Understanding the synergistic potential of phytocannabinoids and umbilical cord-derived mesenchymal stem cells for regenerative medicine

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Preface

The work presented in this thesis was performed at the Research Institute of Medicines (iMed.ULisboa), Faculdade de Farmácia of Universidade de Lisboa (Lisbon, Portugal), during the period of March 2022-April 2023, under supervision of Prof. Joana Miranda. The thesis was co-supervised by Dr. Miguel Santos from ITQB-UNL (Universidade NOVA de Lisboa) and Prof. Cláudia Lobato da Silva from Instituto Superior Técnico.

Declaration

"I declare that this document is an original work of my own authorship and that it fulfills all the requirement of the Code of Conduct and Good Practices of the Universidade de Lisboa"

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Abstract

The therapeutical properties of *Cannabis* and its derived phytocannabinoids have been investigated across the globe for therapeutic applications thanks to their anti-inflammatory, anxiolytic or anti-epileptic effects. Furthermore, mesenchymal stem cells (MSCs) and their derived secretome are already commonly used in regenerative applications, due to their anti-inflammatory and immunomodulatory properties. Therefore, the combination of phytocannabinoids and MSCs seems to be an excellent strategy for tissue regeneration.

Hence, this thesis has the main objective of assessing the synergistic potential between phytocannabinoids, specifically cannabidiol (CBD) and cannabidivarin (CBDV), and umbilical cord-derived MSCs (ucMSCs). To achieve this, the work is divided into two main parts. The first consists in characterizing the cannabinoid receptors in ucMSCs, while the second consists in the evaluation of the therapeutic potential that the secretome of ucMSCs presents when these cells are modulated with the phytocannabinoids.

The results obtained confirm the presence of cannabinoid receptors in ucMSCs and that these receptors can be induced when cells are exposed to an inflammatory stimulus. Furthermore, CBD and CBDV modulate ucMSCs' gene expression profile and secretion of cytokines involved in tissue repair, showing that phytocannabinoids can influence these cells therapeutic potential.

Ultimately, this thesis fills the gap of the lack of characterization of the cannabinoid receptors in ucMSCs, showing that these receptors are present and can be targeted, and that these cells can be modulated by phytocannabinoids, opening the discussion about the potential therapies that can be formulated from the interaction between these factors.

Keywords: Phytocannabinoids, Cannabinoid receptors, Mesenchymal stem cells, Secretome, Tissue regeneration.

Resumo

As propriedades terapêuticas da *Cannabis* e dos seus fitocanabinóides têm sido investigadas em todo o mundo para aplicações terapêuticas devido aos seus efeitos anti-inflamatórios, ansiolíticos ou antiepiléticos. As células estaminais mesenquimais (MSCs) e o seu respetivo secretoma já são amplamente exploradas em aplicações regenerativas, devido às suas propriedades anti-inflamatórias e imunomoduladoras. Desta forma, a combinação entre fitocanabinóides e MSCs parece ser uma excelente estratégia para regeneração de tecidos.

Assim, esta tese tem como objetivo principal a avaliação do potencial sinergístico entre os fitocanabinóides, nomeadamente canabidiol (CBD) e canabidivarina (CBDV), e as MSCs derivadas do cordão umbilical (ucMSCs). Para alcançá-lo, este trabalho está dividido em duas partes principais. A primeira consiste em caraterizar os recetores canabinóides nas ucMSCs, enquanto a segunda se baseia na avaliação do potencial terapêutico que o secretoma derivado das ucMSCs apresenta quando estas células são moduladas por fitocanabinóides.

Os resultados obtidos confirmam a presença de recetores canabinóides nas ucMSCs e que estes recetores podem ser induzidos quando estas células são expostas a um estímulo inflamatório. Além disso, o CBD e a CBDV modulam o perfil de expressão génica das ucMSCs e a secreção de citocinas envolvidas na reparação de tecidos, mostrando que os fitocanabinóides conseguem influenciar o potencial terapêutico destas células.

Em última análise, esta tese mostra que os recetores canabinóides estão presentes e podem ser induzidos nas ucMSCs, e que estas células podem ser moduladas por fitocanabinóides, abrindo a discussão para as potenciais terapias que poderão ser formuladas através da interação entre estes fatores.

Palavras-chave: Fitocanabinóides, Recetores canabinóides, Células estaminais mesenquimais, Secretoma, Regeneração de tecidos.

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List of abbreviations

2-AG – 2-arachidonoyl glycerol

α-MEM – minimum essential medium alpha modification

AEA – anandamide

atMSCs – adipose tissue derived mesenchymal stem cells

ATMPs – advanced therapy medicinal products

bmMSCs – bone marrow derived mesenchymal stem cells

CB1 – cannabinoid receptor 1

CB2 – cannabinoid receptor 2

CBD – cannabidiol

CBDA – cannabidiolic acid

CBDV – cannabidivarin

CBDVA – cannabidivarinic acid

CBG – cannabigerol

CBGA – cannabigerolic acid

CBGVA – cannabigerovarinic acid

CBN – cannabinol

cDNA – complementary deoxyribonucleic acid

CM – conditioned medium

C_{max} – maximum plasmatic concentration

CNS – central nervous system

CO_2 – carbon dioxide

DAGL – diacylglycerol lipase

DMEM – Dulbecco's modified Eagle medium

DMSO – dimethyl sulfoxide

ECL – enhanced chemiluminescence

ECS – endocannabinoid system

EGF – epithelial growth factor

ESCs – embryonic stem cells

FAAH – fatty acid amino hydrolase

FBS – fetal bovine serum

FGF2 – fibroblast growth factor 2

G-CSF – granulocyte-colony stimulating factor

GPCR – G-protein coupled receptor

GVHD – graft versus host disease

HRP – horseradish peroxidase

IFN-γ – interferon-γ

IL – interleukin

iPSCs – induced pluripotent stem cells

ISCT – International Society for Cellular Therapy

KGF – keratinocyte growth factor

K_i – binding affinity

LPS – lipopolysaccharides

MAGL – monoacylglycerol lipase

MAPK – mitogen-activated protein kinases

MSCs – mesenchymal stem cells

NAPE-PLD – N-arachidonoyl phosphatidyl ethanolamine phospholipase D

OA – osteoarthritis

PBMC – peripheral blood mononuclear cells

PBS – phosphate-buffered saline

PGE2 – prostaglandin E2

PPAR – peroxisome proliferator activated receptor

qRT-PCR – quantitative real-time polymerase chain reaction

RNA – ribonucleic acid

SDS-PAGE – sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SEM – standard error of the mean

THC – Δ^9-tetrahydrocannabinol

THCA – Δ^9-tetrohyrocannabinolic acid

THCV – Δ^9-tetrahydrocannabivarin

THCVA – Δ^9-tetrahydrocannabivarinic acid

TGF-β – transforming growth factor β

TNF – tumor necrosis factor

Tregs – T regulatory cells

TRP – transient receptor potential

TRPA – transient receptor potential ankyrin

TRPM – transient receptor potential melastatin

TRPV – transient receptor potential vanilloid

ucMSCs – umbilical cord derived mesenchymal stem cells

VEGF – vascular endothelial growth factor

WB – western blot

WJ-MSCs – Wharton's Jelly derived mesenchymal stem cells

1. Introduction

1.1. Cannabinoids

1.1.1. The endocannabinoid system

The endocannabinoid system (ECS) is involved in important physiological processes, such as the maintenance of homeostasis, the regulation of anxiety and feeding behaviors, pain sensation, amongst others[1]. The ECS consists of various components, such as the cannabinoid receptors, namely cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2), which are two 7-transmembrane-domain G-protein coupled receptors (GPCR) that bind to cannabinoids, as well as their endogenous ligands, called endocannabinoids, of which the best known are anandamide (AEA) and 2-arachidonoyl glycerol (2-AG). Moreover, the ECS is also composed by the enzymes responsible for the synthesis and degradation of endocannabinoids, with diacylglycerol lipases (DAGL), DAGL-α and DAGL-β, and monoacylglycerol lipase (MAGL) being responsible for 2-AG synthesis and degradation, respectively, while N-arachidonoyl phosphatidyl ethanolamine phospholipase D (NAPE-PLD) and fatty acid amino hydrolase (FAAH) are responsible for AEA formation and breakdown, respectively[2,3]. The levels of 2-AG are markedly higher than AEA and whilst 2-AG has full agonist activity at both cannabinoid receptors[4], AEA is a partial agonist with high affinity to CB1 and low affinity to CB2[5].

Endocannabinoids function as “retrograde” signals in brain central synapses (Figure 1). After their production in the postsynaptic neuron, believed to be *de novo* and on demand following elevation of intracellular calcium concentration, endocannabinoids travel “backwards”, activating the CB1 receptors located in the presynaptic nerve terminals and contributing to processes of synaptic plasticity, including depolarization-induced suppression of excitatory or inhibitory currents, or long-term depression of both excitatory and inhibitory signaling. Both the endocannabinoids and the enzymes responsible for their synthesis and degradation interact with several signaling pathways outside of the ECS, namely those regulated by transient receptor potential (TRP) channels or the receptor GPR55[6]. Moreover, macrophages, neutrophiles, T-cells and other immune cells that express cannabinoid receptors can be modulated by the ECS, showing that a therapeutic strategy that interacts with the respective receptors may also present immunologic effects[5].

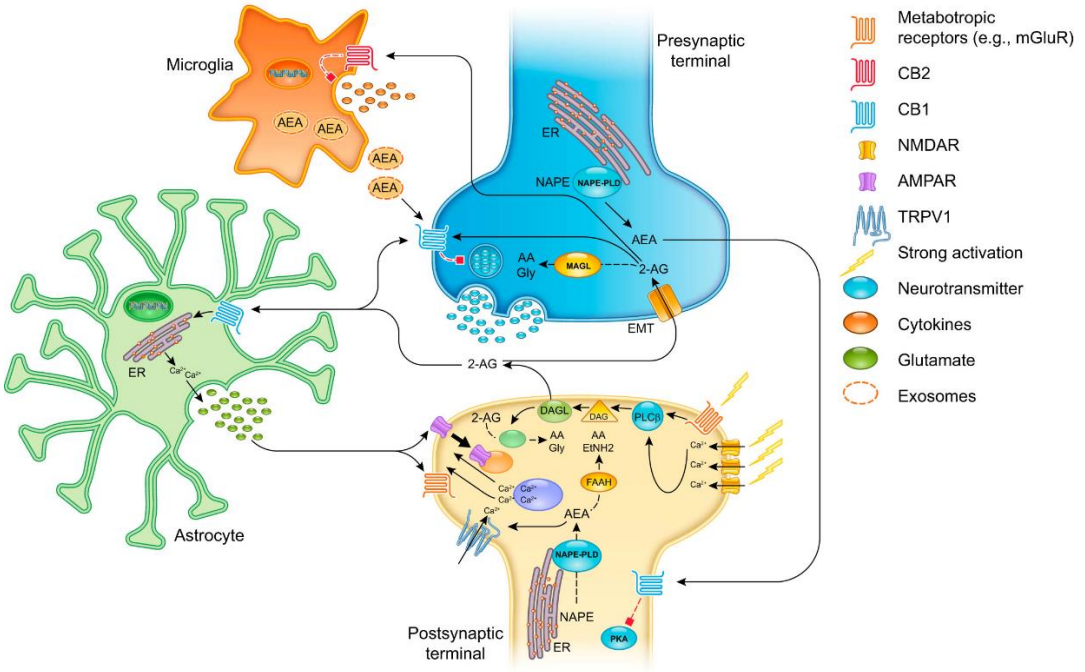

Figure 1- The endocannabinoid system modulates synaptic plasticity through retrograde signaling at synapses. Endocannabinoids are generated in the postsynaptic neuron by intracellular calcium (Ca^{2+}) mobilization. Anandamide (AEA) induces synaptic strength through transient receptor potential vanilloid 1 (TRPV1) channels, triggering long-term depression. 2-arachidonoyl glycerol (2-AG) travels to the presynaptic neuron and inhibits neurotransmitter release. Cannabinoid receptor 1 (CB1) in astrocytes is activated by endocannabinoids with elevation of Ca^{2+} and stimulation of glutamate release and its participation in synaptic plasticity. AEA can also be produced in microglia, inhibiting presynaptic transmission via CB1 receptors. Black arrows indicate stimulation, and red blunted arrows indicate inhibition. Dotted black arrows indicate enzymatic transformation[6].

1.1.1.1. Cannabinoid Receptors

As previously mentioned, the cannabinoid receptors, namely CB1 and CB2, are members of the GPCR family, both coupling to inhibitory G proteins and modulating various intracellular signaling pathways important for the control of cell survival, differentiation and apoptosis, such as the inhibition of adenylyl cyclase activity, stimulation of mitogen-activated protein kinases (MAPK) and recruitment of beta-arrestins[2,5]. Both receptors are expressed in the cells of various organs, such as skin, immune cells, lung, heart, among others. Nevertheless, CB1 receptors are mainly present in the brain and central nervous system (CNS), mediating excitatory and inhibitory neurotransmitter release in GABAergic and glutamatergic neurons, while CB2 receptors are mainly expressed in the peripheral nervous system, immune cells and tissues, like spleen and thymus[7]. Therefore, the activation of CB1 receptors can regulate memory, cognition, and mood processes, inhibit nociception and activate reward pathways, since it is present in regions of the CNS responsible for those sensory and motor functions, like basal ganglia, substantia nigra, globus pallidus, cerebellum, hippocampus, and brain stem[6,8,9]; while CB2

activation influences mainly processes related with inflammation and immunomodulation, suppressing immune cell activation through modulation of T-helper cells, inhibition of pro-inflammatory cytokine production, and nuclear factor-B dependent apoptosis[10]. Importantly, contrary to CB1 activation, CB2 receptor activation does not show psychotropic activity[11], turning this receptor into a prime target for regenerative therapies where the occurrence of psychoactive effects is not advantageous. Not only endocannabinoids, but also phytocannabinoids, which are cannabinoids produced by *Cannabis* and other plants, can interact with these receptors. Moreover, synthetic cannabinoids, the so called "spice", which are produced *in vitro*, can specifically bind to a certain or both receptors with high binding affinity, usually functioning as an agonist of those receptors, and can therefore cause more pronounced psychoactive effects[12,13].

