na planta *Cannabis*, e o composto mais estudado é  $\Delta^9$ -tetra-hidrocanabinol ( $\Delta^9$ -THC), embora mais de 60 canabinóides naturais tenham sido descritos. Os endocannabinóides foram descobertos *in vivo* e estão envolvidos em vários processos fisiológicos. Os canabinóides sintéticos são moléculas sintetizadas em laboratório com efeitos canabinomiméticos, utilizados principalmente na investigação, mas também como drogas de abuso. O consumo de canábis aumentou em todo o mundo, em particular entre os jovens, embora a sua posse e consumo sejam proibidos. Estas proibições levaram ao aparecimento e aumento do abuso de canabinóides sintéticos, conhecidos como "drogas legais" devido à falta de legislação. Apesar destas substâncias terem maior afinidade para os recetores canabinóides do que  $\Delta^9$ -THC, pouco se sabe sobre o modo de atuação destas moléculas. Uma gravidez de sucesso é a combinação de uma série de eventos altamente regulados e já foi demonstrado que a é necessária uma sinalização endocanabinóide rigidamente controlada. Assim, o consumo de substâncias exógenas pode afetar negativamente estes eventos reprodutivos. Neste trabalho, estudou-se a influência de canabinóides sintéticos em células do estroma endometrial. Estas células foram tratadas durante 24 e 48 h com os canabinóides sintéticos THJ-2201, JWH-122, AB-FUBINACA, UR-144, 5F-PB-22 e WIN 55,212-2 (0,01-50  $\mu$ M). Tratamentos com JWH-122, AB-FUBINACA, UR-144 e 5F-PB-22 induziram um aumento da atividade mitocondrial, embora não se tenham observado alterações na proliferação celular ou ainda na relação entre o DNA mitocondrial (mtDNA) e DNA nuclear (nDNA). Curiosamente, o WIN 55,212-2 causou uma diminuição na viabilidade celular, o que foi acompanhado por um aumento da atividade da caspase -3/-7. Além disso, o WIN 55,212-2 induziu condensação da cromatina como observado com a coloração de Giemsa e H $\ddot{o}$ chst. Os efeitos destes compostos na função das células do estroma endometrial foram também investigados através da determinação da atividade de metaloproteinases e dos níveis de prostaglandina E<sub>2</sub>, no entanto não foram observadas alterações significativas. Este trabalho mostra o impacto que os canabinóides sintéticos podem ter no ciclo endometrial e no estabelecimento de gravidez, ao influenciar a proliferação e/ou morte das células do estroma uterino. Com o aumento do consumo destas substâncias, especialmente por mulheres em idade fértil, é crucial compreender o impacto destas drogas na reprodução.

Palavras-Chave: canabinóides, canabinóides sintéticos, endométrio, gravidez

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## List of abbreviations

<b><math>\Delta^9</math>-THC</b>	$\Delta^9$ -tetrahydrocannabinol
<b>2-AG</b>	2-arachidonoylglycerol
<b>AA</b>	arachidonic acid
<b>AB-AM</b>	antibiotic-antimycotic solution
<b>AC</b>	adenylyl cyclase
<b>AEA</b>	arachidonylethanolamine, anandamide
<b>AM</b>	Alexandros Makriyannis
<b>BrdU</b>	5-bromo-2'-deoxyuridine
<b>cAMP</b>	cyclic adenosine monophosphate
<b>CB<sub>1</sub></b>	cannabinoid receptor 1
<b>CB<sub>2</sub></b>	cannabinoid receptor 2
<b>CBD</b>	cannabidiol
<b>CBG</b>	cannabigerol
<b>CBN</b>	cannabinol
<b>CBR</b>	cannabinoid receptors
<b>CP</b>	Charles Pfizer
<b>DAG</b>	1,2-diacylglycerol
<b>DAGL</b>	diacylglycerol lipase
<b>DMEM</b>	dulbecco's modified eagle medium
<b>E2</b>	estrogen
<b>ECM</b>	extracellular matrix
<b>EMCDDA</b>	European Monitoring Centre for Drugs and Drug Addiction
<b>EMT</b>	endocannabinoid membrane transporter
<b>ERK1/2</b>	extracellular signal-regulated kinase -1 and -2
<b>EtNH<sub>2</sub></b>	ethanolamine
<b>EU</b>	European Union
<b>FAAH</b>	fatty acid amide hydrolase
<b>FAN</b>	neutral sphingomyelinase activation
<b>FBS</b>	fetal bovine serum
<b>FBS-CT</b>	fetal bovine serum-charcoal treated
<b>GPCR</b>	G-protein-coupled receptors
<b>GPR55</b>	G protein-couple orphan receptor 55
<b>HU</b>	Hebrew University
<b>JNK</b>	Jun N-terminal kinase

<b>JWH</b>	John W. Huffman
<b>LDH</b>	lactate dehydrogenase
<b>LSD</b>	lysergic acid diethylamide
<b>MAGL</b>	monoacylglycerol lipase
<b>MAPK</b>	mitogen-activated protein kinases
<b>MDMA</b>	3,4-methylenedioxy-methamphetamine
<b>MMP</b>	matrix metalloproteinase
<b>MMP-9</b>	matrix metalloproteinase 9
<b>mtDNA</b>	mitochondrial DNA
<b>MTT</b>	[3-(4,5-dimethylthiazol-2-yl)-2,5-dipheniltetrazolium bromide]
<b>NADA</b>	N-arachidonoyl-dopamine
<b>NAPE</b>	N-acyl phosphatidylethanolamine
<b>NAPE-PLD</b>	N-acylphosphatidylethanolamine-specific phospholipase D
<b>NAT</b>	N-acyltransferase
<b>nDNA</b>	nuclear DNA
<b>NPS</b>	new psychoactive substances
<b>P4</b>	progesterone
<b>PG</b>	prostaglandins
<b>PGE2</b>	prostaglandin E2
<b>PI</b>	phosphatidylinositol
<b>PI3K</b>	phosphatidylinositol- 3-kinase
<b>PKA</b>	protein kinase A
<b>PKB/Akt</b>	protein kinase B
<b>PLC</b>	phospholipase C
<b>PLC-β</b>	phospholipase C-β
<b>PPAR</b>	peroxisome proliferator-activated receptor
<b>PPAR-γ</b>	peroxisome proliferator-activated receptor-γ
<b>RLU</b>	relative light luminescence units
<b>RT-PCR</b>	real-time polymerase chain reaction
<b>SM</b>	sphingomyelin
<b>SMase</b>	sphingomyelinase
<b>SPT</b>	serine palmitoyltransferase
<b>SRB</b>	sulforhodamine B
<b>TRPV<sub>1</sub></b>	transient receptor potential vanilloid 1
<b>UPLC-MS/MS</b>	ultra-performance liquid chromatography-mass spectrometry

## Chapter I - Introduction

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# 1. Cannabinoids

## 1.1 Historical Background

The cultivation of the *Cannabis* plant with medicinal purposes was originally described in ancient China, where it was considered effective treating pain (1, 2). However, in the 19<sup>th</sup> century, the research of the chemistry and pharmacology of *Cannabis* constituents increased, culminating in 1964 with the discovery of its major psychoactive component, the  $\Delta^9$ -tetrahydrocannabinol ( $\Delta^9$ -THC) (3, 4).

*Cannabis* is a flowering plant with three species: *sativa*, *indica* and *ruderalis*, with the plants being assorted according to the levels of  $\Delta^9$ -THC they contain. It is easily cultivated outdoors in most parts of the world and anywhere indoors under proper conditions. From a *Cannabis* seedling, distinct products can be obtained: herbal *Cannabis* (marijuana) and *Cannabis* resin (hashish). The psychoactive effects can cause changes in memory, perception, psychomotor activity and consciousness, which are probably the reasons why *Cannabis* is still one of the most popular recreational drugs in Western societies, even though its use is forbidden in some countries (2, 5-7).

Cannabinoids were, until recently, characterized as terpenophenolic constituents of *Cannabis sativa* plant. Currently, cannabinoids can be divided in three types: the plant-derived natural products, the “phytocannabinoids”; the synthetic cannabinoids; and the animal-derived, the “endocannabinoids” (8, 9). Thus, cannabinoids include all the molecules that activate cannabinoid receptors (9, 10).

## 1.2 Cannabinoid Receptors

The discovery of  $\Delta^9$ -THC increased the study of cannabinoid pharmacology, which culminated in the discovery of cannabinoid receptors (CBR). The first cannabinoid receptor to be described was the cannabinoid receptor type 1 (CB<sub>1</sub>), which was cloned after screening of previously characterized orphan G-protein-coupled receptors (GPCRs) for their affinity for  $\Delta^9$ -THC, followed by a second one, the cannabinoid receptor 2 (CB<sub>2</sub>) identified by homology cloning (11-13).

The CB<sub>1</sub> and CB<sub>2</sub> are transmembrane receptors coupled to protein G and present different sequences of amino acids and tissue distribution. The CB<sub>1</sub> seems to be mainly expressed in the brain, although it can be found in peripheral tissues such as the placenta, fetal membranes, myometrium (uterus) and testes (1, 11, 14, 15). The CB<sub>2</sub>

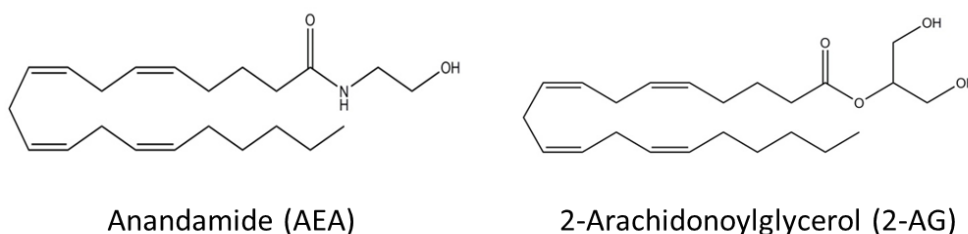
receptor is primarily expressed by the immune system cells, even though it is expressed in the central nervous system and also in peripheral tissues (12, 16-18).

The cannabinoid receptor binding may cause the activation of different signalling pathways (11, 12, 19, 20). Compounds with high binding affinity for cannabinoid receptors are considered direct ligands that can act as agonist or inverse agonist. Indirect ligands can exert their effects by targeting key proteins that regulate tissue levels of endocannabinoids or allosteric sites of CB<sub>1</sub> (9).

### 1.3 Endocannabinoids

Endocannabinoids were discovered *in vivo* after the identification of  $\Delta^9$ -THC. The two endocannabinoids whose biological activity has been better characterized are *N*-arachidonylethanolamine (anandamide; AEA) and 2-arachidonoylglycerol (2-AG) (21). Both chemical structures are represented in Figure 1. Like  $\Delta^9$ -THC, AEA and 2-AG, bind to the extracellular site of the CB<sub>1</sub> and CB<sub>2</sub> (11, 12, 22, 23). Despite these two well-studied endocannabinoids, there are also the 2-arachidonyl-glycerol ether (noladin-ether) (24), the *N*-arachidonoyl-dopamine (NADA) (25, 26) and virodhamine (27), whose metabolism and pharmacological activity are yet to be fully investigated (13). More recently, it was identified the first potent endogenous antagonist of CB<sub>1</sub>, a nonapeptide known as hemopressin, found in numerous tissues including the brain, but more studies of its metabolism and regulatory levels are needed (13).

AEA, an endogenous eicosanoid derivative, is released by the cleavage of the phospholipid precursor *N*-acyl phosphatidylethanolamine (NAPE). The synthesis of this precursor is catalysed by the *N*-acyltransferase (NAT), whereas a specific phospholipase D, the *N*-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) is responsible by the release of AEA from NAPE.



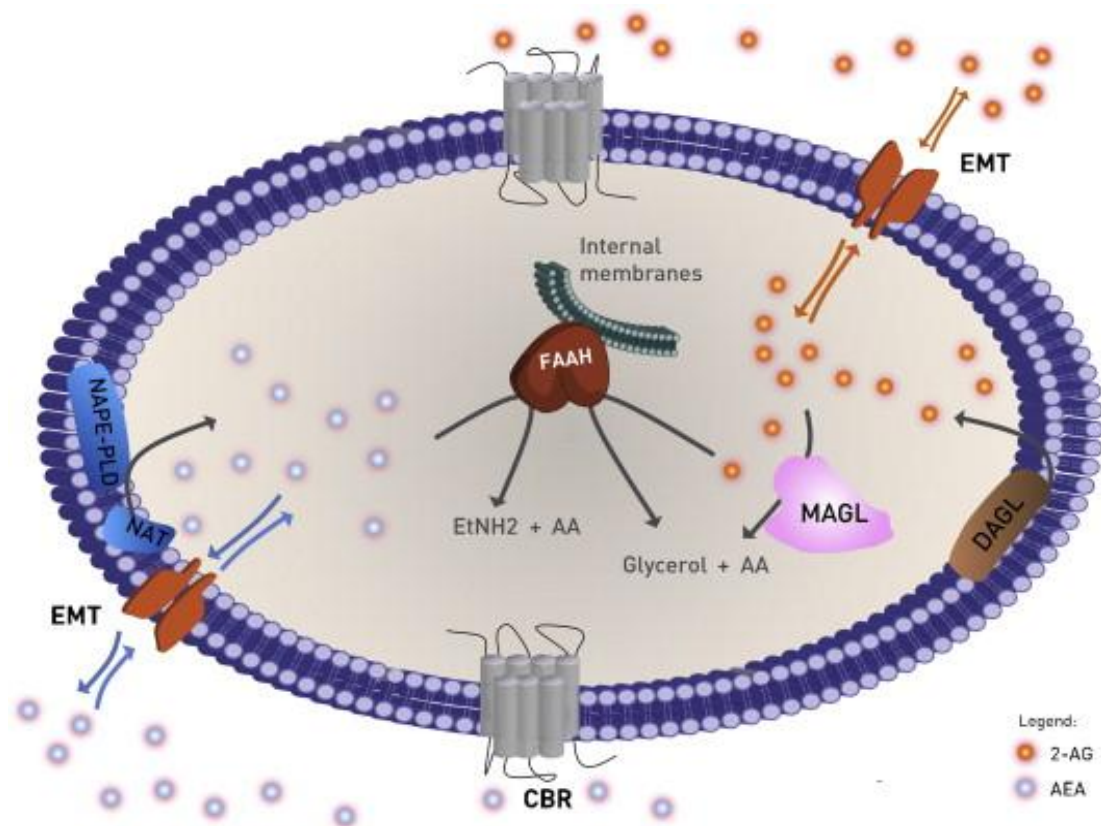
**Figure 1 – The chemical structures of the major endocannabinoids.** AEA and 2-AG have different biosynthetic pathways, although both are produced on demand by cleavage of membrane phospholipids precursors.

2-AG is produced by the cleavage of a membrane phospholipid, by phosphatidylinositol (PI) and, sometimes, from the hydrolysis of phosphatidic acid (28) to produce 1,2-diacylglycerol (DAG). The formation of this compound is catalysed by phospholipase C (PLC), that after conversion via a membrane-bound diacylglycerol lipase (DAGL), gives rise to 2-AG.

These endocannabinoids are suppressed by enzymatic hydrolysis of their amide and ester bonds by a membrane fatty acid amide hydrolase (FAAH) and/or by a membrane-associated serine hydrolase, monoacylglycerol lipase (MAGL) (29-31). The AEA hydrolysis is mainly mediated by FAAH to generate ethanolamine (EtNH<sub>2</sub>) and arachidonic acid (AA), whereas 2-AG is degraded to AA and glycerol by MAGL or also by FAAH, though the former is considered the main degrading enzyme.

AEA binds to both cannabinoid receptors and, like  $\Delta^9$ -THC, it has lower efficacy and affinity for CB<sub>2</sub> than for CB<sub>1</sub>. AEA can also activate other receptors, such as the orphan G protein-couple receptor 55 (GPR55), the transient receptor potential vanilloid 1 (TRPV<sub>1</sub>) and the peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) (2, 32-34). 2-AG affinity and efficacy to CB<sub>1</sub> and CB<sub>2</sub> is higher than AEA and it can also interact with PPAR- $\gamma$ , but not with TRPV<sub>1</sub> or GPR55 (21, 35).

