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Handling Inflammation in Cartilage Lesions: The Chondroprotective Effects of Ibuprofen Loaded PLGA Nanoparticles

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Abstract

Cartilage lesions are one of the leading causes of disability worldwide. Since articular cartilage is a complex, and avascular connective tissue, almost incapable of self-renewal after injury, untreated defects tend to expand and can evolve towards osteoarthritis. The damage of cartilage triggers an inflammatory response that disturbs the balance between anabolic and catabolic factors, leading to the degradation of the articular cartilage. The control of the established proinflammatory environment presents a promising strategy to inhibit ECM destruction, and promote cartilage repair. Several anti-inflammatory drugs have been successfully tested in cartilage lesions, leading to a decrease in the inflammation at defect site and, subsequently, to a decrease in the ECM degradation.

Nowadays, the treatments available in the clinic, such as abrasion arthroscopy or osteochondral autograft transplantation, are very invasive and ineffective procedures that lead to the formation of a fibrocartilaginous tissue with mechanical properties different from the native tissue. Additionally, their efficacy is limited, since after a short period of time symptoms, like pain or sweeling, reappear. Therefore, novel and innovative therapeutic approaches to effectively treat cartilage lesions are deeply needed.

In this study we propose the use of ibuprofen loaded Poly-Lactic-co-Glycolic Acid (PLGA) nanoparticles as a strategy to locally deliver an anti-inflammatory treatment to the damaged cartilage. Specifically, the aim of this work was to evaluate the chondroprotective effects of the ibuprofen loaded PLGA nanoparticles in 3D cultures of human chondrocytes under inflammatory conditions. For this purpose, human chondrocytes were cultured as 3D pellets, submitted to a proinflammatory condition and to a treatment with the ibuprofen loaded PLGA nanoparticles.

As a first step, to set the inflammatory environment, two different cytokines were tested in different concentrations, 10 and 100ng/mL of TNFα and/or IL1β. Based on the results obtained concerning the effects on chondrocytes proinflammatory response and matrix degradation, 100ng/mL of IL1β was chosen as the more reliable proinflammatory condition and was used in the subsequent experiments. The obtained results showed that this condition did not induce cytotoxicity and triggered the greatest increase in the synthesis of other proinflammatory cytokines. Furthermore, it induced higher extracellular matrix (ECM) degradation.

Regarding the effects of the PLGA nanoparticles loaded with 15 and 30μg/mL of ibuprofen on the activity of chondrocytes cultured under proinflammatory conditions, the obtained results indicate a decrease in the levels of the proinflammatory cytokine IL1β, and in the degradation of collagen type II and aggrecan (important components of cartilage ECM). It is also suggested a decrease in the synthesis of matrix metalloproteinases (MMPs).

Overall, the obtained data suggest that treatment with ibuprofen loaded PLGA nanoparticles diminishes chondrocytes' proinflammatory response and ECM degradation activity induced in an inflammatory situation. Therefore, this is a promising strategy to overcome the catabolic effects triggered by the inflammatory response caused by a cartilage injury.

Further experiments will allow to conclusively establish the chondroprotective effects of ibuprofen loaded PLGA nanoparticles.

Resumo

A cartilagem articular é um tecido conjuntivo complexo e avascular, com uma baixa capacidade de autorrenovação após uma lesão. As lesões da cartilagem provocam uma resposta inflamatória por parte do organismo, que irá perturbar o equilíbrio homeostático entre anabolismo e catabolismo, levando degradação do tecido. Assim, é importante controlar o ambiente inflamatório que se estabelece após uma lesão, de forma a impedir a destruição da matriz extracelular e a promover a reparação da cartilagem. Vários fármacos anti-inflamatórios têm sido testados e os resultados obtidos mostram a sua eficácia na redução da inflamação e na consequente redução da degradação da matriz extracelular da cartilagem.

Atualmente, o tratamento de lesões da cartilagem articular requer procedimentos muito invasivos e ineficazes, que em muitas situações levam à formação de um tecido fibrocartilaginoso com propriedades mecânicas diferentes do tecido nativo. Além disso, a eficácia destes tratamentos é limitada, uma vez que, após um curto período de tempo, sintomas como dor e inchaço reaparecem na articulação. Por conseguinte, é extremamente necessário que se desenvolvam novas abordagens terapêuticas para tratar eficazmente as lesões da cartilagem.

Neste estudo propomos o uso de nanopartículas de Ácido Poliláctico-co-Glicólico (PLGA) carregadas com ibuprofeno como forma de fornecer localmente um tratamento anti-inflamatório. Este trabalho tinha como objetivo avaliar o efeito condroprotetor das nanopartículas de PLGA carregadas com ibuprofeno, em culturas 3D de condrócitos humanos, sujeitas a condições inflamatórias.

Em primeiro lugar, e no sentido de definir as condições necessárias para induzir um ambiente inflamatório, foram testadas citocinas em duas concentrações, 10 e 100ng/mL de TNFα e/ou IL1ß. Os resultados obtidos permitiram definir a condição 100ng/mL de IL1ß, como sendo a que definiu um ambiente inflamatório adequado. Esta condição não induziu citotoxicidade, e desencadeou um maior aumento na síntese de outras citocinas pró-inflamatórias e na degradação da matriz extracelular.

Relativamente ao efeito das nanopartículas de PLGA carregadas com 15 e 30µg/mL de ibuprofeno na atividade dos condrócitos cultivados em condições inflamatórias, os resultados obtidos indicam uma diminuição dos níveis da citocina pro-inflamatória IL1ß, da degradação do colagénio tipo II e do agrecano (componentes fundamentais da matriz extracelular da cartilagem), e sugerem uma possível diminuição da síntese de metaloproteínases de matriz.

No geral, os resultados obtidos sugerem que o tratamento com as nanopartículas de PLGA carregadas com ibuprofeno, numa situação inflamatória, diminui a resposta pró-inflamatória dos condrócitos e a degradação da matriz extracelular. Por conseguinte, esta é uma estratégia

promissora para superar os efeitos catabólicos causados pela inflamação após uma lesão da cartilagem.

O aumento do número de experiências irá permitir estabelecer definitivamente os efeitos condroprotetores das nanopartículas de PLGA carregadas com ibuprofeno.

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Abbreviations, Acronyms, and Symbols

List of abbreviations and acronyms (sorted alphabetically)

ACI	Autologous chondrocyte implantation
ADAMTS	Disintegrin and metalloproteinase with thrombospondin motifs
AR	Aspect Ratio
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
COX2	Cyclooxygenase enzyme 2
CSPC	Chondrocyte stem/progenitor cells
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extracellular matrix
ESC	Embryonic stem cells
FBS	Fetal bovine serum
FDA	Food and Drug Administration
PCL	Polycaprolactone
GAGs	Glycosaminoglycans
iPSC	Induce pluripotent stem cells
IL1β	Interleukin 1 beta
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-17	Interleukin-17
IL-18	Interleukin-18
ITS	Insulin/Transferrin/Selenium
LDH	Lactate Dehydrogenase
MACI	Matrix-induced autologous chondrocyte implantation
MMPs	matrix metalloproteinases
MSC	Mesenchymal stem cells
NO	Nitric oxide

OA	Osteoarthritis
PCL	Poly(ε-caprolactone)
PEG	poly(ethylene glycol)
PGE_2	Prostaglandin E2
PLA	Poly(L-lactic acid)
PLGA	Poly(lactic-co-glycolic acid)
P/S	Penicillin/Streptomycin
qRT-PCR	Quantitative real-time polymerase chain reaction
TNFα	Tumor necrosis factor alpha
TGF-β1	Transforming growth factor-beta 1
TGF-β3	Transforming growth factor-beta 3

Chapter 1

1. Introduction

Synovial joints, also known as diarthrosis, are the most common joint in the body, allowing most body movements [1]. They are composed of a fluid-filled joint cavity in which the articulating surfaces of the adjacent bones contact with each other [2]. These surfaces are covered with articular cartilage, allowing the bones to move gently against each other and enabling the bearing and transmission of loads between adjacent bones [3]. Articular cartilage is a complex, flexible, and avascular connective tissue, almost incapable of self-renewal after damage [4]. Damages can occur after trauma, such as falling onto the knee, deceleration injuries that happen when someone jumps, falls, or quick changes direction due to aging or induced by disease [5]. Because of cartilage low self-healing capacity, untreated defects tend to expand and can evolve towards osteoarthritis (OA) [6].

Osteoarthritis, a degenerative condition associated with the articular cartilage [6], is the most prevalent form of musculoskeletal disease worldwide [7]. OA affects people's physical and mental health, presenting a high personal and socio-economic impact [7]. It is a progressive disease that affects mostly joints, such as the knee, spine, hip, and hand [8] [9]. Several risk factors have been identified [10]. Among them, the increase in life expectancy, genetic predisposition, obesity, and joint disorders have been highlighted [10]. Genetic factors include polymorphisms and mutations in genes that encode matrix components and signaling molecules, contributing to OA susceptibility [8]. The aging population and the increase in obesity are anticipated to contribute significantly to the rise of the OA prevalence in the coming years [8]. The main clinical symptoms of OA are swelling, stiffness, and crackling of the affected joint [6]. These symptoms are translated into pain and are caused by joint cartilage destruction, subchondral bone alterations, and synovitis [8].

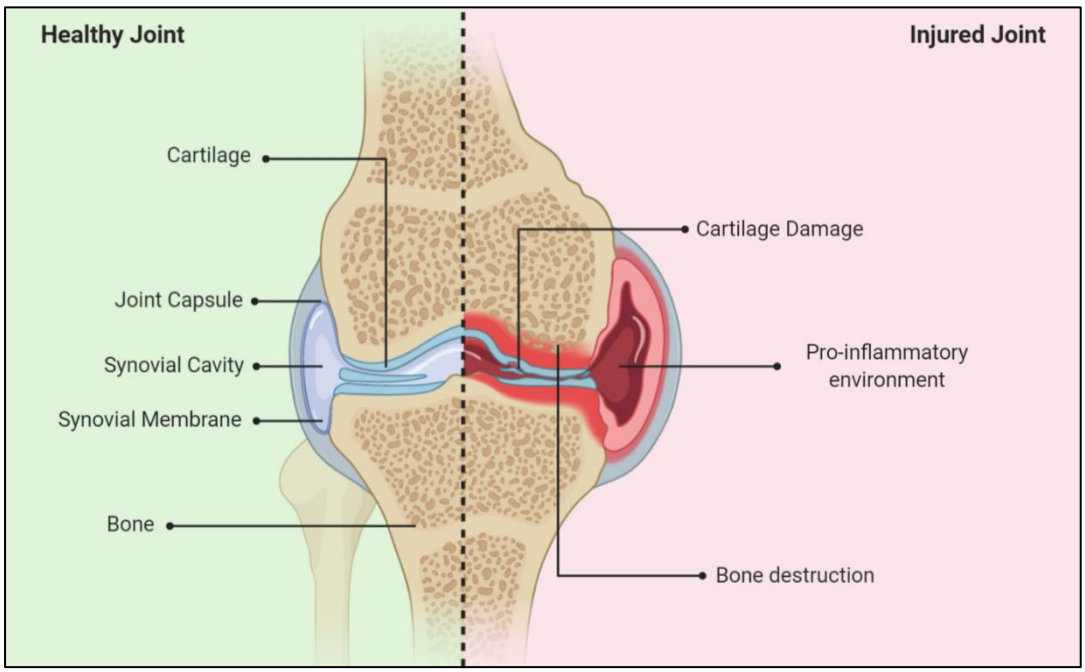

Figure 1.1 - Differences between healthy and injured joints: in healthy situations, tissue homeostasis is maintained. However, after an injury, there is the destruction of articular cartilage and bone erosion, due to the proinflammatory environment surrounding the tissue. This inflammatory environment, also known as synovitis, will increase cartilage turnover and matrix destruction [8].

The currently available approaches to treat OA are palliative and do not avoid further joint deterioration, leading to a situation where most patients need a total joint arthroplasty [11]. Due to its irreversible nature, this procedure is recommended only in patients over 60 years of age and for whom other modalities of treatment have failed or are contraindicated [12].

The surgical techniques to promote cartilage repair in current use, such as abrasion arthroscopy or osteochondral autograft transplantation, are very invasive and ineffective procedures, with minimal capacity to induce tissue regeneration, producing only a short-term relief of symptoms [11], [13]. An utterly successful solution is still not found, and novel and effective therapies are of upmost necessity.

The advances in materials science are pushing tissue engineering to the vanguard of cartilage repair procedures [14]. Its main tools are scaffolds, cells, biochemical and biomechanical stimuli [15]. Nevertheless, cost-effective design methods are not yet in place to ensure the viability of the novel products [15].

Currently, the ultimate goal is to develop approaches based on biomaterials that will allow cell and/or drug (such as growth factors, cytokines, or signalling factors) delivery to the defect site, promoting tissue repair, relieving patients pain, and restoring joint mobility [16].

1.1 – Cartilage

1.1.1 – Cartilage Tissue

Cartilage is a connective tissue composed of a single cell type, the chondrocytes, (which differentiate from mesenchymal stem cells (MSC), from the bone marrow [17]), embedded within a dense extracellular matrix (ECM) [16]. It is characterized by having no blood or lymphatic vessels, no nerve fibres, and for being a flexible connective tissue with a low self-renewal ability after injury [18] or disease [19]. Its composition evolves throughout human growth: the cartilage generated throughout the embryonic development has a high cell-matrix ratio, when compared with adult cartilage (that only has 2% of cells) [20]. Moreover, cartilage matrix composition and biomechanical properties depend on its location [21].

1.1.1.1 – Chondrocytes

Chondrocytes are characterized by their unique ability to produce matrix and highly developed cytoskeleton, composed of a large number of actin filaments [21]. The actin filaments contribute not only to the biomechanical properties of chondrocytes, but also to connect cells to the ECM [21]. The interactions with the ECM are fundamental for the development and maintenance of cartilage. Chondrocytes are responsible for maintaining and remodelling the ECM by secreting both the components that make up the matrix, like collagen type II or aggrecan, and the enzymes that degrade it, such as matrix metalloproteinases (MMPs), and disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) [17], [22].

Chondrocytes have a small proliferative capacity and low ability to migrate to injured areas [23]. Consequently, cartilage is almost incapable of repairing itself [23]. Due to the avascular condition of cartilage, chondrocytes obtain nutrients mostly through diffusion from the synovial fluid (rich in proteins derived from the blood plasma) and from the joint tissues [4].

1.1.1.2 – Cartilage Extracellular Matrix

Cartilage ECM is composed by a complex of macromolecules such as collagens (types II, VI, IX, X, XI), hyaluronan, proteoglycans [24], and other smaller amounts of several different classes of molecules, like lipids, phospholipids, and glycoproteins [17]. Water is the most abundant component of the ECM, located in the intrafibrillar space within collagen, and in the pore space of the matrix [25]. The water flow through cartilage facilitates the transportation and distribution of nutrients to chondrocytes [25].

1.2 – Articular Cartilage

There are three types of cartilage that present different structures, elasticity, and strength: i) the elastic, ii) the fibrous, and iii) the hyaline [26]. The elastic cartilage has a yellowish colour and is commonly found in the larynx, ear, epiglottis, and eustachian tube [26]. Its matrix is composed mainly by a dense network of elastin fibres [26]. It is surrounded by a layer similar to a perichondrium, and it is resistant to pressure, providing a certain flexibility to the tissue [26]. The fibrous cartilage is composed of a large amount of type I collagen and a small concentration of proteoglycans, being resistant to high tension and compression rates [26]. It is located on tendons, ligaments, intervertebral discs, and meniscus [26]. Unlike elastic cartilage, it has no perichondrium [26].

Hyaline cartilage is the most abundant cartilaginous tissue in the body, covering the articulating ends of bones, serving as a lubricated, wear-resistant, and friction-reducing surface, which is slightly compressible to distribute forces evenly into the bone [17], [27]. Articular cartilage is a specialized form of hyaline cartilage [26]. Before birth and in the early stages of life, articular cartilage is a compact tissue with a high percentage of cells and poor in matrix [28]. After birth, articular cartilage grows in thickness, chondrocytes grow in size, and the ECM becomes abundant [28].

The articular cartilage can be found in the synovial joints that allow the movement of articulating bones [2]. These joints are considered a solid organ composed of articular cartilage, synovium perichondrium, and subchondral bone, as illustrated in figure 1.1 [2]. Healthy articular cartilage is known to support intensive physical stress. However, the continuous use of the joint leads to a progressive loss of joint function and a possible chronic degeneration [2].

Although the cartilage tissue is composed only by one type of cells, its morphology differs from layer to layer of the articular cartilage [29], depending on the shape of chondrocytes, ECM composition, and type II collagen fibers orientation [30]. Typically, it is divided into four zones, as illustrated in figure 1.2. The superficial zone is the thinnest layer, containing flattened chondroprogenitor cells, and is in contact with the synovial fluid [29], [30]. The collagen fibres are parallel to the surface of the joint [30]. The middle or transition zone is the thickest layer and contains more rounded chondrocytes. The collagen fibres are randomly organized [29], [30]. The deep or radial zone contains spherical chondrocytes, and collagen fibres are perpendicular to the joint [30]. Lastly, the calcified zone is in contact with the subchondral bone by anchoring the collagen fibres of the deep zone [29]. Chondrocytes are scarce and hypertrophic [25].