Besides the two main cannabinoid receptors CB1 and CB2, cannabinoids can also interact with other receptors, such as TRP channels, the so called "ionotropic cannabinoid receptors", since they function as ion channels. Inside this channel family, only three subfamilies have been shown to be modulated by cannabinoids. These are the TRP vanilloid (TRPV), the TRP ankyrin (TRPA) and the TRP melastatin (TRPM) subfamilies, inside of which only TRPV1, TRPV2, TRPV3, TRPV4, TRPA1 and TRPM8 are capable of interacting with cannabinoids[14,15]. These channels are involved in many functions, working as sensors for several physiological and pathological processes such as itch, temperature and pain sensation and regulation, besides playing an important role on inflammation[16,17]. For TRPV1, after its activation by calcium, a series of calcium-dependent processes are stimulated, ultimately leading to the desensitization of this channel. Once desensitized, the channel enters a refractory period when it can no longer respond to further stimulation, causing a paradoxical analgesic effect[14]. Phytocannabinoids have been shown to activate these channels, desensitizing some, such as TRPV1 and TRPA1[18], denoting their anti-hyperalgesic effects. Unlike what occurs in the other TRP channels, cannabinoids possess antagonistic activity at TRPM8, suppressing the intracellular calcium elevation caused by the activation of this channel[18]. Antagonists to this receptor have also shown analgesic properties, being capable to reduce cold-related pain sensation[19].

Beyond TRP channels, phytocannabinoids can interact with other receptors, such as the nuclear peroxisome proliferator activated receptor (PPAR) family, namely PPARγ, a key regulator of adipogenesis[20,21]. Other GPCRs, such as GPR55, which is responsible for energy homeostasis, can be modulated by some cannabinoids as well, possessing important roles in nociception and neuropathic or inflammatory pain[7].

1.1.2. Phytocannabinoids

The use of *Cannabis* plants as a medicinal product has been documented since ancient times to alleviate pain and treat infections[6,22]. Phytocannabinoids are plant-derived cannabinoids, mainly from plants of the genus *Cannabis*, such as *Cannabis sativa* or *Cannabis indica*, indigenous from Asia. These compounds are especially interesting for their many therapeutic effects, turning them into a prime target

for regenerative medicine. The major phytocannabinoids are Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD). However other cannabinoids have also been shown to possess therapeutic properties, such as cannabidivarin (CBDV), cannabinol (CBN), cannabigerol (CBG), Δ^9-tetrahydrocannabivarin (THCV), among others[15,23]. This work will study both CBD and one of its homologs CBDV. Therefore, the actions and properties of these two phytocannabinoids, as well as THC, will be further explored in the following paragraphs.

The biosynthesis pathway that leads to the formation of the main phytocannabinoids is derived from fatty acids, that generate hexanoyl-CoA and butyl-CoA. These compounds are converted into olivetolic acid and divarinic acid, respectively, through polyketide cyclase enzymes. Olivetolic acid and divarinic acid give rise to cannabigerolic acid (CBGA) and cannabigerovarinic acid (CBGVA), respectively, mediated by cannabigerolic acid synthase. These compounds generate the acid precursors of cannabinoids with CBGA forming acids with a pentyl side chain, such as cannabidiolic acid (CBDA) and Δ^9-tetrohyrocannabinolic acid (THCA), while CBGVA is involved in the creation of acids containing a propyl side chain like cannabidivarinic acid (CBDVA) and Δ^9-tetrahydrocannabivarinic acid (THCVA). These acidic forms are commonly found in fresh plants, however by exposure to factors such as heat or light, decarboxylation can occur which causes loss of its unstable carboxylic group. Following this reaction, the final cannabinoid compounds are synthesized, with CBD deriving from CBDA, CBDV deriving from CBDVA and THC deriving from THCA, and most of the remaining phytocannabinoids deriving from their respective acidic precursors[24–26]. The formation process of the main phytocannabinoids is represented in Figure 2.

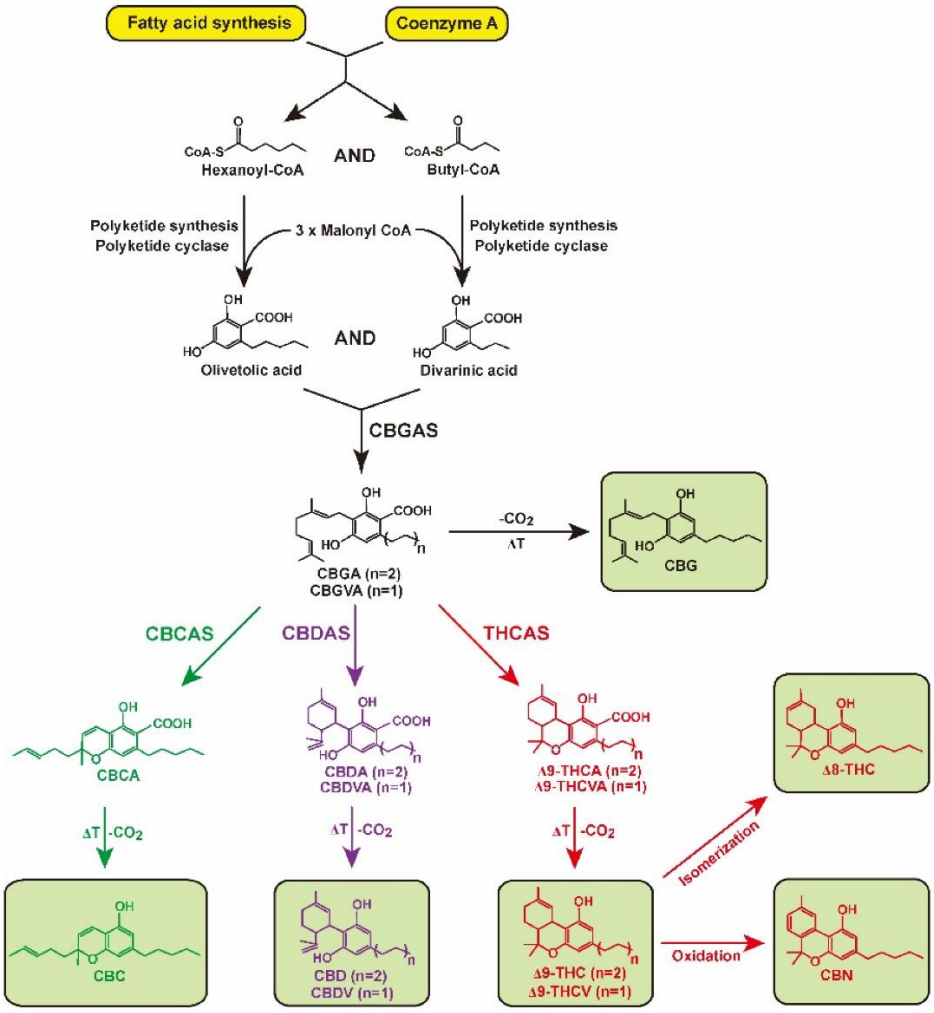

Figure 2- Biosynthetic pathways of phytocannabinoids. Olivetolic acid and divarinic acid are synthesized by hexanoyl-CoA and butyl-CoA, respectively. Through the action of cannabigerolic acid synthase (CBGAS), olivetolic acid will give rise to cannabigerolic acid (CBGA), which generates cannabichromenic acid (CBCA), cannabidiolic acid (CBDA) and Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA); while divarinic acid forms cannabigerovarinic acid (CBGVA), which gives rise to cannabidivarinic acid (CBDVA) and Δ^9-tetrahydrocannabivarinic acid (Δ^9-THCVA). These acids are converted into neutral cannabinoids by decarboxylation processes, like cannabigerol (CBG), cannabichromene (CBC), cannabidiol (CBD), cannabidivarin (CBDV), Δ^9-tetrahydrocannabinol (Δ^9-THC), or Δ^9-tetrahydrocannabivarin (Δ^9-THCV). Δ^9-THC can also suffer isomerization or oxidation, forming Δ^8-tetrahydrocannabinol (Δ^8-THC) or cannabinol (CBN), respectively[25].

The different molecular structure that phytocannabinoids possess may be responsible for their different effects at the cannabinoid receptors. While THC binds to CB1 and CB2 with relatively high affinity, CBD and CBDV have much lower binding capacity to these receptors. Even between CBD and CBDV, the presence of a propyl side chain in the latter may cause this compound to interact with cannabinoid receptors differently from the other phytocannabinoid, which could cause higher affinity of CBDV to CB2 and lower affinity to CB1, in comparison with CBD[23].

THC is a partial agonist for the cannabinoid receptors CB1, leading to its known psychotropic effects, as well as CB2[27]. CBD, and similarly CBDV, function as inverse agonists or negative allosteric

modulators for these two receptors, blocking its access to their agonists, what causes these compounds to lack psychoactive effects and antagonize the effects caused by THC, like anxiety, hunger, sedation and tachycardia[23], making them look promising for the use in medicinal applications. Besides the interaction with CB1 and CB2, both CBD and CBDV can interact with TRP channels, working as agonists for TRPV1, TRPV2, TRPA1, and as antagonists of TRPM8[18,28], potentiating some of the effects caused by the activation of these receptors, such as pain sensation and inflammatory responses[29]. These phytocannabinoids can desensitize these channels following their activation[30], creating a consequent analgesic effect. Besides those channels, CBD is also able to interact with the PPAR receptors family[15], namely with PPARγ, which has roles in adipogenesis[20], fatty acid storage, cell differentiation and growth. It can also function as a GPR55 antagonist, a receptor that has roles in inflammatory pain[6].

CBD also affects processes in the ECS, namely increasing the tissue levels of AEA by suppressing the activity of FAAH[18], and therefore can possess an indirect agonist activity at cannabinoid receptors[6]. Nevertheless, as mentioned before, it functions as a negative allosteric modulator at CB1, reducing efficacy and potency of endocannabinoids and THC for this receptor[31], and impeding the occurrence of psychotropic effects characteristic of the binding of these compounds to CB1. Thanks to the wide array of target receptors that can interact with these phytocannabinoids, there is a high potential use of these compounds in a range of pathological conditions.

1.1.3. Therapeutical applications of cannabinoids

Given the different therapeutical properties of phytocannabinoids, researchers express great interest in their use for several applications. Both CBD and CBDV, as well as THC, have purported anxiolytic, analgesic, anti-epileptic and anti-inflammatory effects[32], therefore their clinical applications are aplenty. Phytocannabinoids are being tested to help ameliorate the inflammatory conditions in several diseases[33], such as allergic contact dermatitis[34] and other skin disorders[35–38], osteoarthritis[39,40], ulcerative colitis[41], Duchenne muscular dystrophy[42], among others. CBD has been applied in the treatment and alleviation of chronic pain[43], although further studies and clinical trials are still necessary to assess with certainty the effects of this compound in these situations[44]. Due to not possessing psychoactive activity, CBD also shows a high therapeutic potential in the treatment of symptoms related with neuropsychiatric disorders, such as anxiety, depression, Alzheimer's, Parkinson's, multiple sclerosis, schizophrenia, posttraumatic stress disorder, among others[1,22,33,45–47]. THC has been shown to possess positive effects in some of these conditions[11], however there is a risk of adverse effects originated from its psychotropic activity, such as hypolocomotion, hypothermia, catalepsy, and analgesia[48]. CBDV is tested for its anticonvulsive properties, majorly in epilepsy and autism spectrum diseases[28], with varying effects obtained that could be explained by the low oral bioavailability of the compound and the heterogeneity of these diseases. A study that approached the effect of CBDV in autism spectrum disorders, showed that this compound can modulate glutamate levels in the human brain[49]. This compound, together with CBD, have been tested as a mean to reduce neuronal hyperexcitability in rat hippocampus, due to their ability to desensitize TRP channels, leading to a

possible prevention of epileptic activity[30]. Moreover, it has been shown that CBD possesses anti-hyperalgesic effects in rat models with thermal hyperalgesia due to its activation and desensitization of TRPV1[50].

Since cannabinoids have a wide span of effects, there are already several commercially available therapies containing these compounds. Epidiolex® has as active principle CBD and was the first drug approved by the Food and Drug Administration, being orally administered for the treatment of seizures in Lennox-Gastaut or Dravet syndromes[51]. Sativex®, also called Nabiximol, is another approved drug that contains equimolar concentrations of THC and CBD, available as a sublingual spray to relieve symptoms of neuropathic pain in patients with multiple sclerosis[11,51]. Other approved medicines contain synthetic analogues of THC, such as dronabinol (Marinol®) and nabilone (Cesamet®), to control nausea and vomiting in oncologic patients[23].

Similar to phytocannabinoids, endocannabinoids and other modulators of the ECS, like synthetic cannabinoids, also present several applications in different fields, testing the effects caused by the binding of these compounds to cannabinoid or other receptors involved in this system, and the respective modulation of the signaling pathways influenced by them. Thanks to these properties, the ECS can be targeted in many areas of research, like inflammatory and skin diseases[52–54], neurodegenerative disorders[6,46], cartilage diseases[55–57], pain management, and many other conditions[58] with different levels of severity.

1.2. Mesenchymal stem cells

Stem cells are undifferentiated cells characterized by their capacity of self-renewal and differentiation into different types of tissues. These cells have different levels of potency depending on the variety of tissues they can differentiate into. Pluripotent cells can differentiate into cells from any of the three germ layers: endoderm, mesoderm or ectoderm, while multipotent stem cells can only differentiate into tissues from one layer[59]. There are different types of stem cells, for example embryonic stem cells (ESCs), which are pluripotent cells originated in the inner cell mass of the embryo, induced pluripotent stem cells (iPSCs), which are originated from somatic cells that are reprogrammed by pluripotency factors *in vitro*, giving rise to pluripotent stem cells, or mesenchymal stem cells (MSCs), which are multipotent stem cells with the capacity to differentiate in different tissues originated from the mesoderm, such as bone, cartilage and adipose tissue. Thanks to the properties these cells present, they have become extremely useful in the regenerative medicine field, being commonly investigated to tackle tissue and organ regeneration. The use of MSCs has advantages compared with the other types of stem cells, due to not generating ethical issues with its use, contrary to ESCs, given their embryonic origin, or not having to undertake a reprogramming process *in vitro* before its application, like iPSCs, besides having lower risk of clinical tumorigenesis than pluripotent stem cells[60].