The AEA and 2-AG pharmacological effects depend on their concentration in the extracellular space, which is limited by the transport through the cell membrane, either by passive diffusion or by endocannabinoid membrane transporter (EMT) (22, 36). The endocannabinoids, together with the respective metabolic enzymes and cannabinoid receptors constitutes the endocannabinoids system, a lipid signalling network in which different proteins can control or modulate various physiological and pathophysiological processes (9, 37, 38). The endocannabinoid system elements are represented in Figure 2.

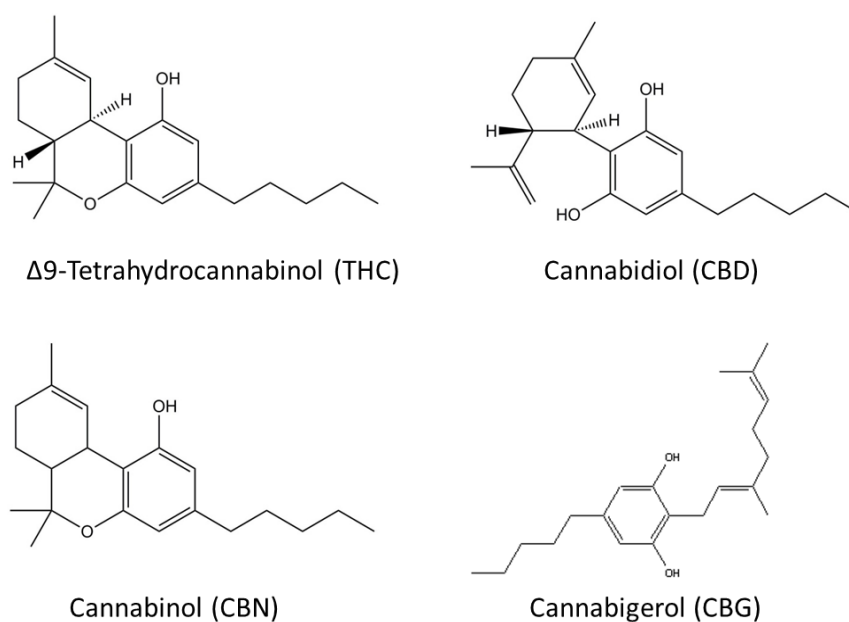


**Figure 2 – The components of the endocannabinoids system.** The biosynthesis of AEA (blue circles), is catalysed by NAT followed by NAPE-PLD, and the biosynthesis of 2-AG (orange circles) occurs through DAGL. Endocannabinoids are transported in both directions through cell membrane by diffusion or selective transport via the EMT. In the extracellular space can interact with CBR or be internalized and degraded. AEA is hydrolyzed by FAAH into EtNH<sub>2</sub> and AA. 2-AG is hydrolyzed through MAGL or FAAH into glycerol and AA. Adapted from (21).

## 1.4 Phytocannabinoids

Plant derived natural products that are able to directly interact with cannabinoid receptors or share chemical similarities with cannabinoids, or both, are known as phytocannabinoids (9).

There are more than 60 naturally occurring substances found in *Cannabis sativa* such as  $\Delta^9$ -THC, cannabidiol (CBD), cannabinol (CBN) and cannabigerol (CBG) (Figure 3). Besides its psychoactive properties,  $\Delta^9$ -THC also presents muscle relaxant, analgesic and antispasmodic characteristics. CBD is a nonpsychotropic constituent of *Cannabis*, being a versatile pharmacological agent, causing some of the effects of  $\Delta^9$ -THC, such as sedation, and it has the ability to act as an antagonist of CB<sub>1</sub>, evidenced by reducing  $\Delta^9$ -THC psychomimetic effects. CBN, like CBD, has a low affinity for the cannabinoid receptors and causes sedation when combined with  $\Delta^9$ -THC (9, 39-41).

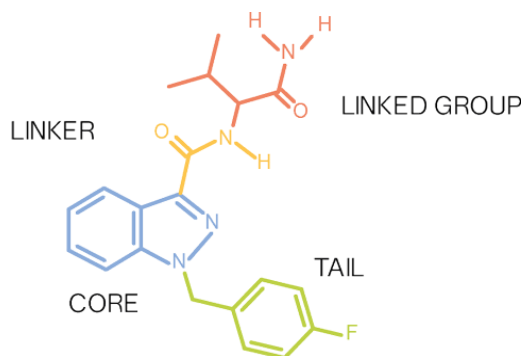


**Figure 3 – The chemical structures of the main phytocannabinoids.** The psychoactive component,  $\Delta^9$ -THC, and other natural substances found in *Cannabis* plant, such as CBD, CBN and CBG.

## 1.5 Synthetic cannabinoids

Synthetic cannabinoids began being developed in the 1960s after the discovery of  $\Delta^9$ -THC, to help investigate the endocannabinoid system pharmacology and its potential therapeutic effects, emphasizing in the analgesic and anti-inflammatory properties and eliminating the psychoactive effects. The majority of these new synthetic cannabinoids were synthesized in four laboratories: John W. Huffman, Alexandros Makriyannis, Pfizer and Hebrew University, and now are known by their initials (JWH, AM, CP and HU) (42-45).

The structure of the major synthetic cannabinoids can be divided into four parts: the core and substituents, the link section, the ring and substituents and the tail section (46) (Figure 4).



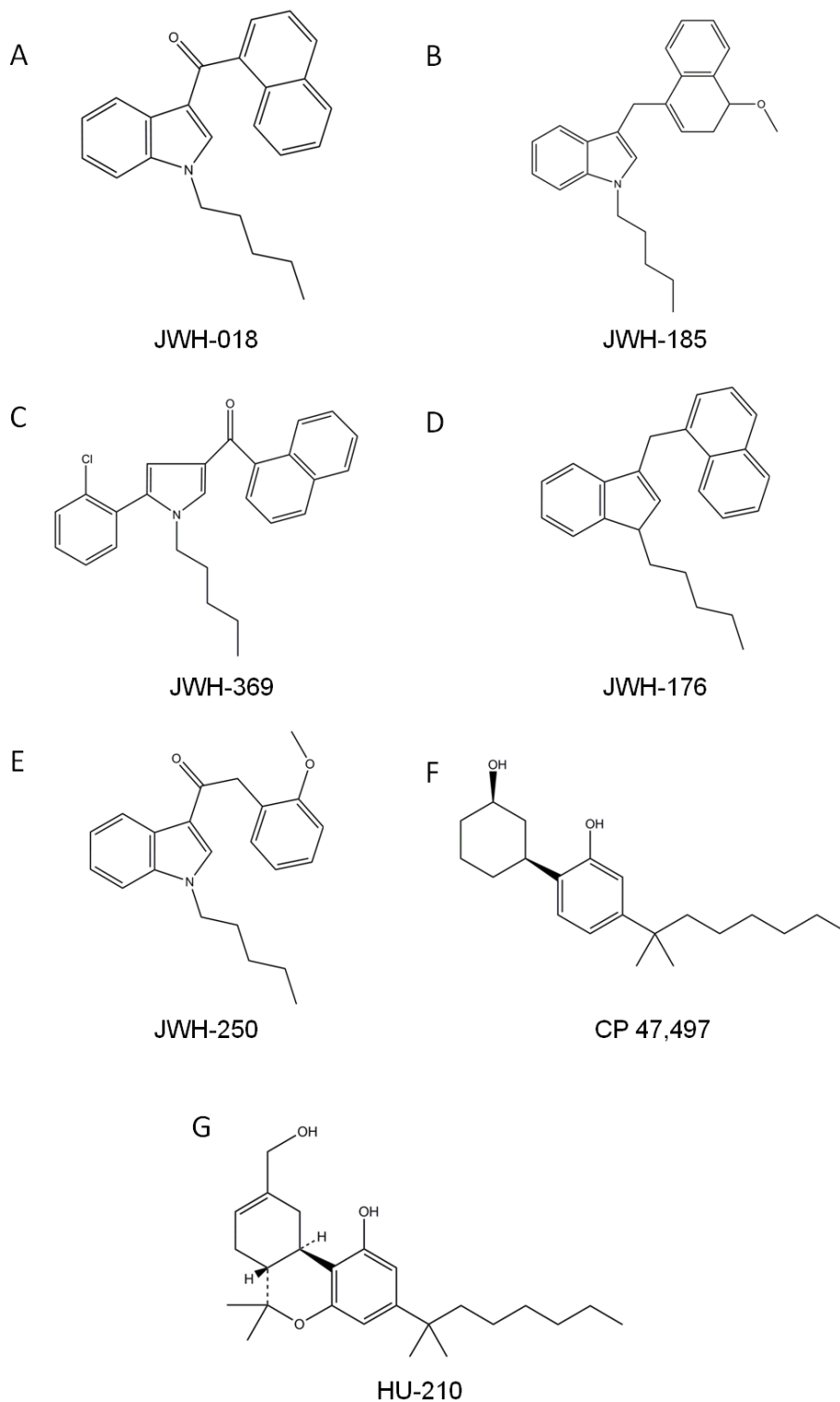
**Figure 4 – The general chemical structure of the main synthetic cannabinoids.** The majority of synthetic cannabinoids follow this general structure. From (46).

These new substances can be classified by their chemical structure, as suggested by Howlett *et al.* and Thakur *et al.* (16, 47) and the Advisory Council on the Misuse of Drugs and the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) (48, 49) divides synthetic cannabinoids into seven groups: naphthoylindoles, naphthylmethyloindoles, naphthoylpyrroles, naphthylmethyloindenes, phenylacetylindoles, cyclohexylphenols and classical cannabinoids (Table 1).

**Table 1 – Classification of synthetic cannabinoids.** The generic definition of the seven major groups of synthetic cannabinoids and respective examples.

Cannabinoids	Generic definition	Example	Reference
<b>Naphthoylindoles and Naphthylmethyloindoles</b>	Any compound structurally derived from 3-(1-naphthoyl)indole or 1H-indol-3-yl-(1-naphthyl)methane by substitution of the indole ring	JWH-018 (Figure 5A); JWH-185 (Figure 5B)	
<b>Naphthoylpyrroles</b>	Any compound structurally derived from 3-(1-naphthoyl)pyrrole by substitution of the pyrrole ring	JWH-369 (Figure 5C)	
<b>Naphthylmethyloindenes</b>	Any compound structurally derived from 1-(1-naphthylmethyl)indene by substitution of the indene ring	JWH-176 (Figure 5D)	(48, 49)
<b>Phenylacetylindoles</b>	Any compound structurally derived from 3-phenylacetylindole by substitution of the indole	JWH-250 (Figure 5E)	
<b>Cyclohexylphenols</b>	Any compound structurally derived from 2-(3-hydroxycyclohexyl)phenol by substitution of the phenolic ring	CP 47,497 (Figure 5F)	
<b>Classical Cannabinoids</b>	Any compound structurally related to $\Delta^9$ -THC and other constituents of cannabis	HU-210 (Figure 5G)	

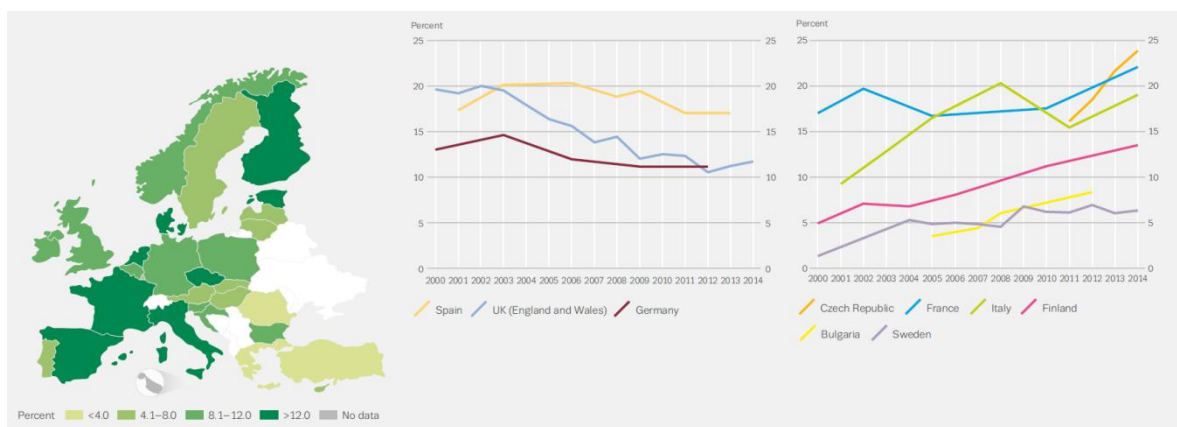
These new substances have psychoactive effects as  $\Delta^9$ -THC. Also the chemical properties are similar, sharing with  $\Delta^9$ -THC high lipophilicity. Nonetheless, though the ability to bind to the cannabinoid receptors is higher, these are not structurally similar to classic cannabinoids (48). In fact, some of these synthetic cannabinoids have even higher affinity to the receptors than  $\Delta^9$ -THC due to their full agonist capability and active metabolites. In addition, the possibility that they may also interact with other receptors could explain why they are more potent than  $\Delta^9$ -THC (40, 50-52). This can cause a variety of symptoms that can go from mild to moderate, like nausea, emesis and agitation to more severe such as cardiac arrhythmias, psychosis, respiratory depression to coma and even death (51, 53, 54).



**Figure 5 – The chemical structures of synthetic cannabinoids.** Examples of each one of the seven major classes of synthetic cannabinoids. A – Naphthylindoles; B – Naphthylmethylindoles; C – Naphthylpyrroles; D – Naphthylmethylindenes; E – Phenylacetylindoles; F – Cyclohexylphenols; G - Classical Cannabinoids.

## 1.6 Cannabinoids and drug abuse

*Cannabis* use, in such different forms, is prevalent in every European Union (EU), with an upward trend in young adults, as seen in Figure 6. A recent analysis made by the EMCDDA revealed that over a quarter of 15- to 64-years olds in EU is thought to have consumed the drug at least once in their lifetime. On the other hand, the use amongst young individuals (15-34 years) within the last 12 months is about 13,3 % or 16.6 million EU individuals, which makes *Cannabis* derivatives the most widely consumed illicit drug by both adults and youths in the world (55).

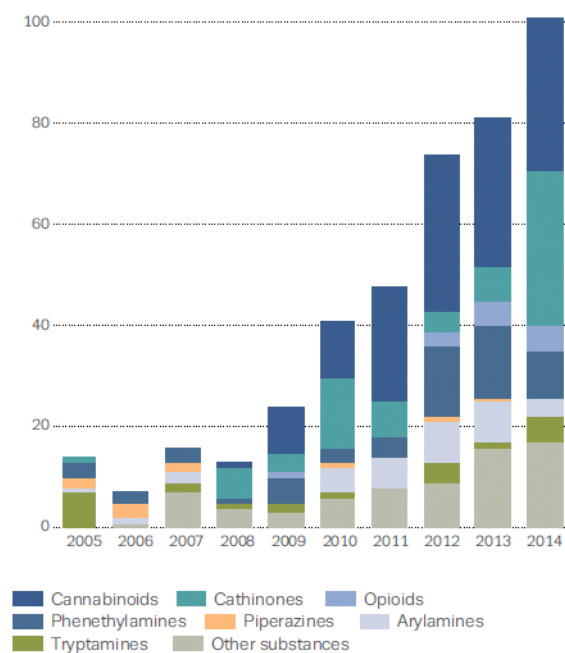


**Figure 6 – Cannabis use in Europe by young adults (15-34).** In the left, the countries with significant statistically trends in *Cannabis* use are represented and, in the right is the most recent data (2015) about *Cannabis* use in Europe. It was observed an increase in consumption in France, Italy, Finland, Czech Republic and Sweden, but with different prevalence. However, Spain, Germany and the United Kingdom showed a decrease in *Cannabis* use in the last decade. Bulgaria, with less data available, has upward trends. From (55).