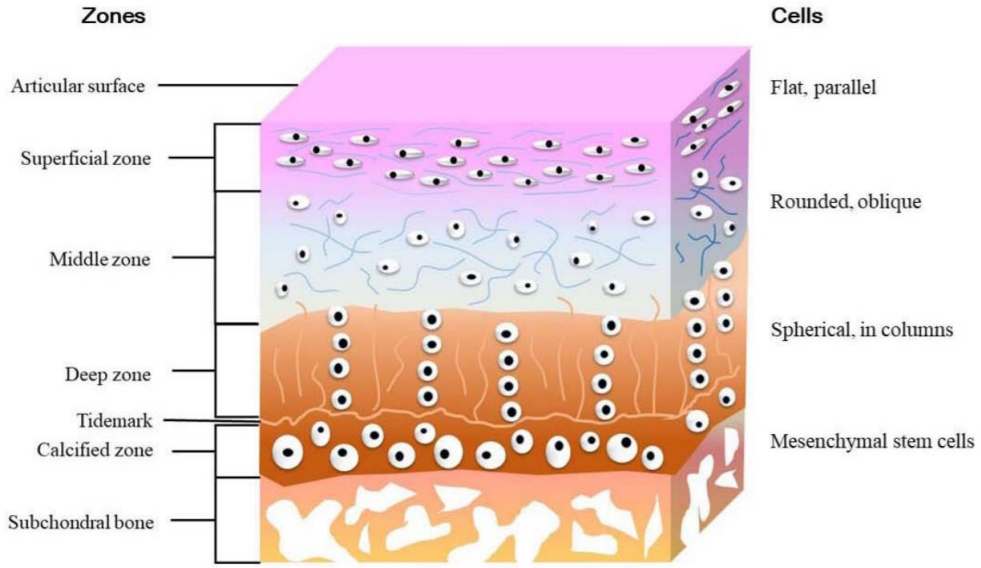

Figure 1.2 - Structure of mature articular cartilage zones [31].

Collagen type II is the main collagen present in the articular cartilage matrix [16]. It can be found in two different variants, IIa and IIb [16]. Of these two, IIb is ranked as a highly differentiated form, specific to articular cartilage [16]. Since collagen turnover is a very slow process, the increase in its synthesis indicates an unbalance in tissue homeostasis [21]. Collagen fibre's single structure makes this macromolecule insoluble and resistant to degrading enzymes [16]. Furthermore, their horizontal orientation makes cartilage very resistant and capable of absorbing shear forces produced by joint movement [16]. Proteoglycans fill the majority of the extracellular interstitial space and are composed of glycosaminoglycans (GAGs) chains covalently linked to a specific protein core [32]. Their function is to absorb shocks, while collagens are resistant to shear stress, provide structure and tensile strength [16]. The main proteoglycan in cartilage is aggrecan, which forms large aggregates with hyaluronan that can hold water in the tissue, contributing to its hydration [17].

1.3 – Cartilage Lesions

Damage to the articular cartilage is a common occurrence caused by several factors, including trauma and deterioration by disease and aging, leading to joint pain, loss of mobility, and progressive joint destruction [19].

Acute or repetitive trauma to the joint might damage the articular cartilage, leading to the development of isolated defects [20]. These defects can be superficial or can widen up until the subchondral bone [33]. Superficial defects are usually not associated with clinical complications. However, if not adequately treated, they may be the first step in the progression to a more severe injury [33]. Articular cartilage lesions can be classified as (i) chondral lesions, injuries that occur in the superficial/middle zone, or (ii) osteochondral lesions; in this category,

lesions can be limited to the subchondral bone cortical plate or can go deeper into the bone marrow [34].

When the biomechanical properties surrounding the injured area are affected, the remaining chondrocytes will be subjected to new mechanical forces, resulting in their death or apoptosis [20].

Osteoarthritis is a progressive disease that affects mostly joints, such as the knee, spine, hip, and hand [8], [9].

Among the common symptoms, joint swelling, stiffness, and crackling [6], pain is the most common disturbing symptom caused by joint cartilage destruction, subchondral bone alterations, and synovitis [8]. Synovial inflammation, also known as synovitis, can trigger a cascade of events driven by inflammatory mediators that increase cartilage turnover and matrix degradation [8]. Angiogenesis is also described to contribute to OA, specifically to the OA pain symptoms [8]. New vessels, that bring new nerve fibres associated, can enter the degenerated cartilage in the repair effort [35]. The formation and innervation of these vessels are important pathophysiological events that can cause deep pain, even when the inflammation is low [35]. Additionally, angiogenesis may perpetuate chondrocyte hypertrophy, endochondral ossification and the formation of osteophytes [35].

In most cases, OA emerges without an apparent cause [36]. However, the destruction of articular cartilage can be triggered by a number of events, including changes in the mechanical environment, biochemical changes in the ECM, and chondrocytes biological responses [36]. The development process of OA is initiated by the modification in the composition and organization of the ECM [7]. Chondrocytes, that normally have a low regenerative capacity and low metabolic activity, start to exhibit a proliferative response, increasing the ECM synthesis, in an attempt to initiate a repair process [9]. However, changes in composition and structure of the articular cartilage stimulate chondrocytes to produce catabolic factors, such as MMPs and aggrecanases, that will degrade the ECM [7]. In case of injury or disease, there is a disturbance in the balance of some regulatory factors, which leads to difficulties in the maintenance and repair of the tissue, tissue degeneration and accelerated erosion of the articular surface [37]. Cartilage integrity is compromised, cells become apoptotic and cartilage will be lost, leading to friction between bones, which will induce pain and joint mobility impairment [9].

1.4 – Inflammation in cartilage damage

After a joint injury, there is an increase in the levels of proinflammatory cytokines at the defect site, as illustrated in figure 1.3 [37]. This increase will disturb the balance between anabolic and catabolic factors, leading to articular cartilage degeneration [37]. Proinflammatory cytokines, such as interleukin 1 beta (IL1β), tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), interleukin-17 (IL-17), and interleukin-18 (IL-18), are considered critical mediators of this disturbed metabolism by enhancing the catabolic activity of the articular cartilage [37], [38]. Among the proinflammatory cytokines present in the damaged site, IL1β and TNFα are considered the key regulators of the inflammatory environment [38].

IL1β and TNFα are both associated with cartilage destruction [38]. They are produced by chondrocyte, inducing the production of other inflammatory cytokines, and the release of several proteolytic enzymes, such as MMPs, namely MMP-1, MMP-3, and MMP-13, which are

important regulators of cartilage destruction [38]. In patients with articular cartilage lesions, the levels of IL1ß and TNFα were found to be elevated in the synovial fluid, synovial membrane, subchondral bone and cartilage, inhibiting the synthesis of major ECM components, namely collagen type II and aggrecan [38].

IL1ß activation is mediated by its cell-surface receptor IL-1 receptor type I (IL1RI), whose expression is increased in inflamed joints [38]. IL1 receptor antagonist (IL1Ra), that inhibits IL-1 activity, is also produced by chondrocytes [38], however, its production was shown to be compromised in an inflammatory scenario [38].

Regarding TNFα, it binds to two receptors: TNF receptor I (TNFRI) and TNF receptor II (TNFRII), both overexpressed in case of inflammation [38].

The increased levels of both cytokines, induces the expression of nitric oxide (NO) and prostaglandin E_2 (PGE_2) in chondrocytes, increasing the production of MMPs, leading to cartilage destruction [39], [40]. These MMPs will degrade collagen and proteoglycans, important molecules present in the ECM, and will interfere with the production of IL1Ra, promoting chondrocyte death [38].

Several chondroprotective drugs have been successfully tested to minimize the inflammation within the defect site [37]. Glucosamine sulfate, chondroitin sulfate, hyaluronic acid, and diacerein have shown to inhibit he synthesis of NO, reduce the blood markers of inflammation, the synthesis of MMPs, and the formation of IL1ß, and TNFα [37].

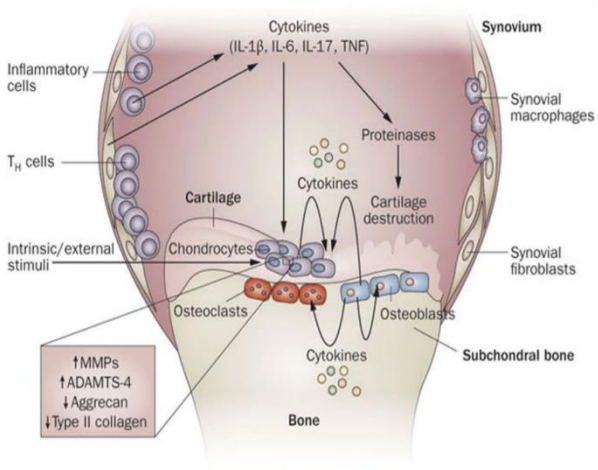

Figure 1.3 - Proinflammatory cytokine environment, at the injured site, that leads to the upregulation of catabolic factors and to the downregulation of anabolic processes, resulting in tissue degradation. Cartilage degradation products and proinflammatory signals will induce further the inflammation, enhancing the desregulation of typical chondrocyte function. Adapted from [41].

The control of this inflammatory environment presents an important strategy to inhibit the ECM destruction, and, consequently, promote cartilage repair.

1.5 – Therapeutic approaches for cartilage repair

1.5.1 – Non-surgical techniques

Non-surgical therapies are usually the first treatment option for early stage injuries [42]. These include exercise, physical therapy, and pharmacological treatments [42].

1.5.1.1 – Non-Pharmacological Therapies

Conservative measures play an important role in the management of pain and disability associated with articular cartilage damage [43]. The use of conservative strategies such as self-management programs, social support, weight loss, physical therapy and appropriate footwear are important factors that will contribute to improving patients quality of life [43].

1.5.1.2 – Pharmacological Therapies

1.5.1.2.1 – Analgesics

The use of analgesic treatments is one of the most frequent methods applied to improve the patients' quality of life [42].

Acetaminophen (Tylenol®, Paracetamol®, and Panadol®) is the first line of treatment used to relieve pain by inhibiting the cyclooxygenase enzyme 2 (COX2). COX2 is easily induced by proinflammatory stimuli, catalysing the production of prostaglandins that mediate inflammation [44].

The use of non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin and ibuprofen, are known to reduce the prostaglandin biosynthesis [42]. Other analgesics used are the opiates such as tramadol, and selective serotonin and norepinephrine reuptake inhibitor antidepressants (SSNRI), such as duloxetine hydrochloride (Cymbalta™). [43].

1.5.1.2.2 – Intra-articular injection

Intra-articular injections are used to deliver certain compounds directly to the defect site. Their application is minimally invasive and can be easily performed as an outpatient procedure [29]. The administered compounds include corticosteroid, hyaluronic acid, autologous platelet-rich plasma, and bone marrow aspirate concentrate. The corticosteroids are used as anti-inflammatory agents to reduce pain, however its efficacy is short over time. Hyaluronan is an important compound of the healthy synovial fluid, contributing to joint homeostasis. In case of injury or disease, its concentration is decreased. Therefore, the hyaluronic acid injections are used as viscosupplementation therapies. The autologous platelet-rich plasma injections contain

platelets, growth factors, proteases and cytokines that will activate several signalling pathways for cartilage repair [29]. The bone marrow aspirate concentrate contains bone marrow collected from large bones [45]. It is rich in stem cells, cytokines, and growth factors that are believed to decrease the inflammation at the injured site, as well as to promote tissue repair [45].

1.5.2 – Surgical techniques

Currently, surgical interventions are performed in an attempt to stop cartilage degradation and eventually promote its repair [46]. Some of the techniques used are illustrated in figure 1.4. Yet, these approaches have been reported to provide only short term solutions [46].

When treating articular cartilage lesions, there are several factors that should be taken into account: depth, surface and location of the injury, associated injuries, knee stability, meniscal injuries, limb mechanical axis and patient's age [46]. Nowadays, the surgical methods used to treat cartilage defects are: arthroscopic lavage and debridement, bone marrow stimulation, osteochondral allograft or/and autograft transplantation and autologous chondrocyte implantation (ACI) or matrix-induced autologous chondrocyte implantation (MACI) [46].

Arthroscopic lavage and debridement is considered a palliative technique that aims to debride the loose chondral tissue, in order to remove the damaged cartilaginous tissue [47]. The removal of this tissue and the inflammatory mediators that were generated by the synovial coating provides satisfactory short-term effects for acute and degenerative chondral lesions [47]. This treatment is performed in older sedentary patients, and in patients with early OA [47]. Nevertheless, this is not a definitive treatment since the lesions are not repaired.

Abrasion arthroplasty consist on the debridement of the joint defect, to remove 1 to 3 mm of subchondral bone, with the aim of accessing the vasculature [47], [48]. Due to the blood perfusion, a fibrin clot is generated, initiating a repair process [48]. The removal of the subchondral bone is performed by a motorized burr, which as the disadvantage of causing trauma and thermal necrosis in the underlying bone [47]. This technique is, therefore, not widely used [47].

Drilling is similar to the abrasion arthroplasty procedure but, in this case, the subchondral bone is perforated with a drill in multiple holes in order to penetrate the bone marrow, starting a blood perfusion and, consequently, a fibrin clot that will initiate the repair process [47]. These holes allow a consistent cellular migration between them, promoting a repair process, which will form a mixed cartilage of hyaline and fibrocartilage [48]. Like abrasion arthroplasty, this technique may also cause thermal necrosis [48].

Microfracture is an invasive technique that stimulates bone marrow [48]. Damaged cartilage is debrided until the subchondral bone [47], using small awls to make multiple holes [48]. The drills are approximately 3 to 4 mm apart, and have 4 mm depth [47]. Consequently, there is a bleeding and the formation of a blood clot, leading to the development of fibrous tissue repair with low biomechanical and viscoelastic properties [48]. Consequentially the symptoms will reappear [48]. The procedure is similar to drilling, but because the holes are made with an arthroscopic drill, there is less thermal effects [48].

In osteochondral autograft transplantations, articular cartilage from the patient is collected, from nonload bearing regions, and transplanted into the injured area [48]. One advantage of

this technique is that it implies only a single surgery: the tissue is collected from one site and immediately transplanted to the lesion site [48]. It has a low complication rate and it is cost effective [47] This type of grafting is performed when the lesions are small or medium (less than 4 cm^2) [48].

Osteochondral allograft transplantation corresponds to the transplantation of a composite cadaveric graft from the subchondral bone overlaid with hyaline cartilage in the patient's injured area [48]. This transplants are used for medium to large articular lesions (up to 3 cm^2) [47]. Allografts should be collected from young, and healthy individuals [48] with high bone and cartilage quality [47]. Since the allografts should be fresh, there is an increased risk of disease transmission [48]. Cost and size mismatch are problems that should also be considered [47].

Autologous chondrocyte implantation involves two stages [47]. The first stage requires an arthroscopic assessment of the chondral lesion and a biopsy to obtain chondrocytes [47]. The cartilage samples collected are sent to the laboratory and chondrocytes are isolated and expanded [47]. The second phase includes the implantation of the expanded cells into the damaged site by an arthrotomy [47]. After implantation, cells are covered with a periosteal patch with the size of the defect, and sealed with fibrin glue [47]. The purpose is to obtain a repair tissue as similar as possible with hyaline articular cartilage [47].

A matrix induced chondrocyte implant is a layer of porcine derived collagen, where chondrocytes, collected from the patient, are seeded [47]. The implementation of this matrix includes two phases: the arthroscopic evaluation of the chondral lesion, and a biopsy to collect chondrocytes, and the implantation of the matrix through arthroscopic surgery [47]. During the implantation, the graft is fixed to the defect only by fibrin glue, without sutures [47].

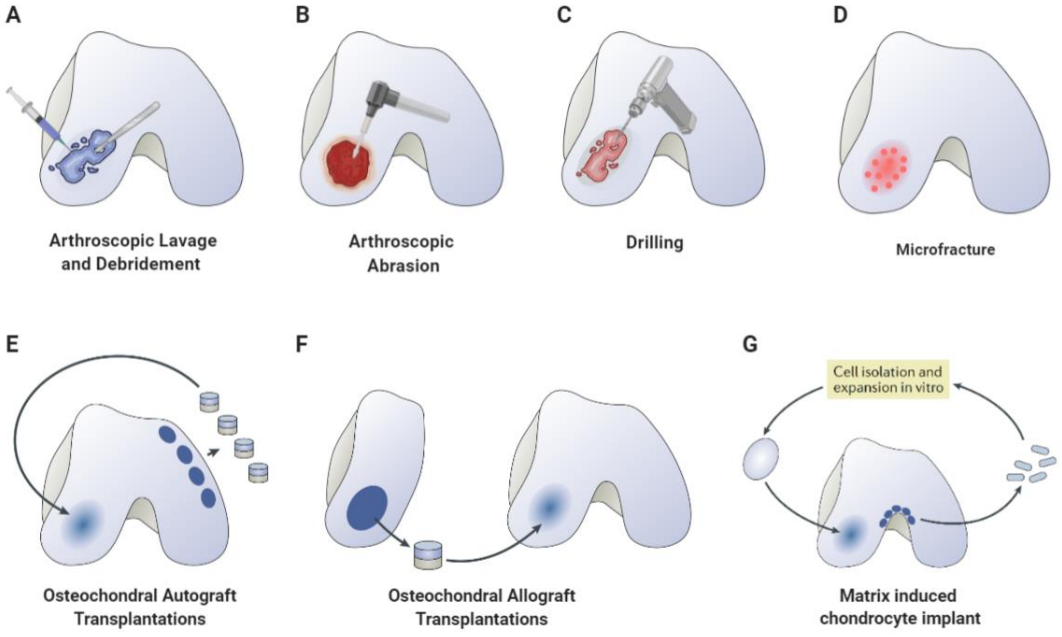

Figure 1.4 - Surgical techniques used to treat articular cartilage defects: (A) arthroscopic lavage and debridement, used to debride the loose chondral tissue; (B) arthroscopic abrasion, that involves the debridement of the joint defect up to the subchondral bone; (C) drilling, a similar technique to abrasion arthroscopy, with the difference that the multiple holes are performed using a drill; (D) microfracture, a minimal invasive technique, that stimulates bone marrow by debriding the damaged cartilage up to the subchondral bone; (E) osteochondral autograft transplantation, where human articular cartilage is collected, from nonload bearing regions, and transplanted into the injured area; (F) osteochondral allograft transplantation, which corresponds to the transplantation of a composite cadaveric graft from

subchondral bone overlaid with hyaline cartilage in the patient's injured area, and (G) matrix induced chondrocyte implant, where a layer of porcine derived collagen is seeded with chondrocytes collected from the patient, and subsequently implemented at the damaged site. Adapted from [11].

