Sistema Endocanabinoide na Patofisiologia da Fibrose Dérmica
Endocannabinoid System in the Pathophysiology of Skin Fibrosis

Inês Bastos Correia de Sá

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TÍTULO **Sistema Endocanabinoide na Patofisiologia da Fibrose Dérmica**
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Aos meus pais, Mário e Maria Angelina
Ao meu irmão Mário Pedro

Ao Professor Doutor
Daniel Filipe de Lima Moura

À Professora Doutora
Maria Augusta Vieira Coelho

Prefácio

Decorria o ano de 2011 quando fui confrontada pelo **Professor Doutor Daniel Moura**, na época regente das disciplinas de Farmacologia do curso de Medicina da Faculdade de Medicina da Universidade do Porto, sobre a possibilidade de ingressar num Doutoramento em Medicina. Na altura, estando a iniciar o 2º ano de internato em Cirurgia Plástica, Reconstrutiva e Estética, pareceu-me um plano demasiado ousado. No meio do bloco operatório, bisturis e fios de sutura, agulhas e facas de enxerto, retalhos e reconstruções e todos os outros desafios e especificidades com que uma especialidade cirúrgica consegue deslumbrar qualquer jovem recém-licenciado em Medicina, pareceu-me difícil repartir tempo, interesse e empenho num projeto tão exigente como o é um Doutoramento. No entanto o repto estava lançado. Foi ele, portanto, quem me deu a oportunidade de iniciar este trabalho. Por isso, a sua amizade e apoio prestado no início dos meus trabalhos, e o interesse pelas minhas actividades nunca serão esquecidos. Não posso também deixar de lembrar o convite feito pelo **Professor Doutor Daniel Moura**, para ser primeiro Monitora e mais tarde Assistente da sua Cadeira pelo que o interesse pelas áreas básicas da Medicina, pelo ensino e investigação foram também por ele promovidos.

O **Professor Doutor Daniel Moura**, para além de um homem de ciência, sempre foi também um excelente avaliador de carácter. Acredito que por isso me sugeriu a **Professora Doutora Maria Augusta Vieira-Coelho** para guiar esta dissertação. Não poderia ter encontrado melhor orientadora. O seu espirito contagiante e jovial, a sua energia e o seu entusiasmo pela ciência, rapidamente não só despertaram a minha vontade por prosseguir com este trabalho, como foram também uma força motivadora em vários momentos de desanimo, desgaste ou cansaço. À minha orientadora atribuo grande parte do sucesso deste trabalho. A ela agradeço todo o entusiamo, incentivo, motivação e presença atenta e dedicada às minhas necessidades enquanto aluna. Agradeço o rigor científico e as ideias ousadas e criativas que contribuíram para um trabalho "fora da caixa" aos olhos de vários Farmacologistas e muitos Cirurgiões Plásticos. Agradeço a flexibilidade e compreensão demonstrada nos vários momentos em que as minhas prioridades clinicas se sobrepuseram aos interesses laboratoriais. Agradeço, por último, toda a amizade e apoio constante que sempre demonstrou em todos os momentos.

O segundo desafio surgiu pouco depois: escolher um tema que aliasse duas disciplinas tão distintas e até opostas da Medicina: a Farmacologia e a Cirurgia Plástica. Assim surgiu este projeto que une a Cicatrização, um tema tão relevante para qualquer

Cirurgião Plástico e o Sistema Endocanabinoide, tema tão contemporâneo para qualquer Farmacologista.

Paralelamente ao desenvolvimento desta dissertação, dei continuidade ao meu percurso clínico e neste caminho tive a oportunidade de colaborar diretamente com a **Professora Doutora Marisa Marques**, a minha orientadora de formação específica e coorientadora deste trabalho. Agradeço-lhe sobretudo a amizade e todo o incentivo, apoio e confiança dados para que estes dois caminhos fossem conciliáveis.

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Ao **Professor Doutor José Amarante** agradeço o incentivo e apoio dado na prossecução do meu trabalho universitário, docente e hospitalar.

Às **Investigadoras Cláudia Carvalho**, **Vera Machado** e **Sofia Carvalho**, dirijo um agradecimento especial por toda a amizade e apoio no trabalho laboratorial executado. Sem a sua ajuda este trabalho dificilmente teria saído do papel para o laboratório.

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trabalho ao serviço do Sistema Nacional de Saúde. Não posso deixar de lhes reconhecer ainda o papel formador fundamental enquanto interna de Cirurgia Plástica.

À **equipe de Enfermagem do Serviço de Cirurgia Plástica, Reconstrutiva, Estética e Unidade de Queimados**, da **Consulta Externa de Cirurgia** e do **Bloco Operatório Central** agradeço pelo empenho e apoio logístico nos estudos clínicos, nomeadamente nas colheitas de sangue e tecidos realizadas. Dirijo um agradecimento especial à **Srª Enfermeira Alda Maria Ribeiro**, enfermeira chefe do Serviço de Cirurgia Plástica, Reconstrutiva, Estética e Unidade de Queimados, pela dedicação que ofereceu a este empreendimento, ao colocar a sua equipe inteiramente ao dispor dos *timings* e necessidades deste projeto.

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List of abbreviations

ABHD – Alpha-beta hydrolase domain protein
AEA – N-arachidonoyl ethanolamine, Anandamide
2-AG – 2-Arachidonoylglycerol
AjA – Ajulemic acid
cAMP – Cyclic adenosine monophosphate
BMI – Body mass index
BSA – Bovine serum albumin
CB1 – Cannabinoid 1
CB2 – Cannabinoid 2
CBr1 – Cannabinoid receptor 1
CBr2 – Cannabinoid receptor 2
CBD – Cannabidiol
CCT – Corticosteroid
Chloroform:MeOH – Chloroform methanol
COX-2 – Cicloxigenase-2
CRP – C-reactive protein
CTGF – Connective tissue growth factor
DAG – Diacylglycerol
DAPI – 4',6-Diamidine-2'-phenylindole dihydrochloride
DMEM – Dulbecco's modified eagle medium
ECM – Extracellular matrix
EDTA – Ethylenediamine tetraacetic acid
EGF – Epidermal growth factor
ERK – Extracellular signal-regulated kinase
FAAH – Fatty acid amide hydrolase
FGF – Fibroblast growth factor
FBS – Fetal bovine serum
GF – Growth factors
GPCR – G protein-coupled receptor
GPR – G protein-coupled receptor
hCAP18 – Cathelicidin antimicrobial proteins
HE – Hematoxylin-eosin
HPLC – High-performance liquid chromatography
HRP – Horseradish peroxidase
HT – Hypertrophic
IGF-1 – Insulin-like growth factor 1
IL – Interleukin
Ig – Immunoglobulin
ISTD – Internal standard spiking solution
JNK – Jun N-terminal kinase
KGF – Keratinocyte growth factor
LC-MS/MS – Liquid chromatography–mass spectrometry
LC-MS/MS TQ – Liquid chromatography–mass spectrometry technology
LTA – Lipoteichoic acid
MAGL – Monoacylglycerol lipase
MAPK – Mitogen-activated protein kinase
MCP-1 – Monocyte chemoattractant protein-1
MCV – Mean cell volume
MMP-1 – Matrix metalloproteinase 1
N – Normal

NAAA – N-acylethanolamine-hydrolyzing acid amidase
NAEs – N-acylethanolamines
NAPE – N-acyl-phosphatidylethanolamine
NMDA – N-methyl-D-aspartate
NO – Nitric oxide
OEA – Oleoylethanolamine
1,25(OH)2D3 – 1,25-Dihydroxyvitamin D3
25(OH)D – Hydroxyvitamin D
2-OG – 2-Oleoylglycerol
PBS – Phosphate-buffered saline
PDGF – Platelet-derived growth factor
PEA – Palmitoylethanolamide
Pen-Strep – Penicillin-Streptomycin
PFA – Paraformaldehyde
PKA – Protein kinase A
PLC – Phospholipase C
PMSF – Phenylmethylsulfonyl fluoride
PPAR – Peroxisome proliferator-activated receptor
PPAR-α – Peroxisome proliferator-activated receptor alpha
PPAR-β/δ – Peroxisome proliferator-activated receptor beta/delta
PPAR-γ – Peroxisome proliferator-activated receptor gamma
PRISMA – Preferred reporting items for systematic reviews and meta-analyses
RIPA – Radioimmunoprecipitation assay
SDS/PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM – Standard error of the mean
α-SMA – Alfa smooth actine
SR – Sirius red
SSc – Systemic sclerosis
TBS – Tris-buffered saline
TBS-T – Tris-buffered saline with tween
TGF-β – Transforming grown factor beta
TGF-β1 – Transforming grown factor beta 1
TGF-β2 – Transforming grown factor beta 2
TGF-β3 – Transforming grown factor beta 3
THC – Δ(9)-Tetrahydrocannabinol
TNC – Tenascin C
TNF-α – Tumor necrosis factor alfa
TRP – Transient receptor potential
TRPA – Transient receptor potential cation channel subfamily ankyrin member
TRPM – Transient receptor potential ion channel subfamily melastatin member
TRPV – transient receptor potential cation channel subfamily vanilloid member
TSK-1 – Tight-skin-1
VCAM1 – Vascular cell adhesion molecule 1
VDR – Vitamin D receptor
VEGF – Vascular endothelial growth factor
VSS – Vancouver scar scale

Introduction and Aims

WOUND HEALING

Wound Healing Evolution

Disruption of a normal anatomic structure or wounding, is a threatening event for every living being, from simple bacteria to complex multicellular organisms. The ability of these organisms to regenerate or repair wounded tissues provides them the capacity to survive in adverse environments. This is a significant selective advantage in nature. Two processes can achieve tissue repair: regeneration and wound healing. While regeneration describes the specific substitution of the tissue (i.e. superficial epidermis, liver, mucosa, or fetal skin), wound healing represents an unspecific form of repair in which the wound heals by fibrosis and scar formation. The latter, unfortunately, represents the main form of human skin repair (Reinke and Sorg 2012).

Skin wound healing is a dynamic and highly regulated physiological process that begins immediately after wounding and lasts for up to a year, and is responsible for at least partial reconstruction of the wounded area. It has conceptually been divided into three consecutive but overlapping phases: the haemostasis/inflammatory phase, the proliferative phase and the remodelling phase (Schilling 1976). Within each phase, different cells, growth factors (GF) and cytokines are involved, and different cellular, humoral and molecular mechanisms take place. Each phase of wound healing is briefly described below and a representative image is given in **Figure 1** (Werner and Grose 2003, Broughton, Janis et al. 2006, Wang, Huang et al. 2018).

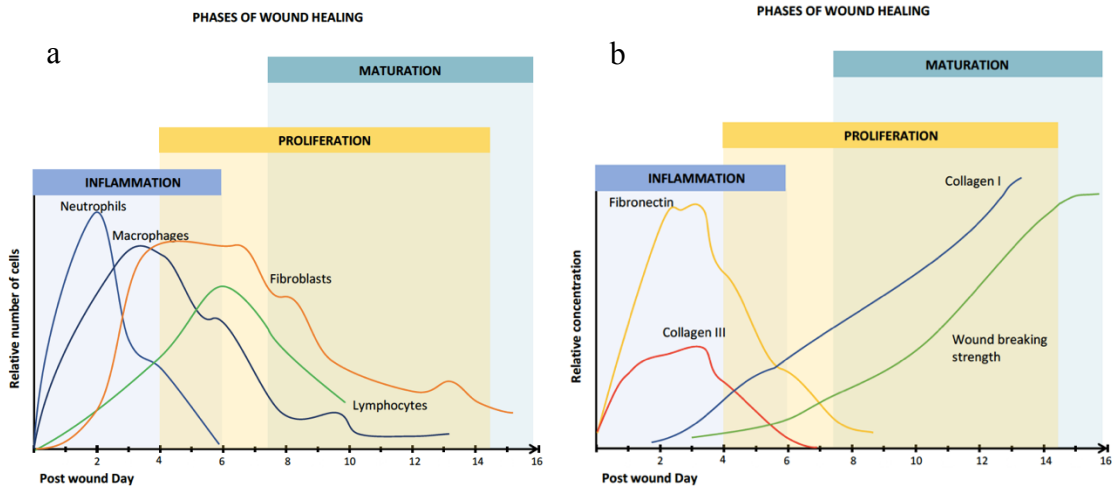

Figure 1. Main cellular mediators (a) and wound matrix components (b) over time, during inflammatory, proliferative and remodelling/maturating phases of wound healing. Note that macrophages and neutrophils are the predominant cells during inflammation, whereas lymphocytes peak later and fibroblasts are the predominant cells during the proliferative phase. Although fibronectin and collagen type III constitute the early matrix, collagen type I accumulates later, matching with the increase in wound-breaking strength. Adapted from Broughton, G., *et al.* The basic science of wound healing. *Plast Reconstr Surg*. 2006;117(7 Suppl):12S-34S.

- ***Hemostasis/Inflammation Phase:*** This first stage begins immediately after injury and lasts up to 4 to 6 days. After the injury, the sub-endothelium is exposed and actions are taken to control bleeding. Vasoconstriction, platelet aggregation, and activation of the intrinsic and extrinsic pathways of blood coagulation will form the clot. The clot, composed of collagen, platelets, thrombin, and fibronectin, provides a barrier against invading microorganisms; serves as a scaffold for invading cells, such as neutrophils, monocytes, fibroblasts, and endothelial cells and releases cytokines and GF that initiate the inflammatory response (interleukin (IL)-1, IL-6, tumor necrosis factor alfa (TNF-α) and transforming grown factor beta (TGF-β)). Neutrophils are the first inflammatory cells arriving at the injured site, peaking at 24h and are present for 2 to 5 days. They produce a wide variety of proteases, metalloproteinases, and reactive oxygen species as a defense against contaminating microorganisms, and they are involved in the cell debris phagocytosis. Forty-eight to 96 h after injury, monocytes enter the site of injury and differentiate into macrophages and support the ongoing process by performing phagocytosis of pathogens and cell debris. They also mediate angiogenesis (by synthesizing vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and TNF-α) and fibroplasia (by synthesizing TGF-β, epidermal growth factor (EGF), platelet derived growth factor (PDGF), IL-1, and TNF-α) and synthesize nitric oxide (NO). Macrophages will activate the next phase of wound healing.

- ***Proliferative phase:*** This phase lasts approximately from day 4 till day 14. It is characterized by epithelization, angiogenesis, and provisional matrix formation. It begins with migration and proliferation of keratinocytes located at the wound edge and epithelial stem cells from hair follicles or sweat glands. This process is activated by different cytokines and GF (keratinocyte growth factor (KGF), insulin-like growth factor 1 (IGF-1), EGF, IL-6 and TNF-α). It is then followed by fibroblasts proliferation from wound neighborhood (mediated by PDGF and EGF, secreted from platelets and macrophages). Fibroblasts acquire a contractile phenotype and transform into myofibroblasts, a cell type that plays a major role in wound contraction. They are responsible for deposit extracellular matrix (ECM) composed of collagen, glycosaminoglycan and fibronectin. Fibroblast activation is strongly induced by TGF-β. At same time, restoration of the vascular system occurs. Endothelial cells located at intact venules, are induced by VEGF to form new

capillary tubs. VEGF is secreted by keratinocytes, macrophages, fibroblasts, platelets, and other endothelial cells in response to IL-1, TNF-α, TGF-β1, KGF and NO. The resulting wound connecting tissue is known as granulation tissue. During this phase fibroblasts and endothelial cells are the predominant cells.

- ***Remodelling phase:*** This final phase lasts from day 8 till up to one year after injury. During the remodelling phase collagen III, produced in the proliferative phase, is replaced by collagen I. This type of collagen is aligned in small parallel bundles, different from the basket weave collagen in uninjured dermis. Concurrently, myofibroblasts contract and help to decrease the surface of the developing scar, and angiogenesis diminish.

The collagen organization and tensile strength of a scar will never be the same found in uninjured skin. At 1 week, the wound only has 3 percent of its final strength; at 3 weeks, it is 30 percent; and at 3 months, it is approximately 80 percent. Additionally, there are certain skin components that will never fully recover. Epidermal appendages such as hair follicles or sweat glands have no potential to heal or regrow. Lastly, if patients have matrix deposition problems wound healing can be compromised, and a chronic wound can occur. Excessive scarring can also occur, leading to a hypertrophic scar or keloid (Reinke and Sorg 2012).

Excessive Wounding: Hypertrophic Scars and Keloids

Keloids and hypertrophic scars represent an exuberant healing response, characterized by an overabundant accumulation of ECM components, especially collagen (Sephel and Woodward 2001). The underling mechanism that leads to excessive fibrosis is not clear yet but a prolonged or excessive inflammatory phase is believed to be on the onset of the disease.

Excessive scarring was first described in the Smith papyrus about 1700 BC (Berman and Bieley 1995). Centuries later, Mancini (1962) and Peacock (1970) differentiated excessive scarring into hypertrophic and keloid scars. Hypertrophic scars are raised scars that typically remain within the confines of the original wounds and often spontaneously regress. Keloids, on the other hand, appear as firm, mildly tender, bosselated tumours that typically project beyond the original wound limits (**Figure 2**). They rarely regress spontaneously and usually recur after excision. Both lesions are commonly pruritic, but keloids may even be the source of significant pain and hyperesthesia (Gauglitz, Korting et al. 2011). Besides those clinical distinctions, there are important epidemiologic, etiologic and histologic differences between both entities (**Table 1**). A strong genetic component has been associated with keloids. Familiar predisposition, with autosomal dominant and recessive genetic variants, has been identified (Omo-Dare 1975). Individuals with darker skin have an increased risk of developing keloid scarring, as they occur in ~15–20% of individuals of African, Hispanic and Asian ancestry, less commonly in Caucasians and no reported cases in patients with albinism (Gauglitz, Korting et al. 2011). Beside genetic component, other factors like age (10-30 years old), blood type A (Ramakrishnan, Thomas et al. 1974) and hyper-Immunoglobulin (Ig) E syndrome (high allergy risk) (Placik and Lewis 1992) predispose

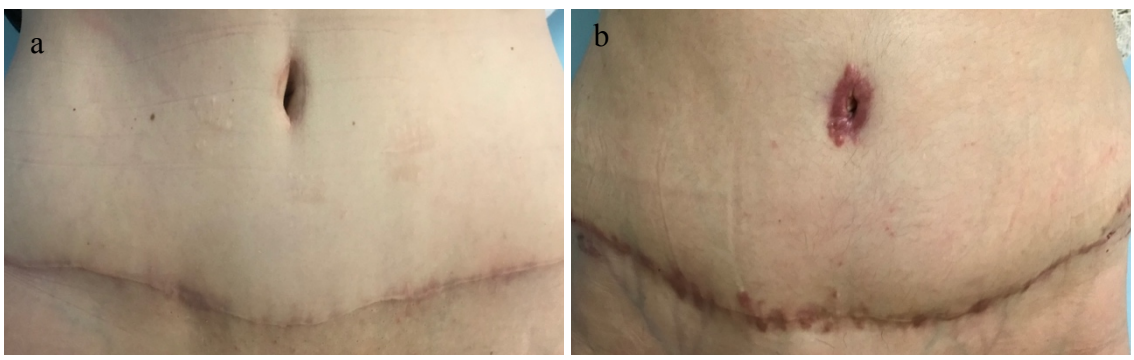

Figure 2. Scars after abdominoplasty surgery: normal (a) and excessive scar formation (b) can occur after skin trauma or surgery. Note that in **Figure 2b** both hypertrophic scar formation (inferior abdominal scar) and keloid scar (umbilicus scar) are found in the same patient. Both pictures are taken 3 months after surgery.

to keloid scar formation. On the other hand, hypertrophic scars do not appear to have a genetic component, and they occur in 40% to 70% of patients following surgery and up to 91% of patients after burn injury. Keloids develop more commonly in anterior chest, shoulders and upper arms, earlobes and cheeks, whereas hypertrophic scars tend to occur in locations with high tension, such as shoulders, neck, presternum, knees and ankles (Niessen, Spauwen et al. 1999). Histologically, both scars contain an excess of dermal collagen and other fibroblast proteins, as fibronectin. Keloids are composed of disorganized and randomly oriented type I and III collagen, containing pale-staining hypocellular collagen bundles with no nodules and few myofibroblasts. Hypertrophic scars, in contrast, are mostly composed of primarily type III collagen, oriented parallel to the epidermal surface and arranged in a wavy pattern, with abundant nodules containing myofibroblasts, large extracellular collagen filaments and plentiful acidic mucopolysaccharides (Rubin, Strayer et al. 2012).

Table 1. Epidemiological, clinical and histological differences between hypertrophic scars and keloids.

	HYPERTROPHIC SCARS	KELOIDS
Appearance	Limited to the initial site of injury	Projects beyond the original wound margins
Incidence	40% to 70% following surgery Up to 91% following burn injury	15% to 20% in African, Hispanic and Asian populations
Gender/age	Equal in gender distribution. Highest incidence in the second to the third decade	
Genetic component	No	Yes
Association with skin pigmentation	No association with skin pigmentation	More common in darker skin types
Predilection sites	Shoulders, neck, presternum, knees and ankles	Anterior chest, shoulders, earlobes, upper arms and cheeks
Symptoms	Less pruritic and rarely painful	Pruritic and extremely painful
Histology	Primarily fine, well-organized, wavy type III collagen bundles oriented parallel to epidermis surface with abundant nodules containing myofibroblasts, large extracellular collagen filaments and plentiful acidic mucopolysaccharides	Disorganized, large, thick and randomly oriented type I and III hypocellular collagen bundles with no nodules or excess myofibroblasts. Poor vascularization with widely scattered dilated blood vessels.
Occurrence	Posttraumatic	Posttraumatic or spontaneous
Evolution	Develop soon: Appear within 4 to 8 weeks following wounding Rapid growth phase for up to 6 months Regression over a few years	Develop later: Appear 3 months or later after initial wounding Indefinitely proliferation
Spontaneous regression	Yes	No
Recurrence rate	Low	High

It has been suggested that a prolonged or excessive inflammatory phase would incite fibroblast activity and increased ECM deposition. Fibroblasts persist longer in keloids and hypertrophic scars than in normal scars and their proliferative activity is significantly higher (Nakaoka, Miyauchi et al. 1995). Fibroblasts phenotype alterations have also been shown to occur: keloid fibroblasts show increased numbers of GF receptors and respond more vigorously to PDGF and TGF-β when compared with normal fibroblasts (Tuan and Nichter 1998). TGF-β is also implicated in the etiology of these fibrotic disorders. TGF-β is the most important stimulator of collagen and proteoglycan synthesis (Szulgit, Rudolph et al. 2002). Overexpression of TGF-β (mainly TGF-β1 and TGF-β2 isoforms) has been observed in keloid and keloid-derived fibroblasts (Lee, Chin et al. 1999). However, it is still unclear how TGF-β is involved in the induction and subsequent perpetuation of collagen deposition. Lastly, keratinocytes and mast cells have also a role in excessive scar pathogenesis. Keratinocytes produce GF that stimulates fibroblast activity (Armour, Scott et al. 2007) and an increased number of mast cells have been reported during hypertrophic scar development (Smith, Smith et al. 1987).

Both of these conditions can be a significant burden for the affected individual. Besides the obvious cosmetic impact, those scars are responsible for functional disability, subjective symptoms such as pruritus, pain and tenderness, and a decrease in quality of life, physical status and psychological health (Chiang, Borovikova et al. 2016).

Available Therapies

Treatment of hypertrophic scars and keloids is not always successful and recurrence rates are high. Since the definitive mechanisms behind excessive scars are still unidentified, most of the available therapies are directed to reduce inflammation. A better understanding of the cellular and molecular mechanisms underlying wound healing will ultimately allow us to influence, accelerate, or attenuate the wound repair/regeneration process. Till now, the prevention of pathologic scarring is unquestionably more effective than treatment.

As high tension predisposes to hypertrophic scars, most of the preventive measures are aimed to reduce wound tension. Tension-free primary closure, massage therapy, pressure therapy, and passive mechanical stabilization with paper tapes or silicone sheets have been used. Silicone sheets are considered superior since they also occlude and hydrate the scar surface. Flavonoids, naturally-derived substances from plants, have also been topically applied to treat or prevent excessive scarring. Although their efficiency is controversial, they are thought to promote antifibrotic actions through matrix metalloproteinase 1 (MMP-1) induction or inhibition of SMAD2, SMAD3 or SMAD4 expression.

Surgical excision is the traditional treatment for these scars. Although recurrence rates for hypertrophic scars revision are low, they can be extremely high (45% and 100%) for keloids.

Intralesional corticosteroid (CCT) injections are considered first-line therapy for keloids and second-line for hypertrophic scars. They improve scar pliability, diminish volume and height, and reduce scar-related itching and pain. The most common CCT used is triamcinolone acetonide. They are believed to suppress inflammation, increase vasoconstriction, and inhibit fibroblast and keratinocyte proliferation (Atiyeh 2007). The response rate for intralesional CCT injections varies from 50% to 100%, with a recurrence rate of 9% to 50% (de Oliveira, Nunes et al. 2001).

Cryotherapy, laser therapy and radiotherapy have been used as monotherapy or in conjunction with other forms of therapy. Radiotherapy is very efficacious in severe cases of keloids, combined with surgical excision, with reported recurrence rates of 5.95% (Shen, Lian et al. 2015).

Lastly, several emerging therapies have been proposed. Interferon, imiquimod 5%, 5- fluorouracil, tranilast, botulinum toxin A, mesenchymal stem cell therapy, fat grafting, and human recombinant TGF-β3/TGF-β1 *or* 2 neutralizing antibodies have been

suggested as possible options for keloid scar management. Many of these treatments have been shown to be effective but more well-defined pre-clinical studies are needed before their approval and widespread use in clinical practice (Gauglitz, Korting et al. 2011, Arno, Gauglitz et al. 2014, Lee and Jang 2018).

Although a brought range of therapeutically and preventive measures for hypertrophic scars and keloids have been proposed, optimal treatment for these lesions remains uncertain. Successful healing may only be achieved with a full understanding of the molecular mechanism involved in the pathogenesis of the diseases.

ENDOCANNABINOID SYSTEM

Endocannabinoids

First descriptions about the medical use of *Cannabis sativa* date from 2700 BC, from ancient China (Mechoulam, Hanus et al. 2014). For years, the plant was used to treat a variety of medical conditions and for recreational purposes due to its already known psychotropic effects. It was only in 1964 that Gaoni and Mechoulam (Gaoni and Mechoulam 1964) isolated the Δ(9)-tetrahydrocannabinol (THC), the main psychoactive constituting of cannabis. Since that, more than 100 cannabinoid compounds and 400 non-cannabinoids have been isolated from the plant. More than 20 years later, in 1988, Devane *et al*. discovered a G-protein-coupled cannabinoid receptor (GPCR) in the brain, named cannabinoid 1 (CB1) receptor (Devane, Dysarz et al. 1988). Soon after that, in 1993, the cannabinoid 2 (CB2) receptor was discovered in the immune system by Munro *et al*. (Munro, Thomas et al. 1993).

Phytocannabinoids (plant extracted cannabinoids) were the first considered. Yet, in 1992, right after CB1 identification, N-arachidonoyl ethanolamine (Anandamide, AEA) was found, leading to the discovery of endocannabinoids. Those are cannabinoids that are naturally produced in mammalian tissues (Devane, Hanus et al. 1992). Latter, 2-arachidonoyl glycerol (2-AG) was identified and evidence has emerged for the existence of additional endocannabinoids (Mechoulam, Benshabat et al. 1995). In total, 11 more compounds have been pointed has possible orthosteric endocannabinoids since they bind to cannabinoid receptors and are detectable in mammalian tissues: noladin ether, dihomo-γ-linolenoylethanolamide, virodhamine, oleamide, docosahexaenoylethanolamide, eicosapentaenoylethanolamide, sphingosine, docosatetraenoylethanolamide, N-arachidonoyldopamine, N-oleoyldopamine and haemopressin (Pertwee 2015). Nevertheless, AEA and 2-AG are still considered the most relevant endocannabinoids.

AEA behaves as a partial agonist for CB1 receptors and exhibits less relative intrinsic activity and affinity for CB2 than CB1 receptors. 2-AG is an agonist for both CB1 and CB2 receptors and shows greater potency and efficacy than AEA as a CB1 agonist and greater potency than AEA as a CB2 agonist. Both endocannabinoids bind and activate non-CB1 and non-CB2 receptors and channels, as the G-protein coupled receptor GRP55, 5-HT_3 and opioid receptors and the transient receptor potential cation channel subfamily vanilloid member (TRPV) 4. AEA activates the TRPV1 and several subtypes

of the peroxisome proliferator-activated receptor (PPAR) family (Pertwee, Howlett et al. 2010).

Unlike most neurotransmitters, AEA and 2-AG are not stored in vesicles but rather synthesized on-demand, in response to increased concentration of intracellular calcium. They act mainly as presynaptic retrograde messengers, with an inhibitory activity over the release of other neurotransmitters, preventing the development of excessive neuronal activity in the central nervous system and thereby, contributing to the maintenance of homeostasis in both health and disease. They are then cleared by a process of cellular intake and enzymatic inactivation (Wilson and Nicoll 2001).

2-AG is produced from diacylglycerol (DAG) as a result of the activation of phospholipase C (PLC) in cells that express diacylglycerol lipase. Diacylglycerol lipase converts the PLC product DAG in 2-acylglycerols, including 2-AG and 2-oleoylglycerol (2-OG) (Hillard 2015). The concentration of 2-OG in human serum is typically 5–10 times higher than 2-AG. It has been suggested that 2-OG is an endogenous ligand for the orphan receptor G protein-coupled receptor (GPR) 119 (Syed, Bui et al. 2012). 2-AG is catabolized by hydrolysis of its ester bond, resulting in the formation of glycerol and free arachidonic acid. Several enzymes are responsible for this hydrolysis, including monoacylglycerol lipase (MAGL), alpha-beta hydrolase domain protein (ABHD)-6, and ABHD-12 (Blankman, Simon et al. 2008).

AEA and other N-acyl ethanolamines (NAEs) are synthesized from a low abundance phospholipid, N-acyl-phosphatidylethanolamine (NAPE), through several different pathways (Hillard 2015). NAPE is synthesized via an enzymatically mediated transfer of an acyl chain from the sn-1 position of a donor phosphatidylcholine to the amine of phosphatidylethanolamine. The relative abundance of the NAEs formed reflects the relative abundance of the acyl chains found in the sn-1 position of the donor phospholipids. The NAEs are catabolized by hydrolysis of the amide bond through the actions of fatty acid amide hydrolase (FAAH) (Cravatt, Giang et al. 1996) and N-acylethanolamine-hydrolyzing acid amidase (NAAA), which is found primarily in peripheral tissues (Ueda, Yamanaka et al. 2001). Both AEA and 2-AG can also serve as a substrate for cyclooxygenase-2 (COX-2), and lipoxygenase and P450 enzymes that utilize arachidonic acid as a substrate (Urquhart, Nicolaou et al. 2015).

Cannabinoid Receptors

Since the identification of CB1 and CB2 receptors, at the beginning of the 90s, a range of pharmacological and genetic tools have been developed to characterize their predicted amino acid sequence, signalling mechanisms, tissue distribution, and sensitivity to certain potent agonists and antagonists. Furthermore, other non-CB1 and non-CB2 receptors and ion channels have been identified as potential targets for endocannabinoids (Howlett, Barth et al. 2002, Howlett 2005).

CB1 and CB2 receptors are members of the superfamily of GPCRs. They both signal through $G_{i/o}$ proteins ($G_{i1,2}$ and $_3$, and G_{o1} and $_2$), inhibiting adenylyl cyclase and activating mitogen-activated protein (Howlett, Johnson et al. 1988, Matsuda, Lolait et al. 1990, Vogel, Barg et al. 1993, Pinto, Potie et al. 1994). In addition, CB1 receptor $G_{i/o}$ proteins can mediate activation of A-type and inwardly rectifying potassium channels, and inhibition of N- and P/Q-type calcium currents (Deadwyler, Hampson et al. 1995, Hampson, Evans et al. 1995, Childers and Deadwyler 1996). CB1 receptors can also signal through G_s proteins (Glass and Felder 1997, Maneuf and Brotchie 1997, Calandra, Portier et al. 1999, Jarrahian, Watts et al. 2004). Evidence exists for cannabinoid receptor-mediated Ca^{2+} fluxes and stimulation of phospholipases A and C. Stimulation of CB1 and CB2 receptors leads to phosphorylation and activation of p42/p44 mitogen-activated protein kinase (MAPK), p38 MAPK and Jun N-terminal kinase (JNK) 1 and JNK2 as signalling pathways to regulate nuclear transcription factors (Wartmann, Campbell et al. 1995, Sugiura, Kodaka et al. 1996, Sugiura, Kodaka et al. 1997, Rueda, Galve-Roperh et al. 2000). Lastly, the CB1 receptor regulates K^+ and Ca^{2+} ion channels, probably via G_o (Caulfield and Brown 1992, Mackie, Lai et al. 1995). Drugs that activate CB1 and CB2 receptors target orthosteric sites on these receptors. CB1 and CB2 receptors also contain one or more allosteric sites that can be targeted by ligands to enhance or inhibit its activation by direct "orthosteric" agonists (Price, Baillie et al. 2005, Horswill, Bali et al. 2007, Navarro, Howard et al. 2009, Feng, Alqarni et al. 2014)

CB1 receptors are found mainly in terminals of central and peripheral neurons, where they usually inhibit the release of excitatory and inhibitory neurotransmitters. They are commonly located in central nervous areas responsible for cognition and memory, control of motor function, and analgesia. CB1 receptors are now also known to be present in several peripheral tissues as in the immune system, adipocytes, liver, pancreas, skeletal muscle and skin.

CB2 receptors are less abundant than CB1 receptors. They are expressed on immune cells (especially those derived from macrophages, such as microglia, osteoclasts and osteoblasts), neurons and other peripheral tissues (Mackie 2008).