In the last decade the availability of new psychoactive substances (NPS) has increased in the European market (Figure 7). These new drugs, natural or synthetic, are produced to mimic effects of controlled substances and are known as “legal highs”. On the other hand, the rapid modification of chemicals, the unknown quantity of drug used and the unknown herbal components that are difficult to detect in a typical drug screen, and the lack of reference samples to make detection possible, makes these new synthetic cannabinoids potentially dangerous to human health (51, 52).

The majority of these new substances are sold as alternatives to cannabis, heroin, cocaine, amphetamines, 3,4-methylenedioxy-methamphetamine (MDMA), benzodiazepines and lysergic acid diethylamide (LSD), and can be found in either the Internet as in specialized shops – head shops – being advertised as “research chemicals”, “food supplements” or as “not intended for human consumption” (56).

Synthetic cannabinoids is the largest group of NPS with more than 160 different synthetic cannabinoids identified till December 2015. Usually, these substances are produced in China or India and arrive in Europe where they are sold as chemical powders or herbal mixtures similar to potpourri or incense that can be smoked, and also used as infusions or even in e-cigarettes. The common street names of the new drugs are: Spice, K2, Bhang, Yucatan and Mojo. These are not marijuana, but really a combination of various synthetic compounds that can interact with the cannabinoid receptors and induce marijuana-like effects (46, 51, 52, 56).

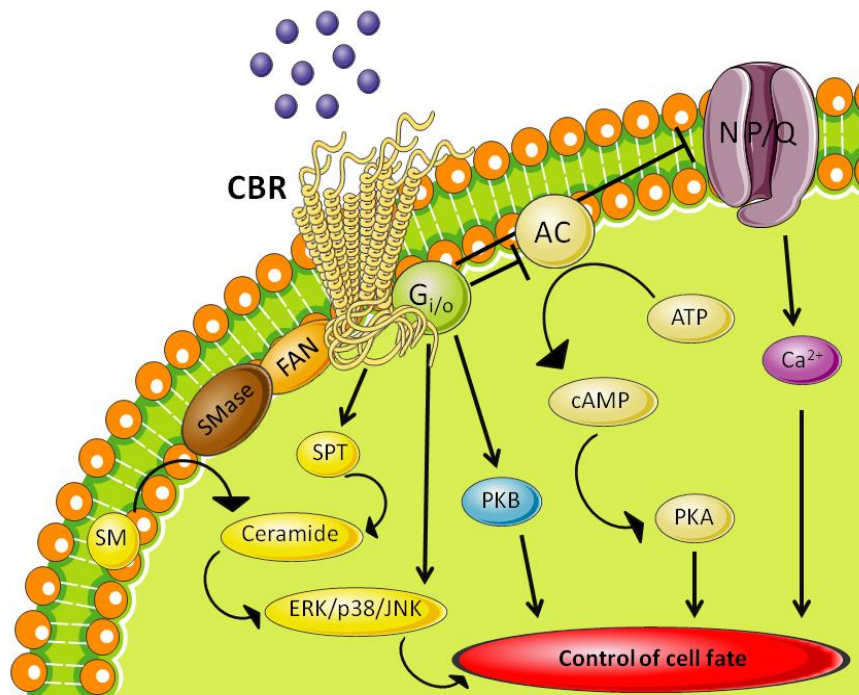


**Figure 7 – New psychoactive substances notified to the EU Early Warning System.** Number and categories of new psychoactive substance reported to the Eu Early Warning System increased 25% compared to 2013, including 30 new synthetic cannabinoids. From (57).

## 2. Cannabinoids and signalling pathways

Cannabinoids exert their effects by binding to specific cannabinoid receptors, usually activated by the endogenous ligands, the endocannabinoids. These receptors are able to regulate several central nervous system functions, such as neuronal development, neuromodulatory processes and several peripheral physiological functions, for instance cardiovascular, respiratory, digestive and reproductive systems. Additionally, they can also modulate proliferation, motility, adhesion and apoptosis of cells (58, 59).

Both cannabinoid receptors belong to the GPCR superfamily, and more specific, the rhodopsin subfamily of GPCRs, and are characterized by 7-transmembrane domains, an extracellular NH<sub>2</sub> terminus and an intracellular COOH terminus (18). They are also associated with G proteins of the Gi/o family (Gi 1, 2 and 3; Go 1 and 2) (11, 12). The activation of cannabinoid receptors leads to inhibition of the adenylyl cyclase (AC) enzyme, activation of mitogen-activated protein kinases (MAPKs) and modulation of ion channels, in this case only through activation of CB<sub>1</sub>, as represented in Figure 8.



**Figure 8 – Cannabinoid signalling pathways after activation of the cannabinoid receptors coupled to G protein.** This leads to inhibition of adenylyl cyclase, inducing a decrease in cAMP levels, which translates in a decrease of PKA. The activation also activates MAPKs, such as PKB, and induces an increase in ceramide levels, through sphingomyelin hydrolysis by SMase, which regulates ERK, p38 MAPK and JNK. Additionally, CB<sub>1</sub> can negatively couple to N- and P/Q-type voltage-operated Ca<sup>2+</sup> channels and induce an increase in intracellular Ca<sup>2+</sup>.

Even before cannabinoid receptors were described, it was already known that cannabinoids caused inhibition of the AC, which leads to a reduction of intracellular cyclic

AMP (cAMP) levels. cAMP is responsible for numerous roles inside the cell, such as the phosphorylation of proteins through protein kinase A (PKA). Essentially, decreased cAMP in cells that express CB<sub>1</sub> and CB<sub>2</sub> will most likely culminate in an inhibition of function of those cells (59, 60).

Both cannabinoid receptors are also able to regulate the phosphorylation and activation of the family of mitogen-activated protein kinase (MAPK). Activation of extracellular signal-regulated kinase-1 and -2 (ERK1/2), also known as p42/p44 MAPK, by CB<sub>1</sub> receptors, requires mobilization of the phosphatidylinositol-3-kinase (PI3K) and protein kinase B (PKB/Akt) which results in the phosphorylation of Raf-1. p38 MAPK and c-Jun N-terminal kinase (JNK) can also be regulated by these receptors (58, 59, 61).

The sphingolipid-metabolising pathways also seem to be modulated by cannabinoids. Neutral sphingomyelinase activation (FAN) is the possible adaptor protein responsible for the hydrolysis of sphingomyelin (SM) via sphingomyelinase (SMase) activation. This activation can cause a peak of ceramide accumulation by enhanced synthesis de novo via serine palmitoyltransferase (SPT), which is important in the regulation of metabolic functions, such as induction of apoptosis, via a ERK/p38/JNK (59, 62).

The activation of cannabinoid receptors is also responsible for the regulation of other key proteins, including the focal adhesion kinase that plays a part in synaptic plasticity (63); activation of phospholipase A<sub>2</sub>, via MAPK, inducing the arachidonic acid cascade and production of prostaglandins (64); is also able to decrease the synthesis of growth factor receptors in some tissues, what could explain the antiproliferative effects of cannabinoids (65).

However, cannabinoid receptors agonists have different affinities to agonists and the activated signalling pathways can differ from cell to cell, which may account to the various cells responses obtained upon binding to receptors (60). CB<sub>1</sub>, additionally, can negatively couple to N- and P/Q-type voltage-operated Ca<sup>2+</sup> channels and positively couple to A-type and inwardly rectifying K<sup>+</sup> channels, and through G protein-dependent activation of phospholipase C-β (PLC-β) induce an increase in intracellular Ca<sup>2+</sup> (58).

The involvement of different mitogen- and stress- activated protein kinase cascades in the control of cell fate, and the evidences that cannabinoid receptors interact with these signalling pathways, show that cannabinoids may play an important role in the cell survival/death decision (59).

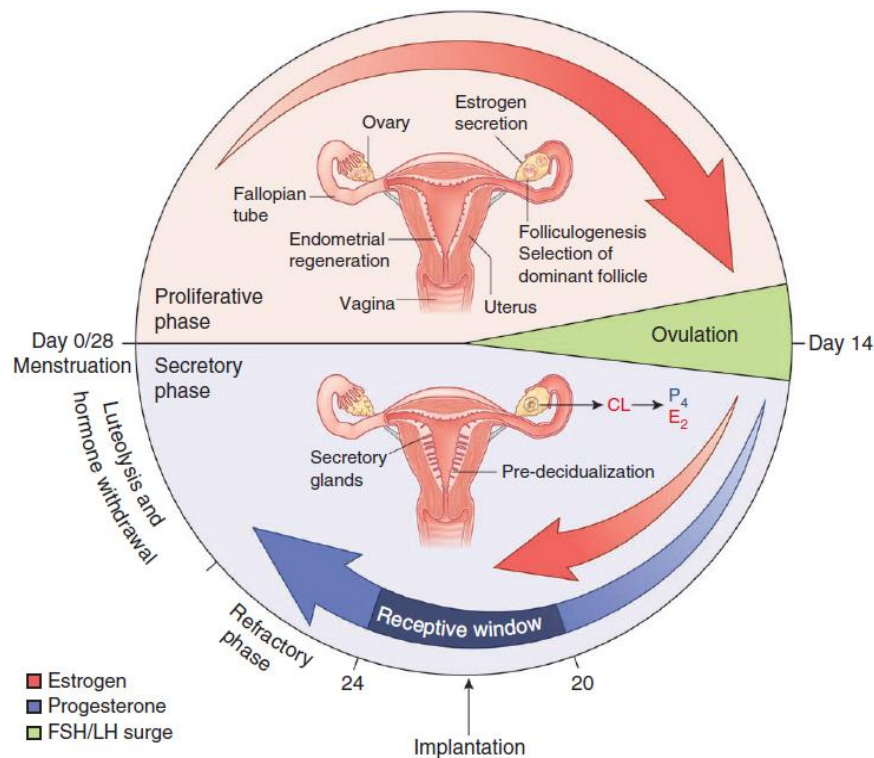
## 3. Cannabinoids and Pregnancy

### 3.1 The endometrium and menstrual cycle

The endometrium is composed of epithelial and mesenchymal elements that suffer different morphological alterations during the menstrual cycle. The superficial epithelium is formed of epithelial cells, and the mesenchyme, or stroma, contains predominantly fibroblasts. It can also be divided into two different layers based on morphological and physiological characteristics, such as, the basalis, the deepest layer with a constant morphology during the menstrual cycle, and the functionalis, the superficial layer, where most of the alterations occur to allow the blastocyst implantation (66).

The two primary steroid hormones are estrogen (E2) and progesterone (P4), which are mainly produced in ovaries and are responsible for the cyclic changes in morphology and function during the menstrual cycle. Due to fluctuations of these hormones, menstrual cycle can be divided into three phases: in the proliferative phase, the levels of estrogen increase, inducing the thickening of the functionalis layer, by proliferation of epithelial, stromal and endothelial cells; in the secretory phase, which occurs after ovulation, the differentiation of the functional layer starts, due to the influence of progesterone, produced by the corpus luteum. It is at the end of this phase that decidualization begins. When no implantation occurs, the levels of estrogen and progesterone decrease, due to the decay of the corpus luteum, leading to shedding of the functional layer and consequent menstrual bleeding, which characterises the menstrual phase (Figure 9) (66, 67).

Studies suggest that menstrual bleeding is a secondary effect of extracellular matrix (ECM) proteolysis (68) and, although numerous proteinases and enzymes expressed in human endometrium seem to participate in menstruation, the most important enzymes for direct ECM proteolysis is the matrix metalloproteinases (MMP) family. MMP are Zn-dependent proteinases capable of degrading the ECM and regulate several signalling molecules like cytokines and chemokines. The endometrium has plenty of MMP transcripts and proteins, particularly during menstruation, and of all MMP studied only MMP-13 was not detected in the human endometrium. The major regulator of MMP expression is progesterone (66).



**Figure 9 – The menstrual cycle in humans.** The 28- to 30 days menstrual cycle begins with menstruation, followed by the proliferative phase (red), which is under the influence of increasing estrogen levels, produce by growing ovarian follicles, which induce the proliferation of the endometrium. By midcycle, a surge of gonadotropins (LH and FSH) occur, leading to ovulation on day 14 (green). In the luteal phase, the thickening of the endometrium, formation of the corpus luteum and subsequent secretion of progesterone is the preparation for implantation of the blastocyst. Decidualization starts and the estrogen levels increasing overlapping on progesterone define the “implantation window”. In the absence of pregnancy, the window progresses to a refractory phase, leading to luteolysis, hormone withdrawal and menstruation (69).

### 3.2 The endometrium and pregnancy establishment

A successful pregnancy, which is the nurturing of the offspring within the womb and the production of a live birth, depends on a sequence of well-timed events since fertilization to parturition. It all starts with the fertilization of an egg by a spermatozoid, the zygote, in the Fallopian tube. Then, it travels through the oviduct, suffering multiple divisions and developing into a blastocyst, until it reaches the uterus, where it will adhere to the decidualized endometrium. This sequence of events starts with proper establishment of a receptive endometrium and a competent blastocyst (67).

Implantation is an intrusive process and occurs during the mid-secretory phase, in a 2- to 4 day period called “implantation window”, during which the endometrium is prepared by estrogen and progesterone. It can be divided into three stages: apposition, adhesion and invasion. During apposition, the first contact between blastocyst and endometrium is made and the location for implantation is found. The invasion of the

endometrium by the blastocyst is when the trophoblast cells infiltrate the endometrial epithelium and into the endometrial stroma until reaching blood vessels (67, 70).

The uterine endometrium is prepared every month for the possible implantation of a blastocyst and this process is known as decidualization. The rapid proliferation of epithelial and endometrial stromal cells is followed by the differentiation of the glandular epithelium into a highly secretory state. Ultimately, the fibroblast stromal cells differentiate into enlarged and glycogenic rich cells with a polygonal morphology, which characterises the decidual cells (71, 72). It also involves a tightly regulated expression of specific adhesion molecules, growth factors and cytokines (72, 73).

Decidua can be classified into three types relative to the developing conceptus: the decidua basalis, the region beneath the site of implantation; decidua capsularis, the region over the developing conceptus, and the remaining is the decidua vera or decidua parietalis (67). Decidua is mostly comprised of decidualized endometrial stromal cells, but it also has other cell types, like hematopoietic cells, macrophages, uterine natural killers and monocytes, associated with blood vessels, and uterine glands (74). To promote the adhesion of the trophoblast cells, the decidua forms a dense cellular matrix that, at the same time, helps to limit the aggressive invasion of those cells. This invasion depends upon proteolytic degradation and remodelling of the decidual extracellular matrix, which is accomplished by the MMPs secreted by the trophoblast cells, and by decidualized stromal cells, as shown by recent studies (75). Thus, decidualization results in these cells acquiring biochemical and cellular properties that facilitates them support implantation (67).

If the decidual process does not occur normally, pregnancy complications may happen, such as miscarriage, preeclampsia, foetal growth restriction and preterm labour (76). Although it was already shown that implantation and even successful pregnancies can occur without the decidua (77, 78), it is the successful decidualization that is important for implantation and success of normal pregnancies (73).

On the other hand, development and function of the placenta is the key to an adequate exchange of nutrients and gas between mother and foetus, which is intimately dependent on endometrial environment.

### **3.3 Endocannabinoids and pregnancy**

It was already described that endocannabinoid system is implicated in several physiological and pathological processes, such as the reproductive system (79).

Evidences of their role in decidualization, embryo development and implantation were already shown (80).

AEA, 2-AG and respective metabolic enzymes were found in the mouse endometrium and their concentration varies during the oestrus cycle (81). In humans AEA levels increase in the menstrual cycle at the time of ovulation, the cannabinoid receptors are present in the ovaries, and NAPE-PLD is expressed by granulosa and theca cells, meaning that AEA is more likely to be produced by granulosa of growing cells than by oocytes. In addition, considering that follicular AEA concentrations are related to follicular size and that are lower in follicles from which oocytes were retrieved, it is suggested that AEA is involved in the maturation of follicles and oocytes (82-84).

Additionally, mouse embryos express cannabinoid receptors and high doses of AEA can induce a dose-dependent suspension of embryo development, and even inhibition of blastocyst hatching (85). Mouse embryos with cannabinoid receptor abnormalities also present delayed development (86). Furthermore, it was shown in mice that CB<sub>1</sub> is important to assure embryo transport through the oviduct as CB<sub>1</sub> knockout mice showed high levels of abortion (87).

