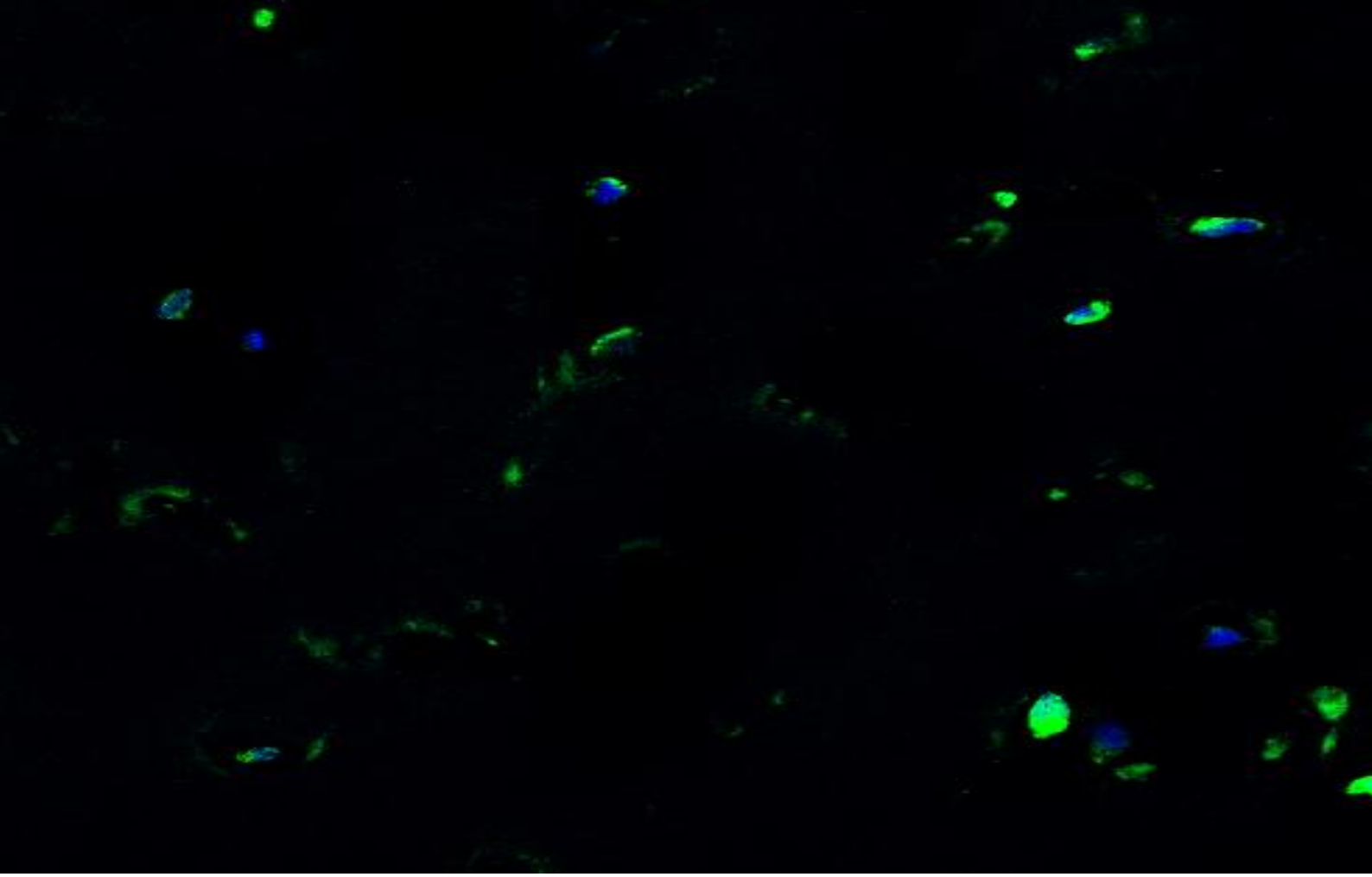

Ana Teresa Oliveira Rufino

# GLUCOSE SENSING AND MODULATION OF HUMAN CHONDROCYTE FUNCTIONS BY HYPERGLYCEMIA: RELEVANCE AS PHARMACOLOGICAL TARGETS FOR DIABETES–ASSOCIATED OA

Tese de Doutoramento em Farmácia, Especialidade de Farmacologia e Farmacoterapia, orientada por
Alexandrina Maria Ferreira dos Santos Pinto Mendes e Carlos Manuel Freire Cavaleiro
e apresentada à Faculdade de Farmácia da Universidade de Coimbra

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UNIVERSIDADE DE COIMBRA

# GLUCOSE SENSING AND MODULATION OF HUMAN CHONDROCYTE FUNCTIONS BY HYPERGLYCEMIA: RELEVANCE AS PHARMACOLOGICAL TARGETS FOR DIABETES – ASSOCIATED OA

Dissertação apresentada à Faculdade de Farmácia da Universidade de Coimbra para prestação de Provas de Doutoramento em Farmácia, na Especialidade De Farmacologia e Farmacoterapia

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Ana Teresa Oliveira Rufino

Universidade de Coimbra

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Capa: Imagem de microscopia confocal de ensaios de imunofluorescência de condrócitos articulares humanos com ampliação de 400x

A verde marcação para a proteína Kir6.2 que faz parte da composição dos canais de potássio dependentes de ATP e a azul núcleos corados com DAPI.

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**ABBREVIATIONS**

**2-DG:** 2-Deoxy-D-Glucose
**ADAMTS:** A Desintegrin and Metalloproteinase with TromboSpondin Motif
**ADAM:** A Desintegrin and Metalloproteinase
**ADP:** Adenosine diphosphate
**AGEs:** Advanced Glycation End Products
**AP-1:** Activation Protein-1
**ATF-2:** Activating Transcription Factor 2
**ATP:** Adenosine triphosphate
**Bay:** Bay-11-7082
**BMP-2:** Bone Morphogenic Protein 2
**BSA:** Bovine Serum Albumin
**COMP:** Cartilage Oligomeric Matrix Protein
**CS:** Chondroitin Sulphate
**DAPI:** 4',6-diamidino-2-phenylindole
**DM:** Diabetes Mellitus
**DMEM:** Dulbecco's Modified Eagle Medium
**DMOAD:** Disease Modifying Osteoarthritis Drugs
**DMSO:** Dimethilsulphoxide
**ECM:** Extracellular Matrix
**ELF3:** E74-Like Factor
**ELK-1:** ETS domain-containing protein
**EMA:** European Medicine Agency
**eNOS:** Endothelial form of Nitric Oxide Synthase
**EO:** Essential oil
**ERK:** Extracellular regulated Kinase
**EULAR:** European League Against Rheumatism
**FBS:** Fetal Bovine Serum
**FDA:** Food and Drug Administration
**GAG:** Glycosaminoglycans
**GlcN:** Glucosamine
**GLUT:** Glucose transporter
**HA:** Hyaluronic Acid
**HIF-2:** Hypoxia- inducible Factor 2
**ICE:** Interleukin-1 Coverting Enzyme (caspase-1)

**IGF-1:** Insulin Growth factor-1

**IKK:** IκB Kinase

**IL:** Interleukin

**IL-1β:** Interleukin-1β

**iNOS:** Inducible form of Nitric Oxide Synthase

**IκBα:** nuclear factor kappa B inhibitor, alpha

**JNK:** c-Jun NH2-terminal Kinase

**K (ATP) channel:** ATP-sensitive potassium channel

**LPS:** Lipopolissacaride

**MAPK:** Mitogen Activated Protein Kinase

**MEF-2:** Myocyte Enhancer Factor-2

**MKK:** Mitogen Activated Protein Kinase Kinase

**MMPs:** Matrix Metalloproteinases

**MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

**NF-κB:** Nuclear Factor κB

**NLS:** Nuclear Localization Sequence

**nNOS:** Neuronal form of Nitric Oxide Synthase

**NO:** Nitric Oxide

**NSAIDS:** Non- Steroidal Anti-inflammatory drugs

**OA:** Osteoarthritis

**OARSI:** Osteoarthritis Research Society International

**OCT:** Optimal Cutting Temperature compound

**PAGE:** Poliacrilamide gel Electrophoresis

**PBS:** Phosphate Buffered Saline

**PGE2:** Prostaglandin E2

**PI9:** Protease Inhibitor 9

**PRAC:** Pharmacovigilance Risk Assessment Committee

**PVDF:** Polyvinylidene fluoride

**RA:** Rheumatoid Arthritis

**ROS:** Reactive Oxygen Species

**RUNX2:** Runt-related Transcription Factor 2

**SASP:** Senescence Associated Secretory Phenotype

**SDS:** Sodium Dodecilsulphate

**Ser:** Serine

**SYSADOA:** Symptomatic Slow Acting Drugs in Osteoarthritis

**TBS-T:** Tris Buffered Saline Tween

**Thr:** Threonine

**TIMPs:** Tissue Inhibitor of Metalloproteinases

**TNF-α:** Tumor Necrosis Factor α

**Tyr:** Tyrosine

**VGCC:** Voltage-gated Calcium Channel

**WHO:** World Health organization

**ABSTRACT**

Osteoarthritis (OA) and Diabetes Mellitus (DM), especially type 2, are age-related diseases with increasing prevalence. Several epidemiological and experimental data support a strong relation between DM and the development and progression of OA which leads to the concept of Diabetes–associated OA. Understanding the underlying mechanisms is essential for developing prevention strategies and targeted therapies that are urgently needed to stop the growing incidence of OA and its huge socio-economic costs.

Hyperglycemia, as a hallmark of DM, is by hypothesis a major trigger of joint degradation in this concept of DM-associated OA. In this context, this work aimed to contribute to elucidate how extracellular glucose levels are sensed by human chondrocytes, in particular by determining the role of potassium channels in this process, and to further understand the mechanisms by which hyperglycemia induces cartilage damage, unraveling possible pathological mechanisms and potential pharmacological targets of DM-associated OA. Moreover, molecules, of plant origin, were studied to assess their anti-inflammatory and chondroprotective activities that can be explored for development of new drugs with disease-modifying osteoarthritis properties.

In general, the results obtained show that:

- Human chondrocytes of any OA grade present heterogeneous K(ATP) channels, with Kir6.2 and SUR2B being the major subunits expressed.
- The activity of K(ATP) channels influences the abundance of the major glucose transporters, GLUT-1 and GLUT-3, in normal chondrocytes, but did not affected glucose transport, suggesting that these channels participate in a glucose-sensing apparatus that regulates GLUT levels which, by themselves, are not sufficient to adjust glucose transport capacity.
- Chondrocytes of increasing OA grade are less responsive to changes in K(ATP) channel activity.
- Exposure of human chondrocytes to high, hyperglycemia-like glucose concentrations induces the expression of pro-inflammatory mediators, namely IL-1β, TNF-α and iNOS, accompanied by increased NO production, in a mechanism, at least partially, mediated by NF-κB.
- Insulin, not only does not prevent the pro-inflammatory effects of hyperglycemia in human chondrocytes, as, in supraphysiologic concentrations, can induce NF-κB activation by itself.

- The essential oils of *Lavandula luisieri* and *Eryngium duriae* subsp. *juresianum* display important anti-inflammatory properties, namely, significant inhibition of IL-1β-induced iNOS expression, NO production and NF-κB activation in human chondrocytes. Likewise, these essential oils may be of great value in the development of new therapies or as sources of active compounds for the treatment of chronic inflammatory diseases, like OA.
- (+)-α-pinene, showed the most potent chondroprotective and anti-inflammatory activities among its isomers, enantiomers and other naturally occurring compounds derived from the pinane nucleus, by significantly inhibiting IL-1β-induced inflammatory and catabolic pathways, namely, NF-κB and JNK activation and the expression of inflammatory (iNOS) and catabolic (MMP-1 and -13) genes.
- Limonene and myrcene, natural compounds found in the essential oils of *Eryngium duriae* subsp. *juresianum* and *Lavandula luisieri*, showed interesting anti-inflammatory and anti-catabolic properties, namely inhibition of IL-1β-induced NF-κB and p38 MAPK activation and expression of pro-inflammatory and catabolic mediators, namely NO, iNOS and MMP-1 and -13.
- Myrcene further inhibits the phosphorylation of JNK and ERK1/2 MAPKs, which possibly contributes to the inhibition of pro-inflammatory, pro-catabolic and chondrocyte dedifferentiation genes, namely collagen type I, as well as to relieve the inhibition of TIMP-1 and -3 expression caused by IL-1β.

In summary, a comprehensive view of the results suggests that if, on one hand, altered glucose sensing mechanisms and high glucose exposure lead to an increased expression of inflammatory mediators and pathways in chondrocytes, establishing a new link between DM and OA and supporting the existence of a DM-induced OA phenotype, on the other hand, those inflammatory and catabolic mediators and signalling pathways are major targets for the development of new drugs with disease-modifying OA properties. Myrcene is an interesting lead compound for the treatment of this disease.

## RESUMO

A Osteoartrite (OA) e a Diabetes Mellitus (DM), especialmente tipo 2, são doenças relacionadas com o envelhecimento e, por isso, cada vez mais prevalentes. Estudos epidemiológicos e experimentais suportam a forte relação entre a DM e o desenvolvimento e progressão da OA, conduzindo ao aparecimento do conceito de OA associada à diabetes. A compreensão dos mecanismos subjacentes é essencial para o desenvolvimento de estratégias preventivas e terapêuticas específicas que são urgentemente necessárias para travar a crescente incidência da OA e os seus enormes custos socioeconómicos.

A hiperglicémia, como característica fundamental da DM, é por hipótese um importante promotor da degradação da articulação, neste conceito de OA associada à diabetes.

Neste contexto, o principal objectivo deste trabalho foi contribuir para o esclarecimento dos mecanismos pelos quais os níveis extracelulares de glucose são detectados pelos condrócitos, em particular a clarificação do papel dos canais de potássio dependentes de ATP [K(ATP)] neste processo e compreender os mecanismos através dos quais a hiperglicemia induz dano nos condrócitos. Compreender estes processos é essencial para evidenciar possíveis mecanismos patológicos e potenciais alvos farmacológicos para a OA associada à DM. Adicionalmente procurou-se identificar compostos com propriedades anti-inflamatórias e anti-catabólicas que justifiquem a sua exploração para o desenvolvimento de novos fármacos anti-osteoartríticos. Como fontes dos compostos a estudar, utilizaram-se extractos de plantas, a maioria constituintes da flora endémica da Península Ibérica, de modo a contribuir também para a valorização do nosso património biológico.

Em geral, os resultados obtidos mostram que:

- Condrócitos humanos de todos os graus de OA apresentam canais de potássio ATP dependentes [K(ATP)] heterogéneos, com múltiplas combinações de subunidades possíveis, mas parecendo a Kir6.2 e a SUR2B as subunidades mais expressas.
- A actividade dos canais K(ATP) influencia a abundância dos principais transportadores de glucose, GLUT-1 e -3, nos condrócitos normais, mas não afecta o transporte de glucose. Isto sugere que estes canais participam num mecanismo sensor de glucose que regula os níveis dos GLUT, mas a modulação da sua actividade isoladamente não é suficiente para ajustar a capacidade de transporte de glucose em condrócitos.

- Os condrócitos de graus de OA mais elevados, isto é, em que a degradação da cartilagem é progressivamente mais extensa, respondem menos eficazmente ou não respondem a alterações na actividade dos canais K(ATP).
- A exposição dos condrócitos a concentrações elevadas de glucose induz a expressão de mediadores pro-inflamatórios, nomeadamente a IL-1β, o TNF-α e a iNOS, acompanhada pelo aumento da produção de NO, através de um mecanismo, pelo menos parcialmente, mediado pelo NF-κB.
- A insulina não só não previne os efeitos pro-inflamatórios da hiperglicemia nos condrócitos humanos mas ainda, em concentrações supra-fisiológicas, induz por si só a activação do NF-κB.
- Os óleos essenciais de *Lavandula luisieri* e *Eryngium duriaei* subsp. *juresianum* apresentam importantes actividades anti-inflamatórias, nomeadamente a inibição significativa da expressão da iNOS, da produção de NO e da activação do NF-κB induzidos pela IL-1β. Assim, estes óleos podem ser de grande valor no desenvolvimento de novas terapias ou como fonte de compostos activos para o tratamento da OA
- O (+)-α-pinene apresenta o maior potencial condroprotector e anti-inflamatório de entre os seus isómeros, enantiomeros e outros compostos naturais de núcleo pinano por inibir significativamente a activação de vias de sinalização inflamatórias e catabólicas induzidas pela IL-1β, nomeadamente a activação do NF-κB e da JNK e a expressão de genes inflamatórios (iNOS) e catabólicos (MMP-1 e -13).
- O mirceno e o limoneno, compostos naturais encontrados nos óleos essenciais de *Eryngium duriaei* subsp. *juresianum* e *Lavandula luisieri*, mostraram interessantes propriedades anti-inflamatórias e anti-catabólicas, nomeadamente a inibição da expressão de mediadores pro-inflamatórios e catabólicos como o NO, iNOS, MMP-1 e -13 e da activação de vias de sinalização como a do NF-κB e a da p38MAPK.
- Adicionalmente, o mirceno ainda inibe a fosforilação das MAPK, JNK e ERK1/2, o que possivelmente contribui para a inibição da expressão de genes pro-inflamatórios, pro-catabólicos e de desdiferenciação do condrócito, nomeadamente o colagénio I, e atenua a inibição da expressão dos genes anti-catabólicos TIMP-1 e -3 causadas pela IL-1β.

Em resumo, uma visão geral dos resultados sugere por um lado, que a alteração dos mecanismos de sensibilidade à glucose e a exposição a concentrações de glucose elevada conduzem a um aumento da expressão de mediadores e vias de sinalização

inflamatórias nos condrócitos, estabelecendo uma nova ligação entre a OA e a DM e suportando a existência de um fenótipo específico de OA induzida pela DM. Por outro lado, estes mediadores e vias de sinalização inflamatórios e catabólicos são alvos importantes para o desenvolvimento de novos fármacos com capacidade de modificar o curso da doença. O mirceno aparece como um composto *lead* interessante a ser explorado para o desenvolvimento de novas terapêuticas para a OA

# CHAPTER 1.
# INTRODUCTION

Rheumatic and musculoskeletal conditions comprise over 150 diseases and syndromes which are usually progressive and associated with pain. The World Health Organization (WHO) classifies them as conditions that mostly affect joints, bones, cartilage, tendons, ligaments and muscles yielding a reduction of the range of motion in one or more areas of the musculoskeletal system. Included in this group are joint diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA); back and neck pain; osteoporosis and fragility fractures; soft tissue rheumatologic diseases as bursitis and tendinitis; injuries due to trauma (sports, accidents) and autoimmune diseases as Lupus erythematosus. Rheumatic diseases associated with age, like OA, are leading causes of morbidity and disability, yielding a great socio-economic burden whose prevalence continues to increase. Recent epidemiologic data [Arden and Nevitt, 2006; Neogi and Zhang, 2013; Zhang and Jordan, 2010] indicate that OA is the most common joint disorder in the world. According to the WHO, by 2050 people aged over 60 will account for more than 20% of the world's population. Of these 20%, it is estimated that about 15% will have symptomatic OA, and one-third of these people will be severely disabled. This means that by 2050, 130 million people will suffer from OA worldwide, of whom 40 million will be severely disabled by the disease [Goldring, 2006; Heidari, 2011; Kaplan et al., 2013].

The diagnosis and definition of OA are complicated; especially due to the discrepancy between the symptomatic and the radiographic evidences of the disease which are the major factors considered for diagnosis. For instance, 50% of subjects in the general population with radiographic evidence of knee OA do not have pain and 50% of subjects who complain of knee pain, and who are at or above the age when osteoarthritis starts to become common(about 55 years), have no definite radiographic evidence of OA [Heidari, 2011; Neogi and Zhang, 2013].

In 1986, the American College of Rheumatology defined OA as "a heterogeneous group of conditions that lead to joint symptoms and signs, which are associated with defective integrity of articular cartilage, in addition to related changes in the underlying bone at the joint margins" [Altman et al., 1986].

Since then, there was consensus that OA is usually a progressive disease affecting the whole synovial joint [Lane et al., 2011] and generally it can be characterized by a local chronic inflammation, pain, cartilage destruction and loss of joint function.

Traditionally, OA was thought to be a normal consequence of mechanical use and ageing. With age it is unequivocal the accumulation of changes in the joint tissue composition and structure, reason why it is considered a primary risk factor for OA. Despite its importance, there are also elderly people that do not develop OA, so the recent paradigm for disease definition considers that, more than just an “age-related” and “wear and tear” disease, OA results from an interplay of multiple factors, including trauma, genetic pre-disposition, local inflammation, congenital malalignement of joints and cellular and biochemical insults resulting from metabolic conditions [Goldring and Goldring, 2007; Lane et al., 2011].

Generally, OA is subdivided by aetiology into either, idiopathic, if no identifiable cause exists for development of the disease, which corresponds to the majority of the cases and does not affect people under 40 years old, or secondary, when it is caused or associated with an identifiable underlying condition that promotes joint vulnerability and development of OA lesions at younger age. In children or younger adults, trauma or joint insults resulting from sports practice or accidents as well as congenital malalignement of joints and consequent biomechanical instability are strongly implicated in the initiation of OA. Also hereditary predisposition, and metabolic and inflammatory disorders, as obesity and gout, can result in an earlier onset of the disease and be considered causes of secondary OA [Buckwalter and Martin, 2006; Goldring, 2006].

It is likely however, that for idiopathic OA, several risk factors contribute to the initiation and progression of the disease, including ageing, hereditary predisposition, mechanical factors (joint malalignement and overuse, trauma, etc.), gender, obesity and other metabolic factors as malnutrition and Diabetes Mellitus [Buckwalter and Martin, 2006; Goldring and Goldring, 2007; Katz et al., 2010].

Even though the clinical manifestations and symptoms [Goldring and Goldring, 2007] are common to the multiple OA aetiologies, it is reasonable that each risk factor influences the joint in a particular way and increases the susceptibility to the disease and its progression through a variety of mechanisms [Felson, 2010; Kerkhof et al., 2010]. This complexity and variability of OA aetiologies suggests the need of identifying risk factors, establishing distinct phenotypes and understanding in each way they contribute to the initiation and progression of the disease. Uncovering the molecular or biomechanical processes affected in the joint and the mechanisms underlying the

primary trigger are critical for the development of new treatments and new preventing strategies for OA [Berenbaum, 2008; Felson, 2010; Kerkhof et al., 2010; Wenham and Conaghan, 2013].

The factors that initiate OA likely vary depending on the joint site [Lane et al., 2011]. They can affect a single joint, a few joints or be generalized. The most commonly affected joints are the knees, hips, spine and hands [Goldring and Marcu, 2009]. Although most tissues of the joint, including subchondral bone, synovial membrane, joint capsule, periarticular muscles and tendons, are definitely involved in and may be affected by the disease pathogenesis [Attur et al., 2010; Brandt et al., 2006; Goldring and Goldring, 2010; Hulejova et al., 2007; Stone et al., 2013], it is the cartilage that is affected to the major extent and its alterations are the most “visible” face of the disease.

In the first part of this chapter, we will make a brief overview to the anatomy and physiology of the synovial joint by presenting the structure and composition of the articular cartilage and the role of chondrocytes, the only cell type in the articular cartilage, in the maintenance of the tissue homeostasis. Then, the known OA risk factors and specific phenotypes already identified were also briefly described. Special emphasis will be given to Diabetes Mellitus (DM) and particularly to glucose physiological and pathological effects and its sensing and transporting mechanisms. Then, the major cartilage changes observed in OA will also be addressed, as well as the function and regulation of the catabolic and anabolic processes involved in OA pathology.

Finally, we will make a short description of the current OA therapies, including the currently available treatments and the current investigation on drugs with capacity to stop OA progression.

The second part of this chapter will be dedicated to presenting the objectives that determined this work, as well as the content and organization of this thesis.

## 1.1 SYNOVIAL JOINTS AND ARTICULAR CARTILAGE

Synovial joints allow movement at the point of contact of articulating bones. Joints of the knees, hips and hands are general examples of synovial joints that are the most affected in OA. These joints, as represented in figure 1.1, are characterized by the presence of a joint cavity surrounded by a capsule around the bone extremities, covered inside by the synovial membrane, and by the presence of a lubricating

synovial fluid. The bones extremities in a synovial joint are covered by a layer of hyaline cartilage, the articular cartilage, which is a stiff but at the same time flexible and elastic tissue.

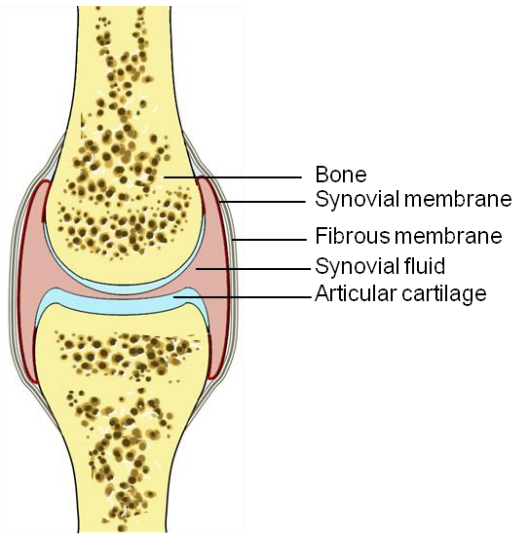


**Figure 1.1** Schematic representation of the major structures of a synovial joint

### 1.1.1 Structure and Composition of Articular Cartilage

Articular cartilage is a specialized connective tissue that covers the bony surface of synovial joints. It is avascular and aneural and serves as a viscoelastic, load-bearing support tissue that absorbs impacts, resists to compression and provides a smooth surface with almost no friction during joint movement, without losing its original shape [Martel-Pelletier et al., 2008]. These unique biomechanical properties are given by its extracellular matrix composition and structure, namely by the collagen and proteoglycan network [Goldring, 2006].

The chondrocyte is the only cell type found in mature articular cartilage, thus it is responsible for the homeostatic process involving the synthesis and degradation of the cartilage matrix components and consequently for its biomechanical properties. The physical function of joints is critically dependent on the integrity of the matrix and so dependents on normal chondrocyte function.

Proteoglycans and collagens are the most abundant macromolecules of the extracellular cartilage matrix and are ordered in distinct layers from the superficial to the deepest zones of the cartilage, reflecting different proportions, structural

organization and function. The superficial layer is the thinnest and consists of fine collagen fibrils with tangential orientation and low proteoglycan content. This zone is in contact with synovial fluid, and is responsible for most of the tensile properties of cartilage. The middle zone represents 40–60% of the total cartilage height. It is formed by proteoglycans and thicker collagen fibrils organized into radial layers. The deep zone contains the largest collagen fibrils in a radial disposition, and the highest aggrecan content. Finally, the calcified cartilage is divided from the other zones by the tidemark, and separates – physically and mechanically – the hyaline cartilage from the subchondral bone [Martel-Pelletier et al., 2008].

Despite the importance of collagen, proteoglycans and other supporting glycoproteins, they only represent 20% of the tissue wet weight. Chondrocytes represent 5% and the remaining is water and inorganic salts. This enormous water content, almost all associated with proteoglycans, is extremely important for the mechanical properties, especially elasticity and capacity to resist to compressive forces, but also for the nutrition and lubrication of the tissue [Martel-Pelletier et al., 2008; Mobasheri, 1998; Muir, 1995].

#### 1.1.1.1 Collagens and Proteoglycans

The collagen network provides the shape and form of the articular cartilage and is composed by several types of collagens, including collagen type II, which represents the major collagen type in the tissue (90-98% of total collagen), and collagens type XI, type IX and type XXVII. Collagen type VI, characteristic of the connective tissue is also present in a small percentage [Eyre, 2002; Goldring and Marcu, 2009]. It constitutes the collagen network close to the cells in the so-called territorial matrix and acts as an interface between the rigid interterritorial cartilage matrix and the chondrocyte. It is also involved in cell anchoring, as well as in matrix-cell signalling [Soder et al., 2002].

The collagen type II macromolecule is composed of 3 identical polypeptide chains – α1 (II) chains – interconnected to form a triple helix, as represented in figure 1.2. Those helixes do not exist in an isolated form, but as fibrils resulting from the association of adjacent collagen molecules within the extracellular matrix and stabilized by crosslinks between the N-terminal telopeptide of one collagen molecule and the triple helix of another [Eyre, 2002]. Collagens type XI and type IX are also associated with the collagen type II fibrils, the first lying within the fibril and the second integrated in the fibril surface, providing protection to collagen type II and enabling interactions with the other non-collagen elements of the matrix, especially proteoglycans [Eyre et al., 2006].

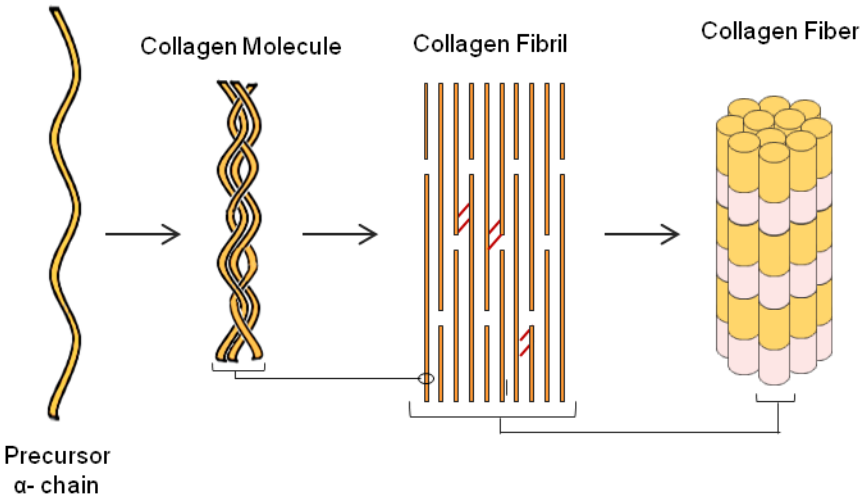


**Figure 1.2** Schematic diagram of collagen type II fiber structure. Adapted from [Eyre, 2002]

The major non-collageneous components of the cartilage matrix are proteoglycans, in particular aggrecan which represents about 5% of the cartilage wet weight. It can be found in association with the polysaccharide, hyaluronic acid (HA), forming large aggregates stabilized by a small link protein through non-covalent bonds. [Hardingham and Fosang, 1995; Heinegard, 2009; Kiani et al., 2002].

Proteoglycans are a family of glycoconjugates formed by a central core protein around which several sulphated glycosaminoglycan (GAG) chains attach, as represented in figure 1.3 [Kiani et al., 2002]. Two types of GAG can link to the central core protein. The most abundant is chondroitin sulphate wherein 100 molecules can link to the core protein. The other is keratan sulphate, smaller and less abundant than chondroitin sulphate. Up to 30 molecules of keratan sulphate can link to the core protein. [Hardingham and Fosang, 1995; Heinegard, 2009].

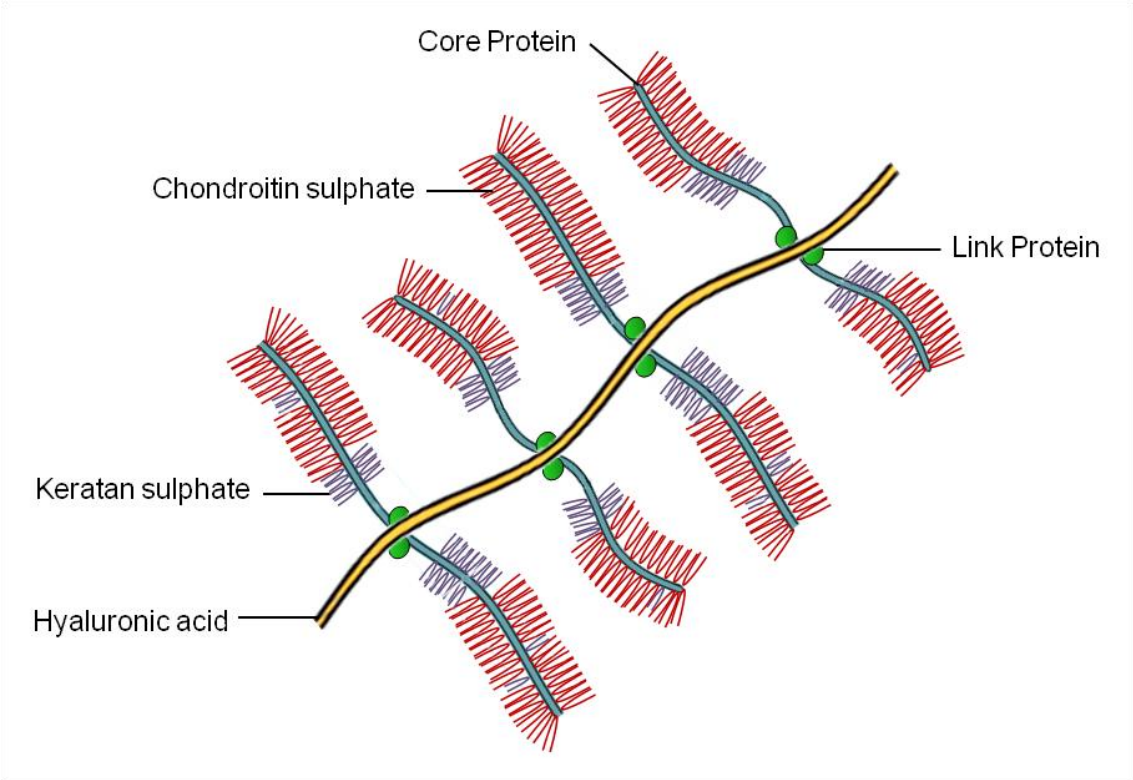


**Figure 1.3** Schematic representation of proteoglycans linked to Hyaluronic acid. Adapted from [Hardingham and Fosang, 1995]

At high concentrations, proteoglycans, and in particular the aggregates formed with HA, create a large osmotic swelling pressure that draws water into the cartilage. This osmotic pressure occurs because all of the negatively charged sulphated groups of chondroitin and keratan sulphates on the GAG chains of aggrecan carry with them positive ions such as $Na^+$, generating a huge difference in ion concentration between the cartilage and surrounding milieu. The osmotic imbalance generated is responsible for water to flow and to be retained within the cartilage. The addition of water causes the aggrecan-rich matrix to swell and expand which is essential to the biomechanical properties of cartilage, namely its visco-elastic properties that allow the impact loading without permanent deformation [Hardingham and Fosang, 1995; Kiani et al., 2002]. In an exclusively biophysic perspective of the process, when the cartilage is submitted to a compressive force, an amount of the water retained in the tissue is released, in proportion to the load applied [Kiani et al., 2002]. Moreover, some authors also postulate that aggrecan, through the chondroitin sulphate chains, can protect collagen type II fibrils from degradation by collagenases, possibly via direct blocking of their binding sites [Hedlund et al., 1999].

Small amounts of other non-collagen molecules, including biglycan, decorin, fibromodulin, the matrilins and cartilage oligomeric matrix protein (COMP), are also present in the matrix. COMP acts as a catalyst in the formation of the collagen fibrils. Interactions between type IX collagen and COMP or matrilin-3 are essential for proper formation and maintenance of the articular cartilage matrix [Shakibaei et al., 2008].
The collagen/aggrecan network is a resilient, viscoelastic load-bearing structure that allows smooth movement and distributes loads applied to the joint. The type II collagen network gives cartilage its shape and tensile strength and provides a framework to resist the swelling pressure of aggrecan. On its hand, aggrecan resists to any fluid flow and is able to efficiently distribute water. Proteolysis of any of the components of the cartilage not followed by its respective resynthesis will imbalance the network compromising the tissue biomechanical properties [Eyre, 2002; Goldring and Marcu, 2009; Hedlund et al., 1999; Kiani et al., 2002; Martel-Pelletier et al., 2008].

#### 1.1.1.2 Chondrocyte

Similarly to the other cartilage components, chondrocytes are distributed unevenly throughout the cartilage depth, being in general more abundant at the surface. Differences in the morphology and organization of chondrocytes can be a reflex of the mechanical environment given by the collagen architecture in the interterritorial matrix, and can be the reason for the different matrix compositions found along the tissue. Chondrocytes in the superficial zone (SZ) are small and flattened; those of the middle zone (MZ) are rounded with no organized orientation while those of the deep zone (DZ) are grouped in columns or clusters, as represented in figure 1.4. These cell distribution patterns are thought to derive from the formation and specific organization of the Extracellular Matrix (ECM) during joint development and maturation [Lotz et al., 2010].

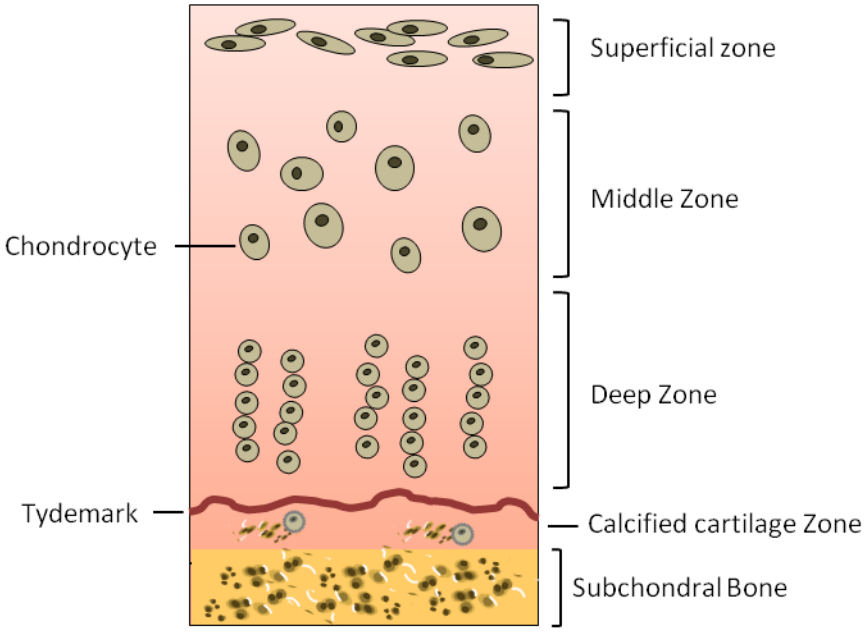


**Figure 1.4** Schematic Representation of the different cartilage zones and chondrocytes distribution. Adapted from [Lotz et al., 2010]

Due to the avascular nature of the articular cartilage, chondrocytes receive oxygen and nutrients mainly by diffusion form the synovial fluid and from the subchondral bone [Levick and McDonald, 1995; Zhou et al., 2004b]. For the same reason, chondrocytes live in a hypoxic environment when compared to most other tissues, with an average of 5 to 6% oxygen tension in the cartilage surface and 2 to 3% at the interface with the bone [Henrotin et al., 2005; Zhou et al., 2004b]. The chondrocytes adapt to these conditions by using glycolysis as their main energy source [Lee and Urban, 1997; Rajpurohit et al., 1996] and give preference to it even when exposed to higher oxygen tensions [Schneider et al., 2007].

How chondrocytes maintain cartilage homeostasis remains somehow unknown, since under physiological conditions, they do not divide, have a low cartilage matrix protein turnover rate and the matrix structure only admits a few chondrocytes adjacent to each other which limits their interactions [Lotz et al., 2010]. However, in order to maintain this homeostasis, chondrocytes have to be able to sense matrix alterations and also to integrate and to be sensible to stimuli addressed to the cartilage. To achieve that, chondrocytes have to be sensible to: 1) direct interaction with the ECM components via different surface receptors, as those of integrin and annexin families, the hyaluronan

receptor, CD44, or the Discoidin Domain receptor 2 (DDR2) which binds collagen II when proteoglycans are depleted [van der Kraan et al., 2002; Xu et al., 2007]; 2) soluble mediators produced by the chondrocytes themselves or by any other articular or non-articular tissue, and, finally, 3) to mechanical stimuli, [Goldring and Marcu, 2009; Shakibaei et al., 2008; van der Kraan et al., 2002].