MSCs are characterized by a fibroblastic-like morphology and must fulfill three minimum criteria, as defined by the International Society for Cellular Therapy (ISCT), namely being able to adhere to a plastic

surface under normal culture conditions, to express specific cell-surface markers, namely CD105, CD73 and CD90, lack the expression of CD45, CD34, CD14, CD19 and HLA class II, and present tri-lineage differentiation potential into osteogenic, chondrogenic and adipogenic lineages[10,61]. These cells can be extracted from various sources, mainly from bone marrow (bmMSCs), adipose tissue (atMSCs), umbilical cord blood or matrix (ucMSCs), amongst others. Furthermore, MSCs present a high therapeutic potential due to their ability to migrate into injury sites (homing) to differentiate and replace damaged tissues, their low immunogenicity and immunosuppressive properties, but mostly because of their paracrine effects leading to the release of specific cytokines and growth factors[62–65]. Although bmMSCs are the most commonly used MSCs for regenerative applications[64], other types such as ucMSCs have been gaining increasing interest in the regenerative field, since these present an easier noninvasive extraction and isolation, as well as more primordial characteristics, given that ucMSCs are extracted from neonatal tissues, with higher capacity for expansion[66,67].

1.2.1. Mesenchymal stem cells on tissue regeneration

MSCs regulate several regenerative processes, contributing as homeostasis regulators and aiding in the resolution of inflammation and tissue repair in various sites. Their therapeutic effect is mostly associated with its differentiation capacity (Figure 3) and paracrine activity.

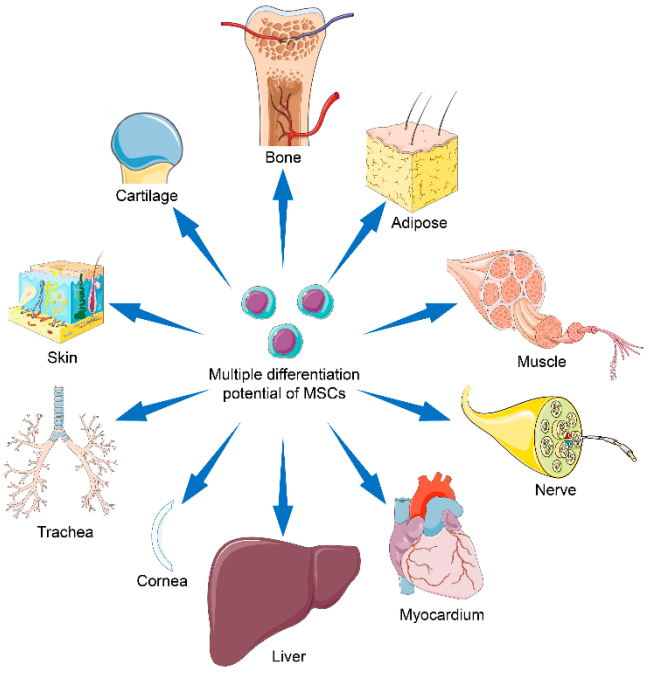

Figure 3- Differentiation potential of mesenchymal stem cells involved in the repair of various tissues, such as cartilage, bone, adipose tissue, muscle, nerve, myocardium, liver, cornea, trachea and skin[68].

MSCs have been applied in bone regeneration, where their differentiation into osteoblasts allows them to heal injured bone tissue, and can be used in varied applications, such as implantation of scaffolds seeded with MSCs into bone defects to help accelerate bone reconstruction[68]. Cartilage is another tissue

that benefits from MSCs' differentiation capacity, since it is a difficult tissue to recover from injury given its avascular and hypoxic nature. Therefore, chondrogenesis of MSCs is an important property in the repair of cartilage and osteochondral injury [40,69] and, together with these cells trophic abilities, can be applied to cartilage degenerative conditions like osteoarthritis (OA)[70].

Moreover, MSCs have neurotrophic and neuroprotective effects, improving nerve injury models by modulating the inflammatory environment on the site, increasing vascularization, thickness of myelin sheets and accelerating fiber regeneration[71], and are tested for treatment of conditions such as spinal cord injury or Parkinson's disease[72]. MSCs can also regenerate the myocardium, by differentiating into cardiomyocytes, secreting angiogenic, anti-apoptotic and mitogenic factors and inhibiting myocardial fibrosis[68]. Liver is another organ that can be regenerated with the aid of MSCs, thanks to their immunomodulatory properties, impeding tissue fibrosis and improving liver function in alcoholic cirrhosis, and through differentiation into hepatocytes, aiding in liver regeneration and impeding its failure[68,72].

Other example of the action of MSCs is skin repair and wound healing, through the secretion of different cytokines and growth factors throughout the different phases of this process [73–75.] The use of MSCs for skin treatment can improve skin regeneration and reduce scarring. MSCs exert their functions through migration into the damaged site, suppressing inflammation, and increasing the growth and differentiation ability of fibroblasts, epidermal cells, and endothelial cells[72].

Given the wide range of applications that MSCs and their derived secretome are being tested on, over the last years, there have been an increasing number of advanced therapy medicinal products (ATMPs) that have been approved across the world, denoting the value of these cells for regenerative medicine. Some of these include Alofistel® for the treatment of Chron's disease, Prochymal® and Temcell® HS for graft versus host disease (GVHD), Cartistem® for knee articular cartilage defects, Stemirac® for spinal cord injury, Stempeucel® for critical limb ischemia, and some others[76,77].

1.2.2. Secretome of mesenchymal stem cells

The secretome, also called conditioned medium (CM), is defined by the set of molecules secreted by MSCs (Figure 4). It includes growth factors, cytokines, exosomes and microvesicles, among other factors, which play important roles in cell communication, signal transduction and inflammatory responses[78]. The use of the secretome is advantageous for therapeutic purposes, since it has shown to improve the regeneration and repair of injured tissue and it overcomes the problems associated with the administration of live cells, such as the risk of rejection, occurrence of GVHD or tumoral transformation. The secretome has the benefit of being able to be stored for long periods of time without loss of product potency or use of toxic cryopreservative agents, and the biological product obtained can be modified for therapeutic applications based on the cell-specific effects desired[71], namely by modulating the environmental cues to which MSCs are exposed.

MSCs can have immune plasticity and possess two distinct secretory profiles depending on the microenvironment they are exposed to: a pro-inflammatory MSC1 or an anti-inflammatory or immunosuppressive MSC2[27]. An inflammatory MSC1 is characterized by the release of interleukin (IL)-6 and IL-8 and promotion of the activation of T cells[60]. On the other hand, when MSCs are exposed to a pro-inflammatory environment, promote the secretion of anti-inflammatory cytokines such as IL-10 and prostaglandin E2 (PGE2), leading to a lower expression of pro-inflammatory factors like IL-6, tumor necrosis factor (TNF)-α and IL-1β[65].

The secretome of MSCs can be used in various applications thanks to its immunosuppressive effects, modulating the release of several factors that influence tissue regeneration and wound re-epithelialization, such as keratinocyte growth factor (KGF), fibroblast growth factor 2 (FGF2), epithelial growth factor (EGF), with roles in the re-epithelialization process, promoting migration and differentiation of fibroblasts and keratinocytes[75], as well as vascular endothelial growth factor α (VEGF-α), a known regulator of angiogenesis[64,74]. This secretome can also promote a faster and scar-free tissue repair process, preventing the differentiation of fibroblasts into myofibroblasts by blocking transforming growth factor β1 (TGF-β1), and since myofibroblasts secrete more collagen, lead to the suppression of wound contraction and scar formation[67]. The secretome can also be an important factor in angiogenesis regulation and has already been demonstrated to promote this process in animal models of cerebral ischemia, myocardial infarction, among others[71].

The secretome of MSCs can be easily modulated through a change in the cues present in their microenvironment, in a process called priming, namely by modulation with other molecules or factors, such as inducing an inflammatory environment, *e.g.,* by increasing concentrations of TNF-α or interferon γ (IFN-γ)[60,71], or by changing environmental aspects and culture conditions, such as mechanical stress or oxygen concentration[77]. Likewise, this secretome can be modulated by phytocannabinoids. THC pretreated bmMSCs, for example, produced a secretome with immunomodulatory properties, namely with increased levels of IL-10, which was capable of reducing the production of inflammatory cytokines by the microglia that was previously induced by exposure to an inflammatory stimulus (lipopolysaccharides; LPS)[79]. Therefore, priming of MSCs has become a useful tool to easily modulate the therapeutic effects desired for a specific application, and the derived secretome possesses several advantages when compared to therapies using stem cells.

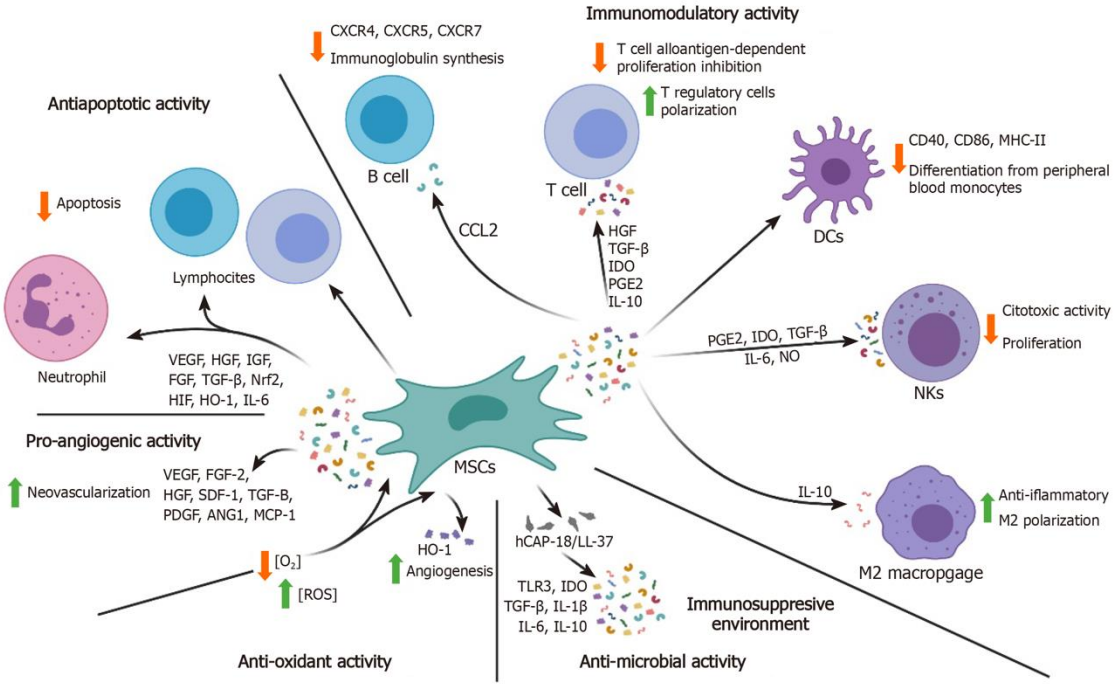

Figure 4- Summary of the various soluble factors secreted by mesenchymal stem cells and their functions: Immunomodulatory, immunosuppressive, anti-oxidant, anti-apoptotic, anti-microbial and pro-angiogenic activities[80].

1.3. Therapeutical applications of MSCs and their combination with cannabinoids

MSCs from the bone marrow[10,79] and adipose tissue[81] have shown the expression of both cannabinoid receptors and can interact with endo- and phytocannabinoids[27,73,82]. Furthermore, it has been observed that the expression of these receptors in bmMSCs changes according to its passage, with CB1 expression decreasing after each passage, while the opposite occurs with CB2 expression[10,79]. CB1 and CB2 activity can regulate their migration and proliferation[21,63], being also essential within the MSCs' inflammatory response, since their absence can increase overall pro-inflammatory cytokines as well as delaying the wound healing process[83]. In fact, CBD modulates the action of MSCs, promoting the anti-inflammatory functions of these cells through the reduction of pro-inflammatory cytokine levels in bmMSCs previously induced by an inflammatory stimulus[82], and can augment tissue regeneration by restoring atMSCs differentiation capacity after exposure to an inflammatory stimulus, without affecting their proliferation capacity[27]. Lastly, AEA and 2-AG have shown to promote the immunosuppressive effects of MSCs, decreasing the levels of the pro-inflammatory cytokines IL-6, IL-8 and TNF-α after an inflammatory stimulus, also presenting similar levels of suppression as the ones obtained from the action of a CB2 agonist, indicating that these effects could be mediated by this receptor[73]. Despite these findings in bmMSCs and atMSCs, there is still a lack of studies on the presence and the action of the ECS and phytocannabinoids in ucMSCs. Thus, research focusing on these elements is imperative in the near future, given the high regenerative potential of the MSCs from this source.

Cannabinoids can interact with MSCs, with the intent to potentiate their action in various applications. MSCs express both cannabinoid receptors, which makes them a useful target for interaction, with several studies targeting and activating CB2 to increase MSCs immunosuppressive properties[10,79]. Stimulation of CB2 receptors by an agonist caused an increase in bmMSCs viability, exerted an anti-inflammatory effect after cells were challenged with LPS and promoted migration of cells towards a 10μM concentration of 2-AG, demonstrating that endocannabinoids are relevant mediators for cell migration and proliferation[10]. Overall, the cannabinoid receptors are essential for the survival, differentiation and regenerative potential of MSCs[40,63,84,85], showing the importance that CB1, CB2 and the rest of the ECS have in these cells. Phytocannabinoids also play a similar role in these cells, with studies concluding that THC and CBD can aid MSCs in differentiation and survival activities[21,40,63,82]. THC has been shown to lead to a decrease in mouse bmMSCs viability and osteogenesis[63], however it increases their survival in stress conditions caused by serum withdrawal, besides increasing their chondrogenic potential[40], showing the contradictory effects that phytocannabinoids can have on MSCs depending on the evaluated outcome. Furthermore, CBD promotes the proliferation of bmMSC colonies and increases adipogenic factors in these cells, promoting adipogenesis[21], indicating that this compound is important in this differentiation process. CBD can also reduce the inflammatory markers induced by LPS stimulation in bmMSCs, together with promoting osteogenic differentiation markers in these cells, through a CB2-dependent p38 MAPK dependent pathway[82].