1.6 – Tissue Engineering Strategies

The main challenge of tissue engineering is to regenerate the damaged tissue as similar as possible to the composition, structure and function of the natural tissue [49]. Therefore, many therapies using cell-based strategies and biomaterials, such as scaffolds (hydrogels, and porous structure scaffolds), or nanomaterials (nanoparticles, nanotubes, and nanofibers), are being developed [49].

1.6.1 – Cell-based therapies

Cell-based strategies are a very promising approach that experimentally can be divided into four main steps, as illustrated in figure 1.5: i) cell isolation from an appropriate tissue, and subsequent cell expansion and differentiation; ii) cell seeding in a 3D scaffold that will mimic the proper environment for cell attachment, proliferation, and differentiation or scaffold-free approaches that will facilitate cell-to-cell interactions, and minimize the toxicity associated with scaffolds degradation; iii) *in vitro* culture in an environment that promotes tissue growth; iv) integration of the created tissue into the lesion site [11], [49].

Chondrocytes are usually the main cell choice for the engineering of articular cartilage [11]. The implantation of mature chondrocytes cultured *in vitro* is a technique used worldwide, however, there are still problems associated with their maintenance in culture [29]. The possible dedifferentiation of chondrocytes and the fact that the differentiated mature cells do not proliferate, hinders there expansion *in vitro* [29].

Therefore, to overcome these problems, the use other cell types with proliferative capacity and that can maintain the chondrogenic phenotype, such as MSCs, embryonic stem cells (ESC), induced pluripotent stem cells (iPSC) and chondrocyte stem/progenitor cells (CSPC), has been explored [29].

Different source of MSCs are used to treat articular cartilage lesions, such as bone marrow, fat tissue, synovial membrane, umbilical cord blood, periosteum and muscle [29]. Bone marrow stem cells are a homogeneous population with high chondrogenic potential that can be easily isolated [13]. However, extracting bone marrow is a painful, invasive and low yield procedure [13]. Adipose derived stem cells, isolated from lipoaspirates, exist in great abundance, have a high yield and its extraction does not cause morbidity to the donor tissue [13]. However, there is still the need to perform more studies regarding its use in cartilage repair [13]. The synovium derived stem cells have a high proliferative rate, a high chondrogenic potential and a high yield [13]. However, there is a limited source of tissue from where these cells can be extracted [13]. The ability of MSCs to differentiate into chondrocytes varies according to the sites from which the cells were obtained, and MSCs transplantation can lead to a mixture of hypertrophic, fibrous and cartilaginous tissues, which in the long term leads to a loss of the repaired tissue [29].

ESC can proliferate and differentiate in almost any somatic cell type [29]. The procedures to convert ESC into chondrocytes include a co-culture with primary joint chondrocytes and mesenchymal stem cell-like cells produced from ESC, that will subsequently differentiate into chondrocytes [29]. There are some disadvantages associated with the use of ESC, such as ethical concerns, immune rejection by the host and the risk of teratoma formation [29].

iPSC are a newly used source of stem cells with a similar capacity for self-renewal and pluripotency as ESC [29]. There are several types of somatic cells from which these cells can be generated, such as keratinocytes, mesenchymal cells, adipose stem cells, melanocytes and postmitotic neurons [29]. However, their clinical used is not legalized, since iPSC may induce tumor formation, and there are some difficulties to control and direct specific differentiation [50], [51]

CSPC can be found in the superficial area of the articular cartilage [29]. These cells are adhered to fibronectin, express stem cell markers, and have the ability to proliferate and differentiate into chondrocytes *in vitro* [29]. The CSPCs can be expanded extensively *in vitro*, and form the entire adult articular cartilage tissue *in vivo*, thus contributing to the physiological healing of small cartilage defects [29]. However, there are still some difficulties in identifying and purifying CSPC from human articular cartilage due to the shortage of well-defined markers [29]. Furthermore, the therapeutic potential of these cells has not yet been tested [29].

Overall, although cell-based therapies are a very promising approach, they still present several drawbacks, such as high cost, ethical concern, the possibility of immune rejection by the host, limited cell sources, and low yield from the cells sources available [13], [29].

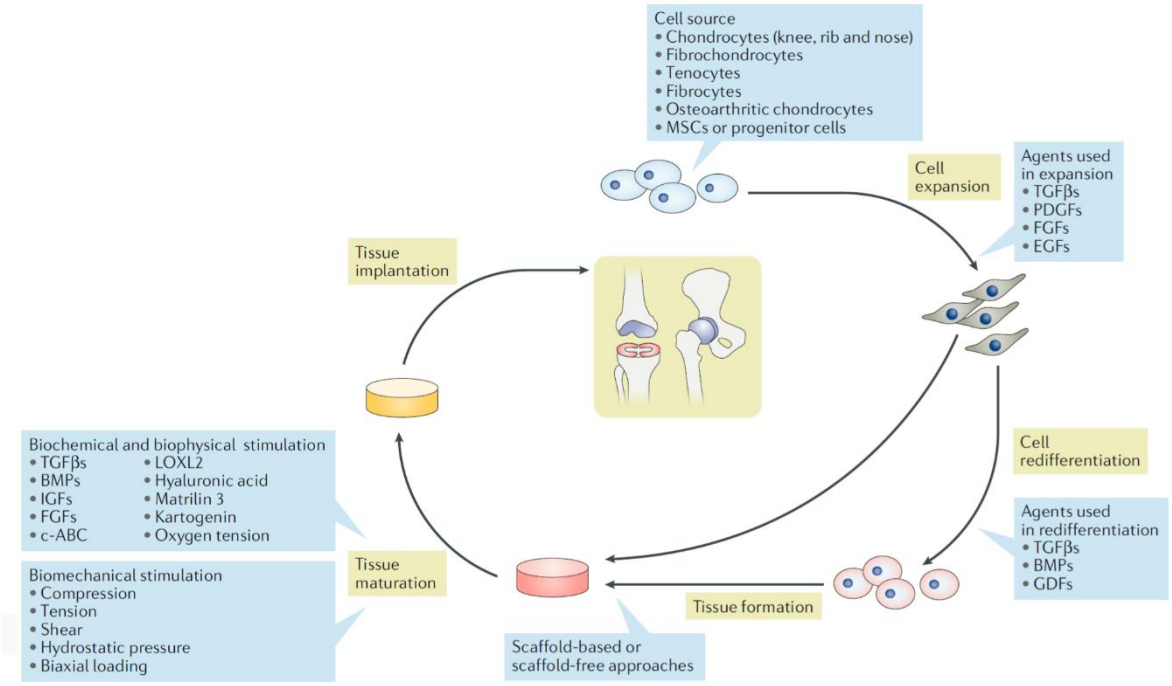

Figure 1.5 - Cell-based strategies include i) cell isolation from an appropriate tissue, ii) cell seeding in a 3D scaffold that will mimic the proper environment for cell attachment, proliferation, and differentiation or a scaffold-free approaches that will facilitate cell-to-cell interactions, iii) *in vitro* culture in an environment that promotes tissue growth, and iv) integration of the created tissue into the lesion site. Adapted from [11].

1.6.2 – Scaffolds

The development of a biomaterial is directly related to both the properties of the materials and the target tissue [49]. Among the desirable characteristics of the biomaterials are biocompatibility, the materials should not induce any immunologic response, and biodegradability, by having an appropriated rate of degradation, providing support while new tissue formation occurs.

For scaffolds, the architecture is also an important parameter [49]. It should mimic the native tissue organization, shape and size, and should have open interconnected porous to enable cell growth, allowing nutrients transport and vascularization, promoting cell adhesion, growth and differentiation [49]. This way, scaffolds offer cells an environment able to promote and support cell viability [52]. Many ECM like scaffolds are available and used in cartilage tissue engineering [52]. Protein or carbohydrate-based polymers (also known as natural polymers), synthetic polymers and composites are used in scaffolds. Compounds like fibrin, collagen or gelatine are being used for the construction of protein-based polymers. Collagen is a macromolecule that has been widely used as biomaterials due to its protein structure (repetitive sequence of amino acids with specific size and structure) and biodegradation, that may be controlled by the level of reticulation of the polypeptide chains [53]. Furthermore, it is a non-toxic, biocompatible material, widely available from a great variety of sources [50]. Chitosan, agarose, poly(ethylene glycol) (PEG) and alginate are examples of carbohydrate-based polymers used to construct scaffolds [50]. These structures are composed of cross-linked polymers with properties similar to the ECM [50]. Chitosan is a biodegradable and biocompatible polysaccharide that derives from chitin, an element that exists in a large scale on nature (exoskeleton of crustaceans, fungi or insects), and that is normally isolated from residues from food industry [53]. It is structurally similar to GAGs present in the ECM [53]. Therefore, chitosan is used as an innocuous component of ECMs produced through tissue engineering [53]. Regarding synthetic polymers, several materials are used in the construction of scaffolds: poly(ε-caprolactone) (PCL), poly(L-lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA), (poly(vinyl alcohol)), polyethylene glycol, pluronics and polyurethane [29]. Synthetic polymers can be easily produced, using several different techniques including lyophilization, moulding, electrospinning, and 3D printing, and their properties can be precisely controlled [29].

Hydrogels are hydrophilic polymer scaffolds that can be loaded with cells [54]. Cell loaded hydrogels are incorporated with resident tissue cells that may trigger chondrogenesis, and/or maintain a chondrogenic phenotype, while encouraging tissue repair [23]. Acellular constructions are used to obtain structural and mechanical properties similar to native cartilage, while enabling efficient load transfer or recruitment of cells for cartilage repair [23].

Scaffolds can be incorporated with molecules, such as cytokines, growth factors or even loaded nanoparticles, which will interact with the damaged tissue to promote chondrogenesis [23].

1.6.3 – Nanobiomaterials

Nanobiomaterials have been explored due to their high surface to volume ratio [49]. Independently of the biological tissue, as referred for the scaffolds there are requirements that have to be considered, such as biocompatibility with the host, biodegradability, safety, and suitable mechanical properties adequate to the tissue being treated [55], [56].

There are several types of nanomaterials used in tissue engineering, including nanoparticles, nanofibers, nanotubes, and nanosheets [57]. Nanofibers were developed with the aim to produce scaffolds that could mimic natural human tissue architecture on a nanometric scale [58]. These nanomaterials have a porous structure and a high surface to volume ratio, allowing cell adhesion, proliferation and differentiation [58]. Recently, nanotubes have been used to obtain a membrane for guided tissue regeneration with a controlled drug supply [59]. These membranes composed of nanotubes with embedded drugs are biocompatible and allow the fixation and subsequent proliferation of cells on their surface [59]. Nanosheets are used in tissue engineering for the construction of 3D structures with mechanical properties similar to the tissue where they will be incorporated [60]. They can also be used as 2D structures composed of collagen, laminin and fibronectin, which have nano or micropores that allow cellular communication [60].

The use of nanoparticles is considered one of the most promising approaches to improve bioactive agents supply to the target tissue, such as drugs, growth factors, genes or cytokines and subsequently increase their therapeutic efficacy [57]. Nanoparticles are 1 to 100 nm sized particles [57]. Due to their small size, they are able to penetrate cells and induce several molecular changes [57]. The use of nanoparticles as a drug delivery system is known to reduce the toxicity associated with the spread of the bioactive agent, since they act directly at the target site, and subsequently to reduce the adverse side effects. In addition, because the medicine is administrated locally, there is an increase in the effectiveness of the drug, since the necessary therapeutic concentration is used almost entirely at the target site, rather than being released into the bloodstream. It also reduces the overall cost, as there is no need to administer several doses of the bioactive agent to reach the desirable therapeutic dose [61].

There are numerous nanoparticles used as carriers, such as liposomes, polymeric nanospheres, polymeric nanocapsules and polymeric micelles [57], [62].

Liposomes are liquid vesicles formed when lipids are added to an aqueous solution [63]. It is possible to produce liposomes with different sizes, composition and characteristics in order to fulfil the need of the target tissue [64]. Nanocapsules are vesicular systems where the bioactive agent is entrapped in cavity surrounded by a polymeric membrane [65]. Nanospheres are matrix systems where the bioactive agent is physically dispersed [66]. Lastly, polymeric micelles are based on amphiphilic block copolymers, that aggregate when in aqueous media [67].

The polymeric nanoparticles are the largest category of nanomaterials used in the drug delivery field [68]. They can be divided into two groups: the synthetic, such as PLGA, PCL, and PEG, and the natural-origin polymers like albumin, alginate, chitosan, dextran and heparin [68].

The PLGA nanoparticles are one of the most successfully developed biodegradable systems, used as vehicles for the delivery of biological factors [69], [70]. Among their advantageous properties are biocompatibility and biodegradability, the fact that they are FDA and European Medicine Agency approved, formulation and production methods are well described, they are known to protect the bioactive agent from degradation, and it is possibility to modify their

surface in order to achieve better and suitable properties compatible with the target tissue [69], [71].

PLGA nanoparticles were already successfully tested as an articular cartilage delivery system [71]. A study performed in rats injected with betamethasone sodium phosphate loaded PLGA nanoparticles has shown that the use nanoparticles targeted to a specific site, improved the delivery of the drug and, consequently, allowed the reduction of the inflammation [72].

1.7 – Objective

The inflammatory response initiated as a consequence of tissue damage imposes a major challenge in cartilage repair. The released proinflammatory factors induce a shift in the chondrocyte activity towards catabolism and promote further tissue degradation [37], [38].

Several anti-inflammatory drugs have been successfully tested for the reduction of the inflammatory environment within the defect site and, subsequently, for the reduction of the ECM degradation [37]. This study proposes the use of ibuprofen loaded PLGA nanoparticles as a strategy to locally deliver an anti-inflammatory treatment. Here the aim was to evaluate the chondroprotective effects of ibuprofen loaded PLGA nanoparticles to inhibit cartilage ECM destruction in 3D primary human chondrocyte cultures submitted to proinflammatory conditions.

To achieve this goal, specific aims were defined:

- establishment of the 3D primary human chondrocyte cultures.
- establishment of the proinflammatory environment in 3D cultures.
- evaluate the capacity of the PLGA nanoparticles loaded with ibuprofen to inhibit the ECM destruction.

Chapter 2

2. Materials and Methods

2.1 - Materials

The following tables summarize the reagents used for *in vitro* cultures of the experimental procedure.

2.1.1 – Primary human chondrocytes isolation

Table 2.1 - Isolation Medium.

Reagents	Reference
Dulbecco's Modified Eagle's Medium (DMEM)	21885; Gibco®
Penicillin/Streptomycin (P/S)	15140-122; Gibco® (Invitrogen)
Fungizone	A9528; Sigma-Aldrich®

Table 2.2 - Digestion Medium.

Reagents	Reference
DMEM	21885; Gibco®
Fetal Bovine Serum (FBS)	10270-106; Gibco® (Invitrogen)
Collagenase B	11 088 815 001; Roche
Trypan Blue solution	T-8154; Sigma-Aldrich®

Table 2.3 - Expansion Medium.

Reagents	Reference
DMEM	21885; Gibco®
FBS	10270-106; Gibco® (Invitrogen)
HEPES buffer, 1 M	15630056; Gibco®
Sodium Pyruvate	11360-039; Gibco®
P/S	15140-122; Gibco® (Invitrogen)
TGF-ß1	100-B-001; R&D
bFGF	0291; Sigma-Aldrich®
Trypan Blue solution	T-8154; Sigma-Aldrich®
Trypan Blue solution	T-0646; Sigma-Aldrich®

2.1.2 – Establishment of three-dimensional chondrocyte cultures

Table 2.4 - Basal Medium.

Reagents	Reference
DMEM	21885; Gibco®
FBS	10270-106; Gibco® (Invitrogen)
P/S	15140-122; Gibco® (Invitrogen)

Table 2.5 - Chondrogenic Medium.

Reagents	Reference
DMEM	21885; Gibco®
Insulin	I3536; Sigma-Aldrich®
Transferrin	T8158; Sigma-Aldrich®
Sodium Pyruvate	11360-039; Gibco®
Selenious Acid	211176; Sigma-Aldrich®
Dexamethasone	D4902; Sigma-Aldrich®
Ascorbic acid 2-phosphate	A8960; Sigma-Aldrich®
Proline	P5607; Sigma-Aldrich®
BSA	A4919; Sigma-Aldrich®
TGF-ß3	243-B3-010 R&D

2.2 - Methods

2.2.1 – Chondrocyte isolation from human articular cartilage

Chondrocytes were isolated from human adult articular cartilage, obtained from osteochondral leftovers collected during knee or hip arthroplasties performed at Centro Hospitalar de São João, Porto, under a protocol established between i3s and Centro Hospitalar de São João after approval by the Ethical Committee of the Hospital. All patients consented the use of their tissue for research purposes. All the procedures were in accordance with the Helsinki Declaration of 1975, as revised in 2000. Chondrocytes were obtained as previously described in [27]. Tissue samples were collected from the distal and/or posterior femoral

condyle as described in figure 2.1 and immediately after the removal, cuts were transferred to Dulbecco's Modified Eagle Medium (DMEM, Gibco), with 5% Penicillin/Streptomycin (P/S) and 5% Fungizone.

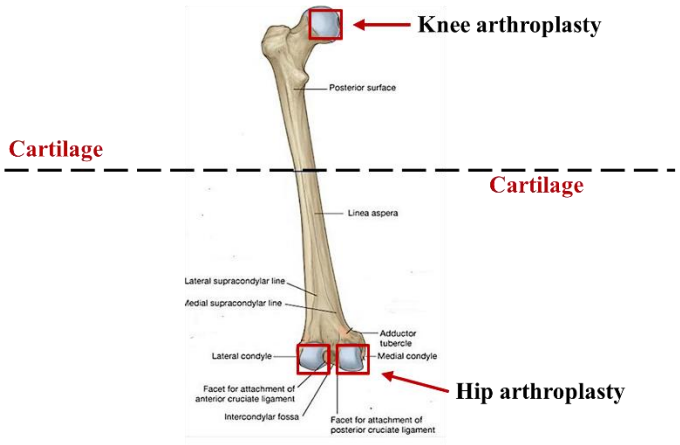

Figure 2.1 - Schematic representation of the distal and posterior femoral condyle from were cartilage samples are collected during knee or hip arthroplasties.