AEA levels are higher in the endometrium than in other reproductive tissues (88, 89). Both cannabinoid receptors, NAPE-PLD and FAAH proteins were also detected in decidua from pregnant women (76). The presence of cannabinoid receptors, endocannabinoids and respective metabolic enzymes in the female reproductive system, with evidences of their presence in maternal and foetal tissues suggests that these are involved in decidualization of the endometrium, oviduct transport and implantation of the embryo (76).

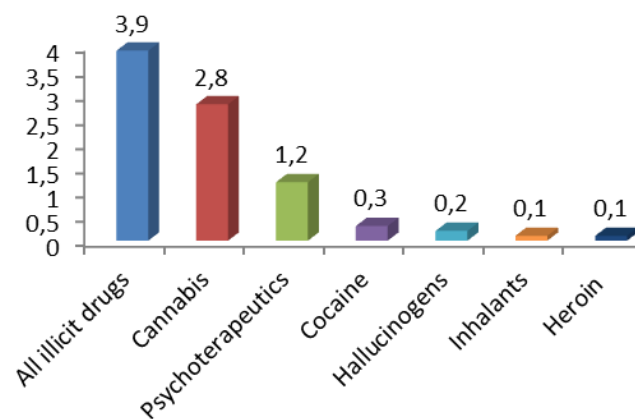
### **3.4 Cannabinoids use by pregnant women**

*Cannabis* is the most consumed illegal substance by pregnant women in Western societies, with nearly 3 % of reported cases by pregnant women in the United States (Figure 10). The lipophilic characteristics of cannabinoids, allows them to cross cell barriers, including transplacental membranes (90). This explains the presence of *Cannabis* metabolites in many human tissues (91), such as the placenta, amniotic fluid and foetus (92), making the study of its effects on reproduction important (7).

Most studies show that continuous use of *Cannabis* is associated with reduced fertility and failed pregnancies. It is also shown that *Cannabis* is correlated with congenital foetal abnormalities and intrauterine growth restriction (93-95), leading to the general conclusion that maternal use of *Cannabis* during pregnancy affects birth outcome and

foetal development. However, contradictory results have also been published, as some studies show that infants exposed to *Cannabis* during pre-natal life did not show increased risks of birth defects (95-97).

The studies to discover the full extent of *Cannabis* use during pregnancy are few due to ethics problems that forbid the experiments in human tissues. In addition, the small number of studies that were published are controversial, due to the fact that women that use *Cannabis* frequently also consume other drugs and have adverse lifestyles (98, 99).



**Figure 10 – Illicit drug use in the United States of America by pregnant women.** Approximately 4% of women admitted the use of illicit drugs while pregnant, with *Cannabis* as the most commonly used drug. Data from 2005. Adapted from (100).

The human studies published to date consist in following pregnant women that admitted the usage of substances that may affect the pregnancy outcome. In most of these studies the parameters that were examined were the ones related to foetal growth. In a study with 1690 children, the birth weight was lower for foetus exposed more than three times per week to *Cannabis* (95). In other study the increased risks of low birth weight was two times higher in foetus exposed several times a month to *Cannabis* (101).

One of these studies was the Generation R, that started in 2002 and consisted in assessing the foetal growth in early, mid and late pregnancy using techniques such as femur length, abdominal and head circumference and transcerebellar diameter (102, 103). In the Generation R study, 8880 pregnant women were enrolled and the results showed that foetal growth, especially birth weight, was reduced in mothers that used *Cannabis* in early pregnancy comparing to tobacco users and non-users. In mothers that used *Cannabis* continuously during pregnancy, the birth weight was even lower comparing to only early usage. The head circumference of foetuses was also reduced in early and continuous use during pregnancy compared to non-exposed foetuses. However, the transcerebellar diameter did not show any differences (103).

In *Cannabis*-exposed pregnant women, the use of Doppler showed an increased foetal pulsatility and resistance index of the uterine artery, which could explain the growing deficit in exposed foetuses (104, 105). Other studies also observed influence on the preterm birth rate, which increased with *Cannabis* use (106).

Studies have already shown that  $\Delta^9$ -THC can cross the placenta in animals and humans (107) and that the behaviour of  $\Delta^9$ -THC concentrations in the foetus are similar to the maternal. However, the concentrations in foetal blood were lower than in maternal blood in several species. The ways of intake can affect the concentrations reached in blood. In oral intake the foetal concentrations can be one-tenth of the maternal (108) comparing to the one-third if the intake occurs by intravenous or inhaled  $\Delta^9$ -THC (109, 110). Involving dizygotic twins, the importance of the placenta in the variability of foetal exposure to  $\Delta^9$ -THC was determined, in which large disparities were found regarding cannabinoid concentrations with undetectable levels in one twin and high levels in the other (40, 111).

The influence of the cannabinoid receptors in the placenta was also a subject of study and it was found that the CB<sub>1</sub> receptor is present in all of the placental layers and that the stimulation of this receptor is responsible for the impaired foetal growth (7, 14, 112). Costa et al. studied the *in vitro* effects of  $\Delta^9$ -THC using human trophoblasts (placental epithelial cells) from term placenta (113). The results showed that  $\Delta^9$ -THC impaired the differentiation of cytotrophoblasts into syncytiotrophoblasts, and in low concentrations demonstrated to be cytoprotective, preventing cell death. Both of these findings occurred through a cannabinoid receptor-dependent mechanism. So,  $\Delta^9$ -THC inhibits trophoblast turnover, impairing placental development by disturbing key processes, such as proliferation, differentiation and apoptosis of cytotrophoblasts (113).

**Table 2 - Retrospective studies on *Cannabis* use by pregnant women.** Published studies analysing the impact of  $\Delta^9$ -THC in pregnancy.

Cannabinoid	Effects observed	Reference
$\Delta^9$ -THC	Decreased birth weight	(101)
	Decreased birth weight	(95)
	Decreased foetal growth Decreased head circumference	(103)
	Increased preterm birth rate	(106)

The effects of synthetic cannabinoids in human reproduction are yet to be fully unveiled, but considering that *Cannabis* use among pregnant women seems to cause

adverse effects in placental development and foetal growth (113) and the fact that synthetic cannabinoids bind to cannabinoids receptors with higher affinity and potency should be of concern (2). It is also important to acknowledge that most of the users of synthetic cannabinoids use others synthetic drugs, such as synthetic cathinones, also known as bath salts (114).

## Chapter II - Aims

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The endocannabinoid system is a signalling network that can regulate several physiological processes, including reproduction. Cannabinoid receptors expression in the placenta, foetal membranes and endometrium, along with the endocannabinoids ability to regulate different signalling pathways involved in cell survival and their role in decidualization, embryo development and implantation shows that a tightly controlled endocannabinoid signalling is necessary for a successful pregnancy.

The emergence and increasing use of new psychoactive substances, like synthetic cannabinoids, which share similar psychoactive effects with  $\Delta^9$ -THC with higher affinity for the cannabinoids receptors and possible interaction with endocannabinoid system, present a new matter of concern. Furthermore, lack of the pharmacological information about the synthetic cannabinoids translated in a variety of symptoms, besides the psychoactive effects, can be quite harmful.

It was already observed that the consumption of *Cannabis* before and during pregnancy affect fertility, birth outcome and foetal growth. These findings together with the higher affinity to cannabinoid receptors of synthetic cannabinoids and increased abuse of these new substances during fertile age may represent a risk for fertility and for the normal progression of a future pregnancy.

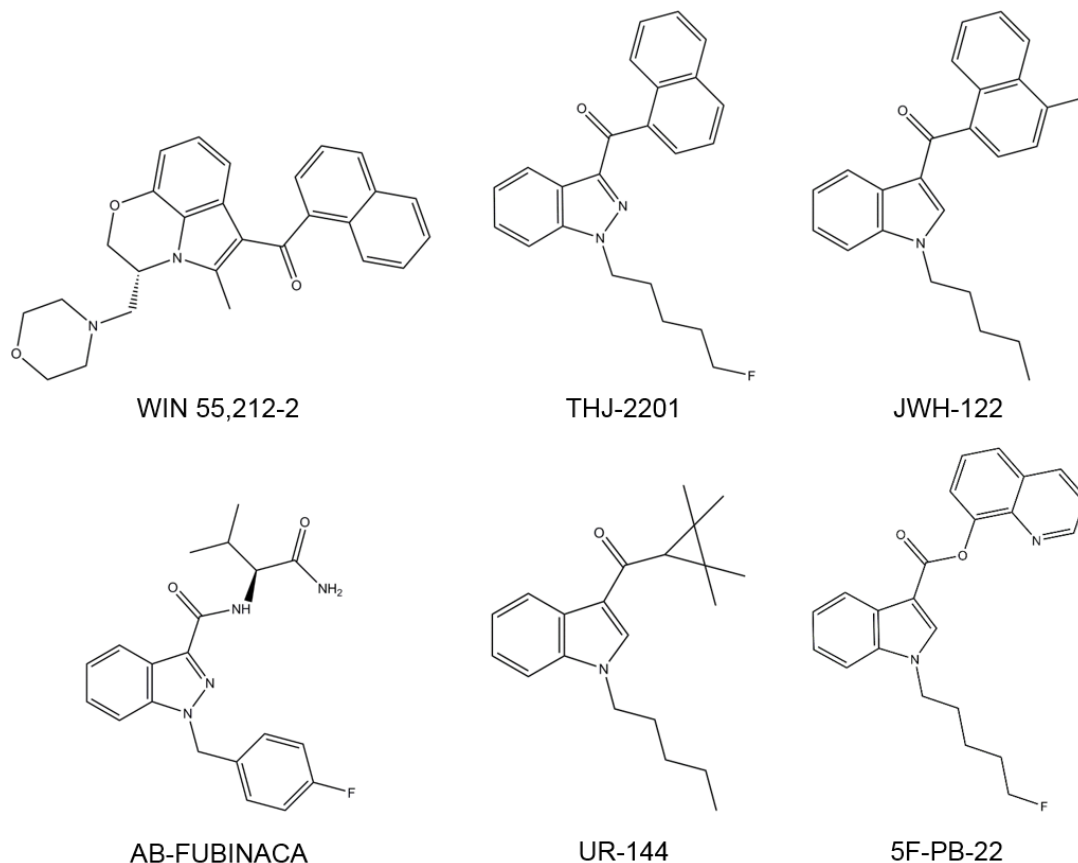
Therefore, with this study we pretend to investigate the influence of different synthetic cannabinoids in endometrial stromal cells, using a cell line model, St-T1b, and primary cultures of human decidual fibroblasts, HdF, hoping to contribute to the understanding of the effects of cannabinoids abuse in endometrium development.

## Chapter III – Materials and Methods

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## 1.1 Materials

Dulbecco's Modified Eagle Medium/F12 (DMEM/F12), foetal bovine serum (FBS), antibiotic–antimycotic solution (penicillin G sodium, streptomycin sulphate and amphotericin B) and trypsin were from Gibco/Invitrogen Corporation, Carlsbad, CA, USA. Trypan blue, activated charcoal, Triton X-100, methylthiazolyldiphenyl-tetrazolium bromide (MTT), Hoechst 33342, 16S and GAPDH primers, Sulforhodamine B sodium salt, gelatin from porcine skin and BrdU antibodies were from Sigma–Aldrich Co. St. Louis, MO, USA. Ethanol and methanol were from Fisher Scientific, Loughborough, UK. DMSO was from VWR, Fontenay-sous-Bois, France. WIN 55,212-2, AM251 and AM630 were from Tocris Bioscience, Bristol, UK. AM281 and broad range molecular markers were from Santa Cruz Biotechnology, CA, USA.  $\Delta^9$ -THC was from Lipomed AG, Swiss. CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit and Caspase-Glo 3/7 were from Promega. Giemsa was from Merck. Bradford assay reagent was from Bio-Rad, Laboratories Melville, NY, USA. TRIsure was from Bioline Reagents Ltd., UK. Prostaglandin E2-d4 was from Cayman Chemical Company. BrdU was from Boehringer Mannheim, Germany. THJ-2201, JWH-122, AB-FUBINACA, UR-144 and 5F-PB-22 (Figure 11) were provided from the Laboratory of Toxicology from the Faculty of Pharmacy of University of Porto, Portugal. Ninety-six-well white plates were from Thermo Scientific, Roskilde, Denmark.



**Figure 11 – Chemical structures of the synthetic cannabinoids used in this work.** These synthetic cannabinoids have different affinities to CB<sub>1</sub> and CB<sub>2</sub>. WIN 55,212-2: CB<sub>1</sub> (1.9 nM); THJ-2201: CB<sub>1</sub> (1.0 nM) and CB<sub>2</sub> (2.6 nM); JWH-122: CB<sub>1</sub> (0.69 nM) and CB<sub>2</sub> (1.2 nM); AB-FUBINACA: CB<sub>1</sub> (0.9 nM) and CB<sub>2</sub> (23.2 nM); UR-144: CB<sub>1</sub> (150 nM) and CB<sub>2</sub> (1.8 nM); 5F-PB-22: unknown.

## 1.2 Cell cultures

### 1.2.1 St-T1b cell line

St-T1b is a telomerase-immortalized human endometrial stromal cell line, well-accepted as an endometrial cell model, kindly provided by Dr Birgit Gellersen from Endokrinologikum Hamburg (Centre for Endocrine, Metabolic and Fertility Disorders), Germany.

St-T1b cells were maintained in culture in DMEM/F12 medium supplemented with 10 % (v/v) of FBS treated with activated charcoal (FBS-CT), 1 % (v/v) of an antibiotic-antimycotic (AB-AM) solution, 1 nM 17- $\beta$ -estradiol and 1  $\mu$ g/ml Insulin and were incubated at 37 °C in 95% air/5% CO<sub>2</sub> humidified atmosphere. After reaching about 80% of confluence, cells were successively sub-cultured to new culture flasks. For this, cells were treated with 0.25 % trypsin/EDTA 1 mM for 4 min at 37 °C, washed with PBS and collected to centrifuge tubes with culture medium containing 10 % FBS-CT (v/v) to

inactivate trypsin. Cells were centrifuged at 180 xg for 5 min at 4 °C. Then cells were counted in a Neubauer chamber and cultured using the cell densities:  $0,5 \times 10^4$  cells/well (final volume 200  $\mu$ L) for 96-well plate,  $2,5 \times 10^4$  cells/well (final volume 500  $\mu$ L) for the 24-well plate and  $5 \times 10^4$  (final volume 2000  $\mu$ L) for the 6-well plate. After adherence (24 hours), the cells were washed with PBS and treated with the compounds, in cell culture medium with 2 % (v/v) FBS-CT.

### **1.2.2 Isolation and primary cultures of human decidual fibroblasts (HdF)**

Term placentas from caesarean section or vaginal delivery, following uncomplicated pregnancies were obtained from Centro Materno-Infantil do Norte – Centro Hospitalar do Porto. All the procedures were reviewed and approved by the Research and Ethics Board of Centro Hospitalar do Porto. After multiple placenta washes, decidua was scraped from the chorionic membrane. The decidual tissue was then dissected and enzymatically dissociated by collagenase (1 mg/ml). Red blood cells were lysed and the resulting suspension was filtered and centrifuged.

The isolated cells were maintained in culture in DMEM/F12 medium supplemented with 10 % (v/v) of FBS treated with activated charcoal (FBS-CT), 1 % (v/v) of an antibiotic-antimycotic (AB-AM) solution, 50 nM  $\beta$ -estradiol and 50 nM insulin and were incubated at 37 °C in 95 % air/5 % CO<sub>2</sub> humidified atmosphere. After 24 h, the non-adherent discarded and the adherent cells were grown to confluence and sub-cultured. The purity of primary HdF was confirmed by immunocytochemical analysis for the cytoskeletal proteins vimentin and cytokeratin-7, a fibroblast and an epithelial cell marker, respectively.