Regardless, the research on the actions of phytocannabinoids and the ECS in MSCs is only now starting to be more extensively explored, and there is a lack of studies elucidating the action of these compounds in ucMSCs. Therefore, this thesis aims to begin filling this gap.

2. Objectives

The final objective of this thesis is to observe a potential synergistic activity between ucMSCs and phytocannabinoids. To achieve such a goal, the work is divided into two integral parts. Due to the lack of characterization of the endocannabinoid system of ucMSCs, it is first needed to characterize the presence and activity of its cannabinoid receptors. The second part consists of the evaluation of the therapeutical potential of the secretome produced by ucMSCs, which were previously primed by phytocannabinoids. More specifically, the tasks are:

1. Characterization of the cannabinoid receptors in ucMSCs
   - Assessment of the basal gene expression of cannabinoid receptors in ucMSCs;
   - Evaluation of the induction ability of the cannabinoid receptors in ucMSCs.

2. Evaluation of the therapeutical potential of the secretome of ucMSCs primed with phytocannabinoids
   - Priming of ucMSCs with CBD and CBDV and secretome production;
   - Evaluation of the effect of the ucMSCs priming on its cannabinoid receptors and regeneration potential.

This work ultimately aims to setup the grounds for future studies regarding the combination of both phytocannabinoids and MSCs, for regenerative therapies.

3. Results and discussion

3.1. Characterization of the cannabinoid receptors in ucMSCs

To understand the capacity of ucMSCs to interact with cannabinoids, namely endocannabinoids and phytocannabinoids, a characterization of its endocannabinoid system is firstly needed. Thus, the first step consists in verifying if there is an expression of not only the cannabinoid receptors, but also other important receptors with which cannabinoids are known to interact with. After verifying that cannabinoid receptors are expressed in ucMSCs, it is needed to understand whether these receptors are inducible.

The basal expression of *CB1, CB2, TRPV1* and *TRPA1* was analyzed in ucMSCs, to verify the presence of these receptors. The results of this analysis are displayed in Figure 5a.

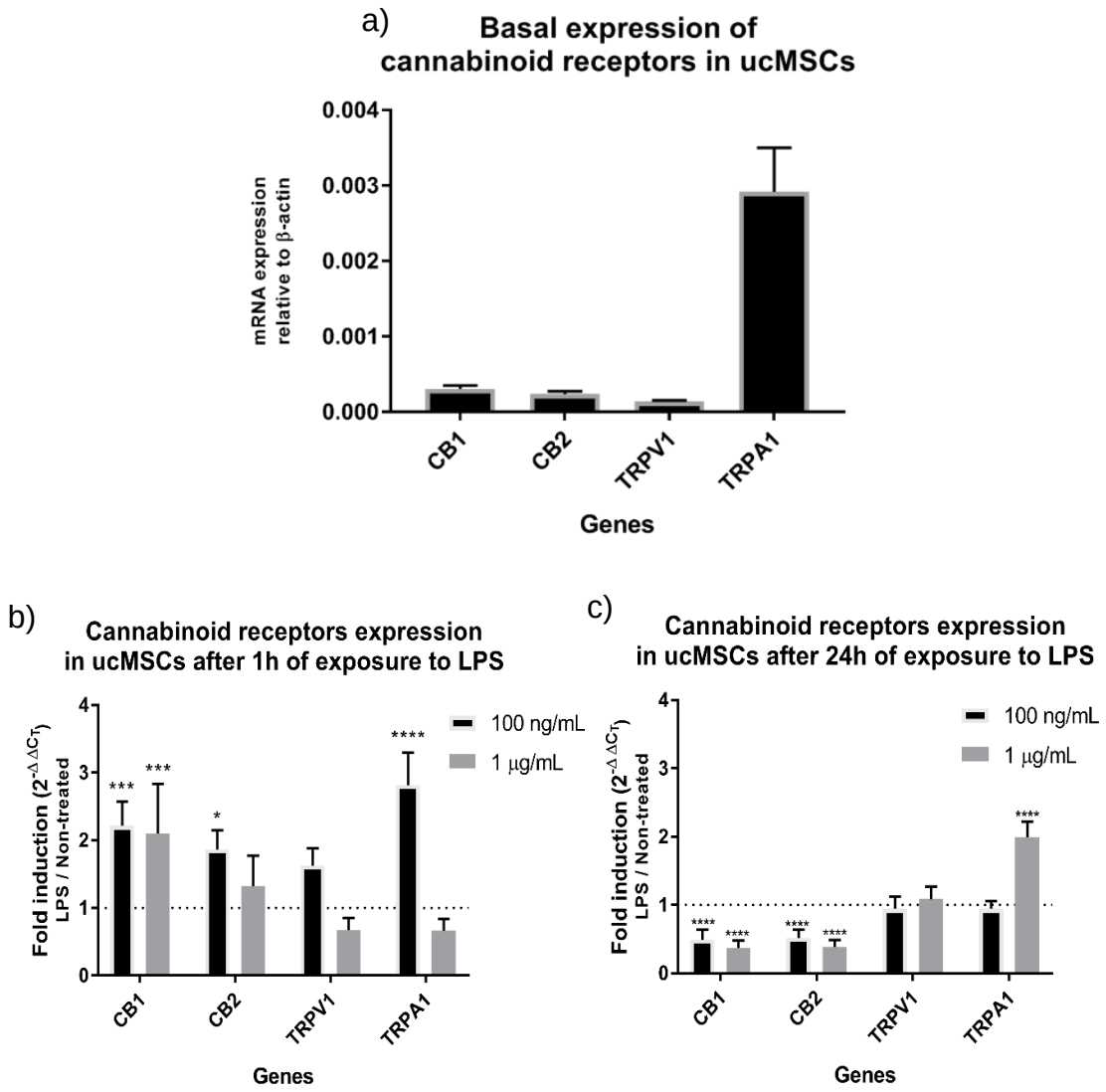

Figure 5- Gene expression levels of the cannabinoid receptors CB1, CB2, TRPV1 and TRPA1 in ucMSCs. a) Basal levels, b) upon 1h induction with LPS, c) upon 24h induction with LPS. Results are presented as the level of mRNA expression of the receptors in non-treated ucMSCs relative to the housekeeping gene β-actin (a) and fold

*induction relative to the non-treated control group (represented by the dotted line); b, c). Data are represented as Average ± SEM and statistically significant results are presented as *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 (n=3-6). Abbreviations: CB, cannabinoid receptor; CBD, cannabidiol; CBDV, cannabidivarin; LPS, lipopolysaccharides. TRPA1, transient receptor potential ankyrin 1; TRPV1, transient receptor potential vanilloid 1.*

As it can be seen, all receptors are expressed in ucMSCs, with *TRPA1* seemingly showing higher basal levels of expression (Figure 5a). Thus, it can be concluded that ucMSCs constitutively express cannabinoid receptors and are potentially able to interact with phyto- and endocannabinoids. In fact, cannabinoid receptors are present in a variety of cells throughout the human body, influencing many vital functions in different tissues. The expression of these receptors has also been detected on bmMSCs, where *CB1* expression has been verified to decrease through each cell passage, while *CB2* expression increases with each passage[10,79]. To account for this possibility, it should be noticed that ucMSCs from similar passages were used throughout this work. *CB1* and *CB2* have also been detected in atMSCs, however their expression significantly increases after the differentiation of these cells into adipocytes[81], which could indicate that these receptors regulate more functions in the differentiated cells originating from MSCs. The results obtained in this work indicate that, much like what happens with MSCs from different origins, ucMSCs also express both *CB1* and *CB2* receptors and therefore can regulate the functions that are mediated by them.

Regarding TRP channels, the expression of several of these receptors has been confirmed in various types of stem cells[86], including MSCs, influencing processes related with their proliferation and differentiation. The results obtained support the existence of these receptors in ucMSCs, specifically for *TRPV1* and *TRPA1* expression, thus allowing for interaction with the receptors by their agonists, like CBD or CBDV, which are then capable of modulating processes regulated by TRP channels, such as inflammation or pain sensation.

In the following steps, it was assessed whether the cannabinoid receptors would be induced by an inflammatory stimulus, as to confirm that these receptors respond to external stimuli and therefore can be potentially activated by the testing compounds (Figure 5b, c). The cannabinoid receptors *CB1* and *CB2* have already been demonstrated to be activated upon exposure to inflammatory stimulus such as LPS, a known inflammatory agent that is a component of the cell wall in gram-negative bacteria, in MSCs[82], keratinocytes and fibroblasts[52,82].

ucMSCs were exposed to two different concentrations of LPS, 0.1 and 1 µg/mL, for two different exposure times, 1h and 24h, after which cannabinoid receptor gene expression was measured (Figure 5b and 5c). As it can be seen in Figure 5b, in the case of exposure to LPS for 1h, there is an increase of expression of all genes exposed to a low concentration of LPS, while for a high concentration of this compound, both TRP channels analyzed present an expression similar to its basal level, which could mean that these receptors are only inducible when exposed to low amounts of LPS. However, after 24h of exposure to LPS (Figure 5c), both *CB1* and *CB2* have a reduced expression in all concentrations tested, which could signify that their response may be time-dependent, namely through their reaction to an inflammatory stimulus immediately after the first contact and reducing their expression thereafter, as

their action may not be needed. For the TRP channels, the inflammatory stimulus seems to minimally affect *TRPV1* expression, while for *TRPA1*, low concentrations of LPS seem to exert the same effect as the one observed in the main cannabinoid receptors. Nevertheless, higher concentrations of this inflammatory agent seem to have the opposite effect, increasing the expression level of *TRPA1* over time, since this receptor seems to be important in the ucMSCs' response to a pro-inflammatory condition.

CB1 and *CB2* have already been demonstrated to be activated upon exposure to inflammatory stimuli such as ultraviolet radiation in mouse embryonic fibroblasts[53]. Even for exposure to LPS, *CB1* and *CB2* expression has been shown to increase in keratinocytes and fibroblasts after exposition to 10 and 5 µg/mL of this agent, respectively, for 24h[52]. It was also observed in a different study that ucMSCs have a lower response to inflammatory stimulus caused by LPS, when compared to bmMSCs or atMSCs[87]. In mice bmMSCs, exposure of these cells to 10 µg/mL of LPS during 12h resulted in a significative decrease in expression of *CB1* and *CB2* receptors[82], which goes according to the results obtained here for 24h of exposure to this inflammation inducer.

TRP channels also affect processes involved in inflammatory responses[88]. Although their role in neurogenic inflammation and as response to LPS in sensory neurons is being studied[89,90], its response in MSCs still remains to be explored. *TRPA1* has been shown to be induced by exposure to LPS for 24h and 48h at a concentration of 100 ng/mL in dental pulp cells[91], proving that this inflammatory promoter can interact with *TRPA1*. Again, the effects of LPS and other inflammatory agents in TRP channels present in MSCs need to be clarified in future works, as their influence and mechanisms of action keep being investigated further.

Importantly, once LPS induces an inflammatory reaction on cells [27,82], depending on the concentration or the time of exposure to this compound, there may be an increase in cell apoptosis, and a correspondent decrease in cell viability. Hence, it is important to understand which conditions, namely which LPS concentrations and exposure times, can favor the expression of the receptors without affecting ucMSCs viability. To confirm this, ucMSCs were subjected to LPS and an MTS viability assay was performed to see how this agent affects the cell viability. The concentrations tested of LPS were 0.1, 0.5, 1 and 5 µg/mL, which correspond to the interval of concentrations usually found in the literature that have shown to induce an inflammatory reaction and increase levels of pro-inflammatory cytokines without affecting ucMSCs characteristics[87,92]. Exposure times to LPS were 1h or 24h to test the response of ucMSCs to a short or a more prolonged inflammatory stimulus, respectively. It was observed that this compound did not significantly alter the viability of these cells for all the conditions analyzed (Figure 6), denoting that LPS can be a useful agent for induction of an inflammatory state without affecting cell viability. These results are in accordance with previous studies, where a concentration of 10 µg/mL of LPS has shown to increase atMSCs proliferation after long-term exposure for 7 days[27], whereas in an experiment using bmMSCs, LPS increased their proliferation at low concentrations of 0.1 µg/mL but decreased it at concentrations of 10 µg/mL[93]. Most importantly, an experiment using Wharton's Jelly-derived MSCs (WJ-MSCs), that is, MSCs extracted from the umbilical cord matrix, exposed to 1.0 µg/mL of LPS showed that there was no alteration on cell proliferation up to 120h of exposure[87].