In the last years, other GPCRs such as GPR18 and GPR55 have emerged as possible members of the cannabinoid family (Pertwee, Howlett et al. 2010). Because of their close phylogenetic relationship, orphan GPCRs, GPR3, GPR6, and GPR12, have also been pointed as possible cannabinoid receptors (Fredriksson, Lagerstrom et al. 2003). Certain endo-, phyto-, and synthetic cannabinoid ligands have displayed activity at other well-established GPCRs, including the opioid (μ and δ) (Kathmann, Flau et al. 2006), adenosine (A_3) (Lane, Beukers et al. 2010), serotonin (5-HT_{1A} and 5-HT_{2A}) (Boger, Patterson et al. 1998), muscarinic (M_1 and M_4) (Christopoulos and Wilson 2001) and dopamine receptor families (Bloomfield, Ashok et al. 2016).

The increasing data regarding cannabinoid interactions with transient receptor potential (TRP) channels have prompted some researchers to consider them as "ionotropic cannabinoid receptors". To date, six TRP channels from three subfamilies have been reported to mediate cannabinoid activity: TRPV1, TRPV2, TRPV3, TRPV4, TRP ankyrin (TRPA) 1, and TRP melastatin (TRPM) 8 (Muller, Morales et al. 2018). Ligand-gated serotonin (5-HT_3), nicotine, glycine (α1, α2, α3, α1β1 and α1β) and N-methyl-D-aspartate (NMDA) ion channels can also be activated by endocannabinoids as AEA (Pertwee, Howlett et al. 2010).

Lastly, AEA and 2-AG have each already been reported to activate two PPAR receptors: PPAR-γ and PPAR-α (AEA) and PPAR-γ and PPAR-β/δ (2-AG). Overall, the potencies of endocannabinoids and their metabolites as PPAR agonists or antagonists are relatively low compared with their potencies as agonists of canonical cannabinoid CB1/CB2 receptors (Pertwee, Howlett et al. 2010).

Cannabinoid CB1 and CB2 receptors, as other GPCRs can couple to different signal transduction pathways in different cell types (termed cell-specific signaling) and can activate different signaling pathways depending on the receptor conformation(s) stabilized by the activating ligand (functional selectivity) (Peters and Scott 2009). Pronounced differences (bias) in the ability of certain agonists to activate distinct signaling pathways (cAMP accumulation, ERK phosphorylation, GIRK activation, GTPγS binding, and β-arrestin recruitment) and to cause off-target effects have been recently identified (Priestley, Glass et al. 2017).

Endocannabinoid System and Skin

More than a 'passive' physical barrier, skin and its adnexal components are now believed to be an 'active' neuro-immuno-endocrine organ. Neuronal network communication, remodelling of non-neuronal cells and 'mini-organs' (i.e. hair follicle, sebaceous gland); inflammatory and immunological activity; thermoregulation, synthesis and release of GF, vasoactive substances and hormones (e.g. vitamin D, steroids and peptide hormones) are some of the skin functions (Biro, Toth et al. 2009).

Recently, the endocannabinoid system and its components (CB receptors, enzymes responsible for synthesis and metabolism of their ligands and endocannabinoids) have been identified in the skin. It has been shown to be responsible for maintaining many aspects of skin homeostasis such as proliferation, differentiation and apoptosis, barrier formation, regeneration, and release of inflammatory mediators (Sheriff, Lin et al. 2019). Dysregulation of this delicate balance was implicated in several highly prevalent skin diseases and disorders. Gathering this data, some authors have proposed a new "C(ut)annabinoid" System (Toth, Adam et al. 2019).

Figure 3 summarizes the main physiological functions of the endocannabinoid system in the skin. These data have been extensively reviewed in recent papers (Biro, Toth et al. 2009, Kupczyk, Reich et al. 2009, Pucci, Pirazzi et al. 2011, Toth, Olah et al. 2014, Caterina and Pang 2016, Mounessa, Siegel et al. 2017, Olah and Biro 2017, Eagleston, Kalani et al. 2018, Marks and Friedman 2018, Rio, Millan et al. 2018, Lim and Kirchhof 2019, Milando and Friedman 2019), and this thesis only provide a general brief overview on the topic. Shortly, CB1 and CB2 receptors are expressed in keratinocytes within the more differentiated epidermal layers, hair follicle cells, sebaceous glands, sweat gland cells, sensory neurons, immune cells, mast cells, and fibroblasts (Casanova, Blazquez et al. 2003, Ibrahim, Porreca et al. 2005, Stander, Schmelz et al. 2005, Blazquez, Carracedo et al. 2006, Karsak, Gaffal et al. 2007, Telek, Biro et al. 2007, Dobrosi, Toth et al. 2008).

Cannabinoid receptor agonists showed to inhibit proliferation of human and murine cell cultures of epidermal keratinocytes, on a CB1 and CB2 independent and dependent mode (Casanova, Blazquez et al. 2003, Wilkinson and Williamson 2007). This growth-inhibitory action was accompanied by CB1 and CB2 dependent apoptosis (Casanova, Blazquez et al. 2003). Endocannabinoids also regulate human epidermal differentiation, probably via CB1 dependent mechanisms. Maccarrone *et al.* (Maccarrone, Di Rienzo et al. 2003) have demonstrated that AEA, locally produced

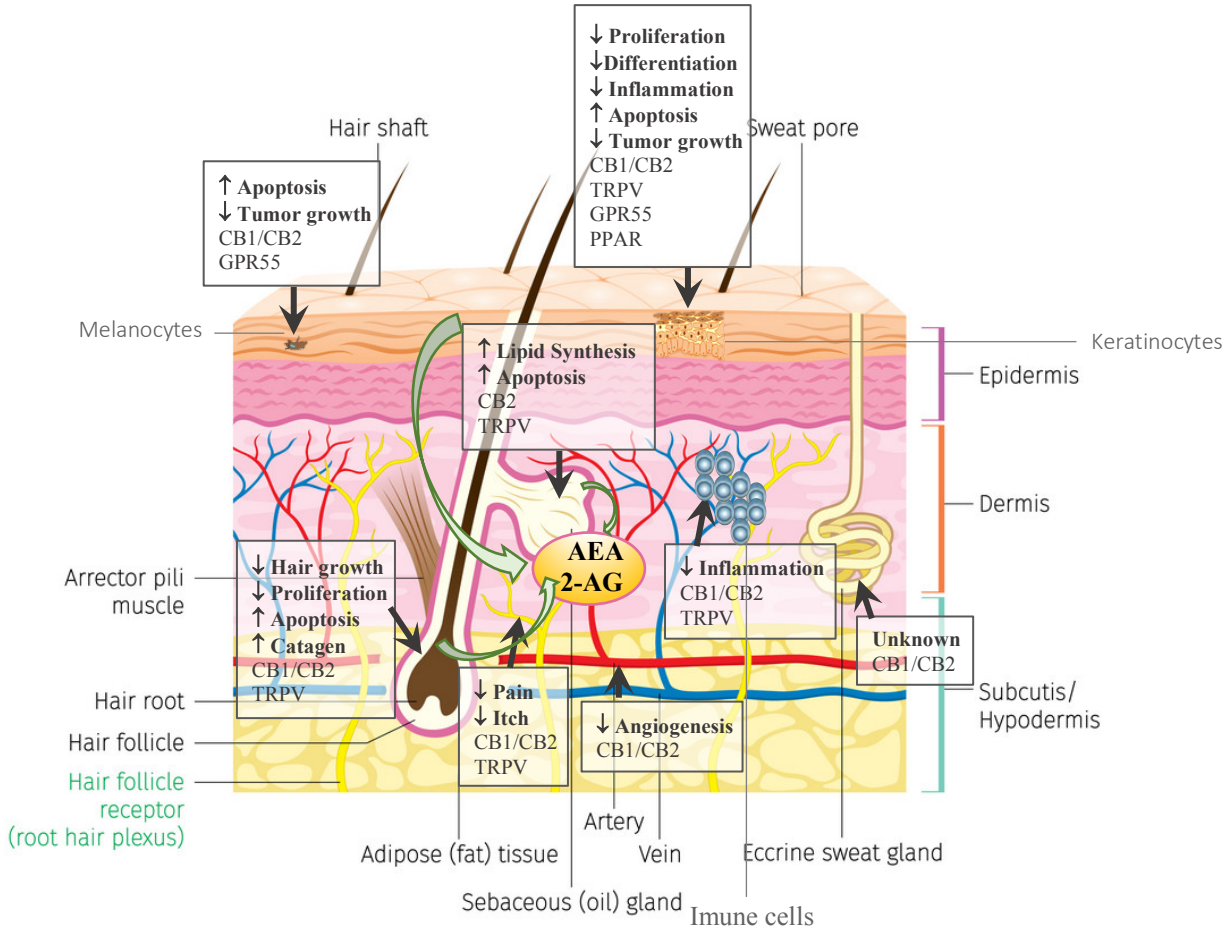

Figure 3. The endocannabinoid system in the skin.
Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are produced locally in different cellular compartments of the skin, as epidermis, sebaceous gland and hair follicles (green arrow). They will activate cannabinoid 1(CB1) and 2 (CB2) receptors, transient receptor potential vanilloid 1 (TRPV1), G protein-coupled receptor (GPR) 55 and peroxisome proliferator activated receptors (PPAR) and control various well-balanced cutaneous functions.

in the cells, inhibited the differentiation of cultured NHEK and HaCaT keratinocytes. The authors have shown that these effects were mediated by increasing DNA methylation through MAPK dependent pathways (p38, p42/44) initiated by CB1 activation (Paradisi, Pasquariello et al. 2008). Consistently with this study, it was also shown that CB1 receptor expression is higher in more differentiated layers of the human epidermis (granular and spinous layers) (Casanova, Blazquez et al. 2003, Stander, Schmelz et al. 2005).

The endocannabinoid system is also highly involved in the pilosebaceous unit. AEA and 2-AG have been shown to be produced in both hair follicle and sebaceous gland cells. AEA and THC dose-dependently inhibit hair shaft elongation and the proliferation of hair matrix keratinocytes, and induce intraepithelial apoptosis and premature hair follicle regression, in a CB1 dependent way (Telek, Biro et al. 2007). On sebocytes, both

AEA and 2-AG enhance lipid production and induce cell death, mainly via CB2 coupled signaling involving the MAPK pathway (Dobrosi, Toth et al. 2008).

Primary human melanocytes produce AEA and 2-AG, and express CB1, CB2 and TRPV1 receptors, and various cannabinoid related enzymes as NAPE, DAGL, FAAH and MAGL (Pucci, Pasquariello et al. 2012). Conflicting evidence is found in functional studies. Cannabinoids were found to inhibit melanogenesis and to induce apoptosis of primary human melanocytes most likely by TRPV1 activation (Yang, Huang et al. 2015). On the other hand, Magina *et al*. (Magina, Esteves-Pinto et al. 2011) found that CB1 agonism reduced both spontaneous and UVB-induced melanogenesis, in a melanocyte-keratinocyte cellular model. However, at lower concentrations, AEA dose-dependently stimulated melanogenesis and tyrosinase activity in a CB1-dependent manner through the activation of p38 and extracellular signal-regulated kinase (ERK)1/2 MAPK, as well as the cyclic adenosine monophosphate (cAMP) response element-binding protein, but without influencing the cAMP level (Pucci, Pasquariello et al. 2012).

Cannabinoid signaling controls local immune responses in the skin. Numerous lines of evidence demonstrate that endocannabinoids can modulate immune functions, and they are generally considered to be anti-inflammatory agents. These effects are exerted on "professional" immune cells, and on non-immune cells (e.g., keratinocytes, sebocytes) (Chiurchiu, Battistini et al. 2015, Chiurchiu 2016).

Lastly, the endocannabinoid system has a crucial role in skin-derived sensory phenomena as pain and itch. Potent analgesic effects in both humans and animals are obtained with CB1, CB2 and TRPV1 receptors activation (Walker and Hohmann 2005, Jhaveri, Richardson et al. 2007).

Given the high involvement of the endocannabinoid system in various key processes in the skin, it is not surprising that the scientific community is turning their focus into the implication of this system in skin disorders, and its potential therapeutic role in different cutaneous diseases.

Cannabinoid drugs have been proposed to treat different inflammatory skin diseases like psoriasis, atopic dermatitis, contact dermatitis, systemic sclerosis (SSc) and hidradenitis suppurativa; sebaceous gland related disorders like acne, seborrheic dermatitis and skin dryness; hair growth disorders as alopecia, effluvium, hirsutism and hypertrichosis; melanocytes and pigmentation disorders as vitiligo, skin hypo and hyperpigmentation; and skin tumours as melanoma, and non-melanoma skin cancers (Biro, Toth et al. 2009, Eagleston, Kalani et al. 2018, Milando and Friedman 2019, Toth,

Adam et al. 2019). The topical application of cannabinoids has also been pointed as a hypothetical useful tool in the treatment of pain and pruritus (Szepietowski, Reich et al. 2005, Hashim, Cohen et al. 2017, Eagleston, Kalani et al. 2018). In fact, randomized phase 1, phase 2 and phase 3 clinical trials (Milando and Friedman 2019, Toth, Adam et al. 2019) with both topical or oral cannabinoid drugs administration for treatment of psoriasis (clinicaltrial.gov identifier: NCT02976779), atopic dermatitis (Pulvirenti, Nasca et al. 2007, Yuan, Wang et al. 2014), SSc (clinicaltrial.gov identifier: NCT02465437, NCT03398837), dermatomyositis (clinicaltrial.gov identifier: NCT02466243), seborrheic dermatitis (clinicaltrial.gov identifier: NCT02818777) and acne (clinicaltrial.gov identifier: NCT03573518), are already being conducted. Moreover, other small reports on the clinical efficiency of topical application of cannabinoids to treat uremic (Szepietowski, Reich et al. 2005) or chronic pruritus (Stander, Reinhardt et al. 2006), facial postherpetic neuralgia (Phan, Siepmann et al. 2010) and acne scars (Palmieri, Laurino et al. 2019) have been published. Systemic administration of cannabidiol (CBD) and THC to treat epidermolysis bullosa (Chelliah, Zinn et al. 2018, Schrader, Duipmans et al. 2019) have also been documented in two short reports, showing faster wound healing and better pain control as principal outcomes.

ENDOCANNABINOID SYSTEM AND WOUND HEALING

First reports on the involvement of cannabinoids in skin fibrosis date from 2009. In that year, Akhmetshina *et al*. (Akhmetshina, Dees et al. 2009) reported, for the first time in literature, that $CB2^{-/-}$ *knock-out* mice were more sensitive to bleomycin-induced dermal fibrosis, and showed increased dermal thickness and leucocyte infiltration than $CB2^{+/+}$ mice. The authors confirmed this result with the administration of AM630, a CB2 selective antagonist, and conversely showed that skin inflammation and skin fibrosis was reduced in animals treated with JWH133, a CB2 selective agonist. They argued that this CB2 effect on fibrosis was secondary to an anti-inflammatory response, rather than by direct effects on the collagen synthesis of fibroblasts, since the phenotype of $CB2^{-/-}$ *knock-out* mice was mimicked by $CB2^{-/-}$ bone marrow transplantation into $CB2^{+/+}$ mice, indicating that leukocyte CB2 expression critically influenced experimental fibrosis. One year later, the same group published a similar work (Marquart, Zerr et al. 2010) showing opposite results when studying CB1 receptor response to fibrosis. Shortly, leukocyte infiltration and dermal fibrosis were reduced in $CB1^{-/-}$ *knock-out* mice treated with bleomycin, and the opposite effect was observed when the CB1 receptor was activated with ACEA. They also showed that, consistent with previous observations, $CB1^{-/-}$ *knock-out* tight-skin-1 (TSK-1) mice were not protected from fibrosis. TSK-1 mice are an animal model for later SSc stages when inflammatory infiltrates are absent and the increased release of collagen by fibroblasts is caused by endogenous activation and not by the release of profibrotic cytokines (Green, Sweet et al. 1976). They proclaimed that CB1 enhancing effect on skin fibrosis was due to an exacerbation in the inflammatory response.

Simultaneously with these reports, Garcia-Gonzalez *et al*. (Garcia-Gonzalez, Selvi et al. 2009) showed that CB1 and CB2 receptor expression was increased in human SSc fibroblasts and that WIN55,212-2, a cannabinoid non-selective agonist, reduced ECM deposition, fibroblast transdifferentiation into myofibroblasts, resistance to apoptosis and ERK-1/2 phosphorylation on cultured fibroblasts, in a non CB1 or CB2 mediated response. It was reported, for the first time, that synthetic cannabinoids could directly influence fibroblast activity. Parallel results were obtained by Balistreri *et al.* (Balistreri, Garcia-Gonzalez et al. 2011) in an *in vivo* study, showing that WIN55,212-2 prevented inflammatory cell infiltration and skin fibrosis in bleomycin treated mice. The authors showed that the synthetic cannabinoid prevents fibroblasts activation induced by bleomycin, paralleled by strong inhibition of TGF-β, connective tissue growth factor

(CTGF) and PDGF expression and downregulation of SMAD2/3 phosphorylation but they did not provide any information regarding the importance of each receptor in this response.

Strengthening the importance of the "classic" cannabinoid receptors in wound healing, murine obtained data suggested that CB1 and CB2 receptors are time-dependently expressed during skin wound healing in fibroblasts and immune cells (Zhao, Guan et al. 2010, Zheng, Yu et al. 2012).

Nevertheless, the last reference found regarding the importance of the CB1 receptor on skin fibrosis dates from 2012. In a Palumbo-Zerr *et al*. publication (Palumbo-Zerr, Horn et al. 2012), pharmacological or genetic inactivation of FAAH showed to exacerbate experimental skin fibrosis in mice. Blocking the CB1 receptor with AM281, a CB1 selective antagonist, completely abrogated the profibrotic effects of FAAH inhibition. In contrast to the antifibrotic effects of the CB1 antagonist, inhibition of the CB2 receptor by AM630 further increased fibrosis but the relative effects were less pronounced than for CB1. The authors then proposed that the CB1 receptor should be considered the dominant receptor for endocannabinoid experimental fibrosis and that this receptor would be the more promising candidate for targeted treatments in fibrotic skin diseases.

Interestedly, various publications are found in the literature regarding the role of CB2 receptor in skin fibrosis or in wound healing, both in mouse and fibroblast cultures. In line with previous reports, Servettaz *et al.* (Servettaz, Kavian et al. 2010) showed that treatment with WIN55,212 or with JWH133 prevented the development of skin and lung fibrosis in mice and reduced fibroblast proliferation *in vitro*. $CB2^{-/-}$ *knock-out* mice were also more responsive to fibrotic stimulus, and their fibroblasts presented higher proliferative rates when cultured *in vitro*. Other studies in wounded mice confirmed that CB2 receptor activation decreases collagen deposition and reduces the levels of TGF-β1, TGF-β receptor I and phosphorylated SMAD3 during wound healing (Li, Wang et al. 2016); attenuates inflammation by inhibiting M1 macrophages (Du, Ren et al. 2018) and by reducing neutrophil and macrophage infiltration; and decreases monocyte chemotactic protein, stromal cell-derived factor 1, IL-6, IL-1β, TNF-α, TGF-β1 and VEGF expression (Wang, Zhao et al. 2016). Moreover, keratinocyte proliferation and migration and wound re-epithelization is shown to be enhanced by CB2 receptor activation and, conversely, fibroblast accumulation, fibroblast to myofibroblast transformation and expression of collagen I showed to be decreased (Wang, Zhao et al. 2016). Taken together these data

suggests that CB2 receptor activation could improve wound healing by reducing inflammation, enhancing re-epithelization and reducing scar formation and that CB2 agonists might be a novel perspective for skin wound therapy. Theoretically, such a dual effect (promotion of keratinocyte migration together with suppression of fibroblast activity) could be desirable to obtain scarless healing.

Other receptors involved in the cannabinoid system signalization have been suggested to have a role in skin fibrosis. Adenosine A2A receptor stimulation showed to increase collagen production in sclerodermic dermal fibroblasts either directly and through a cross-talk with the cannabinoid system. More specifically, it was shown that, in SSc human fibroblasts, the A2A receptor was overexpressed and its activation with an agonist increased collagen production, myofibroblast transdifferentiation, and ERK-1/2 phosphorylation. This receptor can form a heteromer with the CB1 receptor and unselective cannabinoid receptor stimulation with a per se ineffective dose of WIN55,212-2, resulted in a marked anti-fibrotic effect after A2A receptor blockage (Lazzerini, Natale et al. 2012). PPAR-γ activation with ajulemic acid, a non-psychoactive synthetic analogue of THC, significantly prevented experimental bleomycin-induced fibrosis in mice and strongly reduced collagen neosynthesis by scleroderma fibroblasts *in vitro*, an action that was completely reversed by co-treatment with a selective PPAR-γ antagonist (Gonzalez, Selvi et al. 2012). Dual PPAR-γ and CB2 activation with VCE-004.8, a non-psychotropic CBD quinol derivative, inhibited TGF-β-induced Col1A2 gene transcription, collagen synthesis, myofibroblast differentiation, and impaired wound healing activity in human fibroblast cultures. This anti-fibrotic activity was confirmed *in vivo,* in a bleomycin murine model of dermal fibrosis (del Rio, Navarrete et al. 2016). Similar results were obtained with EHP-101 (Garcia-Martin, Garrido-Rodriguez et al. 2018, Garcia-Martin, Garrido-Rodriguez et al. 2019) and VCE-004.3 (Del Rio, Cantarero et al. 2018), two other CBD quinol derivatives with PPAR-γ and CB2 activity.

Concerning the efficiency of cannabinoids on wound healing in humans, only insufficient evidence is available. Basically, as mentioned above, three patients suffering from epidermolysis bullosa reported faster wound healing following self-administration of CBD (Chelliah, Zinn et al. 2018).

Despite significant research on the role of the cannabinoid signaling in wound healing and skin fibrosis formation, the potential role of endocannabinoid system in wound healing in humans is still poorly understood. Moreover, the influence of endocannabinoid tone during wound healing is not yet elucidated.

Introduction

Annexe 1 | *Cutaneous endocannabinoid system: does it have a role on skin wound healing bearing fibrosis?"*
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Review

Cutaneous endocannabinoid system: Does it have a role on skin wound healing bearing fibrosis?

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ABSTRACT

Introduction: Recently, the endocannabinoid system has been identified in skin and it has been linked with the formation of skin fibrosis and wound healing. We aimed to find and analyse reported data on compounds acting in the endocannabinoid system with significant effect in skin fibrosis.

Methods: A literature search on PUBMED was conducted for studies published in English until February 2020 on cannabinoids and skin fibrosis. The initial search was performed with terms: "cannabinoid" AND "skin". This search retrieved 296 publications from which 18 directly related to skin fibrosis or wound healing process were included in this review.

Results: CB1 receptor inactivation and CB2 receptor activation show anti-fibrotic effects on cellular and animal experimental models of cutaneous fibrosis. CB2 receptor activation also promotes re-epithelization. Other cannabinoid related receptors, like adenosine A2A receptors and PPAR-γ, are also involved. Their activation lead to a pro-fibrotic and anti-fibrotic effect, respectively.

Conclusion: Several molecular drug targets for endocannabinoid system were identified in skin. It may be a promising approach for the treatment of excessive skin fibrosis disorders.

1. Introduction

1.1. Skin fibrosis

Wound healing is a dynamic process responsible for repair of disruptive skin injuries. It has been conceptually divided in three subsequent but overlapping phases: inflammation, proliferation and remodelling. Within each phase, different cells, growth factors, cytokines, pro- and anti-inflammatory mediators are involved, and different cellular, humoral and molecular mechanisms take place. In consequence of this form of repair, fibrosis and scar formation always occurs [1].

Often disturbances on wound healing process emerge. In consequence of burns, traumatic injuries or surgical incision, excessive scarring or excessive skin fibrosis can occur leading to hypertrophic scars or keloid formation [2]. Excessive scars present a major biological, psychological, social, and financial burden on both individual patients and the broader health system [3]. Excessive scar formation frequently causes scar contracture, permanent functional impairment and disfiguration [4]. Similarly, some systemic medical conditions, including systemic sclerosis (SSc), are characterized by immunological and vascular abnormalities that lead to anomalous fibrotic formation in skin and other organs [5].

The mechanism underling excessive scaring is not fully understood. Hypertrophic scars and keloids are both characterized by an excessive tissue response to dermal injury characterized by local fibroblast proliferation, overproduction of collagen and formation of non-functional scar tissue. A prolonged or excessive inflammatory phase is believed to incite fibroblast activity and increase extracellular matrix (ECM) deposition [4]. Mast cells, lymphocytes, macrophages, and inflammatory cytokines, including transforming grown factor beta (TGF-β), platelet derived growth factor (PDGF) and epidermal growth factor (EGF) [6] are believed to be involved in the pathogenesis of these disorders. Mechanical stress, age and reepithelization delay are also believed to be predisposing factors [7].

Treatment of hypertrophic scars and keloids is often unsuccessful with high recurrence rates. Since excessive pathological scarring has been shown to occur due to an exaggerated inflammatory response most of the therapies available are directed to reduce inflammation

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Table 1
Published studies regarding CB1 receptor and skin fibrosis. Systemic sclerosis (SSc), Cannabinoid (CB), Cannabinoid 1 receptor (CB1), Fatty acid amide hydrolase (FAAH), arachidonyl-2'-chloroethylamide (ACEA), Tight skin 1 mouse model (TSK-1).

Study	Experimental model	CB1 receptor activation	Outcome of CB1 receptor activation	CB1 receptor inactivation	Outcome of CB1 receptor inactivation
Marquart et al. [39]	Mice $CB1^{+/+}$ $CB1^{-/-}$ TSK-1	ACEA	↑ fibrosis	$CB1^{-/-}$	↓ fibrosis
Palumbo-Zerr et al. [38]	Mice FAAH deficient mice SSc human fibroblasts Human fibroblasts	JNJ1661010 (FAAH inhibitor)	↑ fibrosis	AM281	↓ fibrosis

[8–10]. Better understanding of the cellular and molecular mechanisms underlying wound healing bearing fibrosis will allow to attenuate the wound repair/regeneration process and eventually identify new molecular drug targets.

1.2. The endocannabinoid system

The endocannabinoid system includes cannabinoid receptors (CB), their endogenous ligands such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and enzymes responsible for their synthesis and metabolism [11,12]. Cannabinoid 1 receptor (CB1) and cannabinoid 2 receptor (CB2) are members of the superfamily of G protein-coupled receptors (GPCRs) [13–16] and present different distribution in the human body. CB1 receptors are mainly located in the central nervous system, but can also be found in peripheral tissues like in adipose tissue, liver, pancreas, skin and skeletal muscle [17,18]. On the other hand, CB2 receptors are mainly located in immune system cells, such as macrophages, lymphocytes and natural killer cells, but also present in neurons and other peripheral tissues [17].

Recently, a functional endocannabinoid system was identified in skin [19]. CB1 and CB2 receptors have been found in keratinocytes, hair follicle cells, sebaceous glands, sweat gland cells, sensory neurons, immune cells, mast cells and fibroblasts [20–26]. The endocannabinoid system has been shown to be involved in skin physiologic functions as proliferation, differentiation, apoptosis and regulation of inflammatory and immune responses. Disruption of this system may be involved in skin diseases like systemic sclerosis, acne, seborrheic dermatitis, allergic dermatitis, psoriasis and skin tumours as melanoma, and non-melanoma skin cancers. Activation or inactivation of the CB1 and CB2 receptors with selective agonists or antagonists have been proposed as potential therapeutic agents to treat different skin diseases [19]. Randomized phase 1, phase 2 and phase 3 clinical trials [27,28] with both topical or oral cannabinoid drugs administration for treatment of psoriasis (clinicaltrial.gov identifier: NCT02976779), atopic dermatitis [29,30], SSc (clinicaltrial.gov identifier: NCT02465437, NCT03398837), dermatomyositis (clinicaltrial.gov identifier: NCT02466243), seborrheic dermatitis (clinicaltrial.gov identifier: NCT02818777) and acne (clinicaltrial.gov identifier: NCT03573518), are currently being conducted. Moreover, other small reports have been published in literature on the clinical efficiency of cannabinoids topical application to treat uremic [31] or chronic pruritus [32], facial post herpetic neuralgia [33] and acne scars [34].

Lately, some reports emerged reporting the involvement of endocannabinoid system in skin wound healing and in pathological fibrosis [19,27,28,35]. CB1 and CB2 receptors expression has been shown to be time-dependent during skin wound healing in fibroblasts and immune cells in mice [36,37]. In skin incised wounds, the ratio of the CB1 receptor positive cells increased gradually from 6 h to 3 days, peaked at 5 days, and decreased gradually from 7 to 14 days post-injury. CB1 receptor positive cells during wound healing are mostly mononuclear and fibroblastic cells [37]. Differently, the ratio of CB2 receptor positive macrophages reaches a maximum at 3 days post-wounding, while the ratio of CB2 receptor positive myofibroblasts peaks at 5 days post-wounding [36]. Dynamic distribution and expression of CB1 and CB2 receptors suggest that these receptors are involved in regulating the repair process during skin wound healing.

In the present review, we aimed to find and analyse reported data on compounds acting in the endocannabinoid system with significant effect in skin fibrosis.

1.3. Methods

We searched PUBMED for studies on cannabinoids and skin fibrosis published in English until February 2020. The initial search was performed with broad terms: "cannabinoid" AND "skin". Two authors retrieved separately the most prominent articles and extracted the data while a third author checked for accuracy. Due to the preliminary stage of research in this area, all studies that directly related to skin fibrosis or wound healing process were included in this review. Only minimal exclusion criteria were used and no restrictions on the design of the study were applied. Reviews, letters, editorials and case reports were excluded.

The search produced 296 references. Bibliographies of relevant papers were screened, but no further studies were considered relevant. Based on an initial abstract screening, 268 articles were excluded and 28 were selected for full text examination. From these 28 publications, 7 did not address any of the outcomes of interest, 2 were reviews of the existing literature and 1 showed uncompelling and unsubstantiated results and therefore were excluded. Eighteen publications met our eligibility criteria and were included in our review.

2. Results

2.1. CB1 receptor

Table 1 summarizes relevant data found in studies involving CB1 receptor in skin fibrosis [38,39]. Although references regarding the role of CB1 receptor in skin wound healing are still limited [38,39], they clearly suggest that CB1 receptor activation with CB1 receptor agonists (ACEA) or by fatty acid amide hydrolase (FAAH, an integral membrane enzyme that hydrolyses the endocannabinoid anandamide and related amidated signalling lipids) inactivation, both genetic (in FAAH deficient mice) and pharmacological (FAAH inhibitor JNJ1661010) interventions resulted in fibrosis increment. These results were obtained using an animal model of SSc and bleomycin local injections were used to produce dermal fibrosis.

Consistently, both genetic (in $CB1^{-/-}$ *knock-out* mice) [39] and pharmacological (with the CB1 receptor antagonist AM281) [38] CB1 receptor inactivation showed to protect mice from the bleomycin effect by reducing dermal thickness, leucocyte and macrophages infiltration, hydroxyproline content and myofibroblast count. The authors concluded that CB1 activation exerts profibrotic effects by increasing the

Table 2
Published studies regarding **CB2 receptor** and skin fibrosis or wound healing. Cannabinoid (CB), Cannabinoid 2 receptor (CB2).

Study	Experimental model	CB2 receptor activation	Outcome of CB receptor activation	CB receptor inactivation	Outcome of CB2 receptor inactivation
Akhmetshina et al. [40]	Mice $CB2^{+/+}$ $CB2^{-/-}$	JWH133	↓ fibrosis	$CB2^{-/-}$ AM630	↑ fibrosis
Servettaz et al. [43]	Mice	WIN55,212-2 JWH133	↓ fibrosis	$CB2^{-/-}$	↑ fibrosis
Li et al. [42]	Mice	GP1a	↓ fibrosis	AM630	↑ fibrosis
Wang et al. [44]	Mice HaCaT cells	GP1a	↓ fibrosis ↑keratinocyte proliferation and migration	AM630	↑ fibrosis
Bort et al. [49]	Human fibroblast Human keratinocytes Co-cultures	JWH015	↑ scratch gap closure ↓ inflammatory cytokines	AM630	↑ fibrosis
Du et al. [41]	Mice	JWH133 GP1a	↓ fibrosis	AM630	↑ fibrosis
Koyama et al. [47]	Mice Mice fibroblasts	Beta-caryophyllene	↑reepitelization ↑keratinocyte proliferation and migration	–	–

inflammatory response and secondary fibroblast activation [39]. Supporting these findings, it has been shown that expression of FAAH is reduced in systemic sclerosis human fibroblast [38].

2.2. CB2 receptor

Table 2 summarizes results obtained concerning CB2 receptor and skin fibrosis. It is suggested that CB2 receptor activation is implicated in wound healing, which could improve skin fibrosis by reducing inflammation, enhancing re-epithelization and reducing scar formation [40–44].

It was shown in SSc mice models induced by bleomycin injections that CB2 receptor activation with WIN55,212 (a non-selective CB receptor agonist) or with JWH133 (a CB2 selective agonist) reduces leucocyte infiltration, dermal thickening and skin fibrosis [40,43]. CB2 receptor inactivation in $CB2^{-/-}$ *knock-out* mice or with a CB2 selective antagonist (AM630) abolished the protective CB2 receptor effect, and an increase in dermal thickness, leucocyte infiltration and dermal fibrosis was observed in those animals [40]. Again, in this study, the anti-inflammatory response showed to be essential for CB2 effect on fibrosis. The phenotype of $CB2^{-/-}$ *knock-out* mice was mimicked by $CB2^{-/-}$ bone marrow transplantation into $CB2^{+/+}$ mice, indicating that leukocyte CB2 expression critically influenced experimental fibrosis [40].