Cells were cultured using the cell densities:  $0,5 \times 10^4$  cells/well (final volume 200  $\mu$ L) for 96-well plate,  $2,5 \times 10^4$  cells/well (final volume 500  $\mu$ L) for the 24-well plate and  $5 \times 10^4$  (final volume 2000  $\mu$ L) for the 6-well plate. After adherence (24 h), the cells were washed with PBS and treated with the compounds, in cell culture medium with 2 % (v/v) FBS-CT.

### **1.3 Cell viability assays**

To access cell viability it was used the tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-dipheniltetrazolium bromide] (MTT) assay and measured the activity of the enzyme lactate dehydrogenase (LDH) in cell culture medium. For that, St-T1b cells were plated in 96-well plates. After 24 h for adhesion, the medium was replaced with DMEM/F12 medium with 2 % (v/v) FBS-CT and 1% AB-AM solution in the presence or absence of the

cannabinoids (0.01-50  $\mu\text{M}$ ), and the cells were incubated for 24 and 48 h. Equimolar concentrations of DMSO, the solvent vehicle of the compounds, was tested did not induced any cell viability alterations. The yellow tetrazole MTT (final concentration: 0,5 mg/mL) was added, and the cells were incubated for 3 h at 37 °C. The formed purple formazan was dissolved in a solution of DMSO:isopropanol (3:1) and spectrophotometrically quantified at 540 nm by using a Multiskan Ascent microplate reader. MTT assay relies on the mitochondrial metabolism for the conversion of the yellow dye MTT on a purple dye formazan by viable cells.

Cells supernatants were collected at 24 and 48 h to perform lactate dehydrogenase (LDH) assay. LDH is a cytosolic enzyme that is only released from cells into the extracellular space when the plasma membrane is disrupted. So, the release of LDH into the culture medium was evaluated by measurement of LDH activity by the use of the CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit according to the manufacturer's instructions. Released LDH activity is measured with a coupled enzymatic assay, which results in conversion of a tetrazolium salt into a red formazan product. The intensity of colour formed is proportional to the number of lysed cells. Generation of Formazan is monitored by measuring absorbance at 490 nm using BioTek Power Wave XS plate reader.

All the experiments were performed in at least three independent experiments and results were expressed as percentage of control/untreated cells.

## 1.4 Cell proliferation assays

To determine cell proliferation it was used the Sulforhodamine B (SRB) and BrdU assays. SRB is a fluorescent dye used for cell density determination via quantification of cellular proteins of cultured cells. The method of incubation was similar to the MTT assay till the end of incubation, and then the cells were fixed with trichloroacetic acid (final concentration: 40 %) for 1 h at 4 °C and incubated with SRB (final concentration: 0.4 % in 1 % acetic acid) for 30 min. The bound protein stain was solubilized in Tris-base (final concentration: 10 mM) and absorbance was assessed at 492 nm spectrophotometrically.

Additionally, the thymidine analog BrdU (5-bromo-2'-deoxyuridine) proliferation assay was performed, following its incorporation by the newly synthesized genomic DNA. For that, St-T1b cells were plated in 96-well plates. After 24 h of culture, the medium was replaced with DMEM/F12 medium with 2 % FBS-CT and 1 % AB-AM solution in the presence or absence of cannabinoids (0.01-50  $\mu\text{M}$ ). The cells were labelled by the addition of BrdU (final concentration: 10  $\mu\text{M}$ ) for 28-30h. After the labelling period, the

cells were fixed and the anti-BrdU (final concentration: 1 µg/ml) antibody was added. The absorbance of the reaction product was measured at 450 nm using BioTek Power Wave XS plate reader.

All experiments were performed in triplicate and in at least three independent experiments. Results were expressed as percentage of control/untreated cells.

## 1.5 Morphological studies

The morphological alterations caused by cannabinoids treatment were evaluated by phase-contrast microscopy, Giemsa and Hoechst staining.

The cells were cultured in 24-well culture plates with coverslips and treated with the different cannabinoids (25 µM of  $\Delta^9$ -THC, THJ-2201, JWH-122, AB-FUBINACA, UR-144, 5F-PB-22, 2 and 5 µM of WIN) for 48 h.

Giemsa staining is a variant of the Romanowsky-type stain. This technique allows the evaluation of cell morphology. Giemsa contains a mixture of methylene blue, eosin and Azure B (methylene azure B). The eosin Y dye stains the basic components of the cells. The methylene blue and azure B dyes stain the acidic components in shades between blue and purple. Cells were washed with PBS and fixed with methanol for 30 minutes at 4 °C. Then cells were washed with PBS and stained with Giemsa stain solution, diluted in distilled water (1:10) for 30 min. After washing with tap water, the coverslips with the stained cells were dehydrated and mounted in DPX mounting medium and observed under a bright field microscope (Eclipse E400, Nikon, Japan) equipped with image analysis software LeicaQwin.

Hoechst is a dye that emits blue fluorescence when bound to DNA. This staining allows the evaluation of nuclear morphology and the identification of apoptotic nuclei, which present chromatin condensation and fragmentation. After fixation with methanol for 25 min at 4 °C, the cells were exposed to 0.5 µg/mL Hoechst 33342 (in PBS) for 20 min, washed with PBS, mounted in Fluoroshield mounting medium and observed under a fluorescence microscope equipped with an excitation filter with maximum transmission at 360/400 nm (Eclipse CI, Nikon, Japan). Images were processed by Nikon NIS Elements Image Software.

## 1.6 Real-time Polymerase Chain Reaction (RT-PCR)

To determine the amount of mitochondrial DNA (mtDNA) relative to nuclear DNA (nDNA), a quantitative real-time PCR was performed, by measuring the ratio of the mitochondrial gene, 16S rRNA, and a nuclear gene, GAPDH. For that, St-T1b cells were plated in 6-well plates. After 24 hours for adhesion, the medium was replaced with DMEM/F12 medium with 2 % FBS-CT and 1 % AB-AM solution in the presence or absence of cannabinoids (50  $\mu$ M) for 48 h. Cells were collected in TRIsure reagent, and total DNA was extracted according to the manufacturer's instructions. DNA was quantified in the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). To determine THJ-2201, JWH-122 and AB-FUBINACA impact in the ratio of mtDNA/nDNA, DNA was amplified with specific primers: 16S rRNA and GAPDH. These reactions were carried out with KAPA SYBR FAST qPCR Master Mix 2x Kit (Kapa Biosystems, Woburn, MA, USA) in MiniOpticon Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA), according to kit protocol. Primer sequences and RT-PCR conditions are summarized in Table 1.

**Table 3 – RT-PCR conditions.** Gene, primer sequences and temperatures used to obtain the ratio of mtDNA/nDNA.

Gene	Primer sequence (5'-3')	Annealing temperature (°C)	Melting temperature (°C)
16S rRNA	Sense: ACTTTGCAAGGAGAGCCAAA Anti-sense: TGGACAACCAGCTATCACCA	59 °C	83,5 °C
GAPDH	Sense: GGATGATGTTCTGGAAGAGCC Anti-sense: AACAGCCTCAAGATCATCAGC	59 °C	81,5 °C

## 1.7 Caspase -3/-7 activity

To detect caspase -3/-7 activity, the cells were seeded in a 96-well white plate pre-incubated for 30 min with the CB<sub>1</sub> and CB<sub>2</sub> antagonists, AM251 (1  $\mu$ M), AM281 (2  $\mu$ M) and AM630 (1  $\mu$ M), respectively, and treated with the cannabinoids for 24 h. At the end of the incubation time, Caspase-Glo -3/-7 reagent was added to the cells according to the manufacturer's instructions. The plate was incubated at room temperature for 1 h and the resultant luminescence was measured in relative light luminescence units (RLU) using the 96-well Microplate Luminometer (BioTek Instruments, Winooski, VT, USA). The results are expressed in percentage, comparing caspase -3/-7 activities of cannabinoid-treated cells with the untreated cells.

## 1.8 Zymography

To determine the activity of metalloproteinases (MMPs) a zymography was performed. For that, St-T1b cells were plated in 24-well plates. After 24 h of culture, the medium was replaced by DMEM/F12 medium with 2 % FBS and 1 % AB-AM solution and 12 h before experiment the medium was replaced with DMEM/F12 medium with 2 % FBS-CT and 1 % AB-AM in the presence or absence of cannabinoids (25  $\mu$ M). After 48h the supernatants were collected and the Bradford assay was performed to quantify protein concentration. Then, equal amounts of total proteins (5  $\mu$ g) were separated without heating and without  $\beta$ -mercaptoethanol on a 10 % SDS-polyacrylamide gel containing 1% gelatine. The gels were washed, incubated in developing buffer for 16 h at 37 °C and stained with 5 % Coomassie blue R250. Bands representing gelatinase activity were photographed and analysed by densitometry.

## 1.9 UPLC-MS/MS

To determine the concentration of prostaglandin E2 in term placenta supernatant and tissue, an ultra-performance liquid chromatography-mass spectrometry (UPLC-MS/MS) analysis was performed. Explants of decidua from term placentas were collected within two hours after delivery and plated in 6-well plates with DMEM/F12 medium with 2 % FBS-CT and 1 % AB-AM. After two hours, the medium was replaced in the presence and absence of cannabinoids (25  $\mu$ M). After 24 h, supernatant and tissue were collected and frozen at -80 °C.

### 1.9.1 Sample preparation

In the day of the experiment, prostaglandin extraction was performed. In supernatant, 500  $\mu$ l of sample were added to 0.4 g of MgSO<sub>4</sub>, 0.1 g of NaCl, 500  $\mu$ l of ethyl acetate and 20  $\mu$ l of PGE<sub>2</sub>-d<sub>4</sub>, and centrifuge for 15 min at 3500 xg. At the end of centrifugation, supernatant was collected and evaporated to dryness under nitrogen. The samples were reconstituted with 100  $\mu$ l of methanol. Tissue extraction was performed by adding 500  $\mu$ l of methanol, 20  $\mu$ l of PGE<sub>2</sub>-d<sub>4</sub> and C18 to a small fragment of tissue, and centrifuge for 30 min at 3500 xg. At the end of centrifugation, supernatant was collected and evaporated to dryness under nitrogen. Samples were reconstituted with 100  $\mu$ l of acetonitrile and injected in the UPLC-MS/MS (10  $\mu$ l injection volume).

### 1.9.2 UPLC MS/MS analysis

Separation and quantification of the target analyte was performed by using a liquid chromatography Acquity UPLC system interfaced to a triple quadrupole mass selective detector Micromass Quattro micro API, (Waters, Milford, MA, USA). The injection volume was set at 10  $\mu$ l. The chromatographic separation was carried out with a BEH C18 column (1.6  $\mu$ m, 2.1 mm x 150 mm (i.d.)) from ACQUITY UPLC, at a flow rate of 0.20 ml/min. The mobile phases were (A) 5 % ammonium acetate (5 mM) in ultra-pre MilliQ water and (B) MeOH (UPLC grade, VWR, Radnor, PA, USA) in an isocratic gradient. Mass spectrometry analysis was performed with an electrospray ionization (ESI) source in the negative (ESI-) ion mode for all the analytes. Nitrogen was used as the nebulizer gas. The optimum MS parameters were as follows: capillary, 3.00 kV; extractor, 2 V; RF lens, 0.5 V; source temperature, 150  $^{\circ}$ C; desolvation temperatures, 350  $^{\circ}$ C; desolvation gas flow, 350.0L/h; cone gas flow, 60.0 L/h; LM resolution, 13.0; ion energy, 1.0; entrance, 1; exit, 2; multiplier, 650. All analyses were done in multiple reaction monitoring (MRM) mode.

### 1.10 Statistical analysis

Statistical data analysis was carried out by one or two way ANOVA, followed by the Tukey post hoc test to make pairwise comparisons of individual means when significance was indicated, using Graphpad Prism 6.0 (GraphPad PRISM v. 6.0, GraphPad Software, Inc., San Diego, CA, USA). Graphs were also drawn using Graphpad Prism 6.0. The results are the mean of at least three independent experiments carried out in triplicate. Data are expressed as the mean $\pm$ SEM, and differences were considered to be statistically significant at  $P < 0.05$ .

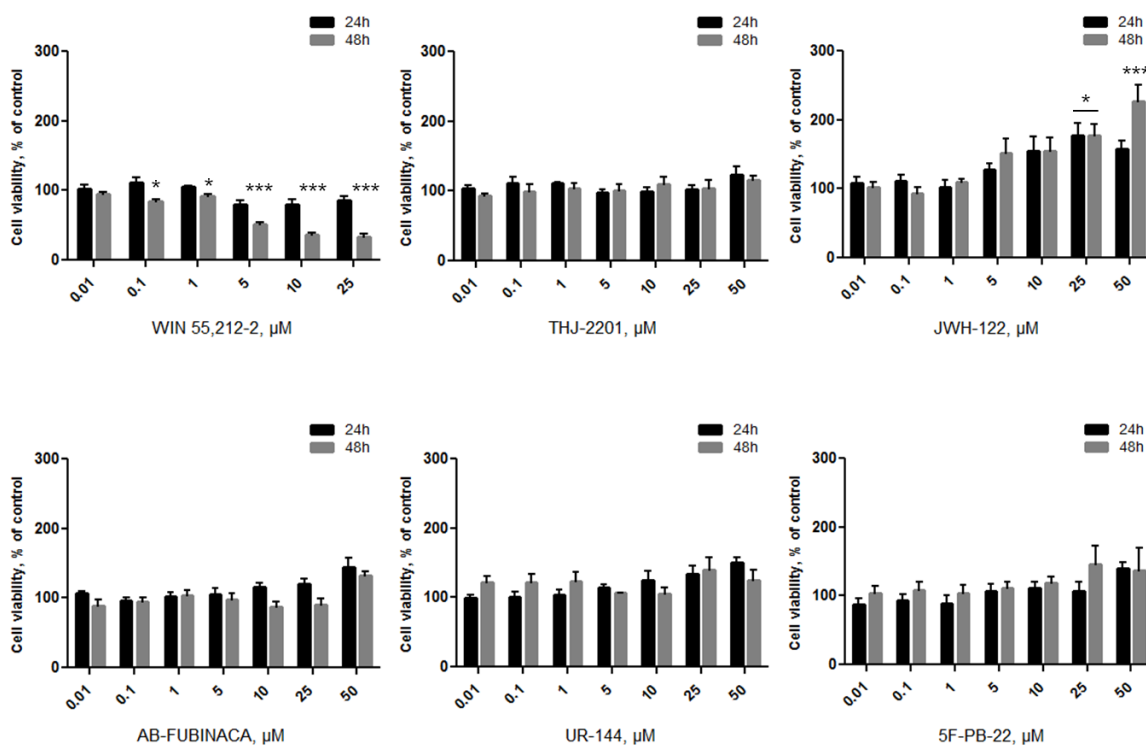
## Chapter IV - Results

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## 1.1 Effects of synthetic cannabinoids on endometrial stromal cell viability

To investigate the effects of synthetic cannabinoids, WIN 55,212-2, THJ-2201, JWH-122, AB-FUBINACA, UR-144 and 5F-PB-22, in the viability of the St-T1b cell line and HdF primary cultures a MTT assay was performed using concentrations ranging from 0.01 to 50  $\mu\text{M}$ , and treatments of 24 and 48 h. This assay quantifies the mitochondrial metabolism that converts the yellow MTT into the purple formazan by viable cells.