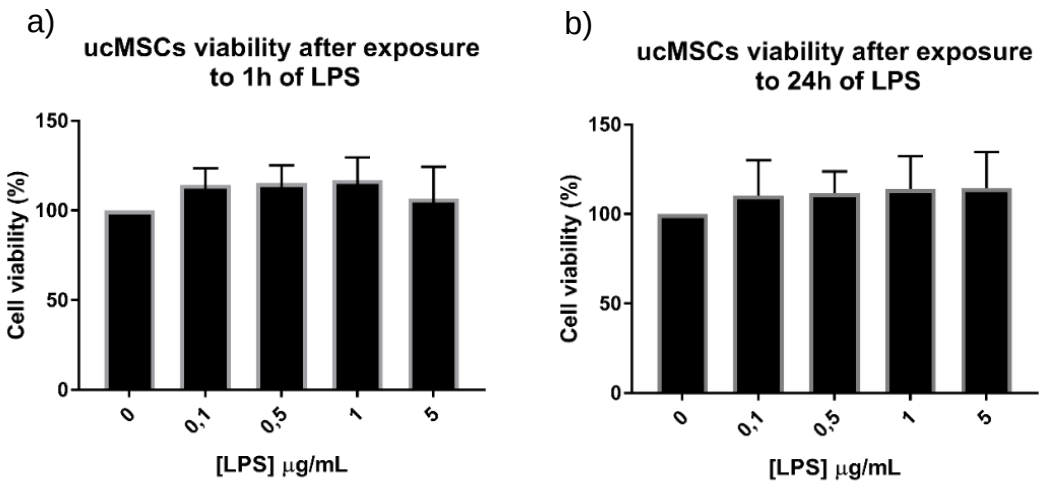

Figure 6- Viability of ucMSCs is not affected after exposure to different concentrations of LPS (0.1, 0.5, 1 and 5 µg/mL). Viability after a) 1h or b) 24h of exposure to LPS. Results are presented as a relative percentage to the non-treated control, with viability for this group being considered as 100% (n=3). LPS, lipopolysaccharides.

Since the condition of exposure to LPS at low concentrations (100 ng/mL) and for a period of 1h was the only to increase expression levels of all receptors analyzed, this same condition was applied in the future experiments for induction of the expression of the cannabinoid receptors in ucMSCs, prior to the application of phytocannabinoids.

3.2. Evaluation of the therapeutic potential of the secretome of ucMSCs primed with phytocannabinoids

After verifying the presence of the ECS in ucMSCs and confirming that cannabinoid receptors can be induced by an inflammatory stimulus, it was studied whether the phytocannabinoids CBD and CBDV could modulate the therapeutic effects of ucMSCs. The influence of CBD and CBDV was thus evaluated not only on ucMSCs in a basal state but also on ucMSCs whose cannabinoid receptors were previously induced, in order to understand whether the response could be modulated through their receptors. Thus, CBD and CBDV concentrations to be tested were firstly defined. Afterwards, the expression of selected genes relevant in tissue regeneration were analyzed to observe the influence of the phytocannabinoids in ucMSCs and enable the choice of the best condition to be used in the next step. Following this, the production of the therapeutic secretome was performed and changes in cell morphology as well as the presence of relevant proteins in the subsequent secretome were assessed.

3.2.1 Selection of the CBD and CBDV concentrations

The activity of each phytocannabinoid varies depending on the receptor it interacts with and the respective signaling pathway it can activate. It is important to know which concentrations of phytocannabinoids to choose when performing a study using these compounds, and how the used concentrations translate into plasmatic concentrations. Given this, the binding properties of several phytocannabinoids to their receptors as well as its maximum plasmatic concentration, measured in several clinical trials or *in vivo* models, was elucidated.

THC is a known agonist of the cannabinoid receptors with a much smaller binding affinity (K_i) for CB1 and CB2 when compared to CBD[23], explaining why THC acts as an agonist for both these receptors, while CBD does not, acting instead as an inverse agonist, consequently inhibiting the effects of THC. CBDV has a similar function as CBD with very low binding affinity for the cannabinoid receptors, acting as inverse agonist of these receptors[23]. The binding affinity values for these phytocannabinoids for CB1 and CB2 receptors are displayed in Table 1. These values of K_i represent the concentration of ligand necessary to occupy half of the receptors, where lower values indicate that less quantity of that compound is required to trigger a correspondent physiological response.

Table 1- Binding affinity (K_i) of different phytocannabinoids (THC, CBD, CBDV) to the cannabinoid receptors CB1 and CB2.[23]

		Receptor Binding affinity (K_i) (nM)	
		CB1	CB2
Compound	THC	40.7 ± 1.7	36.4 ± 10
	CBD	4350 ± 390	2860 ± 1230
	CBDV	14711 ± 5733.87	574.2 ± 146.1

Unlike what occurs at the cannabinoid receptors, CBD and CBDV act as agonists for most TRP channels. Both compounds show values of EC_{50} within the units of µM for TRPV1, with lower values observed for TRPA1[14,18], demonstrating that CBD and CBDV have higher potency for TRPA1 in comparison with TRPV1. EC_{50} values correspond to the concentration of ligand that is needed to produce half of the maximum possible effect caused by these compounds after binding to the receptor. Those values are displayed in Table 2, together with the efficacy of these compounds, represented as a percentage relative to the effect produced by a common agonist for each receptor, where it is seen that both of the studied compounds have a higher efficacy at TRPA1 than at TRPV1, when compared with their respective agonists.

*Table 2- Efficacy and potency of CBD and CBDV at different TRP channels; * Efficacy as % of the effect of 4 µM of ionomycin; ** Efficacy as % of the effect of 100 µM of allyl isothiocyanate.*[14,18]

		Receptor			
		TRPV1		TRPA1	
		Efficacy*	Potency (EC_{50}) (µM)	Efficacy**	Potency (EC_{50}) (µM)
Compound	CBD	44.7 ± 0.02	1.0 ± 0.1	115.9 ± 4.6	0.11 ± 0.05
	CBDV	21.4 ± 0.6	3.6 ± 0.7	105.0 ± 0.7	0.42 ± 0.01

For CBD, there are already several studies that attempt to understand its pharmacokinetic properties, such as Millar *et al.*[94] where the plasmatic concentrations (C_{max}) obtained in clinical trials after the administration of CBD are presented. Some of the values obtained are presented in Table 3, varying from 0.095 nM up to 2181 nM of CBD[94], depending not only on the administered dose but also on the administration route, providing an idea of the range of maximum concentrations of this compound in the blood circulation.

Table 3- Maximum plasmatic concentration (C_{max}), and its correspondent concentration values in nanomolar (nM), obtained after administration of a certain dose of CBD, through different routes of administration.[94]

C_{max} (ng/mL)	Concentration (nM)	Dose (mg)	Route of administration
0.39	1.24	5	Oromucosal Drops/ Spray
2.58	8.2	20	
3.33	10.59	10	
0.93	2.96	5.4	Oral Intake
2.1	6.68	10	
77.9	247.73	800	
221	702.79	800	
0.03	0.095	2	Smoking and Inhalation
9.49	30.18	20	
14.8	47.06	2	
93.3	296.7	1.5	
110	349.81	19.2	
686	2181	20	Intravenous

The most common routes of administration are oral delivery, oromucosal drops/spray and smoking/inhalation[94], but there can be other less used methods like intravenous[94] or transdermal[95] administration, since oral intake can have disadvantages such as the low bioavailability by inactivation of the drugs through hepatic metabolism and its slow absorption, while the inhalation of cannabinoids can have variable effects depending on the volume, length and rate of inhalations[95].

Other study by *Millar et al.*[51] analyzed and compared CBD dosing in clinical populations. More than half of the studies held a positive outcome in several conditions, like schizophrenia or epilepsy, with doses ranging from less than 1 to 50 mg/kg/day, suggesting that this compound has a wide therapeutic range, with varying minimum doses required for effect depending on the clinical outcome and population assessed. Different plasmatic concentrations could be ideal for different endpoints across clinical

populations, where low dosing of CBD may be more effective in anxiety relief, while higher doses could be required for reduction of epileptic seizures[51].

CBDV, on the other hand, has a much less studied pharmacokinetic profile, with plasmatic concentrations values only being obtained for rats and mice as of the present. These values are presented in Table 4, which range between 1.64 μM and 13.97 μM[96]. However, studies of the CBDV effect and concentrations in human plasma are still needed to understand this compound therapeutic effects.

Table 4- Maximum plasmatic concentration (C_{max}), and its correspondent concentration values in micromolar (μM), obtained after administration of a 60 mg/kg dose of CBDV in mice or rats, through oral or intraperitoneal administration.[96]

Animals	C_{max} (μg/mL)	Concentration (μM)
Male Swiss Mice	0.47 (oral) 4 (intraperitoneal)	1.64 13.97
Male Wistar Rats	2.2 (oral) 1.3 (intraperitoneal)	7.68 4.54

As such, the concentrations of 100, 500 and 750 nM for both CBD and CBDV, which are contained well within the range of plasmatic concentrations and binding affinities observed for these compounds, were tested in the following sections.

3.2.2. Phytocannabinoids modify the gene expression profile of the cannabinoid receptors and several cytokines in ucMSCs

To initiate the study of the effects of phytocannabinoids in ucMSCs, it was first assessed how those compounds affect the gene expression profile in these cells. The genes chosen were the previously analyzed cannabinoid receptors, as well as cytokines with different functions in tissue regeneration, with *IL-6* and *TNF-α* possessing pro-inflammatory activity, *G-CSF* working as a recruiting factor for other cells that aid the regeneration process, and *IL-10* presenting anti-inflammatory actions. *EGF*, *FGF2* and *VEGF-α* were also chosen, the first two with roles in cell migration and proliferation and the last being important for the angiogenesis process. Finally, *TGF-β1* too was selected, corresponding to an important factor in the formation of new extracellular matrix, however it can also have a role as an immunomodulatory agent in the initial stages of the regenerative process.

As such, the effect on the gene expression of several cytokines involved in tissue regeneration (Figure 7) and cannabinoid receptors (Figure 8) by incubating, for 24h, ucMSCs with CBD or CBDV, with or without previous induction with LPS for 1h, was analyzed.

Gene expression of cytokines involved in tissue regeneration

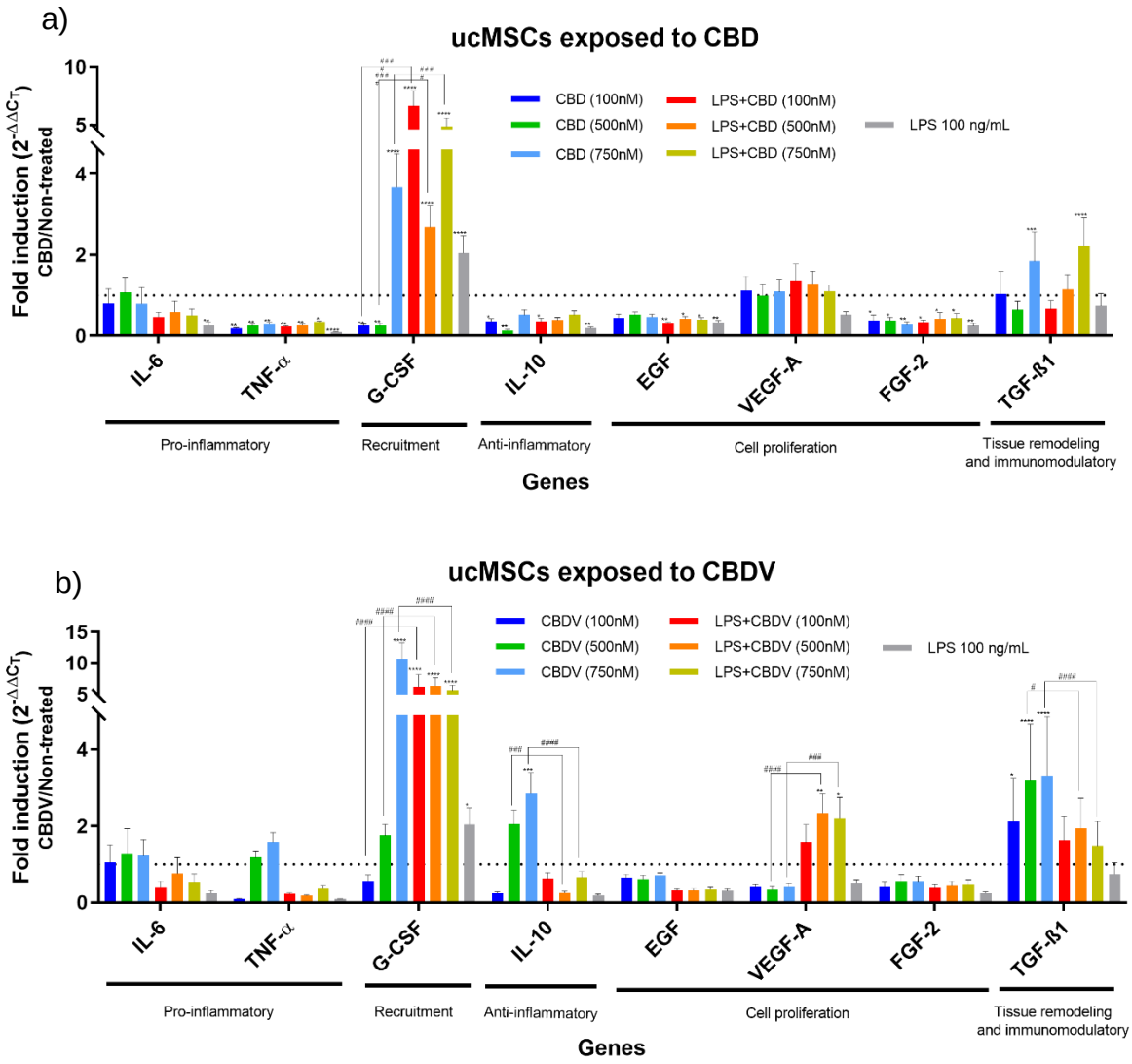

*Figure 7- CBD (a) and CBDV (b) modulate gene expression of several cytokines involved in tissue regeneration, such as IL-6, TNF-α, G-CSF, IL-10, EGF, VEGF-α, FGF2 and TGF-β1, in ucMSCs. Exposure of cells to different concentrations of phytocannabinoids for 24h: 100 nM (blue); 500 nM (green); 750 nM (light blue); 100 ng/mL of LPS for 1h followed by: 100 nM (red); 500 nM (orange); 750 nM (yellow); 24h of culture with α-MEM without FBS (gray). Results are presented as fold induction relative to the non-treated control group (represented by the dotted line). Statistically significant results are presented as *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 in relation with the control, and of $^{\#}$p<0.05; $^{\#\#\#}$p<0.001; $^{\#\#\#\#}$p<0.0001 between the groups with the same concentrations, with or without previous exposure to LPS (n=2-4). Abbreviations: CBD, cannabidiol; CBDV, cannabidivarin; EGF, epithelial growth factor; FGF-2, fibroblast growth factor 2; G-CSF, granulocyte-colony stimulating factor; IL, interleukin; LPS, lipopolysaccharides; TGF-β1, transforming growth factor-β1; TNF-α, tumor necrosis factor-α; VEGF-A, vascular endothelial growth factor α.*