However, it was later shown that synthetic cannabinoids could directly influence fibroblast activity, independently of the anti-inflammatory response [45]. In *in vivo* studies with bleomycin treated mice, WIN55,212-2, prevented inflammatory cell infiltration and reduced dermal thickness and collagen content. Additionally, Fibroblasts activation, TGF-β, connective tissue growth factor (CTGF) and PDGF expression and SMAD2/3 phosphorylation were reduced by WIN55,212-2 [46].

Other studies in wounded mice [41,42,44] showed that CB2 receptor activation decreases collagen deposition and reduces the levels of TGF-β1, TGF-β receptor I and phosphorylated SMAD3 during wound healing [42]; attenuates inflammation by inhibiting M1 macrophages [41] and by reducing neutrophil and macrophage infiltration; decreases monocyte chemotactic protein, stromal cell-derived factor 1, IL-6, IL-1β, TNF-α, TGF-β1 and VEGF expression [44]. Moreover, keratinocyte proliferation and migration and wound re-epithelization was shown to be enhanced by CB2 receptor activation. Conversely, fibroblast accumulation, fibroblast to myofibroblast transformation and expression of collagen I was decreased [44]. On the other hand, AM630, a CB2 selective antagonist, showed to increase collagen deposition and TGF-β1, TGF-β receptor I and P-SMAD3 expression [42] and to inhibit wound closure [44] in wounded mice.

Recently, beta-caryophyllene, a CB2 receptor agonist, has been shown to enhance keratinocytes proliferation and differentiation and to increase re-epithelialization of mouse skin in a full thickness wound generated on the back of mice. CB2 gene was also down-regulated in the beta-caryophyllene treated group. In the same study, primary cultured fibroblasts and keratinocytes from mice exposed to beta-caryophyllene showed higher chemotactic response. However, this effect was not observed in fibroblasts from $CB2^{-/-}$ *knock out* mice suggesting that activation of CB2 could lead to an increase in chemotactic responses. Interestingly, when testing JWH133 (agonist) and AM630 (antagonist) on the full thickness wound model in mice, as controls for beta-caryophyllene, results were not clear. While JWH133 enhanced re-epithelization in the same extent as beta-caryophyllene, AM630 produced variable results. The authors proposed that other pathways than CB2 receptor could be involved in the beta-caryophyllene enhanced wound re-epithelization [47].

In primary human fibroblast and keratinocyte cultures, JWH015 reduced the concentration of major pro-inflammatory factors, such as IL-6 and monocyte chemoattractant protein-1 (MCP-1), and increased the concentration of a major anti-inflammatory factor (TGF-β) in lipopolysaccharide-stimulated cells. JWH015 induced a faster scratch gap closure in co-cultures of human fibroblast and keratinocyte. Although JWH015 is a CB2 preferring agonist [48], the effects studied were blocked by AM281 and AM630 and the authors proposed that those effects were mainly modulated through both CB1 and CB2 receptors [49].

More information regarding CB2 receptor in wound healing was obtained in studies with other cannabinoid related receptors such as PPAR-γ (see below) [50–52]. Some cannabinoid compounds are able to activate those receptors simultaneously.

2.3. Other cannabinoid related receptors

It is suggested that other receptors involved in cannabinoid system signalling have a role in skin fibrosis. Adenosine A2A receptor [53] and PPAR-γ [50–52,54,55] have been shown to have an effect on skin fibrosis, after activation by endo or synthetic cannabinoids. Table 3 summarizes studies describing the effect of activation of other cannabinoid related receptors on wound healing.

The hypothesis that the effects of endocannabinoids may not simply be mediated by CB1/CB2 receptors stems largely from studies in which cannabinoid effect on skin fibrosis was not reverted by antagonists of these classic receptors. It was demonstrated that WIN55,212-2 reduces ECM deposition, fibroblast transdifferentiation into myofibroblasts, resistance to apoptosis and ERK-1/2 phosphorylation in human SSc

Table 3
Published studies on Cannabinoid related receptors and skin fibrosis or wound healing. Systemic sclerosis (SSc), Cannabinoid (CB), Cannabinoid 1 receptor (CB1), Cannabinoid 2 receptor (CB2), Fatty acid amide hydrolase (FAAH). Between parenthesis are ligands for CB related receptors.

Study	Experimental model	Cannabinoid receptors	Other CB related receptors	CB receptor activation	Outcome of CB related receptor activation	CB receptor inactivation	Outcome of CB receptor inactivation
Garcia-Gonzalez et al. [45]	SSc fibroblasts Human fibroblasts	?	?	WIN55,212−2	↓ fibrosis	AM281 AM630	=
Balistreri et al. [46]	Mice	?	?	WIN55,212−2	↓ fibrosis	–	–
Lazzerini et al. [53]	SSc human fibroblasts Human fibroblasts	CB1	A2Ar	WIN55,212−2 (CGS-21,680)	↑ fibrosis	AM630 (ZM-241385)	↑ fibrosis
Garcia Gonzalez et al. [54]	Mice SSc human fibroblasts Human fibroblasts	–	PPAR-γ	Ajulemic acid	↓ fibrosis	(GW9662)	↑ fibrosis
Rio et al. [51]	Mice NIH-3T3 HEK-293T-CB2 Human fibroblasts Mouse embryonic fibroblasts	CB2	PPAR-γ	VCE-004.8 WIN55,212−2 (Rosiglitazone)	↓ fibrosis	AM630 (T0070907)	↑ fibrosis
García-Martín et al. [52]	Mice	CB2	PPAR-γ	EHP-101 (Rosiglitazone)	↓ fibrosis	–	–
Rio et al. [55]	NIH-3T3 fibroblasts Human fibroblasts	CB2 CB1	PPAR-γ	VCE-004.3 WIN55,212−2 (Rosiglitazone)	↓ fibrosis	AM630 SR144528 (T0070709O7)	↑ fibrosis (partially)
García-Martín et al. [50]	Mice	CB2	PPAR-γ	Ajulemic acid EHP-101	↓ fibrosis	–	–

fibroblast cultures. These effects were not prevented by AM281 (CB1 selective antagonist) or AM630 (CB2 selective antagonist). For the first time, it was proposed that other cannabinoid related receptors could be involved in the anti-fibrotic response of cannabinoid ligands.

Besides "orthosteric" sites, CB1 and CB2 receptors also contain one or more allosteric sites that can be targeted by ligands to enhance or inhibit its activation by direct "orthosteric" agonists [56–59]. Adenosine A2A receptor may be one of those allosteric receptors as it was shown to form a heteromer with CB1 receptor. A2A stimulation was shown to increase sclerodermic dermal human fibroblasts activity, either directly or through a cross-talk with the cannabinoid system and the CB1 receptor [53]. A2A was overexpressed in SSc human fibroblasts. Its activation with CGS-21680, a selective A2A agonist, increased collagen production, myofibroblast transdifferentiation, and ERK-1/2 phosphorylation. Cannabinoid receptor stimulation with a low and *per se* ineffective dose of WIN55,212-2 resulted in a marked reduction in collagen production after A2A receptor blockage with ZM-241385 (also used in ineffective doses). Therefore, it was proposed that the synergistic inhibitory effect of WIN55,212-2 and ZM-241385 co-incubation on collagen production is likely the final result of a net cannabinoid CB2-stimulating effect. In these conditions WIN55,212-2 could act on the CB2 receptor since the CB1 receptor was indirectly blocked by ZM-241385. Supporting this hypothesis, AM630 was shown to revert the synergic effect of WIN55,212-2 and ZM-241385 [53].

Endocannabinoids and synthetic cannabinoids can activate PPAR [60] and PPAR-γ is highly involved in skin fibrosis and wound healing. It was demonstrated that PPAR-γ activation with rosiglitazone, an oral antidiabetic agent, reduces inflammation, skin fibrosis and lipoatrophy in animal models of SSc [61]. PPAR-γ activation with ajulemic acid (AjA), a non-psychoactive synthetic analogue of tetrahydrocannabinol (THC), significantly reduced skin fibrosis, skin thickness, subcutaneous leucocyte infiltration, ECM accumulation and fat layer replacement in experimental bleomycin-induced fibrosis in mice. Collagen content and myofibroblasts were also reduced with AjA treatment in an animal model of SSc. Additionally, AjA strongly reduced collagen neosynthesis and TGF-β concentration in scleroderma fibroblast cultures. The inhibitory effect of AjA on collagen production was completely prevented by GW9662, a highly selective PPAR-γ antagonist [54].

Dual PPAR-γ and CB2 activation with VCE-004.8, a non-psychotropic cannabidiol (CBD) quinol derivative, was demonstrated to inhibit TGF-β-induced Col1A2 gene transcription and collagen synthesis and myofibroblast differentiation and impaired wound-healing activity in primary human fibroblast cultures. This anti-fibrotic activity was confirmed *in vivo* in bleomycin murine model of dermal fibrosis. In this model VCE-004.8 reduced dermal thickness, blood vessels collagen accumulation, mast cell degranulation and macrophage infiltration in skin. These effects were impaired by the PPAR-γ antagonists T0070907 and the CB2 antagonist AM630. In addition, VCE-004.8 downregulated the expression of several key genes associated with fibrosis [51].

Analogous results were obtained with EHP-101 [50,52], an oral formulation of VCE-004.8. In bleomycin challenged mice, EHP-101 could prevent macrophage infiltration and Tenascin C (TNC), vascular cell adhesion molecule 1 (VCAM1), alfa smooth muscle actin (α-SMA) and vascular CD31 expression. RNAseq analysis of skin biopsies showed a clear effect of EHP-101 in the inflammatory and epithelial-mesenchymal transition transcriptomic signatures. TGF-β-regulated genes were also reduced by EHP-101 administration. Rosiglitazone served as a positive control for PPAR-γ activation [52]. EHP-101, but not AjA, enhanced the expression of some factors related to angiogenesis and vasculogenesis [50].

VCE-004.3, another non-psychotropic CBD quinol derivative and a dual PPAR-γ and CB2 receptor agonist and CB1 receptor antagonist, inhibited collagen gene transcription and synthesis and ERK1/2 phosphorylation in NIH-3T3 cells. In bleomycin treated mice, VCE-004.3 prevented skin fibrosis, myofibroblast differentiation and myofibroblast differentiation and ERK1/2 phosphorylation. Additionally, it reduced mast cell degranulation, macrophage activation, T-lymphocyte infiltration, and expression of inflammatory and profibrotic factors. The VCE-004.3 anti-fibrotic effect was observed with both intraperitoneal and topical administration. Finally, VCE-004.3 inhibited PDGF and SSc IgG-induced ERK1/2 activation in normal human dermal fibroblasts. The anti-fibrotic activity of VCE.004.3 *in vivo* was partially prevented by the CB2 antagonists AM630 and SR144528. However, the *in vivo* effect of VCE-004.3 on PPAR-γ transactivation could not be blocked by T0070907 (a PPAR-γ selective antagonist) showing that both compounds bind simultaneously to distinct PPAR-γ binding sites [55].

2.4. Evidences in humans

Regarding the efficiency of cannabinoids on wound healing in humans, only anecdotal evidence is available. Three patients suffering from epidermolysis bullosa reported faster wound healing, less blistering and amelioration of pain following self-administration of topical CBD oil [62].

3. Discussion

The endocannabinoid system has been identified in skin and having an important role in many aspects of skin homeostasis [63]. Dysregulation of this system has been implicated in several highly prevalent skin diseases [27]. Moreover, the endocannabinoid system has also been linked to fibrosis in different organs as in liver [64] or pancreas [65]. Promptly, several groups turned their attention to the potential link between this system and skin fibrosis and its potential therapeutic role in fibrotic cutaneous disease or wound healing disorders.

Initial reports on involvement of cannabinoids in skin fibrosis date from 2009 [40]. Since then, other studies were published. Although not much information is published yet, results are consistent and encourage future research. CB1 and CB2 receptors have opposing effects on skin wound healing and skin fibrosis. CB1 receptor activation presents profibrotic effects on skin. Conversely, CB2 receptor activation reduces inflammation and skin fibrosis in experimental models of SSc and on wound healing animal models. Interestingly, CB2 receptor activation also enhances keratinocyte proliferation and migration and re-epithelization. Theoretically, such a dual effect of promoting keratinocyte migration together with suppression of fibroblast activity could be desirable to obtain scarless healing with less fibrotic responses. CB2 receptors are highly expressed in immune cells [17] and have anti-inflammatory effects in mammals [66]. As inflammation is an important step in skin wound healing [67], it is not entirely surprising that CB2 receptor is involved in this skin process. Possibly, this is why studies regarding CB2 receptor are more numerous and robust than those found for CB1 receptor [40–44]. CB2 agonists might be a novel approach for skin wound therapy.

Other cannabinoid related receptors are involved in skin fibrotic process. A2A receptor activation has been shown to increase fibroblast activity, both directly and through a cross-talk with the cannabinoid system. On the other hand, PPAR-γ activation exerts anti-fibrotic effects in animal and cellular models of skin wound healing. Both receptors, A2A and PPAR-γ, are interesting targets in the context of wound healing and fibrotic diseases. Dual PPAR-γ and CB2 activation can be a promising goal for those diseases as they target two receptors implicated in skin fibrosis.

We acknowledge our review's limitations. Firstly, there are still few studies published in literature. We were only able to find 18 published studies revealing the preliminary steps that are still being done in the field. Furthermore, the methodology used in each of the published paper varies significantly making it difficult to summarize all the collected data in a proper systematic review or meta-analysis. However, the published findings are reliable and will certainty direct further research in the near future.

In conclusion, the endocannabinoid system may be an interesting target, not only for systemic fibrotic conditions like SSc, but also for local skin fibrotic disorders as hypertrophic scars or keloids. A multitarget approach involving CB1, CB2, A2A or PPAR-γ may be an interesting strategy to treat these conditions. Further pre-clinical and clinical studies should be conducted to verify efficiency, security and tolerance of those compounds in the treatment of these pathological disorders. Topical skin administration will possibly increase tolerance of cannabinoid compounds in this setting.

Declaration of Competing Interest

There are no potential and real conflicts of interest with this article.

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Aims

The aim of this work was to study a new therapeutic target for the endocannabinoid system. After the literature review, the following question arises: can we treat skin wound healing disorders with cannabinoid ligands?

This work was divided into two chapters. The main objectives of each chapter are described below.

Chapter I

In this chapter, results were obtained from primary cultures of adult human fibroblasts and from human *ex vivo* full skin cultures. The main objectives of this chapter were to:

- Establish a primary culture of adult human fibroblasts, from skin samples obtained from surgical patients.
- Create a model of wound healing in human *ex vivo* skin cultures.
- Assess the presence of cannabinoid receptors (CB1 and CB2) in primary cultures of adult human fibroblasts.
- Evaluate the influence of TGF-β on the expression of CB1 and CB2 receptors.
- Assess the role of cannabinoid receptors in human fibrogenesis.
- Evaluate the influence of cannabinoid drugs in fibroblast activation in primary cultures of adult human fibroblasts.
- Determine the influence of cannabinoid drugs on collagen deposition in primary cultures of adult human fibroblasts.
- Investigate the effect of cannabinoid drugs on the wounded human *ex vivo* skin model.

Chapter II

In chapter II, two clinical studies in patients submitted to routine body-contouring surgery were performed. With this approach, we were able to:

- Measure plasmatic levels of inflammatory biomarkers (inflammatory cells and protein inflammatory biomarkers) during wound healing, in patients submitted to elective surgery for body contouring.

- Quantify circulating plasma levels of 25(OH)D, vitamin A and vitamin E in patients submitted to surgery, and that subsequently acquired normal scars and hypertrophic scars.

- Evaluate the impact of bariatric surgery on wound healing and vitamin status.

- Quantify the most extensively studied endocannabinoids, AEA and 2-AG, and NAEs, oleoylethanolamine (OEA) and palmitoylethanolamide (PEA), during different phases of wound healing in patients that later developed normal and hypertrophic scars.

- Quantify endocannabinoids in skin and in scars.

- Identify differences in plasma and skin endocannabinoid levels in humans with normal scars and hypertrophic scars.

- Identify potential fluctuations in plasmatic endocannabinoid levels before surgery and during the different phases of wound healing (inflammatory, proliferative and remodelling phases).

- Identify correlations between the levels of endocannabinoids found in plasma and found in skin from the same patient.

Chapter I
Cannabinoids on primary cultures of human fibroblasts

AM251, a cannabinoid receptor 1 antagonist, prevents human fibroblasts differentiation and collagen deposition induced by TGF-β – an in vitro study.
Submitted for publication 2020

Targeting cannabinoid receptor 2 (CB2) limits collagen production – an in vitro study in a primary culture of human fibroblasts.
Submitted for publication 2020

AM251, a cannabinoid receptor 1 antagonist, prevents human fibroblasts differentiation and collagen deposition induced by TGF-*β* – an *in vitro* study.

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Abstract
Background: Previous studies showed that the cannabinoid 1 receptor (CB1) is linked with skin fibrosis and scar tissue formation in mice. Therefore, the topical use of cannabinoids may have a role in the prevention or treatment of local fibrotic and wound healing diseases as hypertrophic scars or keloids. In this study, we asked whether CB1 activation or inactivation would change fibroblast differentiation into myofibroblast and collagen deposition in skin human fibroblast.
Methods: Primary cultures of adult human fibroblasts were obtained from abdominal human skin. Cells were stimulated with transforming growth factor beta (TGF-*β*, 10ng/mL) and treated with a CB1 selective agonist (ACEA 1μM) and an antagonist (AM251 1, 5 and 10μM). Alpha-smooth muscle actin (*α*-SMA) was quantified using Immunocytochemistry and Western Blot. Collagen was quantified with Sirius Red staining assay. Significance was assessed by One-way ANOVA. $P<0.05$ was considered significant.
Results: TGF-*β* significantly increases *α*-SMA expression. ACEA 1μM significantly increases collagen deposition but does not change *α*-SMA expression. AM251 10μM added in the absence and the presence of ACEA reduces *α*-SMA expression and collagen content in TGF-*β* treated cells. AM251 shows a concentration-dependent effect over collagen deposition with an IC50 value of 2.9μM (0.4, 24.8). TGF-*β* significantly increases CB1.
Conclusion: CB1 inactivation with AM251 prevents fibroblasts differentiation and collagen deposition, induced by TGF-*β* in human fibroblasts. The outcome supports that CB1 is a molecular target for wound healing disorders and *in vivo* and pre-clinical studies should be implemented to clarify this premise.

Keywords: CB1 receptors, fibroblasts, skin, fibrosis, TGF-*β*, AM251

Introduction

"Will it leave a scar?" Surgeons often face this question even from the most enlightened patient, hungry to get a non-scar surgery. Unfortunately, after a trauma, surgery or burn, if a full-thickness injury occurs in the skin, there is always a scar[1]. And if scars are already a matter of concern and dissatisfaction for patients and surgeons, the distress is greatly higher when they face a hypertrophic scar or keloid.

Both hypertrophic scars and keloids are wound healing disorders characterized by an increase in fibroblast proliferative activity and overabundant accumulation of extracellular matrix components and collagen in the dermis and subcutaneous tissue[2]. The exact etiology and underlying mechanisms of these conditions are unknown but fibroblast phenotype alteration[3] and transforming growth factor beta (TGF-*β*) overproduction[4] have been pointed as possible triggers for the diseases. TGF-*β* is a multifunctional growth factor that exerts pleiotropic effects on wound healing by regulating cell proliferation and migration, differentiation, collagen and proteoglycan synthesis[5].

Hypertrophic scars and keloids can be a significant burden for the affected individual in terms of cosmetic harm, and cause incapacitating symptoms as pruritus, pain and tenderness. In severe cases, they can limit joint movement, produce severe deformities and functional impairment that negatively impact the patient's quality of life[6]. To date, surgical excision remains the mainstay for the treatment of these conditions but recurrence rates of 70–100% are expected when performed in isolation. To reduce this recurrence rate, several adjunct therapies have been described: pressure therapy, CO2 laser ablation, silicone gel, flavonoids, corticosteroids, cryotherapy and radiotherapy. In most instances, effectiveness is controversial and the recurrence rate and adverse effects are high[6]. Targeted therapies for the many key factors crucial to the fibrotic cascade may be the key answer to treat these conditions.

The endocannabinoid system has been described in the early 1990s, when 2 different receptors for the marijuana component, Δ-9-tetrahydrocannabinol, were identified and named cannabinoid 1 receptor (CB1) and cannabinoid 2 receptor (CB2)[7,8]. This system has been involved in a number of physiologic processes including the regulation of neurotransmitter release, pain, energy haemostasis and immune cell control[9]. Endocannabinoids also have a role in fibrotic diseases like liver[10], cardiac[11] or pancreatic fibrosis[12].

The endocannabinoid system has been recently described in the skin: in cutaneous nerve fibers, epidermal keratinocytes and in skin appendages cells[13-19]. They are involved in cell proliferation, survival and differentiation[20], and melanogenesis[21].

Recently, it has been suggested that cannabinoids may behave as profibrotic or antifibrotic agents in skin[22,23]. More detailed, CB1 activation showed to increase inflammation, fibrogenesis and skin fibrosis in bleomycin-treated mice, with an opposite effect when blocking the receptor[23,24]. In this view, the cannabinoid system may be a target to prevent, limit or even reverse tissue damage in pathological fibrosis.

Fibroblasts are key players in the maintenance of skin homeostasis and are the predominant cells during the proliferative and remodelling phases of wound

healing[25]. After differentiation into myofibroblasts, they are responsible for wound contraction and maturation, and collagen production[26]. Although it has been shown in the literature that CB1 is involved in skin inflammation and fibrosis in rodents, there is no evidence of its' role in human wound healing. Therefore, in this study, we have demonstrated that CB1 is involved in fibrogenesis in humans. More specifically we have established that ACEA (a CB1 selective agonist) increases collagen production in a primary culture of adult human fibroblasts and that AM251 (a CB1 selective antagonist) inhibits fibroblast differentiation into myofibroblast and collagen deposition induced by TGF-*β*. Additionally, both ACEA and AM251 were tested in a human *ex vivo* skin culture model supporting these results.

Material and Methods

Primary culture of adult human fibroblasts

The study was approved by the Portuguese Institutional Review Board for Human Subjects and by the Portuguese and Local Hospital Ethical Committee. All patients gave written informed consent to take part in this research.

Abdominal skin samples were obtained from patients submitted to abdominoplasty in the Department of Plastic, Reconstructive and Aesthetic Surgery of Centro Hospitalar de São João. Skin samples from 9 female healthy patients, with a mean age of 37 years old (ranging from 26 to 51years old) were collected. Immediately after surgery, tissue was collected in Dulbecco's Modified Eagle Medium (DMEM) with Fetal Bovine Serum (FBS) 10% and Penicillin-Streptomycin (Pen-strep) 1% and kept at 4ºC for no more than 12h. The skin was then rinsed in DMEM medium with trypsin solution 0.25% and incubated at 4ºC overnight. The dermis was then separated from the epidermis and incubated at 37ºC for 2h, with collagenase 0.1% (5mg Collagenase+5mL DMEM+Pen-strep 1%). DMEM with FBS was added and centrifuged for 5min, at 200g. Cells were then resuspended, rinsed and transferred to 6-wells plates[27]. For all the experiments, fibroblasts were grown to 80% confluence and serum-starved for 24h before treatment. DMEM, antibiotic antimycotic solution, FBS and trypsin were purchased from Gibco (Paisley, UK). ACEA and AM251 were purchased from Tocris Bioscience (Bristol, UK).

Human fibroblasts treatment

Human fibroblasts were seeded in 24-well plates. After 24 hours of serum deprivation, cells were pre-incubated with the CB1 antagonist (AM251 – Batch No: 18B/177059) at time 6 hours and/or the CB1 agonist (ACEA- Batch No: 13A/175908) at time 18 hours. The treatment was maintained until the end of the experiment (5thday). Different conditions were tested: Control (vehicle with Bovine Serum Albumin (BSA) 0.1%); ACEA 1µM; AM251 (1-10µM) and ACEA 1µM + AM251 10µM. At time 48 hours, cells were stimulated with TGF-*β* (10ng/mL) for 72h. **Figure 1** resumes all the steps performed during this task.

Cannabinoid agonists and antagonists were directly applied at a final ethanol concentration <0.01% (v/v). No significant influence of the vehicle was observed on any of the parameters determined.

Cell viability

Cell viability was measured using Trypan blue exclusion method28. Cells were detached with trypsin, cell count was performed by mixing 10µL of the sample with 10µL of 0.4% trypan blue solution, and afterwards, the mixture was loaded into Countess® cell counting chamber slides (Invitrogen, Oregon, USA) and read in the Countess® Automated Cell Counter.

Immunofluorescence staining

Fibroblasts were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) and permeabilized with 0.2% Triton X-100 in PBS for 10min, at room temperature. After blocking, the primary antibody against vimentin (Mouse Ig G1, CloneRV202, BD-Pharmigen, USA) and against *α*-SMA (Mouse monoclonal, Clone1A4, Dako, Denmark) were diluted at 1:100 and 1:200 respectively. Cells were incubated 1h at room temperature, washed with PBS, and then incubated for 1h at room temperature with the secondary antibody (donkey anti-mouse alexafluor546, 1:400, Molecular Probes, USA). Staining for 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) was performed. Slides were examined under ApoTome Axio Z1 imager microscope (ZEISS, Germany), at x10 magnification. The number of cells positive for *α*-SMA was counted.

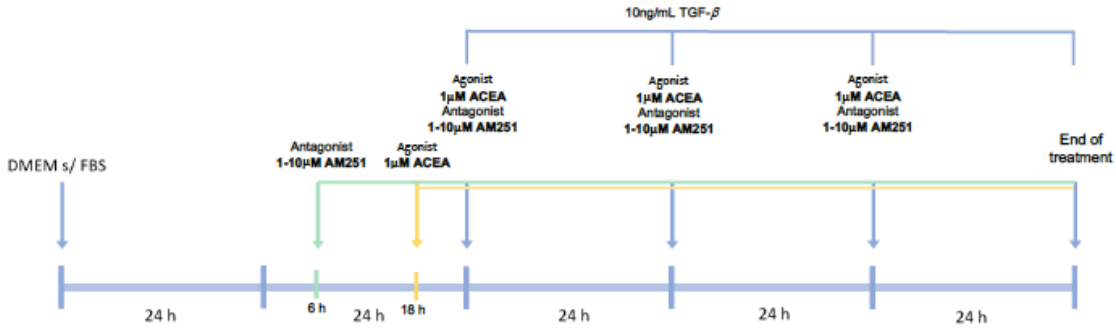

Figure 1. Treatments timeline: After 24 hours of serum privation, cells were pre-incubated with the CB1 antagonist (AM251 - after 6 hours) and/or the CB1 agonist (ACEA after 18h). The treatment was maintained until the end of experiment (5th day). Different conditions were tested: Control (vehicle with Bovine Serum Albumin 0.1%); ACEA 1µM; AM251 (1-10µM) and ACEA 1µM + AM251 10µM. At time 48hours cells were stimulated with TGF-*β* (10ng/mL) for 72h.

Western blot

Fibroblasts were washed with cold PBS and homogenized in Radioimmunoprecipitation assay (RIPA) buffer supplemented with 200mM NaF, 200mM Na3VO4, 10mg/mL leupeptine, 1mg/mL aprotinin, and 100mM Phenylmethylsulfonyl fluoride (PMSF). Protein concentration was determined by the Bradford assay (Bio-Rad, CA, USA) and 12µg of proteins were boiled at 70°C for 10min in Laemmli buffer and electrophoresed in 10% Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS/PAGE gels). Separated proteins were transferred to nitrocellulose membranes (30mA for 1h). Membranes were stained with Ponceau S (Amresco, OH) for 1min and then destained with distilled water to remove non-specific Ponceau staining[29,30]. The total protein content for each sample was quantified using ChemiDoc XRS+ system and Image Lab Software (Bio-Rad, CA, USA). After destaining in TBS-T (Tris-buffered saline with Tween, 0.05M Tris, pH7.4, 0.1M NaCl, 0.05%(v/v) Tween 20) the membranes were blocked in Tris-buffered saline (TBS) solution containing 5%(w/v) non-fat dry milk for 1h at room temperature.

Immunodetection of specific proteins was carried out by incubation with primary antibody against α-SMA (1:200; Mouse monoclonal, Clone1A4, Dako, Denmark), vimentin (1:1000, Mouse Ig G1, CloneRV202, BD-Pharmigen, USA), and CB1 (1:50, mouse monoclonal, Clone2F9, Santa Cruz Biotechnology, USA) overnight at 4°C. After washing membranes, Horseradish Peroxidase (HRP) conjugated secondary antibody was added and incubated for 1h at room temperature and detected by the chemiluminescence system (Clarity Western ECL Substrate, Bio-Rad, CA, USA).

Densitometry analysis of immunoblots was performed using the ChemiDoc XRS+ system and Image Lab Software (Bio-Rad, CA, USA), using the total protein for normalization.

Collagen Quantification

Collagen content was evaluated using the Sirius Red staining assay, performed in 96-well plates[31].

Cell layers were washed twice in PBS before fixation with PFA 4% for 15min. The PFA was removed by suction and the culture plates were washed twice with water. Culture dishes were allowed to air dry before adding the Sirius Red dye. Cells were stained for 1h under mild shaking on a microplate shaker. To remove the non-bound dye, stained cells were washed with acidified water and then dissolved in 0.1N sodium hydroxide for 30min at room temperature using a microplate shaker. Optical density was measured at 550nm against 0.1N sodium hydroxide as blank. Results were expressed as Abs_{550nm}.

Study of Re-Epithelialization

Human skin was collected from routine abdominoplasties and prepared as previously described[32]. Shortly, the skin was disinfected with Betadine and 70% ethanol and then put on the bottom of a nylon mesh cell strainer (BD Biosciences, San Jose, CA, USA) with the epidermis facing up. Four sutures were applied to each piece of skin to fix the skin on the strainer. Then, two different incision wounds were created, cutting completely through the epidermal layer and superficially nicking the dermis. The cell strainer was then placed into a six-well plate. The cell culture medium consisted of DMEM (Mediatech, Herndon, VA, USA) supplemented with 10% FBS (Thermo Scientific HyClone, Logan, UT, USA) and Pen-strep. The skin graft was cultured in the liquid-air interphase and kept in the cell culture incubator at 37°C with 5% CO2. The medium was changed every other day. Different treatments were then applied to the wounded skin grafts (ACEA 10µM, AM251 10µM and respective vehicles) and harvested at day 9 post wounding. Samples were fixed, paraffin-embedded, and cut in semi-serial 3µm-thick sections. The slides were stained with hematoxylin-eosin (HE) and Sirius red (SR).

Statistical analysis

Results are presented as mean±standard error of the mean (SEM) for at least three independent assays with n=6. Significance was assessed by One-way ANOVA. $P<0.05$ was considered significant. Analyses were carried out with Prism7 (Version 7.0; GraphPad Software, Inc.). For IC50 calculation, the parameters of the equation for one site inhibition were fitted to the experimental data[33]. Geometric means are given with 95% confidence limits and arithmetic means are given with SEM. Images were analyzed and quantified using Image J (Version1.52 National Institutes of Health).

Results

ACEA and AM251 do not modify cell viability

Cell viability was not changed by treatments with TGF-β, AM251 and ACEA (Data not shown).

ACEA and AM251 do not modify vimentin expression

Drug treatment did not significantly change vimentin expression in our cell cultures (Data not shown).

TGF-β increases α-SMA

α-SMA expression in fibroblasts culture was evaluated after adding TGF-β at different concentrations (5ng/mL, 10ng/mL and 20ng/mL). There is an increase in the expression of α-SMA when fibroblasts acquire a contractile phenotype. This protein is usually used to detect fibroblast differentiation in their active phenotype[34]. TGF-β produced a concentration-dependent increase in α-SMA expression (20.5±0.5%, 39.5±0.5%, 52.5±0.5%, and 50.5±0.5%, fibroblasts expressing α-SMA in TGF-β treated cells for 0, 5, 10 and 20ng/mL respectively, $p<0.0001$, data not shown). As at higher concentration we found no further effect, we chose 10ng/mL of TGF-β to evaluate the effect of all tested drugs.

AM251 reduces α-SMA

Immunofluorescence staining images of human fibroblasts after treatment with TGF-β 10ng/mL and cannabinoids are shown in **Figure 2a-e**. A red antibody against α-SMA was used and positive cells for α-SMA were counted for every situation. Results are presented in **Figure 2f.**

Immunofluorescence staining revealed a statistically significant increase of α-SMA in cells treated with TGF-β 10ng/mL (8.13%2,46 vs 55.95%±1.80, $p<0.0001$) but no further increase occurred when ACEA 1µM was added (53.37%±4.60, $p=0.94$).