Moreover, several studies indicate that although chondrocytes are normally quiescent, in response to any injury or abnormal stimulus they can shift to an activated form and enter on a remodelling program that includes catabolic (destructive) and anabolic (synthetic) processes. The normal function of chondrocytes and consequently the integrity of the cartilage are dependent on a variety of signals including mechanical forces and chemical mediators (cytokines, growth factors, hormones), and on the capacity of chondrocytes to integrate those signals without disrupting the homeostatic balance of the tissue [Goldring and Marcu, 2009; Martel-Pelletier et al., 2008].

## 1.2 OSTEOARTHRITIS RISK FACTORS AND SPECIFIC PHENOTYPES

Epidemiological studies have been transforming the way how OA is seen. Firstly saw as an age-related and a “wear and tear” disease of the cartilage, OA is now considered a multifactorial disease affecting the whole joint where the identification of several risk factors, both endogenous and exogenous, played an unquestionable role [Michael et al., 2010].

No longer misconceived as a uniform disease, OA is an heterogeneous disease with the failure of the joint function as the major outcome [Goldring and Goldring, 2007]. Different pathophysiological aetiologies resulting in the final common consequence of cartilage destruction have driven the division of OA into different phenotypes according to the respective major related risk factor. The definition of a phenotype should be confined to those factors that have a primary effect on disease biology and aetiology, and that can be significant to treatment and prevention [Felson, 2010]. Traditional risk factors for OA, namely age, trauma, genetic predisposition and obesity by inducing direct effects on cartilage destruction, through destabilizing catabolic and anabolic homeostasis and creating a low grade inflammatory environment, have already been associated with specific OA phenotypes [Neogi and Zhang, 2013]. Although they can be seen as independent, most of the times OA onset and progression does not depend on an individual risk factor alone but on a synergy of multiple factors. Genetics, for instance, plays an indubitable role in OA development and progression [Spector et al., 1996]. However, its role is more likely due to an interaction among multiple genes, in

combination with further risk factors, as ageing. Moreover, the effects of obesity on the joint have been admitted, historically, as simply resulting from excessive mechanical loading. However, there is increasing evidence of multifactorial, systemic links between obesity and OA, since non-loading joints, as elbows and hands, are also affected by OA in obese patients [Sowers and Karvonen-Gutierrez, 2010].

The evidence of systemic factors involved in obesity related-OA also contributed to the definition of a metabolic-induced OA. This phenotype shares several similar biochemical and inflammatory features with the metabolic syndrome. Hypertension, dyslipidemias and conditions with altered glucose metabolism, as diabetes mellitus (DM), and their major characteristics, increased expression of adipokines, varying glucose concentrations and hormonal imbalance characterize the metabolic syndrome. When OA is also present it has been described as metabolic-OA. These characteristics have already been, either directly or indirectly, related with the mechanisms of OA pathology [Zhuo et al., 2012].

Besides its relationship with obesity and the metabolic syndrome, DM is increasingly recognized as an independent risk factor for OA development and progression. Thus, it can define a new specific OA phenotype [Berenbaum, 2011]. Recent epidemiologic data further demonstrate a higher OA prevalence with an earlier onset and more severe manifestations in diabetic vs. non-diabetic patients [King et al., 2013; Martinez-Huedo et al., 2013; Nieves-Plaza et al., 2013; Schett et al., 2012a]. Moreover, several experimental evidences show that hyperglycemia, one of the features of DM, can trigger events that can be related to cartilage destruction, as Reactive Oxygen Species (ROS) production and a low grade systemic inflammation further linking DM and hyperglycemia to OA incidence [Berenbaum, 2011].

### 1.2.1 Diabetes Mellitus and Osteoarthritis: Role of Extracellular glucose concentrations

Diabetes mellitus (DM) is a group of chronic diseases characterized generally by high blood glucose levels that result from defects in the body's ability to produce and/or use insulin efficiently. Together with OA, DM type 2 is one of the most important causes of mortality and morbidity in older individuals [Kaplan et al., 2013]. Injurious effects induced by DM can be related with several factors, including insulin or other pancreatic hormone levels, but hyperglycemia is probably the most significant [Yan and Li, 2013].

At normal physiological conditions, due to the avascular nature of cartilage, chondrocytes are under a particular environment where nutrients and oxygen only

arrive by diffusion from the synovial fluid and subchondral bone. Consequently chondrocytes must have the capacity to survive in an extracellular matrix with limited nutrients and low oxygen tensions. Several studies showed that these particular conditions of low oxygen tension are important for the normal synthesis of matrix components and to the maintenance of the differentiated chondrocytic phenotype [Das et al., 2010; McNulty et al., 2005] and that their disruption results on negative outcomes, namely contributing to the altered synthesis of structural and regulatory proteins [Das et al., 2010; Grimshaw and Mason, 2000; Grimshaw and Mason, 2001]. In terms of energetic needs, chondrocytes adapted to this limited availability of oxygen by using the glycolytic pathway as their major energetic source [Lee and Urban, 1997] and they use it even in the presence of high oxygen tensions [Lee and Urban, 1997; Schneider et al., 2007]. However, glucose serves not only as the major energy source, but also as an essential precursor for glycosaminoglycan synthesis. Thus, a stable supply of glucose is important for the tissue homeostasis [Goldring, 2006].

In pathological conditions, glucose and oxygen supply may be affected by different factors. For example, studies of Otte and Hernvann (1992) relate the importance of glucose as a metabolic substrate and also emphasize the fact that glucose uptake is stimulated by catabolic cytokines in chondrocytes in the concentrations easily found in OA joints [Hernvann et al., 1992; Otte, 1991]. In addition, since chondrocytes receive glucose especially from the synovial fluid whose glucose concentrations reflect, in the majority of cases, the plasma concentrations, conditions like DM that affect glycemia will probably also affect the availability of glucose to articular chondrocytes [Brannan and Jerrard, 2006].

Despite these observations, the role of the extracellular glucose concentrations on the chondrocyte functions is still largely unknown. From the *in vitro* and *in vivo* data available, however, it is possible to understand that both hypo- and hyperglycemic conditions may contribute to cartilage homeostasis breakdown and thus to OA onset and progression. Glucose deprivation has been shown to increase the expression of the Matrix Metalloproteinase 2 (MMP-2) [Richardson et al., 2003], that participates in cartilage degradation in late OA, and was associated with decreased expression of collagen type II and proteoglycans [Yang et al., 2005]. The effects of hyperglycemia in cartilage have been associated with the formation of advanced glycation end products (AGEs) [Verzijl et al., 2003], as mediators of matrix stiffness, subchondral bone destruction and chondrocyte dysfunction. Chronic hyperglycemia increases the occurrence of non-enzymatic glycation reactions, which are associated with oxidative stress, as illustrated in patients with diabetes [Gillery, 2006]. AGEs have been shown to

accumulate within the cartilage in amounts that increase with age [Verzijl et al., 2001]. Hiraiwa and Nah [2011] showed that AGEs mediate the expression of matrix-degrading enzymes and pro-inflammatory and catabolic mediators (MMP-1, MMP-13, COX-2; NO) [Hiraiwa et al., 2011; Nah et al., 2008; Nah et al., 2007] and DeGroot [2001 and 1999] showed that they decrease collagen type II and proteoglycan expression in bovine chondrocytes.

Notwithstanding the importance of AGEs on OA pathogenesis, it is important to note that the development of OA with aging is not inevitable, even though changes in the composition and structure of the cartilage matrix are. So, studies focused only on older individuals and in AGEs, generated in increased amounts during aging, would not completely explain the role of hyperglycemia in OA development [DeGroot et al., 1999; Goldring and Goldring, 2007; Verzijl et al., 2003].

Recent studies have focused on the direct effects of high glucose on the chondrocyte functions. Those studies clearly show that direct exposure of chondrocytes to high glucose affects the cartilage homeostasis, namely by interfering with the synthesis of ECM components. In one study, hypo- and hyperglycemia-like conditions induced resistance to Insulin-like Growth factor (IGF-1), an important anabolic factor in chondrocytes, thus contributing to decreased proteoglycan synthesis [Kelley et al., 1999] and in another, exposure of human chondrocytes to high glucose concentrations, was shown to decrease dehydroascorbate transport, which can compromise the synthesis of collagen type II [McNulty et al., 2005]. On the other hand, another study from our group showed that collagen II mRNA was similarly increased in normal and OA chondrocytes, exposed to hyperglycemia-like conditions, but the increase lasted longer in the OA group [Rosa et al., 2011b]. Moreover, this study also showed that that exposure to high glucose (30 mM) increased the mRNA levels of matrix-degrading enzymes in OA chondrocytes, whereas in normal ones only MMP-1 was increased. Furthermore, exposure of human chondrocytes to high glucose also prevented anti-catabolic effects of TGF-β both in normal and OA chondrocytes [Rosa et al., 2011b]. Finally, culture of chondrocytes under high glucose elicited a increase in ROS production. In OA chondrocytes the increase in ROS production was sustained for a longer period than in normal ones [Rosa et al., 2009]. Since ROS are involved in the pathophysiology of OA, high glucose-induced ROS production is likely to directly contribute to high-glucose induced cartilage damage. In vivo data corroborate these findings. Indeed, diabetic rats were associated with major alterations in matrix composition in the cartilage of the knee joints, namely with decreased expression of proteoglycans and collagen type II, the main structural component of the articular

cartilage, and increased expression of collagen type XI, that is not normally present in articular cartilage and is a marker of chondrocyte progression to hypertrophy and subsequent calcification [Atayde et al., 2012]. This finding may contribute to understanding the functional limitations of diabetic patients and the association of DM with OA. Although these observations of glucose effects on chondrocytes and cartilage may contribute to establish a correlation between DM and OA, strengthening the existence of a specific DM-induced OA phenotype, more studies are needed to understand how DM affects the development and progression of OA. Finally, a better knowledge of the specificities of a DM-induced OA phenotype should allow a targeted approach for the development of preventive and curative treatments for OA [Berenbaum, 2011].

#### 1.2.1.1 Glucose sensing and transport

As glycolytic cells, chondrocytes must be able to sense the quantities of oxygen and glucose available to them in the extracellular matrix and respond appropriately by adjusting cellular metabolism [Mobasheri et al., 2005]. As the concentrations of glucose available may vary, depending for instance on the pathological conditions, immobility and age, glucose transport becomes a critical step on the utilization and on the effects of this nutrient on chondrocyte functions [Mobasheri et al., 2002b; Shikhman et al., 2001].

Glucose consumption seems to be dependent on the extracellular glucose concentrations [Schneider et al., 2007], suggesting that chondrocytes may adjust glucose transport and increase glucose storage, glycolysis, and production of proteoglycans [Lee and Urban, 1997] to the extracellular glucose concentration. Several reports show that high, hyperglycemia-like glucose concentrations play an important role in the development of diabetic complications. High glucose can induce the release of pro-inflammatory cytokines as Interleukin-1β (IL-1β), Interleukin-6 (IL-6), Tumor necrosis factor –α (TNF-α), and interleukin-18 (IL-18) [Asakawa et al., 1997; Esposito et al., 2002; Jiang et al., 2012] and other pro-inflammatory mediators, as Nitric Oxide (NO) [Askwith et al., 2011; Jia et al., 2013], in different cell types, activate pro-inflammatory and pro-catabolic signalling pathways, as those of Nuclear Factor κB (NF-κB) and p38 Mitogen Activated Protein Kinase (MAPK) [Igarashi et al., 1999; Yerneni et al., 1999], and induce the production of ROS [Busik et al., 2008; Yao and Brownlee, 2010] and AGEs [Brownlee, 2005]. Chondrocytes express multiple isoforms of the facilitative glucose or solute carrier (GLUT/SLC2A) family of glucose/polyol transporters

[Mobasheri et al., 2008], including GLUT-1, GLUT-3, GLUT-5, GLUT-9, GLUT-10 and GLUT-11 which are responsible for the majority of the glucose transport capacity of these cells [Mobasheri et al., 2008]. Among those, GLUT-1 and GLUT-3, constitutively expressed in chondrocytes, are the isoforms better characterized, and both have high glucose affinity [Colville et al., 1993]. GLUT-1 seems to be regulated by anabolic and catabolic stimuli, while GLUT-3, is less affected by [Mobasheri et al., 2008; Peansukmanee et al., 2009; Shikhman et al., 2001]. Specifically, GLUT-1 was found to be increased in equine articular chondrocytes stimulated with TNF-α, IL-1β, IGF-I, TGF-β and insulin, while GLUT-3 was only upregulated by IGF-I [Phillips et al., 2005; Richardson et al., 2003]. GLUT-1 and GLUT-3 together with hypoxia-inducible factor alpha (HIF-1alpha) were proposed as potential components of the glucose and oxygen sensing apparatus in chondrocytes by promoting anaerobic glycolysis and favoring oxidative phosphorylation, which allow chondrocytes to survive in the adverse conditions of low glucose and oxygen supply [Mobasheri et al., 2008; Mobasheri et al., 2005]. However, how human chondrocytes sense and modulate glucose transport, in health and disease and in the presence of different extracellular glucose concentrations, remains unclear.

Recent studies showed that normal human chondrocytes respond to high extracellular glucose concentrations by decreasing glucose uptake, which occurs through downregulation of GLUT-1 content [Rosa et al., 2009]. This mechanism seems to protect the cells against excessive intracellular glucose accumulation. Moreover, this study showed that a different effect occurs in OA chondrocytes, which when exposed to high glucose concentrations were unable to downregulate GLUT-1 and glucose transport, leading to increased glucose accumulation and prolonged ROS production [Rosa et al., 2009]. Increased ROS production and, thus oxidative stress, are seen as a major deleterious mechanism in OA pathogenesis by mediating inflammatory and catabolic responses in chondrocytes [Grishko et al., 2009; Mendes et al., 2003a; Mendes et al., 2003b]. Moreover, these observations establish another link between DM and OA development since OA chondrocytes seem to be susceptible to hyperglycemic episodes as those occurring in DM patients, and thus particularly subjected to the deleterious effects of high glucose.

Further research is needed to expand our understanding of glucosensing and glucose signalling in chondrocytes, and other joint tissues, in health and disease.

Understanding how human chondrocytes sense extracellular glucose and adjust its uptake to match the cell's needs and whether such mechanisms remain functional in OA chondrocytes is critical to elucidate the mechanisms by which conditions

associated with impaired glucose metabolism contribute to the development and/or progression of OA, and for further development of new therapies and identification of new therapeutic targets for treating this joint disorder.

## 1.3 CARTILAGE CHANGES IN OSTEOARTHRITIS

Cartilage degradation and loss, one of the OA hallmarks, occurs due to a disturbance of the balance between the reparative and degradative processes of the cartilage. The initiation and progression of OA, however, appear to be enclosed in a cyclic evolution plan where it becomes virtually impossible to understand which are the causes and/or the consequences, i.e., any factor that disturbs the tissue can contribute to the deregulation of the cell's functions and to tissue alterations, which in turn will lead to more cell dysfunction and more ECM alterations. As an example, abnormal mechanical stress induces chondrocytes to produce Matrix Metalloproteinases (MMPs), which degrade matrix components, destroying the cartilage, while the ECM protein fragments generated interact with different receptors to induce the production of inflammatory cytokines, chemokines and more MMPs, expanding further matrix destruction [Fichter et al., 2006; Pulai et al., 2005].

Chondrocytes, because of their location and function, play a central role in OA pathogenesis which can be described as a result of their failure to maintain the homeostatic process, so that the rate of loss of collagens and proteoglycans from the matrix exceeds the rate of deposition of new molecules. This, in turn, involves multiple interactions between local and systemic factors [Buckwalter and Martin, 2006; Goldring and Goldring, 2007; Martel-Pelletier et al., 2008] and even though the aetiology of the disease is still largely unknown, the fact is that several risk factors, lead to the OA phenotype. The similarities observed in the pathological progression of the disease, even when the initiating models are different, indicate that common molecular sequences may underlie OA onset and progression [Castaneda et al., 2013].

Regardless the nature of the factors that initiate the pathology, its progression follows a consistent pattern which includes chondrocyte phenotype alterations, increased general synthetic activity, increased expression of ECM degradative proteinases, gradual loss of proteoglycans and collagen type II, fibrillation and formation of fibrocartilage and osteophytes [Fosang and Beier, 2011]. In non-pathological conditions, the normally quiescent chondrocytes have a very low turnover, which is enough to ensure the replacement of long-lived ECM proteins, like aggrecan with a

half-life of 3-24 years [Maroudas et al., 1998] and collagen whose half-life is even greater of 100 years or more [Verzijl et al., 2000].

In the initial pathological conditions, mechanical stress, cytokines, matrix degradation products, age-related AGEs or any other factor that can disrupt the cartilage homeostasis [Hiraiwa et al., 2011] lead chondrocytes to a changed phenotype where they become “activated”, proliferate and form clusters and suffer an inappropriate hypertrophy-like maturation [Lotz et al., 2010]. Hypertrophic chondrocytes undergo a stress response associated with ECM remodelling in what can be seen as an attempt to repair the damages. In this active remodelling, however, the quality of the ECM is compromised due to the abnormal quick turnover rate, and to the atypical composition of the newly synthesized ECM proteins. In this period, there is expression of non-cartilage specific collagens, like type X, normally confined to the cartilage-calcified zones close to the subchondral bone and only produced by hypertrophic chondrocytes, and collagen type I typically present in fibrocartilage and structurally different from type II. [Boos et al., 1999; Eyre et al., 2006; Lahm et al., 2010; Miosge et al., 2004]. Both collagen types have less capacity to interact with non-collagen cartilage molecules as proteoglycans and contribute to the formation of fibrocartilage, biomechanically less effective than the original one. In the late phases of the disease, this fibrocartilage contributes to further cartilage matrix degradation and loss [Lorenzo et al., 2004; Martel-Pelletier et al., 2008].

Associated with this abnormal renovation, there is decreased expression and higher degradation of the naturally-occurring articular components, namely types IX, XI and II collagens and proteoglycans. It has been shown that there is a significant downregulation of aggrecan gene expression at the onset of OA in a rat model [Nam et al., 2011] and this finding agrees with markedly low proteoglycan synthesis observed in human cartilage samples from an early phase of the disease process, even before a clear fibrillation of the tissue is evident [Lorenzo et al., 2004].

Evidence suggests that the degradative process of the cartilage starts most of the times at its surface. The local loss of collagen type II results in an increased water content and loss of the tensile strength which is often observed in the early phases of the disease. Later, the combination of aggrecan and collagen loss leads to the increased overall stiffness of the tissue. A relationship between this degradation and the increased expression of proteinases, namely those responsible for the degradation of collagen type II and aggrecans, as MMPs (MMP-1, MMP-3 and MMP-13), and the aggrecanases, A Desintegrin And Metalloproteinase with ThromboSpondin motif

(ADAMTS)-4 and -5, was established [Bau et al., 2002; Okada et al., 1992; Shinmei et al., 1990; Yoshihara et al., 2000]

An increasing number of evidences suggest that an important inflammatory process characterized by deregulated cytokine activities that can be triggered by any mechanical, biomechanical or molecular factor involved in OA pathogenesis, yield alterations of chondrocyte functions that are likely to contribute to the described disruption of that balance between the anabolism and catabolism in OA [Goldring and Berenbaum, 2004].

### 1.3.1 Regulation of Catabolic and Anabolic Pathways

#### 1.3.1.1 Matrix Metalloproteinases and Aggrecanases

Degradation of the cartilage ECM is a feature of arthritic diseases and a consequence of the upregulated catabolic process. The destruction seems to be driven by both Matrix Metalloproteinases (MMPs) and aggrecanases, which degrade the two major structural components of the cartilage extracellular matrix, the proteoglycan, aggrecan, and type II collagen.

Several members of the ADAMTs family of enzymes (ADAMTS 1, 4, 5, 8, 9, and 15) have been identified in articular cartilage [Bau et al., 2002] and are known to be capable of cleaving aggrecan in different sites [Troeberg and Nagase, 2012]. ADAMTS4 and ADAMTS5 (aggrecanase-1 and aggrecanase-2, respectively) seem to be the most active aggrecanases and their depletion [Song et al., 2007] was associated with lower proteoglycan degradation and slower OA progression [Majumdar et al., 2007].

Along with aggrecan breakdown, degradation of collagen is a central feature of OA, in a process that is extensively mediated by MMPs. These are a family of zinc-dependent proteinases and although they can, similarly to ADAMTs, participate in the degradation of all the ECM proteins, collagen is their main target [Burrage et al., 2006; Cawston and Wilson, 2006; Patwari et al., 2005]. They can be categorized in different groups according to their preferable substrate and tissue location: the collagenases (MMP-1, -8, -13), which degrade the fibrillated collagens (types I, II and III), the gelatinases (MMP-2, -9), which target denaturated polypeptide chains of types IV and II collagen and, finally, the stromelysins (MMP-3, -10, -11) and the membrane-type MMPs (MMP-14, -15, -16, -17, -24, -25) which degrade non-collagen matrix proteins, as aggrecans, fibronectin and elastin and participate in the activation of other MMPs [Burrage et al.,

2006]. Generally, the collagenases (MMP-1, -8 and -13) cleave the collagen type II triple helix allowing further degradation of the released chains by gelatinases (MMP-2 and -9). Even though all the collagenases were able to effectively cleave the triple helix, they seem to have a substrate preference, with MMP-13 being the most expressed in chondrocytes and preferably cleaving collagen type II. Although MMP-1 preferentially cleaves collagen III, it also effectively cleaves type II collagen and both MMPs were found to be overexpressed in OA joints [Bau et al., 2002; Konttinen et al., 1999; Troeberg and Nagase, 2012; Yoshihara et al., 2000].

Additionally, MMPs participate in other processes that indirectly contribute to further cartilage breakdown, as is the case of MMP-3 that seems to be involved in the activation of MMP-1 and MMP-13 [Troeberg and Nagase, 2012; van Meurs et al., 1999].

Given the destructive potential of MMPs and their abnormal high levels in OA, abolishing or even just reducing their pathologic levels are interesting strategies for development of new disease-modifying OA drugs.

Several factors and mechanisms have been associated with increased expression of the matrix-degrading enzymes observed in OA cartilage. The primary one is probably the abnormal mechanical loading. *In vivo* studies showed that immobilization leads to joint damage by upregulating MMP-3 and ADAMTS-5, while mechanical stimulation of moderate intensity has the opposite effect, increasing aggrecan and decreasing MMP-3 [Leong et al., 2010a; Leong et al., 2010b]. Nevertheless, abnormal mechanical stress can be sensed by chondrocytes resulting in an increased stimulation of matrix-degrading proteins [Fujisawa et al., 1999].

In addition, several other studies showed that activation of mechanical receptors is not the only cause of the upregulated expression of these proteinases. The stronger evidence rests on the role of pro-inflammatory mediators, namely, cytokines (IL-1β and TNF-α) [Fernandes et al., 2002; Kapoor et al., 2011], chemokines [Lotz et al., 1992], prostaglandins [Mohamed-Ali, 1995] and Nitric Oxide (NO) [Abramson, 2008]. Chondrocytes in OA cartilage express receptors that respond to those mediators, produced by chondrocytes themselves or by the synovium and other periarticular joint tissues, resulting in an increased expression of MMP-1,-3, -9 and -13 [Burrage et al., 2006]. Moreover also integrins and matrix proteins fragments (collagen and fibronectin) have been associated with cartilage damage through the production MMPs and aggrecanases and through an amplification feedback by induction of the production of pro-inflammatory cytokines [Fichter et al., 2006; Loeser et al., 2003].

The synthesis of MMPs is highly regulated at the transcriptional level [Burrage et al., 2006; Murphy and Lee, 2005] and regardless of the mechanism that triggers their overexpression, the same signalling pathways seem to be involved. Several evidences showed that, among others, signalling pathways of Activator Protein-1 (AP-1) and NF-κB transcription factors together with those of MAPKs are key pieces on the regulation of the expression of the matrix-degrading enzymes in human chondrocytes [Barchowsky et al., 2000; Mengshol et al., 2000]. At a post-transcriptional level, MMPs are regulated by the Tissue Inhibitors of the Metalloproteinases (TIMPs) [Cawston and Wilson, 2006]. The inhibition of MMP activity by TIMPs involves non-covalent binding in a 1:1 stoichiometry to the active site of the target MMP [Burrage et al., 2006]. Generally, TIMPs-1 to -4 can inhibit all MMPs, but are also effective in inhibiting aggrecanases. For example, TIMP-3 blocks all the MMPs [Burrage et al., 2006] and also ADAM-17 [Amour et al., 2000] and ADAMTS-4 and -5 [Burrage et al., 2006; Kashiwagi et al., 2004].

Regardless of the natural role of MMPs in cartilage homeostasis, in the pathologic environment of an arthritic joint, their elevated levels usually exceed those of TIMPs [Dean et al., 1989] which contributes to cartilage degradation in OA [Martel-Pelletier et al., 1994].

### 1.3.2 Inflammatory Process in OA

OA is not considered a classical inflammatory disease, especially due to the intermittent influx of inflammatory cells into the joint and the sparse characteristic signs of inflammation, such as redness and swelling with heat during the course of the disease when compared to other inflammatory arthritic diseases, namely, rheumatoid arthritis (RA). However, several reports have demonstrated the overproduction and expression of proinflammatory mediators including NO, inducible nitric oxide synthase (iNOS), Cycloxigenase 2 (COX-2), IL-1β, TNF-α, IL-6, and IL-8 in OA cartilage. This inflammatory response, seen as a chronic low-grade inflammation and so called “molecular inflammation”, even though not reflected by clinical signs of inflammation, leads to an imbalance of cartilage homeostasis that results in progressive breakdown [Attur et al., 2000; Fernandes et al., 2002; Sokolove and Lepus, 2013]. It is now significantly clear that inflammation is present in OA joints well before the development of significant radiographic changes, suggesting that the cartilage damage in OA can be driven by an articular inflammatory process [Attur et al., 2000; Ayral, 2005; Fernandes et al., 2002; Haywood et al., 2003; Ishii et al., 2002]. So, any factor that triggers the

excessive production of proinflammatory mediators can contribute to the initiation and progression of this pathology.

Although the mechanism by which production of inflammatory mediators is initiated is unclear, several factors, including mechanical and metabolic, have been related with the induction of cytokines and chemokines, ROS, in particular NO, as well as inflammation-related enzymes, like COX-2, and iNOS in the joint tissues and, particularly, in cartilage. Evidences from *in vivo* and *in vitro* studies indicate that chondrocytes can produce and/or respond to a number of cytokines and chemokines that are present in OA joint tissues and fluids [Berenbaum, 2004; Berenbaum, 2013; Goldring and Goldring, 2007]. Synovial inflammation for instance is a factor that likely contributes to deregulation of chondrocyte functions with the levels of catabolic enzymes and inflammatory mediators, such as prostaglandins and NO, and the levels of IL-1β and TNF-α in OA synovial fluids and joint tissue favoring the imbalance between the catabolic and anabolic activities of the chondrocyte [Loeser, 2006; Loeser et al., 2012].

The major role of the inflammatory mediators in OA pathogenesis is related with their activity on the catabolic and anabolic programs of the chondrocyte [Berenbaum, 2013]. The upregulation of the proinflammatory genes generally results in increased levels of catabolic enzymes, as MMPs and aggrecanases, decreased synthesis of ECM proteins, as collagen type II and aggrecan, and anti-catabolic mediators, as TIMPs contributing, in this way, to the progressive cartilage degradation [Clancy et al., 2004; Goldring and Goldring, 2007; Marcu et al., 2010]. These processes involve the activation of different transcription factors and signalling pathways, in particular those of NF-κB and MAPK.

#### 1.3.2.1 Regulation and Function of Inflammatory Mediators in Osteoarthritis

##### 1.3.2.1.1 Cytokines

Pro-inflammatory cytokines were found to be highly expressed in the synovial fluid, synovial membrane, subchondral bone and cartilage of the joints affected by OA and can be produced not only by chondrocytes, but also by other joint tissue cells [Berenbaum, 2013; Kapoor et al., 2011; Kobayashi et al., 2005; Loeser et al., 2012; Meats et al., 1980].

In OA, pro-inflammatory cytokines are known to induce the catabolic and inhibit the anabolic programs. This translates into an increase of inflammatory mediators and

proteolytic enzymes, and decreased synthesis of matrix components and TIMPs. Additionally, those cytokines also promote the induction of cell death, mainly by apoptosis, at least in part mediated by inflammatory mediators such as NO [Abramson, 2008; Blanco et al., 1995; Fernandes et al., 2002; Kapoor et al., 2011]. These mechanisms make them critical mediators of cartilage breakdown and of disease amplification and progression [Kapoor et al., 2011]

Although several cytokines (i.e. IL-15, IL-17, IL-18) [Fernandes et al., 2002; Kapoor et al., 2011] have been shown to be upregulated in OA joints and definitely contribute to the pathology establishment [Goldring and Goldring, 2007; Melas et al., 2014], both *in vitro* and *in vivo* studies evidence that, IL-1β and at a lower extent TNF-α are major players [Kapoor et al., 2011], by deregulating chondrocyte anabolic and catabolic responses. The deleterious effects of IL-1β and TNF-α are mainly associated with the upregulation of proteolytic enzymes. In response to these cytokines, OA chondrocytes produce a variety of matrix degrading enzymes, including MMP-1, MMP-3, MMP-8, MMP-13 and aggrecanases, as ADAMTS-4 and ADAMTS-5 [Lefebvre et al., 1990; Liacini et al., 2003; Reboul et al., 1996][Maldonado and Nam, 2013; Pelletier et al., 1995; Tetlow et al., 2001] On the other hand, the synthesis and expression of the natural MMPs inhibitors, TIMPs [Dean et al., 1989; Lefebvre et al., 1990], is inhibited, further promoting the degradative process. Moreover, other matrix-degrading enzymes are activated by IL-β in chondrocytes, namely glucosidases, responsible for GAG's degradation, which increases the spectrum of degradation sites activated by this cytokine and that contributes to further damage [Shikhman et al., 2000].

Moreover, the negative effects of IL-1β and TNF-α are also felt in the ECM protein synthesis. TNF-α has been shown to suppress the synthesis of proteoglycans, link protein and collagen type II in chondrocytes [Saklatvala, 1986; Seguin and Bernier, 2003], while the expression of collagens type II and type IX and aggrecan is downregulated in IL-1β-treated chondrocytes from different species [Chadjichristos et al., 2003; Goldring et al., 1988; Nietfeld et al., 1990a; Nietfeld et al., 1990b; Shakibaei et al., 2005; Stove et al., 2000]. At the same time, IL-1β upregulates the expression of the non-cartilage specific collagens type I and type III in human chondrocytes [Goldring et al., 1988].

IL-1β and TNF-α further promote the perpetuation of the disease by inducing the expression of pro-inflammatory cytokines, including their own and IL-17, IL-18 [Kapoor et al., 2011] and IL-6 [Reboul et al., 1996], and chemokines, as IL-8 [Lotz et al., 1992]. Additionally, IL-1β is responsible for the upregulated gene expression and production of other inflammatory mediators implicated in cartilage degradation in OA. Prostaglandin

E2 (PGE2) [Kojima et al., 2004] and NO result from the IL-1β-upregulated expression and activity of COX-2 and iNOS, respectively [Goldring and Goldring, 2007; Taskiran et al., 1994].

Whichever the factor considered that drives OA, the mechanisms underlying its pathogenesis and onset have been linked to the presence or to the production of IL-1β and, to a lower extent, of TNF-α (Figure 1.5). For instance in age-related OA, cell senescence is associated with a high secretory capacity, in a process called senescence-associated secretory phenotype (SASP), that includes the secretion of inflammatory mediators, in particular IL-1β [Coppe et al., 2010; Loeser, 2013]. Also, post-menopausal- [Richette et al., 2007], mechanical- or post-traumatic- and obesity- [Issa and Griffin, 2012] triggered OA subtypes have been associated with the upregulated expression of IL-1β in human chondrocytes.

All these evidences make pro-inflammatory cytokines and the subsequent events they initiate important basis of study in OA. How is the disease triggered and what events, factors and mechanisms can contribute to trigger the inflammatory process that results in structural damage of cartilage are questions of primary importance in order to discover disease-modifying OA drugs capable of stopping the progression of the disease and/or reverse the degradative process.

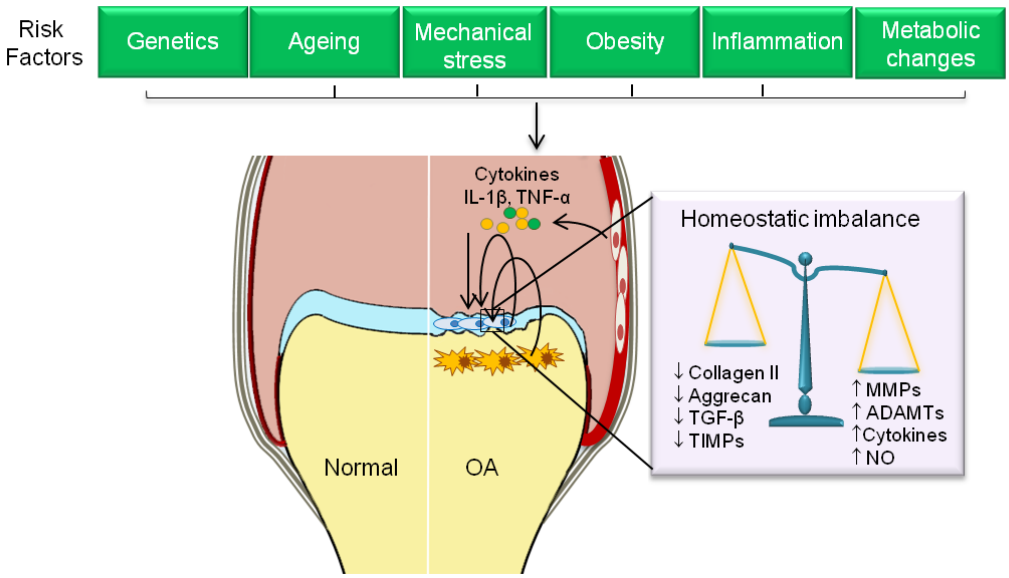


**Figure 1.5** Schematic representation of the cytokine role on the OA pathogenesis. Risk factors contribute to the upregulated levels of the pro-inflammatory cytokines, as IL-1β and TNF-α in the OA joint. Those cytokines contribute to the pathogenesis through several mechanisms including downregulation of anabolic events and upregulation of

catabolic and anabolic responses, effects that lead to the homeostatic imbalance that result on structural damage of the OA joint

#### 1.3.2.1.2 Nitric Oxide

Among the inflammatory mediators upregulated in OA chondrocytes, NO seems to have a prominent role. Several studies suggest that it can have different functions in normal and pathological joint conditions that appear to depend on the concentrations of NO found. In the cartilage of the OA joint, NO production is increased, and this excessive amount of NO has been mainly considered a catabolic factor that may be responsible for perpetuating the release of pro-inflammatory cytokines and other catabolic processes involved in OA process [Abramson, 2008].

Nitric oxide synthesis is catalyzed by one of the three isoforms of the nitric oxide synthase (NOS), iNOS, eNOS and nNOS. The inducible isoform, iNOS, is upregulated in OA chondrocytes, as result for example of mechanical stress, induced by abnormal compressive and shear forces [Fermor et al., 2001; Lee et al., 2003], pro-inflammatory cytokines, like TNF-α, IL-1β and IL-17 [Mendes et al., 2002a; Pacquelet et al., 2002; Palmer et al., 1993] or even as result of the presence of collagen [Chowdhury et al., 2010] and fibronectin fragments [Yasuda et al., 2004]. The upregulation of iNOS results in the overproduction of NO in OA joints [Melchiorri et al., 1998]. NO itself plays a role in perpetuating the release of inflammatory cytokines and other catabolic factors contributing to the positive loop on the inflammatory process found in OA joints. For instance NO is, in part, responsible for the synthesis of caspase 1 or Interleukin-1-coverting enzyme [Boileau et al., 2002], the enzyme responsible for the activation of IL-1β and IL-18 and for downregulation of its natural inhibitor, the protease inhibitor 9 (PI9), thus sustaining the inflammatory response by promoting the maturation of inflammatory cytokines [Boileau et al., 2002]. It also participates in the activation of the AP-1 [Mendes et al., 2002c] and NF-κB [Abramson, 2008], transcription factors that critically mediate the gene expression of inflammatory mediators, like IL-1β, TNF-α and iNOS itself, and catabolic factors, as MMPs [Boileau et al., 2002; Sasaki et al., 1998; Xie et al., 1994].

Apart from its role in sustaining the inflammatory process in OA, NO also participates in the modulation of proteoglycan, collagen and MMPs expression. It has been shown that NO mediates IL-1β-induced suppression of proteoglycans and collagen synthesis in human chondrocytes [Taskiran et al., 1994], increases the gene expression of MMP-

1, -2, -3 and -9 in rabbit articular chondrocytes [Sasaki et al., 1998] and suppresses the endogenous activity of TIMP-1 in human chondrocytes [Ridnour et al., 2007].

On the other hand, several other studies, in part, counteract the observations above showing that NO also have an important role on the maintenance of normal joint conditions. In human chondrocytes, it was shown that the IL-17- induced NO production suppresses the matrix synthesis and protects cartilage from matrix breakdown [Cai et al., 2002]. It also seems to promote procollagen synthesis in tendon cells [Xia et al., 2006]. It is also contradictory the information regarding the pro-inflammatory activity and the cartilage protection yield by NO. Henrotin (1998) showed that, in human chondrocytes, NO reduces the synthesis of $PGE_2$, IL-6 and IL-8 [Henrotin et al., 1998] and Rosa (2008) showed that NO can also help to downregulate NF-κB activity [Rosa et al., 2008]. Moreover, in an animal model of OA where the iNOS gene was silenced, the inflammatory symptoms were exacerbated, as well as cartilage degradation [McInnes et al., 1998; Veihelmann et al., 2001].

Given the variety of catabolic but also protective effects of NO in the chondrocytes, its production, as a potential target for disease-modifying therapy for OA must be carefully managed. Both the inhibition of iNOS, for inhibition of the high amounts of NO associated with catabolic events and the NO donation might have benefits on OA treatment [Abramson, 2008].

#### 1.3.2.1.3 Nuclear Factor – κB (NF-κB)

The upregulation by IL-1β and TNF-α of COX-2, MMP-13, iNOS and other central molecules of the osteoarthritic process is mediated by the induction and activation of transcription factors and signalling pathways including NF-κB and AP-1 which are involved generally in stress and inflammation-induced signalling [Goldring and Goldring, 2007]. Among the signalling pathways involved in the initiation and amplification of inflammatory responses and tissue destruction, the transcription factor NF-κB is regarded as its primary regulator [Tak and Firestein, 2001]. NF-κB represents a family of proteins sharing a Rel homology domain in their N-terminus that include NF-$κB_1$ (p50 and its precursor p105) and NF-$κB_2$ (p52 and its precursor p100) Rel A (p65), Rel B, c-Rel (REL). This Rel domain is responsible for homo- and heterodimer formation, for the interaction with inhibitory molecules and for binding to the promoter sequences of target genes [Ge et al., 2011; Hayden and Ghosh, 2008a; Oeckinghaus and Ghosh, 2009]. This transcription factor takes part of the normal physiology of cells; however, the inappropriate regulation of its activity has been implicated in the pathogenesis of diverse pathologies [Marcu et al., 2010] with several studies showing

that NF-kB dimers are abundant in arthritic joints, especially in those affected by OA [Li et al., 2012; Rosa et al., 2008].