Slices of cartilage were chopped into small pieces, with approximately 2 mm, with a scalpel and digested overnight with 0.15% collagenase B (Roche) in DMEM (Gibco) with 5% FBS (Gibco), at 37ºC under agitation. The digested tissue was filtered through a 100 µm pore size cell strainer. Cells were then pellet down by centrifugation and resuspended in high-glucose DMEM (4.5% glucose; Gibco) supplemented with 10% FBS, 1% N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES buffer, 1 M; Gibco), 1% sodium pyruvate (Gibco), and 1% P/S. In addition, 1 ng/mL transforming growth factor-beta 1 (TGF-ß1; R&D) and 5 ng/mL basic fibroblast growth factor (bFGF; R&D) were freshly added to the medium. This medium, now on referred as expansion medium, allow cell proliferation. Chondrocytes were cultured in monolayer, in the expansion medium, in T75 flasks, at a density of $2 \times 10^6 cells/flask$, in a humidified atmosphere with 5% CO_2 at 37°C. Medium was change every two days. In figure 2.2 there is a schematic representation of the primary chondrocyte isolation procedure.

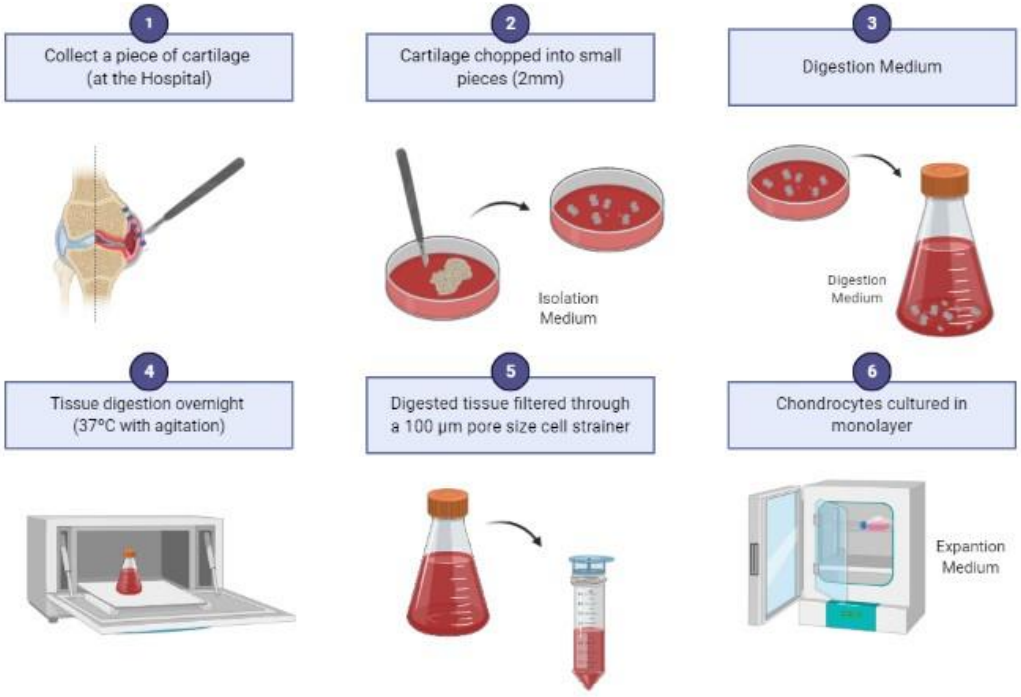

Figure 2.2 - Schematic representation of the primary chondrocytes isolation procedure: (i) cartilage samples were collected during knee or hip arthroplasties; (ii) cartilage samples were chopped into small pieces, with approximately 2 mm, with a scalpel; (iii) the sample was then transferred to the digestion medium containing collagenase B, and (iv) left overnight at 37ºC under agitation; (v) the digested tissue was then filtered through a 100 µm pore size cell strainer, and (vi) cells were pellet down pellet down by centrifugation and cultured in monolayer.

At confluence, cells were detached with 0,05% Trypsin and frozen in basal medium (high-glucose DMEM (Gibco) with 10% FBS (Gibco) and 1% P/S and 10% Dimethyl Sulfoxide (DMSO; which is used as a cryoprotector to protect biological tissue from freeze damages), in liquid nitrogen at a concentration of 1 million cells per cryotube. To create a cell bank, chondrocyte isolations were performed from several different donors.

2.2.2 – Three-dimensional pellet culture of chondrocytes

Due to the limited proliferation capacity of primary chondrocytes and their tendency to de-differentiate under conventional growing conditions (monolayer cultures), long lasting culture conditions can be challenging [73]. Several studies have shown that the culture of expanded primary chondrocytes under 3D culture conditions, with the appropriated supplements, has resulted in increased expression of chondrogenic genes, such as collagen type II, aggrecan and Sox-9 [11].

Therefore, in our experiment, chondrocytes routinely expanded in expansion medium up to passage 4, were seeded at a concentration of 1 million cells/mL/tube in 15mL polypropylene tubes with a lid with filter to allow gas exchanges, as illustrated in figure 2.3. Pellets were formed by centrifugation (5 minutes at 390g) and cultured in basal medium in a humidified atmosphere with 5% CO_2 at 37°C. After 24h, the medium was changed to chondrogenic medium (high-glucose DMEM with 5 µg/mL insulin (Sigma), 5 µg/mL Transferrin (Sigma), 5 ng/mL

selenium acid (Sigma), 0.1 µM dexamethasone (Sigma), 0.17 mM ascorbic acid 2-phosphate (Sigma), 1 mM sodium pyruvate (Gibco), 0.35 mM proline (Sigma), 1.25 mg/mL bovine serum albumin (BSA) (Sigma) and 10 ng/mL transforming growth factor-beta 3 (TGF-ß3; R&D) freshly added) that will promote cell differentiation. As control, pellets were cultured in Basal Medium. Medium was changed every three days. Both pellets and conditioned medium were collected at days 3 and 7 for some experiments, and at days 3,7 14, 21 and 28 for others.

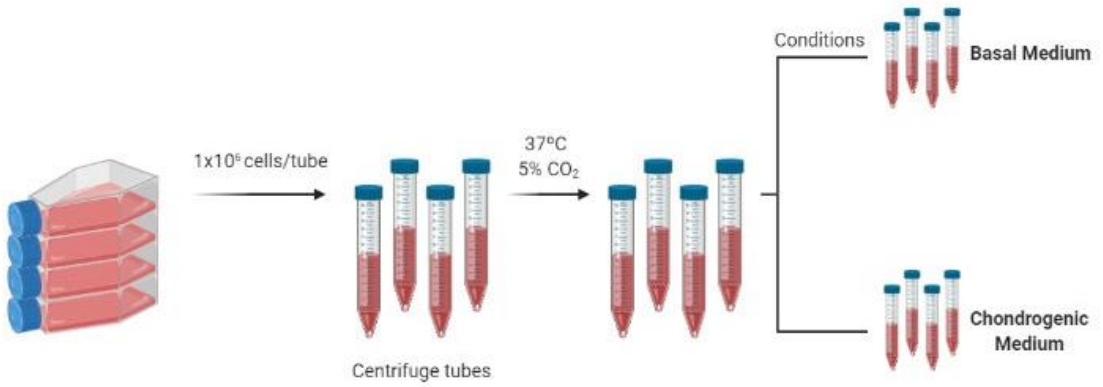

Figure 2.3 - 3D chondrocyte culture experimental design. Firstly, chondrocytes were expanded in monolayer, in expansion medium, and then seeded in 15mL tubes in Basal Medium. After centrifugation, the formed pellets were left in culture at 37ºC, 5% CO2 in chondrogenic medium or in basal medium (non-chondrogenic control condition).

2.2.2.1 – Pellets shape and size analysis

Pellets were photographed, at days 3 and 7 of culture, using a *Canon 1200 D* with a 33-50mm lens and analyzed with ImageJ software. Pellets size was represented by the area delimited by an ellipse. The largest and smallest axes of each ellipse were considered the horizontal and vertical lengths of the pellets, normalized by the length of the tube base. The aspect ratios (AR) of the pellets were determined by the quotient between the horizontal and vertical lengths of the pellets, as illustrated in figure 2.4.

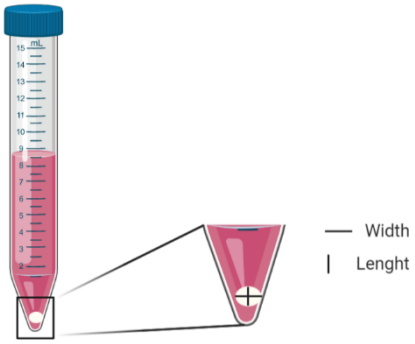

Figure 2.4 - Schematic representation of the horizontal and vertical lengths of the pellets.

2.2.2.2 – Analysis of GAGs deposition by histochemistry

The Alcian Blue staining protocol was used to assess chondrocyte matrix production. Alcian Blue is a basic dye used to stain acidic polysaccharides, like glycosaminoglycans, in cartilage. The staining was performed according to the protocol described below.

At days 3, 7, 14, 21, and 28 pellets were fixed in paraformaldehyde (PFA, 4%) for 30 minutes, and then washed with PBS. Pellets were embedded in paraffin (Microm STP 120, Thermo Fisher Scientific), cut into 5µm sections in the microtome (HistoCore MULTICUT, Leica Bio Systems) and mounted on glass slides.

To perform the staining protocols, slides were immersed three times in xylene for 5 minutes to remove paraffin, rehydrated in an alcohol series (50%, 70%, 95% and 100%) and finally distilled water.

After rehydration, slides were immersed in an Alcian Blue solution for 30 minutes to stain GAGs. After being washed in running tap water for 2 minutes and rinsed in distilled water, nuclei were counterstained with Nuclear Fast Red, followed by a wash in running tap water for 1 minute.

After the staining procedure slides were dehydrated through graded alcohol solutions (50, 70 and 96%, 3 minutes each), cleared in xylene and mounted with mounting medium (DPX mounting medium, VWR). Images were acquired with an Olympus CX31 light microscope equipped with a DP-25 camera (Imaging Software Cell^B, Olympus, PA).

2.2.3 – Establishment of the proinflammatory conditions

After a cartilage injury, an hostile environment is created where several molecules are secreted, namely proinflammatory cytokines, such as TNFα and IL1β [38]. These cytokines are known to promote cartilage destruction [38]. Therefore, the control and/or inhibition of the inflammatory stimuli, might stop further cartilage degeneration.

Three-dimensional pellets of chondrocytes were formed and differentiated for 7 days before being cultured in proinflammatory conditions, like represented in figure 2.5. Six different proinflammatory culture conditions were tested in order to identify the most effective: (i) chondrogenic medium supplemented with 100 ng/mL of TNFα; (ii) chondrogenic medium supplemented with 100 ng/mL IL1β, (iii) chondrogenic medium supplemented with 100 ng/mL of both TNFα and IL1β, (iv) chondrogenic medium supplemented with 10 ng/mL TNFα; (v) chondrogenic medium supplemented with 10 ng/mL IL1β and (vi) chondrogenic medium supplemented with 10 ng/mL of both TNFα and IL1β. Chondrogenic medium without proinflammatory conditions was used as control. At days 3 and 7 of culture under proinflammatory conditions, both pellets and conditioned medium were collected, stored and/or processed for analyses.

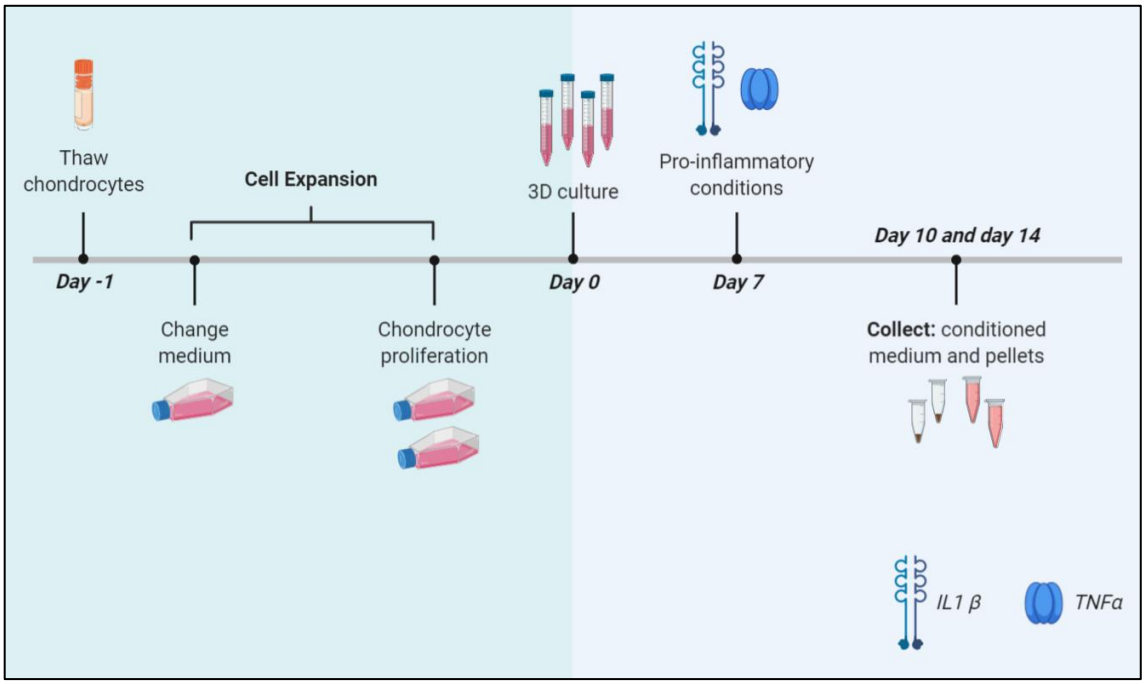

Figure 2.5 - Experimental design used for the establishment of proinflammatory conditions. After defrosted, chondrocytes were expanded in monolayer, in Expansion Medium, for 7 to 14 days. Then, cells were seeded in 15mL tubes in Basal Medium. After 7 days of differentiation, culture medium was supplement with TNFα and/or IL1ß. After 3 and 7 days, pellets and culture medium were collected for analysis.

2.2.3.1 – Cytotoxicity assay

Cytotoxicity was evaluated using a LDH assay (Promega), which quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. LDH released into the culture supernatant was quantified after a 30-minute coupled enzymatic assay, in which there is the conversion of a tetrazolium salt into a red formazan product. A blank control with only medium, and a positive control of total cell death were also included. For each measurement, two replicates per condition were included.

2.2.3.2 – Cytokine quantification by ELISA

The concentration of interleukin-10 (IL-10), IL6, IL1ß and TNFα was determined in the culture medium by ELISA using commercially available kits specific to each cytokine, according to the manufacturer's instructions (ELISA MAX™ Deluxe Set, BioLegend®).

2.2.3.3 – Real Time quantitative PCR

The expression levels of mRNA transcripts for MMPs, and ADAMTSs were measured by quantitative real-time polymerase chain reaction (qRT-PCR). The RNA of the pellets was extracted and purified with Trizol (Invitrogen). Chondrocyte pellets were homogenized by forcing then to pass through needles of various sizes (from 21G to 30 G). RNA concentration was assessed in a NanoDrop spectrophotometer (NanoDrop™ 1000 Spectrophotometer, Thermo Fisher Scientific). The purity of the RNA was obtained by the ratio of absorbance readings at 260 nm to 280 nm. Only ratios between 1,8 and 2 were considered. RNA was reverse transcribed using the NZYtech kit (Direct-zol™, ZYMO Research) following the instructions of the manufacture. Primer design was performed using the Primer-BLAST. Table 3.6 presents the sequence of the primers used for the qRT PCR analysis.

Table 2.6 - Characteristics of the primers used for the qRT PCR.

Primer	Type	Sequence	Size (pb)
MMP 1	Forward	CTGTTCAGGGACAGAATGTGCT	85
MMP 1	Reverse	TCGATATGCTTCACAGTTCTAGGG	
MMP 2	Forward	AGTACCGCTGCTCTCTAACC	200
MMP 2	Reverse	CTGGGGCAGTCCAAAGAACT	
MMP 3	Forward	TTTTGGCCATCTCTTCCTTCA	139
MMP 3	Reverse	TGTGGATGCCTCTTGGGTATC	
MMP 8	Forward	CTCCCTGAAGACGCTTCCAT	108
MMP 8	Reverse	TCCAGGTAGTCCTGAACAGT	
MMP 13	Forward	TCCTCTTCTTGAGCTGGACTCTT	97
MMP 13	Reverse	CGCTCTGCAAACTGGAGGTC	
MMP 14	Forward	TGCCTGCGTCCATCAACACT	84
MMP 14	Reverse	CATCAAACACCCAATGCTTGTC	
ADAM-TS5	Forward	CGCTGCCACCACACTCAA	80
ADAM-TS5	Reverse	CGTAGTGCTCCTCATGGTCATCT	
B2M	Forward	CCAGCGTACTCCAAAGATTCAG	113
B2M	Reverse	AGTCAACTTCAATGTCGGATGG	

The reference gene, ß2 microglobulin (B2M) was used as internal standard for normalization. The qRT-PCR reactions, were performed on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA), using the Itaq (Bio-Rad Laboratories, Hercules, CA), as follows: 1 cycle of 95°C for 15 min followed by 40 cycles of 95°C for 30 sec and 58 °C for 1 min (annealing temperature), ending with a melting curve analysis to control for the amplification of a single gene product. Product fluorescence was detected at the end of the elongation cycle. All melting curves exhibited a single sharp peak at a temperature characteristic of the primer used.