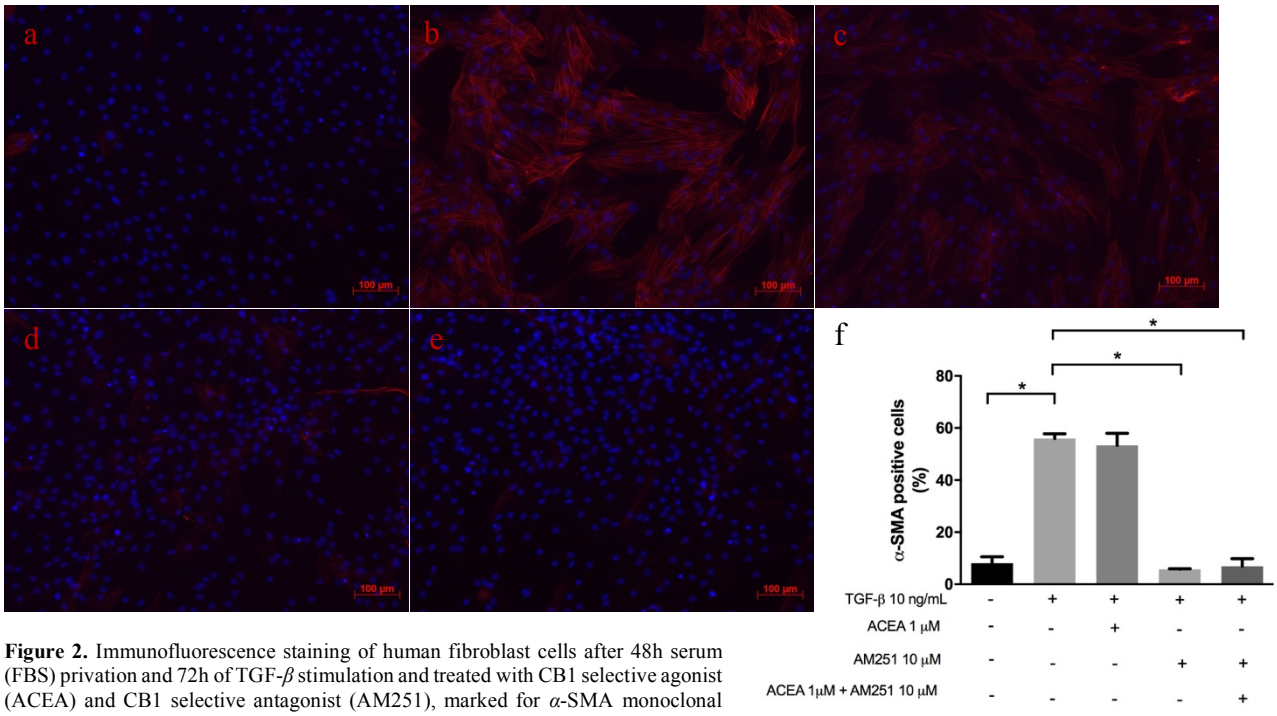

Figure 2. Immunofluorescence staining of human fibroblast cells after 48h serum (FBS) privation and 72h of TGF-β stimulation and treated with CB1 selective agonist (ACEA) and CB1 selective antagonist (AM251), marked for α-SMA monoclonal antibody and DAPI. Results shown are for control cells (a), cells stimulated with TGF-β 10ng/mL (b), cells stimulated with TGF-β 10ng/mL and treated with ACEA 1µM (c), AM251 10µM (d) and ACEA 1µM + AM251 10µM (e). The length of the scale bars is 100µm. The graph shows the percentage of α-SMA positive cells after image analyses (f), *p<0.05.

When cells were treated with the CB1 selective antagonist AM251 10µM, TGF-β stimulation did not increase α-SMA expression (55.95%±1.80 vs 5.72%±0.16, p<0.0001). Similar results were observed in cells treated with the CB1 selective agonist and the CB1 selective antagonist simultaneously (55.95%±1.80 vs 6.88%±2.94, p<0.0001).

In Western Blot analysis (**Figure 3**), we observed that TGF-β at 10ng/mL induces a 24-fold increase in the relative protein expression of α-SMA (1±0.0 vs 24.76±6.43 in optical density, p=0.0145, statistically significant). The CB1 agonist ACEA 1µM, in non-TGF-β activated cells did not increase α-SMA expression compared to control cells (data not shown, 1±0.0vs1.1±0.2, p>0.5). Also, ACEA 1µM did not significantly affect differentiation induced by TGF-β (24.76±6.43 vs 21.43±6.45, p=0.83). However, the TGF-β effect was inhibited in the presence of CB1 agonist ACEA 1µM in association with CB1 selective antagonist AM251 10µM (24.76±6.43 vs 2.03±0.87, p=0.0451, statistically significant). Moreover, CB1 antagonist alone also prevented the TGF-β effect on α-SMA expressions (24.76±6.43 vs 1.74±1.15, p=0.0451, statistically significant). In this context, we evaluated the effect of this drug at concentrations of 1µM, 5µM and 10µM in the fibroblast primary culture activated with TGF-β. AM251 inhibited the TGF-β effect on α-SMA expression in a concentration-dependent manner (**Figure 4**). This effect was significant at concentrations $\geqslant$ 1µM (all p<0.05vsTGF-β), reaching a maximum at the concentration of 5µM.

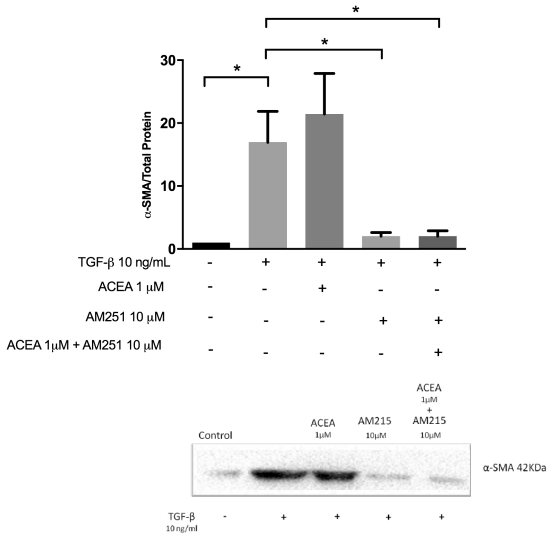

Figure 3. Western blot analysis of α-SMA in human fibroblasts stimulated with TGF-β 10ng/mL and treated with ACEA 1µM, AM251 10µM and ACEA 1µM + AM251 10µM. Results of α-SMA are normalized with total protein and are expressed as relative variation in α-SMA content. Data is expressed in mean±SEM, *p<0.05. Molecular mass markers (MW) are shown on the bottom in kDa. A single band of the expected size (42 kDa) was revealed following incubation with the α-SMA antibody.

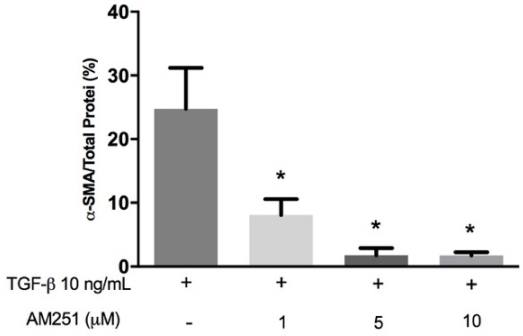

Figure 4. Western blot analysis of α-SMA in human fibroblasts stimulated with TGF-β 10ng/mL and treated with AM251 1μM, 5μM and 10μM. Results of α-SMA are normalized with total protein and are expressed as relative variation in α-SMA content. Results are expressed in mean±SEM, *p<0.05.

ACEA increases and AM251 decreases collagen

Results are presented in **Figure 5a**. Control cells produced 69.77±1.735μg/mL of collagen. Stimulation with TGF-β at 10ng/mL, significantly increased collagen production (82.63±2.177μg/mL, p<0.0001). CB1 selective agonist ACEA 1μM, significantly increased collagen when added to TGF-β stimulated cells (82.63±2.177μg/mL vs 95.62±2.871μg/mL, p=0.0002). On the other hand, CB1 antagonist AM251 1μM significantly decreased collagen deposition either alone or in the presence of CB1 agonist (82.63±2.177μg/mL vs 54.52±1.062μg/mL and 56±2.584μg/mL, p<0.0001, respectively).

AM251 produced a concentration-dependent decrease in collagen deposition with IC50 of 2.9μM (0.4–24.8, 95% CI), shown in **Figure 5b**.

TGF-β increases CB1

TGF-β significantly increased CB1 expression when compared with the control group (1.00±0.00 vs 4.48±0.90, p=0.040; **Figure 6**).

ACEA speeds re-epithelialization

Skin treated with CB1 agonist (ACEA) was totally re-epithelized on day 9, in contrast to CB1 antagonist (AM251) treated skin that still presented a break of the epithelium (**Figure 7**). Furthermore, in both treatments (ACEA or AM251) no evident deleterious effects were observed.

Discussion

We provide the first evidence that cannabinoid CB1 selective antagonist AM251 has an inhibitory effect on fibroblasts differentiation into myofibroblasts and collagen production induced by TGF-β in primary human fibroblasts cultures. Furthermore, we show that this inhibitory effect occurs in a concentration-dependent manner, from 1μM and reaching a maximum at a concentration of 10μM, with an IC50 of 2.9μM.

TGF-β is a well-studied fibrotic cytokine and its role in wound healing has been well characterized[35]. Upon TGF-β stimulation, fibroblasts are activated and undergo a phenotypic transition into myofibroblasts. In healing wounds, myofibroblasts are required for tissue repair; however, in pathologic conditions, activated myofibroblasts become the cellular effectors of fibrosis[34]

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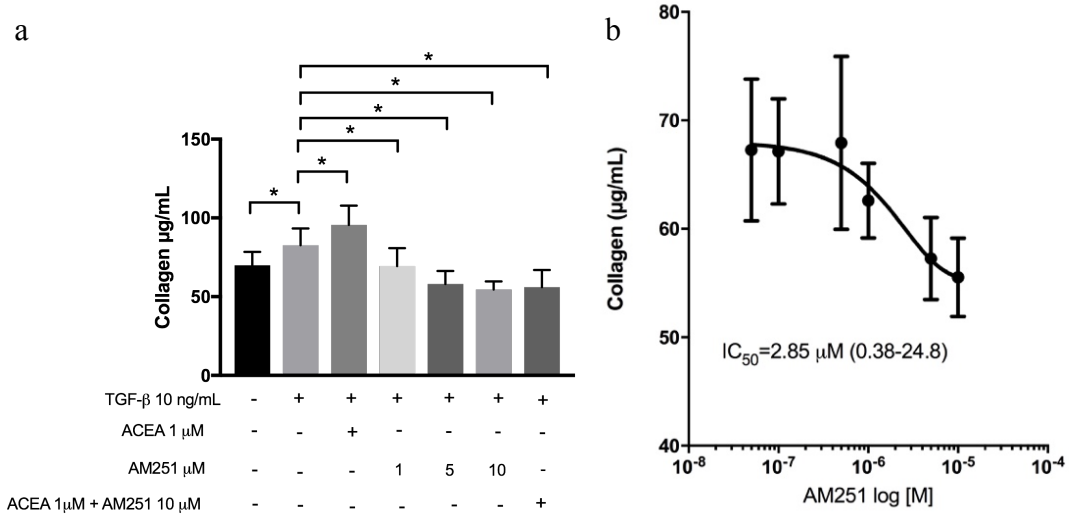

Figure 5. Collagen content in human fibroblasts stimulated with TGF-β 10ng/mL and treated with ACEA 1μM, AM251 1μM, 5μM and 10μM and ACEA 1μM + AM251 10μM. Results are expressed as collagen concentration in μg/mL (a). Data is expressed in mean ± SEM. *p<0.05. Concentration – Response curve for CB1 selective antagonist AM251 0.05μM, 0.1μM, 0.5μM, 1μM, 5μM and 10μM; results are expressed in AM251 log [M] and collagen concentration in μg/mL (b).

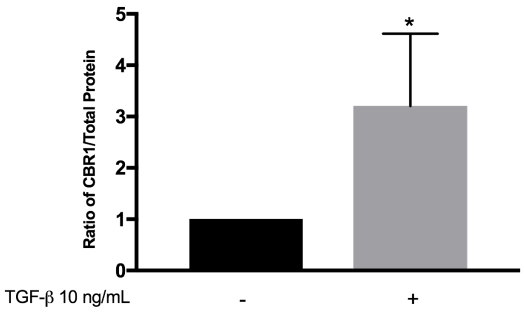

Figure 6. Western blot analysis of CB1 receptor (CBR1) in human fibroblasts stimulated with TGF-β 10ng/mL. Results of α-SMA are normalized with total protein and are expressed as relative variation in α-SMA content. Data is expressed in mean ± SEM, *p<0.05.

There is increasing evidence that the endocannabinoid system may be involved in pathological fibrosis. CB1 inactivation has been shown to decrease wound healing and fibrosis formation in liver[36]. However, there are several differences in the pathogenesis of liver and skin fibrosis: the lack of stellate cells in the skin; the prominent role of epidermal-to-mesenchymal transition in internal organs. The role of the endocannabinoids in the skin has been previously tested in an animal model of Systemic sclerosis (SSc), induced by bleomycin. Subcutaneous injections of bleomycin induce an early inflammatory reaction followed by fibroblast activation and progressive fibrosis that resemble human disease in skin[22,23]. Literature evidence shows that CB2 selective agonists and CB1 selective antagonists significantly decrease subcutis inflammatory cell infiltration on bleomycin-challenged mice and prevents the release of pro-fibrotic mediators. It could be thought that this antifibrotic effect may be due to the anti-inflammatory activity of cannabinoids rather than by direct effect on fibroblasts. Moreover, there is a lack of evidence on the effect of cannabinoids in human skin cells, as much of the existing evidence is obtained from animal models. In our study, we suggest that the inhibition of dermal fibrosis induced by AM251 goes beyond a mere anti-inflammatory activity as this drug showed a direct effect on fibroblast activity. AM251 prevented TGF-β activity over these cells namely, it reduced α-SMA expression and inhibited collagen deposition. Both actions are aligned with the antifibrosis process. Unexpectedly, we did not observe a significant increase in fibroblast differentiation after treatment with CB1 selective agonist ACEA. Two hypotheses can be advanced to explain the effect of the antagonism without an agonist response. As AM251 behave as inverse agonists[37], an effect can be observed in the absence of an agonist. On the other hand, we found that TGF-β at 10ng/mL had a maximum effect on fibroblast differentiation in myofibroblast. Maybe we cannot observe any further stimulating effect in the presence of ACEA, since maximum activation was already present. In fact, a significant increase in collagen deposition after treatment with ACEA was obtained, , which supports this last hypothesis. Although maximum fibroblast activation into myofibroblasts was obtained, more collagen deposition is still observed in ACEA treated cells.

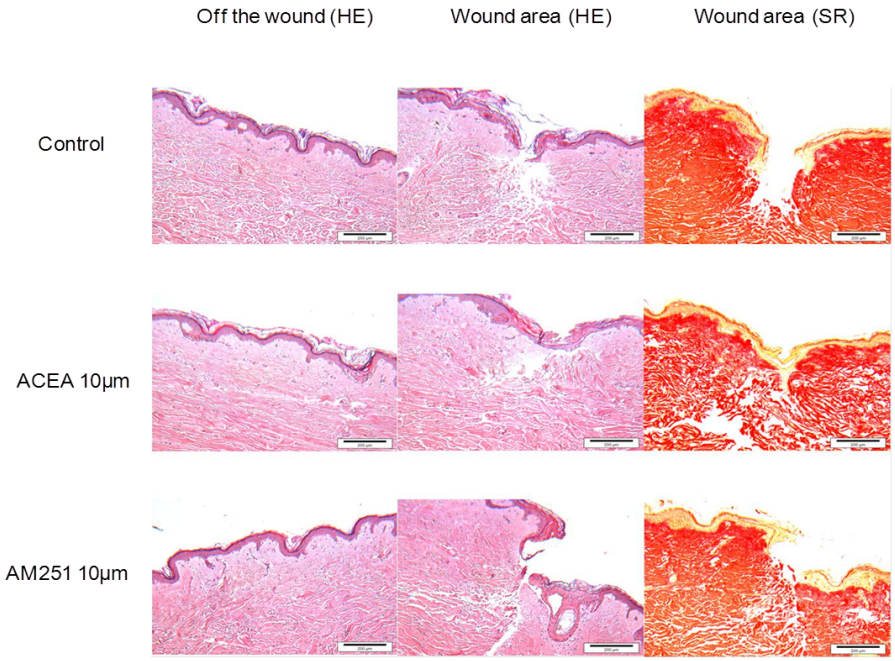

Figure 7. Descriptive histology for human *ex vivo* skin re-epithelialization, 9 days after incisions wounding. Hematoxylin-Eosin (HE) and Sirius Red (SR) staining for control skin and cultured skin with ACEA 10µM and AM251 10µM. The length of the scale bars is 200µ.

Interestingly, WIN55,212-2, a non-selective cannabinoid agonist, was recently reported to decrease the release of collagen in SSc cultured fibroblasts[27]. WIN55,212-2 caused a reduction in extracellular matrix deposition and counteracted several behavioural abnormalities of scleroderma fibroblasts including transdifferentiation into myofibroblasts and resistance to apoptosis. The WIN55,212-2 anti-fibrogenic effect was not reverted by selective cannabinoid antagonists, and authors suggested that other transducing systems may be involved in cannabinoid activity, including inhibition of ERK signaling[27]. Another study involving renal fibrosis also shows the complexity of signal cellular transduction with cannabinoids ligands[38]. The authors described an antifibrotic effect of AM251 with suppression of epithelial-mesenchymal transition that did not involve CB1 or G protein-coupled receptor 55 but acted upstream of SMAD/p38 MAPK in the TGF-β signalling pathway. Moreover, AM251 inhibited the induction of several profibrotic transcription factors such as SNAIL1, and the AP-1 transcription factors FOSB and JUNB. We also can consider the fact that AM251 is known to act as an antagonist/inverse agonist for the cannabinoid receptor type 1 but also can act as an agonist for the G protein-coupled receptor (GRP) 55[37], a common phenome for cannabinoid compounds. Interestingly, GPR55 has been identified as a potential therapeutic target in inflammation[39].

To further investigate the role of CB1 in the wound healing process in human skin, we also tested these compounds in a human *ex vivo* skin culture model. Although this technique is only descriptive, the endpoint (re-epithelization) is clear, and we obtained congruent results: the skin treated with CB1 agonist (ACEA) was totally re-epithelized at day 9, in contrast to CB1 antagonist (AM251) treated skin that still presented a break of the epithelium.

Lastly, TGF-β produced a significant increase in CB1 expression in human fibroblasts.

Our study has some limitations. We only used normal skin from female subjects as a source of human fibroblast. We were not able to collect keloid scars to obtain fibroblasts and test our drugs. We know that keloid fibroblasts phenotype is different and the response of those cells to cannabinoids may be different. Our results cannot oversimplify *in vivo* skin physiology. However, as preliminary data, we could verify that CB1 selective antagonists might represent interesting options to reduce skin fibrosis in disorders characterized by increased fibroblast proliferation activity, as hypertrophic scars and keloids[2]. In the past, rimonabant, a CB1 selective antagonist, was already approved to treat obesity and metabolic syndrome. Due to significant psychiatric adverse effects, it was later withdrawn from the market[40]. Topical administration on the skin will possibly increase tolerance to these drugs but more studies regarding efficacy, local and systemic safety and risk of delayed wounding should be conducted. *In vivo* studies, using animal models of hypertrophic scars and topical CB1 selective antagonist application should be performed to verify this premise and test the tolerance of these compounds.

In conclusion, the present study suggests that AM251 prevents fibroblasts differentiation and collagen deposition induced by TGF-β in human fibroblasts. Inactivation of CB1 by a selective antagonist might be a novel approach for the treatment of skin uncontrolled fibrosis, in pathologic scenarios as hypertrophic scars and keloids. The topical use may be an advantage with these drugs, as the psychotropic effects of the cannabinoids may be avoided.

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Targeting cannabinoid receptor 2 (CB2) limits collagen production – an *in vitro* study in a primary culture of human fibroblasts.

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Abstract

Background: Previous studies showed that cannabinoid 2 receptor (CB2) is involved in skin inflammation, fibrogenesis and re-epithelialization in mice, indicating that this receptor may be implicated in wound healing. Thus, topical use of cannabinoids may have a role in local fibrotic and wound healing diseases as scars or keloids. In this study, we investigate the effect of the CB2 selective agonist JWH133 and the CB2 selective antagonist AM630, on a primary culture of human fibroblasts regarding fibroblast differentiation and activation into myofibroblast, collagen deposition and CB2 expression.

Material and Methods: Primary cultures of adult human fibroblasts were obtained from abdominal human skin samples. Vimentin expression was used to confirm fibroblast culture purity. Human fibroblasts pretreated with JWH133 (3μM) and/or AM630 (10μM) were stimulated with transforming growth factor beta (TGF-*β)* 10ng/mL. Fibroblast activation into myofibroblast was quantified by the expression of alpha-smooth muscle actin (*α*-SMA) using Immunocytochemistry and Western Blot assays. Collagen content was quantified with the Sirius Red staining assay.

Results: When human fibroblasts were stimulated with TGF-*β,* an increase in *α*-SMA and CB2 receptor expression was observed. In these stimulated cells, the CB2 agonist (JWH133) decreased *α*-SMA expression and collagen content. In resting human fibroblasts this effect was not observed. AM630 decreases *α*-SMA expression and collagen content in both resting and stimulated fibroblasts. This effect was time and concentration-dependent, and apparently not mediated by the CB2 receptor.

Conclusion: CB2 is involved in fibroblast fibrotic repair during skin wound healing in humans. JWH133 anti-fibrotic effect is confirmed in human fibroblasts and AM630 also produced a relevant anti-fibrotic effect in these cells, hypothesizing that other cannabinoid receptors, as TRPV, may be involved.

Keywords: Cannabinoid, CB2, fibroblast, myofibroblast, *α*-smooth muscle actin, fibrosis, wound healing, scar

Introduction

The cannabinoids represent a broad class of pharmacologically active chemical compounds that are structurally and biologically similar to the psychoactive compound derived from Cannabis sativa Δ(9)-tetrahydrocannabinol (THC). Although phytocannabinoids (plant extracted cannabinoids), were the first considered, later investigation led to the discovery of endocannabinoids, cannabinoids that are naturally produced in mammalian tissues (such as anandamide (AEA) and 2-arachidonoyl glycerol (2-AG)), as well as a countless number of synthetic cannabinoids[1].

Cannabinoids act by binding to a family of G-protein-coupled receptors, the cannabinoid receptor 1 and 2 (CB1 and CB2), with wide and distinct tissue distribution[2,3], and both present in skin[4]. CB1 and CB2, endocannabinoids and enzymes responsible for their synthesis and catabolism, constitute the endocannabinoid system[1]. This system has been involved in several skin pathophysiological processes like epidermal hemostasis, pain sensation, skin inflammation, melanogenesis and skin appendage regulation[5-7]. Subsequently, cannabinoid products have the potential to treat a variety of skin conditions, including atopic dermatitis, psoriasis, contact dermatitis, pain management, acne vulgaris and skin cancer[8,9]. In fact, in recent years, there has been a growing interest on topical cannabinoid application to treat dermatological disorders[8].

Cannabinoids have also been shown effective in reducing skin and other organs fibrosis in animal models of systemic sclerosis (SSc)[10-14]. More detailed, CB2 activation showed to reduce inflammation, fibrogenesis and promote re-epithelialization on bleomycin-treated mice[15]. This is not entirely surprising, since it is a long time accepted by the scientific community that CB2 activation exerts anti-inflammatory effects in mammals[16]. Skin incisions in mice have also shown dynamic alterations on the expression pattern of CB1[17] and CB2[18] during wound healing in various immune cells and fibroblasts/myofibroblasts. Hence, topical use of cannabinoids may subsist in the future as an interesting approach, not only for this systemic condition, but also for local fibrotic disorders, of which we highlight hypertrophic scars or keloids. Yet, despite promising data in a rodent model, there is a lack of studies on human cells, like human fibroblasts, proving that cannabinoids have a direct effect on fibrogenesis.

Therefore, in this study, we have investigated the effect of a CB2 agonist and the effect of a CB2 antagonist, on a primary culture of human fibroblasts. We specifically evaluated the effect of JWH133 and AM630 on human fibroblast differentiation and activation into myofibroblast, and collagen deposition. CB2 expression on human fibroblast was also quantified before and after activation (induced with transforming growth factor beta (TGF-*β*) treatment).

Material and Methods

Primary culture of adult human fibroblasts

The study was approved by the Portuguese Institutional Review Board for Human Subjects and approved by the Portuguese Ethical Committee and the local Hospital Ethical Committee. All patients gave written informed consent to take part in this research.

Abdominal skin samples were obtained from patients submitted to abdominoplasty surgery in the Department of Plastic, Reconstructive and Aesthetic Surgery of Centro Hospitalar de São João. Skin samples from 9 female healthy patients, with a mean age of 37 years old (ranging from 26 to 51 years old) were collected. Immediately after surgery, tissue was collected in Dulbecco's Modified Eagle Medium (DMEM) with Fetal bovine serum (FBS) 10% and Penicillin-Streptomycin (Pen-strep) 1% and kept at 4ºC for no more than 12h. The skin was then rinsed in DMEM medium with 0.25% trypsin solution 0.25% and incubated at 4ºC overnight. The dermis was then isolated from the epidermis and incubated at 37ºC for 2h, with collagen 0.1% (5mg Collagenase + 5mL DMEM + Pen-strep 1%). DMEM Medium with FBS was added and centrifuged for 5min, at 200g. Cells were then resuspended, rinsed and transferred to a 6cm Petri dish[19]. For all the experiments, fibroblasts were grown to 80% confluence and serum-starved for 24h before treatment.

DMEM, antibiotic antimycotic solution, HEPES, Fetal bovine serum (FBS) and trypsin were purchased from Gibco (Paisley, UK). JWH133 and AM630 were purchased from Tocris Bioscience (Bristol, UK).

Human fibroblasts treatment

Human fibroblasts were seeded in 24-well plates for 48h. 24h before the experiment, cells were serum-deprived. When CB2 antagonist was added, it was present 6h before the agonist. The treatment was maintained with new solutions every day, until the end of the experiment (5thday). Different conditions were tested: Control (vehicle with Bovine Serum Albumin (BSA) 0.1%); JWH133 (3µM); AM630 (10µM) and JWH133 (3µM) + AM630 (10µM). At time 48h, cells were stimulated with TGF-β (10ng/mL) for 72h (**Figure 1**). These experimental conditions for TGF-β were chosen according to previous results with different time and concentration conditions (data not shown).

Cannabinoid agonists and antagonists were directly applied at a final ethanol concentration <0.01% (v/v). No significant influence of the vehicle was observed on any of the parameters determined.

Cell viability

Cell viability was measured using the *Trypan blue* exclusion method. Cells were detached with trypsin, cell count was performed by mixing 10µL of the sample with 10µL of 0.4% trypan blue solution, afterwards, the mixture was loaded into Countess® cell counting chamber slides (Invitrogen, Oregon, USA) and read in the Countess® Automated Cell Counter.

Immunofluorescence staining

Fibroblasts were fixed in 4% paraformaldehyde (PFA) in phosphate buffer saline (PBS) and permeabilized with 0.2%Triton X-100 in PBS for 10min, at room temperature. After blocking, the primary antibody against vimentin (Mouse IgG1, Clone RV202, BD-Pharmigen, USA) and against Alpha-smooth muscle actin (α-SMA; Mouse monoclonal, Clone 1A4, Dako, Denmark) were diluted at 1:100 and 1:200 respectively. Cells were incubated 1h at room temperature, washed with PBS and then incubated for 1h at room temperature with the secondary antibody (donkey anti-mouse alexafluor 546, 1:400, Molecular Probes, USA). Staining for 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) was performed. Slides were examined under ApoTome Axio Z1 imager microscope (ZEISS, Germany) and α-SMA positive cells were counted.

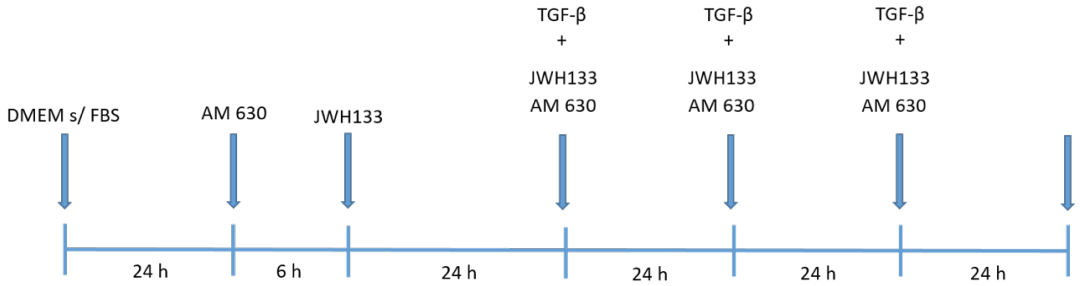

Figure 1. Treatments timeline: After 24 hours of serum privation, cells were pre-incubated with the CB2 antagonist (AM630 - after 6h) and/or the CB1 agonist (JWH133 - after 18h). The treatment was maintained until the end of the experiment (5th day). Different conditions were tested: Control (vehicle with Bovine Serum Albumin 0.1%); JWH133 (3µM); AM630 (1-10µM) and JWH133 (3µM) + AM630 (10µM). At time 48h cells were stimulated with TGF-β (10ng/mL) for 72h.

Western blot

Fibroblasts were washed with cold PBS and homogenized in Radioimmunoprecipitation assay (RIPA) buffer supplemented with 200mM NaF, 200mM Na_3VO_4, 10mg/mL leupeptine, 1mg/mL aprotinin, and 100mM Phenylmethylsulfonyl fluoride (PMSF). Protein concentration was determined by the Bradford assay (Bio-Rad, CA, USA) and 12µg of proteins were boiled at 70°C for 10min in Laemmli buffer and electrophoresed in 10% Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS/PAGE gels). Separated proteins were transferred to nitrocellulose membranes (30mA for 1h). Membranes were stained with Ponceau S (Amresco, OH) for 1min and then destained with distilled water to remove non-specific Ponceau staining. The total protein content for each sample was quantified using ChemiDoc XRS+ system and Image Lab Software (Bio-Rad, CA, USA). After distaining in TBS-T (Tris-buffered saline with Tween, 0.05M Tris, pH 7.4, 0.1M NaCl, 0.05% (v/v) Tween 20) the membranes were blocked in Tris-buffered saline (TBS) solution containing 5% (w/v) non-fat dry milk for 1h at room temperature.

Immunodetection of specific proteins was carried out by incubation with primary antibody against *α*-SMA (1:200; Mouse monoclonal, Clone 1A4, Dako, Denmark), vimentin (1:100, Mouse Ig G1, Clone RV202, BD-Pharmigen, USA) and CB2 (1:50, mouse monoclonal, Clone 3C7, Santa Cruz Biotechnology, USA) overnight at 4°C. After washing membranes, Horseradish Peroxidase (HRP) conjugated secondary antibody was added and incubated for 1h at room temperature and detected by the chemiluminescence system (Clarity Western ECL Substrate, Bio-Rad, CA, USA). Densitometry analysis of immunoblots was performed using the ChemiDoc XRS+ system and Image Lab Software (Bio-Rad, CA, USA).

Collagen Quantification

Collagen content was evaluated using the Sirius Red staining assay, performed in 96-well plates[20]. Briefly, cell layers were washed twice in PBS before fixation with PFA 4% for 15min. The PFA was removed by suction and the culture plates were washed twice with water. Culture dishes were allowed to air dry before adding the Sirius Red dye. Cells were stained for 1h under mild shaking on a microplate shaker. To remove the non-bound dye, stained cells were washed with acidified water and then dissolved in 0.1N sodium hydroxide for 30min at room temperature using a microplate shaker. Optical density was measured at 550nm against 0.1N sodium hydroxide as blank. Results were expressed as Abs_{550}.

Statistical analysis

Results are presented as mean± standard error of the mean (SEM) for at least three independent assays. Significance was assessed by One-way ANOVA. $P<0.05$ was considered significant. All analyses were carried out with the Prism 7 (Version 7.0; GraphPad Software, Inc.)

Results

Cell viability

Cell viability was not changed by TGF-*β*, JWH133 or AM630 in all concentrations used (data not shown).

Immunofluorescence staining for Vimentin

The cultured fibroblasts purity was confirmed using immunofluorescence staining with an antibody against vimentin. Our cultures were marked, as described, with vimentin monoclonal antibody (in green) and DAPI (in blue; **Figure 2**). JWH133 (3µM) and AM630 (10µM) did not significantly change vimentin expression in our cell cultures (data not shown).

Effect of CB2 ligands on the α-SMA expression

Expression of *α*-SMA after treatment with JWH133 (3µM) and AM630 (10µM) was firstly evaluated using immunofluorescence staining. Quantitative results are given in **Figure 3f** and respective representative images in **Figure 3**. An increase in *α*-SMA expression (red) was observed in fibroblasts treated with TGF-*β* 10ng/mL, when compared to non-treated cells (**Figure 3b** and **3a** respectively, 59.25%±1.59 *vs* 6.38%±1.71, $p<0.0001$). When cells were treated with JWH133, the TGF-*β* effect on *α*-SMA expression was decreased by half (**Figure 3c**, 25.47%±4.49, $p<0.0001$). The TGF-*β* effect was also significantly reduced by AM630 (**Figure 3d**, 28.81%±5.725, $p=0.0003$) and when both JWH133 and AM630 were presented (**Figure 3e**, 39.06% ±13.22, $p=0.0179$).

Results obtained by immunodetection for *α*-SMA using Western Blot (**Figure 4**) presented a similar profile, namely TGF-*β* at 10ng/mL increased *α*-SMA expression, (1±0.0 *vs* 73.12±21.65, in optical density), JWH133 (3µM) decreased *α*-SMA expression in TGF-*β* treated cells (47.56±12.48) and AM630 (10µM) used in the absence and the presence of the selective agonist also decreased *α*-SMA expression (54.24±9.037 and 49.31±15.91 respectively).

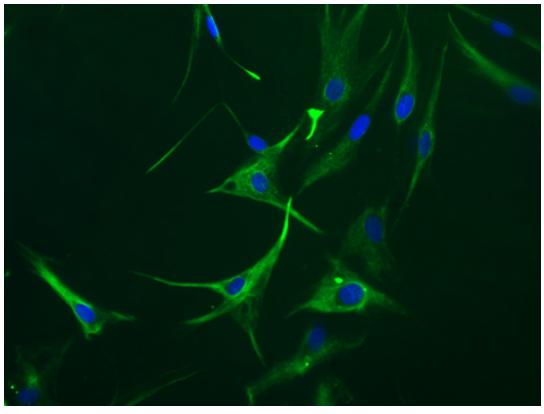

Figure 2. Immunofluorescence staining of human fibroblast cells after 48h serum (FBS) privation, marked for vimentin monoclonal antibody (stained in green) and DAPI (stained in blue). The result shown is for control cells.