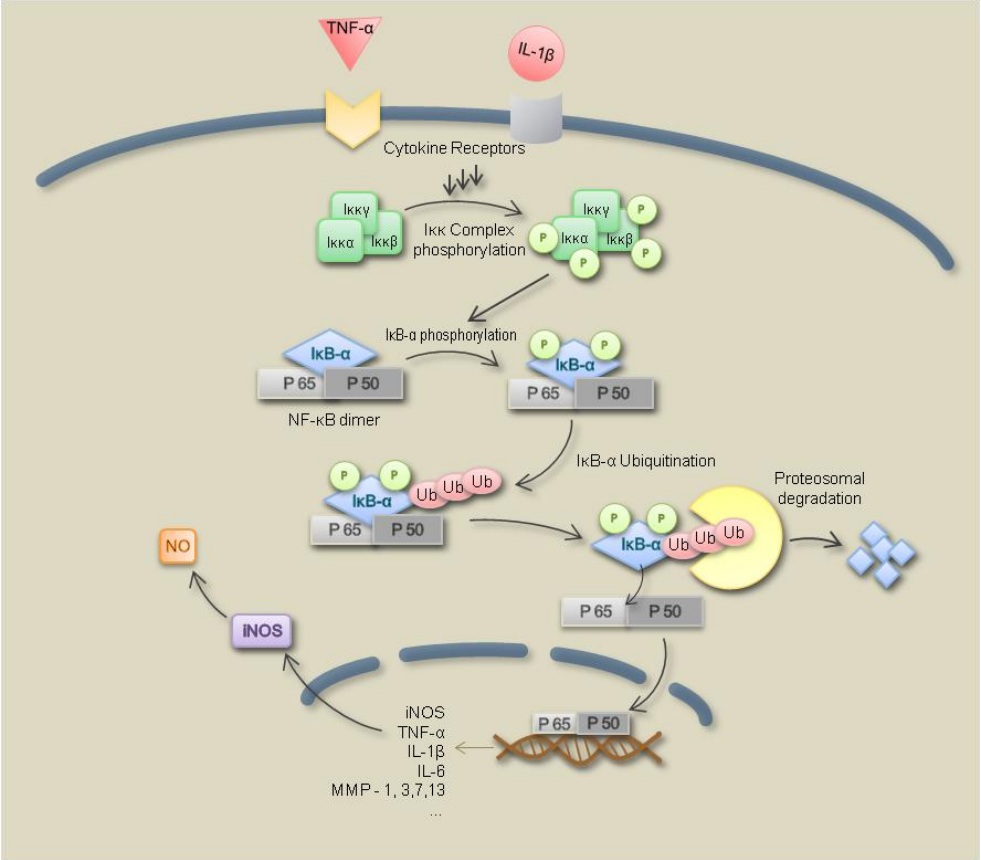


**Figure 1.6** Schematic representation of the canonical NF-κB activation in response to pro-inflammatory cytokines. Adapted from [Hayden and Ghosh, 2008a]

NF-κB belongs to a family of "fast-acting" primary transcription factors, i.e., transcription factors that are present in the cells in an inactive state and that do not require new protein synthesis to become activated. In resting cells, the NF-κB dimers are sequestered by a family of inhibitors, called IκBs, of which IκB-α is the most studied. When bound to NF-κB, these inhibitors function by masking the nuclear localization sequence (NLS) in one of the subunits of the heterodimer, allowing it to cycle between the nucleus and the cytoplasm without binding to DNA [Ghosh and Karin, 2002; Hayden and Ghosh, 2008a].

The canonical NF-κB pathway, as represented in figure 1.6 has been closely related to the inflammatory response. This classical NF-κB activation pathway requires the phosphorylation and subsequent proteossomal degradation of the inhibitory IκB-α,

which releases NF-κB dimers, commonly made of p65 (or RelA) and p50 proteins [Hayden and Ghosh, 2008a; O'Dea and Hoffmann, 2009]. Free dimers translocate to the nucleus to activate the transcription of target genes, particularly those of pro-inflammatory cytokines (e.g. IL-1β, TNF-α), chemokines (e.g. CXCL-8) and inflammation-associated enzymes (iNOS and COX-2) [Hayden and Ghosh, 2008b].

In the context of OA, NF-κB can be triggered by several distinct factors and mechanisms generated or not within the cartilage. Mechanical stress [Agarwal et al., 2004; Ferretti et al., 2006; Knobloch et al., 2008], pro-inflammatory mediators, in particular IL-1β and TNF-α [Frank et al., 2013; Hayden and Ghosh, 2008a], iNOS [Aktan, 2004] or even ECM protein fragments [Chowdhury et al., 2010; Pulai et al., 2005] are known to activate this transcription factor [Uwe, 2008; Yasuda, 2011]. Either acting by itself or in coordination with other mediators or signalling cascades, NF-κB induces additional expression of inflammatory genes, impairs the anabolic activity and directly enhances the secretion of several matrix-degrading proteinases [Fan et al., 2006; Ge et al., 2011; Mengshol et al., 2000; Stove et al., 2000]. More specifically, NF-κB seems to be involved in every step of the cartilage change that occurs in OA. In the OA cartilage, the responses induced by the chondrocytes with a degradative phenotype were NF-κB mediated and included the production of MMP-1, MMP-3, MMP-8, MMP-13, ADAMTS-4 and ADMATS-5 [Liacini et al., 2003; Liacini et al., 2002], increased expression of COX-2 and PGE2, iNOS and its product, NO, IL-1β, IL-6, TNF-α and type X collagen, thus directly contributing to the increased cartilage degradation and further chondrocyte hypertrophy [Goldring and Marcu, 2009]. Thus, it is clear that in OA, NF-κB is part of a positive loop, resulting in a sustained activation of this transcription factor and, in additional expression of inflammatory genes and catabolic mediators further contributing to cartilage degradation.

Moreover, NF-κB activation also results on the expression of other regulatory factors, including E74-like factor 3 (ELF3), hypoxia-inducible factor-2alpha (HIF-2), runt-related transcription factor 2 (RUNX2) and Bone morphogenic protein-2 (BMP-2), which further trigger the hyperthrophic state of the chondrocyte [Goldring, 2012].

Regarding its pivotal role in OA onset and progression, NF-κB is regarded as an important therapeutic target. However, given its importance for cell survival and maintenance, targeted strategies have to be weighted in order to not cause unwanted side effects due to complete inhibition [Uwe, 2008]. Targeting specifically the canonical activation of NF-κB, namely IκB-α phosphorylation and the upstream events may prevent the high activation of this transcription factor that contributes to cartilage degeneration in OA pathology [Marcu et al., 2010]

#### 1.3.2.1.4 Mitogen Activated Protein Kinases (MAPK)

Together with NF-κB, MAPKs pathways are key regulators of the chondrocyte functions, both during development and in adult cartilage [Thalhamer et al., 2008].
MAPK are a family of Ser/Thr kinases formed by 4 subfamilies of kinases with different isoforms, including c-Jun $NH_2$- terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK), each presenting isoforms [Johnson and Lapadat, 2002].
MAPK are activated by phosphorylation of specific Thr and Tyr residues by MAPK kinases (MKKs). Such phosphorylation can be triggered by a great range of factors, namely cytokines, growth factors and/or integrin-mediated signals which activate downstream events that result on the activation of one or more MAPK. Once activated, the MAPK in turn, activate other protein kinases and several transcription regulatory proteins, including ERK activation of Elk-1 and c-Myc, p38 activation of ATF-2 and MEF2, and JNK activation of c-Jun (figure 1.7). These transcription factors regulate the expression of a set of genes relevant to OA, namely some involved in the inflammatory response, regulation of cell proliferation, and production of matrix-degrading enzymes [Berenbaum, 2004; Loeser et al., 2008]. JNK may be particularly relevant due to its role in the activation of c-jun [Han et al., 2001; Johnson and Nakamura, 2007; Kristof et al., 2001], a key AP-1 component essential for the expression of MMPs and pro-inflammatory cytokines, such as TNF-α and IL-1β [Loeser et al., 2008] and other pro-inflammatory mediators, as iNOS and NO [Kristof et al., 2001; Mendes et al., 2003b]

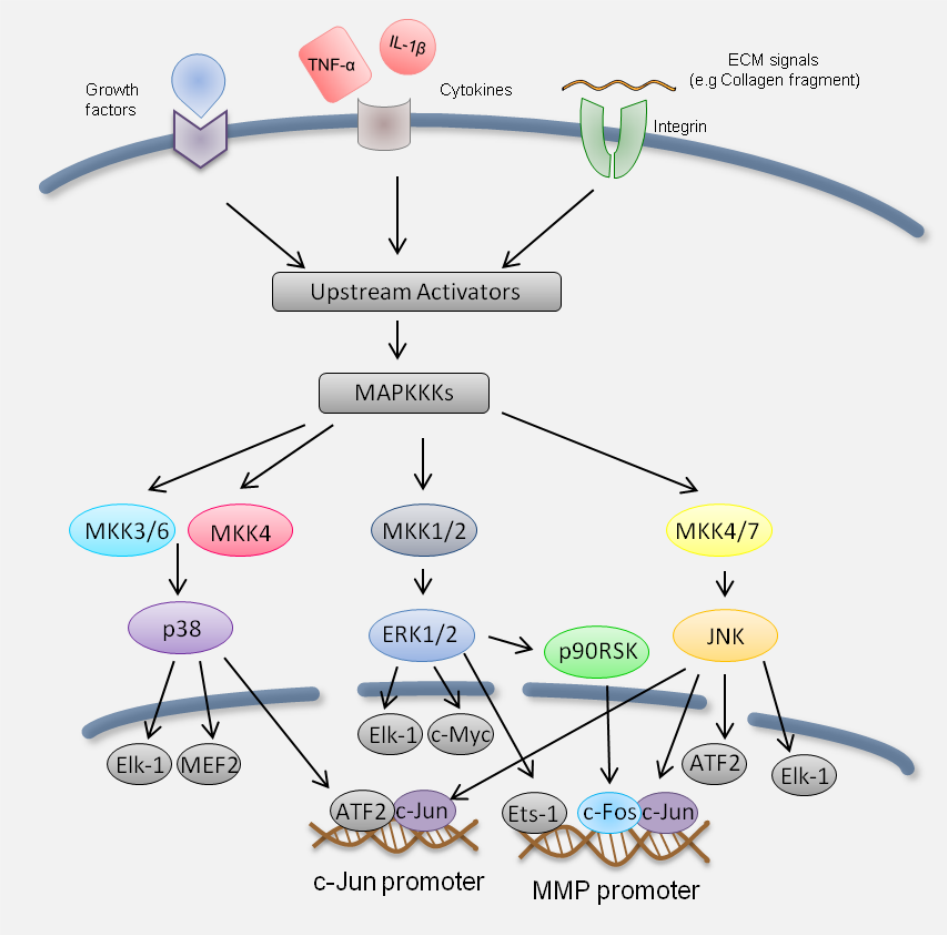


**Figure 1.7** Several receptors, including growth factor, cytokine, and integrin receptors, activate signalling pathways which lead to the activation of the MAP kinase pathways. MAP kinase kinase kinases (MAPKKKs) activate MAP kinase kinases (MKKs; MAP and ERK kinase or MEK), which in turn activate the MAP kinases (p38, ERK1/2, and JNK). The MAP kinases activate other kinases, such as p90RSK, and transcription factors that regulate gene transcription, including Elk-1, MEF2, ATF2, c-Myc, Ets-1, and c-Jun. In this way, all three MAP kinases are involved in the expression of matrix metalloproteinases (MMPs) and other mediators involved in the development of OA. Adapted from [Loeser et al., 2008]

Several evidences attest the activity of all three MAPK in OA. Levels of phosphorylated JNK [Clancy et al., 2001] and p38 [Fan et al., 2007] are higher in human OA cartilage compared to normal cartilage. The higher activation of MAPK has been mainly associated with increased expression of MMPs and aggrecanases, but also with modulation of anabolic activities in the cartilage of several species [Fan et al., 2006; Liacini et al., 2003; Liacini et al., 2002; Liacini et al., 2005; Sylvester et al., 2012]. As examples of the deleterious MAPKs' activity in the cartilage, IL-1β- and TNF-α-induced

expression of MMP-1, MMP-13 and aggrecanase 1 and downregulation of collagen type II in chondrocytes requires JNK and p38 activation [Fan et al., 2006; Liacini et al., 2003; Liacini et al., 2002; Liacini et al., 2005; Sylvester et al., 2012], and the modulation of anabolic and catabolic activities by collagen type II and fibronectin fragments is mediated by MAPK activation [Chowdhury et al., 2010; Loeser et al., 2003; Mengshol et al., 2000; Sondergaard et al.]

All three MAPK are involved in the expression of MMPs and other factors involved in the development of OA. In the majority of cases, the mediation of cartilage destruction did not address a specific MAPK, but two MAPK or the three MAPK signalling pathways. Contrariwise, the inhibition of a single MAPK seems to be, in most cases, enough to inhibit the increased expression of MMPs or aggrecanases. For example, IL-1β stimulation of MMP-13 production requires the activation of both JNK and p38 [Mengshol et al., 2000] and fibronectin fragments activate ERK1/2, p38 and JNK1/2, but inhibition of one of the three MAPK is enough to inhibit the fibronectin fragments-stimulated MMP-13 expression [Loeser et al., 2003].

Besides the role of MAPK in mediating the catabolic function of chondrocytes, several studies also showed that they take part on deregulated anabolic processes observed in OA. Activation of ERK and p38 was associated with inhibition of aggrecan expression [Masuko et al., 2007] and the LPS-inhibited synthesis of proteoglycans in chondrocytes can be partially reversed by inhibition of p38 [Bobacz et al., 2007].

## 1.4 OA TREATMENT

According to the World Health Organization, OA is among the high burden diseases, in Europe particularly, for which the currently available treatment is inadequate. OA is highly common and is increasing not only in the elderly but also among the general population, and available treatment is ineffective in stopping or reversing disease progression [Kaplan et al., 2013; Wieland et al., 2005].

Most of the times, the major disease outcomes, as pain, loss of the joint function and disability [Wieland et al., 2005], are only distinctly observed in late stages of OA, being difficult to establish a correlation between symptoms and pathology in early stages of the disease, which together with the limited biological markers make an early intervention in the disease difficult [Rousseau and Garnero, 2012].

Regarding these difficulties, OA pain and functional impairment of the patient, as the major OA burden on individuals, have been the main focus of OA therapy. In addition

to the first-line non-pharmacological measures, such as weight loss and physical exercise, OA therapy includes pharmacological symptomatic treatment with analgesics, classical non-steroidal anti-inflammatory agents and COX-2 selective inhibitors (NSAIDs and COXIBs), glucosamine and chondroitin sulphate supplements and/or intra-articular administration of steroids or hyaluronic acid, all of them with limited efficacy and/or adverse side effects, especially in the long-term utilization that the disease requires [Wieland et al., 2005]. To date, the approved treatments directed at reducing the symptoms of OA have not been shown to prevent or reverse joint structural damage so, in severe cases, joint replacement surgery is the last choice effective in relieving the painful and debilitating effects of the disease.

Consequently, there is an urgent need for OA disease-modifying therapies which also improve symptoms and are safe for clinical use over long periods of time.

As we described before, OA is not considered only an age-related or a “wear and tear” disease, but a multiplicity of factors and conditions can influence and lead to OA onset and progression. The complexity and variability of OA aetiology is believed to have a further impact in the response to treatment. Thus, a better understanding of particular pathophysiological mechanisms involved in each particular OA phenotype together with an individual evaluation of the patient’s co-morbidities and with the evaluation of specific biomarkers of joint destruction [Poole, 2003] is necessary to found better targeted and effective therapies with capacity to prevent the degenerative joint process and reverse the already established damage, without underestimate the pain [Goldring and Goldring, 2007; Lane et al., 2011; Mobasheri, 2013].

### 1.4.1 Currently Available Treatment

Current treatment options for OA patients are focused on relief from pain and improvement of joint function. Not surprisingly, since these medical interventions do not consider the full OA illness, they have little effect, if any at all, on the structural degradation of the joint tissue. Because of that, many patients have surgery as the only option to improve quality of life [McAlindon et al., 2014].

General recommendations by the European League Against Rheumatism (EULAR), the American College of Rheumatology (ACR) and the Osteoarthritis Research Society International (OARSI) for the management of OA include a combination of non-pharmacological interventions and pharmacological treatments (figure 1.8) [Hochberg et al., 2012; McAlindon et al., 2014; Zhang and Doherty, 2006; Zhang et al., 2008]. OARSI has specifically developed guidelines for the non-surgical treatment of OA that

are based on the stratification of patients into four groups: patients with knee-only OA and no comorbidities, patients with knee-only OA with comorbidities, patients with multi-joint OA and no comorbidities, and patients with multi-joint OA with comorbidities. These comorbidities included diabetes, hypertension, cardiovascular disease, renal failure, gastrointestinal bleeding, depression, or a physical impairment limiting activity, like obesity, and were taken into account based on that those co-morbidities might influence treatment choices for OA [McAlindon et al., 2014].

From the classes of medications currently used to treat OA of the knee we can refer: analgesics/ anti-inflammatory agents, glucocorticoids, opioids and slow-acting drugs for osteoarthritis (SYSADOA).

Acetaminophen (paracetamol) is recommended for initial management of OA, as it is generally safe and particularly good for treatment of mild pain, being the first line therapy for knee OA [Hochberg et al., 2012; Towheed et al., 2006]. Oral and topical non-steroidal inflammatory drugs (NSAIDs) are considered when patients do not respond to acetaminophen. NSAIDs are the most common treatment for rheumatologic conditions, including OA, rheumatoid arthritis (RA) and gout. They present analgesic, antipyretic and anti-inflammatory effects and act mostly through inhibition of the 2 isoforms of COX-1 and COX-2 enzymes, leading to the inhibition of prostaglandin synthesis that is directly related with pain and inflammation [Wieland et al., 2005]. Most conventional NSAIDs inhibit both COX isoforms which is directly related to their gastrointestinal adverse side effects. More recently, these drugs have also been associated with significant cardiovascular side-effects that warrant caution in patients at higher risk [Soubrier et al., 2013]. Selective COX-2 inhibitors were developed in order to avoid those side effects as this isoform is supposed to be induced at the site of inflammation and not on the gastrointestinal system; however, they are not devoid of side effects, as nephrotoxicity and hypertension, which significantly limit their therapeutic use, especially in chronic conditions like OA [Hochberg, 2005]. Despite their side effects, NSAIDs are recommended by EULAR and OARSI treatment guidelines committee recommends NSAIDs for management of hip and knee OA [McAlindon et al., 2014].

Opioid analgesics, such as tramadol, are useful alternatives in patients in whom NSAIDs, including COX2-selective inhibitors, are contraindicated, ineffective and/or poorly tolerated. The increased risk of adverse side effects, particularly in the elderly, has to be taken seriously [Nuesch et al., 2009; Wieland et al., 2005]. Finally, the treatment with glucocorticoids is one of the last pharmacological lines of OA treatment and could be used as alternatives for pain relief in patients who cannot use or do not

respond to other first line therapies [Towheed et al., 2006]. However, they can bring about adverse effects, including mild ones as constipation, nausea and itching, and more serious, as respiratory depression, and addiction [Nuesch et al., 2009].

In addition to the types of medications mentioned above, there is a heterogeneous group of medications that, unlike the COX-2 inhibitors, do not inhibit prostaglandin synthesis. This group includes hyaluronic acid (HA), D-glucosamine sulphate (GlcN), chondroitin sulphate (CS), and diacerhein. These medications are collectively called Symptomatic Slow Acting Drugs for Osteoarthritis (SYSADOA), and have been recommended by EULAR for the treatment of pain in OA [Zhang and Doherty, 2006]. Nevertheless, their usefulness is controversial, being considered as treatments of "uncertain appropriateness" by OARSI on the last update of Knee OA management guidelines [McAlindon et al., 2014]. This heterogeneous group of compounds was the first to be linked to structure-modifying effects in the disease with several evidences showing that these drugs slow the radiographical progression of knee OA [Zhang et al., 2008]. Their activities can range from inhibition of inflammation to a potential alteration of the viscoelastic properties of cartilaginous tissue [Hochberg et al., 2012; Michael et al., 2010; Towheed and Anastassiades, 2007]. Several studies have shown that they have a slow onset of action improving OA symptoms after a couple of weeks, benefits that last for a long period after the end of treatment [Volpi, 2004; Volpi, 2006]. In a randomized, controlled, double-blind trial, patients with knee OA treated with intra-articular injection of hyaluronic acid had significantly less pain and better function for up to three weeks afterward the end of treatment with no severe side effects reported [Petrella and Petrella, 2006]. On the other hand, several studies reported opposite effects regarding the efficacy of CS, GlcN and HA. Among the studies that compare glucosamine with placebo for OA treatment, one review found no statistically significant benefit of glucosamine for pain [Wandel et al., 2010] and the other found a positive effect for pain that did not reach statistical significance [Towheed et al., 2005]. For HA there is only a small effect in the treatment of knee OA compared with placebo injection [Bannuru et al., 2011; McAlindon et al., 2014; Rutjes et al., 2012] and for CS, results differed regarding symptom relief, with some reviews finding no significant benefit over placebo for pain and others finding large effect sizes in favor of chondroitin [Hochberg et al., 2008].

Diacerhein is also a SYSADOA with anti-inflammatory properties used in OA treatment. It works by blocking the action of IL-1β, and is a potent inhibitor of IL-1β-induced NF-κB activation and NO production in chondrocytes [Mendes et al., 2002b; Pelletier et al., 1998]. Several evidences show that diacerhein is slightly, but significantly, more

effective than placebo in improving pain and slowing the progress of hip OA [Bartels et al., 2009; Fidelix et al., 2006]. However the Pharmacovigilance Risk Assessment Committee (PRAC) of the European Medicines Agency (EMA) recommended the suspension of diacerhein-containing medicines across the European Union, in November 2013, due to concerns over gastro-intestinal side effects (diarrhea) and liver toxicity that outweigh the benefits for treating OA.

Despite all the claims of pharmacotherapeutic efficacy of SYSADOAs, to date, none have shown a significant structure modifying effect in placebo-controlled, clinical studies. So, their use in OA treatment is so far debatable [Chard and Dieppe, 2001; McAlindon et al., 2014; Wieland et al., 2005].

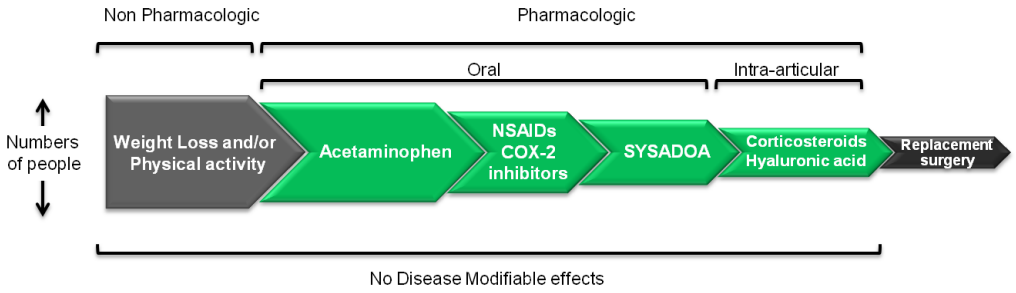


**Figure 1.8** Represent the current treatment options for osteoarthritis as issued by the American college of Rheumatology and OARSI. In addition to non-pharmaceutical measures such as weight loss and physical exercise they include only symptomatic treatment of limited efficacy with analgesics, non-steroidal anti-inflammatory agents, SYSADOAs or intra-articular administration of corticosteroids or hyaluronic acid. None of them present effective disease modifiable effects

More recently, the first steps on anti-cytokine therapy for OA were taken. These therapies are based on the importance of the inflammatory component of OA, especially the increased expression and activity of several secreted proinflammatory cytokines, namely IL-1β and TNF-α, in joint tissues and their importance not only in the inflammatory, but in the degenerative processes of the cartilage. Despite the clear evidence on the role of TNF-α and IL-1β in the pathophysiology of OA, only a few experimental trials have investigated the efficacy of blocking these pro-inflammatory cytokine in the treatment of OA. The currently available biological anti-cytokine therapies are the antibodies against TNF-α, Adalimumab, Infliximab and Etanercept,

which were initially approved for RA treatment [van der Bijl et al., 2008]. For OA, in humans, a case report described the successful treatment of inflammatory knee OA using Adalimumab [Grunke and Schulze-Koops, 2006] with improvement not only in the symptoms, but also on MRI findings with an important reduction of synovitis and synovial effusion. However, the results of the open label study with this drug in 12 patients with erosive OA of the hands revealed only a modest improvement in the number of swollen joints, disability score, pain score, and physician global assessment [Magnano et al., 2007].

### 1.4.2 Disease Modifying OA Treatment

Given the high disability burden among patients, the social-economic impact generated by OA and the limitations of the existing non-pharmacologic/protective measures and pharmacological agents, new treatments for OA are urgently needed. In particular, disease-modifying OA drugs (DMOADs), capable of slowing or halting the structural changes in joint tissues occurring in OA, are crucial to significantly reduce pain and restore the joint function.

Cytokine cascades, nitric oxide, proteinase release and activation, senescence, apoptosis, etc., are all processes or mediators affecting all the cells of the joint, whether they are located in the synovium, bone or in the articular cartilage [Qvist et al., 2008], that can be considered important targets for DMOADs.

In theory, replacing the balance between those processes would stop the progression and eventually favor the reparative process which are the main focus of the DMOADs principals [Alcaraz et al., 2010]. The development of DMOADs presents many challenges that include, firstly, the complexity of the disease, namely the involvement of several joint structures (cartilage, synovium and bone…) the complex etiopathogenesis, the heterogeneity of clinical presentations and the slow progression of OA, which create a complex environment for therapeutic development with disease-modifying objectives [Hunter, 2011]. The second one seems to be the lack of sensitive biomarkers and measurable clinical endpoints that help to connect the structural changes to the patient response and assess the efficacy of new formulations. For instance, the determination of joint space narrowing could be an important radiograph measure of cartilage loss, however, its use as single endpoint considered for the evaluation of the efficacy of a new drug could be critical. The space between the two bones in a joint, in a radiograph view represents the overall thickness of the joint cartilage surfaces of each opposing bone as they articulate, or come into contact with

one another. In conditions like arthritis, the joint space gets narrow because of cartilage degradation. Although this parameter can be relevant on the late phases of OA were the cartilage degradation and loss is obvious, in early stages this parameter is not that relevant as could be, for instance, the presence of inflammatory mediators [Qvist et al., 2008; Sokolove and Lepus, 2013].

Strategies for development of DMOADs include anti-catabolic agents, aiming at slowing or halting OA progression and anabolic agents aiming at inducing cartilage regeneration. Anti-catabolic agents would target: 1) direct inhibition of MMP expression and activity [Clutterbuck et al., 2009], as these proteinases are intrinsically involved in cartilage breakdown. 2) Prevention of excessive proinflammatory cytokine production, especially IL-1β and TNF-α but also IL-6, IL-8, and IL-17, also implicated in OA pathophysiology [Goldring, 2001; Rudolphi et al., 2003] and/or 3) prevention of the activation of major signalling pathways that drive inflammatory and catabolic responses, as those of NF-κB [Costa et al., 2012; Marcu et al., 2010] and MAPKs [Khalife and Zafarullah, 2011], since their activation by stress-related stimuli regulates the expression of many cytokines, chemokines, adhesion molecules, inflammatory mediators, and several matrix-degrading enzymes related with joint damage [Goldring, 2006]. On the other hand, pro-anabolic therapeutic agents, such as growth factors, could be used to target several receptors and signalling pathways that[Pelletier, 2004] increase chondrocyte anabolism [Pelletier, 2004].

Highly specific drugs, small interfering RNAs (siRNAs), or other biological inhibitors are under the focus of current OA research [Mobasheri, 2013], but despite the large number of active research and drug discovery programs to identify structure-modifying approaches to inhibit joint destruction in OA, none has been conclusive or showed significant efficacy as a DMOAD and to date, no DMOADs are approved by the Food and Drug Administration (FDA) or the EMA. Oral administration of PG-116800, a MMP inhibitor effective against MMP-2, -3, -8, -9, -11, -13, and -14 was associated with severe musculoskeletal adverse effects without clear benefits for knee OA [Krzeski et al., 2007]. Similarly, other non-selective MMP inhibitors evaluated for oncology were associated with arthralgia, stiffness and joint swelling, as well as with other severe musculoskeletal complications [Bissett et al., 2005; King et al., 2003; Leff et al., 2003]. Nevertheless, the MMP inhibitor, doxycycline, has demonstrated potential efficacy for disease modification [Brandt et al., 2005; Maher et al., 2013]. Development of more selective MMP inhibitors could potentially improve the balance between benefit and side effects in patients with OA.

Similarly, despite the clear role of inflammation in OA, no anti-inflammatory therapies were found, and those under test, including use of biologic agents to inhibit TNFα and IL-1β, all proved to be disappointing [Hunter, 2008]. IL-1 or IL-1R inhibitors have been tested in preclinical and clinical trials of RA, however only a few of these have been investigated in OA, e.g. Anakinra (IL-1R antagonist) and AMG-108 (anti-IL-1 human antibody, anti-IL-1 huMAb). Their beneficial effects on symptoms and signs in knee OA are still controversial [Chevalier et al., 2005; Yang et al., 2008].

Other DMOADs are under investigation namely the iNOS inhibitor, SD-6010, by Pfizer which rationale for development rests on the inhibition of pro-catabolic and pro-inflammatory effects of effects of NO generated by the upregulation of the synthetic activity of enzyme, iNOS [Abramson, 2008] and the COX-inhibiting NO-donating compound naproxcinod (formerly HCT 3012), from NiCox. Naproxcinod is a derivative of naproxen and is the first compound in the COX-inhibiting NO-donating class, which integrates the anti-inflammatory effects of the NSAID with the potential beneficial effects of NO on the cardiovascular system. It is in phase III trial for the treatment of OA [Anonimous, 2007; Qvist et al., 2008].

Since ever, natural products, as plants, are a source of compounds with pharmacological properties. In the last decades, a great percentage of the new drug entities registered have a natural origin or are inspired in a natural compound which proves them as a believable source of new compounds with pharmacological properties[Newman and Cragg, 2007]. Accordingly, research on OA therapy has focused on these sources to found new compounds with disease modifying properties with very promising results [Henrotin et al., 2013; Khalife and Zafarullah, 2011; Mobasheri, 2012; Seidl, 2002; Shakibaei et al., 2012; Shakibaei et al., 2011; Shakibaei et al., 2005], However, none has yet proceed to clinical development.

The biodiversity of the Portuguese flora opens wide possibilities for identification of compounds to be studied in the context of chronic inflammatory diseases and specifically in OA. From the panoply of compounds that can be obtained from plants, literature and preliminary studies showed that small volatile and hydrophobic plant metabolites can modulate inflammatory signalling pathways [Neves et al., 2009]. A huge amount of compounds with such characteristics can be found in essential oils which are typical extracts from aromatic plants, generally obtained by distillation.

## 1.5 OBJECTIVES AND ORGANIZATION OF THE THESIS

OA and DM are age-related diseases whose prevalence is increasing exponentially. Several studies showed that OA is a heterogeneous disease and that the multiple risk factors associated with it are responsible for a multiplicity of OA phenotypes. Diabetes mellitus, especially type 2, has been considered an independent risk factor for the development and progression of OA in several epidemiological studies which indicate that OA is more prevalent, more severe, has an earlier onset and progresses more rapidly in diabetic- than in non-diabetic patients [King et al., 2013; Martinez-Huedo et al., 2013; Nieves-Plaza et al., 2013; Schett et al., 2012a]. These evidences, as well as *in vitro* and *in vivo* data, including studies by our own group [Rosa et al., 2009; Rosa et al., 2011b], strongly suggest the existence of a specific DM-associated OA phenotype [Berenbaum, 2011]. A better understanding of particular underlying physiological and pathological mechanisms is essential for the design and development of new and better targeted therapeutic strategies effective to halt OA progression, its growing incidence and its associated huge socio-economic costs [Kaplan et al., 2013]. We addressed this issue by hypothesizing that hyperglycemia, a hallmark of DM and other conditions associated with glucose imbalance, is a major effector of chondrocyte damage, accelerating age-related changes that favor OA development. In this context, previous studies from our group [Rosa et al., 2009; Rosa et al., 2011b] showed that hyperglycemia-like glucose concentrations induce catabolic responses and impair the ability of chondrocytes to respond to anabolic agents. Thus, one of the objectives of this thesis was to identify mechanisms by which chondrocytes sense and regulate glucose transport which is the first rate limiting step for glucose utilization and thus can be a target for preventing deleterious effects caused by hyperglycemia. Moreover, and to further identify potential therapeutic targets, we also investigated the role of hyperglycemia in modulating inflammatory responses in chondrocytes. The results obtained and their discussion are presented in chapters 3 and 4.

Since every factor, including hyperglycemia as we demonstrate in chapter 4, that shifts chondrocyte homeostasis towards a pro-catabolic state, induces an inflammatory response in chondrocytes and this is a major effector mechanism of cartilage destruction in OA, our last objective was to identify compounds that can halt inflammation and catabolic responses in chondrocytes. Identification of such compounds is crucial for the development of effective disease-modifying anti-osteoarthritic drugs. For this, we evaluated a series of essential oils and their compounds for their ability to inhibit the inflammatory and catabolic processes in

human chondrocytes, focusing on the major signalling pathways activated in osteoarthritic joints, namely NF-κB and the MAPKs. The results obtained are reported and discussed in chapters 5 to 7.

Finally, even though OA involves the whole joint, cartilage degradation, a multi-faceted and complex process that characterizes all forms of OA, is the hallmark of the progression and irreversibility of the disease reason why this work was specifically focused on this tissue.

The last chapter of this thesis presents a general and integrated overview of the results reported, matching them with the major objectives defined and identifying new questions and future perspectives elicited by this work.

# CHAPTER 2.
# MATERIAL AND METHODS

## 2.1 Cartilage Samples

Human knee cartilage was collected, within 12 h of death, from the distal femoral condyles of multi-organ donors or with informed consent from patients undergoing total knee replacement surgery at the Orthopaedics Department of the University Hospital of Coimbra (HUC). The Ethics Committee of HUC approved all procedures. Where considered relevant for the work goals the samples were classified according to the degree of macroscopic damage, using the classification of Outerbridge (1961). Group 0-1 includes cartilage samples with no macroscopic signs of degradation (score 0) or presenting only a softened surface (score 1); Group 2-3 includes fibrillated or fissured cartilage samples without evident signs of surface erosion (scores 2 and 3); and Group 4 comprises severely damaged cartilage samples, with areas of extensive full thickness erosion that exposed the subchondral bone, corresponding to advanced OA.

## 2.2 Chondrocyte Isolation and Culture

Chondrocytes were isolated by enzymatic digestion from non-pooled cartilage samples. Briefly, cartilage slices underwent sequential digestion with Pronase (Roche, Indianapolis, IN, USA), a non-specific protease for 1h30min and collagenase A (Roche) overnight. To avoid chondrocyte dedifferentiation, non-proliferating monolayer cultures were setup by plating $1x10^6$ chondrocytes/ml in Ham : F-12 medium containing 3% antibiotic/antimycotic solution and 5% Fetal Bovine Serum (Invitrogen, Life technologies, Carlsbad, CA) and allowed to recover for 24 h at 37ºC in a humidified atmosphere supplemented with 5% CO2.

Cells from the human chondrocytic cell line, C28/I2, kindly provided by Prof. Mary Goldring (currently at the Hospital for Special Surgery, New York) and Harvard University, were cultured in DMEM/ Ham's F-12 (1:1, v/v) and passaged every four days, as recommended. For the experiments $0,5x10^6$ chondrocytes/mL were plated and allowed to recover for 24h in similar conditions to that of primary human chondrocytes.

Prior to any treatments, the cells were serum-starved overnight and thereafter maintained in culture medium with 1% antibiotic/antimycotic solution, without serum. The specific treatments conditions are indicated in figure legends.

### 2.3 Assessment of Cell Viability

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT; Sigma) reduction assay, a quantitative colorimetric method based on the reduction of the MTT salt by the mitochondrial enzymes of viable cells [Mosmann, 1983], was used to determine the cytotoxicity and to select non-cytotoxic concentrations of each of the compounds under test. Briefly, the cells were incubated with each compound in different concentrations for 24h h after what the culture medium was replaced by fresh medium containing 5 µg/mL MTT and the cells further incubated for 30 min. The resulting dark blue crystals of formazan were then dissolved in acidified isopropanol and the absorbance of the corresponding solution, directly proportional to the number of living cells, was measured in an automatic plate reader (SLT, Austria) set at a test wavelength of 570 nm and a reference wavelength of 620 nm. The absence of cytotoxic effects in each cell model was defined as the concentration of tested compounds eliciting an absorbance reading of at least 80% of the value measured in the cells treated with IL-1β alone for same time period.

### 2.4 Western Blot

Total, cytoplasmatic and nuclear cell extracts were prepared and subjected to western blot. Briefly, 25 µg of protein were separated by SDS/PAGE [10% (v/v)] and transferred onto PVDF membranes by electroblotting. The membranes were blocked with 5% skim milk for 2 hours in Tris-buffered saline-Tween (TBS-T, 20mM Tris-HCl, 150mM NaCl, 0,1% Tween 20) and probed with the antibodies presented on Table 2.1 and with anti-rabbit, anti-mouse, anti-goat alkaline phosphatase-conjugated secondary antibodies (GE Healthcare, Chalfont St Giles, UK). β-tubulin, actin and lamin B1 antibodies were used to detect those proteins as loading controls. Immune complexes were detected with the Enhanced ChemiFluorescence reagent (GE Healthcare) and the bands were analyzed using ImageQuant™TL (version 7.0, GE Healthcare). The results were normalized by calculating the ratio between the intensities of the bands corresponding to the protein of interest and the protein used as loading control.

Table 2.1 Antibodies used for western blot and immunohistochemistry analysis

| Protein | Source | Purification | MW (KDa) | Purchasing Company |
|---|---|---|---|---|
| Kir 6.1 | Goat | Polyclonal | 51 | Santa Cruz BioTech, Inc. |
| Kir6.2 | Rabbit | Polyclonal | 40 | Santa Cruz BioTech, Inc. |
| SUR 1 | Mouse | Monoclonal | 37 | Abcam® |
| SUR 2A | Goat | Polyclonal | 140 | Santa Cruz BioTech, Inc. |
| SUR2B | Goat | Polyclonal | 140 | Santa Cruz BioTech, Inc. |
| iNOS | Mouse | Monoclonal | 135 | R&D systems |
| GLUT-1 | Rabbit | Polyclonal | 54 | Millipore |
| GLUT-3 | Rabbit | Polyclonal | 54 | Millipore |
| p-IκBα | Mouse | Polyclonal | 40 | Cell Signalling Tech., Inc. |
| IκBα | Rabbit | Polyclonal | 39 | Cell Signalling Tech., Inc. |
| p-JNK | Rabbit | Monoclonal | 54 | Cell Signalling Tech., Inc. |
| p-p38 | Rabbit | Polyclonal | 43 | Cell Signalling Tech., Inc. |
| p-ERK 1/2 | Rabbit | Polyclonal | 42/44 | Cell Signalling Tech., Inc. |
| p65 | Rabbit | Polyclonal | 65 | Abcam® |
| β-Tubulin | Mouse | Monoclonal | 55 | Sigma-Aldrich® |
| Actin | Mouse | Monoclonal | 43 | Millipore |
| Lamin B1 | Rabbit | Polyclonal | 68 | Abcam® |

## 2.5 Immunohistochemistry

Five millimetres diameter full thickness cartilage cylinders were collected from the distal femoral condyles of multi-organ donors, immersed in OCT embedding compound (TAAB Laboratories, UK) and immediately frozen at -80 ºC. Cryostat sections, 10 µm thick, were fixed in acetone at -20 ºC for 10 min and incubated with the primary antibodies against Kir6.1, Kir6.2, SUR1, SUR2A and SUR2B presented on Table 2.1. Secondary antibodies used were Alexa fluor 488-conjugated goat anti-rabbit, Alexa fluor 488-conjugated goat anti-mouse (Molecular Probes, Eugene, OR) and FITC-conjugated rabbit anti-goat (Serotech Laboratories; Toronto, Canada). Counterstaining was performed with DAPI (Sigma-Aldrich) to allow nucleus visualization. Specificity was assessed by omitting the first antibody (negative control). Images of cartilage sections were acquired in a confocal laser scanning microscope (Zeiss LSM 710; Carl Zeiss Co, Germany) using an excitation filter of 500 nm and an emission filter of 520 nm. The acquisition settings were maintained throughout the work.