Linearity and efficiency of PCR amplification reactions were assessed for all pair of primers using standard curves generated by increasing amounts of cDNA. Relationship between the threshold cycle (C_t) and logarithm of the cDNA concentrations were studied according to correlation coefficient and the slopes, calculated by CFX Maestro™ software, version 1.1. For all primer sets, standard curves using four points, diluted over a 100-fold range, always led to a high linearity (correlation coefficients > I- 0.990I).

The PCR efficiency (*Ex*) was calculated using the equation $Ex = 10^{-1/slope}$. Efficiency was presented as a percentage (%) that is the % of template that was amplified in each cycle, calculated by the following equation $\%Ex = (EX-1) \times 100$. Efficiency close to 100% is the best indicator of a robust, reproducible assay. We always work with efficiency between 90-105%. These amplification efficiencies of PCR assays allow the quantification of mRNA with the comparative C_t quantification method (ΔC_t method) using a reference gene. Following this method, the relative expression of a gene was calculated by the expression: $2^{(Ct(reference) - Ct(target))}$. This method assumes that both target and reference genes are amplified with efficiencies near 100% and within 5% of each other.

2.2.3.4 – Detection of Collagen type II and Aggrecan by immunohistochemistry

The expression of chondrogenic markers present in chondrocyte pellets, such as collagen type II, and aggrecan, was also assessed by immunofluorescence staining analysis. Chondrocyte pellets were collected, fixed, and embedded in paraffin. After deparaffinization and dehydration sections were placed in antigen retrieval. The optimal antigen retrieval for each antibody was investigated in a positive control (slices of human cartilage). Three antigen retrieval were tested: Proteinase K (0.2 mg/ml in Tris-EDTA pH 8.0) for 10 minutes at 37ºC, Citrate buffer (pH 6.0), for 10 minutes at 97ºC and Tris-EDTA buffer (pH 9.0), for 10 minutes at 97ºC. Collagen type II and aggrecan did not require antigen retrieval. After endogenous fluorescence blocking with sodium borohydride (0.1% in Tris-EDTA; pH9) for 10 minutes, samples free aldehydes were blocked for 5 minutes with 100 mM NH_4Cl in Tris-EDTA; pH 9.0.

Sections were then incubated with blocking buffer (10% FBS, 1% Bovine serum albumin (BSA), 0.2% Triton X-100). The incubation with the primary antibodies was performed overnight at 4ºC (1:100 of collagen type II, Abcam; 1:50 of aggrecan, Santa Cruz Biotechnology, Inc.), followed by the incubation, for 1 hour, with the secondary antibody anti-rabbit Alexa Fluor 488 antibody (1:1000, Life Technologies). Nuclei were stained with DAPI (Sigma-Aldrich) and tissue sections mounted with Fluoromount Aqueous Mounting Medium (Sigma-Aldrich). Images were acquired on the confocal Leica TCS SP5 microscope (Leica Microsystems).

2.2.4 – Experimental set-up for the evaluation of the effects of ibuprofen loaded PLGA nanoparticles in 3D cultures

Since cartilage damage will lead to joint inflammation and further cartilage deterioration, novel approaches to promote cartilage repair have to take into consideration the effects of inflammation [74]. The three-dimensional pellets of chondrocytes were formed and differentiated for 7 days, and then the culture medium was supplement both with 100ng/mL of IL1β, and PLGA nanoparticles loaded with ibuprofen, as illustrated in figure 2.6. These nanoparticles were provided by the Nanomedicine & Translational Drug Delivery group, headed by Dr. Bruno Sarmento, and produced by the nanoprecipitation method described in [75]. As controls, pellets were cultured under chondrogenic medium only. Four different conditions were tested: (i) medium supplement only with ibuprofen (15μg/mL); (ii) medium supplemented with unloaded PLGA nanoparticles; (iii) medium loaded with 100 μg/mL of ibuprofen-loaded

PLGA nanoparticles (15µg/mL) and (iv) medium loaded with 200 µg/mL of ibuprofen-loaded PLGA nanoparticles (30µg/mL). At days 3 and 7 after treatment both pellets and the conditioned medium were collected, stored and/or processed for analyses.

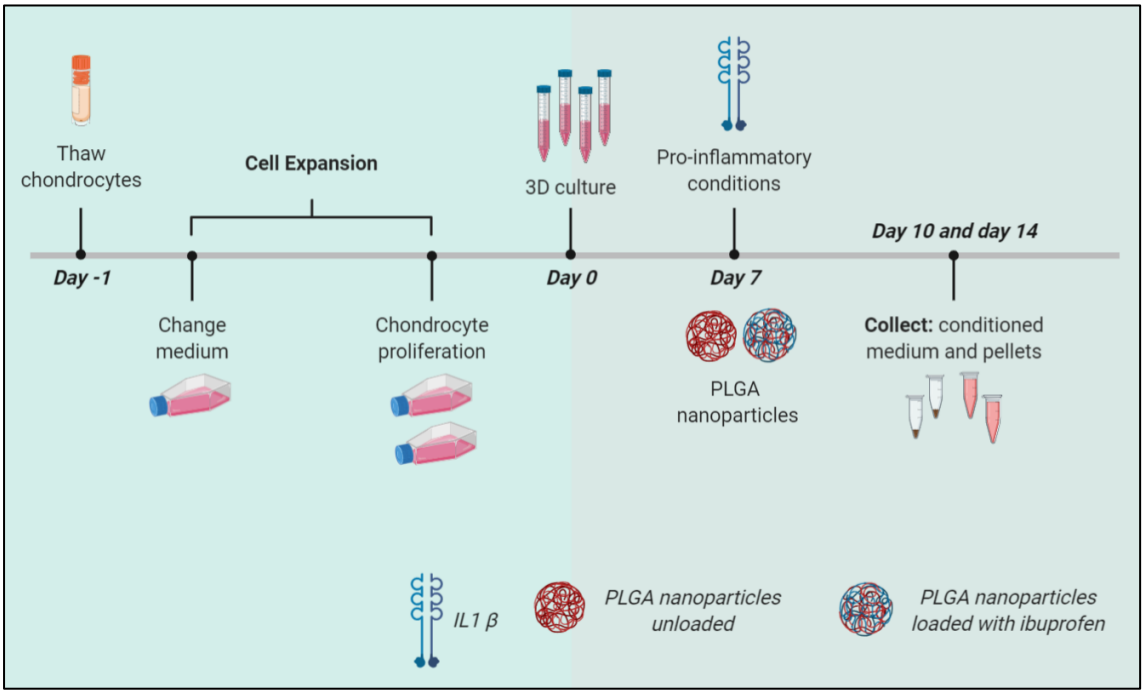

Figure 2.6 - Experimental design used for the PLGA nanoparticles experiment. After being defrosted chondrocytes were expanded in monolayer, in expansion medium. Cells were then seeded in 15mL tubes in basal medium. After 7 days of differentiation, culture medium was supplement both with the proinflammatory cytokine IL1ß, and ibuprofen loaded PLGA nanoparticles. As controls, pellets were cultured under chondrogenic medium only. After 3 and 7 days, pellets and culture medium were collected for analyses.

2.2.5 – Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8 (GraphPad Software, Inc.). All the data were analyzed by non-parametric unpaired Kruskal-Wallis test followed by Dunn's multiple comparison test. Statistical significance was considered for $p < 0.05$ All the data are presented as mean ± SEM.

Chapter 3

3. Results and Discussion

3.1 - Establishment of the 3D primary human chondrocyte cultures

3.1.1 - Chondrocyte isolation from human articular cartilage

To establish the 3D primary human chondrocyte culture, chondrocytes were isolated from human adult cartilage obtained during knee or hip arthroplasties. Tissue samples were collected from the distal and/or posterior femoral condyle of patients, with different gender and age, and with OA. Only cartilage that macroscopically appeared not to be damaged was used to isolate chondrocytes. Collagenase B was used to digest the tissue, in order to remove the chondrocytes from the matrix. This enzyme breaks the peptide bonds of collagen, a major component of cartilage ECM, allowing the chondrocytes to detach from the ECM into the culture medium [76]. The treatment with collagenase B resulted in a mixture of degraded ECM, chondrocytes, and undigested cartilaginous tissue, that was than centrifuged to obtain the chondrocytes.

In each isolation it was obtained around 3 to 4 million cells. As in other chondrocyte extraction protocol, the used protocol has a low yield. The cellular yields of cartilage digestions are typically low, and the results of the procedures may vary due to donor variability, possibly caused by age, gender and/or health status differences among them [77]. The main factor to be taken into consideration during chondrocyte isolation is the exposure time of the cartilage tissue to the enzymatic digestion [77]. The concentration of the enzyme and the incubation time will affect the viability, proliferation and matrix formation capacity of the isolated cells [77]. These parameters were not significantly affected by the used protocol, as dead cells were

almost absent after isolation and as will be described below, cells proliferated and were able to synthetize matrix.

Due to the low yield of the isolation procedure, to obtain the number of cells needed for the experiments to be performed, cells were expanded in monolayer. bFGF, and TGF-ß1 were used to supplement the culture medium, to promote cells proliferation and maintenance of their chondrogenic phenotype [78]. bFGF is a growth factor used in *in vitro* cultures to help chondrocytes maintain their chondrogenic potential when expanded in monolayer [79]. On the other hand, TGF-ß1 is a cytokine that plays an essential role in chondrogenesis, helping to maintain the chondrogenic potential of chondrocytes during *in vitro* cultures, promoting their proliferation, and protecting their morphology by inhibiting the activity of MMPs [80]. As illustrated in figure 3.1, after the isolation, cells presented a round shape morphology, typical of chondrocytes (figure 3.1). However, due to dedifferentiation, during the expansion protocol cells displayed a more fibroblast-like morphology (figure 3.1), and are reported to express markers of pre-chondrogenic mesenchymal cells [81], [82]. Cell dedifferentiation is necessary so that cells are able to proliferate.

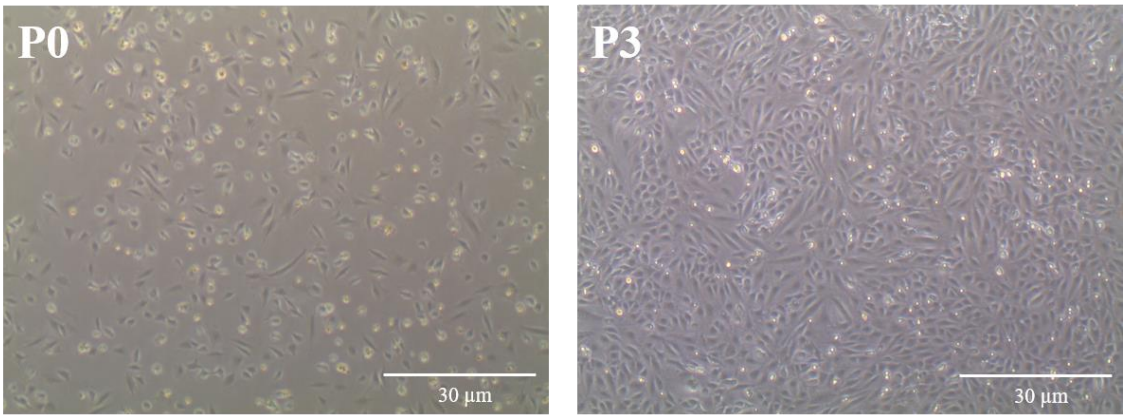

Figure 3.1 - Chondrocyte morphology in monolayer culture. After isolation cells have a rounder shape typical of chondrocytes. During expansion cells morphology evolves into a more fibroblast-like morphology. Scale bar = 30 µm

Chondrocytes were left to expand until they reach sub confluency. It was possible to obtain an average of 15 million cells in each expansion procedure. Freezing the cells after the expansion allowed to create a cell bank, with cells from different donors, to be used in all the planned experiments. This allowed not to be dependent of samples availability from the hospital, to perform the same experience for several donors simultaneously, and to perform different experiments using cells from the same donor.

Overall, the protocol implemented allowed to successfully isolate human chondrocytes, and so far, we were able to isolate chondrocytes from 10 different donors.

3.1.2 - Three-dimensional pellet culture of chondrocytes

Monolayer culture is a simple method for the proliferation of chondrocytes. It is cost-effective and technically simple, allowing the culture of a large number of cells [83]. However, due to the limited proliferation capacity of primary chondrocytes and the tendency to

dedifferentiate after long expansion time and passaging, their culture can be challenging [84] [73]. Chondrocyte dedifferentiation is characterized by the loss of the expression of several ECM molecules, such as collagen type II, and GAGs, and by the increased expression of collagen type I, which indicates an upturn in the fibroblast phenotype [84]. Therefore, cells often expand into a fibrocartilaginous phenotype, rather than articular chondrocytes [29]. Additionally, to gain their ability to expand, cells lose their chondrogenic phenotype, but if culture under appropriated conditions, they are able to maintain their chondrogenic potential [84], [85].

In order to restore the chondrogenic capacity, the culture of expanded primary chondrocytes under 3D culture conditions with the appropriated supplements, such as cytokines and growth factors, has been described to increase the expression of chondrogenic genes, such as collagen type II, aggrecan, and Sox-9 [11], [81], [84]–[87].

In our experiments, cells were routinely expanded up to passage 4, and then cultured in 3D conditions, as pellets, to promote their redifferentiation into chondrocytes. Pellets were formed by centrifugation and cultured in a chondrogenic medium supplemented with several components essential for cell redifferentiation, such as ITS, ascorbic acid 2-phosphate and TGF-β3. The ITS is a complex of insulin, transferrin, and selenium. Insulin, is required for the cellular metabolism of glucose, transferrin, is an essential protein known to transport iron into the cells that will interfere in the hydroxylation of proline during collagen synthesis, and selenium is necessary to protect the cells from oxidative stress. ITS complex is known to regulate articular chondrocytes matrix synthesis [88]. The ascorbic acid 2-phosphate is a requisite cofactor in the production of collagen type II by chondrocytes, and TGF-β3 is a cytokine known to enhance chondrogenesis [89].

To assess whether the cells in the pellets were expressing a chondrogenic phenotype, it was evaluated the size of the pellets, and the expression of typical ECM cartilage compounds, such as collagen type II and aggrecan.

3.1.2.1 - Evolution on pellets shape and size throughout culture of 3D chondrocytes pellets

Due to the production of ECM, pellets are expected to increase in size [90]. Therefore, as an indicator of matrix production pellets size and shape were monitored throughout the culture time. Figure 3.2 illustrates the results obtained for one independent experiment.

To analyze the variations in the pellets size, their surface area was calculated. Figure 3.2B illustrates the size differences of chondrocyte pellets cultured under different conditions. In the basal medium condition (BM-basal medium, without chondrogenic factors), pellets size showed to be smaller and to decrease over the time of culture, when compared with the pellets cultured in the presence of chondrogenic factors present in the chondrogenic medium. These pellets were bigger, and their size increased over the culture time.

Furthermore, the AR was calculated to analyze the pellets shape variations. Figure 3.2C illustrates the AR of the pellets cultured under different conditions. Once again, there was a significant decrease in the AR values for the pellets cultured under basal conditions, and this was not observed in pellets cultured under chondrogenic conditions. Altogether, the data from

size and shape measurements suggests that cells cultured in chondrogenic medium have redifferentiate into chondrocytes, as these cells synthetize higher amounts of ECM.

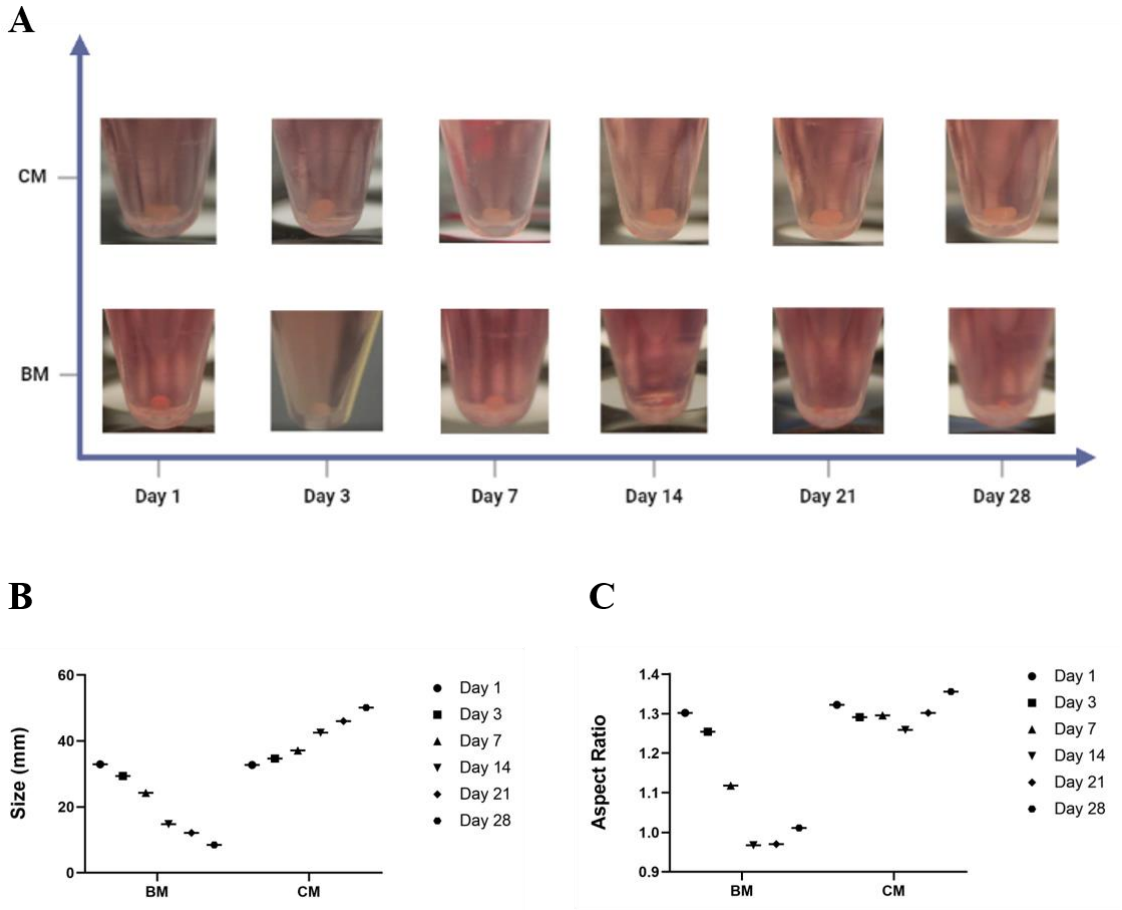

Figure 3.2 – (A) Evolution of the pellets size and shape during the 28 days culture time. (B) The size of the pellets (area delimited by an ellipse) was calculated from the images represented in (A) for the two conditions tested: basal medium (BM) and chondrogenic medium (CM). (C) The AR (quotient between the horizontal and vertical lengths of the pellets) was calculated from the same pellets. Data from one donor is presented.