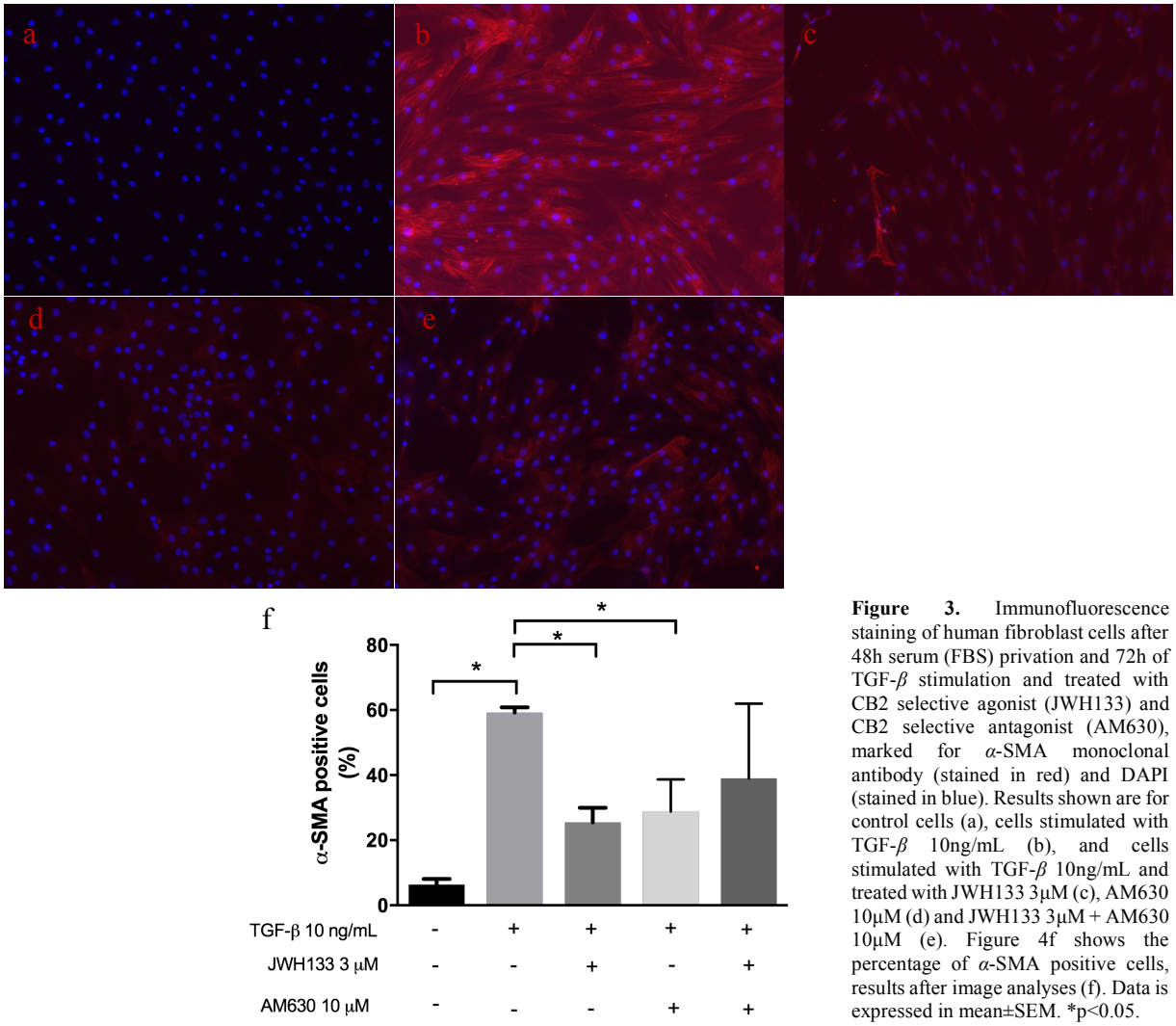

Figure 3. Immunofluorescence staining of human fibroblast cells after 48h serum (FBS) privation and 72h of TGF-*β* stimulation and treated with CB2 selective agonist (JWH133) and CB2 selective antagonist (AM630), marked for *α*-SMA monoclonal antibody (stained in red) and DAPI (stained in blue). Results shown are for control cells (a), cells stimulated with TGF-*β* 10ng/mL (b), and cells stimulated with TGF-*β* 10ng/mL and treated with JWH133 3μM (c), AM630 10μM (d) and JWH133 3μM + AM630 10μM (e). Figure 4f shows the percentage of *α*-SMA positive cells, results after image analyses (f). Data is expressed in mean±SEM. *p<0.05.

Effect of CB2 agonist and antagonist on Collagen

The content of collagen was evaluated in our cultures using the Sirius red assay. Sirius Red is an anionic dye that stains collagen. After morphological analysis of the deposited material, the stain was dissolved and its optical density determined spectrophotometrically using a microtiter plate assay system. This method has been applied to the measurement of collagen content produced by our cultured and treated cells.

Results are presented in **Figure 5**. Control cells produced 67.26±2.119μg/mL of collagen. Stimulation with TGF-*β*, significantly increased collagen production in our human fibroblast cultures (80.08±2.63μg/mL, p<0.0005).

JWH133 (3μM), significantly decreased collagen content when added to TGF-*β* stimulated cells (80.08±2.63μg/mL *vs* 70.58±4.40μg/mL, p=0.001). AM630 (10μM) significantly decreased collagen deposition both alone or in the presence of JWH133 (80.08±2.63μg/mL *vs* 50.71±1.61μg/mL and 56.36±2.29μg/mL, p<0.0001, respectively).

Finally, to assess the role of CB2 in collagen production in non-stimulated human fibroblasts, we tested JWH133 and AM630 at different concentrations (ranging from 1μM to 100μM) in the absence of TGF-*β*. Time-course experiments showed that the effect of AM630 on collagen content is detectable after 24h, it reaches significance after 48h, and decreases after 4 and 5 days of treatment (**Figure 6a-d**). For collagen production experiments, we shortened our treatments to 48h. AM630 produced a concentration-dependent decrease in collagen deposition with IC_{50} of 11.0μM (4.4–27.6, 95%CI; **Figure 7**). This effect was significant at concentrations ≥3μM, reaching a maximum at the concentration of 100μM. In contrast, JWH-133 at 1μM to 100μM (also in the absence of TGF-*β)* did not significantly change collagen content after 48h of treatment in our human fibroblast cultures (**Figure 6e**).

Effect of TGF-β on CB2 expression

Western blotting was preformed to detect CB2 expression on control and TGF-*β* treated cells. Results are presented in **Figure 8**. TGF-*β* significantly increased CB2 expression in human fibroblast culture cells when compared with control group (1.00±0.00 *vs* 3.68±0.81, p=0.0327). JWH133 (3μM), AM630 (10μM) alone or combined did not significantly changed CB2 expression in TGF-*β* treated cells (data not shown).

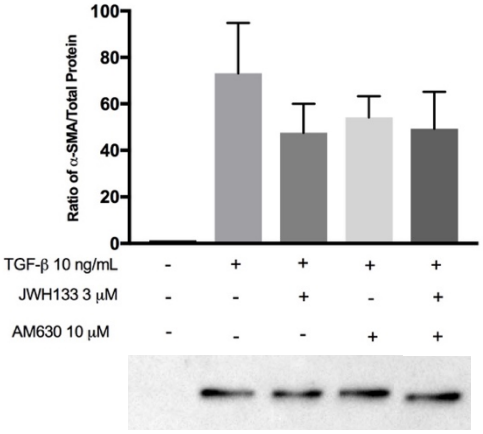

Figure 4. Western blot analysis of α-SMA in human fibroblasts stimulated with TGF-β 10ng/mL and treated with JWH133 3μM, AM630 10μM and JWH133 3μM + AM630 10μM. Results are expressed as variation in α-SMA content, over the total protein content. Data is expressed in mean±SEM. *p<0.05. Molecular mass markers (MW) are shown on the bottom in kDa. A single band of the expected size (42 kDa) was revealed following incubation with the α-SMA antibody.

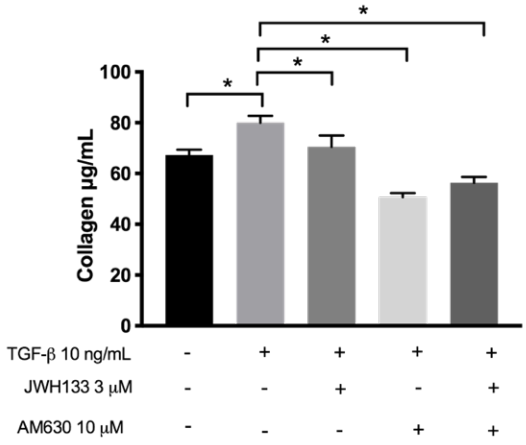

Figure 5. Collagen content in human fibroblasts stimulated with TGF-β 10ng/mL and treated with JWH133 3μM, AM630 10μM and JWH133 3μM + AM630 10μM. Results are expressed as collagen concentration in μg/mL. Data is expressed in mean±SEM. *p<0.05

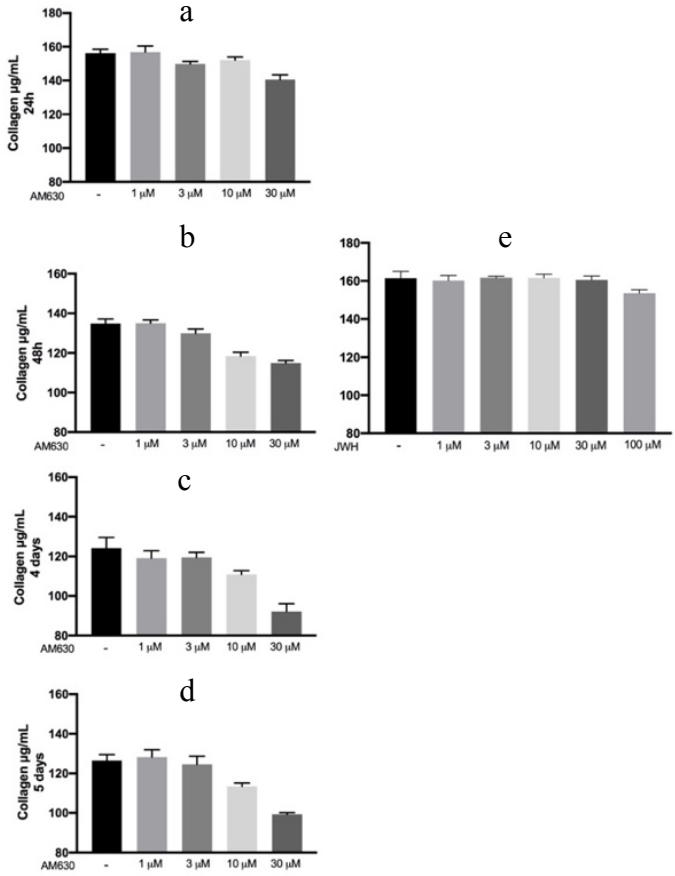

Figure 6. Time-course results for collagen content in human fibroblasts non-stimulated and treated with AM630 (1μM, 3μM, 10μM and 30μM) after 24h (a), 48h (b), 4 days (c) and 5 days (d) and treated with JWH133 1μM, 3μM, 10μM, 30μM and 100μM after 48h (e). Results are expressed as collagen concentration in μg/mL

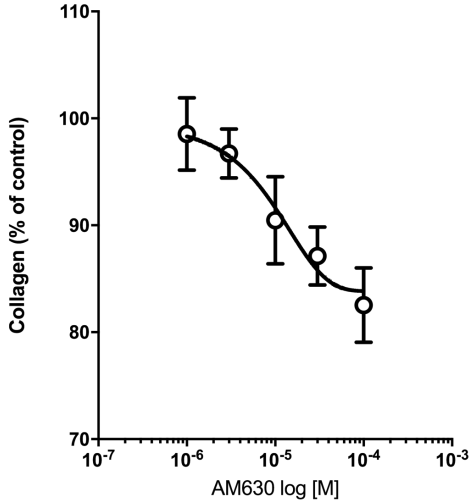

Figure 7. Concentration – Response curve for CB2 selective antagonist AM630 1μM, 3μM, 10μM, 30μM, 100μM; results are expressed in AM630 log [M] and collagen concentration in percentage of control.

Discussion

The present study proves that CB2 is involved in human fibroblast differentiation and collagen production. We report, for the first time in literature, that the receptor expression increases in TGF-*β* stimulated fibroblasts. CB2 activation with JWH133 reduces *α*-SMA expression and collagen production, in human fibroblasts under stimulation with TGF-*β* but this effect is not observed in resting fibroblasts. Unexpectedly, AM630 reduces collagen production in TGF-*β* treated and non-treated cells. The AM630 effect is concentration-dependent.

CB2 ligands have been previously investigated as a potential target for skin fibrotic diseases and wound healing. Most authors show that CB2 agonists decrease inflammation and leukocyte infiltration, followed by a reduction in skin fibrosis[10,12-15,21-23]. Among published data, we only found three studies[10,15,23] testing the CB2 antagonist effect, showing that AM630 increases inflammation and skin fibrosis. It is important to point out that, most of the available data is obtained from experiments with mice, and there is only limited data regarding cannabinoid effect on human cells. Further, most of them approach animal models of SSc. SSc is a systemic inflammatory disease of the connective tissue characterized by immunological, vascular, and fibrotic abnormalities in skin and other organs[24]. Inflammation is critical on the pathophysiology of this disease, and CB2 has shown to have immune-suppressive action[25] and to be widely expressed on a number of immune cells[26]. Yet, although inflammation is a determinant step on wound healing, there are far more than inflammation that can be targeted[27].

Fibroblasts are predominant cells during the proliferative and remodeling phases of wound healing. They acquire a contractile phenotype and transform into myofibroblasts, a cell type that expresses *α*-SMA and that plays a major role in wound contraction. Fibroblasts are also responsible for depositing extracellular matrix composed of high concentrations of collagen. This activation is strongly induced by TGF-*β*[28].

Corroborating earlier findings, we observed that JWH133 reduces *α*-SMA expression and collagen content on human fibroblasts treated with TGF-*β*. Interestingly, this effect was not observed in non-stimulated cells. Since TGF-*β* showed to increase CB2 expression in our cells, it is clear that JWH133 effect only occurs in stimulated cells, with higher CB2 density, and that this effect is CB2 dependent. In our experiment, however, AM630 showed to reduce *α*-SMA expression and collagen. This effect on collagen was similarly observed in TGF-*β* treated and non-treated cells. Briefly, collagen was reduced in about 20μg/mL in both situations. It appears then, that this is effect is not entirely CB2 receptor-dependent and that other non-classical transducing pathways, like TRP channels, may be involved. We point two additional findings supporting that other non-CB2 pathways are involved in AM630 response: a) JWH133 and AM630, CB2 agonist and antagonist respectively, did not exhibit opposite effects and b) AM630 did not block the JWH133 effect when those drugs were added together. AM630 has been shown to activate TRPA1 when the channel is co-expressed with TRPV1[29] and to weakly activate CB1[30]. Interestingly, TRPA1 has been identified as a potential therapeutic target in renal fibrosis via inhibition of TGF-*β*/Smad signalling[31]. In a 2009 research, Garcia-Gonzales *et al.*[19] showed that WIN55,212-2, a non-selective CB1 and CB2 agonist, reduced collagen production in SSc and healthy fibroblast cultures and fibroblasts transdifferentiation into myofibroblasts. This effect was also not reverted by the selective cannabinoid antagonists, and the authors also pointed out that other transducing systems are involved in fibroblast cannabinoid response, including inhibition of ERK signaling. In 2012, Enea Lazzerini *et al.*[32] showed that incubation of SSc fibroblasts with WIN55,212-2 1μM and the selective A2A receptor antagonist ZM-241385 reduced collagen production. This effect on collagen reduction was enhanced at higher AM630 concentrations (80–20μM), unaffected at intermediate concentrations (10μM–5nM), and reduced at lower concentrations (1–0.1nM). The authors fail to explain this outcome but other transduction pathways seem to be involved.

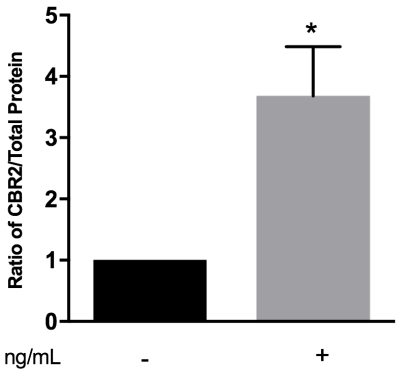

Figure 8. Western blot analysis of CB2 receptor (CBR2) in human fibroblasts stimulated with TGF-*β* 10ng/mL Results are expressed as variation in CBR2 content, over the total protein content. Data is expressed in mean±SEM. *p<0.05.

We point two limitations to our study. First, we only used normal human fibroblasts from female patients in our study. We were not able to collect keloid scars to test our drugs. We know that keloid fibroblasts phenotype is different and the response of those cells to CB2 selective agonist or antagonists may be different. In the future, it would be interesting to test if there is a different behavior of those cells. Second, wound healing is a complex series of reactions and interactions among cytokines, growth factors, inflammatory and mesenchymal cells that cannot be resumed in fibroblast differentiation and collagen deposition[35]. We cannot extrapolate our results to skin wound healing as other cells and mechanisms are also involved[27]. However, as preliminary data, JWH133 and AM630 can be pointed out as interesting drugs to treat wound healing disorders, even on later stages of healing (on proliferative and remodeling phases).

In conclusion, CB2 is involved in fibroblast fibrotic repair during skin wound healing in humans. JWH133 anti-fibrotic effect is confirmed in human fibroblasts and AM630 also produced a relevant anti-fibrotic effect in these cells, hypothesizing that other cannabinoid receptors, as TRPV, may be involved. CB2 might be an interesting molecular target for the treatment of hypertrophic scars or keloids.

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Chapter II
Cannabinoids and inflammation in humans during wound healing

Hypertrophic Scars: Are Vitamins and Inflammatory Biomarkers Related with the Pathophysiology of Wound Healing?
Obes Surg 2017 Dec;27(12):3170-3178

A new role for anandamide: defective link between systemic and skin endocannabinoid systems in hypertrophic human wound healing.
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ORIGINAL CONTRIBUTIONS

Hypertrophic Scars: Are Vitamins and Inflammatory Biomarkers Related with the Pathophysiology of Wound Healing?

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Abstract

Background Hypertrophic scars are a consequence of wound healing.

Objective The objective of the present study is to evaluate vitamin D and inflammatory biomarker plasma levels during wound healing.

Methods A prospective study was performed in patients ($n = 63$) submitted to body contouring surgery. Blood samples were collected before (t_0) and 5 days after surgery (t_5). Blood cell count, protein inflammatory biomarkers, and circulating plasma levels of 25(OH)D, vitamin A and vitamin E were quantified. Six months after surgery, scars were evaluated and classified as normal or hypertrophic.

Results At the end of the study, 73% of the patients developed a normal scar (control group, $n = 46$) and 27% of the patients presented hypertrophic scars (HT group, $n = 17$). The patients in the HT group presented higher eosinophil (0.145×10^9 /L vs. 0.104×10^9 /L, $p = 0.028$) and basophil count (0.031×10^9 /L vs. 0.22×10^9 /L, $p = 0.049$) and C-reactive protein levels (6.12 mg/L vs. 2.30 mg/L, $p = 0.015$) in t_0 than the patients in the control group. At t_5, the patients in the HT group showed a decrease in neutrophil ($3.144 \times 10^{9/L}$ vs. 4.03×10^9/L, $p = 0.031$) and an increase in basophil (0.024×10^9/L vs. 0.015×10^9/L, $p = 0.005$) and lymphocyte count (1.836×10^9 /L vs. 1.557×10^9/L; $p = 0.028$). Before surgery, vitamin D plasma levels were found to be decreased by almost 50% (23.52 ng/mL vs. 15.46 ng/mL, $p = 0.031$) in the patients who developed hypertrophic scars. Thirty-one percent of the patients submitted to bariatric surgery had more hypertrophic scars, versus 24% of the patients with no previous bariatric surgery.

Conclusion There is a different systemic inflammatory profile response in the patients during the formation of hypertrophic scars. Vitamin D plasma levels are marked reduced in these patients. Considering the powerful anti-inflammatory effect of vitamin D, these findings could be related.

Keywords Vitamin D · Wound healing · Scar · Hypertrophic · Inflammation · Bariatric

Introduction

Hypertrophic scars and keloids represent common undesirable consequences of wound healing, characterized by pathologically excessive dermal fibrosis and aberrant scaring. It is thought to be caused by increased or decreased regulation of cellularity during the wound healing in predisposed individuals, but the exact pathogenesis and etiology are still undetermined [1]. Blood type A; hyper-immunoglobulin (Ig) E syndrome (high allergy risk); hormone peaks (puberty, pregnancy); age 10–30 years old; and Hispanic, Afro-American, or Asian ethnicities [2]; and delayed reepithelialization [3–6] due to deep wounding [7] or wound infection background have all been associated with high risk of developing these disorders [5, 7]. Pathophysiology is still complex, with both genetic and environmental factors being involved. Increased fibroblast density and extracellular matrix substances hold activated keratinocytes and myofibroblasts, and a robust inflammatory

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mechanism has been shown to play a key role [6, 8–10]. It is believed that a prolonged inflammatory phase is an important prerequisite for this disorder, with decreased apoptosis and increased inflammation [11, 12].

Vitamin D is a fat-soluble nutrient that humans obtain via the diet and by synthesis in the skin upon exposure to ultraviolet B light [13]. A photochemical reaction with maximum spectral effectiveness at about 297 nm results in the generation of previtamin D3 from 7-dehydrocholesterol in basal and suprabasal layers of the skin [14]. Previtamin D3 is isomerized to vitamin D, and it is then hydroxylated in the liver to hydroxyvitamin D (25(OH)D) and further hydroxylated in the kidney to calcitriol (1,25-dihydroxyvitamin D3 (1,25(OH)2D3)), which is the active hormone involved in calcium absorption in the gut [15].

The critical importance of the skin for the human body's vitamin D endocrine system is not only because vitamin D is firstly synthetized in this tissue, but also because skin is a target of vitamin D action [16]. This has been clearly established by the presence of the vitamin D receptors in keratinocytes, fibroblasts, sebocytes, and dermal papilla cells [17–19]. Calcitriol exerts its biological effects both via non-genomic and genomic mechanisms and induces differentiation and suppresses the growth of keratinocytes and other cell types [20]. Furthermore vitamin D also has a role as an anti-inflammatory mediator, via cytokine expression. It affects inflammatory processes in the skin by altering the synthesis and signaling of several growth factors/cytokines [21]. 1,25(OH)2D3 inhibits the activation of T cells and induces the generation of CD25+/CD4+ regulatory T cells [22]. It also decreases the binding of NF-κB on IL-6 and IL-8 promoter genes in the human fibroblasts, thus decreasing the transcription of these cytokines. With the reduction of these proteins, specifically IL-6, inflammatory responses are attenuated [23]. Inoue et al. [24] have shown that 1,25(OH)2D3 exhibited anti-inflammatory effects through its downregulation of inflammatory cytokines IL-1, IL-6, and IL-8 in keratinocytes stimulated by TNF-α and IFN-γ. 1,25(OH)2D3 has also been showed to increase pivot mediators in wound healing such as production of PDGF [25] and cathelicidin anti-microbial proteins (hCAP18) [26, 27].

Consequently, vitamin D analogues have been proved to be effective drugs in the treatment of hyperproliferative skin diseases as psoriasis [28, 29] and a promising drug for the prevention and treatment of inflammatory skin diseases including atopic dermatitis and allergic contact dermatitis [30].

Although there is an apparent pivotal role of the vitamin D in skin homeostasis as an anti-proliferative and anti-inflammatory agent that may relate this hormone with wound healing, little is publish in literature regarding this topic.

Vitamin D has been proved to be deficient in a general population. Up to 35–80% of children and adults in undeveloped and developed countries are vitamin D deficient, even in those sunniest countries of the world such as Portugal, placing this deficiency and its consequences as a major health problem [31–35].

Bariatric surgery is the successful treatment for obesity in individuals who failed medical weight loss. Although significant improvement is seen with weight loss along with a decrease in comorbid conditions, it is known that all bariatric procedures are capable of producing significant nutritional and metabolic abnormalities. It includes fat-soluble vitamin deficiencies, such as vitamins A, D, E, and K [36].

Based on this, the aim of this study is to evaluate vitamin D and inflammatory biomarker plasma levels during wound healing, in patients submitted to elective surgery for body contouring. The impact of bariatric surgery in wound healing and vitamin status was also evaluated.

Materials and Methods

A prospective hospital-based study was performed. In total, 63 patients submitted to routine body contouring surgery in the Department of Plastic and Reconstructive and Aesthetic Surgery of Centro Hospitalar de São João were included. The study was approved by the Portuguese Institutional Review Board for Human Subjects and carried out in accordance with principles of the Declaration of Helsinki as revised in 2001. All patients gave written informed consent to take part in this research.

Prior to surgery, the patients were asked to answer a questionnaire regarding demographic data, medication use, smoking, alcohol and drug habits, medical history, weight, height, and other surgeries performed (including bariatric surgery).

Blood samples were collected before surgery (t_0) and 5 days after surgery (t_5) corresponding to the inflammatory phase of wound healing. Both blood samples were taken in the morning, in a fasting state, and immediately sent to the laboratory for blood cell count and C-reactive protein (CRP) quantification. Circulating serum levels of vitamin D (25(OH)D), vitamin A, and vitamin E were determined in the sample collected before surgery (t_0).

Serum vitamins A and E were measured by high-performance liquid chromatography; serum vitamin D was measured by electrochemiluminescence.

Patients submitted to another surgery 1 year before this procedure, under immunosuppressive therapy or with postoperative complications, were excluded.

Six months after surgery, the patients were asked to attend a consultation to be further independent evaluated by two trained plastic surgeons to decrease the subjectivity of this evaluation. Scars were evaluated and classified as normal when they did not raised or grow beyond the boundaries of the original wound or hypertrophic when elevated or exceeded

the boundaries of the original wound. Patients were divided into control group and hypertrophic group (HT group) according to scar classification.

Results are presented as mean ± SEM. The significance of differences between the means was assessed by Student's paired *t* test when comparing the same group of patients before and after surgery and Student's unpaired *t* test when comparing independent groups. Categorical data was analyzed using Fisher's exact test. Two-way ANOVA was used to analyze the influence of two different categorical independent variables. $p < 0.05$ was considered significant. All analyses were carried out with the Prism 7 (Version 7.0; GraphPad Software, Inc.).

Results

The baseline characteristics of subjects in normal and hypertrophic groups are listed in Table 1.

In the end of the study, 73% of the patients developed a normal scar (control group, $n = 46$) and 27% of the patients presented hypertrophic scars (HT group, $n = 17$). All patients submitted to surgery were female. The mean age of the patients was 43.67 years (20–65). Mean body mass index (BMI) was 27.39 ± 3.42. There was no significant difference between the ages and BMI in both groups: control and HT group. Fifteen (23.80%) patients reported smoking habits, but none reported drug or alcohol abuse.

Blood Cell Count and CRP

There were no significant differences in erythrocyte count ([N vs. HT] t_0 4.55×10^{12}/L vs. 4.39×10^{12}/L; t_5 3.56×10^{12}/L vs. 3.77×10^{12}/L), hemoglobin levels ([N vs. HT] t_0 13.17 g/100 mL vs. 12.76 g/100 mL; t_5 10.33 g/100 mL vs. 10.84 g/100 mL), hematocrit ([N vs. HT] t_0 39.51% vs. 38.51%; t_5 30.93% vs. 32.78%), mean cell volume (MCV; [N vs. HT] t_0 86.98 μ^3 vs. 87.94 μ^3; t_5 85.85 μ^3 vs. 83.94 μ^3), or platelet cell count ([N vs. HT] t_0 242.10×10^9/L vs. 232.50×10^9/L; t_5 220.70×10^9/L vs. 226.10×10^9/L) between groups before or after surgery (data not shown).

Patients in the HT group presented lower neutrophil (3.008×10^9/L vs. 3.830008×10^9/L, $p = 0.040$) and higher eosinophil (0.145×10^9/L vs. 0.104×10^9/L, $p = 0.028$) and basophil count (0.031×10^9/L vs. 0.22×10^9/L, $p = 0.049$) and CRP levels (6.12 mg/L vs. 2.30 mg/L, $p = 0.015$) at t_0 compared with the control group. Although without significance, leucocyte (5.55×10^9/L vs. 6.43×10^9/L) showed to be lower and monocytes (0.516×10^9/L vs. 0.453×10^9/L) higher in the HT group when compared to the control group, before the surgical procedure (Fig. 1).

At t_5 (corresponding to the inflammatory phase of wound healing), patients in the HT group showed a decrease in neutrophil count (3.144×10^9/L vs. 4.03×10^9/L, $p = 0.031$) and an increase in basophil (0.024×10^9/L vs. 0.015×10^9/L, $p = 0.005$) and lymphocyte count (1.836×10^9/L vs. 1.557×10^9/L; $p = 0.028$) compared with patients with normal scars. Although without significance, leucocytes (5.84×10^9/L vs. 6.41×10^9/L) showed to be lower and eosinophils (0.205×10^9/L vs. 0.184×10^9/L) higher in the HT group in than the control group, 5 days after surgery (Fig. 1).

Furthermore, after surgery, an increase in eosinophils (N group, t_0 0.104×10^9/L vs. t_5 0.184×10^9/L, $p = 0.0002$; HT group, t_0 0.145×10^9/L vs. t_5 0.205×10^9/L, $p = 0.0003$) in both groups and a decrease in basophils (t_0 0.002×10^9/L vs. t_5 0.015×10^9/L, $p = 0.0019$) and lymphocytes (t_0 1.991×10^9/L vs. t_5 1.557×10^9/L $p < 0.0001$) in patients who developed normal scars were observed. The other changes were not observed in patients in this group (Fig. 1).

Both control (2.3 mg/L vs. 37.23 mg/L, $p < 0.0001$) and HT groups (6.12 mg/L vs. 36.89 mg/L, $p = 0.0023$) showed an increase in CRP after surgery (Fig. 2).

Vitamins A, E, and D

No differences were observed in circulating levels of vitamin A and vitamin E between groups. Both groups showed to have sufficient circulating levels of both vitamins (Fig. 3).

Before surgery, 25(OH)D plasma levels were found to be decreased by almost 50% (23.52 ng/mL vs. 15.46 ng/mL, $p = 0.031$) in patients who developed hypertrophic scars.

Overall, patients who developed normal scars showed deficient levels (21–30 ng/dL) of vitamin D, and patients who developed hypertrophic scars showed to be insufficient in vitamin D (8–20 ng/dL; Fig. 3) [37]. Using dichotomous vitamin D deficiency or normal, only 17.4% of the patients

Table 1 Baseline characteristics of patients in control group and hypertrophic group

	Control group	Hypertrophic group	Total
No. of women, No. (%)	46 (73%)	17 (27%)	63 (100%)
Age (years), mean (range)	43.26 (20–65)	44.76 (25–60)	43.67 (20–65)
Body mass index; mean ± SEM	27.35 ± 3.32	27.49 ± 3.78	27.39 ± 3.42
Smoke, No. (%)	13 (28.26%)	2 (11.76%)	15 (23.80%)
Bariatric surgery	18 (39%)	8 (47%)	26 (41%)

analyzed showed normal levels of vitamin D (>30 ng/dL), and all of them developed normal scars. Of the patients in the study, 82.6% showed deficient levels of vitamin D (Table 2).

Bariatric Surgery

Twenty-six (41%) patients had been submitted to bariatric surgery (15 patients submitted to gastric bypass, 9 submitted to gastric band, and 2 patients submitted to gastric sleeve). Patients submitted to bariatric surgery had a higher BMI than patients who were not submitted to bariatric surgery (28.42 kg/m^2 vs. 26.66 kg/m^2, $p = 0.04$; Fig. 4). Those patients had lower levels of vitamin A (34.00 mcg/dL vs. 39.64 mcg/dL, $p = 0.028$), vitamin E (1087 mcg/dL vs. 1238 mcg/dL, $p = 0.043$), and vitamin D (19.68 ng/dL vs. 22.8 ng/dL, although with no statistical significance; Fig. 5). Thirty-one percent of the patients submitted to bariatric surgery had more hypertrophic scars, versus 24% of the patients with no previous bariatric surgery (no statistical difference) (Fig. 6).

We evaluated the interaction of both surgery and wound healing status with vitamin levels (Fig. 7). The influence of those two independent variables and its interaction was evaluated. We confirmed the interaction of vitamin D levels and wound healing in both bariatric and non-bariatric patients. Vitamin E was lower in those patients with HT that were submitted to bariatric surgery.

Discussion

Hypertrophic scars and keloids can be considered an abnormal end point of wound healing, responsible for impairment of quality of life, by causing physical, psychological, and social sequel. The complexity of events involved makes it very difficult to come up with a single explanation to understand the mechanisms that underlie or strategies to prevent this devastating fibrotic condition. Inflammation is considered one predisposing factor, although this phase is a necessary aspect of the wound healing process. Wound healing has traditionally been divided into three distinct phases: inflammation, proliferation, and remodeling [38]. A prolonged or excessive inflammatory phase is believed to be the onset of excessive scarring [11, 12].