## 2.6 Total RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from primary chondrocyte cultures using TRIzol® Reagent (Invitrogen, Life Technologies, Co; Paisley, UK) and quantified using a NanoDrop ND-1000 spectrophotometer at 260nm. RNA purity was assessed as the 240/260 and

280/260 ratios. The cDNA was reverse transcribed using the iScript Select cDNA Synthesis Kit (Bio-Rad), beginning with 1 µg of RNA. Specific sets of primers used throughout the work, including housekeeping genes and summarized in Table 2.2 were designed using Beacon Designer software (PREMIER Biosoft International, Palo Alto, CA). PCR reactions were performed using 25 µg/mL of transcribed cDNA in a final volume of 20 µL.

The efficiency of the amplification reaction for each gene was calculated using a standard curve of a series of diluted cDNA samples, and the specificity of the amplification products was assessed by analyzing the melting curve generated in the process.

Gene expression changes were analyzed using the built-in iQ5 Optical system software v2, which enables the analysis of the results with the Pfaffl method, a variation of the ΔΔCT method corrected for gene-specific efficiencies [Nolan et al., 2006].

The results for each gene of interest were normalized against HPRT-1 or against 18S ribosomal protein, the housekeeping genes found to be the most stable under the different experimental conditions used.

Table 2.2 Oligonucleotide Primer Pairs Used for qRT-PCR

| Gene Name | Genbank Accession number | Sequence |
|---|---|---|
| GLUT-1 | NM_006516.2 | **F:**5'-CGTCTTCATCATCTTCACTG-3'<br>**R**: 5'-CTCCTCGGGTGTCTTATC-3' |
| GLUT-3 | NM_006931.2 | **F:**5'-CGGCTTCCTCATTACCTTC-3'<br>**R:** 5'-GGCACGACTTAGACATTGG-3' |
| Kir6.1 | NM_000352 | **F:**5'-CAACTGCTGTGTCCAGAT-3'<br>**R:** 5'-ATACGAATGGTGATGTTGGA-3' |
| Kir6.2 | NM_ 000525 | **F:**5'-CATAGGCATTAGTGTAGT-3'<br>**R:** 5'-TTATAGAAGAGGCAACTG-3' |
| SUR 1 | NM_000352 | **F:**5'- CAACTGCTGTGTCCAGAT-3'<br>**R:** 5'-ATACGAATGGTGATGTTGGA-3' |
| SUR 2A | NM_005691 | **F:**5'-AAGCATTCGGTCATTGTAG-3'<br>**R:** 5'-GCCACATAGTAGGTCTGA-3' |
| SUR 2B | NM_020297 | **F:**5'-TGGAGAGGATGTGGAGAA-3'<br>**R:** 5'-CTGTAAGAATGGTGAATGTGAT-3' |
| iNOS | NM_000625.4 | **F:**5'-AATCCAGATAAGTGACATAAG-3'<br>**R:** 5'-CTCCACATTGTTGTTGAT-3' |
| MMP-1 | NM_001145938.1 | **F:**5'-GAGTCTCCCATTCTACTG-3'<br>**R:**5'-TTATAGCATCAAAGGTTAGC-3' |
| MMP-13 | NM_002422.3 | **F:**5'-GTTTCCTATCTACACCTACAC-3'<br>**R:** 5'-CTCGGAGACTGGTAATGG-3' |
| TIMP-1 | NM_003254.2 | **F:**5'-TGTTGCTGTGGCTGATAG-3'<br>**R:** 5'-CTGGTATAAGGTGGTCTGG-3' |
| TIMP-3 | NM_000362.4 | **F:** 5'-CCATACACTATCCAC -3'<br>**R:** 5'-TAACAGCATTGAACA -3' |
| Aggrecan | NM_001135 | **F:** 5'-CCTGGTGTGGCTGCTGTC-3'<br>**R:** 5'- CTGGCTCGGTGGTGAACTC-3' |
| Collagen II | NM_001844.4 | **F:**5'-GGCAGAGGTATAATGATAAGG-3'<br>**R:** 5'-ATTATGTCGTCGCAGAGG-3' |
| Collagen I | NM_000088.3 | **F:**5'GGAGGAGAGTCAGGA-3'<br>**R:** 5'-GCAACACAGTTACAC-3' |
| HPRT-1 | NM_000194.2 | **F:**5'-TGACACTGGCAAAACAAT-3'<br>**R:** 5'-GGCTTATATCCAACACTTCG-3' |
| 18S rRNA | NM_022551 | **F:**5'-GAAGATATGCTCATGTGGTGTTG-3'<br>**R:** 5'-CTTGTACTGGCGTGGATTCTG-3' |
| RPL13A | NM_012423 | **F:**5'-GGAAGAGCAACCAGTTACTATGAG-3'<br>**R:** 5'-CAGAGTATATGACCAGGTGGAAAG-3' |
| GAPDH | NM_002046 | **F:**5'-ACAGTCAGCCGCATCTTC-3'<br>**R:** 5'-GCCCAATACGACCAAATCC-3' |

**F:** Forward sequence; **R:** Reverse sequence

## 2.7 2-Deoxy-D-Glucose uptake Assay

Glucose transport was determined by measuring the net uptake of a non-metabolizable analogue of glucose, 2-deoxy-D-glucose (2-DG). Briefly, chondrocytes were pre-incubated for 18 hours with Glibenclamide (10, 20, 100 nM and 20 μM) and then incubated for 30 minutes at 37$^{o}$C in glucose-free DMEM containing 0.5 mM 2-DG and 0.5 μCi/mL of [2,6-$^{3}$H]-2-DG. Cytochalasin B, a glucose transporter inhibitor (10 μM), was used to determine the non-specific uptake. For each sample, the non-specific uptake was subtracted from the total uptake, before normalization to the respective protein concentration.

## 2.8 Nitric Oxide Production

NO production was evaluated as the concentration of nitrite accumulated in the supernatants of primary chondrocyte cultures treated with IL-1β in the presence or absence of the test compounds, as indicated in figures and figure legends. Nitrite concentration was measured using the spectrophotometric method based on the Griess reaction [Green et al., 1982].

## 2.9 NF-κB Transcription Factor Assay

A colorimetric ELISA-based assay (NoShift Transcription Factor Assay kit, Novagen, La Jolla, CA) was used to evaluate the presence of active NF-κB dimers, capable of binding to the cognate consensus DNA sequence. For this purpose, nuclear extracts from C-28/I2 cells were incubated with a biotinylated consensus NF-κB oligonucleotide (NoShift NF-κB Reagents; Novagen) and the assay performed according to the manufacturer's instructions. The absorbance intensity in each sample is directly proportional to the amount of NF-κB-DNA complexes formed and, thus, to the amount of active NF-κB dimers present in each sample. In parallel, the specificity of the reaction was confirmed in competition assays where addition of a 10-fold molar excess of non-biotinylated wild-type or mutant oligonucleotides (NoShift NF-κB Reagents; Novagen) to binding reactions containing nuclear extracts from IL-1β treated cells abrogated or did not affect, respectively, the formation of NF-κB-DNA complexes.

## 2.10 Essential oil identification, Fractionation and Analysis

EOs from the aerial parts of *Eryngium duriaei* subsp. *juresianum* (M. Laínz) M. Laínz (Apiaceae), from the aerial parts of *Laserpitium eliasii* subsp. *thalictrifolium* Sennen & Pau (Apiaceae), from leaves and flowers of *Lavandula luisieri* (Rozeira) Rivas-Martínez (Lamiaceae), from the aerial parts of *Othantus maritimus* (L.) Hoff. & Link (Asteraceae) and from the aerial parts of *Thapsia villosa L.* (Apiaceae) were picked from the collection of plant extracts of the Faculty of Pharmacy, Univ. Coimbra. Voucher specimens of plant material are deposited at the Herbarium of the Botanic Garden, Univ. Coimbra (COI) or at the Herbarium of the Faculty of Pharmacy, Univ. Coimbra. All EOs were prepared at laboratory by water distillation using a Clevenger-type apparatus (EDQM, 2007) (+)-α-Pinene (≥98% purity) was purchased from Sigma Chemical Co. (St. Louis, MO, US).

The composition of each essential oil was established following a combined methodology of GC and GC/MS. Analytical GC was performed in a Hewlett-Packard 6890 (Agilent Technologies, Palo Alto, CA, US) gas chromatograph with a HP GC ChemStation Rev. A.05.04 data handling system, equipped with a single injector and two flame ionization detectors (FID). A graphpak divider (Agilent Technologies, part no. 5021-7148) was used for simultaneous sampling to two Supelco (Supelco, Bellefonte, PA, US) fused silica capillary columns with different stationary phases: SPB-1 (polydimethylsiloxane 30 m × 0.20 mm i.d., film thickness 0.20 μm), and SupelcoWax-10 (polyethyleneglycol 30 m × 0.20 mm i.d., film thickness 0.20 μm). Oven temperature program: 70–220°C (3°C/min), 220°C (15 min); injector temperature: 250°C; carrier gas: helium adjusted to a linear velocity of 30 cm/s; split ratio 1:40; detectors temperature: 250°C. GC-MS was performed in a Hewlett-Packard 6890 gas chromatograph fitted with a HP1 fused silica column (polydimethylsiloxane 30 m × 0.25 mm i.d., film thickness 0.25 μm), interfaced with an Hewlett-Packard mass selective detector 5973 (Agilent Technologies) operated by HP Enhanced ChemStation software, version A.03.00. GC parameters were as described above; interface temperature: 250°C; MS source temperature: 230°C; MS quadrupole temperature: 150°C; ionization energy: 70 eV; ionization current: 60 μA; scan range: 35 – 350 units; scans/s: 4.51.

The essential oils of *E. duriaei* subsp. *juresianum* and *L. luisieri* were fractionated by flash chromatography on silica gel 63-200 μm (Merck) using a 2 cm x 40 cm Omnifit (Sigma-Aldrich) glass columns. Elution was made in step gradients from 100% of *n*-pentane, *n*-pentane / ethyl ether mixtures, till a final concentration of 100% ethyl ether.

Collected fractions were analysed by gas chromatography-mass spectroscopy (GC/MS) and combined if having similar composition. Analysis were performed using a Hewlett-Packard 6890 gas chromatograph fitted with a HP1 fused silica column (polydimethylsiloxane 30 m × 0.25 mm i.d., film thickness 0.25 μm), interfaced with an Hewlett-Packard mass selective detector 5973 (Agilent Technologies) operated by HP Enhanced ChemStation software, version A.03.00. GC-MS parameters: oven temperature program: 70–220 °C (3 °C.min$^{-1}$), 220 °C (15 min); injector temperature: 250 °C; carrier gas: helium, adjusted to a linear velocity of 30 cm.s$^{-1}$; splitting ratio 1:40; interface temperature: 250 °C; MS source temperature: 230 °C; MS quadrupole temperature: 150 °C; ionization energy: 70 eV; ionization current: 60 μA; scan range: 35–350 units; scans.s$^{-1}$: 4.51.

Components of each EO and fraction were identified considering their retention indices (RIs) on both SPB-1 and SupelcoWax-10 columns and their mass spectra. RIs, calculated by linear interpolation relative to retention times of $C_8$–$C_{23}$ *n*-alkanes (Vandendool & Kratz, 1963), were compared with those of authentic samples, laboratory database (CEF / Faculty of Pharmacy, University of Coimbra) data available in digital banks (Linstrom, 2013; El-Sayed, 2012; Acree, 2004 and/ or literature data.. Acquired mass spectra were compared with reference spectra from the laboratory database, Wiley / NIST library[McLafferty, 2009] Wiley / NIST database (EDQM, 2007) and literature data [Adams, 1995; Cavaleiro et al., 2011; Joulain, 1998; Videira et al., 2013] (Adams, 1995; Joulain, 1998). Relative amounts of individual components were calculated based on GC raw data without further correction. Table 5.1 summarizes the major components found in each EO and table 7.1 summarizes the major components found in each fraction of the *Eryngium duriaei* subsp. *juresianum* and *Lavandula luisieri* essential oils

For cell assays, the EOs and isolated compounds were diluted in Dimethylsulfoxide (DMSO) and then dispersed in the culture medium to achieve final concentrations of 10-200 μg/mL. The final DMSO concentration did not exceed 0.1% (v/v).

## 2.11 Statistical Analysis

Results are presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism (version 5.00). SPSS software (version 17.0) was used to assess the normality (Kolmogorov Smirnov test) and homogeneity of variances to determine whether the conditions required to apply parametric tests were satisfied. As in all cases such conditions were observed, the statistical analysis was performed using the paired

Student t-test for comparison of each condition with its respective positive (IL-1β treated) or negative (untreated cells) control. Results were considered statistically significant at $P < 0.05$.

**CHAPTER 3.**

# EXPRESSION AND FUNCTION OF K(ATP) CHANNELS IN NORMAL AND OSTEOARTHRITIC HUMAN CHONDROCYTES: POSSIBLE ROLE IN GLUCOSE SENSING

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**Expression and Function of K(ATP) Channels in Normal and Osteoarthritic Human Chondrocytes: Possible Role in Glucose Sensing**

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**ABSTRACT**

ATP-sensitive potassium [K(ATP)] channels sense intracellular ATP/ADP levels, being essential components of a glucose-sensing apparatus in various cells that couples glucose metabolism, intracellular ATP/ADP levels and membrane potential. These channels are present in human chondrocytes, but their subunit composition and functions are unknown. This study aimed at elucidating the subunit composition of K(ATP) channels expressed in human chondrocytes and determining whether they play a role in regulating the abundance of major glucose transporters, GLUT-1 and GLUT-3, and glucose transport capacity. The results obtained show that human chondrocytes express the pore forming subunits, Kir6.1 and Kir6.2, at the mRNA and protein levels and the regulatory sulfonylurea receptor (SUR) subunits, SUR2A and SUR2B, but not SUR1. The expression of these subunits was no affected by culture under hyperglycemia-like conditions. Functional impairment of the channel activity, using a SUR blocker (glibenclamide 10 or 20 nM), reduced the protein levels of GLUT-1 and GLUT-3 by approximately 30% in normal chondrocytes, while in cells from cartilage with increasing osteoarthritic (OA) grade no changes were observed. Glucose transport capacity, however, was not affected in normal or OA chondrocytes. These results show that K(ATP) channel activity regulates the abundance of GLUT-1 and GLUT-3, although other mechanisms are involved in regulating the overall glucose transport capacity of human chondrocytes. Therefore, K(ATP) channels are potential components of a broad glucose sensing apparatus that modulates glucose transporters and allows human chondrocytes to adjust to varying extracellular glucose concentrations. This function of K(ATP) channels seems to be impaired in OA chondrocytes.

## 3.1 INTRODUCTION

Glucose serves an important anabolic function in chondrocytes, being required for the synthesis of matrix components. Due to the avascular nature of cartilage, chondrocytes are physiologically exposed to low oxygen tensions [Grimshaw and Mason, 2000; Kay et al., 2011; Zhou et al., 2004b], using glycolysis as the main energy source [Rajpurohit et al., 1996], even when exposed to normoxia [Schneider et al., 2007]. The supply of glucose to chondrocytes depends on diffusion from the synovial fluid [Maroudas, 1970] which is facilitated by joint motion and fluid flow in the extracellular matrix (ECM). The synovial fluid glucose concentration is identical to that in the plasma both in normal conditions and in non-inflammatory and inflammatory types of arthritis, except in septic arthritis where it is usually significantly reduced [Brannan and Jerrard, 2006]. Several members of the facilitative glucose transporter family – the GLUT/SLC2A transporters – are expressed in chondrocytes and optimize its uptake [Mobasheri et al., 2008]. GLUT-1 is especially important as both anabolic and catabolic stimuli increase its expression, while others, like GLUT-3, which is also highly expressed constitutively, does not seem to be regulated by those stimuli [Mobasheri et al., 2008; Phillips et al., 2005; Richardson et al., 2003; Shikhman et al., 2004].

On the other hand, extracellular glucose concentrations both below and above physiologic levels, have been predicted and shown to alter the pattern of gene expression [Mobasheri et al., 2002a; Rosa et al., 2011b], as well as the availability of GLUT-1 protein [Rosa et al., 2009] in chondrocytes. Indeed, glucose deprivation increases the expression of GLUT-1, whereas a supra-physiologic glucose concentration has the opposite effect, decreasing GLUT-1 content and glucose uptake in young/healthy chondrocytes, but has no effect on those isolated from osteoarthritic (OA) cartilage [Rosa et al., 2009]. Therefore, normal chondrocytes seem to be able to adjust to the extracellular glucose concentration by modulating the abundance of GLUT-1 and the total glucose transport capacity. Nonetheless, this ability seems to be compromised in OA chondrocytes *in vitro*, leading to intracellular glucose accumulation, increased production of reactive oxygen species [Rosa et al., 2009] and expression of matrix degrading enzymes [Rosa et al., 2011b] when these cells are exposed to supra-physiologic glucose concentrations, as those occurring in diabetes mellitus, metabolic syndrome and other conditions characterized by impaired glucose metabolism. These conditions are increasingly recognized as independent risk factors for OA development and progression [Berenbaum, 2011; Del Rosso et al., 2006; Hart et al., 1995; Katz et al., 2010; Schett et al., 2012b; Sturmer et al., 2001; Velasquez and

Katz, 2010]. OA is the major musculoskeletal disorder - or most likely, a set of disorders with distinct etiological and clinical characteristics [Berenbaum, 2011; Zhuo et al., 2012] and a main cause of physical disability [Goldring and Marcu, 2009; Martel-Pelletier et al., 2008]. Understanding how human chondrocytes sense extracellular glucose and intracellular ATP concentrations and adjust their substrate uptake to match the cell's needs and whether such mechanisms remain functional in OA chondrocytes is, thus, critical to elucidate the mechanisms by which conditions associated with impaired glucose metabolism contribute to the development and/or progression of OA.

ATP-sensitive potassium [K(ATP)] channels are weak inwardly rectifying potassium channels that act as metabolic sensors in a number of cell types, coupling metabolic activity to the membrane potential and to secretory activity, primarily by sensing intracellular ATP levels [Burke et al., 2008; McTaggart et al., 2010; Miki et al., 2002; Minami et al., 2004]. In pancreatic β-cells [Burke et al., 2008] and in subpopulations of mediobasal hypothalamic neurons [Acosta-Martinez and Levine, 2007], for instance, K(ATP) channels have been identified as an essential component of a glucose-sensing mechanism, wherein uptake and metabolism of glucose leads to increased intracellular ATP/ADP levels, closure of the channels, and increase in insulin secretion and neuronal excitability, respectively.

Structurally, K(ATP) channels are heteromultimers of four ATP-binding cassette (ABC) proteins that form the sulfonylurea receptor (SUR), surrounding four pore forming potassium channel subunits (Kir6) [Babenko et al., 1998]. Kir6 and SUR have two isoforms each, Kir6.1 and Kir6.2 and SUR1 and SUR2, respectively. SUR2 has two major splice variants, SUR2A and SUR2B [Burke et al., 2008; Hibino et al., 2010]. ATP binds to the SUR subunits inducing a conformational change of the Kir6 subunits that results in channel closure, cessation of $K^+$ efflux and cell membrane depolarization. In contrast, decreased intracellular ATP availability allows ADP binding and channel opening with the consequent $K^+$ efflux and hyperpolarization [Babenko et al., 1998; Shi et al., 2005]

The subunit composition of K(ATP) channels determines their sensitivity to intracellular ATP and to pharmacological modulators, eliciting the functional diversity of these channels [Acosta-Martinez and Levine, 2007; Babenko et al., 1998; Inagaki and Seino, 1998]. Kir6.1 and Kir6.2 have similar sensitivities to nucleotides (ATP and ADP), so the differences found are believed to be due to the SUR subtype existing in each channel or to the combination of different SUR subunits in the same channel [Chan et al., 2008; Shi et al., 2005]. K(ATP) channels involved in coupling glucose sensing with insulin

secretion in pancreatic β-cells are composed of Kir6.2 and SUR1 [Nielsen et al., 2007], whereas channels containing Kir6.2 and SUR2A, found in cardiomyocytes, and channels composed of Kir6.2 or Kir6.1 and SUR2B, occurring in vascular cells, regulate the duration of the action potential and vasodilatation [Aguilar-Bryan and Bryan, 1999; Burke et al., 2008; Chan et al., 2008; Gribble and Ashcroft, 2000; Hibino et al., 2010].

Information regarding the subunit composition and function of K(ATP) channels expressed in non-excitable cells is much scarcer. Functional K(ATP) channels have been demonstrated in equine chondrocytes and Kir6.1 was detected in normal and OA human cartilage [Mobasheri et al., 2007], but their subunit composition and physiological roles in cartilage are not yet understood, even though these channels are highly likely to be important for regulation of cartilage metabolism and sensing ATP levels within the cell [Barrett-Jolley et al., 2010; Mobasheri et al., 2012]. We hypothesize that K(ATP) channels, by responding to ATP levels, may indirectly act as components of the glucose sensing apparatus in chondrocytes, generating signals that allow the cell to adjust to varying extracellular glucose concentrations by regulating the availability of its facilitative glucose transporters and hence net glucose transport capacity. Therefore, this work was aimed at i) elucidating the subunit composition of K(ATP) channels expressed in human chondrocytes and ii) determining whether K(ATP) channels play a role in regulating the abundance of two major glucose transporters in chondrocytes, GLUT-1 and GLUT-3, and the glucose transport capacity of human chondrocytes exposed to hyperglycemia-like conditions. Furthermore, these processes were evaluated in chondrocytes isolated from cartilage samples with different degradation grades to determine whether they are impaired in OA.

## 3.2 RESULTS

### 3.2.1 Characterization of the subunit composition of (KATP) channels expression in human chondrocytes

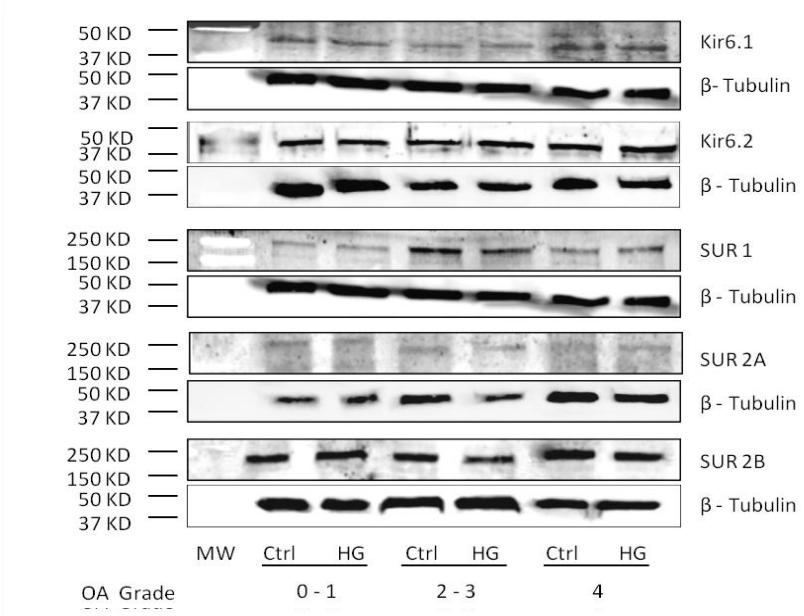
To determine the subunit composition of K(ATP) channels present in human chondrocytes, we evaluated the expression of the different Kir and SUR subunits, both at the protein and mRNA levels, by western blot and qRT-PCR, respectively. The results obtained show that all the pore-forming and regulatory subunits that can compose K (ATP) channels are expressed in human chondrocytes, although with different intensities and inter-individual variability (Fig. 3.1). Kir6.1, SUR1, SUR2A and SUR2B are expressed both at the protein and mRNA levels (Fig. 3.1). Kir6.2 is apparently the most abundant of the pore-forming subunits at the protein level, but its mRNA is absent or, at least, is expressed at very low levels, which may simply indicate that this protein has a low turnover. On the other hand, even though the mRNAs of all the regulatory SUR subunits were detected in human chondrocytes (Fig. 3.1C), SUR2B seems to be expressed at the protein level more intensely than SUR2A, while SUR1 was only detected at very low levels (Fig. 3.1A). Nonetheless, the apparently different intensities of those subunits may simply be accounted for by limitations of the western blot technique and distinct affinities of the antibodies used. Even though it was not possible to gather a sufficient number of cartilage samples of each OA grade to allow an adequate statistical analysis of the expression of each Kir6 and SUR subunit among the three experimental groups defined, the western blot images shown in Fig. 3.1B suggest that OA grade does not affect the protein levels of each subunit.

A

K(ATP) channel subunits protein level in human chondrocytes

| | Control | HG | | |
|---|---|---|---|---|
| Subunits | Mean ± SED | Mean ± SED | P value | N |
| Kir6.1 | 0.341±0.054 | 0.382±0.073 | 0.4322 | 8 |
| Kir6.2 | 2.304±0.239 | 2.775±0.348 | 0.2056 | 6 |
| SUR -1 | 0.099±0.025 | 0.084±0.020 | 0.201 | 8 |
| SUR- 2A | 0,295±0.061 | 0,260±0.059 | 0.6151 | 6 |
| SUR- 2B | 0.890±0.044 | 0.842±0.058 | 0.6513 | 6 |

B




C

K(ATP) channel subunits mRNA levels (ΔΔCt relative to 18S rRNA)

| | Control | HG | | |
|---|---|---|---|---|
| Gene Name | Mean ± SEM | Mean ± SEM | P value | N |
| Kir6.1 | 1.078±0.175 | 1.030±0.168 | 0.877 | 5 |
| Kir6.2 | 0.004±0.002 | 0.028±0.009* | 0.004 | 5 |
| SUR -1 | 0.655±0.314 | 0.370±0.199 | 0.239 | 5 |
| SUR-2A | 1.694±0.252 | 0.968±0.188* | 0.015 | 5 |
| SUR- 2B | 2.503±0.641 | 1.975±0.652 | 0.476 | 5 |

**Figure 3.1** Protein and mRNA levels of K(ATP) channel subunits expressed in human chondrocytes A) Protein content of each Kir6 and SUR subunit in whole cell extracts from human chondrocytes exposed to high glucose (HG) (30 mM) for 18 h or left untreated (control). The protein content of each subunit was evaluated by Western blotting and normalized to the respective β-tubulin band. B) Western blot images

representative of the protein content of each Kir6 and SUR subunit expressed in human chondrocytes according to OA grade, as defined in Materials and Methods Section. MW, molecular weight protein marker. C) mRNA content of each Kir6 and SUR subunit in human chondrocytes exposed to high glucose (HG) (30 mM) for 18 h or left untreated (control). The mRNA content of each subunit was evaluated by qRT-PCR and normalized to the respective 18S rRNA content.

### 3.2.2 Immunofluorescence staining of K(ATP) channels expressed in human chondrocytes

To further confirm the results obtained in isolated chondrocytes (Fig. 3.1), the presence of each subunit in full depth normal (grade 0-1) cartilage samples was assessed by immunofluorescence.

Fig. 3.2 shows the presence of chondrocytes staining positively for Kir6.1, Kir6.2, SUR2A and SUR2B scattered throughout the cartilage sections and in the midst of non-stained chondrocytes. No cells staining positively for SUR1 could be detected in any of the cartilage sections analysed. Although no quantitative analysis of the immunofluorescence images was performed, these results are in agreement with those obtained in cell extracts of isolated chondrocytes where SUR1 was detected at very low levels by western blot, while Kir6.2 and SUR2B seem to be more abundant.

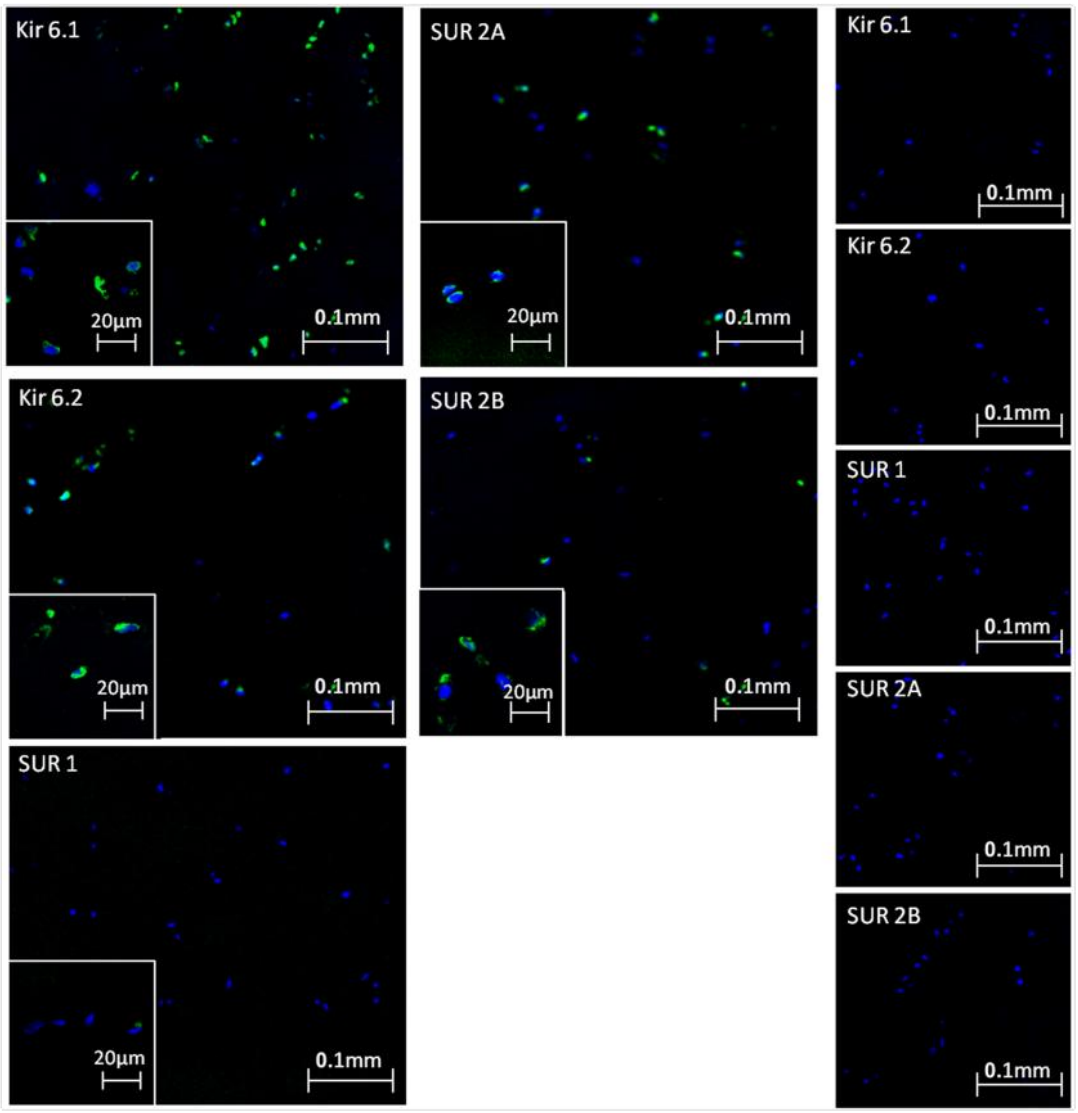



**Figure 3.2** In situ immunofluorescence staining of K(ATP) channel subunits in normal (OA grades 0–1) human cartilage. Each section was stained with an antibody specific for the subunit (green) identified in the upper right corner of the representative image and counterstained with DAPI (blue) to allow visualization of nuclei. Negative controls (column on the right) were obtained by omitting the primary antibody for each subunit. Cartilage sections were viewed and images captured at 100X and 400X (insets) magnifications.

### 3.2.3 Role of exposure to high glucose on K(ATP) channel subunit protein and mRNA expression

To elucidate whether exposure to a hyperglycemia-like glucose concentration influences the subunit composition of K(ATP) channels, chondrocytes, classified according to OA grade, were treated with 30 mM glucose for 18 h and the protein and mRNA levels of each subunit were analysed by western blot and qRT-PCR, respectively. No differences in MTT reduction capacity were found between chondrocytes of either OA grade, exposed or not to high glucose for 18 h (data not shown), thus showing that exposure to high glucose did not affect chondrocyte viability.
The results presented in Fig. 3.1 show that exposure to high glucose did not significantly affect the protein levels of SUR1, SUR2A, SUR2B, Kir6.1 or Kir6.2 relative to chondrocytes maintained in regular culture medium containing 10 mM glucose. However, as shown in Fig. 3.1C, a small, but statistically significant difference was found in the mRNA level of SUR2A between cells treated with 30 mM glucose (0.97±0.19) and control cells maintained in regular medium (10 mM glucose) (1.69±0.25). On the other hand, the mRNA levels of Kir6.2 were approximately 6.5 fold higher in cells cultured in high glucose for 18 h (0.028±0.009) than in those kept in regular medium (0.004±0.002), although the absolute levels in either condition were very low.

### 3.2.4 Regulation of GLUT-1 and GLUT-3 protein levels by the K(ATP) channel blocker Glibenclamide

To determine whether the activity of K(ATP) channels is involved in the regulation of glucose transporters in human chondrocytes, the cells were treated for 18 h with glibenclamide, a widely used SUR inhibitor that blocks K(ATP) channels [Burke et al., 2008], in concentrations ranging from 10 nM to 20 µM.
Data presented on Table 3.1 show that there are no significant differences in the basal protein levels of GLUT-1 and GLUT3 among the three groups defined according to the macroscopic OA grade, as defined in *Materials and Methods*.

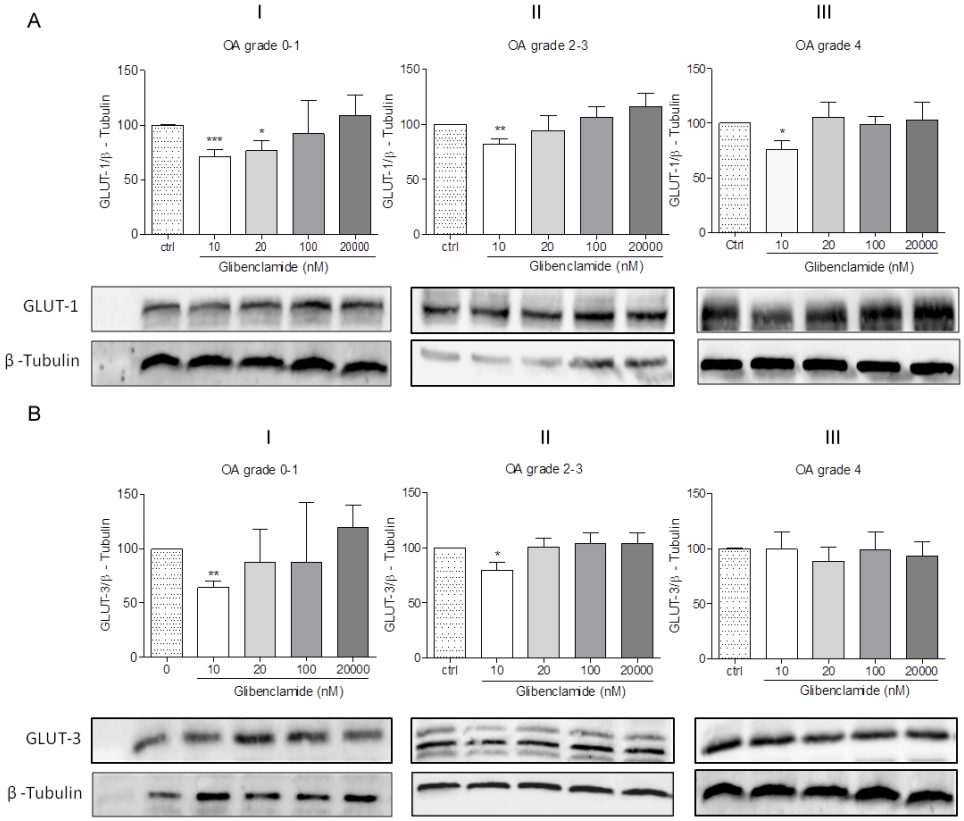
Table 3.1 Basal protein levels of GLUTs-1 and -3 in human chondrocytes according to OA grade

| OA grade | 0 - 1 | 2 - 3 | 4 | | |
|---|---|---|---|---|---|
| | Mean ± SEM | Mean ± SEM | Mean ± SEM | P value | N |
| GLUT-1 | 0.58± 0.097 | 0.70± 0.29 | 0.66± 0.21 | 0.919 | 6 |
| GLUT-3 | 1.85± 0.58 | 1.75± 0.14 | 1.49± 0.34 | 0.766 | 5 |

GLUT-1 protein levels were significantly decreased by treatment of grade 0-1 chondrocytes with glibenclamide 10 nM (71.24 ± 6.59%, *P*=0.0009, *n*=7) and 20 nM (76.55± 9.47%, *P*=0.029, *n*=7), whereas only 10 nM was effective in decreasing GLUT-1 protein levels in chondrocytes of OA grades 2-3 (82.16±4.27, *P*= 0.0024, *n*=7) and 4 (76.3 ± 8.0, *P*=0.025, *n*=7) (Fig. 3.3A).

Fig. 3.3B represents the results obtained for GLUT 3. It shows that a significant decrease of GLUT-3 occurs only in chondrocytes from groups with OA grade 0-1 and 2-3 treated with 10 nM glibenclamide (64.17± 5.89, *P*=0.0038; *n*=3 and 79.77±7.38, *P*=0.0265; *n*=5, respectively), while no significant differences were found in OA grade 4 chondrocytes with any of the glibenclamide concentrations tested.

Regardless of the OA grade, glibenclamide concentrations above 20 nM had no effect either on GLUT-1 or -3 protein content, although for each OA grade the variability between chondrocytes isolated from different cartilage samples was higher than observed by treatment with 10 nM glibenclamide.




C

Relative GLUT-1 and -3 mRNA levels after treatment with 10 nM Glibenclamide

| | 3h | | | 6h | | | 18h | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean±SEM | P value | N | Mean±SEM | P value | N | Mean±SEM | P value | N |
| GLUT-1 | 1.04±0.037 | 0.452 | 4 | 0.91±0.102 | 0.435 | 4 | 0.88±0.151 | 0.489 | 4 |
| GLUT-3 | 1.17±0.089 | 0.134 | 3 | 1.25±0.062 | 0.101 | 3 | 1.17±0.035 | 0.095 | 3 |

**Figure 3.3** Regulation of total GLUT-1 and GLUT-3 protein content by glibenclamide in chondrocytes from different OA grades. A: Relative GLUT-1 protein levels normalized to the respective β-tubulin band, in whole cell extracts from human chondrocytes of OA grades 0–1 (n=7), 2–3 (n=7), and 4 (n=6) treated with the indicated concentrations of glibenclamide for 18 h. B: Relative GLUT-3 protein levels normalized to the respective β-tubulin band, in whole cell extracts from human chondrocytes of OA grades 0–1 (n=3), 2–3 (n=5), and 4 (n=6) treated with the indicated concentrations of glibenclamide for 18 h. Results are expressed in percentage relative to control untreated cells of the same OA grade ***P <0.005; **P <0.01; *P <0.05 relative to the respective control cells. MW, molecular weight protein marker. C: GLUT-1 and -3 mRNA levels in

chondrocytes grades 0–1 treated with 10 nM glibenclamide for the time periods indicated, relative to untreated chondrocytes of the same OA grade.