3.1.2.2 – Analysis of GAGs deposition throughout the culture time of the 3D chondrocyte pellets

To evaluate the capacity of chondrocytes cultured in 3D pellets to produce typical cartilage ECM, an Alcian Blue staining protocol was performed in pellets histological sections. Alcian Blue is a basic dye used to stain acidic polysaccharides, like GAGs, in cartilage. Articular cartilage ECM is composed mainly by a complex of macromolecules such as collagen type II, hyaluronan, and GAGs [24].

As illustrated in figure 3.3, after 3 days of culture it was possible to observe the presence of blue staining in the pellets' section, indicating GAG deposition, and therefore supporting the chondrogenic differentiation of the cells. The blue staining was subsequently observed at least until day 28 of culture (our last evaluation timepoint). Moreover, GAGs deposition appears to be more intense in the pellet's periphery. This may be related to the fact that the cells inside

the pellet are starting to become necrotic, since they do not have free access to the nutrients present in the culture medium.

The replication of this experiment will allow, in the future, to evaluate the differences in the amount of GAGs produced throughout the culture time.

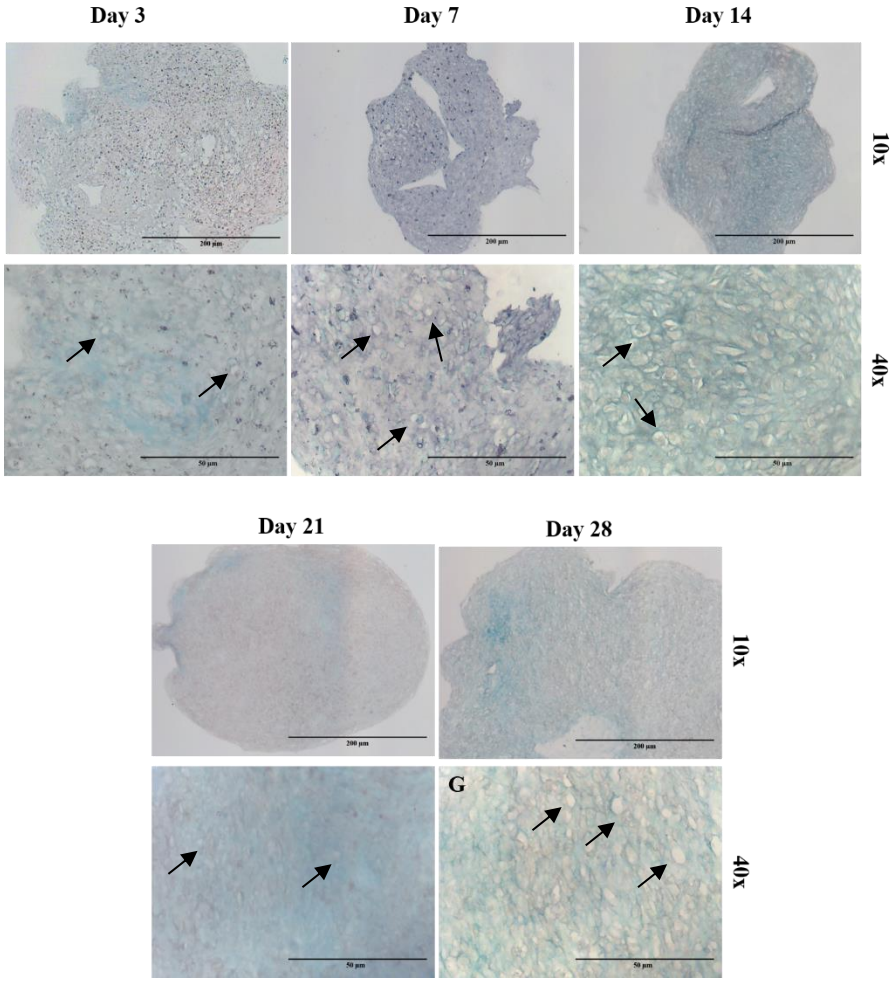

Figure 3.3 - Alcian Blue histological staining in the whole pellet for the 28 days of culture, with a 10x magnificantion (scale bar = 200µm), and 40x (scale bar = 50µm). GAGs deposition was observed by the presence of blue staining. The black arrows highlight rounded-shaped cell morphology.

3.1.2.3 – Detection of Collagen type II and Aggrecan by immunohistochemistry

To confirm pellets chondrogenic phenotype, the expression of chondrogenic markers, such as collagen type II, and aggrecan (major elements of articular cartilage ECM [16], [17]), was assessed by immunofluorescence staining analysis. In figure 3.4 it is possible to observe the positive staining both for collagen type II and aggrecan, indicating that cells were producing cartilage typical ECM.

Moreover, it was also observed an increase in the expression levels of these two chondrogenic markers from day 3 to day 7 of culture. At day 7, the deposition of both collagen type II, and aggrecan is evident in the middle and periphery of the pellet section. The reproduction of this analysis, at longer timepoints of culture, will allow to determine the ability of the culture to maintain the cells' chondrogenic phenotype throughout culture time.

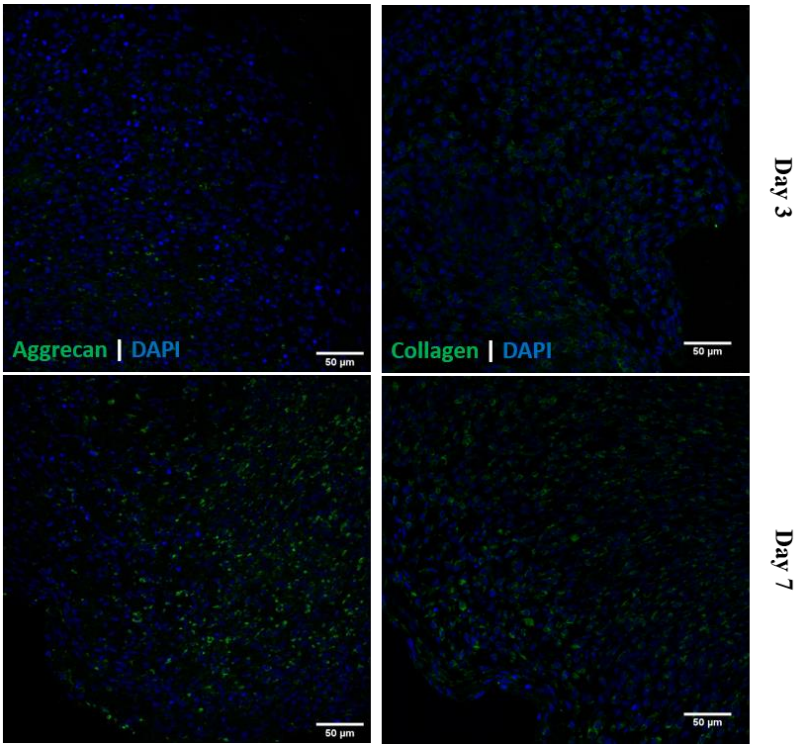

Figure 3.4 - Immunohistochemical staining against aggrecan and collagen type II at days 3 and 7 of chondrocyte pellet cultures. An increase in the stain intensity is observed from day 3 to day 7. Scale bar = 50µm

3.1.2.4 – Evaluation of cytotoxicity throughout the culture time of the 3D chondrocytes pellets

Measuring the activity of cytoplasmic enzymes released by damaged cells is a widely used method to determine cytotoxicity [91]. LDH is a stable cytoplasmic enzyme released into the culture medium when the cells plasma membrane is compromised, has it happens in apoptotic and/or necrotic cells [91].

The LDH assay, is a colorimetric assay characterized by the conversion of a tetrazolium salt into a red formazan product. Therefore, the amount of color formed is proportional to the number of lysed cells. This assay was performed in the pellets cultured for 3, 7, 14, 21, and 28 days, to assess the cytotoxicity throughout the culture time. It was also included a blank control with only medium, and a positive control with 100% cell death. Results obtained for one independent experiment are presented in figure 3.5. The levels of toxicity were maintained throughout the culture time, except for a slight increase from day 21 to day 28, suggesting an increase of the cytotoxicity at later timepoints. The replication of this experiment will allow to confirm the results obtained in this study, and to determine for how long the culture can be maintained without significative cytotoxicity.

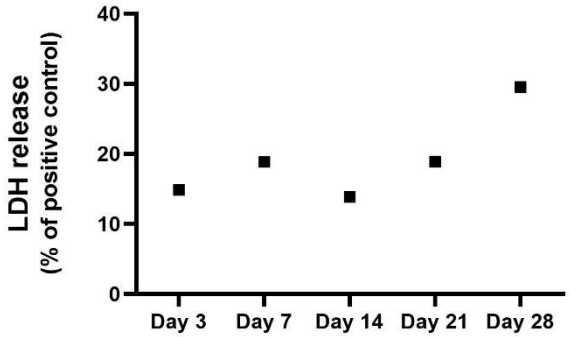

Figure 3.5 - Cytotoxicity assessed by the LDH assay in pellets cultured under two different conditions, basal and chondrogenic medium. The dotted line represents the results obtained for pellets cultured under basal conditions. There is a small increased in the LDH release to the culture supernatant from day 21 to day 28.

Overall, the obtained data support the successful establishment of 3D cultures of human chondrocytes, in which chondrocytes were able to recover their chondrogenic phenotype, and synthesize ECM components, such as collagen type II and aggrecan.

3.2 - Establishment of a proinflammatory environment in the 3D human chondrocyte culture

After an articular cartilage injury, there is an increase in levels of several proinflammatory cytokines, such as TNFα, IL1ß, IL-6, IL-17, and IL-18, at the defect site [38]. The consequent hostile environment disturbs chondrocytes metabolism, inducing a shift from an anabolic metabolism towards catabolism, which will lead to cartilage degradation [37]. TNFα and IL1ß are key players of this proinflammatory environment, promoting cartilage destruction by stimulating the synthesis and release of several MMPs, such as MMP-2 MMP-3, MMP-8, MMP-9, MMP-13, and MMP-14, and ADAMTS, namely ADAMTS4, and ADAMTS5, that will destroy cartilage ECM [92]. Both cytokines are produced by chondrocytes and induce the production of other proinflammatory cytokines [38].

In order to establish a proinflammatory environment, that would mimic a chondral lesion scenario, chondrocytes cultured in 3D pellets were exposed to TNFα and/or IL1ß, in two different concentrations, 10ng/mL, and 100ng/mL, to determine the concentration that would produce the most strong and reproducible proinflammatory environment without affecting cell viability. Overall six conditions were tested i) 10ng/mL of TNFα, ii) 10ng/mL of IL1ß, iii) 10ng/mL of TNFα and of IL1ß, iv) 100ng/mL of TNFα, v) 100ng/mL of IL1ß, and vi) 100ng/mL of TNFα and of IL1ß. The cytokine concentrations used were selected according to the outcomes of previous studies. The use of 10 and 100ng/mL of IL1ß promotes a proinflammatory environment in cultures with macrophages, and chondrocytes from the intervertebral disc, respectively [93]–[95]. TNFα was also tested, since it is reported to be an active player in the proinflammatory environment at the defect site [92].

In this experiment, chondrocytes pellets were formed and differentiated for 7 days before being cultured in proinflammatory conditions. This period of differentiation allowed the cells

to redifferentiate into chondrocytes and produce ECM before being exposed to the proinflammatory stimulus.

3.2.1 - Cytotoxicity induced by proinflammatory stimuli

As a first step, the cytotoxic effect of the proinflammatory stimulation in the pellets culture was assessed.

Once again, the cytotoxicity was evaluated using the LDH cytotoxic assay. The analysis was performed for six different condition, with pellets being cultured in i) chondrogenic medium supplemented with 100 ng/mL IL1β, ii) chondrogenic medium supplemented with 100 ng/mL of TNFα, iii) chondrogenic medium supplemented with 100 ng/mL of both TNFα and IL1β, iv) chondrogenic medium supplemented with 10 ng/mL IL1β, v) chondrogenic medium supplemented with 10 ng/mL of TNFα, and vi) chondrogenic medium supplemented with 10ng/mL of both TNFα and IL1β, for 3 and 7 days of culture. It was also included a blank control with cells cultured without any proinflammatory cytokines. Data presented in figure 3.6 show that the exposure to TNFα or IL1β, in the concentrations of 10 and 100ng/mL, did not induce an increase in LDH release, as compared with the blank control in which no cytokines were added. This suggests that no cytotoxicity was induced in these conditions. However, the conditions in which TNFα and IL1β were used combined, both at 10 and 100ng/mL, presented a significant increase in the LDH release from day 3 to day 7 of culture. Thus, it was concluded that cell death was induced when the cultures were exposed simultaneously to TNFα and IL1β.

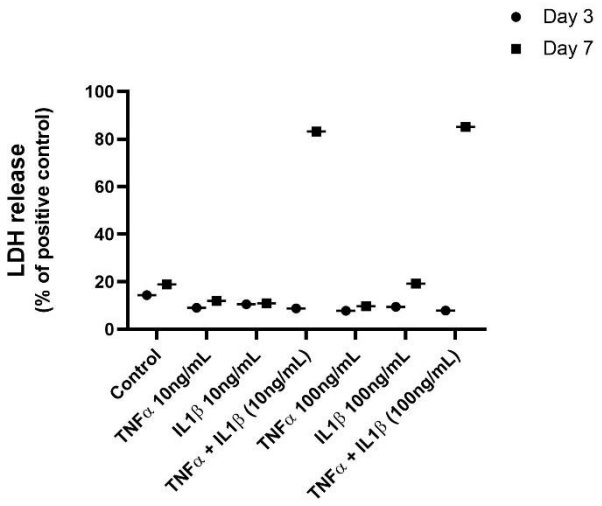

Figure 3.6 – Cytotoxicity assessed by the LDH assay in pellets cultured under inflammatory conditions 3 and 7 days after stimulation with 100 ng/mL IL1β, 100 ng/mL of both TNFα and IL1β, 10 ng/mL TNFα, 10 ng/mL IL1β, and 10 ng/mL of both TNFα and IL1β. The analyses also included a blank control with cells cultured without any proinflammatory cytokines. It is observed that in the conditions where cultures were supplemented with simultaneously with both cytokines there was an increase in the LDH release from day 3 to day 7, indicating that cells are becoming apoptotic and/or necrotic. These data are for one independent experiment.

3.2.2 - Effects of proinflammatory stimuli in the cytokine profile of chondrocyte pellets

Chondrocytes under proinflammatory stimulation have the ability to respond through the production of cytokines, such as IL-10, IL-17, IL-18, IL-6, IL1β, and TNFα [37], [38]. In this study, the IL-6, IL-10, IL1β, and TNFα concentration in the culture medium, at days 3 and 7 after stimulation with the proinflammatory cytokines, was determine by ELISA. TNFα and IL1β are two potent proinflammatory cytokine that stimulate the release of several MMPs that will degrade the ECM [38]. IL-6 is another proinflammatory cytokine and its production is directly stimulated by IL1β and TNFα [38]. It is known to upregulate the expression of MMP-1, and MMP-3, promoting ECM destruction [96]. IL-10 is an anti-inflammatory cytokine produced by chondrocytes in an attempt of reducing the inflammation by antagonizing the action of the matrix degrading enzymes [97].

The quantification of cytokines in the culture medium was performed for all the proinflammatory conditions tested, and for the control with cells cultured without any proinflammatory cytokines. Figure 3.7 illustrates the results obtained for two independent experiments. The obtained data suggest a trend to the increase of the IL1β concentration in the condition 100ng/mL of IL1β, at day 3 (figure 3.7A), when compared with the control. Due to the high variability between experiments, no statistically significant differences were achieved. Concerning TNFα, the concentration levels in the control were found to be below the ELISA kit detection limit for all the tested conditions (figure 3.7B). However, the obtained data indicate no statistically significant differences between the conditions tested. Regarding the IL-6, the obtained data suggest a trend to the increase in these levels in all the proinflammatory conditions, when compared with the control (figure 3.7C). Moreover, it was found that in the condition of stimulation with 100ng/mL of IL1β, the IL-6 concentration levels were significantly higher at day 7, when compared with the control ($p<0,05$), corroborating what has been described in the literature about IL-6 being directly stimulated by IL1β [38]. Lastly, the obtained data indicate that the IL-10 levels were very low for all the conditions (figure 3.7D). Nevertheless, the data seem to suggest an increase in the levels of this cytokine in all the conditions, except for 10ng/mL of TNFα. However, the low number of experiments performed, and the high variability between experiments do not allowed to achieve significant differences. A putative increase in IL-10 synthesis could indicate an attempt of the chondrocytes to minimize the effects of the proinflammatory environment promoted by the proinflammatory stimuli.

Overall, from all the tested conditions the stimulation with 100ng/mL of IL1β was the condition that provided more indication of being able to induce a proinflammatory response by chondrocytes.