Gene expression of cannabinoid receptors

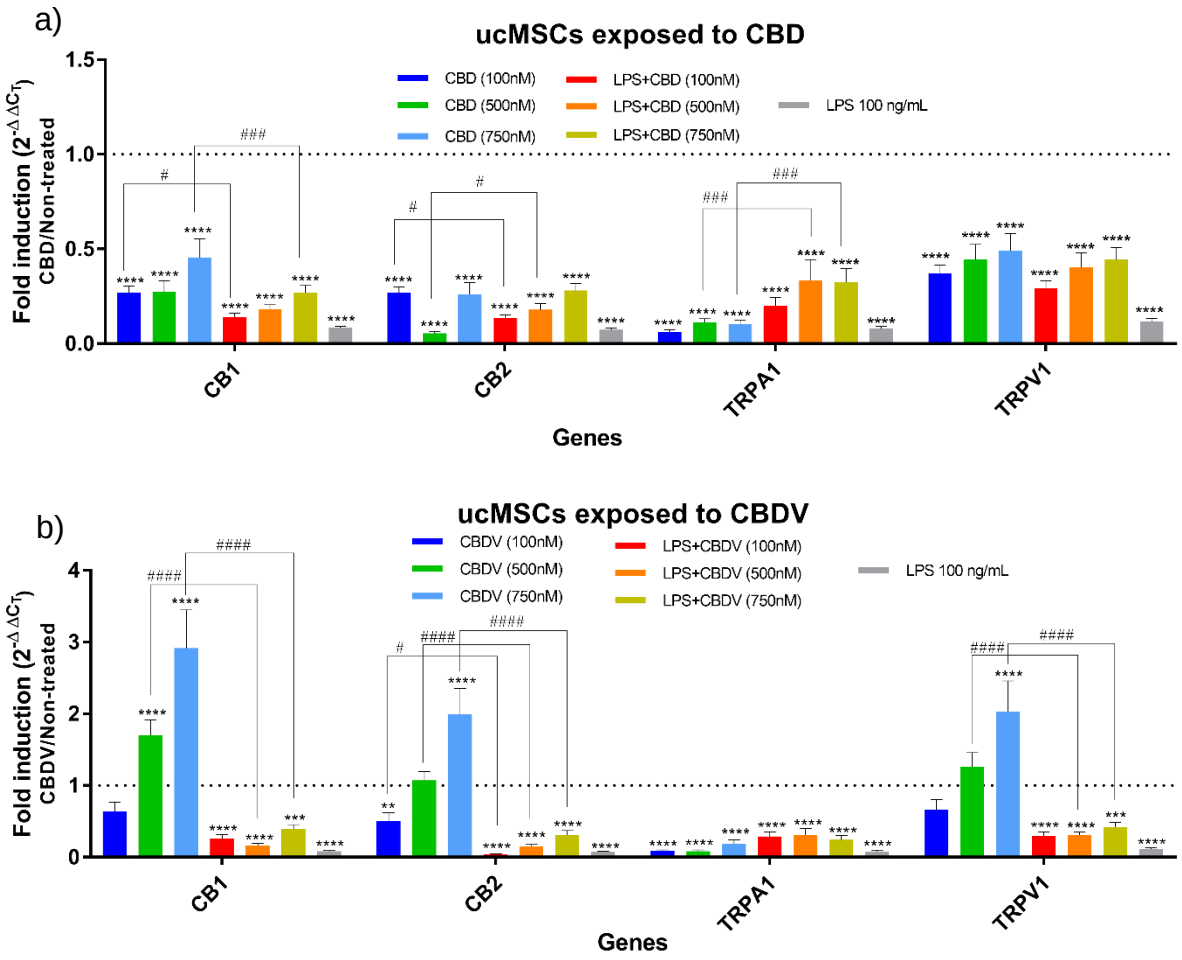

*Figure 8- CBD (a) and CBDV (b) modulate gene expression of cannabinoid receptors, namely CB1, CB2, TRPA1 and TRPV1, in ucMSCs. Exposure of cells to different concentrations of phytocannabinoids for 24h: 100 nM (blue); 500 nM (green); 750 nM (light blue); 100 ng/mL of LPS for 1h followed by: 100 nM (red); 500 nM (orange); 750 nM (yellow); 24h of culture with α-MEM without FBS (gray). Results are presented as fold induction relative to the non-treated control group (represented by the dotted line). Statistically significant results are presented as *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 in relation with the control, and of #p<0.05; ###p<0.001; ####p<0.0001 between the groups with the same concentrations, with or without previous exposure to LPS (n=2-4). Abbreviations: CB, cannabinoid receptor; CBD, cannabidiol; CBDV, cannabidivarin; LPS, lipopolysaccharides; TRPA1, transient receptor potential ankyrin 1; TRPV1, transient receptor potential vanilloid 1.*

As it can be seen, phytocannabinoids exposure to ucMSCs lead to an increase of expression of key genes in the regenerative process. When the cells are exposed to CBD there appears to be an increase in expression of both *G-CSF* and *TGF-β1*, being this simultaneous increase more evident at CBD concentrations of 750 nM, whilst when looking at *G-CSF* in specific there is an increase of expression in all concentrations used if there was a previous induction by LPS. Moreover, a significant decrease in the expression levels of the pro-inflammatory gene *TNF-α* is observed in all conditions. This may signify that CBD could present beneficial effects, such as anti-inflammatory, recruiting, immunomodulatory and

remodeling effects, and that a previous exposure to an inflammatory agent with the activation of the cannabinoid receptors can potentially lead to a higher sensitivity to this phytocannabinoid.

In the case of the exposure of ucMSCs to CBDV, *G-CSF* showed a similar expression profile compared to CBD, but with higher fold induction in the highest concentration tested when cells are solely exposed to this phytocannabinoid. This condition seems to be the one that mostly increased the expression levels of some cytokines, like *G-CSF*, as already mentioned, *IL-10* and *TGF-β1*. Curiously, previous stimulation of receptors by LPS seems either to suppress or not affect the expression of most genes analyzed, excluding *G-CSF* and *VEGF-α*, which were upregulated in these conditions, suggesting that this phytocannabinoid could be important not only on in the recruitment of other cells but also angiogenic processes during inflammatory responses.

In relation to cannabinoid receptor expression, in CBD's case, there is a significant decrease in their expression, which could mean that a 24h exposure of ucMSCs to CBD may deactivate the receptors, or that these receptors are only activated at the initial stages of exposure to this compound. For CBDV, in the highest concentration tested, it was observed a high level of expression for most cannabinoid receptors analyzed, namely for *CB1*, *CB2* and *TRPV1*. These effects appear to be concentration-dependent and suggest that CBDV can more effectively modulate the ECS of ucMSCs when compared with the other phytocannabinoid tested. Ultimately, these results could mean that CBDV increases the expression of cytokines involved in immunomodulatory and anti-inflammatory processes more effectively than CBD, together with the increase of cannabinoid receptors expression levels. Nevertheless, if the cells were previously stimulated by an inflammatory environment, both CBDV and CBD seem to revert the initial induction of the cannabinoid receptors.

The effect that phytocannabinoids have in MSCs have only recently begun to be researched more deeply. Some studies have already focused on the effects of phytocannabinoids on the secretory profile of MSCs from different sources. *Ruhl et al.*[27], tested the change that CBD provoked in the inflammatory profile of atMSCs, induced by exposure to 10 µg/mL of LPS. After 48h of exposure to this compound with or without combination with 3 µM of CBD, the presence of several cytokines in the secreted medium was assessed. It was concluded that co-treatment of LPS and CBD significantly increased the levels of *IL-6* and *VEGF* compared to non-treated atMSCs, however this effect was already seen after sole exposure to LPS[27]. Nevertheless, both the concentrations of LPS and the phytocannabinoid used were higher than the ones tested in this work, besides having used MSCs from adipose tissue and not from umbilical cord, which could explain the differences observed between studies. In a different study using mice bmMSCs, when 12h of exposure to 10 µg/mL of LPS increased the levels of the inflammatory cytokines *TNF-α* and *IL-6*, a follow up 12h exposure to 0.5, 2.5 or 5 µM of CBD reduced the expression of these cytokines in cells, with the two highest concentrations tested almost completely restoring the levels of those cytokines to normal. For the levels of the cannabinoid receptors *CB1* and *CB2*, it was verified that administration of CBD after LPS increased the levels of *CB2*, but not *CB1* expression, indicating that CBD might act via the activation of *CB2* in inflammatory microenvironments[82]. A different study using human gingival MSCs showed that treatment with 5 µM of CBD for 24h reduced expression of pro-inflammatory and apoptosis-related genes, while increasing expression of the *CB1* receptor[97]. A

different study using the same source of MSCs, demonstrated that 5 µM of CBD also increased *TGF-β1* expression in these cells[98]. These results could indicate that depending on the source of MSCs, CBD may have a different effect on the response of those cells to an inflammatory environment.

Regarding CBDV, there have not been any studies that focused on uncovering its roles in MSCs, however several studies researched the function of this compound in inflammatory environments, albeit using other types of cells applied for different contexts. A study researching the therapeutic effect of this phytocannabinoid in ulcerative colitis found that after administration of an inflammatory agent followed by CBDV in mice colon, there was a reduction of pro-inflammatory agents like *IL-6*, although it did not increase the anti-inflammatory *IL-10* levels that were reduced by inflammation. This compound also reduced *TRPA1* channel expression after its induction by inflammation in ulcerative colitis pediatric patients, suggesting that its actions in this condition are mediated by this receptor[41]. Other study focused on the action of phytocannabinoids in Duchenne muscular dystrophy, in which inflammation markers in the skeletal muscles of mice with this disease were analyzed. Administration of 60 mg/kg of CBD or CBDV for 2 weeks was able to reduce expression of these markers, namely *IL-6*, *TNF-α* and *TGF-β1*, but only CBD was able to reduce all of them significantly[42]. Yet another study focused on the anti-inflammatory effects of phytocannabinoids exposed to peripheral blood mononuclear cells (PBMC). They discovered that administration of 1 or 10 µM of CBDV to these cells for 30 minutes followed by 1 µg/mL of LPS for 24h was able to reduce the levels of pro-inflammatory marker *IL-6* in monocytes[99]. Therefore, CBDV appears to present a significant role in the amelioration of inflammatory processes. Regardless, the lack of studies pertaining CBDV and its therapeutic use, especially in MSCs needs to be rectified in the future to prove the potential therapeutic effects of this compound observed here.

Remarkedly, the concentration of 750 nM for both phytocannabinoids tested suggests a significant increase in expression of the aforementioned genes. Therefore, this concentration was used for all conditions tested in secretome production.

3.2.3. Priming with CBD and CBDV does not affect ucMSCs morphology

During the production of the conditioned media or secretome (CM) of ucMSCs, cell imaging was performed to verify if there existed a change in cell morphology. As referred above, the secretome was produced after exposure of ucMSCs to 750 nM of CBD (Figure A1a, b) or CBDV (Figure A1c, d) for 24h, with (Figure A1b, d) or without (Figure A1a, c) previous exposure to 0.1 µg/mL of LPS for 1h. Images were acquired at key points of this process, namely at the beginning of the procedure, after exposure to 1h of LPS, 24h after exposure to either CBD or CBDV, and 48h after conditioning without any compound, at the collection time of the CM. Despite the different compounds applied to the cells, none of them at any of the timepoints analyzed altered the morphology of ucMSCs.

Cell morphology is an important indicator of cell behavior and changes in their conformation could affect their normal functions. In fact, LPS had already been tested in WJ-MSCs and it was concluded that a concentration of 1 µg/mL of this agent did not affect cell conformation up to 72h of exposure[87]. This

confirms the results of this work, in which ucMSCs were exposed to LPS for 1h at concentrations of 100 ng/mL, both values lower than the ones tested in the previous study. Moreover, in atMSCs, CBD was tested to verify if it could protect these cells from endoplasmic reticulum stress, maintaining their cytophysiological properties. It was found that 24h of incubation with 5 µM of CBD succeeded in reverting atMSCs morphology back to normal[100]. Other study using gingival MSCs tested various concentrations of CBD for 24h to see if it altered cell morphology or presented cell toxicity. Cells treated with 5 µM of this phytocannabinoid did not show any morphological changes and it did not cause cell death, however higher concentrations of 10 or 25 µM decreased cell viability[101]. These results are in accordance with the ones obtained here, where exposure of ucMSCs to 750 nM of CBD for 24h did not led to any alteration in cell morphology.

It has yet to be researched how CBDV affects MSCs morphology. However, given the results presented here, it appears that this phytocannabinoid has a similar effect on ucMSCs morphology to that of CBD, indicating that these compounds, at a concentration of 750 nM, are safe to use in these cells.

3.2.4. The protein content of the secretome of ucMSCs is modulated by phytocannabinoids

With the secretome being the desired final product and the one that is going to be applied in therapeutic applications it is important to assess whether the gene expression of the cytokines in ucMSCs before CM production is translated into the respective proteins in the secretome. To assess this, a western blot was performed. The proteins analyzed were TGF-β1 and G-CSF, since their expression was increased in all the conditions chosen, together with IL-10, which expression was increased when it was solely exposed to CBDV.

TGF-β1 is an important cytokine that possesses not only immunomodulatory, but also remodeling effects on injured cells. In the initial stages of tissue repair, platelets secrete this factor, serving as chemoattractant for neutrophiles, macrophages and fibroblasts[75]. This cytokine has also an important role in Tregs formation[102]. It also induces re-epithelialization, increasing the migration of keratinocytes into the wound site[103].