### 3.2.5 Role of Glibenclamide in regulating GLUT-1 and GLUT-3 mRNA levels in OA grade 0-1 chondrocytes

To determine whether the decrease in the protein levels of GLUT-1 and GLUT-3 was due to glibenclamide effects at the transcriptional level, we analyzed the mRNA levels of both glucose transporters by qRT-PCR. Fig. 3.3C shows that there were no statistically significant differences in the mRNA levels of either glucose transporter in glibenclamide-treated groups compared to the control group at any treatment period considered.

### 3.2.6 Role of glibenclamide in regulating cellular glucose transport

To determine whether the effects of glibenclamide on GLUT-1 and -3 protein levels had functional consequences in terms of glucose transport capacity by chondrocytes of various OA grades, the uptake of [$^3$H]2-DG was measured after treatment with glibenclamide for 18 h. The results obtained (Table 3.2) show no significant differences in 2-DG uptake by cells treated with any of the glibenclamide concentrations tested, compared with the corresponding control untreated chondrocytes of the same OA grade.

Table 3.2 : Relative 2-DG uptake (% of untreated cells)

| Glibenclamide (nM) | OA grade 0-1 | | | OA grade 2-3 | | | OA grade 4 | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean±SEM | P value | N | Mean±SEM | P value | N | Mean±SEM | P value | N |
| 10 | 92.63±9.79 | 0.4962 | 6 | 106.9±5.98 | 0.2979 | 5 | 94.03±3.14 | 0.2466 | 3 |
| 20 | 97.92±1.53 | 0.2347 | 6 | 108.6±8.73 | 0.3777 | 4 | 100.7±4.53 | 0.8422 | 3 |
| 100 | 93.95±5.36 | 0.3322 | 6 | 102.2±8.03 | 0.7749 | 5 | 87.33±5.37 | 0.1629 | 3 |
| 20000 | 100.6±3.24 | 0.8203 | 6 | 113.7±8.10 | 0.1785 | 4 | 91.41±8.36 | 0.4120 | 3 |

## 3.3 DISCUSSION

Although the presence of Kir6.1 in human and equine chondrocytes has already been demonstrated [Mobasheri et al., 2007], this study represents the first comprehensive analysis of Kir6.x and SUR subunits expressed in human articular chondrocytes, both at the protein and mRNA levels. The results obtained (Figs. 3.1 and 3.2) suggest that Kir6.2 and SUR2B are major K(ATP) channel subunits expressed in human chondrocytes, while Kir6.1 and SUR2A are likely present at much lower levels and SUR1 is barely detectable or even undetectable. How the various Kir and SUR subunits expressed in human chondrocytes assemble to form functional K(ATP) channels is beyond the scope of this study and was not investigated. Nonetheless, it is possible that K(ATP) channels containing more than one SUR subtype co-exist with others containing only one SUR isoform. The relative abundance of each channel type would depend on the expression level of each subunit, as observed in other cells [Chan et al., 2008; Wheeler et al., 2008]. Since Kir 6.2 and SUR2B seem to be more abundant than the other Kir6 and SUR subunits, it seems likely that K(ATP) channels containing these subunits, namely octamers composed of Kir6.2 and SUR2B, predominate in human articular chondrocytes, although all other possible combinations of Kir6 and SUR subunits can also be present. Even though the scarcity of human cartilage samples did not allow us to determine the influence of the OA grade on the subunit composition of K(ATP) channels in human chondrocytes, the results presented in Fig. 3.1 suggest that none of the Kir6 or SUR subunits is affected at the protein level. The composition of those channels, at least at the protein level, was not significantly affected by culture under a supra-physiologic glucose concentration (30 mM), unlike demonstrated at the mRNA level in other cells, namely mediobasal hypothalamic neurons [Acosta-Martinez and Levine, 2007] and pancreatic β-cells [Moritz et al., 2001].

The nucleotide binding affinity of SUR2B is much higher than that of SUR2A, but lower than that of SUR1, although its channel gating activity is similar to that of SUR1 [Matsuo et al., 2000]. Therefore, SUR2B-containing K(ATP) channels are functionally very similar to those that contain SUR1, namely those expressed in pancreatic β-cells [Burke et al., 2008] and in mediobasal hypothalamic neurons [Acosta-Martinez and Levine, 2007] that readily bind ATP in response to augmented glucose availability. To test the hypothesis that K(ATP) channels expressed in human chondrocytes behave similarly, we attempted to measure changes in membrane potential induced by culture under a supra-physiologic glucose concentration (30 mM) which is in the range of

plasma glucose concentrations found in diabetic patients. For this, we loaded the cells with the potential-sensitive fluorescence dye, bis-(1,3-dibutylbarbituric acid)trimethine oxonol [DiBAC 4 (3)] that becomes fluorescent upon depolarization. Unfortunately, we were unable to detect any changes using fluorescence spectroscopy, probably because changes in membrane potential occur in a very dynamic and fast manner. Possibly, *in vivo* fluorescence imaging may be required to detect such fast and transient changes. Therefore, we were unable to directly demonstrate whether glucose availability affects the chondrocyte membrane potential.

To further test the hypothesis that K(ATP) channels function as metabolic sensors in human chondrocytes playing a role in the modulation of glucose transport capacity, we used glibenclamide, a widely used SUR inhibitor, to block these channels and measured the whole cell content of major glucose transporters, GLUT-1 and GLUT-3. Glibenclamide displays differential selectivity towards distinct SUR subunits, being moderately selective for SUR1 [Burke et al., 2008]. The sensitivity of equine chondrocytes to glibenclamide is lower than that found in cells expressing SUR1 and in the same range of that presented by cells in which K(ATP) channels contain SUR2B [Mobasheri et al., 2007] which further suggests that K(ATP) channels containing this subunit are relevant in human chondrocytes. Nevertheless, as K(ATP) channels composed of other subunits may also exist in human chondrocytes, we used glibenclamide concentrations ranging from 10 nM to 200 μM in order to cover the whole range of channel sensitivities to this SUR inhibitor. Interestingly, only the lowest concentration was effective in reducing GLUT-1 protein content in chondrocytes from all OA grades, whereas 20 nM was effective only in grade 0-1 OA chondrocytes (Fig. 3.3A). GLUT-3 content, however, was only reduced by treatment of grades 0-1 and 2-3, but not grade 4 OA chondrocytes with glibenclamide 10 nM (Fig. 3.3B). These results indicate that closing K(ATP) channels, at least partially, is sufficient to reduce the cell protein content of two major glucose transporters, GLUT-1 and GLUT-3, without affecting their mRNA levels, in normal human chondrocytes, in a way similar to the reduction induced by exposure to 30 mM glucose [Rosa et al., 2009].

The inability of OA chondrocytes to downregulate the protein levels of GLUT-1 and GLUT-3 in response to glibenclamide (Fig. 3.3), mimicking their inability to adjust to high extracellular glucose concentrations [Rosa et al., 2009], further indicates that either K(ATP) channels are functionally defective or some other downstream component or process is impaired in OA chondrocytes. Nonetheless, the possibility that ATP production may simultaneously be impaired in advanced OA chondrocytes cannot be excluded.

Another intriguing question arises from the observation that the response to glibenclamide in terms of GLUT-1 and GLUT-3 protein content, occurred only with the lowest glibenclamide concentrations tested. In equine chondrocytes, similar glibenclamide concentrations (10-20 nM) elicited a partial reduction in current amplitude, while much higher concentrations in the range of 100-300 nM were required to completely block K(ATP) channel activity [Mobasheri et al., 2007]. Therefore, it is possible that different ranges of channel activity elicit distinct changes in membrane potential that operate diverse signalling pathways and cell responses. This is further supported by the observation that K(ATP) channels with distinct subunit compositions and therefore with different glibenclamide and nucleotide sensitivities, may coexist in human chondrocytes (Figs. 3.1 and 3.2).

Nonetheless, glibenclamide-induced changes in the total protein content of GLUT-1 and GLUT-3 were not accompanied by corresponding decreases in glucose transport capacity by chondrocytes of any OA grade (Table 3.2). This suggests that even though K(ATP) channel activity regulates the availability of GLUT-1 and GLUT-3, other mechanisms are involved in the regulation of the overall glucose transport capacity of human chondrocytes. On one hand, other glucose transporters known to be expressed in chondrocytes [Mobasheri et al., 2008] may not be regulated by K(ATP) channels and compensate for changes in GLUT-1 and -3. On the other hand, GLUT-1 has been shown to exist in a dynamic equilibrium involving the plasma membrane, intracellular storage in the trans Golgi network and lysosomal degradation [Ortiz et al., 1992]. Our previous study indicated that high glucose-induced GLUT-1 downregulation involved lysosomal degradation [Rosa et al., 2009]. Thus, it is possible that K(ATP) channel activity affects total GLUT-1 and possibly GLUT-3 protein content by increasing lysosomal degradation of intracellular stores, in agreement with studies in other cells suggesting that GLUT-1 degradation and functional membrane expression may be regulated through independent pathways [Ortiz et al., 1992].

In summary, this study contributes to elucidate the subunit composition of K(ATP) channels expressed in human chondrocytes and establishes a role for these channels as components of the glucose sensing apparatus in these cells by demonstrating that their activity influences the abundance of two major glucose transporters, GLUT-1 and GLUT-3. Nonetheless, since glucose transport was not similarly affected, additional mechanisms must be involved in the adaptation of human chondrocytes to high extracellular glucose concentrations. Moreover, this study also shows that chondrocytes isolated from cartilage of increasing OA grade are less responsive to changes in K(ATP) channel activity, which may contribute to the inability of OA

chondrocytes to downregulate glucose transporters and avoid the deleterious effects of high extracellular glucose concentrations that we have previously observed [Rosa et al., 2009; Rosa et al., 2011b]. Taken together, the results presented highlight the importance of K(ATP) channels as metabolic sensors and potential components of a broad glucose sensing apparatus that allows human chondrocytes to adjust to varying extracellular glucose concentrations.

**CHAPTER 4.**

# ROLE OF HIGH GLUCOSE AS MODULATOR OF INFLAMMATION IN HUMAN CHONDROCYTES

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**Role of High Glucose as a Modulator of Inflammation in Human Chondrocytes**
Submitted for publication to Osteoarthritis and Cartilage (July 2014)

**ABSTRACT**

Objective: This work aimed at determining whether high glucose induces inflammatory responses in human chondrocytes. Since hyperinsulinemia often accompanies DM type 2, we also evaluated the ability of insulin to modulate inflammatory responses induced by high glucose.

Method: We analyzed IL-1β, TNF-α and inducible nitric oxide synthase (iNOS) expression by qRT-PCR and nuclear NF-κB p65 accumulation by western blot in human chondrocytes cultured in medium containing 10 or 30 mM glucose.

Results: Culture in high glucose (30 mM) for 24h increased iNOS mRNA and protein levels and NO production by 61% ($P$=0.0017), 148% ($P$=0.0089) and 70% ($P$=0.049), respectively, relative to human chondrocytes maintained in 10 mM glucose. Similarly, IL-1β and TNF-α mRNA levels increased by 97 ($P$=0.0066) and 85% ($P$=0.0045), respectively. NF-κB p65 nuclear levels increased in a time-dependent manner, peaking at 1h. Treatment with 100 nM insulin also increased nuclear p65 levels, independently of the glucose concentration.

Conclusion: This study shows that hyperglycemia and hyperinsulinemia induce inflammatory responses in human chondrocytes. This can be a relevant mechanism by which DM type 2, obesity, the metabolic syndrome and other conditions associated with impaired glucose and insulin homeostasis contribute to the development and progression of OA.

## 4.1 INTRODUCTION

Aging is the major risk factor for osteoarthritis (OA), but other factors have been recognized [Berenbaum, 2011; Zhang and Jordan, 2010], including diabetes mellitus (DM), especially type 2, and impaired glucose homeostasis  Recent epidemiologic studies demonstrated that DM is an independent risk factor for OA development and progression [Nieves-Plaza et al., 2013; Schett et al., 2012a], supporting the notion of a specific diabetes-induced OA phenotype [Berenbaum, 2011; Zhang and Jordan, 2010]. How DM and impaired glucose homeostasis contribute to cartilage and joint destruction is largely unknown.

Chronic low grade inflammation is a major OA driver, being associated with other mechanisms involved in OA pathogenesis, including cell senescence, oxidative stress, mitochondrial dysfunction and impaired autophagy, which together contribute to cell depletion and imbalanced anabolic and catabolic responses, especially in chondrocytes [Lotz and Carames, 2011].

All the above mechanisms, including inflammation, have been associated with the effects of hyperglycemia and have been implicated in DM pathogenesis and its complications [Donath and Shoelson, 2011].

Only a few studies have been conducted so far to identify direct effects of glucose in articular chondrocytes, collectively indicating that hyperglycemia-like glucose concentrations favor catabolic processes and impair anabolic responses. Our previous studies showed that exposure to high glucose causes oxidative stress and induces catabolic responses in human chondrocytes, especially from OA/aged articular cartilage [Rosa et al., 2009; Rosa et al., 2011a].

Given the role of inflammation in OA and in mediating hyperglycemia-induced effects in other cells, this study aimed at determining whether high glucose induces inflammatory responses in human chondrocytes. Since hyperinsulinemia often accompanies DM type 2, we also evaluated the ability of insulin to modulate inflammatory responses induced by high glucose.

## 4.2 RESULTS

### 4.2.1 Induction of pro-inflammatory cytokines, iNOS expression and NO production by high glucose

Figure 4.1 shows that culture of human chondrocytes in medium containing 30 mM glucose for 24 h is sufficient to increase IL-1β and TNF-α mRNA levels relative to those found in chondrocytes maintained in 10 mM glucose medium. Moreover, 30 mM glucose also induced iNOS expression, both at the mRNA and protein levels, and activity as shown by the augmented NO production.

**A**

Role of high glucose on pro-inflammatory gene expression

Relative mRNA levels (% relative to cells in 10 mM glucose)

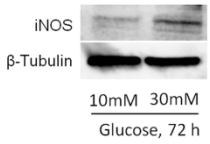
| | Mean (95% CI) | *P** | *N* |
|---|---|---|---|
| iNOS | 161.4 (124.5 – 198.2) | 0.0017 | 5 |
| IL-1β | 196.8 (89.8 – 303.8) | 0.0066 | 4 |
| TNF-α | 285.4 (133.9 – 436.8) | 0.0045 | 5 |

**B**

Role of high glucose on iNOS protein levels

Relative iNOS protein levels (% relative to cells in 10 mM glucose)

| | Mean (95% CI) | *P** | *N* |
|---|---|---|---|
| iNOS | 248 (114 – 382) | 0.0089 | 4 |




**C**

Role of high glucose on NO production

Relative NO levels (% relative to cells in 10 mM glucose)

| | Mean (95% CI) | *P** | *N* |
|---|---|---|---|
| NO | 170.2 (79.0 – 261.5) | 0.0498 | 4 |

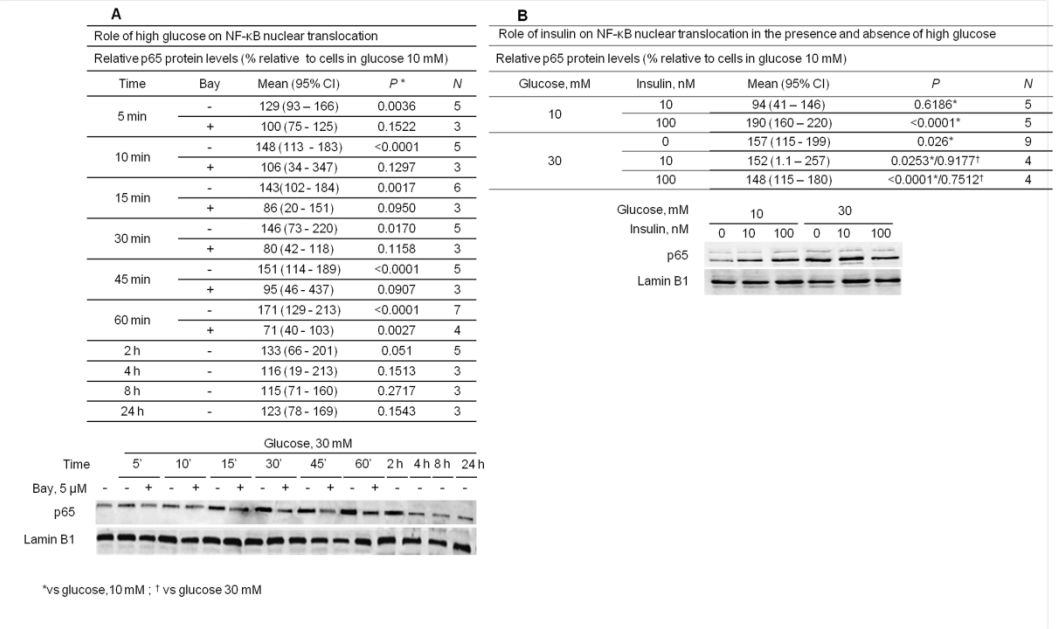
**Figure 4.1** Role of high glucose in modulating pro-inflammatory cytokines, iNOS expression and NO production. mRNA levels of iNOS, IL-1β and TNF-α (A), protein levels of iNOS (B) and NO production (C) were measured in human chondrocytes cultured for 24 (A) or 72 h (B, C) in medium containing 10 or 30 mM glucose. The results represent the means and 95% confidence intervals relative to cells in 10 mM glucose.

### 4.2.2 High glucose induces NF-κB p65 translocation to the nucleus

As human cartilage samples are limited and a large number of cells was required, the human chondrocytic cell line, C-28/I2, was used to evaluate the ability of high glucose to induce NF-κB activation. This requires the phosphorylation and subsequent ubiquitination and proteassomal degradation of an inhibitory protein, IκB-α (NF-κB Inhibitor-α), which, in basal conditions, retains NF-κB dimers in the cytoplasm. Once IκB-α is degraded, the NF-κB dimers translocate to the nucleus promoting the transcription of target genes. Therefore, activation of this transcription factor leads to its nuclear translocation that can be detected as the nuclear levels of p65 (Rel A), a major component of NF-κB dimers found in chondrocytes [Mendes et al., 2002c].

Figure 2A shows that nuclear p65 levels gradually increased during culture in 30 mM glucose, peaking at 1h. After 2h, nuclear levels of p65 were identical to those found in cells maintained in 10 mM glucose and remained as such until up to, at least, 24h. Co-treatment with the specific IκB-α phosphorylation inhibitor, Bay-11-7082, completely reversed the high glucose-induced effect.

To rule out osmotic effects, 20 mM mannitol, a non-absorbable polysaccharide, were added to the medium containing 10 mM glucose. No significant changes were observed in cells cultured in this medium for 1h (mean=80.5, 95% CI: 61.0-100.0, *P*=0.348) relative to those cultured in 10 mM glucose, indicating that the effects detected with 30 mM glucose are not due to osmotic stimulation.



**A**

Role of high glucose on NF-κB nuclear translocation

Relative p65 protein levels (% relative to cells in glucose 10 mM)

| Time | Bay | Mean (95% CI) | *P* * | *N* |
|---|---|---|---|---|
| 5 min | - | 129 (93 – 166) | 0.0036 | 5 |
| | + | 100 (75 - 125) | 0.1522 | 3 |
| 10 min | - | 148 (113 - 183) | <0.0001 | 5 |
| | + | 106 (34 - 347) | 0.1297 | 3 |
| 15 min | - | 143(102 - 184) | 0.0017 | 6 |
| | + | 86 (20 - 151) | 0.0950 | 3 |
| 30 min | - | 146 (73 - 220) | 0.0170 | 5 |
| | + | 80 (42 - 118) | 0.1158 | 3 |
| 45 min | - | 151 (114 - 189) | <0.0001 | 5 |
| | + | 95 (46 - 437) | 0.0907 | 3 |
| 60 min | - | 171 (129 - 213) | <0.0001 | 7 |
| | + | 71 (40 - 103) | 0.0027 | 4 |
| 2 h | - | 133 (66 - 201) | 0.051 | 5 |
| 4 h | - | 116 (19 - 213) | 0.1513 | 3 |
| 8 h | - | 115 (71 - 160) | 0.2717 | 3 |
| 24 h | - | 123 (78 - 169) | 0.1543 | 3 |



*vs glucose,10 mM ; † vs glucose 30 mM

**B**

Role of insulin on NF-κB nuclear translocation in the presence and absence of high glucose

Relative p65 protein levels (% relative to cells in glucose 10 mM)

| Glucose, mM | Insulin, nM | Mean (95% CI) | *P* | *N* |
|---|---|---|---|---|
| 10 | 10 | 94 (41 – 146) | 0.6186* | 5 |
| | 100 | 190 (160 – 220) | <0.0001* | 5 |
| 30 | 0 | 157 (115 - 199) | 0.026* | 9 |
| | 10 | 152 (1.1 – 257) | 0.0253*/0.9177† | 4 |
| | 100 | 148 (115 – 180) | <0.0001*/0.7512† | 4 |



**Figure 4.2** Role of glucose and insulin concentrations on nuclear NF-κB p65 levels. A) C28/I2 cells were cultured in 10 or 30 mM glucose, with or without 5 μM Bay-11782, a specific IκB-α phosphorylation inhibitor, for the indicated time periods. B) Insulin, 10 or 100 nM, was added to C28-I2 cultures in medium with 10 or 30 mM glucose for 1h. The results represent the means and 95% confidence intervals relative to cells in 10 mM glucose.

### 4.2.3 High Insulin modulates NF-κB translocation to the nucleus

To determine whether insulin modulates the inflammatory state induced by high glucose, we evaluated the effect of physiologic (10 nM) and supraphysiologic (100 nM) insulin concentrations on p65 nuclear levels in C28-I2 cells cultured in 10 or 30 mM

glucose. Figure 4.2B shows that treatment of cells in 10 mM glucose medium with 100 nM insulin increased nuclear p65 levels to the same extent as 30 mM glucose. Simultaneous treatment with high glucose (30 mM) and 100 nM insulin did not further increase nuclear p65 levels. On the other hand, 10 mM insulin did not affect p65 nuclear levels, regardless of the glucose concentration in the medium.

## 4.3 DISCUSSION

The present study aimed at determining whether high, hyperglycemia-like, glucose concentrations induce inflammatory responses in human chondrocytes and thus, contribute to unveil pathological mechanisms by which DM and impaired glucose homeostasis can favor OA initiation and progression. For this purpose, we exposed human chondrocytes to culture medium containing 10 or 30 mM glucose and compared the expression of pro-inflammatory mediators known to be relevant in OA pathogenesis, namely the pro-inflammatory and pro-catabolic cytokines, IL-1β and TNF-α, and iNOS, the enzyme responsible for the production of large amounts of NO [Stadler et al., 1991] To further elucidate the mechanisms involved, we also evaluated the ability of high glucose (30 mM) to activate the transcription factor, Nuclear Factor-κB (NF-κB), which plays a central role in inducing inflammatory and catabolic gene expression, including IL-1β, TNF-α and iNOS, in chondrocytes [Rosa et al., 2008].

The results obtained show that culture under high glucose is sufficient to induce NF-κB activation and the expression of IL-1β, TNF-α and iNOS. These observations, along with the pro-catabolic and anti-anabolic effects that we reported previously [Rosa et al., 2009; Rosa et al., 2011a], support the hypothesis that hyperglycemia can drive cartilage damage and, thus, OA.

These results are consistent with several reports that demonstrated the induction of exacerbated inflammatory responses by hyperglycemia in various human tissues and cells [Donath and Shoelson, 2011; Jia et al., 2013].

iNOS mRNA and protein levels were similarly upregulated by high glucose, suggesting that this effect occurs at the transcriptional level, since it is the major mechanism of iNOS regulation. Nonetheless, upregulated iNOS mRNA levels were detected upon culture in high glucose for 24h, whereas measurable amounts of the protein and NO were detected after 72h and required culture periods longer than, at least, 48h. Moreover, NF-κB activation was maximal after exposure to high glucose for 1h and by 2h the effect had returned to levels found in the control. This kinetics is similar to NF-κB activation by other stimuli, like IL-1β, which on the contrary allows iNOS protein to be detected much earlier than observed here with high glucose [Mendes et al., 2002c]. This suggests that other mechanisms may contribute to induce iNOS expression in response to high glucose. One possibility is that high glucose-induced IL-1β and TNF-α, which are potent iNOS inducers in chondrocytes [Stadler et al., 1991] are required to mediate iNOS expression. On the other hand, since we did not evaluate the protein levels of those cytokines, we cannot exclude the possibility that unknown mechanisms

lead to a large gap between mRNA transcription of the three genes and synthesis of the corresponding proteins.

The rapid nuclear translocation of p65 induced by high glucose, suggests a direct effect on the NF-κB signalling pathway in chondrocytes. This is further supported by the finding that the IκB-α phosphorylation inhibitor, Bay 11-7082, completely abolished high glucose-induced nuclear p65 accumulation. This suggests that IκB-α phosphorylation or another upstream event on the NF-κB activation pathway is sensitive to high glucose.

Insulin, especially in supraphysiologic concentrations, was shown to induce anabolic and inhibit catabolic responses in adult chondrocytes and cartilage explant cultures from various species [Kellner et al., 2001; Rosa et al., 2011a]. Thus, we hypothesized that insulin might counteract the deleterious pro-inflammatory effects of high glucose that we observed in chondrocytes. Surprisingly, 100 nM insulin were sufficient to induce nuclear p65 accumulation, either alone or in combination with high glucose, whereas 10 nM insulin, which is at the upper physiologic limit had no effect. These results are in agreement with a recent study showing that insulin, in concentrations similar to those we used, induced NF-κB activation and synergized with TNF-α in cardiac myoblasts [Madonna et al., 2014]. Moreover, accumulating evidence is unravelling a role for hyperinsulinemia in driving or at least contributing to the low-grade inflammation characteristic of metabolic disorders, from obesity and DM type 2 to the metabolic syndrome and their complications [Guo, 2014]. The results presented here also point to the possibility that hyperinsulinemia can induce inflammatory responses in chondrocytes by activating NF-κB. Nonetheless, further studies are required to confirm the proinflammatory effects of hyperinsulinemia in primary human chondrocytes.

In summary, this study shows for the first time that hyperglycemia and hyperinsulinemia can induce inflammatory responses in human chondrocytes. This can be a relevant mechanism by which DM type 2, obesity, the metabolic syndrome and other conditions associated with impaired glucose and insulin homeostasis contribute to the development and progression of OA.

**CHAPTER 5.**

# DIFFERENTIAL EFFECTS OF *LAVANDULA LUISIERI* AND *ERYNGIUM DURAEI* SUBSP *JURESIANUM* ESSENTIAL OILS IN CELL MODELS OF TWO CHRONIC INFLAMMATORY DISEASES

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**Differential Effects of *Lavandula Luisieri* and *Eryngium Duriaei* Subsp. *Juresianum* Essential Oils in Cell Models of Two Chronic Inflammatory Diseases**
Submitted for publication to the Pharmaceutical Biology (July 2014)

**ABSTRACT**

Context: Effective drugs to treat osteoarthritis (OA) and inflammatory bowel disease (IBD) are needed.

Objective: To identify essential oils (EOs) with anti-inflammatory activity in cell models of OA and IBD.

Material and methods: EOs from *Eryngium duriaei* subsp. *juresianum* (M. Laínz) M. Laínz (Apiaceae), *Laserpitium eliasii* subsp. *thalictrifolium* Sennen & Pau (Apiaceae), *Lavandula luisieri* (Rozeira) Rivas-Martínez (Lamiaceae), *Othantus maritimus* (L.) Hoff. & Link (Asteraceae) and *Thapsia villosa L.* (Apiaceae) were analyzed by GC and GC/MS. The anti-inflammatory activity of the EOs (5-200 µg/mL) was evaluated by measuring iNOS and NF-κB activation (total and phosphorylated IκB-α), in primary human chondrocytes and the intestinal cell line, C2BBe1, stimulated with IL-1β or IFN-γ, IL-1β and TNF-α, respectively.

Results: The EO of *L. luisieri* significantly reduced iNOS (by 54.9 and 81.0%, respectively) and phosphorylated IκB-α (by 87.4% and 62.3%, respectively), in both cell models. The EO of *E. duriaei* subsp. *juresianum* caused similar effects in human chondrocytes, but was inactive in intestinal cells, even at higher concentrations. The EOs of *L. eliasii* subsp. *thalictrifolium* and *O. maritimus* decreased iNOS expression by 45.2±8.7% and 45.2±6.2%, respectively, in C2BBe1 cells and were inactive in chondrocytes. The EO of *T. villosa* was inactive in both cell types.

Discussion and conclusion: This is the first study showing anti-inflammatory effects of the EOs of *L. luisieri* and *E. duriaei* subsp. *juresianum*. These effects are cell type-specific and may be valuable to develop new therapies or as sources of active compounds with improved efficacy and selectivity towards OA and IBD.

## 5.1 INTRODUCTION

An increasing number of reports describes anti-inflammatory properties of many natural products and compounds. Nonetheless, very few studies have been dedicated to evaluate their therapeutic potential on chronic inflammatory diseases. These remain important therapeutic targets due to their high prevalence and lack of effective therapies. Indeed, most studies concerning anti-inflammatory properties of natural products have been performed in cell or animal models of acute inflammation. These models do not entirely reflect pathological mechanisms and cells involved in chronic inflammatory diseases.

Among natural products, essential oils (EOs) are particularly interesting to look for compounds with pharmacological activities, since they are complex mixtures of a huge diversity of low molecular weight (<300 Da) lipophilic compounds. These characteristics may represent favorable pharmacokinetic properties [Miguel, 2010].

Recent reviews on the anti-inflammatory potential of EOs fully highlight that these plant extracts and some of their components are useful therapeutic alternatives, modulating several molecular targets of the acute inflammation cascades, namely in cells of the immune system, like monocytes and macrophages [Adorjan and Buchbauer, 2010; Miguel, 2010]. There is also an evident lack of information on the potential of EOs as modulators of chronic inflammatory diseases, especially involving cells unrelated to the immune system. Exceptions are two reports from our group [Neves et al., 2009; Rufino et al., 2014] describing anti-inflammatory effects of the EO of *Juniperus oxycedrus* L. subsp. *oxycedrus* (Cupressaceae) and $\alpha$-pinene in a cell model of osteoarthritis (OA).

Chronic inflammatory diseases, in general, lead to the up-regulation of a series of enzymes and signalling molecules that bring about the characteristic inflammation and tissue destruction. Likewise, OA and Inflammatory Bowel Disease (IBD) share many features and mechanisms and are largely driven and perpetuated by pro-inflammatory cytokines, such as Interleukin-1β (IL-1β), Tumor necrosis factor-α (TNF-α) and Interferon-$\gamma$ (IFN-$\gamma$) [Goldring and Otero, 2011; Goldring et al., 2008; Jobin and Sartor, 2000; Wielockx et al., 2004]. In chronic inflammatory diseases, the onset and perpetuation of inflammatory responses and tissue destruction are primarily dependent on the transcription factor, Nuclear Factor-κB (NF-κB) [Tak and Firestein, 2001]. This promotes the expression of inflammation-related genes, including cytokines (IL-1β, TNF-α, IL-6, etc.) and enzymes. One of these, the inducible nitric oxide synthase (iNOS), produces large amounts of nitric oxide (NO), a potent and destructive inflammatory mediator that plays an important role in the development and progression

of OA and IBD. For instance, our previous study showed that iNOS expression, NO production and NF-κB activity are spontaneously increased in chondrocytes isolated from OA patients compared to those isolated from non-affected human cartilage [Rosa et al., 2008]. Increased mucosal and plasma NO concentrations associated with augmented iNOS activity have also been demonstrated in active IBD [Quenon et al., 2012]. Moreover, NF-κB promotes the expression of specific proteases that degrade the extracellular matrix, causing the characteristic tissue destruction of OA and IBD [Goldring and Otero, 2011; Jobin and Sartor, 2000; Wielockx et al., 2004].

Therefore, this transcription factor constitutes an attractive target for anti-inflammatory therapeutic interventions both in OA and IBD [Berenbaum, 2004; Goldring and Otero, 2011; Jobin and Sartor, 2000; Marcu et al., 2010]. Hence, we proposed to study the ability of EOs to inhibit NF-κB activation in cell models of these diseases.

To address this purpose, EOs to be screened were selected considering two major criteria: i) to collectively ensure the highest diversity of compounds from the chemical classes usually found in EOs; and ii) availability of etnopharmacological information or previous evidence of anti-inflammatory activity. For this, several EOs, isolated at laboratory from native or endemic species of the Iberian flora, were first fully characterized by identification and quantification of their components. Upon composition elucidation, the EOs were assayed in cellular models relevant for the study of OA and IBD [Csaki et al., 2009; Megias et al., 2007]. Since we found previously that α-pinene inhibits inflammatory and catabolic responses in human chondrocytes, namely IL-1β-induced NF-κB activation [Neves et al., 2009; Rufino et al., 2014], we also tested this compound in the cellular model of IBD.

Primary human chondrocyte cultures stimulated with IL-1β were used as an in vitro cartilage degradation model that emulates the damage seen in OA and cultures of the human colorectal adenocarcinoma cell line, C2BBe1, stimulated with a mixture of pro-inflammatory cytokines composed of IFN-γ, TNF-α and IL-1β, were used as a model of IBD.

## 5.2 RESULTS

### 5.2.1 Composition of the essential oils

Chemical characterization of the EOs of *L. luisieri*, *E. duriaei* subsp. *juresianum, O. maritimus*, *L. eliasii* and *T villosa* allowed the identification and quantification of 94, 25, 30, 42 and 14 compounds, respectively, comprising at least 85% of the respective composition. Table 5.1 summarizes the composition of each EO, indicating the relative amounts of grouped components and the most representative compounds (≥ 2%).

Thirty-eight compounds, comprising seven monoterpene hydrocarbons, twelve oxygen-containing monoterpenes, including four necrodane derivatives, eight sesquiterpene hydrocarbons, nine oxygen-containing sesquiterpenes, the phenylpropanoid, methyleugenol, and the aliphatic, *n*-nonane, were found in concentrations over 2.0% in, at least, one EO.

Table 5.1 Classes of compounds and major constituents of the essential oils tested

| | *L. luisieri* | | *E. duriaei* subsp. *juresianum* | | *O. maritimus* | | *L. eliasii* | | *T. villosa* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Major compounds (≥2.0%) | Total % | Major compounds (≥2.0%) | Total % | Major compounds (≥2.0%) | Total % | Major compounds (≥2.0%) | Total % | Major compounds (≥2.0%) | Total % |
| Monoterpene hydrocarbons | α-pinene 2.3<br>3,5-dimethyllene-1,4,4-trimethylcyclopentene 2.5 | 6.4 | - | 1.9 | α-pinene 6.7 | 10.3 | α-pinene 30.5<br>sabinene 11<br>β-pinene 21.7<br>myrcene 3.5<br>limonene 2.7 | 73.5 | limonene **57.6** | 58.5 |
| Oxygen containing monoterpenes | 1,8-cineol 2.5<br>*trans*-α-necrodyl acetate 16.0<br>*trans*-α-necrodol 8.4<br>lavandulyl acetate 6.1<br>2,3,4,4-tetramethyl-5-methyllene-cyclopent-2-enone 5.2<br>lyratyl acetate 3.5<br>linalool 3.1<br>1,1,2,3-tetramethyl-4-hidroxymethyl-2-ciclopentene 2.4 | 61.8 | - | - | filifolone 12.2<br>chrysanthenone 57.2<br>chrysanthenyl acetate 10.1 | 85.6 | - | 3.3 | - | 0.4 |
| Sesquiterpene hydrocarbons | - | 7.1 | α-neocallitropsene 26<br>*E*-caryophyllene 6.3<br>bicyclogermacrene 3.8<br>β-selinene 3.0 | 41.4 | - | 0.1 | - | 3.9 | - | t |
| Oxygen containing sequiterpenes | viridiflorol 3.3<br>α-cadinol 2.0 | 12.4 | isocaryophyllen-14-al 16.2<br>14-hydroxy-β-caryophyllene 13.4<br>caryophyllene oxide 7.6 | 40.1 | - | t | - | 0.7 | - | - |
| Phenylpropanoids | - | - | - | - | - | - | - | - | methyleugenol | **35.9** |
| Aliphatic compounds | - | 0.2 | - | 1.2 | - | - | *n*-nonane 7.4 | 7.7 | - | 0.1 |
| Total % | | 87.9 | | 84.6 | | 96 | | 89.1 | | 95.8 |

### 5.2.2 Evaluation of cytotoxicity and selection of non cytotoxic concentrations of the essential oils

Neither the EOs nor α-pinene were cytotoxic to C2BBe1 cells in concentrations up to 200 µg/mL, either in the presence or absence of the pro-inflammatory cytokine mixture (Fig. 5.1C and D). On the contrary, in human chondrocytes, only the EO of *T. villosa* had no significant cytotoxic effects at that concentration (Fig. 5.1A and B). In concentrations up to 50 µg/mL for 24 h, the EOs of *E. duriaei* subsp. *juresianum*, *L. eliasii* and *L. luisieri* did not affect chondrocyte viability either in the presence or absence of IL-1β. The EO of O*. maritimus* showed no cytotoxicity at concentrations up to 100 µg/mL (Fig. 5.1A and B). Therefore, subsequent experiments were performed using the non-cytotoxic concentrations identified for each EO in each cell model, as shown in Fig. 5.1.

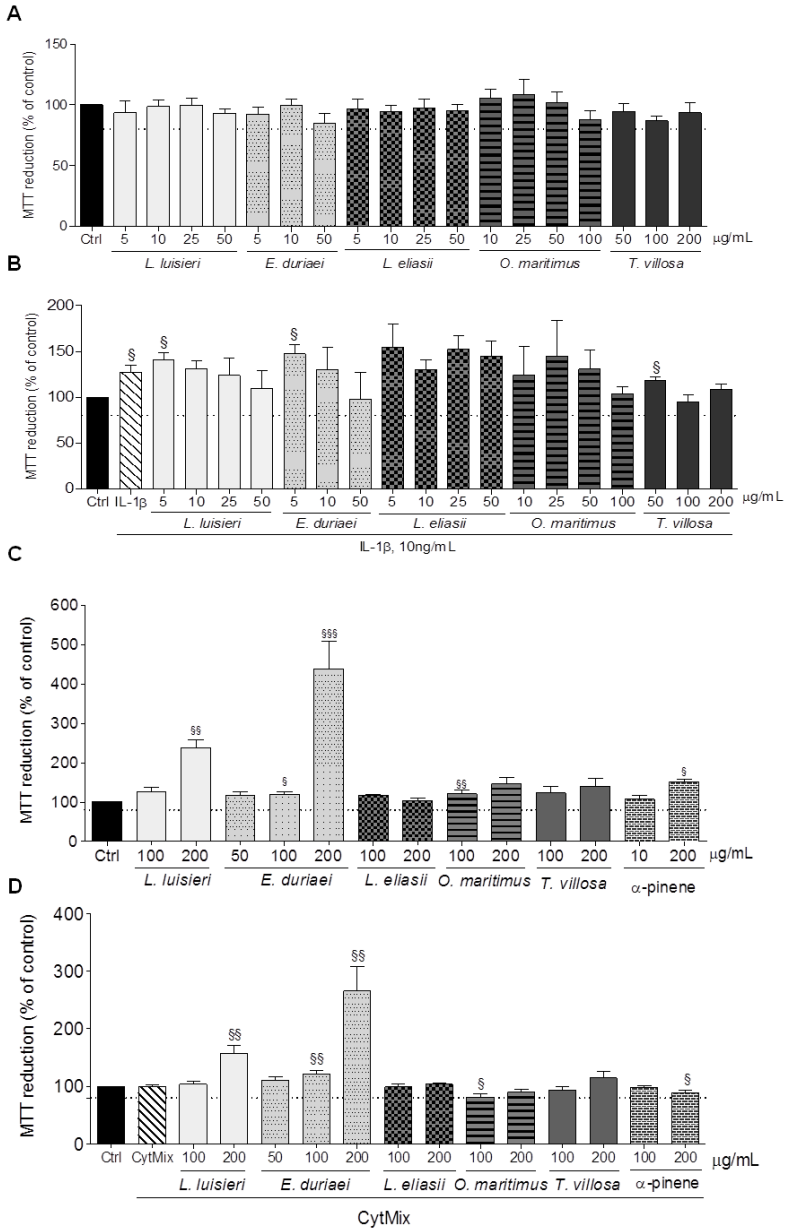


**Figure 5.1** Viability of human chondrocytes (A and B) and C2BBe1 cells (C and D) treated with the EOs for 24 h in the absence (A and C) or presence (B and D) of the respective pro-inflammatory stimulus. Each column represents, at least, 4 independent experiments. The dotted line represents the limit below which cell viability is impaired. $^{\S}P$ <0.05, $^{\S\S}P$ <0.01 relative to the respective control (untreated) cells.