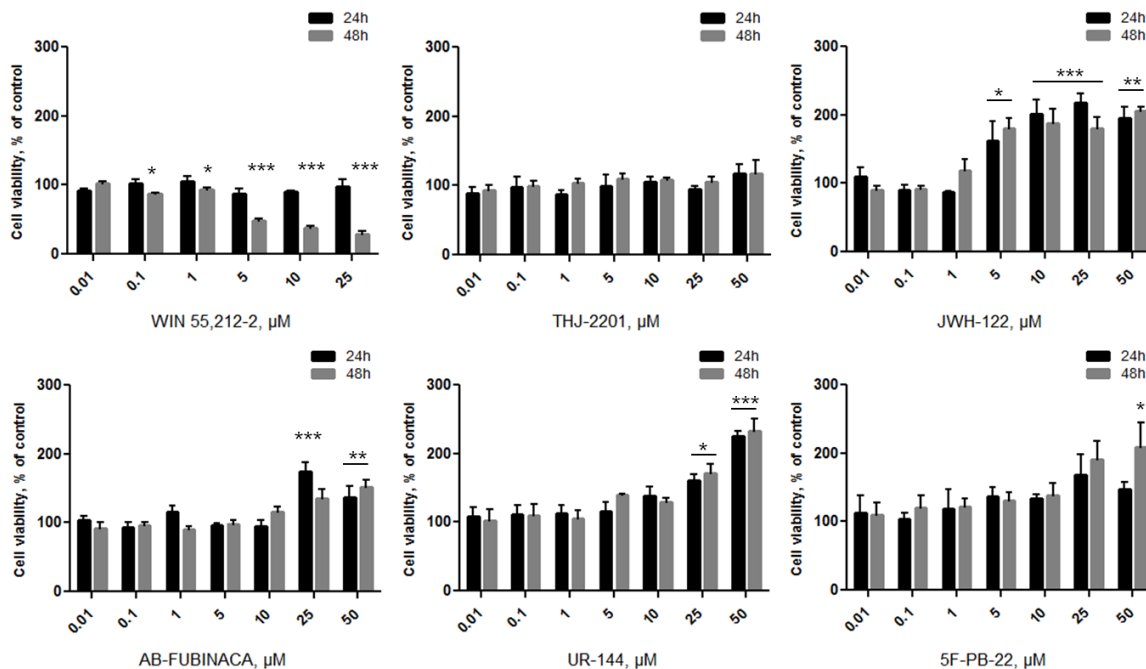
The treatment of St-T1b cells with THJ-2201, UR-144 and 5F-PB-22 did not affect cell viability. On the other hand, JWH-122, AB-FUBINACA and WIN 55,212-2 induced different effects in cell viability. The first two treatments led to an increase in mitochondrial activity in treatments with the higher concentrations, while WIN induced a significant decrease that was dose and time-dependent (Figure 12).



**Figure 12 – Effects of synthetic cannabinoids in St-T1b cells.** Cell viability of treated cells with different concentrations (0.01- 50  $\mu\text{M}$ ) of synthetic cannabinoids, at 24 and 48 h of treatment, assessed by MTT assay. (\*\* $P < 0.01$ , \* $P < 0.05$  vs. Control).

In primary cultures, contrary to THJ-2201, that did not cause any alterations in cell viability, JWH-122, AB-FUBINACA, UR-144 and 5F-PB-22 induced an increase in mitochondrial activity dependent on time and concentration. WIN, like in the St-T1b cell

line, caused a reduction in cell viability. After 48 h it was obtained a drastic reduction for the concentrations between 0.1 and 25  $\mu\text{M}$  (Figure 13).

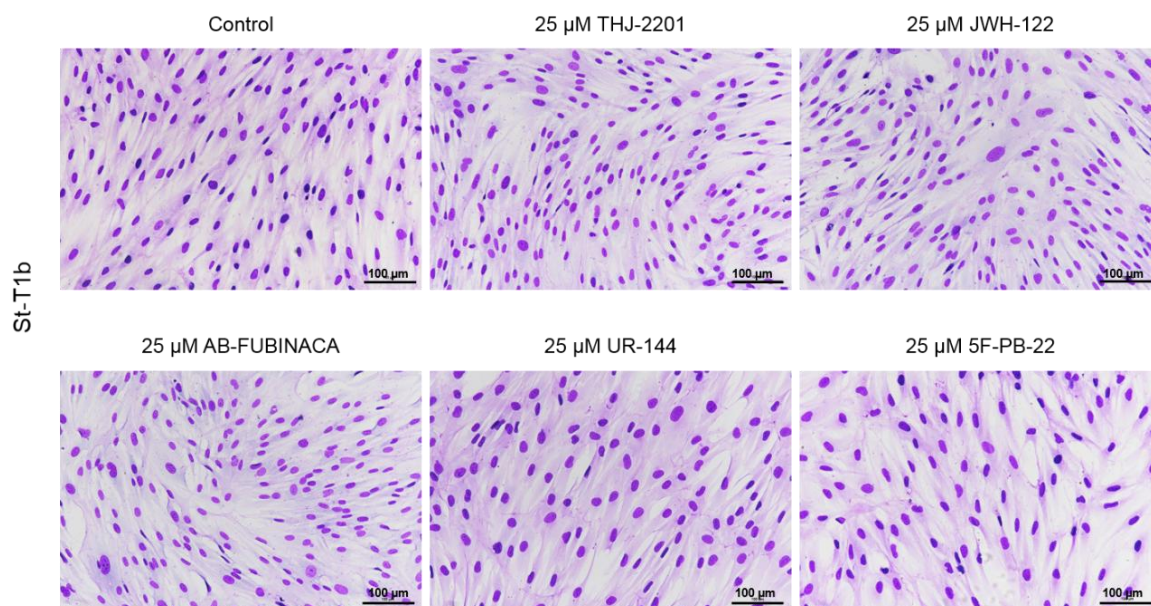


**Figure 13 – Effects of synthetic cannabinoids in HdF cells.** Cell viability of treated cells with different concentrations (0.01- 50  $\mu\text{M}$ ) of synthetic cannabinoids, at 24 and 48 h of treatment, assessed by MTT assay. (\*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.05$  vs. Control).

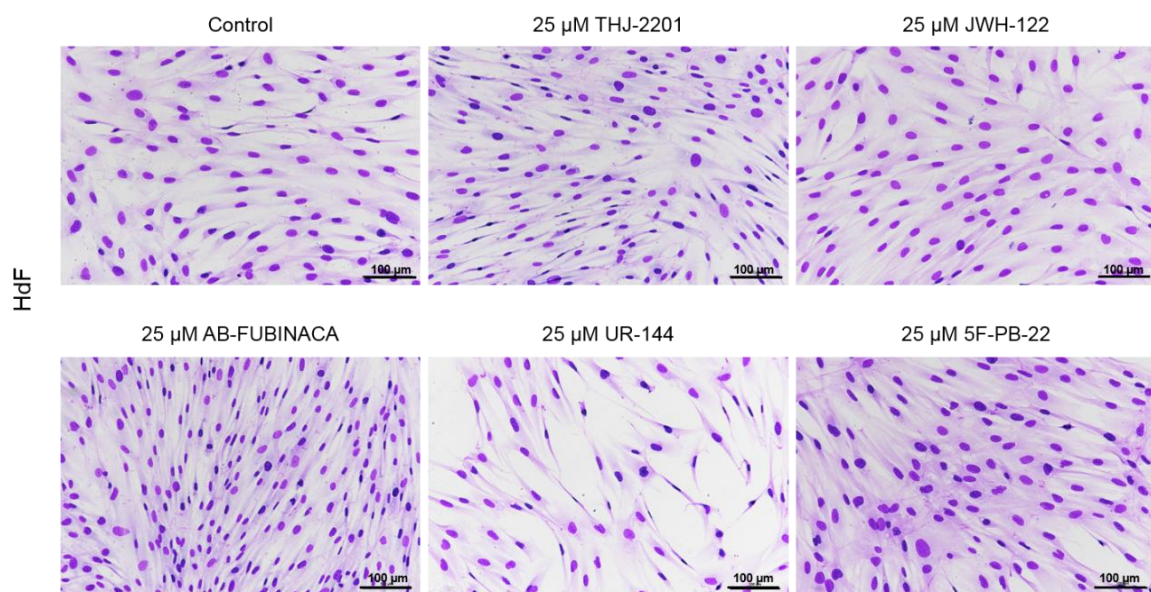
## 1.2 Effects of synthetic cannabinoids in endometrial stromal cell morphology

Giemsa staining was performed to study if the synthetic cannabinoids treatments affected cell morphology. St-T1b and HdF cells were treated for 48 h with 25  $\mu\text{M}$  of THJ-2201, JWH-122, AB-FUBINACA, UR-144 and 5F-PB-22 and with 2 and 5  $\mu\text{M}$  of WIN 55,212-2 (Figure 14 and 15).

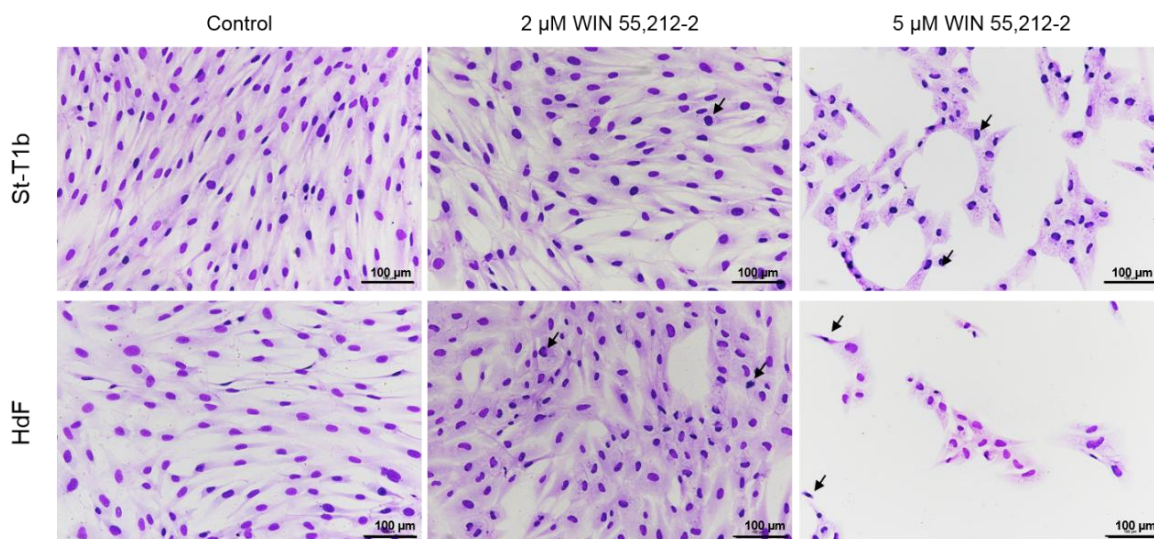
Contrary to THJ-2201, JWH-122, AB-FUBINACA, UR-144 and 5F-PB-22, WIN 55,212-2 induced a dramatic reduction in cell density and chromatin condensation (Figure 16).



**Figure 14 – Effects of synthetic cannabinoids in St-T1b cell morphology.** Cell morphology of treated cells with 25 μM of different synthetic cannabinoids for 48 h was assessed by Giemsa staining.



**Figure 15 – Effects of synthetic cannabinoids in HdF cell morphology.** Cell morphology of treated cells with 25 μM of different synthetic cannabinoids for 48 h was assessed by Giemsa staining.

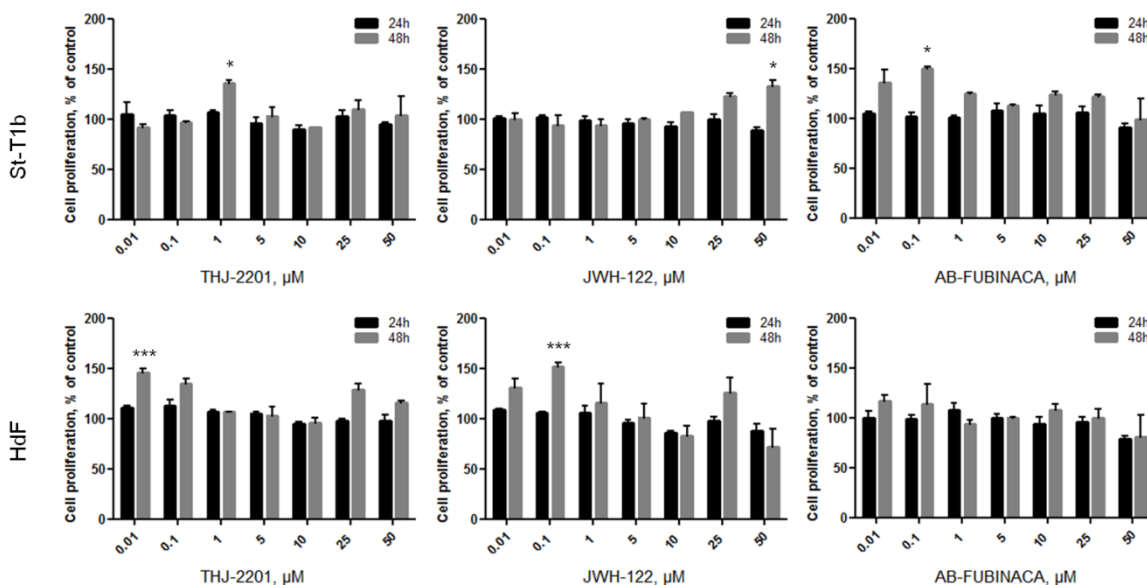


**Figure 16 – Effects of WIN 55,212-2 in cell morphology.** Cell morphology of treated cells with 2 and 5  $\mu\text{M}$  for 48 h was assessed by Giemsa staining. Arrows represent chromatin condensation.

### 1.3 Effects of synthetic cannabinoids on endometrial stromal cell proliferation

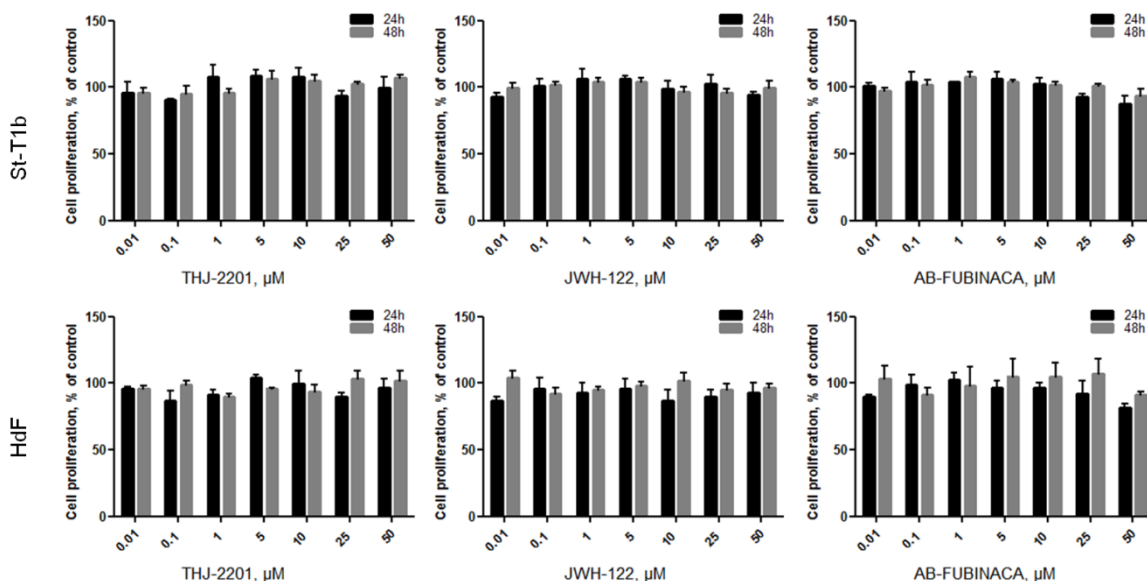
The Sulforhodamine B assay was performed in order to clarify the results obtained with the MTT assay, since it is based on the measurement of cellular protein content, and thus used for cell density quantification. Similarly to MTT assay, cells were treated with synthetic cannabinoids for 24 and 48 h (Figure 17).

In the cell line, THJ-2201, JWH-122 and AB-FUBINACA showed an increase in cell proliferation with the concentrations of 1, 50 and 0.1  $\mu\text{M}$  at 48 h of treatment, respectively. In the HdF cells, only THJ-2201 and JWH-122 caused changes in cell proliferation after 48 h of treatment.



**Figure 17 – Effects of synthetic cannabinoids in cell density.** Cell protein quantification of treated cells with different concentrations (0.01- 25 μM) of different synthetic cannabinoids, at 24 and 48 h of treatment, assessed by SRB assay. (\*\*\*) $P < 0.001$ , \* $P < 0.05$  vs. Control).