In our study, we found, before surgery, lower neutrophil (3.008×10^9/L vs. 3.830008×10^9/L, $p = 0.040$) and higher eosinophil (0.145×10^9/L vs. 0.104×10^9/L, $p = 0.028$) and basophil count (0.031×10^9/L vs. 0.22×10^9/L, $p = 0.049$) and CRP levels (CRP 6.12 mg/L vs. 2.30 mg/L, $p = 0.015$; Fig. 2) and higher monocyte count (without statistical significance) in patients who developed hypertrophic scars. Monocytes are important elements in the first, inflammatory phase, of wound healing. Upon arrival to the wound site, those cells are stimulated to transform into macrophages. Macrophages are the "orchestra leader" of wound healing because of their important role directing the wound healing process: they influence

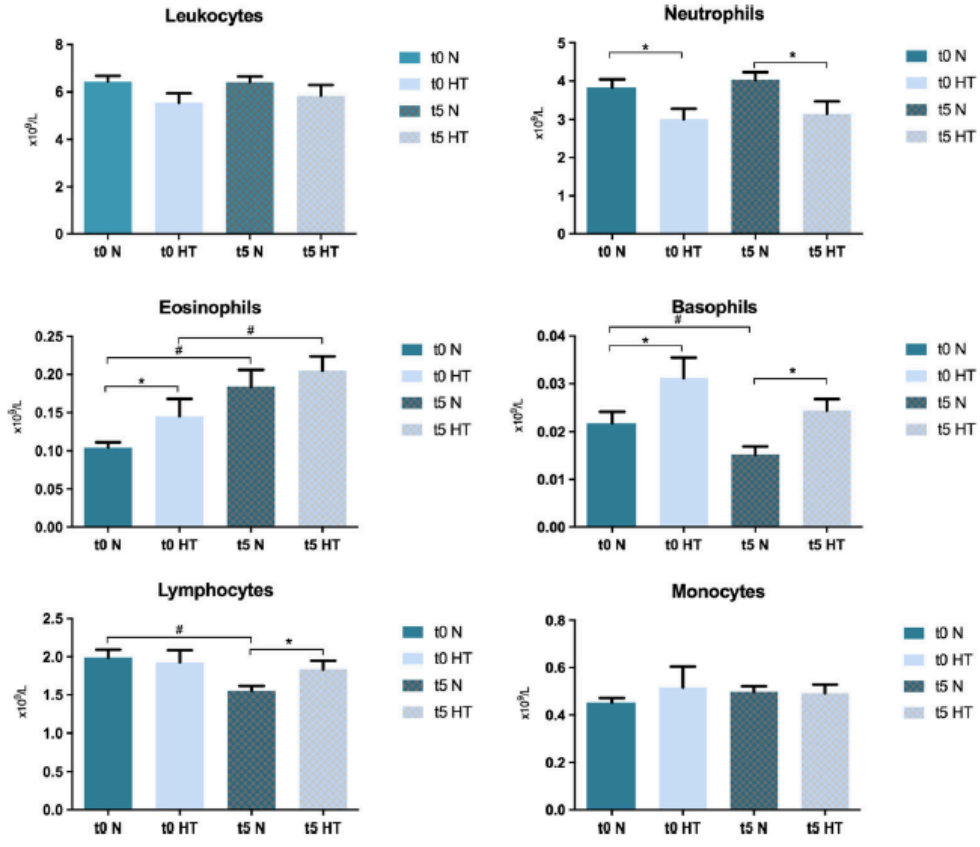

Fig. 1 Inflammatory cells at t_0 and t_5 in patients who develop normal scars (N) and hypertrophic scars (HT). Mean ± SEM; #$p < 0.05$, *$p < 0.05$

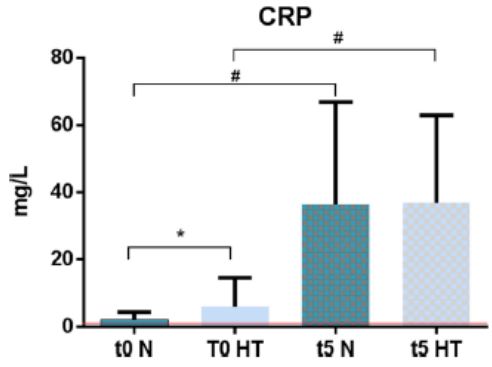

Fig. 2 C-reactive protein (CRP) quantification at t_0 and t_5 in patients who develop normal scars (N) and hypertrophic scars (HT). Mean ± SD; #$p < 0.05$, *$p < 0.05$

angiogenesis, fibroplasia, and extracellular matrix synthesis [39]. Finally, T lymphocytes are known to migrate into the wound after inflammatory cells and macrophages on the fifth day after injury, during the proliferative phase, and peak at day 7 [40]. It appears that T lymphocytes may control the proliferation phase of wound healing. The higher levels of monocytes, eosinophil, basophil, and CRP in the circulation of our patients in the HT group may indicate a preoperative systemic proinflammatory state in these individuals that will predispose them to a prolonged or excessive inflammatory phase and hypertrophic scars. On the other hand, higher lymphocyte count may not only predispose these patients to an exuberant inflammatory phase (as they are responsible to tissue monocyte–macrophage transformation), but may also predispose them to an exuberant proliferation phase, with more fibroblast migration, collagen deposition, and epithelialization [41].

Five days after surgery, corresponding to the inflammatory phase of wound healing, a relative increase in eosinophils (N group, t_0 0.104 × 10^9/L vs. t_5 0.184 × 10^9/L, $p = 0.0002$; HT group, t_0 0.145 × 10^9/L vs. t_5 0.205 × 10^9/L, $p = 0.0003$) and a decrease in basophils (t_0 0.002 × 10^9/L vs. t_5 0.015 × 10^9/L, $p = 0.0019$) and lymphocytes (t_0 1.991 × 10^9/L vs. t_5 1.557 × 10^9/L $p < 0.0001$) in patients who developed normal scars were observed. This is possibly due to marginalization of inflammatory cells to the wounded tissues. CRP showed to be higher in this phase in both groups, as expected, as the individuals are beneath an inflammatory process.

After surgery, patients in the HT group showed a decrease in neutrophil (3.144 × 10^9/L vs. 4.03 × 10^9/L, $p = 0.031$) and leucocyte count and an increase in basophil (0.024 × 10^9/L vs. 0.015 × 10^9/L, $p = 0.005$), lymphocyte count (1.836 × 10^9/L vs. 1.557 × 10^9/L; $p = 0.028$), and eosinophil count compared with patients with normal scars. Possibly neutrophil and leucocyte counts are lower due to a higher marginalization in these patients, reflecting a higher inflammatory response in wound site. Lymphocytes, peaking in the wound at day 7, during the proliferation phase, are still in the circulation, but already in higher levels, in these patients.

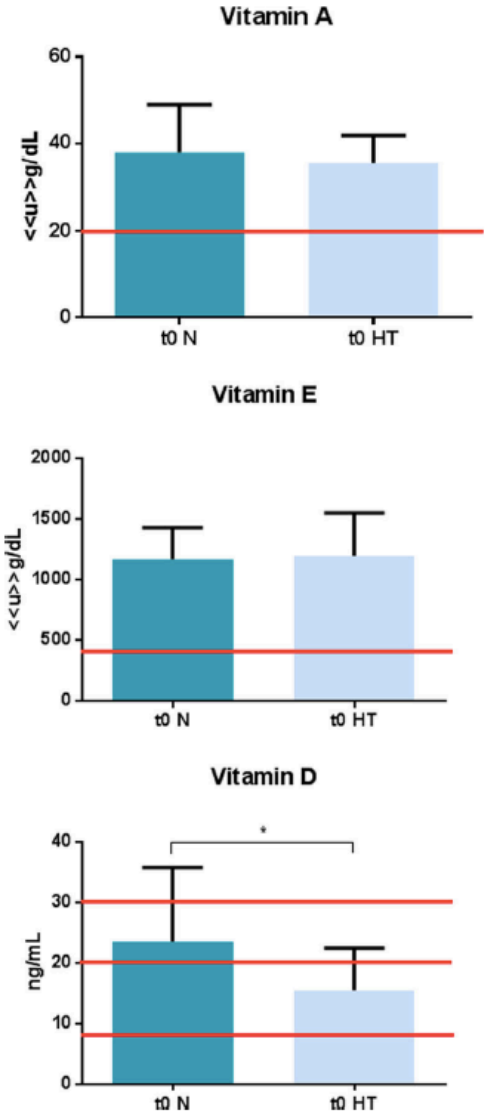

Fig. 3 Serum levels of vitamins A, E, and D at t_0 of patients who develop normal scars (N) and hypertrophic scars (HT). Mean ± SD; *$p < 0.05$

Vitamin D has been reported to exhibit properties of skin hormones such as organized metabolism, activation, inactivation, and elimination in specialized cells of the tissue; exertion of biological activity; and release in the circulation [16]. Vitamin D has also shown to be a powerful anti-inflammatory agent [23]. It has some potential role in skin health and maintenance [17–19], and its analogues have been used for years to treat hyperproliferative and inflammatory skin diseases [28–30].

We have demonstrated that 25(OH)D plasma levels were found to be decreased by almost 50% in patients who developed hypertrophic scars. When hypertrophic scars emerge, they show psoriasis-like features, such as increased acanthosis, higher keratinocyte proliferation, and abnormal keratinocyte differentiation compared with normal scars [42–44]. A prolonged inflammatory phase is also observed in this type of wounding [11, 12]. During this prolonged

Table 2 Vitamin D levels in control group and hypertrophic group

	Control group (%)	Hypertrophic group (%)	Total (%)
Normal (>30 ng/dL)	24.2	0	17.4
Deficiency (21–29 ng/dL)	24.2	23.1	23.9
Insufficiency (8–20 ng/dL)	48.6	61.5	52.2
Severe insufficiency (<8 ng/dL)	3.0	15.4	6.5

Source: [36]

inflammatory phase, relatively high concentrations of proinflammatory cytokines derived from macrophages keep the keratinocytes activated, whereas fibroblasts remain stimulated to produce extracellular matrix. 1,25(OH)2D3 binds to an intranuclear vitamin D receptor, and exerts its antiproliferative and anti-inflammatory actions, reducing the keratinocyte proliferation and inflammatory phase [16, 23] and possibly reducing hypertrophic wounding. Patients with low circulatory plasma levels of 25(OH)D would develop hypertrophic scars due to the reduced ability to cut down these proliferative and inflammatory mechanisms. This is also suggested by the higher CRP levels found before surgery, in patients who developed hypertrophic scars. The patients in the HT group showed to have a higher systemic inflammatory profile when compared to the control subjects, predisposing them to abnormal wound. Low 25(OH)D plasma levels may be responsible for that inflammatory profile. We only measured 25(OH)D plasma levels before surgery because of its serum half-life of 2–3 weeks.

This hormone has already proved to be essential for wound healing. Zubair et al. [45] and Burkiewicz et al. [46] showed that the median plasma level of 25(OH)D was lower in patients with leg ulcers, in both diabetic and non-diabetic patients, compared to control subjects. In addition, the authors showed a trend toward better healing in those with vitamin D reposition: [46]. Heilborn et al. [47] showed that topical treatment with the vitamin D analogue calcipotriol enhances the upregulation of the anti-microbial protein hCAP18/LL-37, during wounding in human skin in vivo. Cathelicidin antimicrobial proteins (hCAP18) are strongly upregulated by epithelium shortly after wounding and then declined slowly to preinjury levels when the wound closes [48]. Accumulating evidence indicates that hCAP18/LL-37 may serve a key role in protecting the integrity of the epithelium and actively promoting reepithelialization and tissue repair [49].

Other groups have shown that in other organ systems, including the intestine, kidney, liver, lung, skin, and heart,

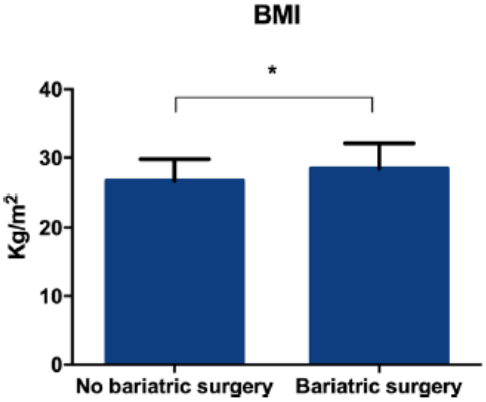

Fig. 4 Body mass index (BMI) of patients with no history of bariatric surgery and previously submitted to bariatric surgery. Mean ± SD; *$p < 0.05$

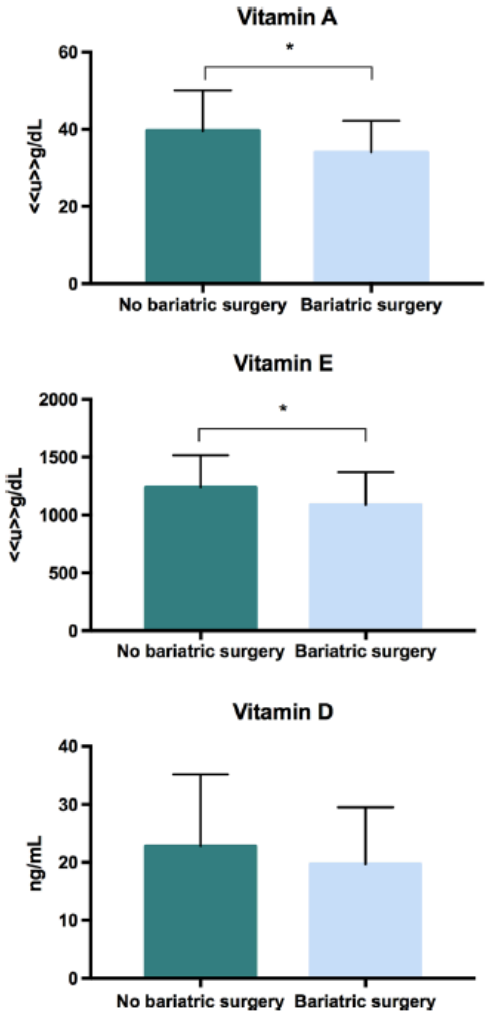

Fig. 5 Serum levels of vitamins A, E, and D at t_0 in patients with no history of bariatric surgery and patients previously submitted to bariatric surgery. Mean ± SD; *$p < 0.05$

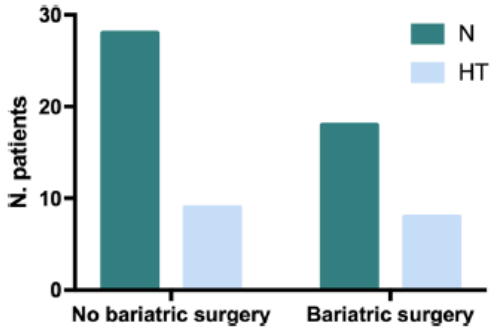

Fig. 6 Number of patients submitted to bariatric surgery vs. patients with no previous bariatric surgery (no statistical difference)

vitamin D deficiency is associated with fibrosis [50–55]. Pharmacological activation of vitamin D receptor (VDR) attenuates the progression of liver fibrosis in an experimental animal model while genetic abrogation of VDR expression results in the spontaneous development of liver fibrosis [56].

Yet, we are the first to link hypertrophic wound healing in the skin with diminished circulating levels of vitamin D.

It is known that hypertrophic and keloids are more common in African descent [57] and in burn scars. The prevalence of hypertrophic scarring following burns is up to 67%, and excessive scarring represents the first morbidity cause in burn survivors [58]. An increase in pigmentation has been shown to decrease the amount of vitamin D3 synthesis in the skin [57]. Similarly, burn scar and normal skin adjacent to the wound showed a fivefold decrease in the ability to transform 7-dehydrocholesterol into previtamin D3 and burn individuals often injury present vitamin D deficiency caused by low endogenous production of vitamin D [59]. Thus, this lights up the hypothesis linking the propensity of inflammation and subsequent scarring in darker-skinned and in burned individuals to the reduced levels of vitamin D3 production. In our study, we only included one African descendent subject who developed a normal scar, so we cannot deepen this question, but this should be further investigated.

Van der Veer et al. [8], in a randomized, double-blind, placebo-controlled trial, failed to prove the preventive effect of topical application of calcipotriol, during the first 3 months in hypertrophic scar formation, in 30 women submitted to bilateral reduction mammoplasty. This concerns the topic application performed. The effects of 25(OH)D3 shown in our study may be systemic (as also suggested by CRP elevation) and not local. Systemic supplementation effects of vitamin D in wound healing should be further evaluated, as this compound has shown to be safe, well tolerated, and inexpensive.

As expected, patients submitted to bariatric surgery had lower levels of vitamins A, E, and D (although this last has no statistical difference). These surgeries produce a mechanical restriction and malabsorption syndrome that are known to lead to nutritional deficiencies. Although having these nutritional impairments, patients submitted to surgery did not have significantly more

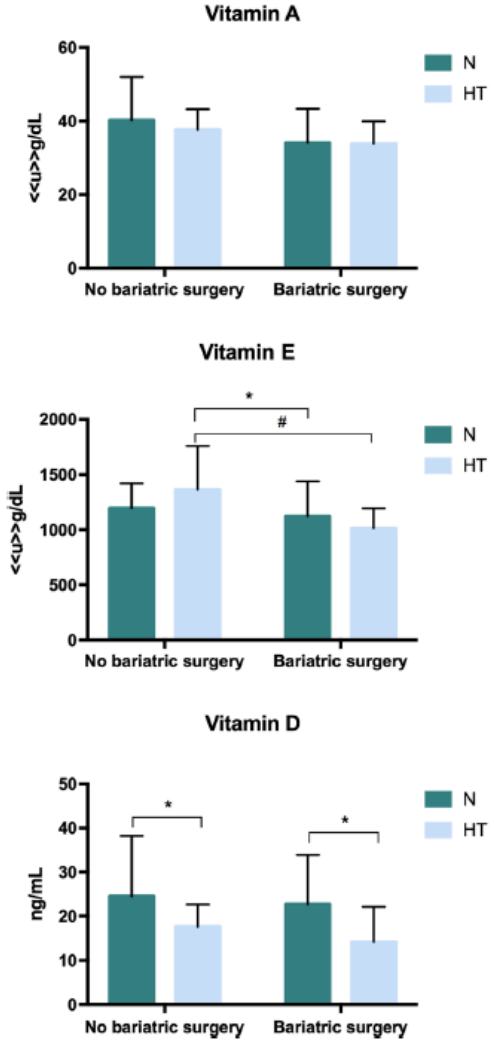

Fig. 7 Serum levels of vitamins A, E, and D at t_0 in patients with normal scars (N) and hypertrophic scars (HT), with no previous bariatric surgery and with previous bariatric surgery. Mean ± SD; #$p < 0.05$; *$p < 0.05$

hypertrophic scars than those with no surgery. Vitamin D levels were lower in the HT group when analyzing bariatric and non-bariatric patients, independently highlighting the role of this vitamin in the wound healing process. Yet, our groups are small when evaluated independently to take further conclusions.

Vitamin A is necessary for growth, differentiation, and maintenance of epithelial tissues [16]. We did not find any relation between vitamins A and E and wound healing. And it should be noted that both of our groups presented normal serum levels of both vitamins.

Conclusion

Patients who develop hypertrophic scars have a different systemic inflammatory profile response. Even before wounding, they already present elevated circulating levels of monocytes

and CRP levels. After surgery, neutrophils and leucocytes are decreased but basophils increased.

Furthermore, vitamin D plasma levels are marked reduced in these patients. Considering the powerful anti-inflammatory and anti-proliferative effects of vitamin D, these findings could be related. Bariatric surgery leads to vitamin A and E deficiency.

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Compliance of Ethical Standards The study was approved by the Portuguese Institutional Review Board for Human Subjects and carried out in accordance with principles of the Declaration of Helsinki as revised in 2001. All patients gave written informed consent to take part in this research.

Conflict of Interest The authors declare that they have no conflicts of interest.

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A new role for anandamide: defective link between the systemic and skin endocannabinoid systems in hypertrophic human wound healing

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The use of cannabinoids to treat fibrotic skin diseases is an emergent issue. Therefore, we aimed to evaluate systemic and skin endocannabinoid responses in the wound-healing process in humans. A prospective study was performed in 50 patients who underwent body-contouring surgery. Anandamide (*N*-arachidonoylethanolamine, AEA), 2-arachidonoylglycerol (2-AG), palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) were quantified using LC–MS/MS. Ten (20%) patients developed hypertrophic (HT) scars. No significant changes were observed between the normal (N) scar and HT scar groups in terms of plasma and skin endocannabinoids. Nevertheless, a positive correlation between plasma and skin AEA concentrations was found in the N group ($r = 0.38$, $p = 0.015$), which was absent in the HT group. Moreover, the AEA concentration was significantly lower in HT scar tissue than in normal scar tissue (0.77 ± 0.12 ng/g vs 1.15 ± 0.15 ng/g, $p < 0.001$). Interestingly, in all patients, the surgical intervention produced a time-dependent effect with a U shape for AEA, PEA and OEA plasma concentrations. In contrast, 2-AG plasma concentrations increased 5 days after surgery and were reduced and stabilized 3 months later. These results suggest crosstalk between systemic and local skin endocannabinoid systems during human wound healing. AEA appears to be the most likely candidate for this link, which is deficient in patients with HT scars.

Endocannabinoids are the endogenous ligands for cannabinoid receptors CB1 and CB2, which are two G-protein coupled receptors that have a widespread distribution throughout the body[1,2]. The most studied endocannabinoids are the arachidonic acid derivatives N-arachidonoylethanolamine (AEA)[3] and 2-arachidonoylglycerol (2-AG)[4]. Palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) are N-acylethanolamines (NAEs) that act by influencing AEA metabolism and binding to peroxisome proliferator-activated receptor alpha (PPAR-α) and to transient receptor potential cation channel subfamily V member 1 (TRPV1)[5–7]. Endocannabinoids and related NAEs play an essential role in many physiological central and peripheral processes. These include emotional responses, cognition, memory, motor behaviour, immune function, feeding, energy consumption and metabolic regulation at the systemic and cellular levels[8–13].

Endocannabinoids are present in human blood, and their concentrations are dynamic. Food consumption, obesity, exercise, sleep pattern, time of the day, stress, anxiety, inflammation and pain are known to modify the endocannabinoid concentrations in the circulation[14]. They have also been quantified in other biological samples obtained from humans, including saliva[15], hair[16], semen[17], breast milk, and amniotic fluid[18].

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	N	HT	Total
No. of women, n (%)	40 (80%)	10 (20%)	50 (100%)
No. of surgeries, n	49	13	62
Age in years, mean ± SD	43 ± 11	43 ± 11	43 ± 11
Body mass index; mean ± SD	27.21 ± 3.43	28.05 ± 3.47	27.38 ± 3.45
Smoking, n (%)	11 (27.5%)	1 (10%)	12 (24%)
Bariatric surgery, n (%)	16 (40%)	3 (30%)	19 (38%)

Table 1. Patient characteristics at baseline in subjects who later developed normal scars (N) or hypertrophic scars (HT).

In the skin, the endocannabinoid system has been identified in epidermal keratinocytes, melanocytes, mast cells, fibroblasts, sebocytes, sweat gland cells and hair follicle cells[19–25]. Here, it is involved in a large number of biological processes, such as proliferation, growth, differentiation and survival, immunocompetence, tolerance[26] and melanogenesis[27]. It was recently shown that abuse of synthetic cannabinoids can result in dermatologic disorders, such as premature skin ageing, hair loss and greying, or acne[28], indicating that cannabinoid signalling can influence skin biology.

In fact, some authors have proposed a new "C(ut)annabinoid" system[29]. This "C(ut)annabinoid" system has been linked to skin fibrosis and wound healing in animals. CB2 selective agonists and CB1 selective antagonists significantly decrease subcutis inflammatory cell infiltration (T cells and macrophages), fibroblast activation and experimental fibrosis in bleomycin-challenged mice[30,31]. It has also been demonstrated in a murine model that skin incisions produce dynamic alterations in the expression pattern of CB1[32] and CB2[33] receptors during wound healing in various immune cells as well as in fibroblasts/myofibroblasts. Regarding the clinical efficiency of cannabinoids in human skin fibrotic diseases, only scarce evidence is available. A short literature report including three patients exhibiting epidermolysis bullosa described faster wound healing following the self-administration of cannabidiol (CBD)[34]. Recently, a small clinical study described a beneficial effect of topical cannabidiol in acne scars[35].

Medical cannabis is now legal in several countries. In addition, persuasive advertisement for cannabis products, namely, for dermatological treatments, and easy availability have led to an increase in consumption. In contrast, knowledge concerning the role of the endocannabinoid system in the pathophysiological responses in human skin fibrosis is missing.

In view of this fact, we quantified the most extensively studied endocannabinoids, including AEA, 2-AG and related NAEs, OEA and PEA, during different phases of wound healing in patients who later developed normal and hypertrophic scars. Our specific aims were to (1) quantify endocannabinoids and related NAEs in skin and in scars; (2) identify differences in the concentration of endocannabinoids and related NAEs in plasma and skin in humans with normal and hypertrophic scars; (3) identify potential fluctuations in endocannabinoid and related NAEs concentrations in plasma before surgery and during the different phases of wound healing (inflammatory, proliferative and remodelling phases); and (4) identify correlations between the concentration of endocannabinoids and related NAEs found in plasma and in skin from the same patient.

Results

Patient characteristics. All the patients included in the study were female. The mean age was 43 ± 11 (20–65) years. The mean body mass index (BMI) was 27.38 ± 3.45 kg/m^2, and nineteen (38%) patients had previously undergone bariatric surgery. There were no significant differences in age and BMI between the two groups. Twelve (24%) patients reported smoking habits, but none reported alcohol or other drug abuse. The results are presented in Table 1.

In total, 40 abdominoplasties, 5 arm lifts and 5 thigh lifts were performed. Of the patients submitted to an abdominoplasty, 32 developed N scars, and 8 developed HT scars. Of the patients who underwent arm lifts and thigh lifts, 4 developed normal scars, and 1 developed HT scars after each surgery. The concentrations of endocannabinoids and related NAEs from skin collected from these different locations were compared, and no differences were found (data not shown). As a result, all the collected samples were studied together.

Quantification of endocannabinoids and related NAEs in skin and scar samples. The concentrations of endocannabinoids and related NAEs in human skin samples collected during body-contouring surgery at time 0 are listed in Table 2. Six months after surgery, all patients were reviewed by two plastic surgeons for scar classification[36]. Patients were then classified into two different groups: those who developed normal scars (N group, n = 40) and those who developed hypertrophic scars (HT group, n = 10).

No significant differences were observed between the N and HT groups for all the endocannabinoids and related NAEs quantified in the skin collected at the time of surgery, namely, AEA, PEA, OEA and 2-AG. Large individual variability was observed for PEA, OEA and 2-AG, contributing to the high SEM values in some groups. 2-AG was the most abundant endocannabinoid found in human skin (120.82 ± 13.24 ng/g), with concentrations 119-fold higher than that of AEA (1.09 ± 0.05 ng/g, $p < 0.001$; ratio 2-AG/AEA in skin: 118.50 ± 13.69), sevenfold higher than that of PEA (22.90 ± 2.16 ng/g, $p < 0.001$; ratio 2-AG/PEA in skin: 6.88 ± 0.75) and fivefold higher than that of OEA (27.98 ± 2.18 ng/g, $p < 0.001$, ratio 2-AG/OEA in skin: 5.16 ± 0.56).

	n	AEA (ng/g)	PEA (ng/g)	OEA (ng/g)	2-AG (ng/g)
N	40	1.05 ± 0.06	22.81 ± 2.49	27.73 ± 2.45	115.75 ± 13.75
HT	10	1.30 ± 0.15	23.31 ± 4.41	23.31 ± 5.06	140.64 ± 39.29
Total	50	1.09 ± 0.05	22.90 ± 2.16	27.98 ± 2.18	120.82 ± 13.24

Table 2. Concentration of endocannabinoids (AEA, 2-AG) and related NAEs (PEA, OEA) in human skin samples collected during body-contouring surgery (t0). Later, 40 patients exhibited a normal healing process (N), and 10 patients developed a hypertrophic scar (HT).

	N	HT	Total
No. of women, n (%)	15 (60%)	10 (40%)	25 (100%)
Age in years, mean ± SD	49 ± 10	52 ± 17	50 ± 13
Body mass index; mean ± SD	27.78 ± 3.74	29.13 ± 4.14	28.38 ± 4.45
Smoking, n (%)	4 (27%)	1 (10%)	5 (20%)
Bariatric surgery, n (%)	7 (47%)	1 (10%)	8 (32%)

Table 3. Patients characteristics that underwent scars correction surgery with normal (N) and hypertrophic (HT) scars.

In the end of the study protocol, since bariatric patients are frequently submitted to several surgeries (see Table 1), we had the opportunity to collect a small amount of the original scar in 12 patients (N group: n = 9; HT group: n = 3), to further study the endocannabinoid system in the scar.

Considering these 12 patients, we evaluated the individual paired changes from time 0 (skin) to time 1 (scar). Endocannabinoids and related NAEs present in scar tissue were in the same range as those found in the normal skin, indicating a full and active cannabinoid function in the scar tissue (data not shown). However, no other conclusions were obtained, since we had only a low number of scars included in each group.

Taking these results into account, we decided to collect scars from other patients who did not pertain to this protocol and were undergoing scar correction surgery. In this group, we also classified the scars as normal or hypertrophic[36]. Table 3 resumes demographic data regarding those 25 patients. All of the included patients had their scar-inducing surgery within one year before the scar correction surgery. The period of time elapsed since the scar-inducing surgery and scar sample extraction was not different between the N and HT groups. In total, we concluded our study with 15 normal scars and 10 hypertrophic scars. In this larger sample, the AEA concentration was lower in HT scars than in normal scars (0.77 ± 0.12 ng/g vs 1.15 ± 0.15 ng/g, respectively, $p < 0.001$; unpaired t-test; Fig. 1a). There were no significant changes in PEA, OEA, or 2-AG between normal and hypertrophic scars (Fig. 1b–d).

Quantification of endocannabinoids and related NAEs in plasma. Before surgery, concentration of endocannabinoids (AEA and 2-AG) and related NAEs (PEA and OEA) in plasma were similar between patients who developed N scars and those who developed HT scars (Table 4).

Regarding the relative abundance, the endocannabinoids and related NAEs followed the same profile in skin and in plasma: 2-AG > OEA = PEA > AEA ($p < 0.001$). However, the ratio between 2-AG and the other compounds differed between skin and plasma. 2-AG was 119-fold higher than AEA in skin but only fourfold higher in plasma (118.50 ± 13.69 vs 4.08 ± 0.35, $p < 0.001$).

As shown in Fig. 2, no significant differences were observed in endocannabinoid and related NAE concentrations between the N and HT groups at any time after surgery. However, it was clear that a time-dependent effect along with a U shape was found for AEA, PEA and OEA (Fig. 2a–c). This profile was more evident in AEA concentrations, with a significant and sequential decrease at 5 and 12 days after surgery (956 ± 9 pg/mL vs 729 ± 8 pg/mL, $p = 0.003$ and 702 ± 12 pg/mL, $p < 0.001$) and a significant increase 3 months after surgery (1,040 ± 22 pg/mL, $p < 0.001$). In contrast, 2-AG concentrations significantly increased 5 days after surgery (3,891 ± 404 pg/mL vs 11,194 ± 2,193 pg/mL, $p = 0.023$) and became significantly lower and stabilized 12 days after surgery (3,882 ± 306 pg/mL, $p = 0.009$), reaching the lowest concentrations at 3 months after surgery (3,289 ± 265 pg/mL, $p = 0.003$).

Relationship between endocannabinoids and related NAEs in plasma and in skin. To investigate a possible association between the systemic and local skin endocannabinoid systems, we tried to find a correlation between the concentrations of the endocannabinoids and related NAEs measured in plasma and in skin for each patient. At time 0, we collected plasma and skin samples from each patient. In Fig. 3a, b, we show the results for AEA in patients who developed normal and hypertrophic scars. A positive correlation between the concentrations of AEA in plasma and in skin with a Pearson r of 0.38 (a significant $p = 0.015$) was found. A linear regression with a slope of 0.44 ± 0.17 is shown in Fig. 3a, including a 95% confidence limit. In contrast, this correlation was lost in patients who developed hypertrophic scars (Fig. 3b), where the Pearson r was 0.13 (not

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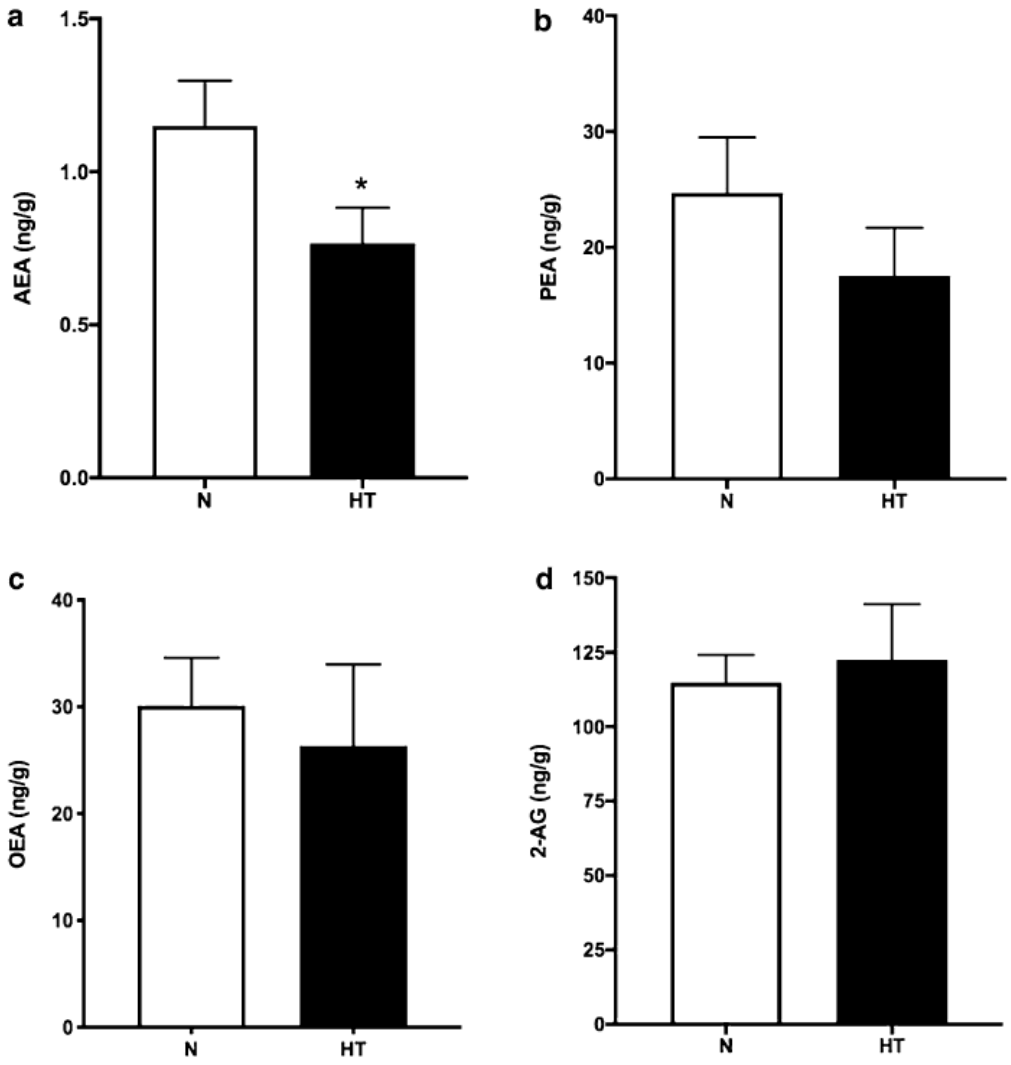

Figure 1. Concentration of endocannabinoids and related NAEs in scars. AEA (**a**), PEA (**b**), OEA (**c**), 2-AG (**d**) in normal scars (N, n = 15) and hypertrophic scars (HT, n = 10). *$p < 0.05$.

	n	AEA (pg/mL)	PEA (pg/mL)	OEA (pg/mL)	2-AG (pg/mL)
N	40	950 ± 48	2,839 ± 164	2,954 ± 155	3,763 ± 378
HT	10	961 ± 155	2,971 ± 376	3,049 ± 357	4,401 ± 1,401
Total	50	952 ± 49	2,865 ± 150	2,973 ± 141	3,891 ± 404

Table 4. Concentration of endocannabinoids and related NAEs in plasma collected immediately before surgery (time 0), in patients who later exhibited a normal healing process (N, n = 40) and in patients who developed a hypertrophic scar (HT, n = 10).

significant). Concerning PEA (Fig. 3c, d), OEA (Fig. 3e, f) and 2-AG (Fig. 3 g, h), no significant correlation was found between plasma and skin endocannabinoid concentrations for normal or hypertrophic patients.