### 5.2.3 Effect of the EO's on cytokine induced iNOS expression and NO production

Treatment of human chondrocytes (Fig. 5.2) and C2BBe1 cells (Fig. 5.3) with IL-1β or CytMix, respectively, for 24 h, strongly induced the expression of iNOS. Accordingly,

human chondrocytes stimulated with IL-1β produced almost six-fold more NO than control cells, as shown by the concentration of nitrite accumulated in the respective culture medium (15.6 ± 1.9 μM and 2.7 ± 0.8 μM, respectively, *P*<0.001, Fig. 5.4).

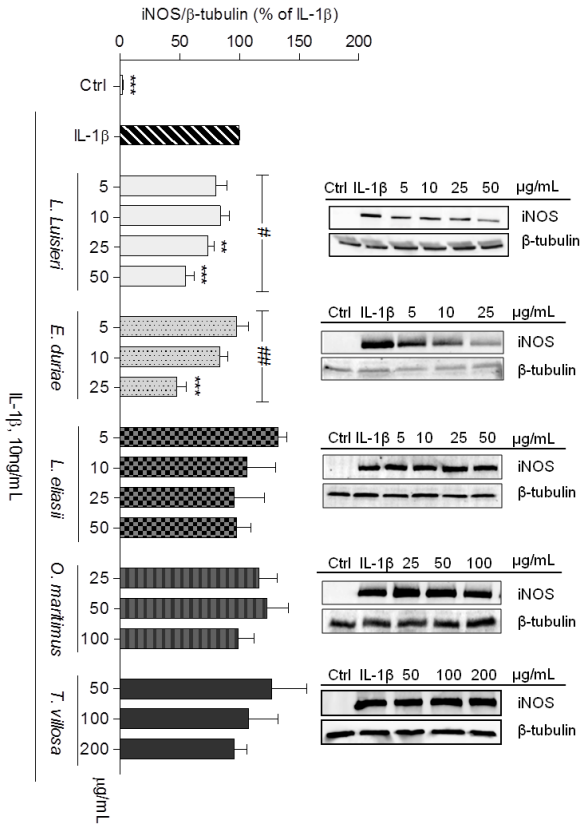


**Figure 5.2** Effect of the EOs on iNOS protein expression in human chondrocytes left untreated (Ctrl) or treated with IL-1β, 10 ng/mL, for 24 h, after pre-treatment with each EO. The images shown are representative of, at least, 3 independent experiments. ***P*<0.01, ****P*<0.001 relative to cells treated with IL-1β. $^{\#}$*P*<0.05 and $^{\#\#}$*P*<0.01 between different concentrations of the same essential oil (one-way ANOVA).

Surprisingly, no differences in nitrite concentration were detected in C2BBe1 cell cultures treated with the CytMix for various periods (6-48 h) in comparison with untreated cells (data not shown). As measurement of NO production was used just as a rapid screening assay and iNOS protein was readily identified, no other methods were used to detect NO production in C2BBe1 cells.

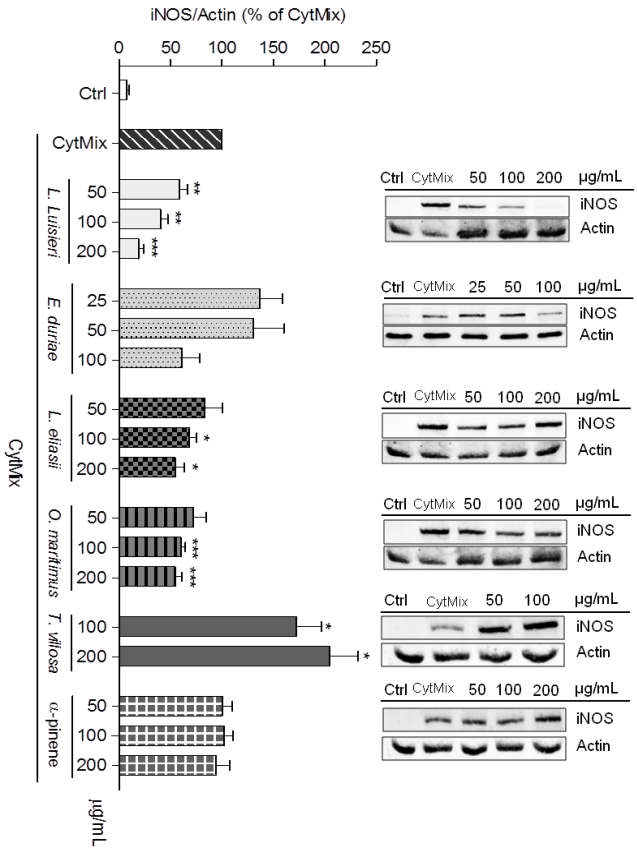


**Figure 5.3** Effect of the EOs on iNOS protein expression in C2BBe1 cells left untreated (Ctrl) or treated with CytMix, for 24 h, after pre-treatment with each EO. The images shown are representative of, at least, 3 independent experiments. **P*< 0.05, ***P*<0.01, ****P*<0.001 relative to cells treated with CytMix.

Of the EOs tested, only those of *L. luisieri* and *E. duriaei* subsp. *juresianum* were effective in decreasing iNOS expression (Fig. 5.2) and the subsequent NO production (Fig. 5.4) in human chondrocytes. The EOs of *L. eliasii* and *O. maritimus* produced a statistically significant, but very modest decrease of NO production (Fig. 5.4), even though no significant effects were detected on iNOS protein levels (Fig. 5.2). The EO of *T. villosa* had no significant effects, either on iNOS protein levels (Fig. 5.2) or NO production (Fig. 5.4).

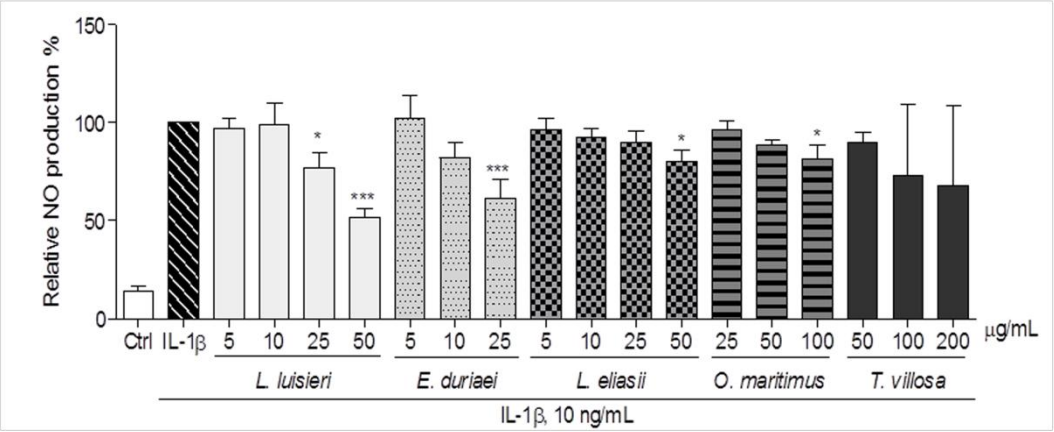


**Figure 5.4** Effect of the EOs on IL-1β-induced NO production in human chondrocytes left untreated (Ctrl) or treated with IL-1β, 10 ng/mL, for 24h, after pre-treatment with each EO. Each column represents, at least, 4 independent experiments. **P*<0.05, and ****P*<0.001 relative to IL-1β-treated cells.

In C2BBe1 cells, the EO of *L. luisieri*, at a concentration of 200 µg/mL, achieved the highest inhibition of iNOS expression (81.0 ± 5.2%). At a concentration of 50 µg/mL, iNOS levels were similarly reduced in C2BBe1 cells and in chondrocytes (Fig. 5.3). The EOs of *L. eliasii* and *O. maritimus* significantly reduced iNOS protein levels (by 45.2 ± 8.7% and 45.2 ± 6.2%, respectively) in C2BBe1 cells, but were much less effective than the EO of *L. luisieri* (Fig. 5.3). Noticeably in these cells, the EO of *E. duriaei* subsp. *juresianum* had no significant effect on iNOS expression (Fig. 5.3), even at a concentration of 100 µg/mL which is several fold higher than those tested in human chondrocytes. Neither the EO of *T. villosa* nor α-pinene had any significant effect (Fig. 5.3).

### 5.2.4 Effect of the EO's of *Lavandula luisieri* and *Eryngium duriaei* subsp. *juresianum* on NF-κB activation

In response to appropriate stimuli, the NF-κB inhibitory protein, IκB-α, is phosphorylated and subsequently degraded, which releases NF-κB and allows its translocation to the nucleus to induce the expression of target genes [Hayden and Ghosh, 2008a; O'Dea and Hoffmann, 2009]. To assess NF-κB activation, we evaluated the cytoplasmic levels of phosphorylated and total IκB-α.

The EOs more effective in inhibiting iNOS expression and NO production were selected to evaluate their ability to inhibit IL-1β-induced NF-κB activation in human chondrocytes and C2BBe1 cells.

In human chondrocytes, the EOs of *L. luisieri* and *E. duriaei* subsp. *juresianum*, at the concentration of 200 µg/mL, completely inhibited IL-1β-induced IκB-α phosphorylation, achieving an effect similar (*P*=0.82 and *P*=0.25, respectively) to that elicited by Bay 11-7082, a specific inhibitor of IκBα-phosphorylation [Mendes Sdos et al., 2009] used as a positive control (Fig. 5.5). Total IκB-α levels relative to those in control cells increased from 4.3 ± 1.5% (n=4) in IL-1β-treated chondrocytes to 47.9 ± 10.7% (n=4) and 55.6 ± 9.9% (n=4) in cells treated with 200 µg/mL of the EOs of *L. luisieri* and *E. duriaei* subsp. *juresianum*, respectively. Moreover, total IκB-α levels in chondrocytes treated with 200 µg/mL of the EO of *E. duriaei* subsp. *juresianum* were not significantly different from those observed in control cells, indicating that this concentration completely prevented the response induced by IL-1β.

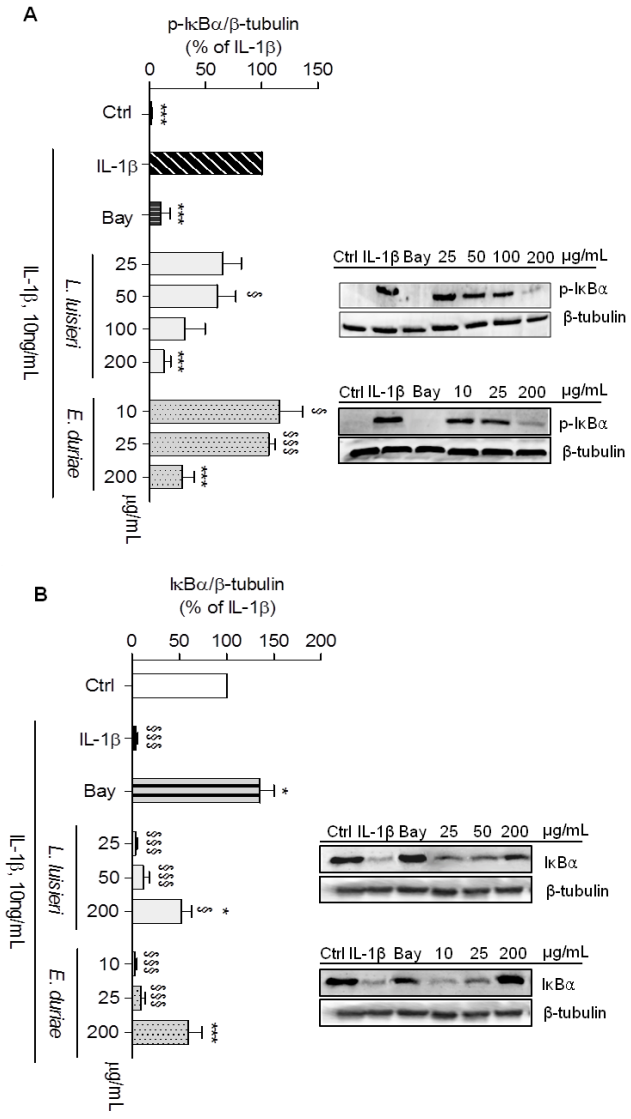


**Figure 5.5** Effect of the EOs of *L. luisieri* and *E. duriaei* subsp. *juresianum* on IL-1β-induced IκB-α phosphorylation and degradation in chondrocytes left untreated (Ctrl) or

treated with IL-1β, 10 ng/mL, for 5 (A) or 30 min (B) after pre-treatment with the EOs or Bay 11-7082 (5 μM). The images shown are representative of, at least, 4 independent experiments. *$P$<0.05, and ***$P$<0.001 relative to IL-1β-treated cells. $^{§}P$<0.05, and $^{§§§}P$<0.001 relative to Ctrl.

In C2BBe1 cells, the EO of *L. luisieri*, at concentrations of 50 and 200 μg/mL, significantly reduced IκB-α phosphorylation to 77.3 ± 8.7% (n=7) and to 37.7 ± 3.0% (n=7), respectively, of the CytMix-induced response (Fig. 5.6). Total IκB-α levels increased from 11.7 ± 1.2% in CytMix-treated cells to 30.4 ± 4.3% (n=8) in *L. luisieri*-treated cells relative to the control, indicating that the EO effectively decreased CytMix-induced NF-κB activation. Moreover, 200 μg/mL of this EO inhibited IκB-α phosphorylation to even a larger extent than the positive control, Bay 11-7082 ($P$=0.028), while IκB-α degradation was similarly inhibited ($P$=0.31).

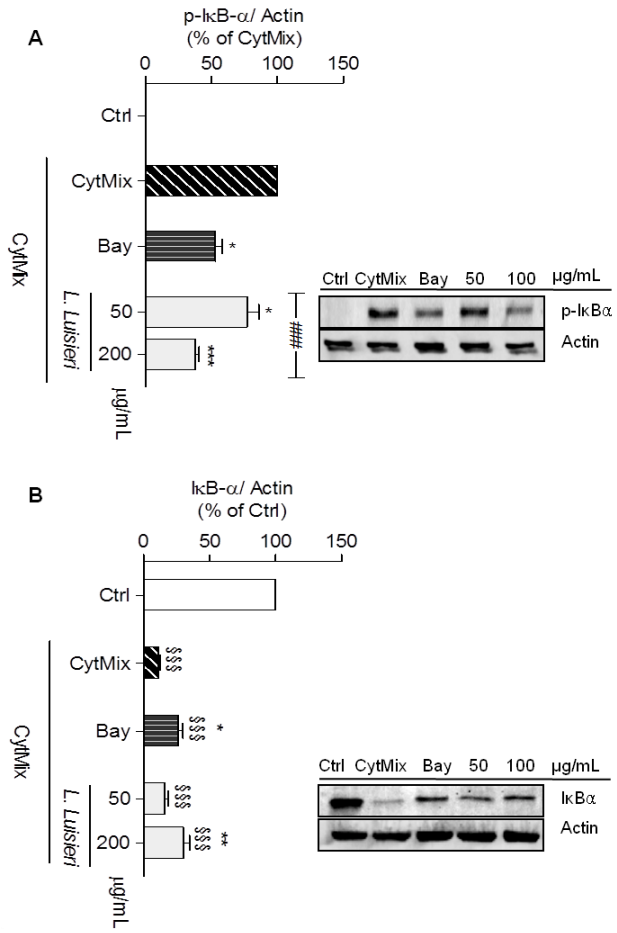


**Figure 5.6** Effect of the EO of *L. luisieri* on CytMix-induced IκB-α phosphorylation and degradation in intestinal C2BBe1 cells left untreated (Ctrl) or treated with CytMix for 5 min (A) or 30 min (B) after pre-treatment with the EO or Bay 11-7082 (5 μM). The images shown are representative of, at least, 4 independent experiments. *$P$<0.05,

**$P$<0.01 and ***$P$<0.001 relative to CytMix-treated cells. $^{§§§}P$<0.001 relative to Ctrl and $^{###}P$<0.001 between different concentrations of the same essential oil (one-way ANOVA)

## 5.3 DISCUSSION

The results presented show clear differences in the ability of the EOs tested to inhibit relevant mediators of inflammation in the two cell models of OA and IBD used. Such differences are evident among distinct EOs, as well as comparing each one in the two cell models. Indeed, only the EO of *L. luisieri* was capable of significantly inhibiting inflammatory markers (iNOS expression and NF-κB activation) both in human chondrocytes and intestinal C2BBe1 cells. These results confirm our previous observation that this EO inhibits IL-1β-induced NO production in human chondrocytes [Neves et al., 2009]. On the other hand, the EO of *E. duriaei* subsp. *juresianum* did not significantly inhibit iNOS expression in intestinal cells over a wide range of concentrations, but was effective in human chondrocytes, even at much lower concentrations. Conversely, the EOs of *O. maritimus* and *L. eliasii* showed some activity in intestinal cells, but were inactive in chondrocytes. These results indicate that the EOs of *L. luisieri* and *E. duriaei* subsp. *juresianum* have cell type-specific anti-inflammatory effects.

To our knowledge, this is the first study showing anti-inflammatory effects of the EOs of *L. luisieri* and *E. duriaei* subsp. *juresianum.* Indeed, EOs from *Lavandula ssp*, namely *L. angustifolia* *L. stoechas*, *L. multifida* and *L. viridis*, have demonstrated antibacterial, antifungal, analgesic and anti-inflammatory effects [Amira et al., 2012; Ghelardini et al., 1999; Kirmizibekmez et al., 2009; Zuzarte et al., 2011a; Zuzarte et al., 2011b], while the EO of *L. luisieri* has only been reported to have antifungal effects [Zuzarte et al., 2012]. On the other hand, EOs from *Eryngium* ssp, including *E. duriaei* subsp. *juresianum*, have been shown to have anti-fungal and anti-bacterial properties [Cavaleiro et al., 2011; Celik et al., 2011], whereas anti-inflammatory effects were only reported for aqueous or alcoholic extracts from *Eryngium* species [Dawilai et al., 2013; Kupeli et al., 2006] not including *E. duriaei* subsp. *juresianum*.

α-Pinene from a commercial source (purity ≥ 98%) was unable to decrease iNOS expression in intestinal cells, while our previous studies showed that both commercial α-pinene[Rufino et al., 2014] and the EO of *J. oxycedrus* subsp. *oxycedrus* (76.4% α-pinene) and one of its fractions (93% α-pinene) [Neves et al., 2009], were effective in human chondrocytes. Interestingly, the EOs of *L. luisieri*, *O. maritimus* and *L. eliasii*, the only ones containing significant amounts of α-pinene (2.3%, 6.7% and 30.5%, respectively), were all effective in intestinal cells, but only that of *L. luisieri* was also effective in human chondrocytes, even though it presents the lowest α-pinene content. Taken together, these results suggest that α-pinene is unlikely the compound

responsible for the inhibitory activities of the EOs of *L. luisieri*, *O. maritimus* and *L. eliasii* observed in this study.

Similar considerations can be made about other compounds present in two or more of the EOs tested. Limonene, for instance, represents 58.8% of the EO of *T. villosa*, but only 2.7% of the EO of *L. eliasii.* Nevertheless, this EO was effective in intestinal cells while that of *T. villosa* was inactive in both cell types.

On the other hand, the EOs of *L. luisieri* and *E. duriaei* subsp. *juresianum* have distinct compositions, the first being rich in oxygenated monoterpenes, while the second is mainly composed of oxygenated and non-oxygenated sesquiterpenes. Nonetheless, they were both effective in reducing markers of inflammation in human chondrocytes, suggesting that distinct compounds are involved in the observed activities of these EOs.

Moreover, we cannot discard the possibility that different components of the EOs act in synergy or in antagonism to modulate their overall activity, contributing to the apparent discrepancies described above. This possibility is even more plausible as some of these compounds, like limonene [Chi et al., 2012], have been shown to exert anti-inflammatory effects in various cell and animal models.

Taken together, the results presented indicate that two of the EOs studied, those of *L. luisieri* and *E. duriaei* subsp. *juresianum*, have significant cell type-specific anti-inflammatory effects that can be useful for the development of tissue-selective anti-inflammatory therapies.

The results obtained also show that the EOs of *L. luisieri* and *E. duriaei* subsp. *juresianum* which were the most effective in inhibiting iNOS expression in human chondrocytes and/or intestinal epithelial cells, also decreased cytokine-induced NF-κB activation. Since NF-κB is essential for iNOS expression [Taylor et al., 1998], these results strongly suggest that the observed inhibition of iNOS is mediated, at least in part, by the inhibitory effects of these EOs on NF-κB activation. The concentrations of the EOs of *L. luisieri* and *E. duriaei* subsp. *juresianum* effective in inhibiting IL-1β-induced NF-κB activation in human chondrocytes were substantially higher than those that inhibited iNOS expression and NO production, suggesting that other mechanisms may contribute to the inhibitory activity of these EOs, at least in human chondrocytes. Since none of the EOs showed NO scavenging activity (data not shown), this can be discarded as a potential contributing mechanism. Other possibilities include direct inhibition of iNOS activity and inhibition of other signalling pathways that are required for iNOS expression in human chondrocytes [Mendes et al., 2002c]. More studies are required to identify the specific molecular targets of the EOs

of *L. luisieri* and *E. duriaei* subsp. *juresianum*. On the other hand, since EOs are complex mixtures of chemically diverse compounds, it is possible that distinct components have different targets, so that the effects observed result from the combined actions of individual compounds. Future work will be directed at fractionating each of these EOs and elucidating the chemical composition and pharmacological activity of each fraction in order to identify the active compound(s) and their specific molecular targets, as well as potential pharmacological interactions.

In summary, this study shows for the first time that the EOs of *L. luisieri* and *E. duriaei* subsp. *juresianum* efficiently inhibit NF-κB activation and the expression of its target genes, namely iNOS, in cells unrelated to the immune system. Moreover, these EOs display differential effects in relevant cell models of OA and IBD and in response to distinct inflammatory stimuli. These properties may be of great value in the development of new therapies with improved efficacy and selectivity towards distinct chronic inflammatory diseases, namely OA and IBD.

# ANTI-INFLAMMATORY AND CHONDROPROTECTIVE ACTIVITY OF (+)-A-PINENE: STRUCTURAL AND ENANTIOMERIC SELECTIVITY

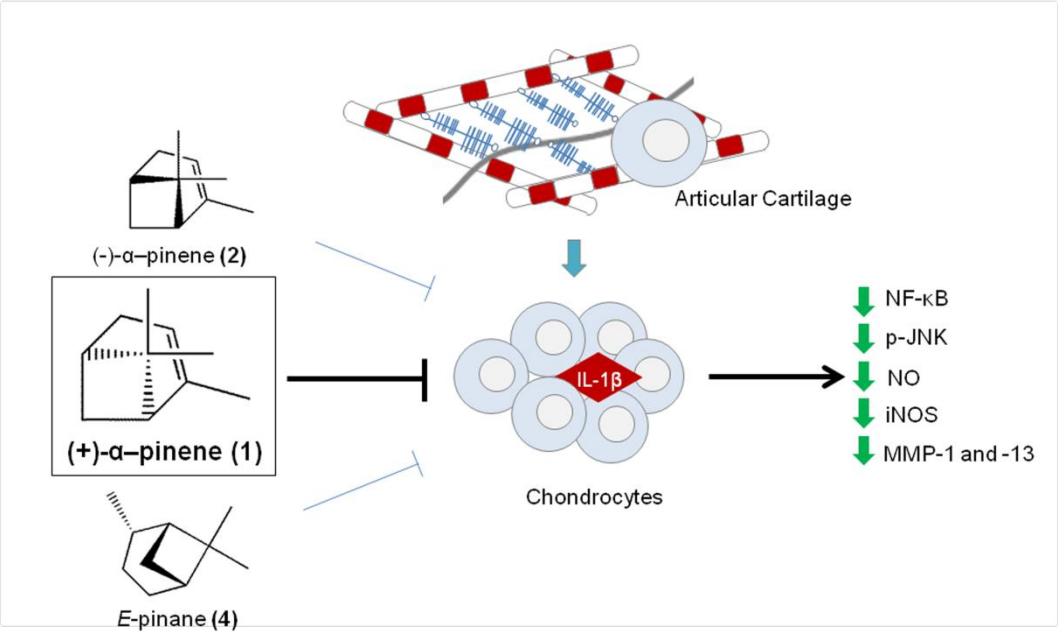


**Figure 6.0** Graphical Abstract

Ana T. Rufino, Madalena Ribeiro, Fernando Judas, Lígia Salgueiro, M. Celeste Lopes, Carlos Cavaleiro and Alexandrina F. Mendes

**Anti-Inflammatory and Chondroprotective Activity Of (+)-α–Pinene: Structural and Enantiomeric Selectivity**

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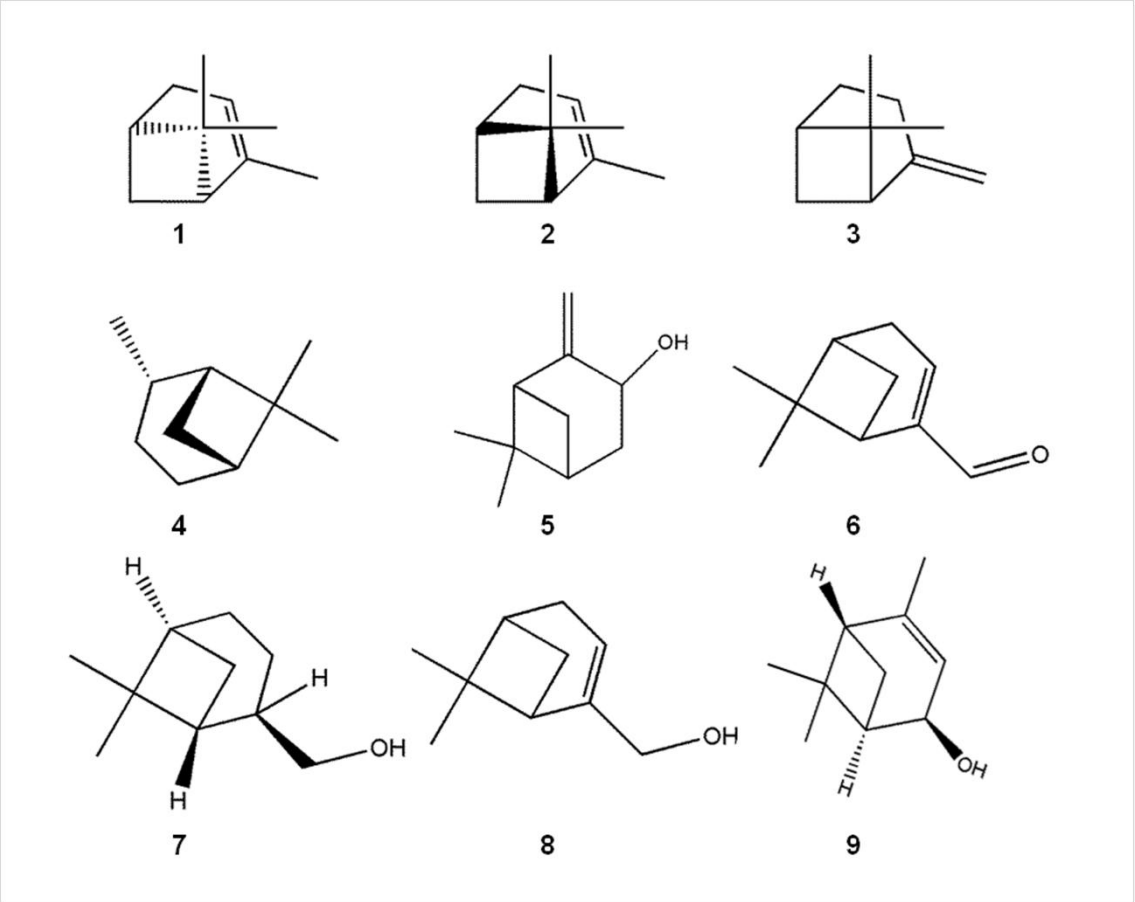
**ABSTRACT**

Previous studies have suggested that α-pinene, a common volatile plant metabolite, may have anti-inflammatory effects in human chondrocytes, thus exhibiting potential anti-osteoarthritic activity. The objective of this study was to further characterize the potential anti-osteoarthritic activity of selected pinene derivatives by evaluating their ability to modulate inflammation and extracellular matrix remodelling in human chondrocytes and to correlate the biological and chemical properties by determining whether the effects are isomer- and/or enantiomer-selective. To further elucidate chemicopharmacological interactions, the activities of other naturally occurring monoterpenes with the pinane nucleus were also investigated. At non-cytotoxic concentrations, (+)-α-pinene (**1**) elicited the most potent inhibition of the IL-1β-induced inflammatory and catabolic pathways, namely, NF-κB and JNK activation and the expression of the inflammatory (iNOS) and catabolic (MMP-1 and -13) genes. (−)-α-Pinene (**2**) was less active than the (+)-enantiomer (**1**), and β-pinene (**3**) was inactive. E-Pinane (*4*) and oxygenated pinane-derived compounds, pinocarveol (**5**), myrtenal (**6**), (*E*)-myrtanol (**7**), myrtenol (**8**), and (*Z*)-verbenol (**9**), were less effective or even completely inactive and more cytotoxic than the pinenes tested (**1**−**3**). The data obtained show isomer- and enantiomer-selective anti-inflammatory and anti-catabolic effects of α-pinene in human chondrocytes, (+)-α-pinene (**1**) being the most promising for further studies to determine its potential value as an anti-osteoarthritic drug.

## 6.1 INTRODUCTION

Osteoarthritis (OA) is estimated to affect 10−15% of the world population, being the leading cause worldwide of chronic disability in the elderly and also in those in the working age range.[Kaplan et al., 2013; Zhang and Jordan, 2010] OA is a multifactorial joint disease characterized by a progressive loss of the articular cartilage extracellular matrix and local inflammation that lead to cartilage destruction, loss of joint function, and pain.[Goldring and Otero, 2011] The huge socioeconomic burden of the disease and the absence of effective disease-modifying OA drugs have led to its inclusion in a group of priority diseases selected in the World Health Organization report “Priority Medicines for Europe and the World”, as commissioned by the European Commission.[Kaplan et al., 2013] In view of this, our previous study identified the essential oil from the leaves of Juniperus oxycedrus L. subsp. oxycedrus (Cupressaceae) as a promising source of compounds with potential anti-osteoarthritic activity. In particular, it was found that two fractions of this essential oil, one rich in α-pinene (93%) and the other containing oxygenated mono- and sesquiterpenes, effectively inhibited nuclear factor-κB activation and the production of nitric oxide (NO),[Neves et al., 2009] which play major roles in promoting the inflammatory and catabolic responses in human articular chondrocytes that drive OA development and progression.[Fernandes et al., 2002; Goldring and Otero, 2011; Goldring et al., 2008] Several studies have attributed biological properties to α- pinene, including antimicrobial, anti-inflammatory,[Zhou et al., 2004a] apoptotic, and anti-metastatic effects,[Matsuo et al., 2011] but information concerning the activities of its enantiomers, as well as of the β-isomer, is scarce. To our knowledge, this is restricted to antibacterial, antifungal,[da Silva et al., 2012] and insecticide[Michaelakis et al., 2009] activities. Therefore, this study aimed to confirm that the previously described inhibitory effects of the essential oil of *J. oxycedrus* L. subsp. *oxycedrus* in human chondrocytes are due primarily to α- pinene and especially to determine whether those effects are enantio- and/or isomer-selective. For this purpose, (+)-α- pinene (**1**), (−)-α-pinene (**2**), the racemic α-pinene (**1** and **2**) OA,[Akhtar and Haqqi, 2011; Csaki et al., 2009] consisting of human articular chondrocytes treated with the pro-inflammatory and pro-catabolic cytokine interleukin-1β (IL-1β).[Fernandes et al., 2002; Goldring and Otero, 2011; Goldring et al., 2008] To further elucidate possible relationships between chemical structure and potential anti-osteoarthritic activity, other naturally occurring pinane derivative compounds were also included in this study, namely, (*E*)-pinane (**4**), pinocarveol (**5**), myrtenal (**6**), (*E*)-myrtanol (**7**), myrtenol (**8**), and (*Z*)-verbenol (**9**). Some

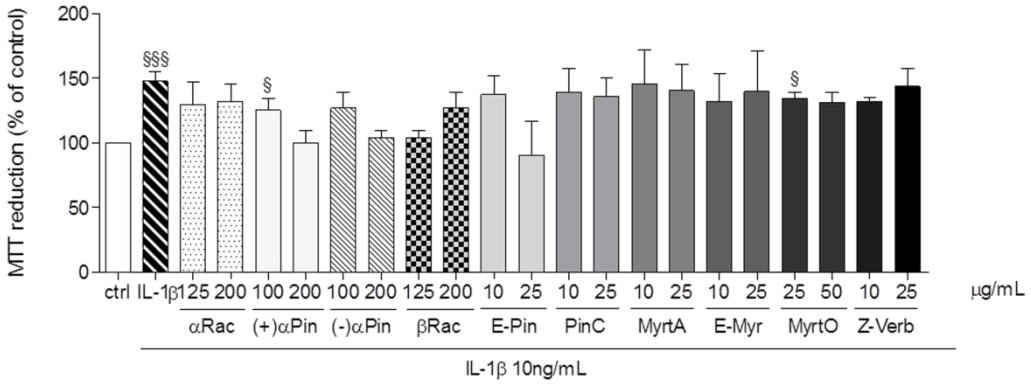



of these pinane derivatives were chosen since they are present in the oxygenated monoterpene containing fraction of *J. oxycedrus* subsp. *oxycedrus* essential oil found to be active on human chondrocytes.[Neves et al., 2009]

Finally, and to further assess the potential anti-osteoarthritic activity of the test compound(s) found to be more active, their ability to modulate signalling pathways that lead to inflammation and articular cartilage degradation was evaluated. Given their central role in those pathways, nuclear factor-κB (NF-κB) and the mitogen-activated protein kinases (MAPKs), JNK, p38, and ERK, are attractive targets for the development of new drugs to effectively block OA progression.[Berenbaum, 2004; Goldring and Otero, 2011] Likewise, we evaluated the ability of the most active test compound to modulate the activity of these mediators and the expression of genes relevant in OA pathogenesis. The genes studied included inflammatory (inducible nitric oxide synthase, iNOS), catabolic (matrix metalloproteases, MMP-1 and -13), and anticatabolic mediators (tissue inhibitor of metalloproteases, TIMP-1) and extracellular matrix components (collagens I and II).

## 6.2 RESULTS AND DISCUSSION

Since NO is a major inflammatory mediator and plays an important role in OA pathogenesis, while simultaneously representing a final end point of NF-κB activation,[Abramson, 2008; Berenbaum, 2004; Marcu et al., 2010] inhibition of its production in human chondrocytes is a facile screening method for the identification of NF-κB inhibitors with potential antiosteoarthritic activity. Thus, inhibition of IL- 1β-induced NO production was used to discriminate active compounds at noncytotoxic concentrations as defined by evaluation of cell viability with the MTT reduction assay (Supporting Information).




**Supporting Information Figure.** Chondrocyte viability in human chondrocytes

Chondrocyte treatment with 10 ng/mL IL-1β increased NO production significantly from 10.6 ± 0.8 µg/mL in untreated control cells to 40.9 ± 3.6 µg/mL ($n$ = 11; $p$ < 0.0001). Treatment of the chondrocyte cultures with noncytotoxic concentrations (Supporting Information) of the test compounds revealed that only myrtenol (**8**) and (Z)-verbenol (**9**) had no effect on IL-1β-induced NO production, while the other compounds decreased NO production to a varying degree, at least, at the highest concentration tested (Figure 6.1A). (+)-α-Pinene (**1**) decreased NO production in a concentration- dependent manner ($p$ = 0.015 between the two concentrations tested), eliciting the highest inhibition of NO production at a concentration of 200 µg/mL, with a decrease to 31.5 ± 2.6% ($n$ = 10, $p$ < 0.0001) relative to cells treated with IL-1β alone. At the same concentration, racemic α-pinene (**3**) decreased NO production to only 40.6 ± 1.9% ($p$ = 0.0011).

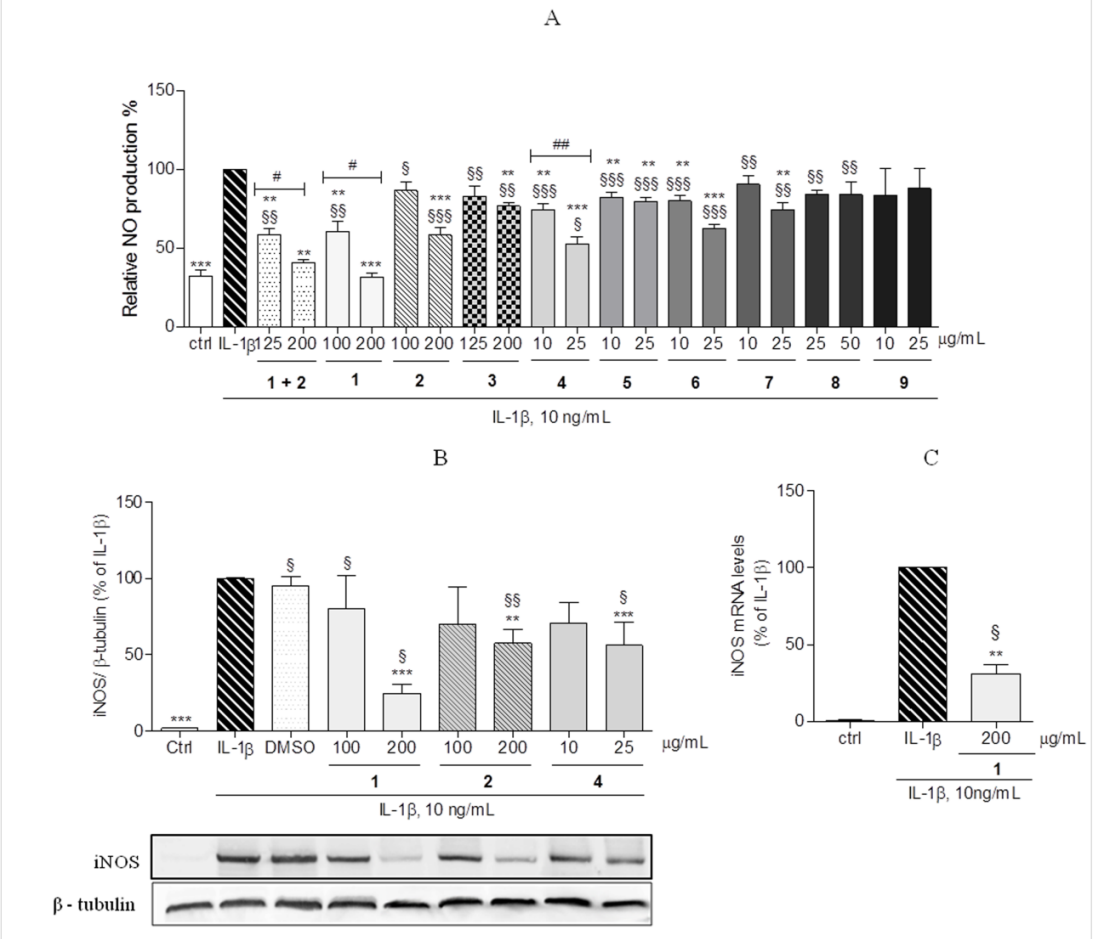



**Figure 6.1** Effect of α–pinene (1), β-pinene (2) and other pinane derivatives (3 – 9) on IL-1β-induced NO production (A) and effect of the active test compounds on iNOS protein (B) and mRNA (C) expression levels in primary human chondrocytes. Human chondrocyte cultures were left untreated (control, Ctrl) or treated with IL-1-β, 10 ng/mL,

for 24 h (A and B) or 6h (C), following pre-treatment for 30 min with the indicated concentrations of the test compounds or DMSO. Each column represents the mean ± SEM of at least, four independent experiments. $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001 relative to IL-1β-treated cells; $^{§}p$ <0.05, $^{§§}p$ <0.01, $^{§§§}p$ <0.001 relative to Ctrl; $^{\#}p$ <0.05, $^{\#\#}p$ <0.01 between the conditions indicated.