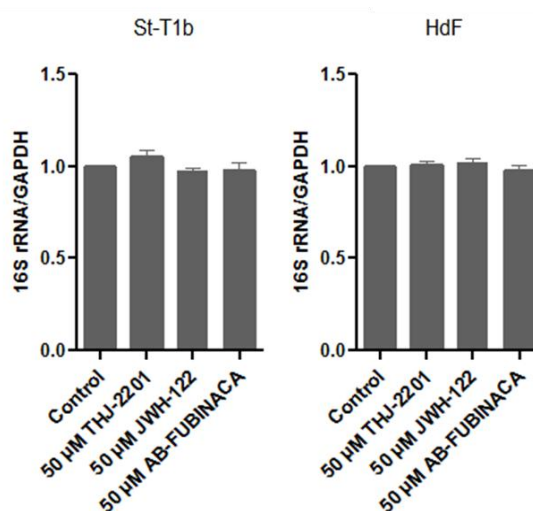
Due to the discrepant results obtained in the MTT and in the Sulforhodamine B assay, another proliferation assay was performed, the BrdU assay in which this nucleoside incorporates in the DNA synthesized by new cells. After incorporation, the nucleoside is detected with an anti-BrdU antibody, allowing to detected cellular proliferation. The synthetic cannabinoids THJ-2201, JWH-122 and AB-FUBINACA did not induce alterations in cell proliferation in both cells models (Figure 18).



**Figure 18 – Effects of synthetic cannabinoids in cell proliferation.** Cell proliferation of treated cells with different concentrations (0.01- 25 μM) of different synthetic cannabinoids, at 24 and 48 hours of treatment, assessed by BrdU assay.

## 1.4 Effect of synthetic cannabinoids in endometrial stromal cells mitochondrial number

In order to understand the results obtained by the MTT assay and by the proliferation assays, it was investigated the effects of THJ-2201, JWH-122 and AB-FUBINACA in mitochondrial number after 48 h of treatment. Thus the ratio of mtDNA/nDNA was assessed, through RT-PCR using specific primers for 16S rRNA and GAPDH as indicated in Table 3. None of the synthetic cannabinoids caused alterations in the mitochondrial number (Figure 19).

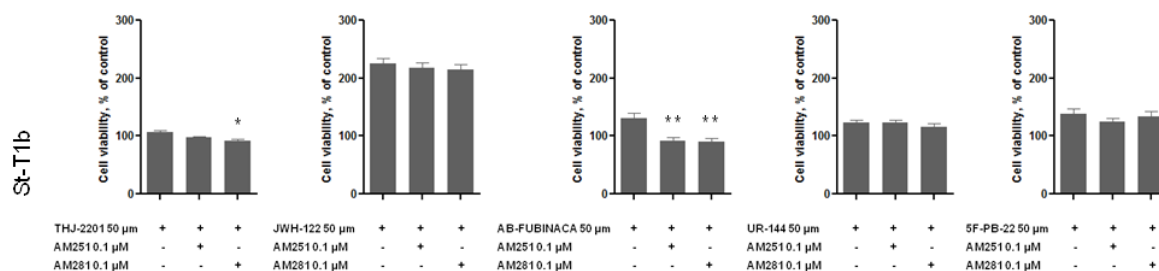


**Figure 19 – Effects of synthetic cannabinoids in mitochondrial number.** The ratio of mtDNA and nDNA in endometrial stromal cell line and in primary cultures of human decidual fibroblast cells obtained by RT-PCR.

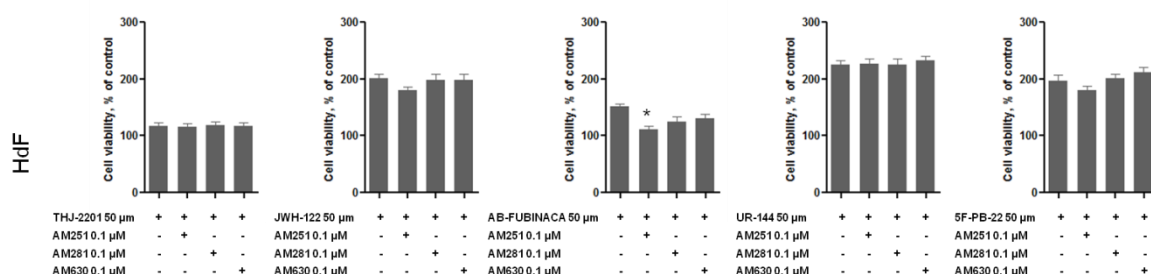
## 1.5 Effect of synthetic cannabinoids in endometrial stromal cells cannabinoid receptors

To investigate if the effects induced by the synthetic cannabinoids were through the cannabinoid receptors, cells were treated with these molecules in the presence and absence of CB<sub>1</sub> and CB<sub>2</sub> antagonists. The CB<sub>2</sub> antagonist was only used in the primary cells cultures because the St-T1b cell line does not express CB<sub>2</sub> receptor.

The increase in mitochondrial activity induced by the synthetic cannabinoid AB-FUBINACA seem to be through the cannabinoid receptor 1, since it appears to be reverted by CB<sub>1</sub> antagonists in both cell lines (Figures 20 and 21).



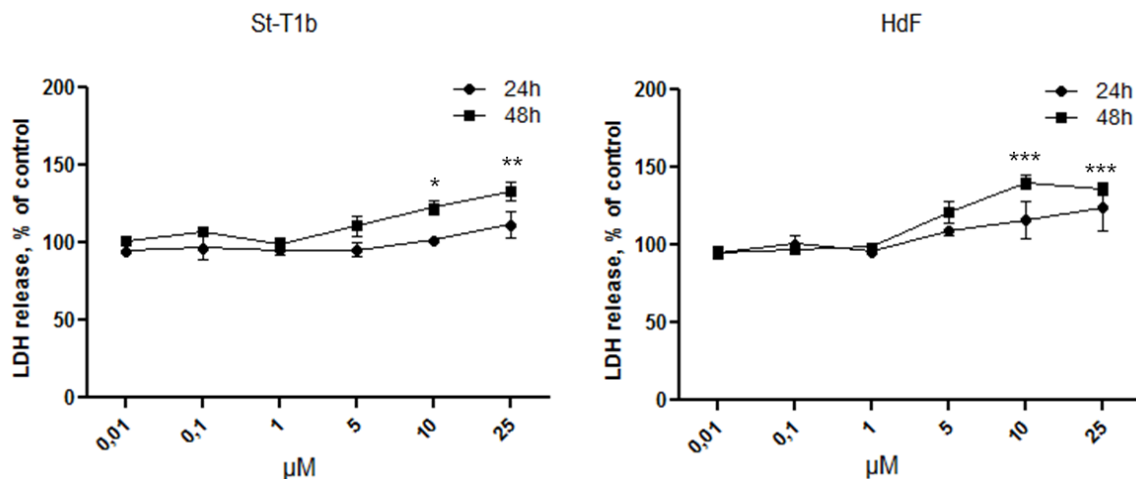
**Figure 20 – Effects of synthetic cannabinoids in St-T1b cell viability in combination with CBR antagonists.** Cell viability of treated cells with 50 μM after 30 min of incubation with CB<sub>1</sub> antagonists (AM251; AM281).



**Figure 21 – Effects of synthetic cannabinoids in HdF cell viability in combination with CBR antagonists.** Cell viability of treated cells with 50 μM after 30 min of incubation with CB<sub>1</sub> antagonists (AM251; AM281) and CB<sub>2</sub> antagonists (AM630). (\*\* $P < 0.01$ , \* $P < 0.05$  vs. Control).

## 1.6 Effects of synthetic cannabinoids in endometrial stromal cell LDH release

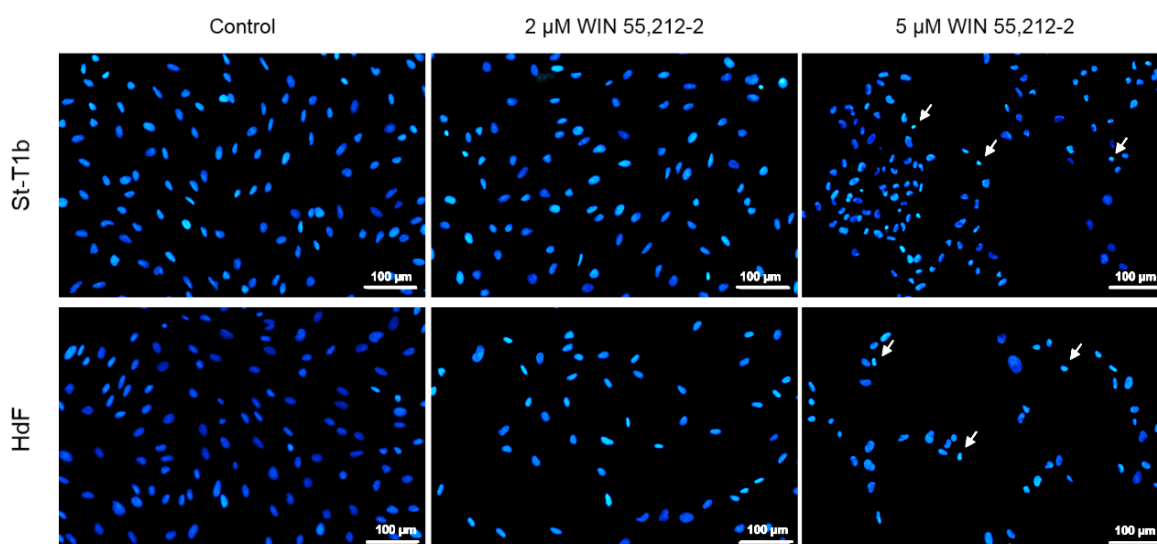
As WIN 55,212-2 caused a drastic reduction in cell viability it was evaluated if this cannabinoid was cytotoxic, by determining the activity of LDH. LDH is a cytosolic enzyme that is only released from cells after membrane disruption, being a biomarker of cell cytotoxicity. WIN 55,212-2 only caused an increase in LDH release, in both cells at 10 and 25 μM (Figure 22).



**Figure 22 – Effects of WIN 55,212-2 in LDH activity.** LDH activity of treated cells with different concentrations (0.01-25 µM) at 24 and 48 h of treatment. (\*\*\*) $P < 0.001$ , (\*\*) $P < 0.01$ , (\*) $P < 0.05$  vs. Control).

### 1.7 Effect of WIN 55,212-2 in endometrial stromal cells chromatin condensation

To confirm the chromatin condensation and nuclei alterations detected in cells stained with Giemsa, H $\ddot{o}$ chst staining was performed. The chromatin condensation, which suggests an apoptotic process, was also observed and was accompanied by a decrease in cell density (Figure 23).

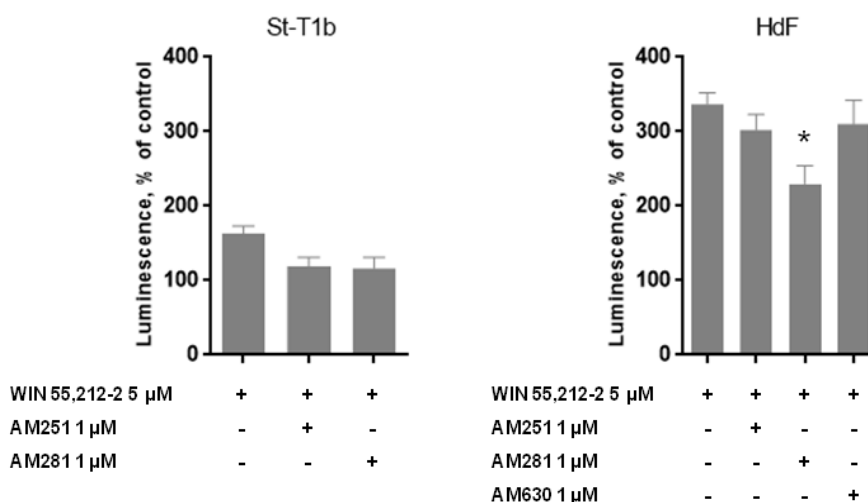


**Figure 23 – Effects of WIN 55,212-2 in chromatin condensation.** Cell morphology of treated cells with 2 and 5 µM after 48 h of treatment was assessed by H $\ddot{o}$ chst staining. Arrows represent chromatin condensation.

## 1.8 Effects of WIN 55,212-2 in endometrial stromal cell apoptosis

Caspases are endoproteases that play essential roles in programmed cell death, acting as initiator or executioner caspases. Caspase -3 and -7 are executioner caspases.

To investigate the reduction in cell viability induced by WIN 55,212-2 and apoptosis suggested by the Höechst staining, caspase -3/-7 activities were quantified at 5  $\mu$ M of WIN 55,212-2, since this concentration caused a decrease in mitochondrial activity but did not induce an increase in LDH release. The reduction in cell viability caused by WIN 55,212-2 in St-T1b cells was not accompanied by an increase in caspase -3/-7 activities nor reverted by the pre-treatment with CB<sub>1</sub> antagonists. Interestingly, in primary cultures, 5  $\mu$ M of WIN 55,212-2 caused an increase in caspase activity, which was partially reversed by co-incubation with CB<sub>1</sub> antagonist AM281 (Figure 24).

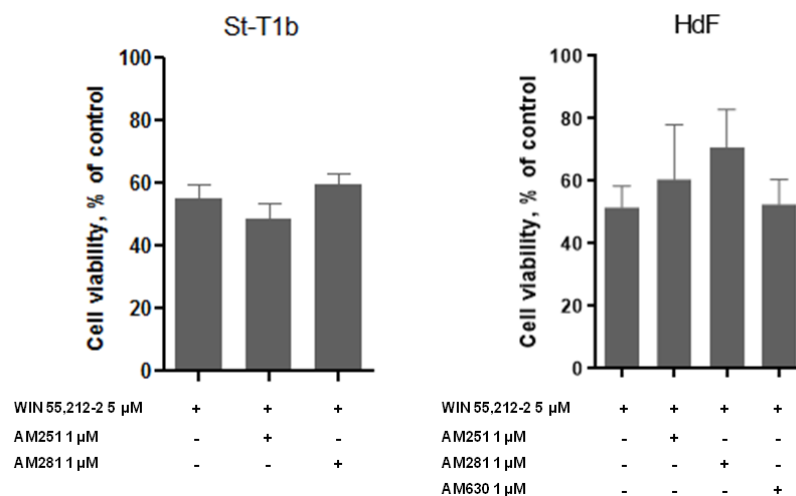


**Figure 24 – Effects of WIN 55,212-2 in caspase -3/-7 activities.** Cells were treated with WIN 55,212-2 in combination with CB<sub>1</sub> and CB<sub>2</sub> antagonists, AM251 or AM281 and AM630, respectively. (\**P* < 0.05 vs. Control).

## 1.9 Effect of WIN 55,212-2 in endometrial stromal cells cannabinoid receptors

To investigate if WIN 55,212-2 affected cell viability through the cannabinoid receptors, cells were treated in the presence and absence of CB<sub>1</sub> and CB<sub>2</sub> antagonists.

The decrease in cell viability induced by WIN 55,212-2 seems to be influenced through the cannabinoid receptor 1, as treatment with AM281 attenuated the effects induced by WIN 55,212-2 (Figure 25).