Discussion

Wound healing has been conceptually divided into three distinct phases: inflammation, proliferation and remodelling. Different cells and cytokines are involved in each wound-healing phase[37]. The endocannabinoid system has been recently implicated in wound healing and skin fibrosis in mice[30,31]. Despite the growing interest in this topic, its role in human wound healing has not yet been described. Therefore, this is the first study measuring

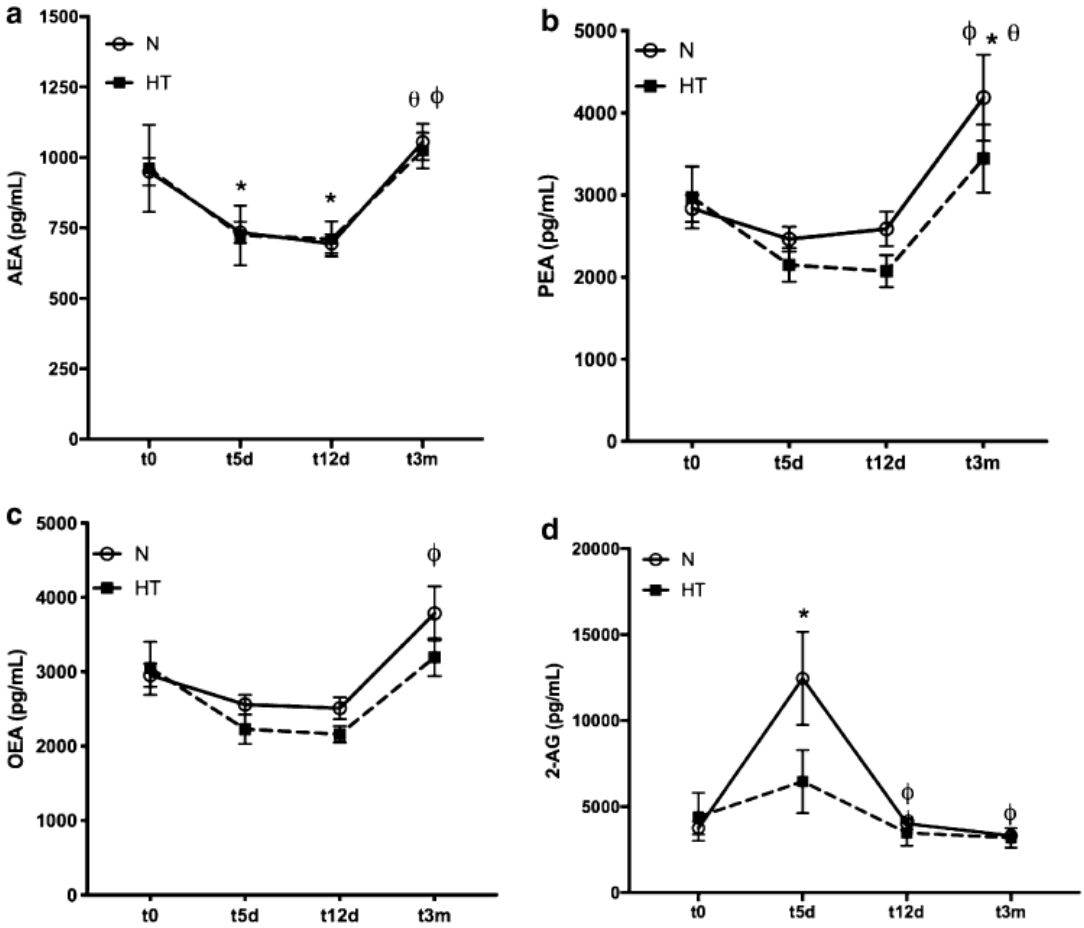

Figure 2. Concentration of endocannabinoids and related NAEs in plasma. AEA (**a**), PEA (**b**), OEA (**c**), 2-AG (**d**) in patients who developed normal scars (N, n = 40) and patients who developed hypertrophic scars (HT, n = 10). Samples were collected immediately before surgery (t0), 5 days after surgery (t5d), 12 days after surgery (t12d) and 3 months after surgery (t3m). Four plasma samples for each patient were collected at different times. *$p < 0.05$ comparing t0 values in the control group; Φ $p < 0.05$ comparing t5d values. Θ Comparing t12d values (Tukey's multiple comparison test).

endocannabinoids in skin in the context of surgery and wound healing. With two plastic surgeons in our team, we were able to collect skin from patients, aiming to quantify endocannabinoids and related NAEs in human skin and scars. Concerning the basal concentrations of endocannabinoids and related NAEs in skin, no significant differences were observed between the N and HT groups. We also found that 2-AG is the most abundant endocannabinoid in human skin, with concentrations 119-fold higher than that of AEA, 7-fold higher than that of PEA and 5-fold higher than that of OEA. This is the first time that endocannabinoids and related NAEs have been quantified in full-thickness human skin, so we cannot compare our findings with those of previous studies; however, the AEA concentrations in these tissues are in the same range of AEA concentrations reported in human hair[38]. The relative proportion of endocannabinoids and related NAEs that we observed in human skin (2-AG > OEA = PEA > AEA) is similar to reported data obtained from different human tissues, namely, the uterus and plasma[39,40], but different ratios have also been described in the liver[41] and plasma[42]. Interestingly, the concentrations of the studied endocannabinoids and related NAEs in scar tissue are in the same range as those found in normal skin, demonstrating the presence of these active molecules in scar tissue. We also found that AEA is significantly reduced in hypertrophic scars compared to normal scars. This finding supports the hypothesis of a significant role for AEA in the pathophysiological process of skin fibrosis. Pathologically excessive dermal fibrosis and aberrant scarring characterize hypertrophic scars. Although the exact pathogenesis and aetiology are still unsettled[43], it is believed that a sustained inflammatory phase is an essential prerequisite for this disorder, with a decrease in apoptosis and an increase in inflammation[44,45]. Endocannabinoids can regulate immune function and are generally considered to be anti-inflammatory agents[46]. Fatty acid amide hydrolase (FAAH) inhibitors have been proven to inhibit lipoteichoic acid (LTA)-induced pro-inflammatory responses in a CB1 and CB2 receptor-dependent manner. Topical application of a FAAH inhibitor reduced dust mite-induced skin inflammation in NC/Tnd mice with the same efficiency as the positive control tacrolimus[47]. FAAH is responsible for AEA and other NAE metabolism, and its inhibition locally increases this endocannabinoid concentration[48]. Moreover, AEA was recently shown to suppress the production and release of key Th1- and Th17-polarizing

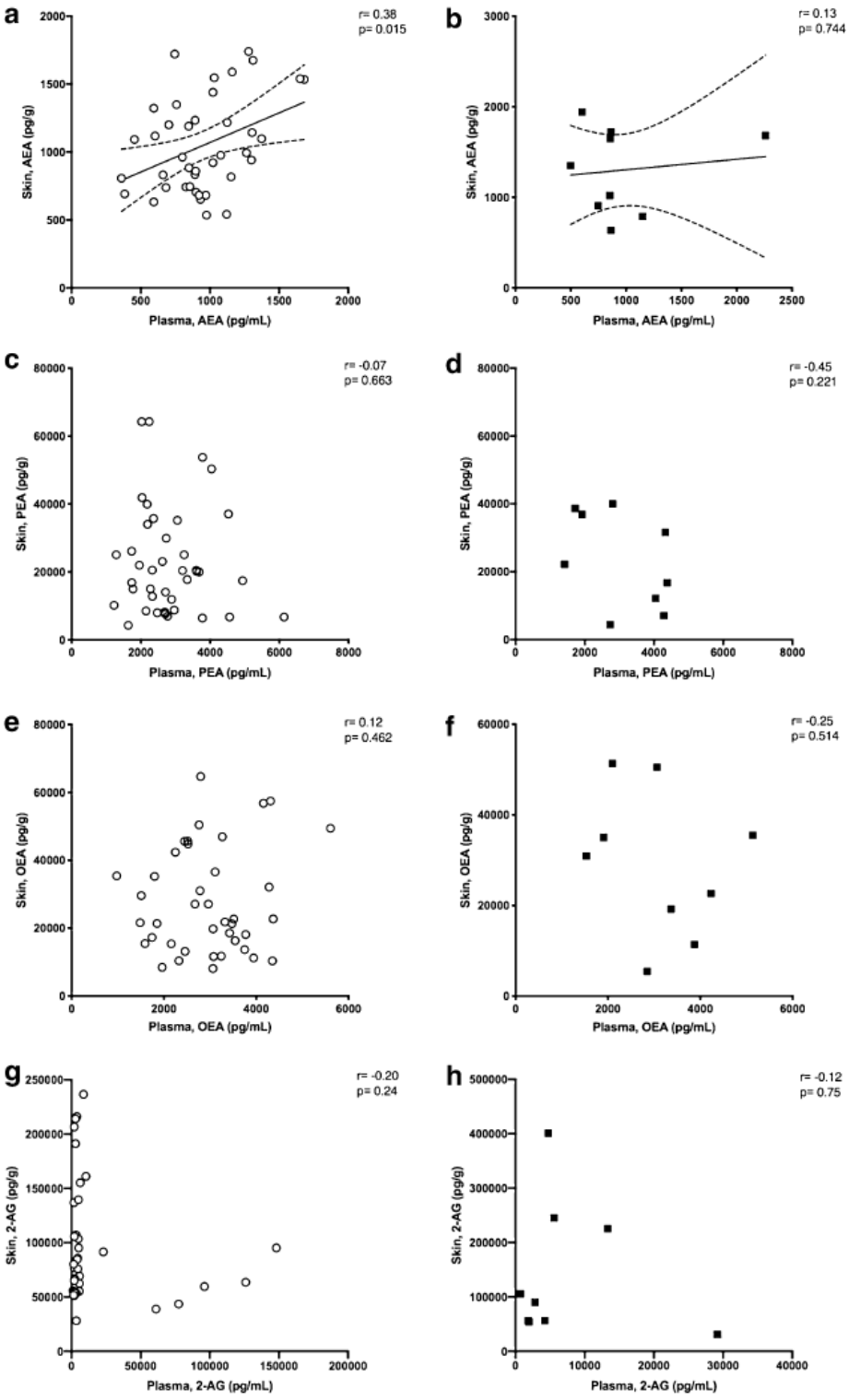

Figure 3. Concentration of endocannabinoids and related NAEs in plasma and skin. Relationship between the concentrations of AEA in (**a**) normal scars and (**b**) hypertrophic scars; PEA in (**c**) normal scars and (**d**) hypertrophic scars; OEA in (**e**) normal scars and (**f**) hypertrophic scars; and 2-AG in (**g**) normal scars and (**h**) hypertrophic scars in the plasma and skin of patients who developed normal and hypertrophic scars. A positive correlation with a Pearson r of 0.38 ($p = 0.0152$) with a linear regression slope of 0.44 ± 0.17, including a 95% confidence limit, was found for AEA in patients who developed normal scars (**a**). No significant correlation was found for AEA in patients who developed HT scars (**b**). No significant correlation was found for PEA (**c**, **d**), OEA (**e**, **f**), and 2-AG (**g**, **h**) in either group of patients.

cytokines (IL-12 and IL-23) via CB1-mediated inhibition of mammalian target of rapamycin (mTOR) in human keratinocytes[49]. We suggest that reduced AEA in hypertrophic scars may be related to increased inflammation or a prolonged inflammatory phase that predisposes patients to this condition. It would be interesting to measure FAAH activity in both hypertrophic and normal scars. More studies are needed to confirm this hypothesis, but if corroborated, the topical administration of AEA, other non-psychotropic cannabinoids or FAAH inhibitors could be an interesting tool to treat or prevent this condition.

In addition to skin tissue and scars, we also had the opportunity to collect blood samples from all patients before and after wounding at three different time points, corresponding to the different phases of wound healing: 5 days for inflammation (t5d), 12 days for proliferation (t12d) and 3 months after surgery/wounding for remodelling (t3m). This allowed us to take a glimpse on what is happening in circulatory endocannabinoids and related NAEs in the four perioperative periods. Although no differences were observed at any time between the two different studied groups (N or HT group), a systematic fluctuation pattern in the concentration of all the endocannabinoids and related NAEs was observed. Interestingly, while AEA and NAEs presented a U shape after surgery, characterized by significantly lower concentrations in circulation at t5d and t12d and with the normal concentration restored at t3m, 2-AG showed a completely different pattern, with an increased concentration at t5d and a progressive decrease at t12d and t3m. It is not surprising that different endocannabinoids have diverse responses to the same stimulus since they have specific synthesis and metabolism pathways. AEA and other NAEs are produced from a low abundance phospholipid, namely, N-acyl-phosphatidylethanolamine (NAPE)[50], and are catabolized by hydrolysis of the amide bond through the actions of FAAH[48] and N-acylethanolamine-hydrolysing acid amidase (NAAA), found primarily in peripheral tissues[51]. The relative proportion of the NAEs produced reflects the relative proportion of the acyl chains found in the sn-1 position of the donor phospholipids; therefore, the concentrations of AEA are commonly lower than that of PEA and OEA in human biological samples, like plasma and serum[14], as confirmed in our samples. Hypothetically, we could say that the decrease in circulating NAEs 5 days and 12 days after surgery could reflect a common global response of these molecules to local skin injury. On the other hand, 2-AG might act as a different metabolic pathway: 2-AG is synthetized in cells that express diacylglycerol lipase, by activation of phospholipase C (PLC), and is catabolized by hydrolysis of its ester bond by several enzymes, such as alpha–beta hydrolase domain protein (ABHD)-6, ABHD-12 and monoacylglycerol lipase (MAGL)[52]. Regarding 2-AG, an increase in circulating concentrations was observed 5 days after surgery. This increase was also accompanied by extremely high variability between patients. These findings highlight that variance in 2-AG can be attributed to wound healing or surgery since most differences occurred immediately after surgical intervention. However, other common factors in patients undergoing hospitalization can cause 2-AG fluctuations, such as acute stress, anxiety or sleep disorders. It was shown in a previous study[53] that 2-AG plasma concentrations increased significantly immediately after the beginning of cardiac surgery and reached maximal concentrations during cardiopulmonary bypass. However, in contrast to our results, after termination of cardiopulmonary bypass, 2-AG concentrations decreased significantly and were close to preoperative values at the time of admission in the cardiovascular intensive care unit. The authors suggest that the increase in 2-AG after initiation of cardiopulmonary bypass should be part of the inflammatory response. In our study, we were not able to collect plasma samples during surgery or immediately after the induction of general anaesthesia, but the 2-AG concentration in our study certainly remained elevated for at least 5 days after surgery. An inflammatory response is known to occur after surgery or skin injury[37] and can also explain our results regarding 2-AG plasma fluctuation.

Several studies have reported that many personal characteristics, such as gender[54], age[55], BMI and the presence of metabolic dysfunction[56], can influence circulating endocannabinoid concentrations. Food consumption and circadian rhythms also influence the endocannabinoid system[39,57]. Coincidentally, both of our groups (N and HT group) exhibited no significant differences in any of these features, and blood was collected on the same morning after an overnight fasting period for all patients. However, other physiologic and pathologic disorders, such as behavioural regulation of feeding[58], psychiatric disorders such as anxiety and depression[59] and fertility[60], are involved in the endocannabinoid system. As no psychometric tests were performed before the study and fertility was not evaluated, these may have also contributed to the high variability in concentrations of plasma endocannabinoids observed between patients and may constitute a limitation to the study.

Regarding the relationship between circulating endocannabinoids and the skin, we found a positive correlation between the concentrations of AEA in plasma and in skin of patients who later developed normal scars. Curiously, this correlation was not present in patients who developed hypertrophic scars. Somehow it appears that regarding AEA, there is a link between both endocannabinoid systems (skin and systemic), and this link is lost in patients who develop hypertrophic scars. This was already observed before surgery. It should be noted that the number of patients included in the HT group (n = 10) was smaller than that included in the N group (n = 40). This may cause limitations in the interpretation of the lack of correlation in the HT group. It would be interesting to measure endocannabinoids and related NAEs in the skin during all wound-healing phases in all 50 patients to clarify the role of endocannabinoids. This would allow us not only to evaluate local variations during wound healing but also to understand whether this relation changes over time. However, this evaluation was not possible due to ethical implications, as it is not permitted to perform a surgery or inflict a wound in a patient for research purposes only. It remains to be established whether changes in peripheral concentrations reflect similar modifications in skin or if circulating changes may affect cutaneous functions, since these compounds, due to their lipophilicity, are believed to act as an autocrine/paracrine mediator[61]. Nevertheless, the present study demonstrates that in humans, AEA circulatory concentrations can reflect AEA concentration in the skin, and this is not true for PEA, OEA and 2-AG. It seems that AEA shares the same origin in both skin and systemic systems, in contrast to 2-AG, which appears to be under distinct local control.

In conclusion, female humans submitted to body-contouring surgery presented a time response pattern of plasma endocannabinoids and related NAEs, and the concentration of AEA in skin was positively correlated

with the concentration of AEA in plasma. Patients who developed HT scars did not present this correlation, and AEA was significantly reduced in HT tissues compared to normal scar tissues. The current study adds to the available literature and increases knowledge on the role of the endocannabinoid system in wound healing and hypertrophic scarring of human skin.

Currently, patients frequently question their dermatologists about the effects of cannabis-derived products in the skin, but clinicians usually fail to find robust clinical evidence for their efficacy[62]. In fact, the data reported herein should certainly encourage researchers to further explore cannabinoid effects in human skin, namely, as an adjunct treatment strategy for hypertrophic scars or other wound-healing disorders.

Material and methods

Subjects. A prospective hospital-based study was conducted for 18 months. Fifty women submitted for routine body-contouring surgery (abdominoplasty, arm lift and thigh lift) in the Department of Plastic, Reconstructive and Aesthetic Surgery of Centro Hospitalar e Universitário de São João in Porto were selected. Exclusion criteria included additional surgeries 1 year before inclusion in this study, immunosuppressive therapy or postoperative complications.

Before surgery, all the subjects were asked to answer a survey concerning demographic data, alcohol, smoking and drug habits, medical and medication-use history, weight, height and history of past surgeries (including bariatric surgery).

Six months after surgery, patients were asked to attend a consultation to be evaluated by two independent trained plastic surgeons to decrease subjectivity. Scars were classified according to the Vancouver Scar Scale (VSS). Scars scoring ≥ 1 were classified as hypertrophic (HT group, n = 10), and scars scoring 0 were classified as normal scars (control group, N = 40)[63].

Blood and tissue samples. Blood samples of every subject included in the study were collected immediately before surgery (t0d), 5 days after surgery (t5d; corresponding to the inflammatory phase of wound healing), 12 days after surgery (t12d; corresponding to the proliferation phase of wound healing), and 3 months after surgery (t3m; corresponding to the remodelling phase of wound healing). All blood samples were taken in the morning after an overnight fasting state. Blood samples were collected by vein suction into a vacutainer containing EDTA. Phenylmethyl-sulfonyl-fluoride (PMSF) (100 µM final concentration), an inhibitor of fatty acid amide hydrolase (FAAH), was added to blood samples to prevent endocannabinoid and related NAEs degradation. Samples were then placed on ice and centrifuged within 1 h at 1,500 × g for 10 min at 4 °C. Plasma was removed to a fresh plastic tube and immediately stored at − 80 °C until processing and endocannabinoid analysis.

At time 0, skin samples (200 to 250 mg) taken from the abdomen, arm or thigh were surgically removed from skin flaps at the site of surgery. In detail, abdominal skin was collected from the left corner of the abdominal flap resected (left hypogastrium); arm skin was collected from the corner located near the elbow; and thigh skin was collected from the anterior corner in the resected inguinal flap. The subcutaneous fat was removed using surgical preparative scissors, and the skin was cut by a scalpel into pieces of 1 × 1 cm and immediately frozen in liquid nitrogen. Samples were stored at − 80 °C for posterior endocannabinoid and related NAEs quantification. Most bariatric patients undergo several body countering or revision surgeries. In total, of the 50 patients first included in the study, 12 underwent another surgery within the first postoperative year. During this second procedure, we were able to collect 12 scar samples from those patients (time 1, normal scars, n = 9 and hypertrophic scars, n = 3). Scars were collected and processed as described for skin samples.

Endocannabinoid and related NAEs quantification. Anandamide, PEA, OEA and 2-AG were quantified in human plasma and skin for every collected sample using LC–MS/MS following extraction, as described below. All procedures were performed in the dark to protect the samples from degradation.

Human skin sample extraction. Skin samples were thawed at 4 °C in ice. After weighing, 500 µL of phosphate buffer 0.1 mM pH 5.6 and 2 µL ISTD spiking solution containing AEA-d8, PEA-d4, OEA-d2 and 2AG-d8 (Cayman Chemical) were added to all samples. Chloroform:MeOH (2:1) 500 µL was added, and the samples were then vortexed vigorously for 2 cycles of 5 s at 5,000 rpm using a bead beater and centrifuged at 20,000 × g for 10 min at 4 °C, after which the organic layer was removed. This procedure was repeated three times, and all the organic phases were pooled. Then, the organic phase was evaporated in a CentriVap concentrator at 50 °C until dryness and reconstituted in 100 µL of acetonitrile. The supernatant was then transferred to HPLC vials to be injected (5 µL) into an LC–MS/MS device.

AEA, PEA and OEA plasma sample extraction. Aliquots of human plasma (50.0 µL) were added to 400 µL of 1.0 µg/mL ISTD working solution containing AEA-d8, PEA-d4 and OEA-d2 in acetonitrile 0.1% formic acid for protein precipitation[64,65]. The samples were vortex-mixed and centrifuged for 10 min at 14,000 rpm at 4 °C, and the supernatant was injected (7 µL) into the LC–MS/MS machine.

2-AG plasma sample extraction. Aliquots of human plasma (500.0 µL) were added to 500 µL of internal standard working solution containing 10 ng/mL 2-AG-d8 in Milli-Q water. Samples placed into (16*125 mm) glass culture tubes were vortex-mixed and loaded (900 µL) into Oasis cartridges (HLB, 30 mg, 1 mL waters) previously conditioned with 1 mL of methanol and with 1 mL of water. After being loaded with the sample, the cartridges were washed twice with 0.5 mL of 40% aqueous methanol, and after the second wash, the cartridges were

flushed with an air push of 2 mL at 1 mL/min. The samples were eluted twice with 1,000 µL of methanol with an air push of 2 mL at 1 mL/min. The eluate was placed under vacuum until reaching dryness for up to 2 h and then was reconstituted in 100 µL of acetonitrile. The samples were then injected (1 µL) into the LC–MS/MS device.

LC–MS method. The analysis of sample extracts for AEA, PEA, OEA and 2-AG was performed using LC–MS/MS TQ (6,470, Triple Quad LC–MS Agilent Technologies, Santa Clara, California, EUA) with electrospray ionization and an Agilent jet stream. Separation for AEA, PEA and OEA was performed on an Agilent Poroshell 120 Phenyl-Hexyl, 4.6 × 50 mm; 2.7 µm, using water (A) and acetonitrile 0.1% formic acid (B) as the mobile phase and a stop time of 5 min. The separation for 2-AG was performed on a Waters XSelect CSH Phenyl-Hexyl, 3.5 µm, 4.6 × 50 mm column, using water 0.1% formic acid (A) and acetonitrile 0.1% formic acid (B) as the mobile phase and a stop time of 7 min. The flow rate was 0.5 mL/min, and samples were maintained at 4 °C throughout. The ionization mode was electrospray, polarity positive. Electrospray jetstream conditions were as follows: capillary voltage, 3,500 V; drying gas flow, 10 L/min nitrogen; drying gas temperature, 300 °C; nebulizer pressure, 30 psi; sheath gas temperature, 400 °C; and sheath gas flow, 11 L/min. The mass spectrometer was operated in the multiple reaction monitoring mode. The multiple reaction monitoring pair was m/z 326.5 → 62.1 for OEA; m/z 300.5 → 62.1 for PEA; m/z 328.5 → 62.1 for OEA-d2; m/z 304.5 → 62.1 for PEA-d4; m/z 348.3 → 62.1 for AEA; and m/z 356.6 → 62.1 for AEA-d8. The collision energy used for all compounds was 12 eV. For 2-AG, the multiple reaction monitoring pair was m/z 379.6 → 287.2 and m/z 387.6 → 294.3 for 2-AG-d8, with a collision energy of 14 eV. Peaks from standards and analyses were integrated using MassHunter Workstation software version B.04.00 (Agilent, Santa Clara, California, EUA), and the concentration of each compound was calculated using calibration curves of concentration against relative response. Together with the tissue samples, quality control (QC) samples were also extracted, evaporated and injected. A set of QC samples was placed at the beginning and at the end of the analytical run, demonstrating the good precision and accuracy of the overall process.

In plasma, the results are presented in pg/mL for AEA, PEA, OEA and 2-AG. The linearity ranged from 100 pg/mL to 10,000 pg/mL for AEA, PEA and OEA quantification and from 500 pg/mL to 50,000 pg/mL for 2-AG quantification. In human skin samples, the results are presented in pg/g, using a linear range from 100 pg/mL to 10,000 pg/mL for AEA quantification, from 200 pg/mL to 20,000 pg/mL for OEA and PEA quantification and from 1.0 ng/mL to 1,000 ng/mL for 2-AG quantification.

Statistical analyses. The sample size was determined using G Power (Version 3.1). We considered a 30% effect size on the primary outcome (concentration of plasma AEA) to be clinically relevant and estimated a 20% standard deviation from previously published data[66]. Furthermore, we decided that the ratio between the control and experimental group size would be 4:1, according to the natural occurrence of the phenomenon. Using these parameters, we obtained a total sample size of 48 subjects for a 5% significance level and a statistical power of 90%.

The results are presented as the mean ± SEM. Analyses were carried out with Prism 7 (Version 7.0; GraphPad Software, Inc.). The Kolmogorov–Smirnov test was used to test for normal distribution. To analyse individual changes over time points in skin samples, Student's paired t-test was used. To analyse differences between groups in skin samples, an unpaired t-test was used. Two-way ANOVA followed by Tukey's multiple comparison test was used to compare concentrations of plasma endocannabinoids and related NAEs in each group. For correlation analyses, Pearson r was calculated with a 95% confidence value, and a linear regression was fitted for data concerning AEA plasma and skin data. $p < 0.05$ was considered significant.

Statement of ethical approval. The study was approved by the Portuguese Institutional Review Board for Human Subjects (Comissão de Ética para a Saúde – Centro Hospitalar de São João) and carried out in accordance with principles of the Declaration of Helsinki as revised in 2001. All patients gave written informed consent to participate in this research.

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Conceptualization, I.B.C.-S. and M.A.V.-C.; Methodology, C.M.C., P.V.S., A.I.L. and C.F.-L.; Formal, Analysis, I.B.C.-S.; Writing—Original Draft Preparation, I.B.C.-S.; Writing—Review and Editing, I.B.C.-S., C.M.C. and M.A.V.-C.; Supervision, M.A.V.-C. and M.M.

Competing interests
The authors declare no competing interests.

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Discussion and Conclusions

Discussion

Our work intends to clarify the role of the endocannabinoid system in human wound healing. To answer this issue, this work was divided into two phases. Firstly, we established a role for CB1 and CB2 ligands *in vitro*, on human fibroblasts. Secondly, we expanded our knowledge regarding circulating and skin endocannabinoid responses during wound healing *in vivo*, on humans, focusing on those that developed hypertrophic scars.

In **chapter I** we reported, for the first time in literature, the role of CB1 and CB2 agonists and antagonists on primary cultures of human fibroblasts. To achieve this goal, our first task was to isolate human fibroblasts from abdominal skin samples from patients submitted to surgery. CB1 and CB2 receptors were then both identified in our cultures, by western blotting. To create a simplified *in vitro* experimental model of wound healing, we used TGF-β to stimulate our cultures and simulate the fibroblast behaviour in the context of wound healing. As already detailed in the **Introduction section**, TGF-β is a well-studied fibrotic cytokine (Brunner and Blakytny 2004) and a crucial regulator of fibroblast phenotype and function. It is responsible for fibroblast chemotaxis and proliferation, myofibroblast differentiation, and collagen (Sporn, Roberts et al. 1986, Lawrence and Diegelmann 1994), fibronectin and proteoglycan synthesis (Ignotz, Endo et al. 1987, Bassols and Massague 1988). TGF-β also has the ability to organize the ECM and is involved in scar remodelling and wound contraction (Fukamizu and Grinnell 1990). This makes TGF-β a central mediator of fibrogenesis (Biernacka, Dobaczewski et al. 2011). Moreover, TGF-β has been shown to be upregulated and activated in fibrotic diseases (Leask and Abraham 2004, Pohlers, Brenmoehl et al. 2009). Interestedly, we found that this cytokine, when added to primary cultures of human fibroblast, significantly increases the expression of both CB1 and CB2 receptors.

In the first study of **chapter I,** we studied CB1 receptor ligands in our *in vitro* experimental model of wound healing. We showed, for the first time, that CB1 receptor inactivation with AM251, a selective CB1 receptor antagonist, reduces primary human fibroblast differentiation into myofibroblast and collagen production induced by TGF-β, *in vitro*. These results are in accordance with previous reports, in which CB1 receptor inactivation was reported to reduce skin fibrosis in murine models of SSc (Marquart, Zerr et al. 2010, Palumbo-Zerr, Horn et al. 2012). However, our study gives new insight

information regarding the CB1 receptor role in human fibrosis. In fact, previous studies only report a murine model of SSc and inflammation (Marquart, Zerr et al. 2010, Palumbo-Zerr, Horn et al. 2012). Although both studies described a decrease in skin fibrosis after CB1 receptor pharmacological and/or genetic inactivation, as the primary endpoint, they both emphasize that this effect was dependent on the inflammatory response. In Marquart *et al.* work (Marquart, Zerr et al. 2010), authors even claim that the inactivation of the CB1 receptor does not prevent inflammation independent fibrosis and that the anti-fibrotic effect is due to leucocyte infiltration inhibition instead of a direct inhibitory effects on fibroblasts. Supporting this observation, they demonstrated that $CB1^{-}$ *knock-out* mice phenotype was mimicked by $CB1^{-/-}$ bone marrow transplantation into $CB1^{+/+}$ mice, and that $CB1^{-/-}$ *knock-out* TSK-1 mice were not protected from fibrosis. In our work, however, we clearly showed that AM251, a CB1 receptor antagonist, prevents fibroblast activation and collagen deposition induced by TGF-β, entirely independently of inflammation. Strengthening our results, we showed that the AM251 inhibitory effect on human fibroblast collagen production is drug concentration-dependent, with significant effect at 1µM and reaching a maximum at 10µM with an IC50 of 2.9µM.

Interestedly, in the same study, ACEA, a CB1 selective agonist, did not increase fibroblast differentiation in fibroblasts treated with TGF-β. As already discussed in our first paper, different hypothesis can be advanced to explain how AM251, the CB1 selective antagonist, can affect human fibroblast cultures without an opposite agonist response: 1) AM251 is now known to be an inverse agonist, instead of a pure selective antagonist (Pertwee 2005); 2) our primary cultures of human fibroblasts seem to be fully activated with the 10ng/mL TGF-β, and possible no further effect is observed with ACEA treatment; 3) other transducing systems are involved in cannabinoid activity of AM251 as inhibition of ERK signaling (Garcia-Gonzalez, Selvi et al. 2009), inhibition of the induction of profibrotic transcription factors such as SNAIL1, and the AP-1 transcription factors FOSB and JUNB (Yoshinaga, Uwabe et al. 2016), or activation of GRP55 (Pertwee 2005, Yang, Zhou et al. 2016). Of note, that ACEA significantly increased collagen deposition in our model, supporting, the pro-fibrotic effect of CB1 receptor activation.