However, these results were not statistically different and are in the same range reported for the α-pinene-rich fraction of the essential oil from the leaves of *J. oxycedrus* subsp. *oxycedrus*. [Neves et al., 2009]

On the other hand, (−)-α-pinene (**2**) and the racemic β- pinene (**3**) were active only at the highest concentrations tested (200 μg/mL), with much lower inhibitions (to 58.1 ± 4.9%, $p$ = 0.0001 and 76.8 ± 2.3%, $p$ = 0.0098) than those elicited by either racemic α-pinene (**1** and **2**) or its (+) enantiomer (**1**) at the same concentration. In contrast, (*E*)-pinane (**4**), pinocarveol (**5**), myrtenal (**6**), and (*E*)-myrtanol (**7**), at the highest non-cytotoxic concentration, elicited only relatively small decreases of IL-1β-induced NO production.

These results show clear differences in activity among the compounds tested (Figure 6.1A). In particular, it is noticeable that α-pinene is the most active, showing not only selectivity concerning the β-isomer (**3**), almost devoid of activity, but also enantiomeric selectivity, as (+)-α-pinene (**1**) was found to be more effective than the (−) enantiomer (**2**).

To confirm these results and further elucidate the mechanism of action of the compounds that more effectively reduced NO production, the α-pinene enantiomers (**1** and **2**) and (*E*) - pinane (**4**), effects on iNOS protein levels induced by IL-1β were evaluated. Paralleling the results relative to NO production, Figure 6.1B shows that at 200 μg/mL (+)-α-pinene (**1**) elicited the greatest inhibition, decreasing iNOS protein levels to 24.4 ± 14.9% ($p$ < 0.0001) relative to those found in cells treated with IL-1β alone. The highest concentrations tested of (−)-α-pinene (**2**) and (*E*)-pinane (**4**) elicited a smaller, but statistically significant, decrease of iNOS protein levels. (+)-α-Pinene (**1**) also extensively decreased IL-1β- induced iNOS mRNA levels (Figure 6.1C), indicating that reduction of iNOS protein and enzymatic activity likely occur through effects at or upstream of gene transcription.

As human cartilage samples are limited, both in number and quantity, and a large number of cells is required, the human chondrocytic cell line, C-28/I2, was used to evaluate the ability of α-pinene enantiomers to inhibit IL-1β-induced NF-κB activation. This requires the phosphorylation and subsequent ubiquitination and proteassomal

degradation of an inhibitory protein, IκB-α (NF-κB inhibitor-α), which, in basal conditions, retains NF-κB dimers in the cytoplasm. Once IκB-α is degraded, the freed NF-κB dimers translocate to the nucleus and bind to specific sequences in the promoter region of target genes promoting their transcription.[Hayden and Ghosh, 2008a] Treatment of the cells with 10 ng/mL IL-1β, for 5 min, induced a pronounced phosphorylation of IκB-α, indicating the activation of NF-κB, which, as expected, is barely detected in the untreated control cells (Figure 6.2A).

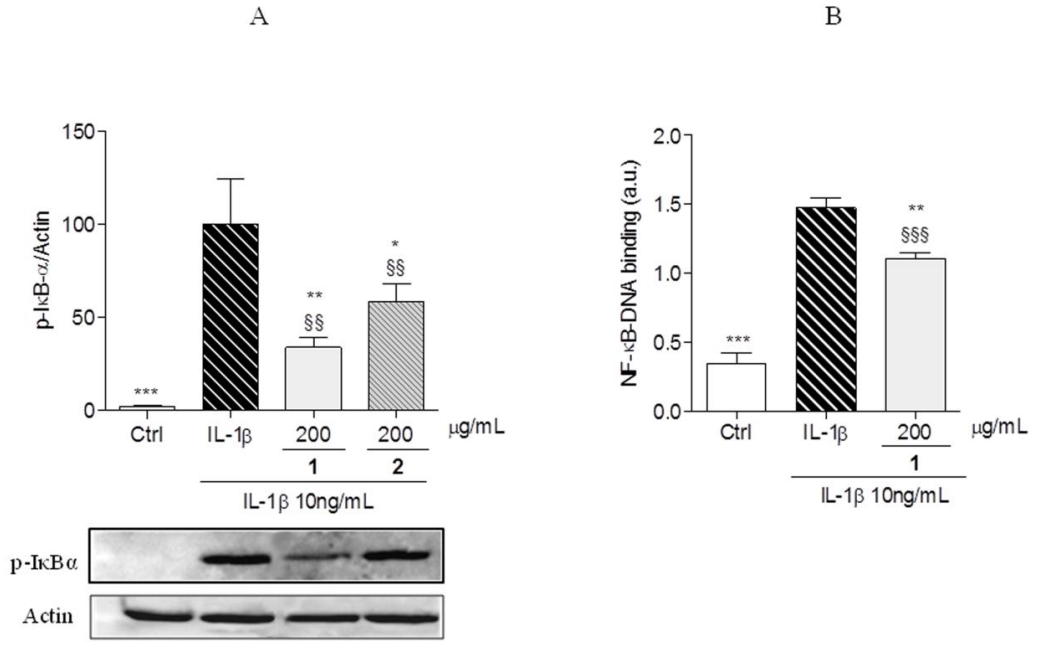



**Figure 6.2** Effect of α–pinene enantiomers (1 and 2) on IL-1β-induced NF-κB activation, evaluated as the levels of phosphorylated IκB-α (A) and NF-κB–DNA complexes (B). C28-I2 chondrocytic cells were left untreated (control, Ctrl) or treated with 10 ng/mL IL-1β, for 5 (A) or 30 min (B), following pre-treatment with or without 200 μg/mL of the test compounds for another 30 min. Each column represents the mean ± SEM of three (A) or four (B) independent experiments. $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001 relative to IL-1β-treated cells and $^{§§}p$ <0.01, $^{§§§}p$ <0.001 relative to Ctrl cells.

The results presented in Figure 6.2A show that treatment with (+)-(**1**) and (−)-α-pinene (**2**) reduced IL-1β-induced IκB-α phosphorylation to 33.6 ± 3.1% ($p$ = 0.002) and 58.4 ± 5.4% ($p$ = 0.017), respectively, relative to cells treated with IL-1β alone. Interestingly, each enantiomer inhibited IκB-α phosphorylation to approximately the same extent as iNOS expression and NO production, further corroborating that these two events are dependent on NF-κB activation and that the (+)-α enantiomer (**1**) is the most effective.

To confirm that inhibition of IκB-α phosphorylation by (+)-α-pinene (**1**) results in decreased NF-κB activity, the formation of specific NF-κB−DNA complexes was evaluated by an ELISA assay, as described in the Experimental Section. The results obtained indicated that this process suffers a dramatic increase upon stimulation with 10 ng/mL IL-1β for 30 min (Figure 6.2B) and is effectively reduced by pre-treatment with 200 μg/mL (+)-α-pinene (**1**) ($p$ = 0.0081).

As other transcription factors, namely, AP-1,17,18 and signalling intermediates, such as JNK and p38 MAPKs,[Kristof et al., 2001; Marks-Konczalik et al., 1998; Mendes et al., 2002c] can be involved in the expression of iNOS, MMPs, and other genes relevant in OA pathogenesis,[Berenbaum, 2004; Han et al., 2001; Mengshol et al., 2000] the possibility that such pathways are also modulated by α-pinene enantiomers was investigated. For this, both compounds (**1** and **2**) were tested for their capacity to modulate IL-1β-induced JNK and p38 MAPK phosphorylation. The results obtained show that only the highest concentration of the α-pinene enantiomers (**1** and **2**) elicited a statistically significant decrease of IL-1β-induced JNK phosphorylation (51.3 ± 6.3%; $p$ = 0.0015 and 65.8 ± 12.9%, $p$ = 0.0452) (Figure 6.3A), while none of them affected p38 phosphorylation (Figure 6.3B).

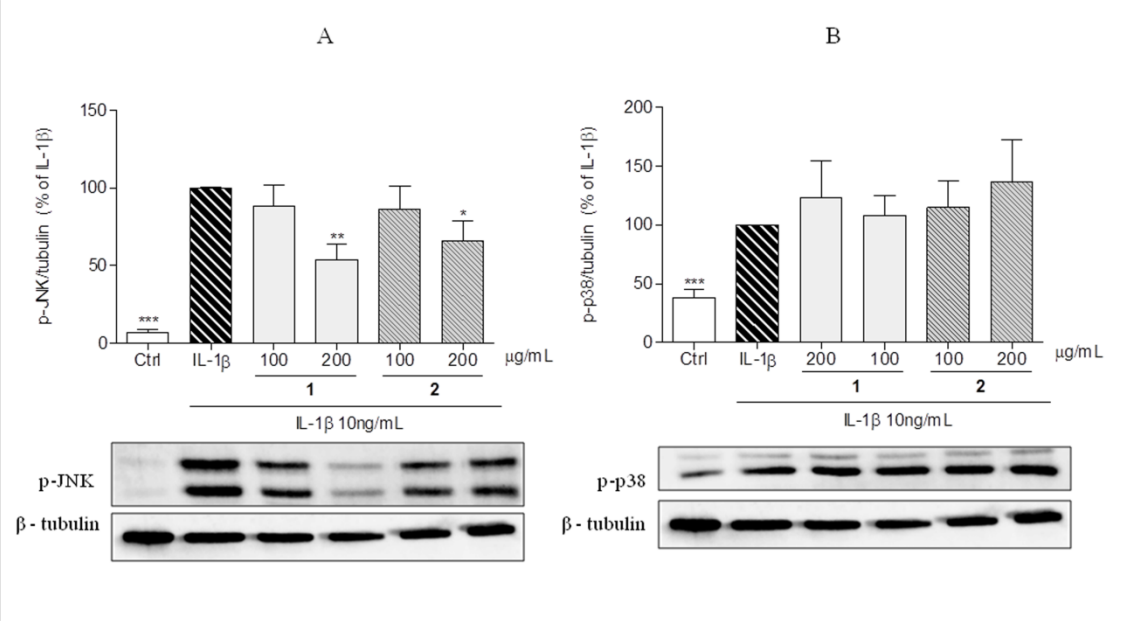



**Figure 6.3** Effect of the α–pinene enantiomers on IL-1-induced JNK (A) and p38 MAPK (B) phosphorylation. Primary chondrocytes were left untreated (control, Ctrl) or were treated with 10 ng/mL IL-1β, for 5 min, following pre-treatment with or without the indicated concentrations of the α-pinene enantiomers. Each column represents the

mean ± SEM of four independent experiments. $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001 relative to IL-1β-treated cells.

Since (+)-α-pinene (**1**) demonstrated the highest ability to inhibit IL-1β-induced NF-κB and JNK activation, which are required for the expression of MMPs-1 and -13 induced by IL- 1β,[Han et al., 2001; Mendes et al., 2002c] its ability to decrease these effects was also evaluated. As expected the expression of both genes increased significantly in response to IL-1β and was completely abolished by pre-treatment with 200 μg/mL (+)-α-pinene (**1**) (Figure 6.4A).

To further investigate its potential anti-osteoarthritic activity, the effect of (+)-α-pinene (**1**) was also evaluated on the expression of TIMP-1, collagen I, and collagen II genes.

Treatment of chondrocytes with 10 ng/mL IL-1β significantly decreased TIMP-1 (Figure 6.4B) and collagen II (Figure 6.4C) mRNA levels to 59.5 ± 7.0% and 32.2 ± 3.8%, respectively, relative to untreated control cells, while the mRNA levels of the non-cartilage specific collagen I were significantly increased to 378.39 ± 82.9% (Figure 6.4C). Treatment with 200 μg/mL (+)-α-pinene (**1**) did not affect the expression of any of these genes, significantly (Figure 6.4B and C). Nevertheless, the mean mRNA levels of TIMP-1 and collagen I showed a tendency to reach control levels on (+)-α- pinene (**1**) treatment (Figure 6.4B and C).

Several studies suggest that inhibition of MMP expression is sufficient to prevent the release of matrix components, like collagen II, and thus retard OA progression.[Piecha et al., 2010; Sabatini et al., 2005] Therefore, the ability of (+)-α-pinene (**1**) to decrease MMP-1 and -13 expression, even if not increasing TIMP-1 and collagen II expression, suggests that this compound can be effective in blocking cartilage destruction and, thus, OA progression. To determine further whether (+)-α-pinene (**1**) can restore the ability of chondrocytes to produce and maintain a normal articular cartilage matrix, the ratio of collagen II/collagen I mRNA levels was calculated as an index of chondrocyte differentiation, where the higher the ratio value, the more differentiated the phenotype.[Marlovits et al., 2004; Martin et al., 2001] Even though treatment with this compound increased the ratio 2.6-fold, indicating a tendency toward a more differentiated phenotype in (+)-α- pinene (**1**)-treated cells, the difference relative to the same ratio in cells treated with IL-1β alone did not reach statistical significance.

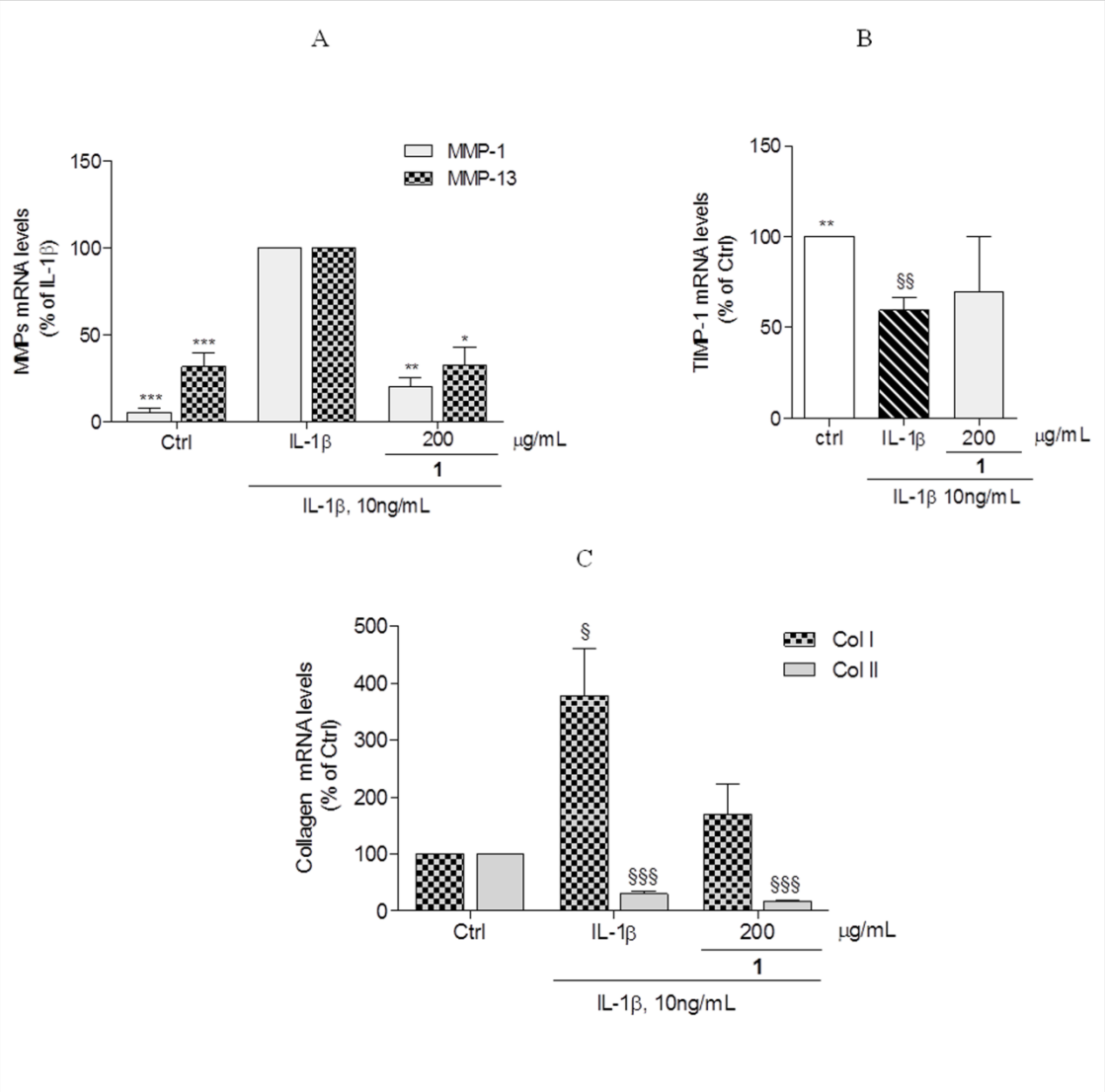



**Figure 6.4** Effect of (+)-α-pinene (1) on IL-1β-induced changes in gene expression. mRNA levels of (A) MMP-1 and -13, (B) TIMP-1 and (C) collagen I and collagen II were evaluated by RT-PCR. Each column represents the mean ± SEM of, at least, four independent experiments in which human chondrocytes were left untreated (control, Ctrl) or treated for 12 h (A) or 24 h (B and C) with 10 ng/mL IL-1β, in the presence or absence of 200 µg/mL (+)-α-pinene (1). $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001 relative to IL-1β-treated cells; $^{§§}p$ <0.01 and $^{§§§}p$ <0.001 relative to Ctrl cells.

In summary, the results reported in this study showed enantiomeric-selective anti-inflammatory and anticatabolic effects of (+)-α-pinene and derivatives in human chondrocytes. Of the compounds tested, (+)-α-pinene (1) showed the greatest efficacy to inhibit IL-1β-induced inflammatory and catabolic pathways, namely, NF-κB and JNK activation and the expression of inflammatory (iNOS) and catabolic (MMP-1 and -13) genes. Thus, (+)-α-pinene (**1**) deserves to be further investigated, namely, by

evaluating its pharmacokinetic and pharmacodynamic in vivo properties, in order to determine its potential as an antiosteoarthritic drug. Moreover, this study also shows that oxygenated pinane-derived compounds, namely, pinocarveol (**5**), myrtenal (**6**), (*E*)-myrtanol (**7**), myrtenol (**8**), and (*Z*)-verbenol (**9**), are less effective or even completely inactive and more cytotoxic than the non-oxygenated counterparts tested.

# EVALUATION OF THE ANTI-INFLAMMATORY, ANTI-CATABOLIC AND PRO-ANABOLIC EFFECTS OF *E*-CARYOPHYLLENE, MYRCENE AND LIMONENE IN A CELL MODEL OF OSTEOARTHRITIS

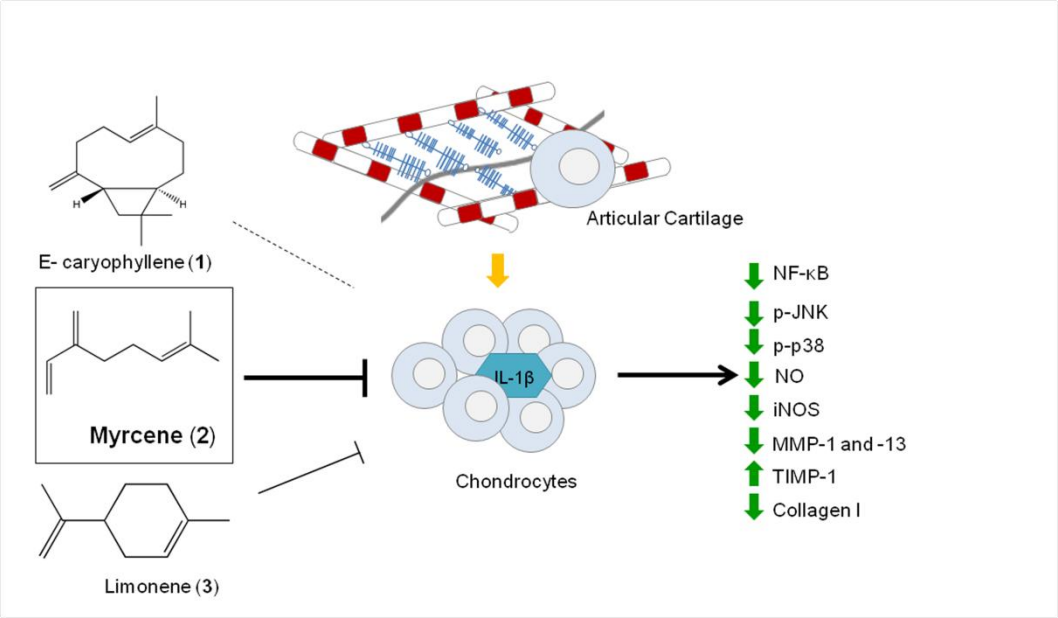



**Figure 7.0** Graphical Abstract

Ana T. Rufino, Madalena Ribeiro, Cátia Sousa, Fernando Judas, Lígia Salgueiro, Carlos Cavaleiro and Alexandrina F. Mendes

**Evaluation of the Anti-Inflammatory, Anti-Catabolic and Pro-Anabolic Effects of *E*-Caryophyllene, Myrcene and Limonene in a Cell Model of Osteoarthritis**

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**ABSTRACT**

The essential oils of *Eryngium duriaei* subsp. *juresianum* and *Lavandula luisieri* demonstrated anti-inflammatory properties in human chondrocytes. This study aimed at identifying active compounds and characterizing their antiosteoarthritic properties. *E*-caryophyllene (**1**), myrcene (**2**) and limonene (**3)**, present in the active fractions of these essential oils, were evaluated for their ability to modulate inflammatory, catabolic and anabolic processes in human chondrocytes. Myrcene (**2)** ($IC_{50}$=37.3 µg/mL) and limonene (**3)** ($IC_{50}$=85.3 µg/mL) inhibited IL-1β-induced nitric oxide production, but *E*-caryophyllene was inactive. Myrcene, and limonene to a lesser extent, also decreased IL-1β-induced NF-κB, JNK and p38 activation and the expression of inflammatory (iNOS) and catabolic (MMP-1 and MMP-13) genes, while increasing the expression of anti-catabolic genes [TIMP-1 and -3 by myrcene (**2**) and TIMP-1 by limonene (**3**)]. Neither myrcene (**2**) nor limonene (**3**) increased the expression of cartilage-specific genes (collagen II and aggrecan), but both compounds prevented the increase in the non-cartilage specific, collagen I. Limonene (**3**) increased ERK1/2 activation by 30% which may be unfavorable, while myrcene (**2**) decreased it by 26%, relative to IL-1β-treated cells.

These data show that myrcene (**2**) has significant anti-inflammatory and anti-catabolic effects and, thus, may halt or, at least, slow down cartilage destruction and osteoarthritis progression.

## 7.1 INTRODUCTION

Osteoarthritis (OA) is a multifactorial degenerative joint disease characterized by inflammation and progressive loss of the articular cartilage. It affects 10-15% of the world population and is a major cause of disability in the elderly, as well as in the workforce population.[Zhang and Jordan, 2010] Existing therapeutic approaches are mainly symptomatic, thus novel drugs with disease-modifying and chondroprotective properties are required to halt disease progression and decrease its huge socio-economic impact.[Goldring and Goldring, 2007; Kaplan et al., 2013]

Plant-derived compounds show important biological properties that can be explored in the context of OA for identification of compounds with potential anti-osteoarthritic activity.[Calixto et al., 2004; Khalife and Zafarullah, 2011] Among compounds of plant origin, those found in essential oils present favorable pharmacokinetic properties, namely lipophilicity and low molecular weight.[Miguel, 2010] Our previous studies have been focused in identifying essential oils with anti-inflammatory and anti-catabolic properties in human chondrocytes to be used as sources of compounds with potential anti-osteoarthritic activity[a][Neves et al., 2009] In this context, we identified the essential oils of *Eryngium duriaei* subsp. *juresianum* and *Lavandula luisieri* as possessing anti-inflammatory properties in human chondrocytes.[a] The current study aims at identifying the active compounds of these essential oils and further characterizing their pharmacological properties in human chondrocytes.

For this, mechanisms relevant as pharmacological targets for the development of anti-osteoarthritic drugs need to be addressed. Although OA etiology is not yet completely understood, pro-inflammatory cytokines, like interleukin-1β (IL-1β), play a central role in disease development and progression by inducing the expression of cartilage matrix-degrading enzymes and impairing anabolic and anti-catabolic responses.[Goldring et al., 2008] The consequent upregulated degradative process, together with impaired reparative responses, results in progressive cartilage loss, the hallmark of OA, accompanied by alterations in other joint tissues.

[a] Rufino, A. T.; Ferreira, I. V.; Judas, F.; Salgueiro, L.; Lopes, M. C.; Cavaleiro, C.; Mendes, A. F. (submitted at Journal of Natural products _ Chapter 5).

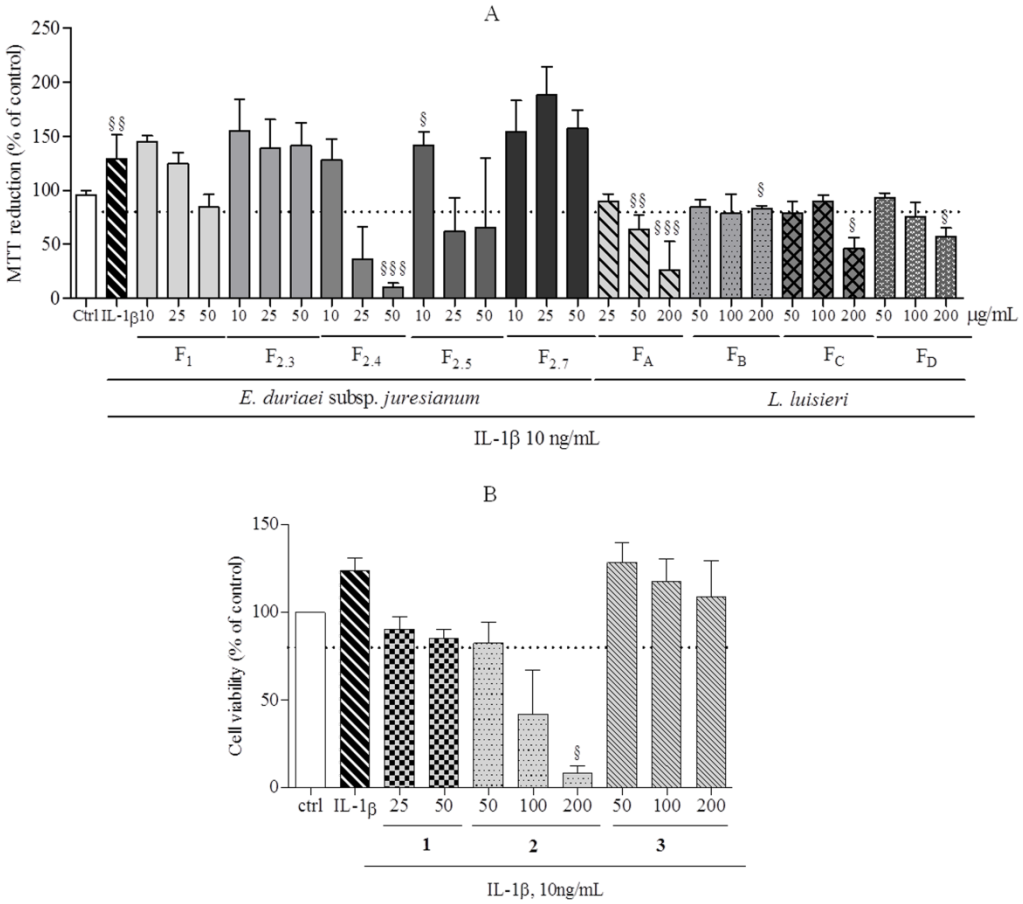
Matrix metalloproteases (MMPs) and aggrecanases are the main enzymes responsible for hydrolyzing the major articular cartilage-specific matrix components, collagen II and aggrecan. This is accompanied by impaired reparative responses involving downregulation of the natural MMP inhibitors, the tissue inhibitor of metalloproteases (TIMP) family, and decreased synthesis of collagen II and aggrecan.[Troeberg and Nagase, 2012] Moreover, increased production of pro-inflammatory and pro-catabolic mediators, like nitric oxide (NO), amplifies and perpetuates cartilage destruction.[Boileau et al., 2002; Rosa et al., 2008; Sasaki et al., 1998] The transcription factor, Nuclear Factor-κB (NF-κB), and the family of mitogen-activated protein kinases (MAPK) play a central role in modulating the expression of those catabolic and inflammatory mediators [Goldring and Otero, 2011] and, thus, agents that suppress their activity have the potential to effectively decrease cartilage destruction and, therefore, OA progression.[Berenbaum, 2004] Furthermore, compounds that can also restore anabolic and anti-catabolic gene expression have the potential to promote cartilage repair.

Therefore, we used primary human chondrocyte cultures stimulated with IL-1β as an *in vitro* cartilage degradation model that emulates the damage seen in OA. Using this model, we evaluated the inhibition of IL-1β-induced NO production as a simple screening assay to rapidly identify active fractions and compounds of the essential oils of *E. duriaei* subsp. *juresianum* and *L. luisieri*. Moreover and to further assess the anti-osteoarthritic potential of the active compounds identified, we evaluated their ability to modulate IL-1β-induced signaling pathways involved in the expression of inflammatory and catabolic genes, namely activation of NF-κB and the MAPK family members, Jun terminal Kinase (JNK), p38 and Extracellular signal-Regulated Kinase 1/2 (ERK1/2). Then, we evaluated the ability of those compounds to counteract the effects of IL-1β on the expression of those genes, including inflammatory (iNOS), catabolic (MMP-1 and -13), anti-catabolic (TIMP-1 and -3) and extracellular matrix (collagen I, and the cartilage matrix-specific, collagen II and aggrecan) genes in human articular chondrocytes.

## 7.2 RESULTS AND DISCUSSION

In the current study, various fractions of the essential oils of *E. duriaei subsp. juresianum* and *L. luisieri*, whose full chemical compositions we previously reported, [a] were separated in amounts sufficient for pharmacological characterization. The composition of these fractions was fully elucidated and is reported in table 7.1.

The fractions obtained were then tested at non-cytotoxic concentrations (Supporting Information) ranging from 10-50 µg/mL. The results (Figure 7.1A) show that the hydrocarbon-containing fractions ($F_1$ and $F_A$) of both essential oils decreased IL-1β-induced NO production in a concentration-dependent manner, the highest concentrations achieving an inhibition of 55 and 75%, respectively, relative to cells treated with IL-1β alone. The other three fractions of the essential oil of *L. luisieri* also showed some inhibitory activity which, nonetheless, did not exceed 35% relative to IL-1β-treated cells. Fractions $F_{2.4}$ and $F_{2.5}$ of the essential oil of *E. duriaei* subsp. *juresianum* were only tested at a concentration of 10 µg/mL, as higher concentrations were found to be cytotoxic (Supporting Information). These fractions, as well as fraction $F_{2.7}$ at a concentration of 50 µg/mL, also significantly inhibited IL-1β-induced NO production, although to a lesser extent than found with $F_1$. No significant effects were obtained with $F_{2.3}$ at any of the concentrations tested. Therefore, the hydrocarbon-containing fractions ($F_1$ and $F_A$) of both essential oils were considered the most promising for selection of compounds for further studies.




**Supporting Information Figure:** Effect of the fractions isolated from the essential oils of *Eryngium duriaei* subsp. *juresianum* and *Lavandula luisieri* (A) and of the isolated compounds E-Caryophyllene (**1**), myrcene (**2**) and limonene (**3**) (B) on human chondrocyte viability. Each column represents the mean ± SEM of at least four independent experiments where chondrocytes were treated with the indicated concentrations of the test compounds for 24 h together with IL-1β (10ng/mL) and cell viability accessed as described above. $^{\S}P<0.05$, $^{\S\S}P<0.01$ relative to untreated control cells (Ctrl).

Table 7.1 Composition of *E.duriae*i subsp *juresianum* and *L. luisieri* Essential Oils Fractions

| | *E. duriaei* subsp *juresianum* | % |
|---|---|---|
| $F_1$ | *E*- caryophyllene | 29.5 |
| | α-neocallitropsene | 50.2 |
| | germacrene D | 2.7 |
| | β- selinene | 2.7 |
| | Bicyclogermacrene | 6.3 |
| | Limonene | 0.1 |
| | Myrcene | T |
| $F_{2.3}$ | Octanal | 9.3 |
| | caryophyllene oxide | 31.6 |
| | isocaryophyllene-14-al | 44.4 |
| $F_{2.4}$ | unknown 1 | 22.8 |
| | Spathulenol | 9.8 |
| | 14-hydroxy-β-caryophyllene | 38.0 |
| | unknown 2 | 6.6 |
| $F_{2.5}$ | unknown 1 | 8.8 |
| | Spathulenol | 21.4 |
| | 14-hydroxy-β-caryophyllene | 45.2 |
| | unknown 2 | 2.9 |
| | unknown 3 | 3.7 |
| $F_{2.7}$ | caprylic acid | 8.1 |
| | buthyhidroxytoluene (solvent contaminant) | 8.6 |
| | tetradecanoic acid | 40.5 |
| | hexadecanoic acid | 8.5 |

| | *Lavandula luisieri* | % |
|---|---|---|
| $F_A$ | 3.5-Dimethylene-1.4.4-trimethylcyclopentene | 10.4 |
| | α-pinene | 26.9 |
| | β-pinene | 3.5 |
| | Δ-3-carene | 5.2 |
| | Limonene | 3.0 |
| | *E*-β-ocimene | 1.6 |
| | Cyclosativene | 2.0 |
| | α-copaene | 2.2 |
| | *E*-caryophyllene | 3.9 |
| | Alloaromadendrene | 1.2 |
| | β-selinene | 2.9 |
| | α-selinene | 3.5 |
| | δ-cadinene | 6.7 |
| | selina-3.7(11)-diene | 4.5 |
| $F_B$ | *trans*-α-necrodyl acetate | 30.5 |
| | lavandulyl acetate | 8.2 |
| | *cis*-α-necrodyl acetate | 3.8 |
| | 1.8-cineole | 32.4 |
| | lyratyl acetate | 2.4 |
| $F_C$ | 1.10-di-*epi*-cubenol | 4.8 |
| | 2.3.4.4-tetramethyl-5-methylene-cyclopent-2-enone | 8.6 |
| | Linalool | 11.9 |
| | *epi*-cubenol | 1.3 |
| | *trans*-α-Necrodol | 20.1 |
| | Lavandulol | 2.2 |
| | viridiflorol | 8.2 |
| | *T*-cadinol | 2.4 |
| | *T*-muurolol | 4.4 |
| | *cis*-linalool oxide (THP) | 3.2 |
| | unkown ($C_{10}H_{16}O$) | 2.3 |
| | 1.1.2.3-tetramethyl-4-hidroxymethyl-2-ciclopentene | 7.2 |
| | 14-norcadin-5-ene-4-one (Isomer) | 4.3 |
| $F_D$ | *trans*- linalool oxide (THF) | 12.6 |
| | α-terpineol | 7.1 |
| | Verbenone | 2.5 |
| | α-muurolol | 1.3 |
| | *trans*-verbenol | 3.1 |
| | globulol | 2.5 |
| | α-cadinol | 48.7 |

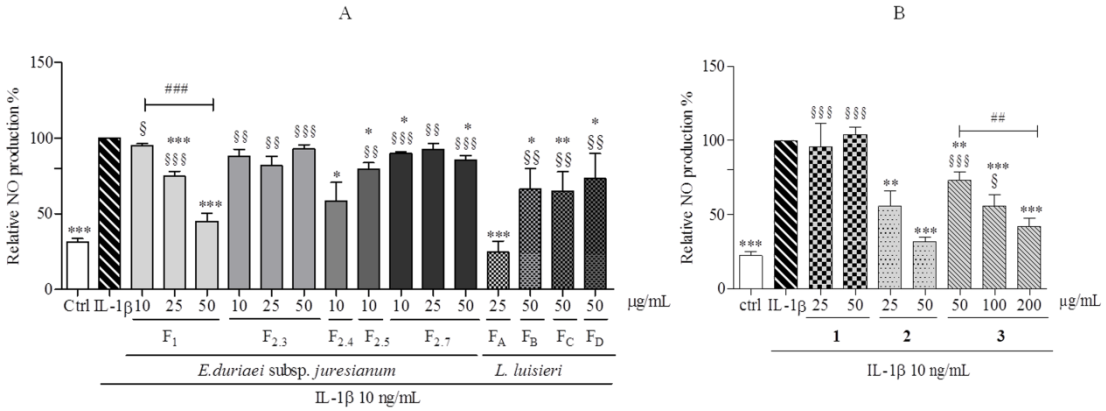
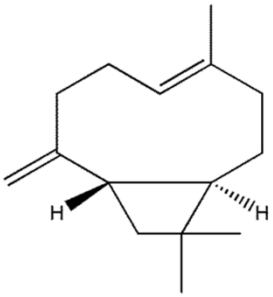



**Figure 7.1** Effect of the fractions isolated from the essential oils of *Eryngium duriaei* subsp. *juresianum* and *Lavandula luisieri* (A) and of the isolated compounds *E*-caryophyllene (1), myrcene (2) and limonene (3) (B) on IL-1β-induced NO production. Human chondrocytes were left untreated (Control, Ctrl) or treated with IL-1-β, 10ng/mL, for 24 h, following pre-treatment for 30 min with the indicated concentrations of each fraction or compound. Each column represents the mean ± SEM of, at least, four independent experiments. $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001 relative to IL-1β-treated cells; $^{\S}p$ <0.05, $^{\S\S}p$ <0.01, $^{\S\S\S}p$ <0.001 relative to Control cells; $^{\#\#}p$ <0.01, $^{\#\#\#}p$ <0.001 between the conditions indicated.

As shown in table 7.1, these fractions ($F_A$ and $F_1$) are composed of monoterpene and sesquiterpene hydrocarbons, chiefly α-pinene and 3,5-dimethylene-1,4,4-trimethylcyclopentene, and *E*-caryophyllene (**1**), α-neocallitropsene, germacrene D, β-selinene and bicyclogermacrene, respectively. Of these compounds, only α-pinene and *E*-caryophyllene (**1**) are readily available from commercial sources and we have recently reported the differential activity of α-pinene enantiomers as inhibitors of pro-inflammatory and catabolic pathways in human chondrocytes.[Rufino et al., 2014] Nonetheless, both essential oils have other minor components in common, namely the monoterpene hydrocarbons, myrcene (**2**) and limonene (**3**), which were thus selected for pharmacological evaluation and comparison with the effects of α-pinene previously reported.[Rufino et al., 2014] Thus, *E*-caryophyllene (**1**), myrcene (**2**) and limonene (**3**), obtained from commercial sources, were screened for their ability to inhibit IL-1β-induced NO production.