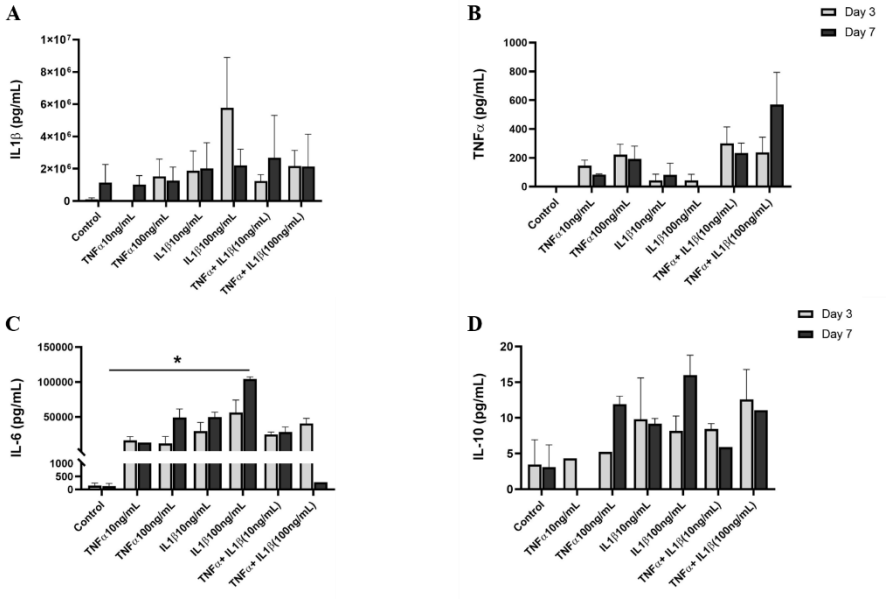

Figure 3.7 – The concentration levels of (A) IL1β, (B) TNFα, (C) IL-6, and (D) IL-10 were assessed under inflammatory conditions 3 and 7 days after stimulation with 100 ng/mL IL1β, 100 ng/mL of both TNFα and IL1β, 10 ng/mL TNFα, 10 ng/mL IL1β, and 10 ng/mL of both TNFα and IL1β. The analyses also included a blank control with pellets cultured without any proinflammatory cytokines. Results are presented as mean ± SEM, n= 2 per group

3.2.3 - Effects of the proinflammatory stimuli in the expression of MMPs and ADAMTS5

As previously mentioned, after an articular cartilage lesion, there is an increase in the levels of proinflammatory cytokines at the defect site [38]. In turn, cytokines, such as TNFα and IL1β, will enhance the synthesis of several proteolytic enzymes, such as MMPs and ADAMTS, that will destroy the ECM [38]. It is described that both MMP-1, MMP-2, and MMP-8 are involved in the degradation of collagen type II, MMP-3 is capable of degrading a wide array of ECM molecules, MMP-13 is employed as a matrix MMP of choice in the detection of OA, and MMP14, has the ability to activate MMP-2 and MMP-13 [92].

ADAMTS are also proteolytic enzymes involved in the cleavage of aggrecan [98]. ADAMTS5, is the major aggrecanase present in articular cartilage involved in the pathogenesis of OA [98].

The expression levels of mRNA for MMPs and ADAMTS-5, for all the proinflammatory conditions tested, were measured by qRT-PCR.

Figure 3.8 illustrates the results obtained for two independent experiments. Regarding the mRNA expression levels of the MMPs investigated, there were no significant changes between the tested proinflammatory conditions, when compared with the control. These results might be a consequence of the low number of experiments performed, and of the variability between donors. Nevertheless, the obtained results showed that chondrocytes were in fact producing MMPs, and that MMP1 and MMP3 were being expressed at higher levels. The mRNA expression levels of ADAMTS5 also presented no significant changes between the conditions, when compared with the control. Once again, this may be due to inter-experiments variability. The number of independent experiments must be increased to overcome this variability constrain, allowing to obtain statistically significant results.

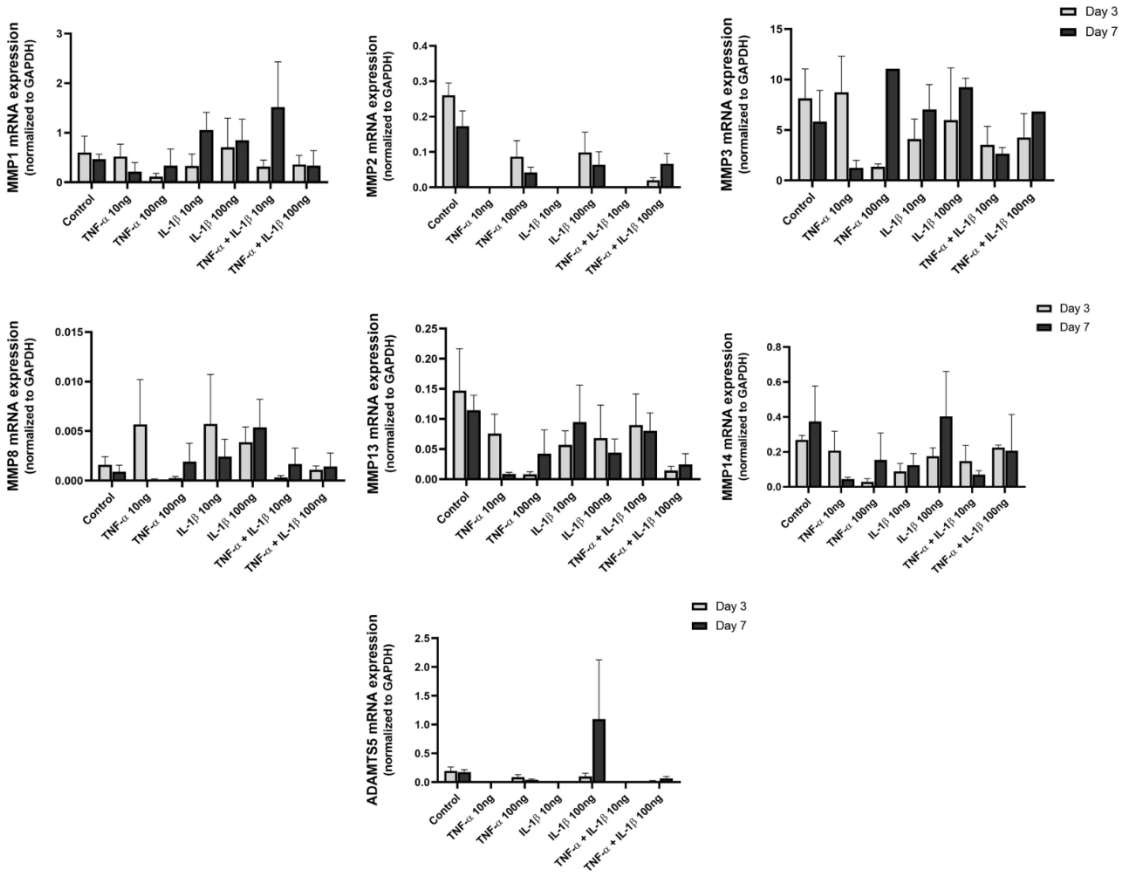

Figure 3.8 - The mRNA expression levels of (A) MMP1, (B) MMP2, (C) MMP8, (D) MMP13, (E) MMP14, and (F) ADAMTS-5 were assessed under inflammatory condition 3 and 7 days after treatment with 100 ng/mL IL1β, 100 ng/mL of both TNFα and IL1β, 10 ng/mL TNFα, 10 ng/mL IL1β, and 10 ng/mL of both TNFα and IL1β. The analyses also included a blank control with cells cultured without any proinflammatory cytokines.There were no significant changes in the mRNA expression levels for all the conditions. Results are presented as mean ± SEM, n= 2 per group.

3.2.4 - Expression of Collagen type II and Aggrecan under proinflammatory stimuli

To evaluate how the proinflammatory stimuli were impacting chondrocytes ECM production, and degradation, the expression of the chondrogenic markers collagen type II, and aggrecan, was assessed in the 3D pellets by immunofluorescence staining analysis.

At early inflammatory stages, chondrocytes can respond to tissue destruction by increasing the synthesis of proteoglycans and collagen type II up to a certain levels of tissue damage [99].

Figure 3.9 and 3.10 illustrate the results acquired for aggrecan and collagen type II, respectively. The results obtained for aggrecan, showed a decrease in the stain intensity, from day 3 to day 7 of culture, in all the conditions, in opposition to the control where there was an increase in stain intensity between the two timepoints (figure 3.9). The decrease in the expression is consistent with the literature about the capacity of IL1β and TNFα to promote cartilage ECM destruction [37]. It was observed that after 3 days of culture the 100ng/mL of IL1β condition presented the lower aggrecan expression levels, and this was also the condition

that induced the lowest aggrecan expression at day 7. These results support the condition 100ng/mL of IL1β as having the highest indicator of ECM degradation.

The results obtained for the collagen type II expression (figure 3.10) show a decrease of the stain intensity from day 3 to day 7 in all the conditions, as observed for the aggrecan expression. In the control, as expected, there was an increase in the collagen type II expression over time. It was observed that the conditions that presented lower collagen type II expression, after 3 days of culture, were the stimulated with IL1β, and the effect was higher in the condition 100ng/mL of IL1β (figure 3.10). In fact, very low levels of positive staining for collagen were observed in the sample after 7 days of culture in this condition.

Overall, the obtained data indicate, for all the proinflammatory conditions, a decrease in the levels of collagen type II and aggrecan throughout the culture time. However, the condition 100ng/mL of IL1β seems to be the most effective in promoting matrix degradation.

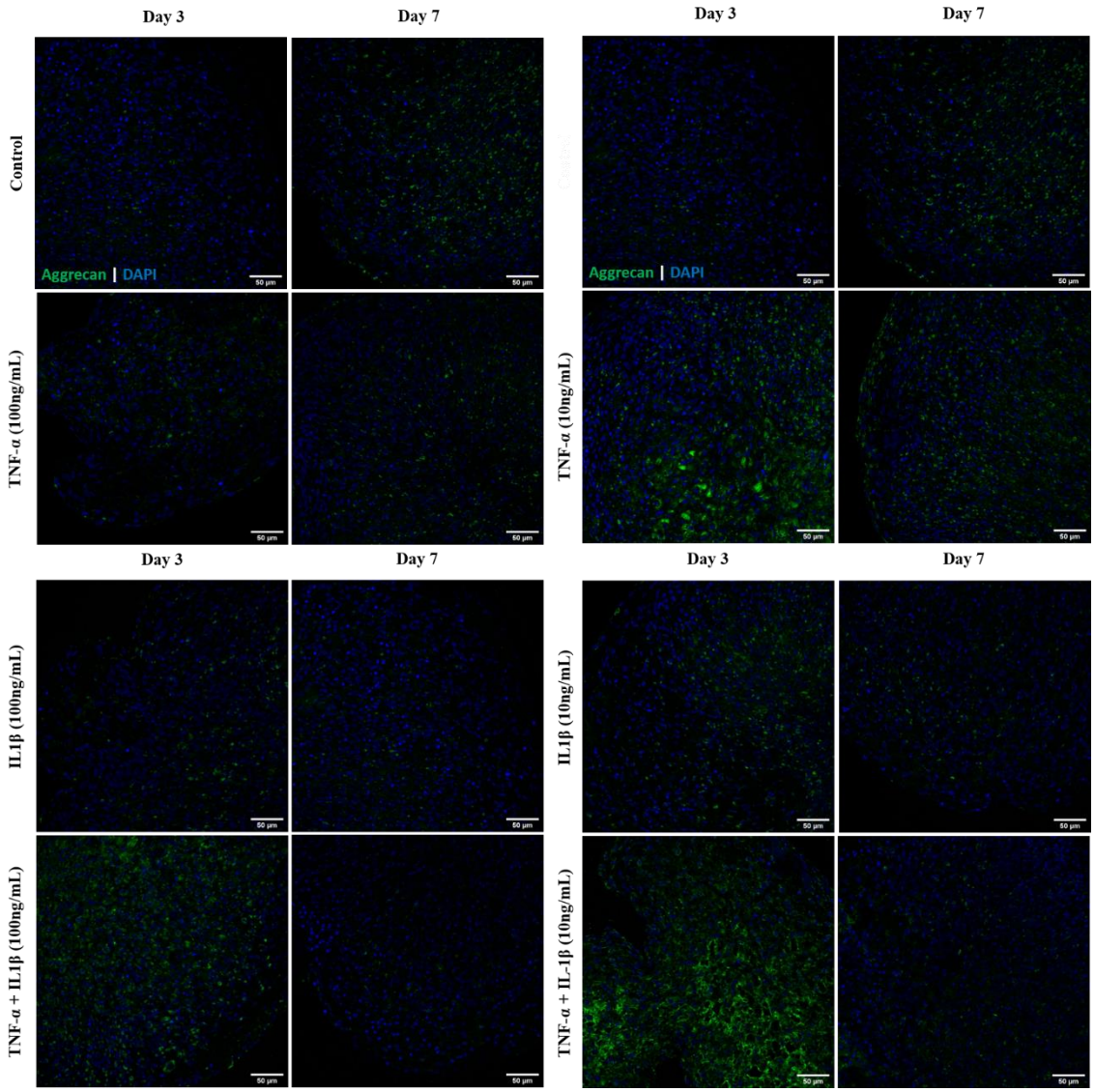

Figure 3.9 - The expression of aggrecan was assessed under inflammatory conditions 3 and 7 days after stimulation with 100 ng/mL IL1β, 100 ng/mL of both TNFα and IL1β, 10 ng/mL TNFα, 10 ng/mL IL1β, and 10 ng/mL of both TNFα and IL1β. The analyses also included a blank control with cells cultured without any proinflammatory cytokines. For both concentrations there was a decrease in the stain intensity from day 3 to day 7 in all the conditions. The condition that presented the lower stain intensity was the 100ng/mL of IL1β. Scale bar = 50μm

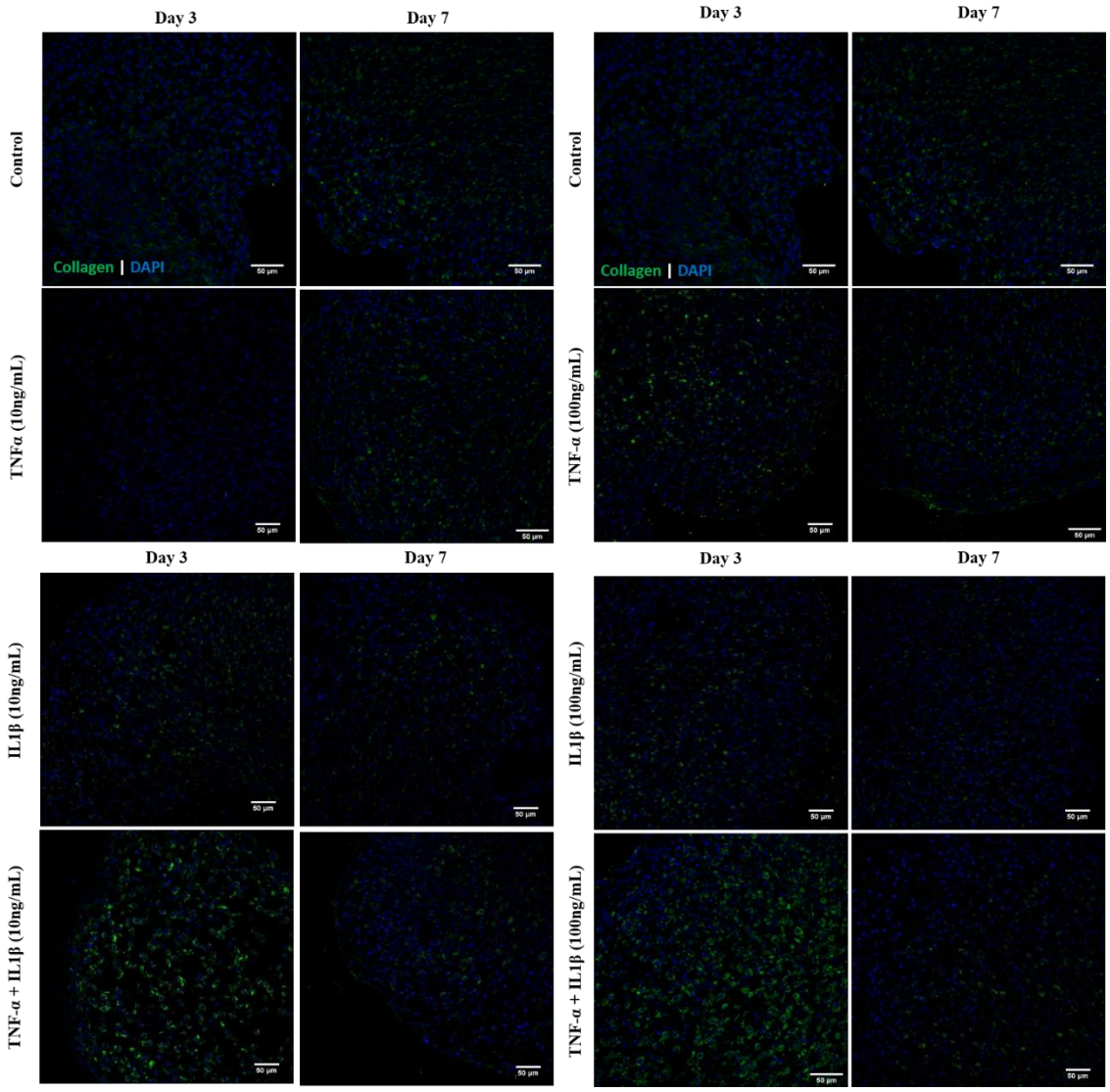

Figure 3.10 - The expression of colagen type II was assessed under inflammatory conditions 3 and 7 days after stimulation with 100 ng/mL IL1ß, 100 ng/mL of both TNFα and IL1ß, 10 ng/mL TNFα, 10 ng/mL IL1ß, and 10 ng/mL of both TNFα and IL1ß. The analyses also included a blank control with cells cultured without any proinflammatory cytokines. For both concentrations there was a decrease in the stain intensity from day 3 to day 7 in all the conditions. The condition that presented the lower stain intensity is the 100ng/mL of IL1ß. Scale bar = 50µm

Altogether, after the analysis of all the outcomes from this experiment, the stimulation with 100ng/mL of IL1ß seems to be the most effective condition for establishing a proinflammatory environment. This condition was not cytotoxic and was the most efficient in promoting matrix degradation (according to the results obtained by the immunohistochemistry analysis).