IL-10 presents an anti-inflammatory role in tissue regeneration, being important in the termination of the inflammatory response, through inhibition of pro-inflammatory cytokines and infiltration of neutrophiles and macrophages into the wound site[75]. This cytokine also influences the formation process of Tregs, being crucial for the maintenance of a healthy immune response in different diseases[102].

Lastly, G-CSF is an important cytokine that regulates immunomodulatory functions and the recruitment of cells to the wound site, controlling proliferation, differentiation and survival of neutrophiles[104]. Similarly to the other two cytokines analyzed, it shows an important role in the formation of Tregs, specifically γδTregs, and may be important for the induction of their function in the treatment of GVHD[105]. Moreover, CM from ucMSCs was discovered to induce migration of bmMSCs, effect which was mediated by the

action of G-CSF[66]. These studies show the importance of this cytokine in immune and inflammatory responses. The results of the western blot executed are displayed in Figure 9.

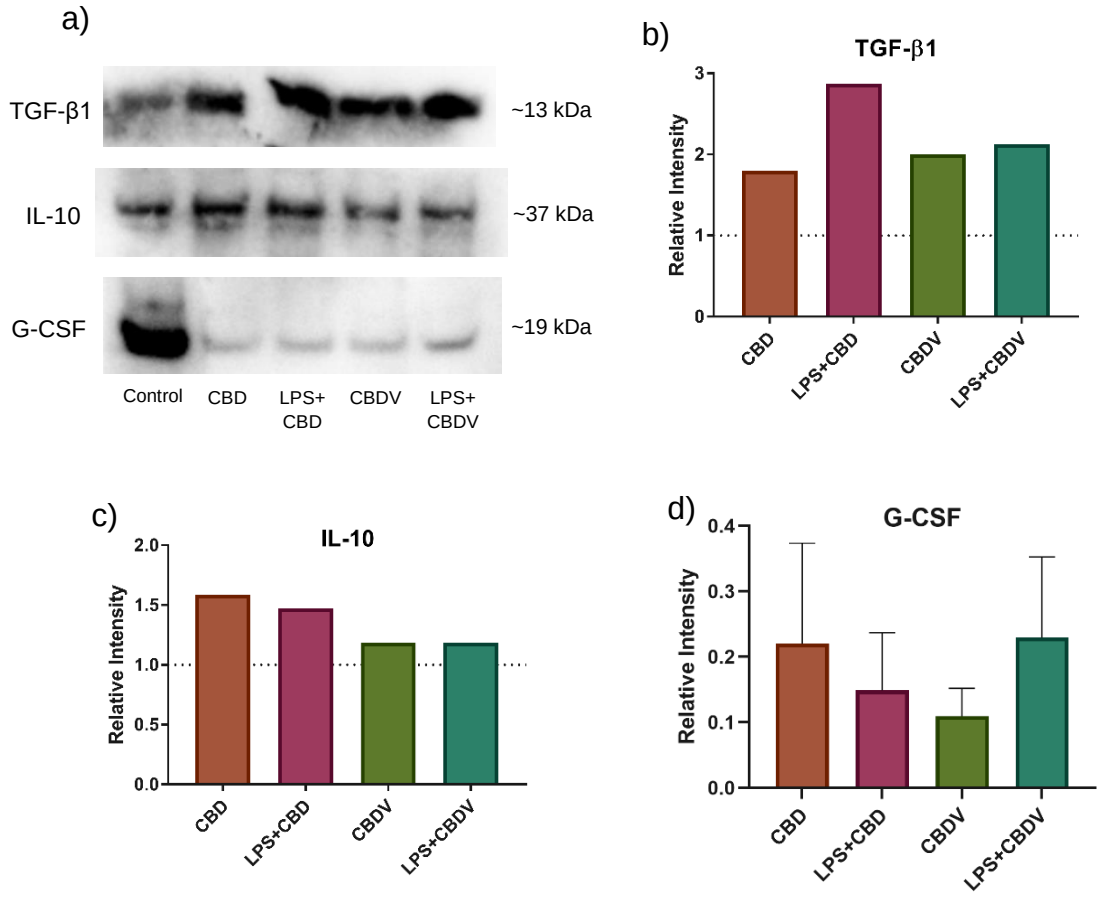

Figure 9- The secretome of ucMSCs modulated by phytocannabinoids contains several cytokines of interest. a) Western Blot (WB) bands of each of the proteins analyzed, namely TGF-β1, IL-10 and G-CSF in ucMSCs non-treated (control); exposed to 750 nM of CBD for 24h; to 100 ng/mL of LPS for 1h, followed by 750 nM of CBD during 24h; to 750 nM of CBDV for 24h; and to 100 ng/mL of LPS for 1h, followed by 750 nM of CBDV during 24h. Relative intensity of the WB bands of b) TGF-β1 (n=1), c) IL-10 (n=1) and d) G-CSF (n=3) in relation to the control (relative intensity equal to 1). Abbreviations: CBD, cannabidiol; CBDV, cannabidivarin; G-CSF, granulocyte-colony stimulating factor; IL-10, interleukin-10; LPS, lipopolysaccharides; TGF-β1, transforming growth factor-β1.

The western blot results enable the verification of the presence of the analyzed proteins in the ucMSCs secretome, presenting bands for the proteins for TGF-β1 in its mature form, at 13 kDa, for IL-10 in its biological active conformation, at 37 kDa, and for G-CSF, in the range of 19 kDa of molecular weight. For TGF-β1, there is an increase in the presence of this protein for all tested conditions, larger in the case of exposure to CBD after induction with LPS, indicating that phytocannabinoids can be involved in the stimulation of the release of this cytokine by these cells. These results are mostly concordant with the cytokine's gene expression profile observed previously, although the highest values were observed in the sole exposure to CBDV. For IL-10, an increase was observed in all groups tested, with higher levels of secretion for cells exposed to CBD when compared to CBDV. These results are in accordance

with what was obtained previously in gene expression analysis, although the increase was only in the case of sole exposure to CBDV. Interestingly, in the case of G-CSF, the untreated control condition possesses a greater amount of this protein compared to the other conditions tested, unlike what was observed in the gene expression analysis, suggesting the existence of potential post-transcriptional alterations after gene expression, where this increased expression was not translated in the protein secretion.

A possible explanation for the results obtained could be the different experimental setup of each analysis. In the case of the protein analysis, the cells were left in culture for a further 48h without exposure to any compound, to produce CM. Within this time, ucMSCs could be responding to the withdrawal of phytocannabinoids, compared to what was observed in the gene expression analysis, where the expression observed was right after cells were exposed for 24h to the phytocannabinoids, therefore resulting in different secretory profiles and induced genes.

The presence of these cytokines in the secretome produced by MSCs is relevant for its therapeutic potential. Previous studies have already derived a few results based on the secretome of MSCs. In a study researching the potential of ucMSCs-CM on wound healing, it was observed that its secretome reduced the secretion levels of TGF-β1 in dermal fibroblasts, with a higher reduction for more concentrated CM. This resulted in a reduced fibroblast differentiation potential, showing the anti-fibrotic properties achieved by this secretome[67]. Other study assessed the expression of this cytokine in gingival MSCs pretreated with 5 µM of CBD, observing an increase in TGF-β1 protein expression compared with the control condition[98]. The results obtained here corroborate the increase in secretion of TGF-β1 when MSCs are exposed to CBD.

Regarding IL-10, a study using bmMSCs observed a reduction of secretion of this cytokine by these cells after exposure to 500 ng/mL of LPS for 24h. However, this reduction was reverted when cells were treated with 1 µM of JWH-133 for 24h, a CB2 receptor agonist, suggesting this receptor mediated anti-inflammatory actions in bmMSCs[10]. A different study also using bmMSCs but from murine origin, concluded that treatment with 1 µM of THC for 24h increases the levels of IL-10 in the secretome, through a CB2 mediated pathway[79], showing again the important function of this receptor for immune regulation. In this work, it was seen that CBD leads to a higher secretion of IL-10 in ucMSCs, both when these cells were stimulated with or without LPS, which can be linked to the important role of this compound in the modulation of ucMSCs anti-inflammatory effects.

For G-CSF, a study explored the secretory profile of bmMSCs stimulated by LPS, for 72h, in which the respective CM was collected and analyzed right after exposure. Exposing these cells to different concentrations of LPS resulted in a CM with induced release of G-CSF for all concentrations tested, indicating that the secretion of this cytokine increases when cells are exposed to an inflammatory environment[106]. Nevertheless, in the results observed in this thesis, there is a decrease in secretion of this cytokine, suggesting that the secretory profiles may vary depending on the time of CM collection. Further studies will be conducted to explore this hypothesis, to understand whether there is a potential post-transcriptional change that influences G-CSF secretion, or if it depends on the timepoint of analysis of protein secretion.

4. Conclusion

The use of MSCs in therapeutic applications has become common due to the properties that these cells possess, like their paracrine effects and differentiation capacities, facilitating tissue repair and recovery in various conditions. The actions of phytocannabinoids and modulation of the ECS in regenerative medicine, although less explored, have also been proved to be useful thanks to anti-inflammatory and analgesic roles, amongst other effects of these compounds. The combination of these two therapies has only recently started to be researched, but it appears to be advantageous in regenerative medicine fields, making use of the combination of the characteristics of both MSCs and phytocannabinoids. Despite this, studies regarding the influence of phytocannabinoids specifically in ucMSCs are currently inexistent.

Therefore, this thesis aimed to assess whether there is a synergistic activity between ucMSCs and phytocannabinoids, specifically CBD and CBDV, by verifying the modulatory effects of these phytocannabinoids in the secretome of ucMSCs. This work intended to bridge the gap currently existent, firstly regarding the absence of studies characterizing the endocannabinoid system of ucMSCs as well as regarding the combination of phytocannabinoids and this type of MSCs, serving as a stepping-stone for future research that intends to further explore these interactions.

The results obtained here showed that cannabinoid receptors *CB1* and *CB2*, and TRP channels, namely *TRPV1* and *TRPA1*, are expressed in ucMSCs and are inducible by an inflammatory stimulus. Thus, these cells can interact with phytocannabinoids. In fact, after receptor induction by the inflammatory agent, CBD and CBDV were able to reverse the expression levels of cannabinoid receptors, whereas without inflammatory stimulus, only CBDV was able to induce cannabinoid receptor expression. Moreover, these phytocannabinoids have shown to affect ucMSCs functions and secretory profile by modulating the expression of pro-regenerative genes. CBD increased the expression level of genes involved in immunomodulatory actions and reduced the expression of pro-inflammatory factors, whilst CBDV, besides increasing expression of immunomodulatory genes also induced genes related with anti-inflammatory actions. This was confirmed by the protein content of the secretome, where TGF-β1 and IL-10 proteins have shown to be increased. On the other hand, the secretion of the recruiting agent G-CSF was decreased, which opens the door to further studies. Furthermore, the stimulation of MSCs with an inflammatory agent before CBDV exposure further increased the expression of angiogenic markers. These results ultimately show the potential that these phytocannabinoids have in influencing ucMSCs and its secretome, showing the great potential of the combination of these two strategies for regenerative medicine, and demonstrating the possible use of this secretome-based therapy in future clinical applications.

The next steps in this research should consist in performing functional assays to assess the effect of the ucMSCs secretome primed with CBD or CBDV on other cells, namely in the production of T cells, since phytocannabinoids promote ucMSCs' secretion of cytokines involved in the formation of these cells. Moreover, more research is required in the near future to solidify the results obtained here,

specifically by testing other cannabinoids, e.g., receptor agonists, that interact with the ECS in ucMSCs to understand the mechanism behind their function.

5. Materials and Methods

5.1. Reagents

Minimum essential medium alpha modification (α-MEM), Dulbecco's modified Eagle medium (DMEM), Lipopolysaccharides (LPS; LPS from Escherichia coli O55:B5) and trypan blue were obtained from Sigma-Aldrich® (St. Louis, MO, USA). Fetal bovine sera (FBS) and trypsin/ethylenediamine tetraacetic acid (Trypsin-EDTA) solution were obtained from Gibco® (Thermo Fisher Scientific™, Waltham, MA, USA). 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was purchased from Promega (Madison, WI, USA). Cannabidiol and cannabidivarin were obtained from Cayman Chemical (Ann Arbor, MI, USA). TRIzol® reagent was purchased from Invitrogen™ (Thermo Fisher Scientific™, Waltham, MA, USA). Dimethyl sulfoxide (DMSO) was obtained from Fisher Chemical™ (Fisher Scientific, Hampton, NH, USA). Bradford reagent was purchased from Bio-Rad® (Hercules, CA, USA).

5.2. Mesenchymal stem cells culture

This study was approved by the Ethics Committee of the Hospital Dr. José de Almeida (Cascais, Portugal), in the scope of a research protocol between ECBio (Research & Development in Biotechnology, S.A.) and HPP Saúde (Parcerias Cascais, S.A.). Umbilical cord donations, with written informed consents, as well as umbilical cord procurement, were made according to Directive 2004/ 23/EC of the European Parliament and of the Council of 31 March 2004 on setting standards of quality and safety for the donation, procurements, testing, processing, preservation, storage and distribution of human tissues and cells. Human umbilical cord matrix-derived mesenchymal stem cells were isolated as described by *Santos et al.*, according to a patented method (PCT/IB2008/054067; WO 2009044379), designed to produce a highly homogeneous population of cells that comply with the MSC standards defined by the ISCT[107]. ucMSCs were cultured and expanded in specific culture medium containing α-MEM, with 2 mM of L-Glutamine and 1 g/L of D-Glucose (11900-073, Gibco™), supplemented with 2.2 g/L of $NaHCO_3$ (131638, ITW Reagents©) and 10% (v/v) of heat inactivated fetal bovine serum (FBS) (10500-064, Gibco™), in a humidified atmosphere chamber at 37 ºC with 5% carbon dioxide (CO_2). ucMSCs were seeded in monolayer in t-flasks (Nunc™ EasYFlask™, Thermo Scientific™) at 0.7 to $1.0x10^4$ cells/cm^2 and routine passages were performed every 2 to 3 days when cells reached up to 90% confluence. Cells were used from passage 12 up to 16. During each passage, cells were washed with phosphate-buffered saline (PBS) solution followed by trypsinization with 0.05% of Trypsin-EDTA solution (25300-062, Gibco™) for 5 minutes. Cell counting and viability assessment were performed using the trypan blue (T8154, Sigma-Aldrich®) exclusion method and an Olympus CK30 inverted phase contrast microscope. Cells were cryopreserved in FBS with 10% DMSO (Fisher Chemical™) and stored in liquid nitrogen until next use.