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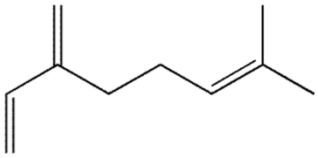

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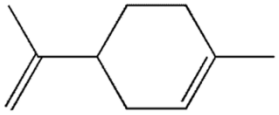

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The results obtained (Figure 7.1B) show that, at non-cytotoxic concentrations (Supporting Information), myrcene (**2**) and limonene (**3**) effectively inhibited IL-1β-induced NO production, while *E*-caryophyllene (**1**) had no significant effect at any of the concentrations tested. This finding was somewhat unexpected as this compound has been reported to exert anti-inflammatory effects by activating cannabinoid CB2 receptors,[Bento et al., 2011; Medeiros et al., 2007] while endogenous and synthetic cannabinoids have been reported to decrease inflammation in animal models of arthritis[Sumariwalla et al., 2004] and to inhibit IL-1-induced NO production in bovine chondrocytes.[Mbvundula et al., 2005]

Since myrcene (**2**) and limonene (**3**) showed inhibitory activity towards IL-1β-induced NO production, various non-cytotoxic concentrations were then tested to determine the respective concentration required to inhibit NO production by 50% ($IC_{50}$) and thus, to compare their relative potencies. We found that myrcene (**2**) has an $IC_{50}$ value (37.3 $\pm$ 1.1 µg/mL) lower than that of limonene (**3**) ($IC_{50}$ = 85.3 $\pm$ 1.2 µg/mL), thus indicating a higher potency.

To determine whether the observed inhibition of NO production by myrcene (**2**) and limonene (**3**) is due to inhibition of iNOS expression, its mRNA (Figure 7.2A) and protein (Figure 7.2B) levels were evaluated. Treatment with myrcene (**2**), 50 µg/mL, significantly diminished IL-1β-induced iNOS mRNA and protein levels by 78% and 69%, respectively, while inhibition by limonene (**3**), even at a concentration four fold higher, did not exceed 39% and 60%, respectively, further confirming the higher potency of myrcene (**2**).

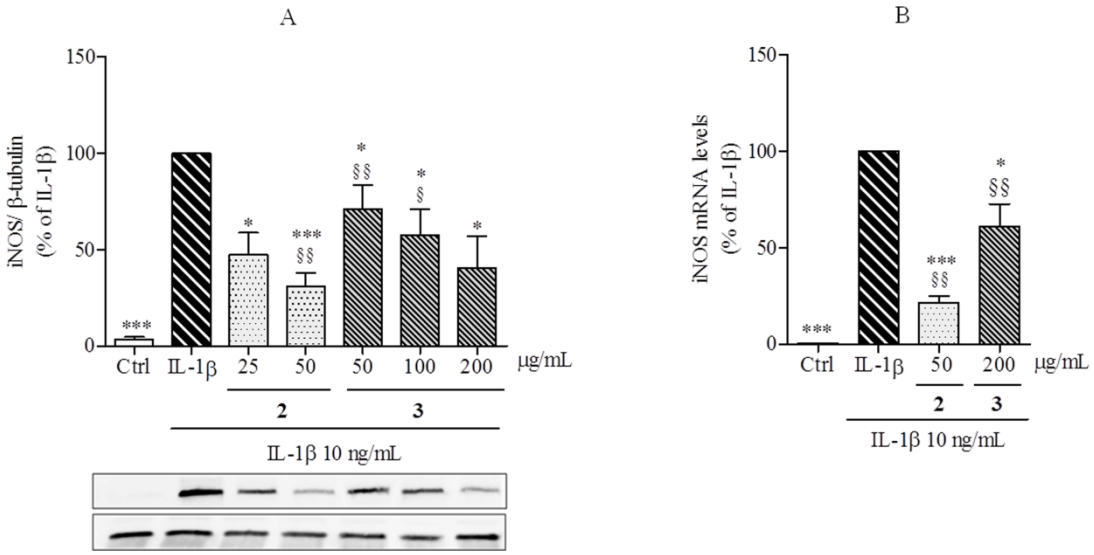


**Figure 7.2** Effect of myrcene (2) and limonene (3) on IL-1β-induced iNOS protein (A) and mRNA (B) levels in human chondrocytes left untreated (Control, Ctrl) or treated with IL-1-β, 10 ng/mL, for 24 h (A) or 6 h (B), following pre-treatment for 30 min with the indicated concentrations of the test compounds. Each column represents the mean ± SEM of, at least, four independent experiments. A representative image is shown. $^{*}p$ <0.05, $^{***}p$ <0.001 relative to IL-1β-treated cells; $^{\S}p$ <0.05, $^{\S\S}p$ <0.01 relative to Control cells.

To further elucidate the mechanisms by which the two compounds (**2** and **3**) inhibit iNOS expression and to evaluate their potential as anti-osteoarthritic drugs, their ability to inhibit IL-1β-induced NF-κB activation was determined. NF-κB activation requires the phosphorylation, ubiquitination and proteassomal degradation of its natural inhibitor, NF-κB Inhibitor-α (IκB-α), which, in unstimulated cells, retains NF-κB dimers in the cytoplasm. Once IκB-α is degraded, the freed NF-κB dimers translocate to the nucleus and bind to specific sequences in the promoter region of target genes promoting their transcription.[Hayden and Ghosh, 2008b] Thus, we evaluated the protein levels of phosphorylated and total IκB-α by western blot and the binding of the freed NF-κB dimers to a specific DNA sequence by ELISA.

Since human cartilage samples are scarce and a large number of cells is required, the ability of the test compounds to inhibit IL-1β-induced NF-κB-DNA binding was evaluated in the human chondrocytic cell line, C-28/I2, while the levels of phosphorylated and total IκBα were evaluated in primary human chondrocytes. The results show that treatment with IL-1β dramatically increased IκB-α phosphorylation

(Figure 7.3A) and decreased total IκB-α levels, reflecting its almost complete degradation (Figure 7.3B), followed by increased NF-κB-DNA binding (Figure 7.3C). Treatment with Bay 11-7082 (Bay), a specific NF-κB inhibitor that selectively prevents IκB-α phosphorylation, or with the test compounds markedly reduced IL-1β-induced IκB-α phosphorylation (Figure 7.3A) and degradation (Figure 7.3B), as well as NF-κB–DNA binding (Figure 7.3C). Interestingly, although the degree of inhibition of IκB-α phosphorylation and of NF-κB-DNA binding achieved with Bay was significantly higher than that obtained with myrcene (**2**) and limonene (**3**), IκB-α degradation was similarly decreased by the three compounds.

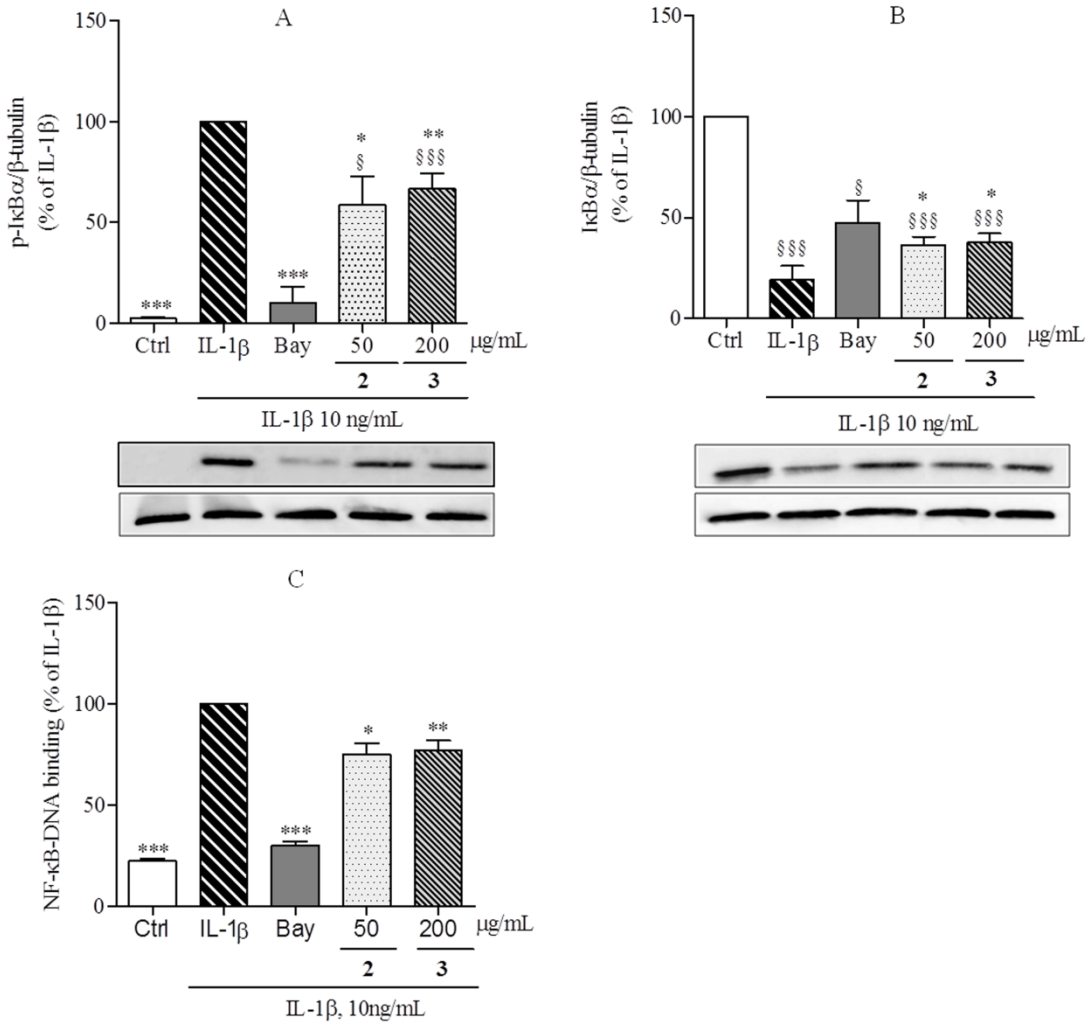


**Figure 7.3** Effect of myrcene (2) and limonene (3), on IL-1β-induced NF-κB activation, evaluated as the levels of phosphorylated (A) and total (B) IκB-α and NF-κB–DNA complexes (C). C28-I2 cells were left untreated (Control, Ctrl) or treated with IL-1β, 10 ng/mL, for 5 min (A) or 30 min (B and C) following pre-treatment with or without the test compounds or the specific NF-κB inhibitor, Bay 11-7082 (Bay, 5 µM). Each column represents the mean ± SEM of three to five independent experiments. $^{*}p$ <0.05, $^{**}p$

<0.01 $^{***}p$ <0.001 relative to IL-1β-treated cells; $^{§}p$ <0.05, $^{§§§}p$ <0.001 relative to Control cells.

Together with NF-κB activation, signalling pathways involving MAPK activation also play an important role in the proteolytic cartilage degradation process, namely in the expression of MMPs.[Mengshol et al., 2000] Thus, the ability of the test compounds (**2** and **3**) to inhibit IL-1-induced MAPK activation was assessed by evaluating their phosphorylation levels.

Myrcene (**2**) and limonene (**3**) showed quite distinct effects on IL-1β-induced JNK (Figure 7.4A), p38 (Figure 7.4B) and ERK1/2 (Figure 7.4C) phosphorylation. Myrcene (**2**) significantly reduced IL-1β-induced phosphorylation of the three MAPKs by approximately 33%, 26% and 26%, respectively, while limonene (**3**) effectively reduced the phosphorylation of p38 by near 39%, but increased that of ERK1/2 by 30% and had no significant effect on JNK phosphorylation.

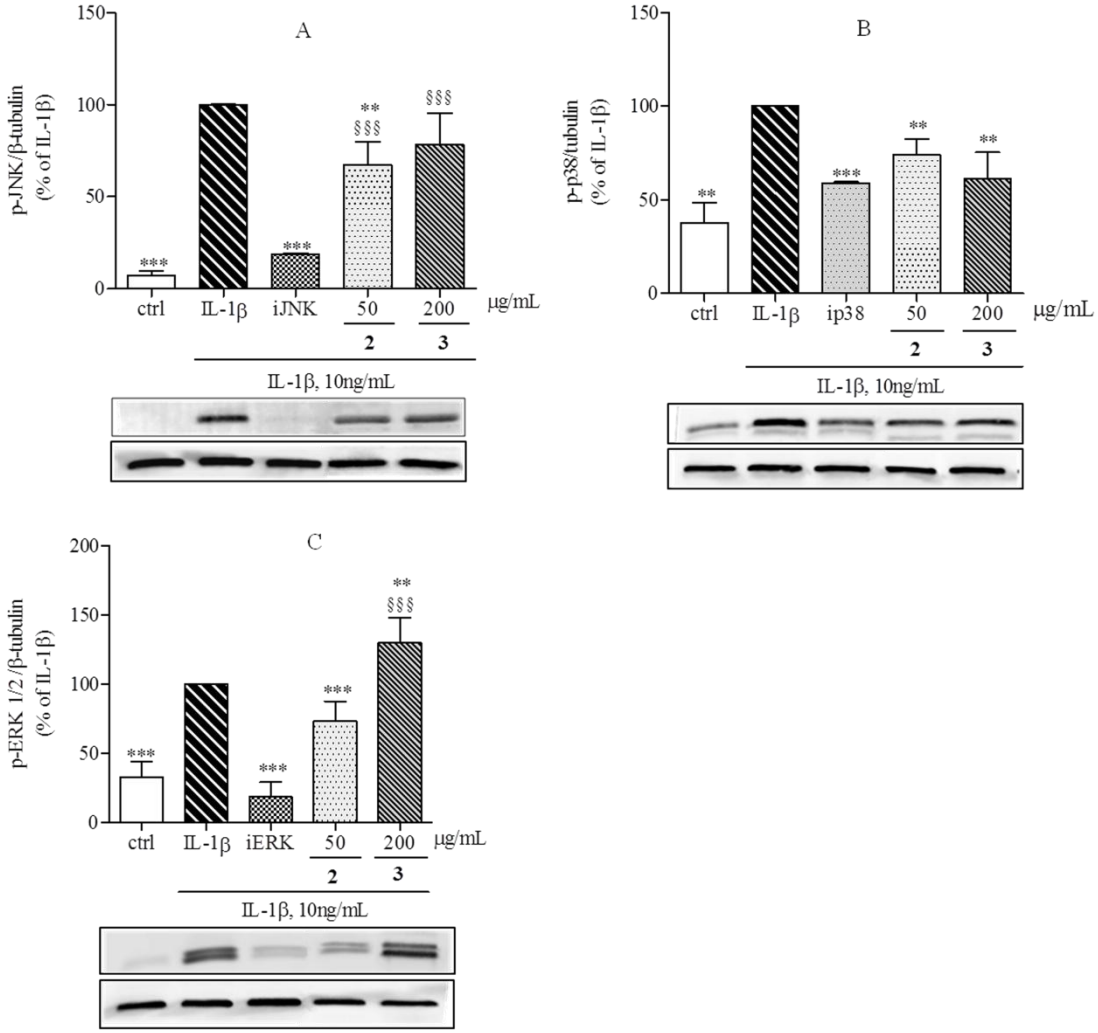

**Figure 7.4** Effects of myrcene (2) and limonene (3) on IL-1β- induced activation of JNK, p38 and ERK1/2 in human chondrocytes. Phosphorylated levels of JNK (A), p38 (B) and ERK (1/2) (C) were analyzed in total cell extracts of human chondrocytes left untreated (Control, Ctrl) or treated for 5 min with IL-1β, 10 ng/mL, following a pre-treatment for 30 min with the indicated concentrations of myrcene, limonene or a specific inhibitor of each MAPK. Each column represents the mean ± SEM of, at least, four independent experiments. Representative images are shown. $^{**}p$ <0.01, $^{***}p$ <0.001 relative to IL-1β-treated cells and $^{§§§}p$ <0.001 relative to Control cells. iJNK: SP600125 (20 μM); ip38: SB203580 (20 μM); iERK: U0126 (10 μM).

Reports on pharmacological properties of limonene (**2**) are scarce, but it has been shown to have antimicrobial properties [Bevilacqua et al., 2010] and anti-inflammatory effects in a mouse model of LPS-induced acute lung injury by suppressing MAPK and NF-κB pathways[Chi et al., 2012]. The results presented here only partially agree with this study, since limonene (**3**) inhibited NF-κB and p38 activation, but did not affect IL-1β-induced JNK and actually potentiated ERK1/2 activation, suggesting that this compound (**3**) has cell and/or stimulus specific effects. On the other hand, ERK1/2 is required for a number of cellular processes, including activation of *c-fos* expression which, among other functions, is involved in cell survival.[Karin et al., 1997; Shaulian and Karin, 2002] Whether increased activation of ERK1/2 by limonene (**3**) contributes to enhance chondrocyte survival was not addressed in this study, but is an interesting possibility to study further, as increased chondrocyte death is a relevant feature of OA.[Johnson et al., 2008] Nonetheless, since ERK1/2 has also been shown to inhibit proteoglycan synthesis and promote inflammatory responses in chondrocytes,[Scherle et al., 1997] the net effect resulting from its induction by limonene (**3**) is likely undesirable, compromising its potential utility as a therapeutic agent in OA.

Then, we evaluated the ability of the compounds tested (**2** and **3**) to counteract the effects of IL-1β on the expression of catabolic, anti-catabolic and extracellular matrix genes, which, at least in part, are mediated by NF-κB and the MAPKs.[Goldring and Otero, 2011] As expected, treatment of human chondrocytes with IL-1β (10 ng/mL) increased MMP-1 and -13 mRNA levels by nearly 9- and 5-fold, respectively (Figure 7.5A), while decreasing TIMP-1 and -3 expression (Figure 7.5B). Myrcene (**2**) decreased the mRNA levels of both MMPs by nearly 60%, relative to IL-1β, while limonene (**3**) reduced MMP-1 and -13 levels by 51% and 39%, respectively.

Nevertheless, the reduction of MMP-13 levels elicited by limonene (**3**) did not reach statistical significance.

On the other hand, limonene (**3**) did not significantly change the inhibitory effect of IL-1β on TIMP-1 and -3 mRNA levels, even though it showed a tendency to increase TIMP-1 levels that did not reach statistical significance. On the contrary, myrcene (**2**) not only completely reversed the inhibitory effect exerted by IL-1β, as it effectively increased TIMP-1 and -3 levels approximately 2- and 1.3-fold above those in untreated control cells, respectively, which correspond to even larger increases if compared to TIMP-1 and -3 mRNA levels in cells treated with IL-1β alone.

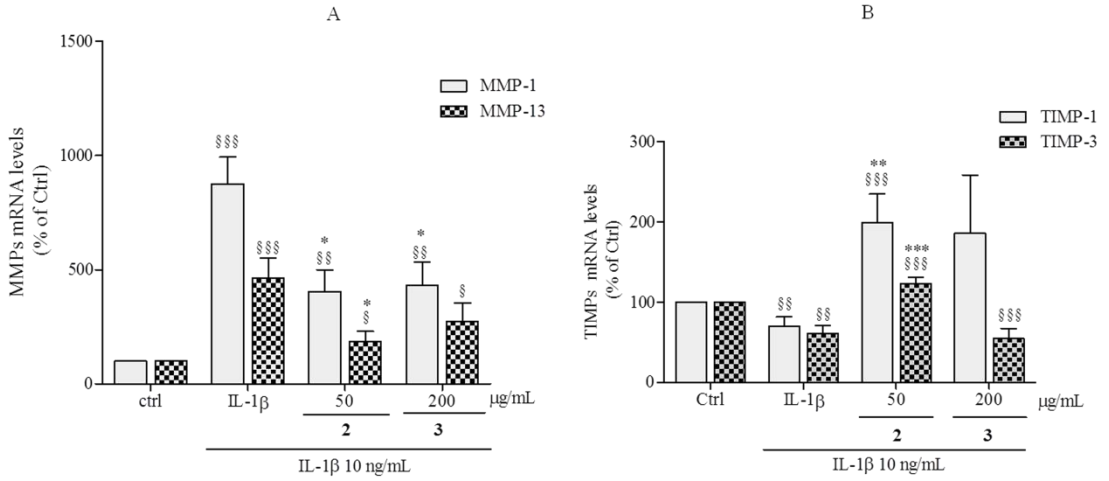


**Figure 7.5** Effect of myrcene (2) and limonene (3) on IL-1β-induced changes in the expression of catabolic and anti-catabolic genes. mRNA levels of MMP-1 and -13 (A) and TIMP-1 and -3 (B) were evaluated by RT-PCR. Each bar represents the mean ± SEM of, at least, four independent experiments in which human chondrocytes were left untreated (Control, Ctrl) or treated for 12 h (A) or 24 h (B) with IL-1β, 10 ng/mL, in the presence or absence the indicated compounds. $^{*}p$ <0.05, $^{**}p$ <0.01, $^{***}p$ <0.001 relative to IL-1β-treated cells and $^{\S}p$ <0.05, $^{\S\S}p$ <0.01, $^{\S\S\S}p$ <0.001 relative to Control cells.

To assess the potential ability of the test compounds to inhibit the deleterious effects of IL-1β in anabolic responses that are essential for repair of damaged articular cartilage, the expression of collagen II and aggrecan was evaluated. Furthermore, the ability of the test compounds to decrease the expression of the non-cartilage specific, collagen I gene, induced by IL-1β, was also evaluated. Chondrocyte treatment with 10 ng/mL IL-1β, for 24 h, significantly increased collagen I mRNA levels, while decreasing those of collagen II and aggrecan, relative to untreated control cells (Figure 7.6). Treatment of human chondrocytes with myrcene (**2**) or limonene (**3**) caused no significant changes

on collagen II (Figure 7.6A) and aggrecan (Figure 7.6B) mRNA levels compared to those in cells treated with IL-1β alone. Nonetheless, both treatments were able to completely abolish or even reverse the increase in collagen I mRNA levels induced by IL-1β.

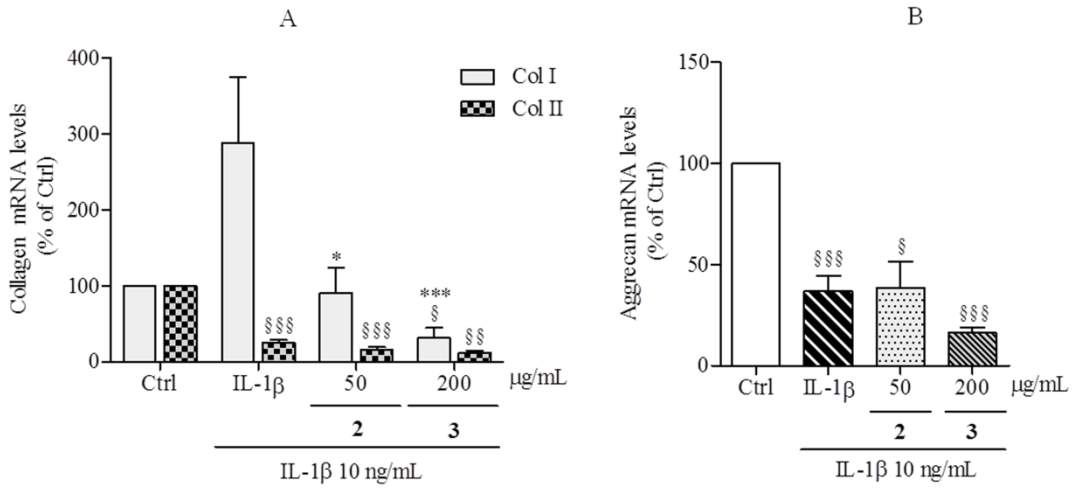


**Figure 7.6** Effect of myrcene (2) and limonene (3) on IL-1β-induced changes in the expression of extracellular matrix genes. mRNA levels of collagen I and collagen II (A) and aggrecan (B) were evaluated by RT-PCR. Each bar represents the mean ± SEM of, at least, four independent experiments in which human chondrocytes were left untreated (Control, Ctrl) or treated for 24 h with IL-1β, 10 ng/mL, in the presence or absence of the indicated compounds. $^{*}p$ <0.05, $^{***}p$ <0.001 relative to IL-1β-treated cells and $^{§§}p$ <0.01, $^{§§§}p$ <0.001 relative to Control cells.

Taken together, the results presented indicate that myrcene (**2**) is more effective than limonene (**3**) in preventing inflammatory and catabolic responses, namely iNOS expression and NO production, and MMP-1 and -13 expression, induced by IL-1β in human chondrocytes, likely reflecting, at least in part, the stronger inhibition of NF-κB and the three MAPKs. These findings are in agreement with another study that reported anti-inflammatory properties of myrcene (**2**) in a mouse model of pleurisy induced by zymosan and bacterial lipopolysaccharide where it inhibited the production of NO and inflammatory cytokines.[Souza et al., 2003] Moreover, myrcene (**2**), but not limonene (**3**), caused a net increase in the expression of the anti-catabolic genes, TIMP-1 and -3, which in combination with the decrease in MMP-1 and -13 expression can cause a significant reduction of the catabolic milieu characteristic of OA.

On the other hand, myrcene (**2**), unlike limonene (**3**), also completely prevented the increase in collagen I induced by IL-1β. Collagen I is not normally found in articular

cartilage and its expression is associated with chondrocyte dedifferentiation, a process that involves several alterations of chondrocyte gene expression and morphology and leads to the formation of fibrocartilage, increasing in OA.[Martin et al., 2001] Therefore, even though it did not increase the specific anabolic responses of human chondrocytes, myrcene (**2**) may be effective in preventing chondrocyte dedifferentiation associated with increased collagen I expression, while decreasing inflammatory and catabolic processes directly involved in cartilage destruction.

In comparison with (+)-α-pinene,[Rufino et al., 2014] myrcene (**2**) shows potential advantages as, besides inhibiting iNOS expression and activity and NF-κB and JNK activation to a similar extent, this compound can further decrease ERK1/2 and p38 activation and increase anti-catabolic responses, namely TIMP-1 and -3 expression, while promoting the maintenance of the differentiated chondrocyte phenotype, as it also decreased collagen I expression. Nonetheless, none of the compounds tested (**2** and **3**), nor (+)-α-pinene, seem effective in promoting the expression of articular cartilage matrix-specific genes.

In summary, the results presented suggest that myrcene (**2**) may be useful to halt or, at least, slow down cartilage destruction and, thus, OA progression, although it is unlike to promote the repair of areas already damaged.

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# CHAPTER 8.
# DISCUSSION AND CONCLUSION

## 8.1 GENERAL DISCUSSION

Research on osteoarthritis in the last few years have been focused in particular mechanisms underlying the course of the disease that can be related to particular risk factors or events. This is essential for a better understanding of the intrinsic mechanisms of specific phenotypes and, in that way, to enable the development of new drugs targeted to the deregulated molecular processes responsible for disease onset and progression that can prevent or halt OA and, consequently, reduce its individual and socio-economic costs.

The association between Diabetes and the development and progression of OA is increasingly clear. Independently of the glucose requirements for the plastic and metabolic needs of chondrocytes, hyperglycemia has been shown to induce deleterious effects in human chondrocytes. In diabetes, especially in uncontrolled type 2 diabetes, chronic hyperglycemia is the major hallmark and has been shown to directly lead to diabetic complications, including those with inflammatory characteristics as retinopathy, atherosclerosis or β-cell deterioration. The inflammatory process also plays a central role in OA pathogenesis since the deregulated pro-inflammatory mediators and, in particular, cytokine activities likely contribute to the disruption of the balance between anabolic and catabolic processes of chondrocytes that lead to the joint destruction characteristic of OA.

In this context, this work aimed to contribute to elucidate how extracellular glucose levels are sensed by human chondrocytes, in particular by determining the role of potassium channels in this process, and to further understand the mechanisms by which hyperglycemia induces cartilage damage, unraveling in that way possible pathological mechanisms and potential pharmacological targets of DM-associated OA. Moreover, molecules, from plant origin were studied to assess their anti-inflammatory and chondroprotective activities that can be explored for development of new drugs with disease-modifying osteoarthritis properties.

In general, the results presented in chapters 3 and 4 show that:

- Human chondrocytes of any OA grade present heterogeneous K(ATP) channels, with Kir6.2 and SUR2B being the major subunits expressed.
- The activity of K(ATP) channels influences the abundance of the major glucose transporters, GLUT-1 and GLUT-3, in normal chondrocytes, but did not affected

glucose transport, suggesting that these channels participate in a glucose sensing apparatus that regulates GLUT levels which, by themselves, are not sufficient to adjust glucose transport capacity.

- Chondrocytes of increasing OA grade are less responsive to changes in K(ATP) channel activity.
- Exposure of human chondrocytes to high, hyperglycemia-like glucose concentrations induces the expression of pro-inflammatory mediators, namely IL-1β, TNF-α and iNOS, accompanied by increased NO production, in a mechanism, at least partially, mediated by NF-κB.
- Insulin, not only does not prevent the pro-inflammatory effects of hyperglycemia in human chondrocytes, as, in supraphysiologic concentrations, it can induce NF-κB activation by itself

Finally, the results presented in chapters 5, 6 and 7 show that:

- The essential oils of *Lavandula luisieri* and *Eryngium duriaei* subsp. *juresianum* display important anti-inflammatory properties, namely, significant inhibition of IL-1β induced iNOS expression, NO production and NF-κB activation in human chondrocytes. Likewise, these essential oils may be of great value in the development of new therapies or as sources of active compounds for the treatment of chronic inflammatory diseases, like OA.
- (+)-α-pinene, showed the most potent chondroprotective and anti-inflammatory activities among its isomers, enantiomers and other naturally occurring compounds derived from the pinane nucleus, by significantly inhibiting IL-1β-induced inflammatory and catabolic pathways, namely, NF-κB and JNK activation and the expression of inflammatory (iNOS) and catabolic (MMP-1 and -13) genes.
- Limonene and especially Myrcene, natural compounds found in the essential oils of *Eryngium duriaei* subsp *juresianum* and *Lavandula luisieri*, showed interesting anti-inflammatory and anti-catabolic properties, namely inhibition of IL-1β-induced expression of pro-inflammatory and catabolic mediators, namely NO, iNOS, MMP-1 and -13, and pathways as NF-κB and p38 MAPK.
- Myrcene further inhibits the phosphorylation of JNK and ERK MAPKs which possibly contributes to the inhibition of pro-inflammatory, pro-catabolic and chondrocyte dedifferentiation genes, namely the expression of collagen type I, as well as to relieve the inhibition of TIMP-1 and -3 expression caused by IL-1β.

Considering the objectives proposed, the findings that either hyperglycemia or hyperinsulinemia, as well as the two combined, activate NF-κB, the major pro-inflammatory signalling pathway, are by themselves main mechanisms that can shift the chondrocyte homeostasis towards the catabolic program leading to OA development and/or progression. Among the specific molecular events that may underlie this response, the results obtained demonstrate that K(ATP) channels are involved in glucose sensing, playing a role in the regulation of glucose transporter availability in human chondrocytes which, nonetheless, is not sufficient to adjust glucose transport in response to changes in its extracellular concentration. Thus, the observed lack of response of chondrocytes of increasing age/OA grade to modulators of K(ATP) channel activity, namely glibenclamide, a sulfonylurea drug used in DM 2 therapy as an insulin secretagogue, implies that in patients under this kind of therapy, chondrocytes may still be susceptible to the deleterious effects caused not only by hyperglycemia episodes, even occurring sporadically, as by hyperinsulinemia. Nonetheless, glibenclamide has also been shown to decrease high glucose-induced IL-1β production in pancreatic β cells [Zhou et al., 2010] which, if also occurring in chondrocytes, may counteract the pro-inflammatory effects of hyperglycemia and hyperinsulinemia. More studies are clearly required to elucidate the effects of anti-diabetic drugs in human chondrocytes, which will contribute to fully assess their risk/benefit and inform the rationale therapy of both conditions.

On the other hand and even though the specific molecular events were not elucidated in this work, several other mechanisms may regulate glucose transport and metabolism and mediate the effects of hyperglycemia in chondrocytes. Such mechanisms deserve to be further investigated as they may disclose relevant targets both for prevention and treatment of DM-associated OA. Among those potential mechanisms, AMP-activated protein kinase (AMPK) and Silent Information Regulator 2 type 1 or sirtuin 1 (SirT1) may play important roles.

AMPK is a metabolic sensor in a wide variety of cells that becomes activated during metabolic stress. At least in pancreatic β cells, K(ATP) channels and AMPK cooperate to regulate the energetic status [Lim et al., 2009]. Moreover, AMPK also seems to play an important role in regulating the mechanisms involved in glucose transport in response to hyper or hypoglycemic conditions [Lim et al., 2009; McCrimmon et al., 2004; Ning et al., 2011]. Activation of AMPK has been shown to increase glucose transport in various cells by promoting the recruitment of GLUT-4 [Li et al., 2004] or GLUT-1 [Xi et al., 2001] to the plasma membrane. AMPK is expressed and constitutively active in normal, but is decreased in human OA knee articular

chondrocytes and cartilage [Terkeltaub et al., 2011]. So, it is possible that AMPK participates in the glucose sensing apparatus in chondrocytes that also includes K(ATP) channels, regulating, at least in part, GLUT trafficking to the plasma membrane and glucose uptake. Further studies will be necessary to elucidate the role of AMPK in the regulation of glucose transport in chondrocytes in normal and osteoarthritic conditions and whether modulation of its activity can have beneficial effects on chondrocyte functions under hyperglycemia.

SirT1 is a deacetylase that fine tunes many cellular biochemical processes through its capacity to interact and modify various histone and non-histone proteins, playing important roles in the coordination of the response to low calorie intake and in cellular survival. On the other hand, under high glucose conditions, SirT1 expression and activity are decreased in many cells, contributing to cell senescence and dysfunction [Ceolotto et al., 2014; Servillo et al., 2013]. Accordingly, SirT1 has been associated with metabolic and aging-related diseases, including DM 2 [Donath, 2014] and OA [Dvir-Ginzberg and Steinmeyer, 2013]. SirT1 has been shown to promote cartilage extracellular matrix synthesis [Dvir-Ginzberg et al., 2011] and chondrocyte survival [Gabay et al., 2012], even under pro-inflammatory stress conditions. Furthermore, SirT1 has been associated with anti-inflammatory effects induced by various stimuli, including high glucose, in many cells through its ability to inhibit NF-κB [Kauppinen et al., 2013; Liu and McCall, 2013]. Recent studies showed that the same mechanism is also relevant in cell and animal models of OA [Lim et al., 2012] and inflammatory arthritis [Wang et al., 2014]. Inhibition of NF-κB by SirT1 seems to occur through, at least, two distinct mechanisms. The first is deacetylation of the p65 subunit of NF-kB directly by active SirT1 while the second involves activation of other intermediates, namely AMPK, PPAR-α and PGC-1α, which stimulate oxidative energy production and also inhibit NF-κB activity [Kauppinen et al., 2013]. Thus, by impairing SirT1 expression and activity, high glucose can induce NF-κB and the subsequent expression of pro-inflammatory genes, like IL-1β and TNF-α, as we observed. Nonetheless, other mechanisms are also likely involved as SirT1 impairment may not be sufficient to explain the inability of OA/aged chondrocytes to downregulate glucose transport and prevent its intracellular accumulation when exposed to hyperglycemia-like conditions. Nonetheless, the role of SirT1 and its downstream effectors, namely AMPK, in the adaptation of chondrocytes to high glucose deserves to be further investigated as it can disclose an important therapeutic target for DM-associated OA.

From another point of view, our results show that the inflammatory response induced by high glucose in human chondrocytes is not distinct from that induced by other

stimuli, from ageing of chondrocytes to abnormal mechanical forces [Goldring and Otero, 2011], that play significant roles in OA pathophysiology. These triggering factors not only contribute to irreversible joint damage (progression), but importantly, also to the initiation/onset phase, wherein the homeostatic balance between anabolic and catabolic functions becomes disrupted. Our work thus points to pro-inflammatory cytokines, namely IL-1β and TNF-α, as common mediators of DM-associated OA.

Given their central role in OA development and progression, these mediators and the signalling pathways that they initiate are likely main targets for the development of new drugs with disease-modifying properties for OA treatment. Therefore, we used IL-1β to imitate those processes *in vitro*, so as to evaluate the anti-inflammatory, anti-catabolic and pro-anabolic effects of compounds of plant origin.

The initial screening assays showed that essential oils have interesting anti-inflammatory effects and thus can be important sources of compounds with anti-osteoarthritic properties. Among the essential oils tested, those of *Lavandula luisieri* and *Eryngium duriaei* subsp. *juresianum* presented the best properties, reason why they were further studied. Screening of fractions of these essential oils containing distinct families of compounds showed that in both cases, the most active fractions are composed of mono- and sesquiterpene hydrocarbons, respectively, while fractions containing oxygenated mono- and sesquiterpenes were less or completely inactive. Finally, the screening of some commercially available compounds found in the active fractions, allowed to identify myrcene and limonene as potential anti-inflammatory and anti-catabolic compounds in human chondrocytes. Also (+)-α-pinene was shown to have anti-inflammatory properties in human chondrocytes being the active enantiomer of the α-pinene racemic mixture, previously presented by our group as showing anti-inflammatory properties in this cells [Neves et al., 2009].

Among those compounds, myrcene showed to be the most promising as a lead candidate for further development of new drugs that can halt OA progression. Even though the 3 compounds showed ability to significantly reduce some of the major pro-inflammatory and pro-catabolic mediators (NO, iNOS, MMP-1 and MMP-13) and pathways (NF-κB) involved in the pro-inflammatory stress-related responses of chondrocytes that contribute to OA onset and progression, myrcene was additionally able to increase the anti-catabolic expression of the MMP inhibitors, TIMP-1 and -3, and to further decrease the activation of the MAPK pathways (p38 and ERK1/2 in relation to (+)α-pinene and JNK and ERK1/2 in relation to limonene)deeply involved in the expression of catabolic mediators.

Moreover, even though none of the compounds seemed to be effective in restoring the synthesis of ECM components, myrcene may contribute to the maintenance of the differentiated chondrocyte phenotype and to prevent the formation of fibrocartilage by decreasing the expression of type I collagen. Further studies are needed to determine if the effects observed result from the action of those compounds on a common single molecular target upstream of all the signalling pathways evaluated or, on the other hand, if they result from the combined action on various components of those signalling pathways. Moreover, it will also be interesting to understand whether these compounds, belonging to the same broad class of organic compounds, the terpenes, act on the same molecular target or not. Future work can also involve the evaluation of the observed myrcene properties *in vivo* using appropriate animal models of OA, as the model induced by intra-articular injection of mono-iodoacetate in rats or the surgical induction by transecting the anterior cruciate ligament [Bendele, 2001].

Furthermore, to establish a proof of concept for the efficacy of myrcene in Diabetes-associated OA, it would also be interesting to induce OA in a model of non-obese type 2 diabetes, as the Goto-Kakizaki rat model which presents mild hyperglycemia, insulin resistance and hyperinsulinemia [Akash et al., 2013], and evaluate the ability of the compound to prevent OA development and/or to stop its progression.

In summary, a comprehensive view of the results suggests that if, on one hand, altered glucose sensing mechanisms and high glucose exposure lead to an increased expression of inflammatory mediators and pathways in chondrocytes, establishing a new link between DM and OA and supporting the existence of a DM-induced OA phenotype, on the other hand, those inflammatory and catabolic mediators and signalling pathways are major targets for the development of new drugs with disease-modifying OA properties, myrcene being an interesting lead compound for that purpose.

## CHAPTER 9.

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