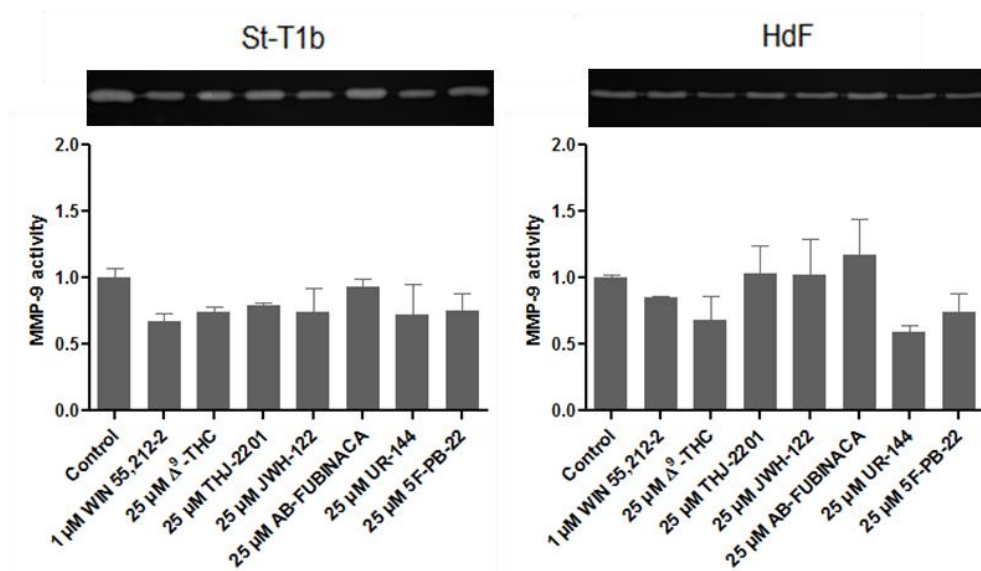
**Figure 25 – Effects of WIN 55,212-2 in cell viability in combination with CBR antagonists.** Cell viability of cells pre-incubated with CB<sub>1</sub> antagonists (AM251; AM281) and CB<sub>2</sub> antagonists (AM630) for 30 minutes were treated with 5  $\mu$ M of WIN 55,212-2 during 48 h.

## 1.10 Effects of cannabinoids in endometrial stromal cells function

### 1.10.1 Matrix metalloproteinase 9 activity by zymography

Various proteinases and enzymes are expressed in the human endometrium, being the MMP family responsible for the extracellular matrix proteolysis during the menstrual cycle. This family of proteinases is also responsible for the degradation and remodelling of the endometrium necessary to implantation.

To determine the activity of matrix metalloproteinase 9 after 48 h of treatment with different synthetic cannabinoids, and the phytocannabinoid  $\Delta^9$ -THC as a control, a zymography was performed. Although, a decrease in MMP-9 activity occurred in cells treated with WIN 55,212-2,  $\Delta^9$ -THC, UR-144 and 5F-PB-22 in both cells, none of the different treatments caused significant changes in the activity of MMP-9 as observed by the densitometric analysis (Figure 26).



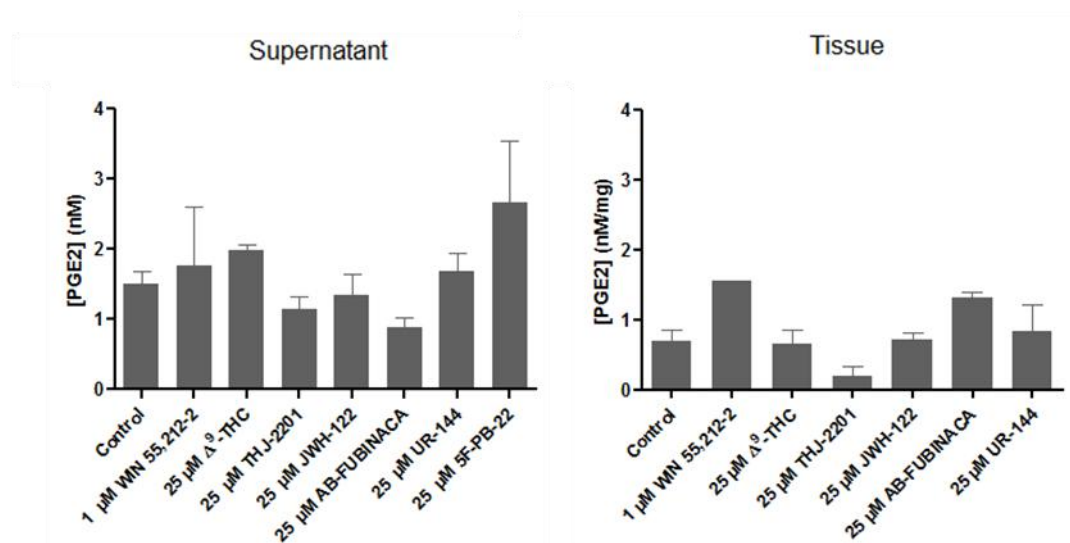
**Figure 26 – Effects of synthetic cannabinoids in MMP-9 activity.** A zymography was performed with six different synthetic cannabinoids and a phytocannabinoid. Densitometric analysis is graphically represented and representative figures of zymography results are shown.

### 1.10.2 Prostaglandin E2 levels by UPLC-MS/MS

As prostaglandins (PGs) are synthesized by the endometrial stromal (the precursor of the decidual cell), epithelial and endothelial cells across the menstrual cycle, experiments were performed to determine whether synthetic cannabinoids may affect prostaglandin E2 production in the supernatant and tissue from term placenta explants.

Preliminary results (n=3) showed that, after 24 h of treatment, in explants supernatant, contrary to the  $\Delta^9$ -THC that caused an increase in PGE2, THJ-2201 and AB-FUBINACA induced a reduction in its production. In the tissue, WIN 55,212-2 and AB-FUBINACA induced an increase in PGE2 production, whilst THJ-2201 caused a decrease in PGE2 levels. The increase in the PGE2 levels caused by AB-FUBINACA, in the tissue, may explain the reduction in the supernatant.

Although statistically significance was not achieved, probably due to the small number of samples, these effects were more evident within tissue PGE2 production (Figure 27).



**Figure 27 – Effects of synthetic cannabinoids in PGE2 concentration in supernatant and tissue of term placenta.** After 24 h of treatments, no significant alterations in PGE2 levels were observed, even though cannabinoids caused changes in PGE2 production.

## Chapter V - Discussion

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*Cannabis* is the most abused illicit substance in the world, having nearly 80 million people of the EU consumed at least once. Its use is spread across all ages, from the occasional to regular users. Cannabis abuse is the most frequent reason for seeking drug treatment, and it is responsible for a fair amount of drug-related emergencies (57). However, restrictions to its use has raised new concerns about the consumption of new psychoactive substances with unclear legislation, like synthetic cannabinoids (56).

The drug most commonly used by women in childbearing age is *Cannabis*, and this substance seems to affect negatively female endocrinologic and reproductive functions (115). In addition, synthetic cannabinoids production and consumption has been increasing in the past years. However, their effects are unknown to health and in particular to reproduction.

In this work, the effects of six synthetic cannabinoids in endometrial stromal cells were studied in a cell line, St-T1b, and in primary cultures of human decidual fibroblasts, HdF. These two cell types complement each other, which makes them good cell models to study these effects. THJ-2201, an analog of the synthetic cannabinoid AM-2201, did not induce any alterations in cell viability, morphology, as well as in cell proliferation of St-T1b cell line and primary decidual fibroblasts. Cytotoxicity studies with the synthetic cannabinoid AM-2201 are scarce, nevertheless, a recent study described that this compound induces cytotoxicity after 2 hours of treatment but at a concentration of 30  $\mu\text{M}$  in primary cultures of neuronal cells (116).

On the other hand, AB-FUBINACA, UR-144, 5F-PB-22 and JWH-122, analogs of JWH-018, induced an increase in MTT metabolism at high concentrations (10-50  $\mu\text{M}$ ). Interestingly, this effect was not accompanied by an increase in cell proliferation as determined by the SRB and BrdU assays. So, as the MTT assay measured mitochondrial activity, the involvement of mitochondria was investigated by quantifying the ratio of mtDNA versus nDNA, to verify if the number of mitochondria varies between treatments. However, no significant differences were observed, which suggests that no alterations in the mitochondrial number occurred and the effects observed in the MTT assay may result from increased mitochondrial activity.

Most of the synthetic cannabinoids studied in this work are analogs of JWH-018. Couceiro *et al.* studied the cytotoxicity of JWH-018 in cells with and without CB<sub>1</sub>, a human kidney cell line (HEK293T) and human cell line of neuroblastoma (SH-SY5Y), and observed that this compound did not significantly affect cell viability in both cells, although a dose-dependent increase in MTT metabolism was also observed (117). A similar effect was described for WIN 55,212-2 by Aguirre-Rueda *et al.*, in primary cultures of cortical

astrocytes (118). This effect was associated to a cytoprotective role attributed to cannabinoids.

Our results suggest that mitochondrial activity may be influenced by the compounds, particularly by activation of the cannabinoid receptor CB<sub>1</sub> in mitochondrial membranes, as described in brain neuronal mitochondria membrane. In this study, it was suggested that it directly regulates mitochondrial metabolism (119), through modulation of complex I activity, regulating cAMP levels and PKA activity. These molecules may modulate the organelle metabolism. Nevertheless, these effects may also be due to activation of other non-CB<sub>1</sub>/CB<sub>2</sub> cannabinoid receptors (120) that are yet to be fully understood. This may also be one explanation for the results obtained with the CB<sub>1</sub>/CB<sub>2</sub> antagonists. CB<sub>1</sub> antagonist was used in both the cell line and primary cells, however CB<sub>2</sub> antagonist was only tested in HdF, because, as referred, the St-T1b cell line does not express this receptor (121). In fact, the co-incubation with CB<sub>1</sub>/CB<sub>2</sub> antagonists did not reverse the effects induced by synthetic cannabinoids in our study. Fišar *et al.* also described a non-receptor-mediated cannabinoid mechanisms involved in the regulation of mitochondrial respiration (122).

The other synthetic cannabinoid, WIN 55,212-2 is an aminoalkylindole-type and a full agonist of CB<sub>1</sub> receptor (123). In our work, WIN 55,212-2 caused a decrease in cell viability as revealed by MTT assay in a dose and time-dependent way. For high concentrations, 10 µM in primary human decidual fibroblast and 25 µM in St-T1b cell line, these effects were accompanied by an increase in LDH release, suggesting the occurrence of a cytotoxic effect for these concentrations. WIN 55,212-2 also induced morphological alterations, in both models, such as chromatin condensation, accompanied with decreased cell density.

In order to confirm the apoptotic cell death suggested by the morphologic alterations, caspase -3/-7 was quantified. It was observed a dramatic increase in this effector caspase activity, being this increment more significant in primary cells. It was also verified that the caspase activity was partially reversed by the CB<sub>1</sub> antagonist AM281, suggesting that WIN 55,212-2 induced apoptosis through CB<sub>1</sub> activation. These effects are in concordance with a previous study of Kessler *et al.*, which described apoptosis in decidualized fibroblasts induced by WIN 55,212-2 in concentrations ranging from 0.1 to 10 µM. These affects were also dependent on CB1 activation (124). Furthermore, the cellular morphological alterations induced by WIN 55,212-2 in both cell line and primary cultures were also observed by Giuliano *et al.* in liver hepatocellular cells (125).

In accordance to our results, WIN 55,212-2 has already been shown to induce apoptosis in other cell models, like hepatocellular carcinoma cells (126) and HepG2 cells

(125). In hepatocellular carcinoma cells, cell death was via mitochondrial pathway, with a significant decrease in mitochondrial membrane potential, and increase in caspase -3, -8 and -9 activities, suggesting the involvement of mitochondria. In HepG2 cells, a human liver cancer cell line, apoptosis was accompanied by the up-regulation of the death signalling factors Bax, which, subsequently, led to a mitochondrial dysfunction and activation of executioner caspase -3 and -6, and activation of JNK/p38 MAPK pathway.

As no significant effects seem to be induced by synthetic cannabinoids in the viability and proliferation of endometrial cells, we investigated if these compounds caused any interference at the functional level, interacting with enzymes or proteins important to the normal function of the endometrium. Regulation of MMPs production and activation, and subsequent degradation and tissue remodeling, is crucial for the fast and extensive tissue destruction occurring during the menstrual cycle (127), as the receptivity of the endometrium to implantation is one of the most important processes to accomplish a successful pregnancy. So, normal proliferation of endometrial stromal cells and subsequent decidualization, along with extracellular decidual matrix remodeling are necessary (67). Another important part for a normal maintenance of the endometrium is the production of Prostaglandins (PGs), which have a key role in numerous aspects of pregnancy, including term and preterm delivery. Its importance is due to arachidonic acid, a precursor of prostaglandin production and a final product of endocannabinoid hydrolysis, which means that any imbalance in the endocannabinoid system may affect PG production (128).

One of the most important enzymes responsible for remodeling of the extracellular matrix is the MMP family, which is in charge of degradation of ECM to allow implantation. In this work, the activity of MMP-9 did not present significant differences between treatments and the control group, although a slight decrease in gelatinase activity was detected in the cells treated with WIN 55,212-2,  $\Delta^9$ -THC, UR-144 and 5F-PB-22. The decrease of MMPs in the endometrium may affect the decidualization of the stromal cells and even the implantation of the blastocyst, as described by Rechtman *et al.* They demonstrated that inhibition of MMPs affected decidualization, causing a reduction in the total decidua area (129).

Even though most studies were performed in cancer cells, it was observed a decrease in MMP -2 and -9 activities. Blázquez *et al.* using rat glioma cells and human tumor samples showed that the expression of MMP-2 was inhibited by  $\Delta^9$ -THC and by a synthetic cannabinoid (JWH-133), via the sphingolipid ceramide regulation (130). Also, Preet *et al.* demonstrated that MMP-9 secretion and activity was significantly inhibited by WIN 55,212-2 and JWH-015 in non-small cell lung cancer cell line (NSCLC) and

adenocarcinomic human alveolar basal epithelial cells (A549). However, this inhibition was reversed by a GPCR inhibitor, implicating the involvement of CB<sub>1</sub> and CB<sub>2</sub> (131). Nevertheless, no studies have addressed the role of synthetic cannabinoids on MMP in the endometrium.

PGs that are members of the eicosanoid family and their synthesis involve the cyclooxygenase enzyme (COX), which is present in numerous tissues and cell types. So, this enzyme is responsible for production of PGs which are essential to normal physiological functions. COX-2 expression and PGE<sub>2</sub> synthesis were already associated to the modulation of cellular proliferation, resistance to apoptosis and cell differentiation. PGE<sub>2</sub> is also important for a normal physiological function of the endometrium with maximum synthesis during the menstrual and proliferative phases, and is produced in epithelial, stromal and endothelial cells, across the menstrual cycle (132).

In our work, WIN 55,212-2,  $\Delta^9$ -THC, UR-144 and 5F-PB-22 induced an increase in PGE<sub>2</sub> production, whereas THJ-2201, JWH-122 and AB-FUBINACA induced a reduction, with a more evident effect in tissue than in the supernatant of decidua explants from term placenta. These results may be explained by the results described by Mitchell *et al.*, who studied the effects of endocannabinoids and CP 55,940, a synthetic cannabinoid, in decidua explants. They showed that AEA significantly reduced PGE<sub>2</sub> production while CP 55,940 caused a slight increase. This study also suggested that the effects of cannabinoids on PG production in placenta were through activation of CB<sub>1</sub> and induction of COX-2 (128). Mestre *et al.* also studied the effects of WIN 55,212-2 in murine endothelial brain cells, showing that PGE<sub>2</sub> production increased. It was also observed that WIN 55,212-2 induced the production of PGE<sub>2</sub> that was due to COX-2, and that these effects were dose-dependent and mediated by mechanisms independent of CB<sub>1</sub> and CB<sub>2</sub> activation (133). These studies demonstrate that these new substances may interact with the production of PGs, affecting fertility.

In summary, we present evidences for the first time that synthetic cannabinoids may affect endometrial stromal cell stability, as well as have implications in PGs production, which are important for a myriad of physiological functions in the endometrium and in the placenta. We also describe that these substances may affect MMP-9 activity, influencing endometrial extracellular matrix degradation. Furthermore, WIN 55,212-2 induced apoptosis through CB<sub>1</sub> activation, and this may interfere with endometrial stromal cell remodeling. These effects may be dependent on the chemical structure and thus, the CB affinity that induce activation of different signaling pathways, justifying the effects observed by WIN 55,212-2 in relation to cell viability.

The increased use of synthetic cannabinoids by young people is a today's society problem with important impact on fertility. Although most of the problems that may result from its consumption are unknown, in this work we evidenced some signaling pathways that may affect fertility. Thus, this work may be a starting point to begin to understand the impact of this emerging class of substances and their effects in human fertility.

## Chapter VI - References

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