Lastly, we tested these compounds in a human *ex vivo* skin culture model (Xu, Jong Hong et al. 2012). Although descriptive, this technique allowed us to confirm our results in a more complex model of human wound healing. A full-thickness human skin

graft was wounded and cultured in the liquid-air interphase for 9 days. ACEA treated skin grafts were totally re-epithelized after the experiments, whereas AM251 treated skin grafts still presented a break of the epithelium. This not only confirms our results, but also points that further pre-clinical and clinical studies should be conducted, regarding the role of CB1 ligands on skin wound healing.

Still in **chapter I**, when we focus on experiments with CB2 ligands intriguing results were obtained. Once more we have shown that this receptor is expressed in primary cultures of human fibroblasts and it has a role in response to fibrosis modulation. As stated above, TGF-β increased CB2 receptor expression in our cultures. Previous studies showed that CB2 receptor activation decrease inflammation and leukocyte infiltration, followed by a reduction in skin fibrosis (Akhmetshina, Dees et al. 2009, Servettaz, Kavian et al. 2010, Balistreri, Garcia-Gonzalez et al. 2011, del Rio, Navarrete et al. 2016, Wang, Zhao et al. 2016, Del Rio, Cantarero et al. 2018, Garcia-Martin, Garrido-Rodriguez et al. 2018, Garcia-Martin, Garrido-Rodriguez et al. 2019). As for the CB1 receptor, most of the studies focusing on CB2 are conducted in animal models of inflammation rather than on human models of wound healing. Scarce information is known from research conducted with human fibroblasts treated with CB2 agonists (Servettaz, Kavian et al. 2010), and most of this information is obtained for drugs capable of PPAR-γ and CB2 dual activation rather than for CB2 pure agonists (del Rio, Navarrete et al. 2016, Del Rio, Cantarero et al. 2018). In agreement with previous reports, however, in our experiments JWH133, a selective CB2 receptor, prevented α-SMA expression and collagen deposition induced by TGF-β in our simplified model of human wound healing. Curiously, this effect was not observed in non-stimulated cells. Since TGF-β increased CB2 receptor expression in our cells, it is clear that the JWH133 effect only occurs in stimulated cells, with higher CB2 receptor density.

This agonist effect was not, however, antagonized when adding AM630, a CB2 antagonist. In fact, further reduction in α-SMA expression and collagen deposition was observed in our cells when adding AM630. Different from the agonist, this effect was observed in both TGF-β stimulated and non-stimulated cells. We suggested that other receptor or transduction pathways, rather than the CB2 receptor, are responsible for this response. TRPA1 and TRPV1 ion-channels may be potential targets for this effect. AM630 has been shown to activate TRPA1 when the channel is co-expressed with TRPV1 (Patil, Patwardhan et al. 2011) and TRPA1 has been identified as a potential therapeutic target in renal fibrosis via inhibition of TGF-β/SMAD signalling (Wang and

Wang 2011). Intriguingly, there is a lack of information regarding CB2 receptor inactivation on human wound healing or fibroblast activity. Most of the previous studies focus their experiments on the agonist outcome. Garcia-Gonzales *et al* and co-workers (Garcia-Gonzalez, Selvi et al. 2009) were not able to block the WIN55,212-2 effect in their SSc and healthy fibroblast cultures with AM630. Enea Lazzerini *et al.* (Lazzerini, Natale et al. 2012), on the other way, showed an inconsistent effect of AM630 on collagen production in SSc fibroblasts with WIN55,212-2 and the selective A2A receptor antagonist ZM-241385. In their study, as in ours, AM630 also induced a reduction of collagen production but only at higher concentrations (80–20μM). AM630 produced no changes in intermediate concentrations (10μM–5nM) but increases collagen deposition at lower concentrations (1–0.1nM). Strengthening our results regarding the effect of AM630 on collagen production, we also obtained a Concentration – Response curve. In our cells, AM630 significantly decreases collagen deposition when added at concentrations ≥3μM, reaching a maximum at the concentration of 100μM. The IC50 for AM630 on collagen production is 11.0μM.

Of note that in our fibroblast cell culture, neither CB1 nor CB2 agonists and antagonists or TGF-β treatments had an effect on viability or vimentin expression.

Afterwards, we decided to study inflammation and cannabinoid system *in vivo* (**Chapter II**). For that, we conducted two prospective clinical studies. In the first phase, we evaluated the inflammatory background of patients submitted to body contouring surgery and analyzed existing disparities between patients with different scars (hypertrophic and normal scars). Additionally, we also measured plasmatic vitamins A, D and E in those patients. This topic has arisen as an important issue to be addressed since inflammation is an important component of wound healing (Broughton, Janis et al. 2006) and for hypertrophic and keloid scar development as we detailed described above (Andrews, Marttala et al. 2016, Ogawa 2017).

In this first study, we found that patients that developed hypertrophic scars after surgery presented, immediately before surgery, lower levels of circulating neutrophils but higher levels of monocytes, eosinophils, basophils and C-reactive protein (CRP). Blood samples collected five days after surgery, aligned with the inflammatory phase of wound healing, revealed lower levels of circulating neutrophils and leukocytes but higher circulating levels of basophils, lymphocytes and eosinophils, in that group of patients. Five days after, CRP increased in both groups. Interestingly, hydroxyvitamin D (25(OH)D) plasma levels were preoperatively reduced by almost 50% in our patients that

developed hypertrophic scars. The differences in the inflammatory background of those patients may explain, at least in part, the different scar outcome. We hypothesized that those patients may present a preoperative systemic pro-inflammatory state that predisposes them to a longer or excessive inflammatory phase of wound healing. A higher number of lymphocytes may turn surgical patients more susceptible for an excessive inflammatory phase (as these cells are responsible to tissue monocyte – macrophages transformation), and an exuberant proliferation phase (lymphocytes peak in the wound at day 7), with more fibroblast migration, collagen deposition and epithelization (Broughton, Janis et al. 2006).

Vitamin D, on the other hand, is a fat-soluble vitamin produced in the skin after ultraviolet B light exposure, and obtain in the diet (Ross 2014). Apart from other organs, the skin is a target of vitamin D action (Reichrath, Lehmann et al. 2007). Vitamin D is a powerful anti-inflammatory agent (Harant, Wolff et al. 1998), and its analogues have been used in the treatment of hyperproliferative and inflammatory skin diseases as psoriasis atopic dermatitis and allergic contact dermatitis (Kragballe, Gjertsen et al. 1991, Ashcroft, Po et al. 2000, Zugel, Steinmeyer et al. 2002). This vitamin is responsible for modifying the expression of different GF, cytokines and mediators of inflammation (Zhang, Maruyama et al. 1995, Harant, Wolff et al. 1998, Inoue, Matsui et al. 1998, Gurlek, Pittelkow et al. 2002, Adorini, Penna et al. 2003, Wang, Nestel et al. 2004, Schauber and Gallo 2007). This hormone has also been shown to be essential for wound healing in the skin: patients with higher levels of vitamin D revealed better healing ability in different studies (Heilborn, Weber et al. 2010, Burkiewicz, Guadagnin et al. 2012, Zubair, Malik et al. 2013). In other organs, vitamin D deficiency has also been associated with fibrosis (Weishaar, Kim et al. 1990, Li, Spataro et al. 2005, Tan, Li et al. 2006, Ramirez, Wongtrakool et al. 2010, Zhang, Cheng et al. 2011, Johnson, Sauder et al. 2012). The markedly reduced 25(OH)D plasma levels observed in our group of patients with hypertrophic scars, could be a predisposing factor for developing those scars. Of note, the Portuguese population in our study showed overall deficient levels of vitamin D. From the 63 patients included, only 17.4% presented normal levels of this hormone (>30 ng/dL), and none of those developed hypertrophic scars. Finally, we did not find any relation between vitamin A and E and wound healing. It would be interesting to measure 25(OH)D levels in the skin and normal and hypertrophic scars, as we did for endocannabinoids in the second phase of **Chapter II**, and determine possible correlations between circulating and local levels of this vitamin. Unfortunately, we do not have the

necessary resources to proceed with this quantification, and it was also not the main objective of our work. We are, however, the first linking hypertrophic skin wound healing and reduced circulating levels of vitamin D. Topical or systemic vitamin D supplementation could be an interesting approach to treat this fibrotic condition.

In a second phase of **Chapter II**, we investigated the endocannabinoid system in plasma, in skin and scars during wound healing in humans. For that purpose, a prospective study was developed, involving 50 patients submitted to body countering surgery in the Department of Plastic, Reconstructive and Aesthetic Surgery. This is the first study in literature measuring endocannabinoid levels in the context of wound healing and surgery. It is also the first work quantifying endocannabinoid levels in human skin.

When we focus on the data obtained from skin, 2-AG was found to be the most abundant endocannabinoid, followed by OEA, PEA and AEA. The relative proportion of endocannabinoids that we found in the human skin (2-AG>OEA>PEA>AEA) is similar to previous results with human samples, namely uterus and plasma (Ayakannu, Taylor et al. 2019, Hanlon 2020), but different ratios were described as well in liver (Yang, Tian et al. 2019) and plasma (van Eyk, van Schinkel et al. 2018). Although we also observed this sequence (2-AG>OEA>PEA>AEA) in plasmatic levels in our population, the relative amounts of cannabinoids were different, as we discuss later. In skin, 2-AG levels are 100-fold higher than AEA and 5-fold higher than OEA and PEA. No significant differences were found in skin endocannabinoid levels between patients that later developed normal and hypertrophy scar.

Remarkable results were also observed from endocannabinoid quantification in scar samples. Curiously, endocannabinoid levels in scar tissue showed to be in the same range of those found in the normal skin, demonstrating the presence of these active molecules in scars. AEA was, however, significantly reduced in hypertrophic scars when compared to normal scars. This finding supports the hypothesis of a significant role for AEA in the pathophysiological process of skin fibrosis. As pointed before in this dissertation, endocannabinoids are generally considered to be anti-inflammatory agents (Turcotte, Chouinard et al. 2015). More specifically, AEA was shown to suppress the production and release of key Th1- and Th17-polarizing cytokines (IL-12 and IL-23) via CB1-mediated inhibition of the mammalian target of rapamycin (mTOR) in human keratinocytes (Chiurchiu, Rapino et al. 2016). Hypertrophic scars and keloids, on the other hand, are believed to occur in an increased and prolonged inflammatory environment (Castagnoli, Trombotto et al. 1997, Niessen, Schalkwijk et al. 2004). We

suggest that reduced AEA levels in hypertrophic scars may be related to increased inflammation or a prolonged inflammatory phase that will predispose to this condition. In fact, in our first phase of **Chapter II**, we already observed an increased inflammatory background in patients that developed hypertrophic scars, supporting this assumption. Conflicting with our results, Palumbo-Zerr *et al* (Palumbo-Zerr, Horn et al. 2012) found that FAAH inactivation exacerbates experimental skin fibrosis in mice, through CB1 receptor activation. FAAH is responsible for AEA and other NAEs metabolism and its inhibition will locally increase this endocannabinoid concentration (Cravatt, Giang et al. 1996). As in results obtained by us in **Chapter I**, the authors state that CB1 receptor activation exerts a pro-fibrotic effect, and that this effect is avoided by CB1 inactivation. In our **Chapter II** study, however, increased local levels of AEA, seem to protect, rather than predispose, from fibrosis. We point three hypothesizes that, altogether, could justify the differences obtained from Palumbo-Zerr *et al* publication. 1) Our results were obtained from humans, and Palumbo-Zerr *et al* from mice. 2) In humans, the anti-inflammatory action of AEA on the skin is potentially more important than the CB1 activation pro-fibrotic effect. 3) AEA is a CB1 receptor partial agonist; in hypertrophic scar tissue, reduced levels of AEA will leave more unoccupied CB1 receptors; 2-AG showed, in our study, to be 100-fold higher in the skin than AEA and is an agonist with greater potency and efficacy than AEA for CB1 receptor (Pertwee, Howlett et al. 2010); AEA increased levels may block CB1 receptors for 2-AG activity and reduce fibrosis; on the other hand, with reduced levels of AEA, 2-AG will be able to activate more CB1 receptors, increasing collagen deposition and fibroblast differentiation and predispose individuals for hypertrophic scars. It would be interesting to measure FAAH activity in both hypertrophic and normal scars. More studies are needed to confirm this hypothesis but, if corroborated, topical administration of AEA, other non-psychotropic cannabinoids or FAAH inhibitors could be an interesting tool to treat or prevent this condition, maybe taking advantage of a dual AEA action: anti-inflammatory and anti-fibrotic.

We also collected blood samples for every studied subject before wounding and after wounding in three different times, corresponding to the different phases of wound healing, namely: 5 days for inflammation, 12 days for proliferation and 3 months after surgery/wounding for remodeling. Endocannabinoid levels were then measured in different moments. Although no differences were observed at any time between the two different studied groups (normal or hypertrophic scar group), a systematic pattern fluctuation on all the endocannabinoid levels was observed. While AEA and NAEs levels

presented a U shape after surgery, 2-AG levels showed to be higher before and 5 days after surgery, and lower 12 days and 3 months after surgery. Different metabolic pathways, already detailed, between AEA and NAEs and 2-AG may explain these differences. We further suggest that the decrease in circulating NAEs 5 days and 12 days after surgery could reflect an increase in their demanding for local inflammatory responses to skin injury. On the other hand, variance in 2-AG cannot be attributed to wound healing or a specific surgery, since most differences were obtained before any surgical procedures, except venous punting. The extremely high variability between patients observed in 2-AG levels at t0 and t5 can also be justified by acute stress, anxiety and sleep disorder caused by hospitalization and surgery (Jumpertz, Wiesner et al. 2010). Lastly, we analyzed possible correlations between endocannabinoid plasma and skin levels. A positive correlation was found between AEA in patients that later developed

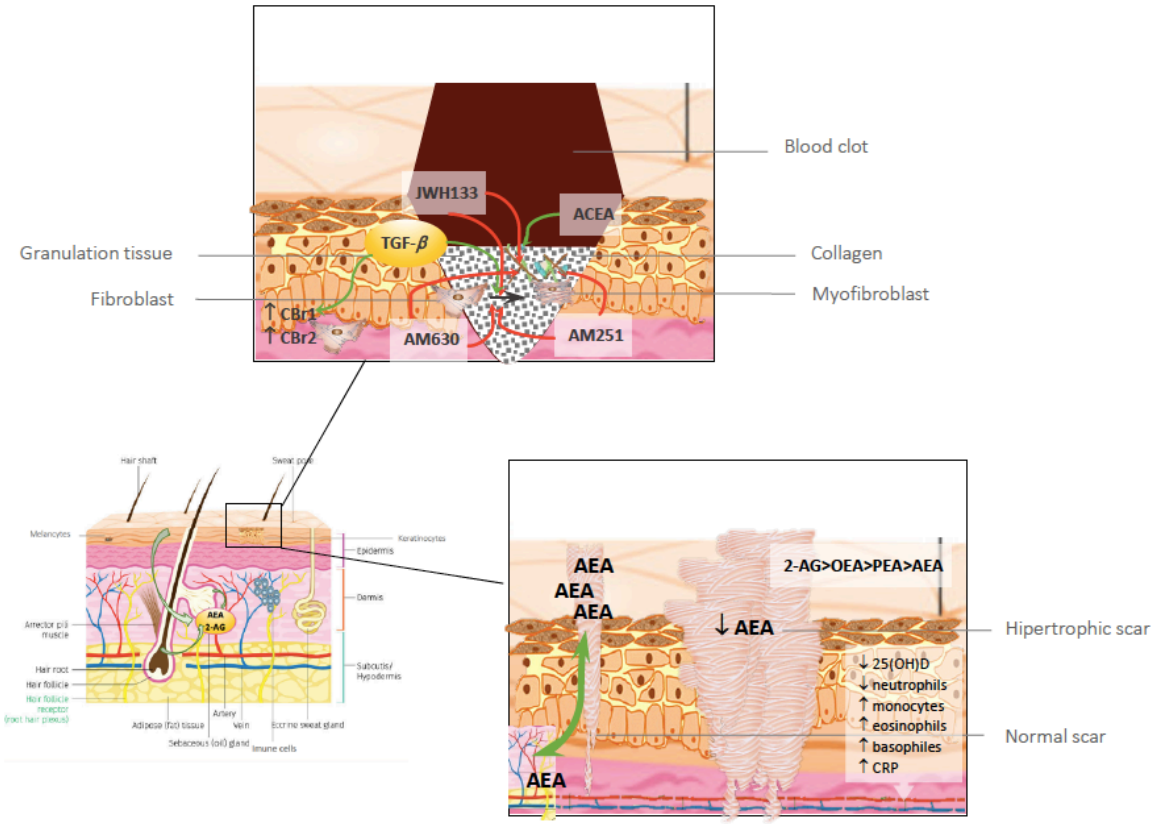

Figure 4. Major findings in our studies. Red arrows represent negative stimulus and green arrows represent positive stimulus. Transforming growth factor beta (TGF-*β)* increases cannabinoid 1 and cannabinoid 2 receptors (CBr1 and CBr2) expression on primary cultures of adult fibroblasts. CB1 receptor pharmacological inactivation with AM251 reduces fibroblast differentiation into myofibroblast and collagen production. Collagen production is increased by CB1 receptor activation with ACE JWH133 (a CB2 receptor agonist) and AM630 (a CB receptor antagonist) reduce fibroblast differentiation and collagen deposition. Patients with hypertrophic scar present lower hydroxyvitamin D (25(OH)D) plasma levels and lower circulating levels of neutrophils but higher levels of eosinophils, basophils and CRP before surgery. 2-Arachidonoylglycerol (2-AG) is the most abundant endocannabinoid in human skin (2-AG>oleoylethanolamine (OEA)> palmitoylethanolamide (PEA)> anandamide (AEA)). AEA is significantly reduced in hypertrophic scars when compared with normal scars. There is a positive correlation between circulating endocannabinoids and the skin AEA levels in patients that develop normal scars, lost in patients with hypertrophic scars.

normal scars, but not in patients with hypertrophic scars neither for the other endocannabinoids studied. We proposed that, concerning AEA, there may be a link between both endocannabinoid systems (skin and systemic), and that this link is lost in patients that developed hypertrophic scars. Moreover, when calculating the ratio between skin and plasma endocannabinoid levels, we found that, although the sequence in relative proportion is maintained (2-AG>OEA>PEA>AEA), levels are different for endocannabinoids between both systems, but not for AEA. More detailed, 2-AG is 40-fold higher in the skin than in plasma but AEA levels are very similar in those two tissues. Taken together, it seems that AEA shares the same origin in both skin and systemic systems and, in contrast, 2-AG appears to be under a distinct local control. We propose a new role for AEA with crosstalk between systemic and local skin endocannabinoid systems during human wound healing, defective in the hypertrophic wound.

Taken together, data obtained in **Chapter I** and **Chapter II**, point a role for the endocannabinoid system in human wound healing. The main findings of our study are represented in **Figure 4**. Both CB1 and CB2 receptors affect fibroblast activity and their ability to produce collagen. On the other hand, endocannabinoids, namely AEA and NAEs undergo systematic fluctuation patterns after surgery and during wound healing. AEA levels are reduced in hypertrophic scars and a positive correlation is found between plasmatic and skin levels of this cannabinoid in patients that later developed a normal scar. Skin excessive wound healing is a topic of concern for the medical community in general, but particularly for Plastic Surgeons that have to deal with the consequences of excessive scaring in their everyday practice. Hypertrophic scars and keloids frequently lead to cosmetic and functional impairment complaints from patients, seeking for immediate and successful treatment for those conditions (Slemp and Kirschner 2006). As described before, although several therapies have been proposed, most of them present unacceptably high recurrence rates (Gauglitz, Korting et al. 2011). Our findings suggest that the cannabinoid system may be an interesting target to treat or to prevent these skin fibrotic diseases. CB1 receptor antagonist, AM251, seems to be the more promising candidate as it is able to reduce fibroblast differentiation and collagen deposition in a concentration-dependent manner, with no cytotoxic effects on fibroblasts.

In the past, rimonabant, a CB1 selective antagonist, was already approved to treat obesity and metabolic syndrome. But, due to substantial psychiatric adverse effects, it

was later withdrawn from the market (Cheung, Cheung et al. 2013). Randomized clinical trials with cannabinoid drugs administration for treatment of various dermatological disorders are already been conducted (Milando and Friedman 2019, Toth, Adam et al. 2019), and a few compounds based on THC, like Marinol®, Cesamet®, and Sativex®, have already been FDA approved for limited indications (Eagleston, Kalani et al. 2018).

The low selectivity of the cannabinoid ligands available and the marked differences in the ability of certain agonists to activate distinct signaling pathways depending on the cell type or receptor conformation (Peters and Scott 2009), were the major difficulties in our study. It certainly makes it challenging to study cannabinoid effects on multilayer models as in the skin.

Our work definitely encourages further explore cannabinoids effects in human skin, namely as an adjunct treatment strategy for hypertrophic scars or other wound healing disorders. Topical administration on the skin of cannabinoids with high tolerance rates and low psychotropic or other side effects (Grotenhermen and Muller-Vahl 2012, Fraguas-Sanchez and Torres-Suarez 2018, Pergolizzi, Lequang et al. 2018), will assuredly allow other pre-clinical and clinical studies, on the use of cannabinoids on the treatment of wound healing and fibrotic diseases.

Conclusions

1. Adult human fibroblasts express cannabinoid 1 and cannabinoid 2 receptors.

2. TGF-β increases CB1 and CB2 receptor expression on primary cultures of adult human fibroblasts.

3. CB1 receptor activation increases collagen production. CB1 receptor pharmacological inactivation, on the other hand, reduces *in vitro* primary human fibroblast differentiation into myofibroblast and collagen production induced by TGF-β.

4. Experiments in human *ex vivo* skin culture confirmed that CB1 receptor activation speeds re-epithelization and wound healing.

5. CB2 receptor activation reduces *in vitro* α-SMA expression and collagen deposition induced by TGF-β, in primary cultures of adult human fibroblasts but it does not affect non-stimulated cells.

6. Unexpectedly, inactivation of the CB2 receptor with AM630 reduces α-SMA expression and collagen deposition in adult human fibroblasts treated and non-treated with TGF-β. This effect is concentration-dependent, for concentrations $\geq$3µM. This suggests that other cannabinoid related receptors or transduction pathways, as the TRPA1 and TRPV1 may be involved in the anti-fibrotic effect of this system.

7. The inflammatory background is different in patients with normal and hypertrophic scars. Patients with hypertrophic scars present a distinctive plasmatic inflammatory profile before and five days after surgery.

8. 25(OH)D plasma levels are preoperatively reduced by almost 50% in patients that developed hypertrophic scars. Portuguese population studied, show overall deficient levels of vitamin D: only 17.4% of studied subjects presented 25(OH)D

normal levels (>30 ng/dL), and none of those developed hypertrophic scars. No relation between vitamin A and E and wound healing is found.

9. Bariatric surgery has no impact on vitamin A, E and 25(OH)D or in wound outcome in the population studied.

10. Endocannabinoids (AEA, PEA, OEA and 2-AG) were quantified for the first time in skin. 2-AG is the most abundant endocannabinoid in human skin, with 100-fold higher levels than AEA and 5-fold higher levels than PEA and OEA (2-AG>OEA>PEA>AEA). We find no differences in endocannabinoid skin levels, in patients that develop normal or hypertrophic scars but a large individual variability between subjects is observed.

11. Scars have endocannabinoids in the same range levels than normal skin. AEA is significantly reduced in hypertrophic scars when compared to normal scars. We suggest a role for AEA in the pathophysiological process of wound healing.

12. Plasma endocannabinoid levels are no different, at any time before or after surgery, between the two different studied groups. However, a systematic pattern fluctuation on all the endocannabinoid levels is observed: while AEA and NAEs presented a U shape after surgery, 2-AG shows a different pattern, raised and extremely variable in the first-week surgery but reduced and stabilized 3 months later.

13. There is a positive correlation between circulating endocannabinoids and the skin AEA levels in patients that develop normal scars. This correlation is lost in patients with hypertrophic scars. Additionally, skin and plasma AEA levels are very similar, while there are marked differences for other endocannabinoids levels in those systems (e.g. 2-AG is 40-fold higher in the skin than in plasma). We suggest a possible link for AEA between local and systemic endocannabinoids.

14. We propose a new role for AEA, with crosstalk between systemic and local skin endocannabinoid systems during human wound healing, defective in hypertrophic wounding.

Summary
Resumo

Summary

Skin wound healing is a dynamic and highly regulated physiological process, responsible for the reconstruction of a wounded area. In pathologic scenarios, an exuberant healing response, characterized by an overabundant accumulation of extracellular matrix (ECM) components, especially collage can occur, leading to hypertrophic scars or keloids. In the present work, cannabinoid 1 (CB1) and cannabinoid 2 (CB2) receptors and its response to stimulation with the multifunctional cytokine transforming growth beta (TGF-β), in primary cultures of adult human fibroblasts, are described.

We report that TGF-β increases the expression of CB1 and CB2 receptors in cultures of human fibroblasts. Moreover, CB1 receptor activation increases collagen production induced by TGF-β, *in vitro*, in the same cellular cultures. CB1 receptor pharmacological inactivation, on the other hand, reduces fibroblast differentiation into myofibroblast and collagen production. Corroborating these results, CB1 receptor activation enhances re-epithelization and wound healing in human *ex vivo* skin cultures.

Conversely, CB2 receptor activation reduces alfa smooth actine (α-SMA) expression and collagen deposition induced by TGF-β, in human fibroblasts but it does not affect non-stimulated cells. Inactivation of the CB2 receptor with AM630 also reduces α-SMA expression and collagen deposition in adult human fibroblasts but contrasting with the agonist, these results are observed in both TGF-β stimulated and non-stimulated cells.

Our findings suggest that the endocannabinoid system has a role on fibroblast activity during wound healing, through CB1 and CB2 receptor but that other cannabinoid related receptors or transduction pathways, as the transient receptor potential ankyrin (TRPA) 1 or transient receptor potential vanilloid (TRPV) 1 may also be involved in the anti-fibrotic effect of this system.

We also investigated the inflammation and endocannabinoid system during wound healing and in response to surgery *in vivo*. For that, patients submitted to body contouring surgery were selected.

In the population studied, we show that the inflammatory background at the time of surgery is different in patients that later develop normal and hypertrophic scars: hypertrophic scar patients present lower levels of circulating neutrophils but higher levels of eosinophils, basophils and CRP before surgery, and lower levels of circulating

neutrophils and leukocytes but higher levels of basophils, lymphocytes and eosinophils five days after surgery. We hypothesized that those patients may present a preoperative systemic pro-inflammatory state that predisposes them to a longer or excessive inflammatory phase of wound healing and hypertrophic scar formation. CRP is increased after surgery in both groups. Hydroxyvitamin D (25(OH)D) plasma levels are also preoperatively reduced by almost 50% in patients that develop hypertrophic scars. Portuguese population, show overall deficient levels of vitamin D in this study: only 17.4% of studied subjects presented 25(OH)D normal levels (>30 ng/dL), and none of those developed hypertrophic scars.

We quantify, for the first time, endocannabinoids (anandamide (AEA), palmitoylethanolamide (PEA), oleoylethanolamine (OEA) and 2-arachidonoylglycerol (2-AG) in human skin. We demonstrated that 2-AG is the most abundant endocannabinoid in human skin, with 100-fold higher levels than AEA and 5-fold higher levels than PEA and OEA (2-AG>OEA>PEA>AEA). However, no differences in endocannabinoid plasmatic or skin levels, in patients that develop normal or hypertrophic scars are found. AEA is, however, reduced in hypertrophic scars when compared to normal scars. Additionally, a positive correlation between plasma and skin AEA levels exists in the normal group that was absent in the hypertrophic group.

Interestingly, in all patients, the surgery intervention produced a time-dependent effect with a U shape for AEA, PEA and OEA plasma levels. In contrast, 2-AG plasma levels were higher and extremely variable in the first-week surgery but reduced and stabilized 3 months later. We propose a new role for AEA, with crosstalk between systemic and local skin endocannabinoid systems through human wound healing, defective in hypertrophic healing.

In conclusion, the results obtained demonstrate that the endocannabinoid system activity as a role in skin human wound healing.

Resumo

A cicatrização é um processo fisiológico dinâmico e altamente regulado, responsável pela reconstrução ou substituição de tecidos lesados. Em cenários patológicos, uma resposta cicatricial exuberante, caracterizada por acumulação excessiva de componentes da matriz extracelular nomeadamente colagénio pode ocorrer, conduzindo à formação de cicatrizes hipertróficas ou quelóides. Neste trabalho descreve-se a presença de receptores canabinoides (CB1 e CB2) em culturas primárias de fibroblastos de humanos adultos, e a sua resposta à estimulação com a citocina multifuncional transforming growth beta (TGF-β).

Verificou-se que o TGF-β aumenta a expressão de receptores CB1 e CB2 em culturas de fibroblastos humanos. Adicionalmente demonstrou-se que a activação do receptor CB1 induz a produção de colagénio induzida pelo TGF-β, *in vitro*, nestas culturas celulares. Por outro lado, a inativação farmacológica do receptor CB1 reduz a diferenciação dos fibroblastos em miofibroblastos e a produção de colagénio. A activação do receptor CB1 aumenta ainda a reepitelização e a cicatrização em culturas de pele humanas *ex vivo*.

Em contrapartida, a activação do receptor CB2 em fibroblastos humanos, reduz a expressão da proteína alfa smooth actine (α-SMA) e a deposição de colagénio induzida pelo TGF-β, mas não altera a actividade de células não estimuladas. A inactivação do receptor CB2 com o antagonista selectivo AM630 também reduz a expressão de α-SMA e a produção de colagénio, mas, diferente do efeito do agonista, este efeito é observado em células estimuladas e não estimuladas pelo TGF-β.

Estas observações sugerem que o sistema endocanabinoide tem um papel na actividade dos fibroblastos durante a cicatrização, através dos receptores CB1 e CB2, mas que outros receptores ou vias de sinalização relacionados com os canabinoides, como o transient receptor potential ankyrin (TRPA) 1 ou o transient receptor potential vanilloid (TRPV) 1 poderão também estar envolvidos no efeito anti-fibrótico deste sistema.

De seguida analisou-se a resposta inflamatória e o sistema endocanabinoide durante a cicatrização e em resposta à cirurgia, *in vivo*. Para isso foram selecionados doentes submetidos a cirurgia de contorno corporal.

Na população estudada, observou-se que o perfil inflamatório no momento da cirurgia é diferente em doentes que vieram a desenvolver cicatrizes normais e cicatrizes hipertróficas: doentes com cicatrizes hipertróficas apresentam níveis circulatórios reduzidos de neutrófilos, mas aumentados de eosinófilos e basófilos e de Proteína C

Reactiva (CRP), em colheitas realizadas imediatamente antes da cirurgia. Estes indivíduos apresentam adicionalmente níveis reduzidos de neutrófilos circulantes, mas níveis mais elevados de basófilos, linfócitos e eosinófilos em colheitas realizadas cinco dias após a cirurgia. Colocou-se a hipótese de que estes doentes poderão apresentar um estado de inflamação sistémico pré-operatório, que os predispõe a uma fase inflamatória da cicatrização mais longa ou exuberante, e à formação de cicatrizes hipertróficas. Os níveis de CRP aumentaram 5 dias após a cirurgia em ambos os grupos estudados. Os indivíduos que desenvolveram cicatrizes hipertróficas apresentam também níveis plasmáticos pré-operatórios de hidroxivitamina D (25(OH)D) reduzidos em cerca de 50%. A população portuguesa estudada mostrou-se, de uma forma geral, deficiente em vitamina D: apenas 17.4% dos indivíduos estudados apresentam níveis considerados normais de 25(OH)D (>30 ng/dL), e nenhum destes indivíduos desenvolveu cicatrizes hipertróficas.

Foram quantificados, pela primeira vez, endocanabinoides (anandamida (AEA), palmitoylethanolamida (PEA), oleoylethanolamina (OEA) e 2- arachidonoylglycerol (2-AG)) na pele humana. Demonstrou-se que o 2-AG é o endocanabinoide mais abundante na pele, com níveis 100 vezes superiores aos da AEA e 5 vezes superiores aos do PEA e do OEA (2-AG>OEA>PEA>AEA). Não foram encontradas, no entanto, diferenças significativas nos níveis de endocanabinoides plasmáticos ou cutâneos entre doentes que desenvolveram cicatrizes normais e cicatrizes hipertróficas. A AEA está, no entanto, reduzida em tecido de cicatriz hipertrófica em relação ao tecido cicatricial normal. Adicionalmente, existe uma correlação positiva entre os níveis plasmáticos e cutâneos de AEA nos doentes do grupo controlo, e esta correlação está ausente no grupo de cicatrizes hipertróficas.

Surpreendentemente, em todos os pacientes, a intervenção cirúrgica produziu uma curva em U, para as determinações de níveis plasmáticos AEA, PEA e OEA, realizadas nos diferentes tempos. Por outro lado, observaram-se níveis elevados e extremamente variáveis de 2-AG antes e após a primeira semana de cirurgia, e níveis reduzidos e estáveis 3 meses após a cirurgia. Propomos assim um novo papel para a AEA, com um "sistema de comunicação" entre os sistemas endocanabinoides sistémico e local durante a cicatrização humana. Esta "comunicação" é inexistente em situações de cicatrização hipertrófica.

Em conclusão, os resultados aqui apresentados demonstram que o sistema endocanabinoide tem um papel na cicatrização em humanos.

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