5.3. Cell morphology assessment

At the different stages of the conditioned medium (CM) production protocol, contrast-phase images of the cells were captured with the Moticam 2500 5.0M Pixel USB 2.0 (Motic®) mounted on an Olympus CK30 inverted phase contrast microscope and viewed using the Motic Images Plus 3.0 software (Motic®).

5.4. MTS cell viability assay

ucMSCs were seeded in 96-well plates (83.3924, Sarstedt) at a density of 1.0x 10^4 cells/cm^2 and kept in a humidified atmosphere at 37 ºC and 5% CO_2. Cells were cultured in α-MEM supplemented with 5% FBS until they reached confluence. LPS (L6529-1MG, Sigma-Aldrich®) was sequentially diluted to the final testing concentrations of 5, 1, 0.5 and 0.1 µg/mL. Cell medium was renewed and LPS was added to each well and incubated for 1h or 24h. After this, cell culture medium was replaced by 100 µL of DMEM (D5523, Sigma-Aldrich®) and 20 µL MTS (CellTiter 96® AQ_{ueous} MTS Reagent Powder; G111A, Promega Corporation©) *per* well. After 2h of incubation at 37 ºC, absorbance was measured at 490 nm using a microplate spectrophotometer (SPECTROstar® Omega, BMG LABTECH). 10 % (v/v) DMSO was used as negative control and α-MEM supplemented with 5% FBS as positive control. Experiments were performed in triplicates, and results were expressed as percentage relative to positive control, which was considered 100% cell viability.

5.5. RNA extraction and gene expression analysis (qRT-PCR)

ucMSCs were seeded in 6-well plates (83.3920, Sarstedt) at a density of 0.7 to 1.0 cells/cm^2, cultured in α-MEM supplemented with 5% FBS and kept in a humidified atmosphere at 37 ºC and 5% CO_2 until confluence was reached. Following this, culture medium was renewed and LPS 0.1 or 1 µg/mL was added to the wells, and incubated for 1h or 24h, after which total ribonucleic acid (RNA) of cell samples was isolated with TRIzol® reagent (15596-018, Invitrogen™) and extracted according to manufacturer's instructions. For the experiments using phytocannabinoids, after cells were seeded in 6-well plates and confluence was reached, medium was changed to α-MEM supplemented with 5% FBS with LPS 0.1 µg/mL for 1h, after which LPS was removed and wells were washed with PBS. Then, medium was replaced for α-MEM without FBS, and CBD or CBDV were added to the wells at concentrations of 100, 500 or 750 nM in 0.1% DMSO. Cells were exposed to these concentrations of CBD or CBDV during 24h, after which total RNA of cell samples was isolated with TRIzol® reagent and extracted according to manufacturer's instructions.

For RNA quantification, absorbance was measured at 260 and 280 nm using LVis Plate mode on SPECTROstar® Omega. Purity measures were determined with standard 260/280 nm, for protein

presence, considering ratios between 1.8 and 2.0. Complementary DNA (cDNA) was synthesized from samples of 0.7 to 2.5 µg RNA using the NZY First-strand cDNA Synthesis kit (MB12502, NZYTech®) following manufacturer instructions. qRT-PCR was performed using 7.5 µL PowerUp™ SYBR™ Green Master Mix (A25741, Applied Biosystems™) which was prepared for a final reaction volume of 15 µL, using 2 µL of template cDNA, 1 µL of forward and 1 µL of reverse primers (Annexes; Table A1). As to assure the inexistence of contamination, blank controls were also prepared without template cDNA. Reaction was performed on QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems™). The comparative Ct method ($2^{-\Delta\Delta Ct}$) was used to quantify gene expression, normalized to the reference gene *β-actin*. Results were expressed relative to the non-treated control condition.

5.6. Conditioned Media Production

ucMSCs were seeded in 175 cm^2 t-flasks at a density of 0.7 to $1.0x10^4$ cells/cm^2, cultured in α-MEM supplemented with 5% FBS and kept in a humidified atmosphere at 37 ºC and 5% CO_2 until reaching 70% confluence. Upon this, medium was renewed and LPS 0.1 µg/mL was added to the t-flasks and incubated during 1h. After priming with LPS, t-flasks were washed with PBS to remove any vestigial remaining LPS. α-MEM without FBS and CBD or CBDV at concentrations of 750 nM were added to the t-flasks with a total volume of 18 mL, followed by their incubation at 37 ºC for 24h. After this, medium was removed and t-flasks were washed with PBS to remove residual CBD or CBDV. α-MEM without FBS was added to t-flasks with total volume of 25 mL and incubated for 48h. For the conditions where cells were only exposed to phytocannabinoids, CBD or CBDV were added to t-flasks with α-MEM without FBS right after cells reached 70% confluence. For the control group, a total volume of 25 mL of α-MEM without FBS was added when cells reached 90% confluence and were incubated for 48h. Post conditioning, the conditioned media (CM) produced was collected with each condition belonging to one of five different groups: i) Control; ii) CBD; iii) LPS+CBD; iv) CBDV; and v) LPS+CBDV. The corresponding CM was collected under sterile conditions, submitted to a cycle of centrifugation of 300 x g for 10 minutes at 25 °C and a following cycle of 2700 x g for 30 minutes at 4 °C to remove cell debris. CM was concentrated in 3 kDa cut-off centrifugal concentrators, Amicon® Ultra-15 Centrifugal Filter Unit (UFC900396, Millipore®), as *per* manufacturer's recommendations. All samples were stored aseptically at -80 ºC until further use. Cells were harvested for posterior qRT-PCR and total protein quantification.

5.7. Total Protein quantification

Total protein quantification of the secretome of ucMSCs was determined with a colorimetric assay based on the Bradford method, with protein dye reagent concentrate (500-0006, Bio-Rad®) according to manufacturer's instructions for standard procedure for microtiter plates. Absorbance at 595 nm was measured using microplate reader on SPECTROstar® Omega.

5.8. Western Blot

A total of 30 µg of each condition was resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12% polyacrylamide gels according to manufacturer's recommendations (Bio-Rad®). Gels were blotted onto PVDF transfer membranes (88518, Thermo Scientific™), which were incubated with primary antibody diluted in 5% BSA blocking buffer overnight at 4ºC, namely anti-G-CSF (ab9691, Abcam plc©) diluted 1:2500, anti-TGF beta 1 (ab92486, Abcam plc©) diluted 1:100 and anti-IL10 (sc-8438, Santa Cruz Biotechnology©) diluted 1:200. Following this, membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (Jackson ImmunoResearch©) dilution 1:20000, for the first two, or anti-mouse antibody (R&D Systems, Bio-Techne®) dilution 1:1000, for the last, for 1h at room temperature. Protein loading control was performed with Ponceau S staining. Western blot bands were detected by enhanced chemiluminescence (ECL) (Immobilon® Western Chemiluminescent HRP Substrate; WBKLS0100, Immobilon®) according to the manufacturer's instructions, and images were recorded using an iBright™ CL750 Imaging System (A44116, Invitrogen™).

5.9. Statistical Analysis

All statistical analysis of data was performed in GraphPad Prism 7.04 (GraphPad Software®) and Microsoft Excel (Microsoft Office®). Comparisons were analyzed by two-way ANOVA followed by Tukey's post hoc test. Results were expressed as average ± standard error of the mean (SEM), and *p*-values were presented for statistically significant results as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

6. Bibliography

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7. Annexes

Table A1- List of primers used in qRT-PCR.

Primer	Sequence (5'-3')
βactin_F	AAGTCCCTCACCCTCCCAAAAG
βactin_R	AAGCAATGCTGTCACCTTCCC
IL-6_F	ACTCACCTCTTCAGAACGAATTG
IL-6_R	CCATCTTTGGAAGGTTCAGGTTG
TNFα_F	AAGCACACTGGTTTCCACACT
TNFα_R	TGGGTCCCTGCATATCCGTT
G-CSF_F	GCTGCTTGAGCCAACTCCATA
G-CSF_R	GAACGCGGTACGACACCTC
IL10_F	GACTTTAAGGGTTACCTGGGTTG
IL10_R	TCACATGCGCCTTGATGTCTG
EGF_F	TGGATGTGCTTGATAAGCGG
EGF_R	ACCATGTCCTTTCCAGTGTGT
VEGFα_F	AGGGCAGAATCATCACGAAGT
VEGFα_R	AGGGTCTCGATTGGATGGCA
FGF2_F	AGAAGAGCGACCCTCACATCA
FGF2_R	CGGTTAGCACACACTCCTTTG
TGFβ1_F	AAGGACCTCGGCTGGAAGTG
TGFβ1_R	CCCGGGTTATGCTGGTTGTA
CB1_F	CAAGCCCGCATGGACATTAGGTTA
CB1_R	TCCGAGTCCCCCATGCTGTTATC
CB2_F	GACACGGACCCCTTTTTGCT
CB2_R	CCTCGTGGCCCTACCTATCC
TRPV1_F	GGCTGTCTTCATCATCCTGCTGCT
TRPV1_R	GTTCTTGCTCTCCTGTGCGATCTTGT
TRPA1_F	TGGTGCACAAATAGACCCAGT
TRPA1_R	TGGGCACCTTTAGAGAGTAGC

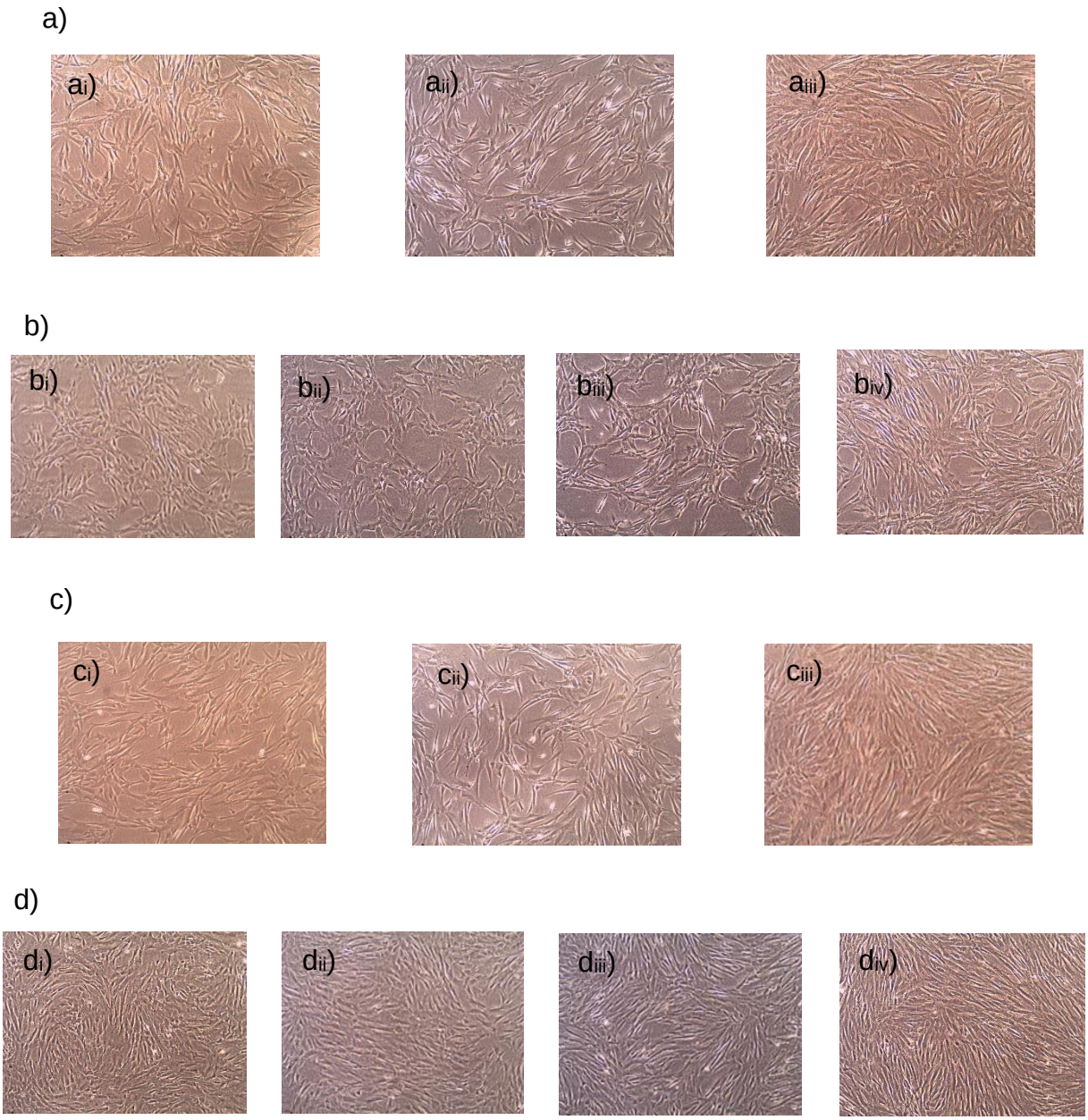

Figure A1- ucMSCs morphology is not affected during key steps of CM production. Photos were taken during cell exposure to a) CBD; b) LPS and CBD; c) CBDV; d) LPS and CBDV. Steps of secretome production correspond to: a_i), b_i), c_i), d_i)- before application of any compound; b_{ii}), d_{ii})- 1h after exposure to LPS; a_{ii}), b_{iii}), c_{ii}), d_{iii})- 24h after exposure to phytocannabinoids; a_{iii}), b_{iv}), c_{iii}), d_{iv})- before medium collection, 48h after removal of all compounds.