3.3 - Capacity of the ibuprofen loaded PLGA nanoparticles to inhibit ECM destruction

Articular cartilage lesions lead to joint inflammation and, consequently, to further cartilage deterioration [74]. It is crucial that novel approaches to promote cartilage repair take into consideration chondrocytes inflammatory response [74]. In fact, several anti-inflammatory drugs have been successfully tested, and proved to diminish the inflammatory environment and, subsequently, the ECM destruction [37].

Appropriated drug delivery systems can improve the drug activity, by delivering it directly to the defect site, reducing the toxicity, and improving the diffusion capacity [57], [59].

Polymeric materials have been used for the construction of nanoparticles suitable for targeted delivery of therapeutic drugs [99]. PLGA-based nanoparticles have been shown to be a powerful delivery system [71]. PLGA nanoparticles are biodegradable and biocompatible, protect the drug from degradation, their surface properties can be modified, and are FDA and European Medicine Agent approved as a drug delivery system [71].

In this study the use of ibuprofen loaded PLGA nanoparticles is proposed as a strategy to locally deliver an anti-inflammatory treatment. Ibuprofen is a frequently used anti-inflammatory drug to treat inflammatory pain [43].

Here we evaluated the potential chondroprotective effects of ibuprofen loaded PLGA nanoparticles under inflammatory conditions.

After the establishment of the most effective proinflammatory condition (100ng/mL of IL1ß), 3D chondrocytes pellets were formed and differentiated for 7 days, before being exposed to 100ng/mL of IL1ß, and treated with ibuprofen loaded PLGA nanoparticles. Two different concentrations of ibuprofen loaded PLGA nanoparticles were tested: 100μg/mL of PLGA nanoparticles (15μg/mL of ibuprofen), and 200μg/mL of PLGA nanoparticles (30 μg/mL of ibuprofen).

3.3.1 - Cytotoxicity induced by the ibuprofen loaded PLGA nanoparticles

The cytotoxicity of the PLGA nanoparticles and the ibuprofen in the 3D pellets was evaluated using the LDH assay. The analysis was performed for four different conditions: pellets in inflammatory conditions treated with ibuprofen (15ng/mL), with 100 μg/mL of unloaded PLGA nanoparticles, with 100 μg/mL of PLGA nanoparticles loaded with ibuprofen (15μg/mL of ibuprofen), and with 200μg/mL of PLGA nanoparticles loaded with ibuprofen (30μg/mL of ibuprofen). As controls, pellets were cultured with chondrogenic medium only, or with chondrogenic medium supplemented with 100ng/mL of IL1ß (inflammatory conditions). Figure 3.11 illustrates the obtained results. No significant differences were observed between the tested conditions and the controls, indicating that the PLGA nanoparticles, the ibuprofen, and the combination of both, at the tested concentrations, were not toxic for the chondrocytes.

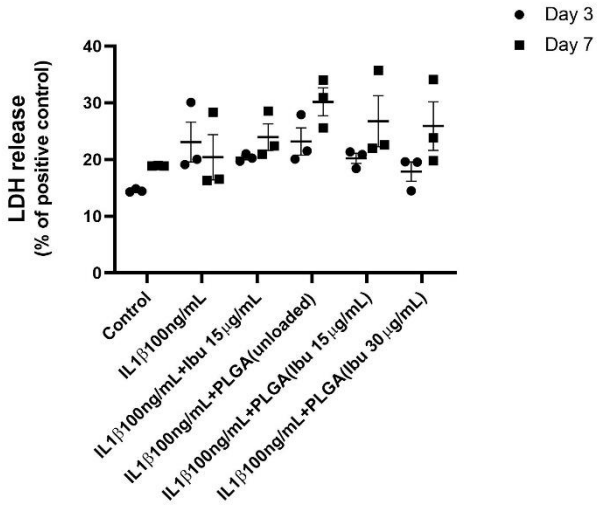

Figure 3.11 – Cytotoxicity assessed by the LDH assay in pellets cultured under inflammatory conditions, and treated with (i) ibuprofen (15μg/mL), (ii) 100 μg/mL of unloaded PLGA nanoparticles, (iii) 100 μg/mL of ibuprofen loaded PLGA nanoparticles (15μg/mL of ibuprofen), and (iv) 200μg/mL of ibuprofen loaded PLGA nanoparticles (30μg/mL of ibuprofen). These results were compared with those obtained in the control conditions: pellets cultured only with chondrogenic medium, and pellets cultured with 100ng/mL of IL1ß (inflammatory condition) Ibuprofen loaded PLGA nanoparticles were not toxic for the chondrocyte. Results are presented as mean ± SEM, n= 3 per group.

3.3.2 - Effects of the treatment with ibuprofen loaded nanoparticles on the cytokine profile

The levels of IL1ß, TNFα IL-6, and IL-10 were assessed by ELISA for the four conditions tested, pellets cultured under proinflammatory conditions treated with i) ibuprofen (15ng/mL), ii) 100ng/mL of unloaded PLGA nanoparticles, iii) 100 ng/mL of ibuprofen loaded PLGA nanoparticles (15μg/mL of ibuprofen), and iv) 200ng/mL of ibuprofen loaded PLGA nanoparticles (30μg/mL of ibuprofen). It was also included a blank control with cells cultured without any proinflammatory cytokines, and a control with chondrogenic medium supplemented with 100ng/mL of IL1ß. Figure 3.12 illustrates the obtained results. The analysis was performed for three independent experiments.

As showed in figure 3.12, although there is a trend to the increase in the IL-10 concentration, at day 7 of culture, in the conditions treated with 100 or 200μg/mL of PLGA nanoparticles loaded with ibuprofen, when compared with the controls no statistically significant differences were observed. The increase in the number of experiments may allow to confirm these observations. Therefore, if this increase is confirmed, it may indicate that the PLGA is protecting the ibuprofen and mediating its release in the target cells, promoting the production of the anti-inflammatory cytokine. In the conditions with the ibuprofen and the PLGA nanoparticles alone, the presence of IL-10 was not detected.

The levels of IL-6 were low in the control, and the stimulation with 100ng/mL of IL1ß largely increased its release (figure 3.12). However, the treatment with ibuprofen or with ibuprofen loaded PLGA nanoparticles did not affect the increase in the release of IL-6 induced by the stimulation with IL1ß.

Concerning the TNFα, the levels were found to be below the ELISA kit detection limit for all the tested conditions.

The IL1ß levels were very low in the control, and there was a huge increase, at day 3, in the condition stimulated with IL1ß ($p<0,05$). It could be argued that the quantified IL1ß could correspond to the concentration that was added as a proinflammatory stimuli, however, the levels measured in this analysis were much higher than the added amount. Interestingly, the treatment with ibuprofen and ibuprofen loaded PLGA nanoparticles induced a decrease in release of IL1ß, at day 3 (Ibu 15µg/mL, PLGA+Ibu 15µg/mL, PLGA+ Ibu 30µg/mL, $p<0,05$), when compared with the condition 100ng/mL IL1ß. However, it was also observed that unloaded PLGA nanoparticles have the same effect ($p<0,05$). Studies have shown that PLGA reduces the synthesis of TNFα by macrophages stimulated with LPS, indicating that PLGA has an anti-inflammatory role [100], [101]. Our findings, showing the reduction of the IL1ß release in condition with unloaded nanoparticles, also support the anti-inflammatory role of the PLGA nanoparticles.

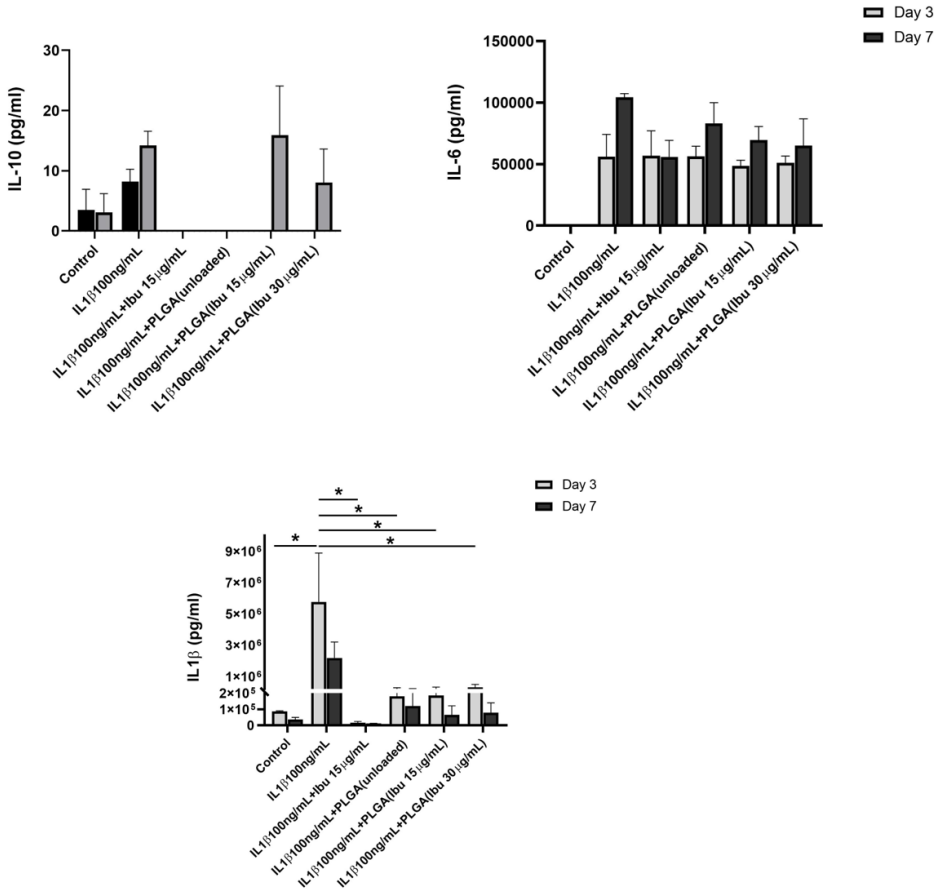

Figure 3.12 - The concentration levels of IL-10, IL-6, and IL1ß, were assessed under inflammatory condition 3 and 7 days after treatment with ibuprofen (15µg/mL), 100 µg/mL of unloaded PLGA nanoparticles, 100 µg/mL of ibuprofen loaded PLGA nanoparticles (15µg/mL of ibuprofen), 200µg/mL of ibuprofen loaded PLGA nanoparticles (30µg/mL of ibuprofen). IL1ß concentration levels decreased in all the conditions, the levels of IL-6 presented no significant changes, and the IL-10 concentration levels increased at day 7 in the conditions with ibuprofen loaded PLGA nanoparticles. Results are presented as mean ± SEM, n= 3 per group.

3.3.3 - Effects of Ibuprofen loaded PLGA nanoparticles in the mRNA expression of MMPs and ADAMTS5 under a proinflammatory condition

The effects of ibuprofen loaded PLGA nanoparticles in the expression levels of mRNA transcripts for MMPs and ADAMTS under a proinflammatory condition (100ng/mL IL1ß) were evaluated by qRT-PCR.

Figure 3.13 presents the results obtained for three independent experiments. The obtained data suggest that the treatment with the ibuprofen loaded nanoparticles, the ibuprofen alone and with the unloaded nanoparticles reduces the mRNA expression levels of all the MMPs tested (MMP-1, MMP-2, MMP-8, MMP-13 and MMP-14). The increase in the number of experiments might allow to achieve statistically significant differences. Not significant effects in the mRNA expression levels of ADAMTS5 were observed for all the treatments.

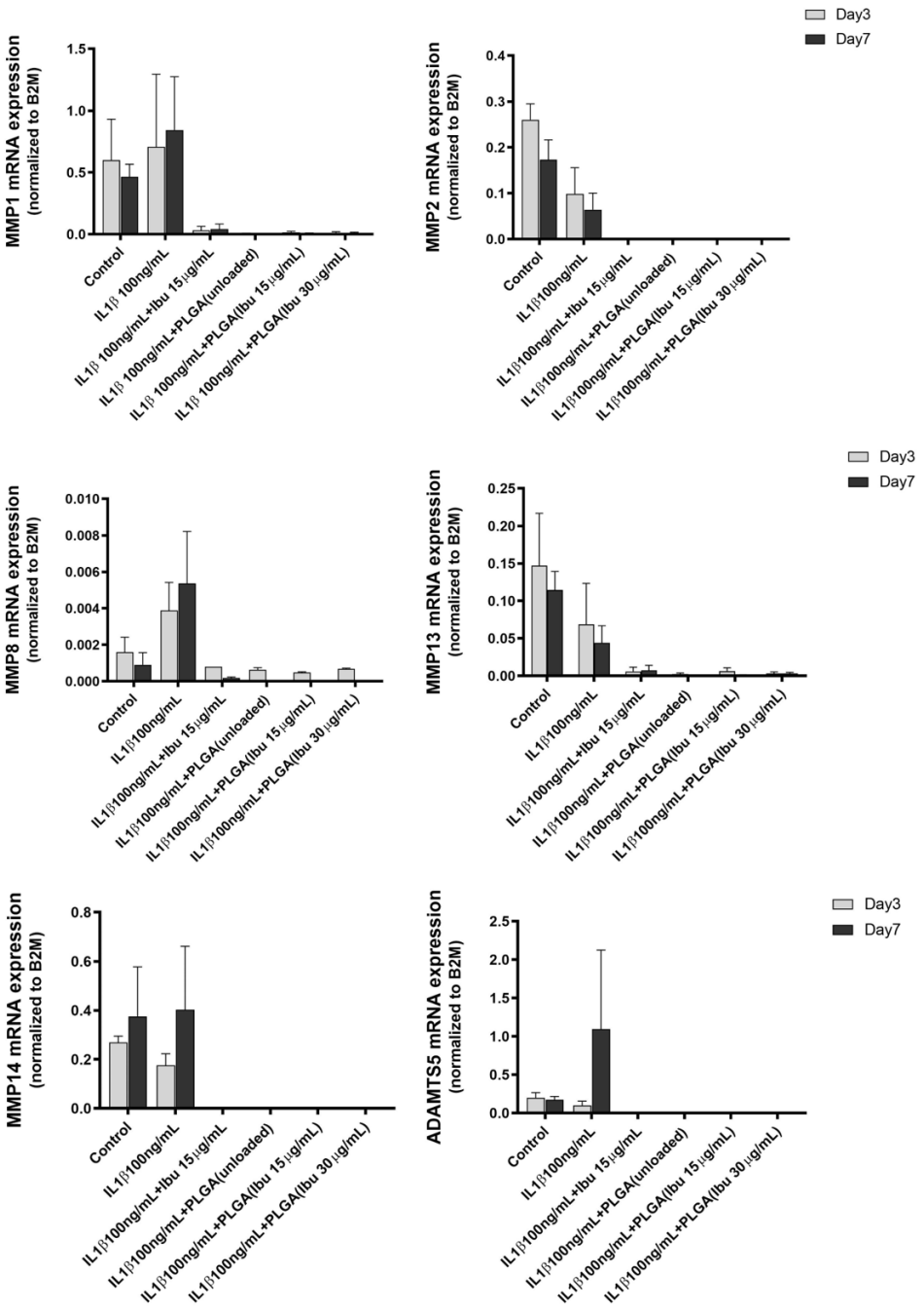

Figure 3.13 – The mRNA expression levels of (A) MMP1, (B) MMP2, (C) MMP8, (D) MMP13, (E) MMP14, and (F) ADAMTS-5 were assessed under inflammatory condition 3 and 7 days after treatment with (i) ibuprofen (15μg/mL), (ii) 100 μg/mL of unloaded PLGA nanoparticles, (iii) 100 μg/mL of ibuprofen loaded PLGA nanoparticles, and (iv) 200μg/mL of ibuprofen loaded PLGA nanoparticles. The MMPs mRNA expression levels decreased in all the treatment conditions tested.There were no significant changes in the mRNA expression levels of ADAMTS5. Results are presented as mean ± SEM, n= 3 per group.

Overall, the obtained results indicate that the treatment with ibuprofen loaded nanoparticles (15 or 30 μg/mL of ibuprofen) leads to a decrease in the mRNA expression levels of MMPs by the chondrocyte pellets cultured under a proinflammatory stimulus (100ng/mL of IL1ß).

3.3.4 - Effects of Ibuprofen loaded PLGA nanoparticles in the expression of Collagen type II and Aggrecan under proinflammatory stimuli

The chondroprotective effects of ibuprofen loaded PLGA nanoparticles under a proinflammatory condition were assessed through the evaluation of their impact on the expression of collagen type II and aggrecan. The analysis was performed by immunohistochemistry.

Figures 3.14 and 3.15 show the results obtained for aggrecan and collagen type II, respectively. In Figure 3.14 it was possible to observe an increase in the aggrecan expression, at day 3, in all the conditions, when compared with the proinflammatory control (supplemented with 100ng/mL of IL1β). This was also observed at day 7, except for the condition with 200μg/mL of ibuprofen loaded PLGA nanoparticles. Nevertheless, at day 7 the stain intensity did not reach the one observed in the control condition.

The results regarding the expression of collagen type II are presented in figure 3.15. Once again, there was an increase in the stain intensity in the non-inflammatory control from day 3 to day 7, and a decrease in the stimulation with IL1β (proinflammatory condition). At day 3, all the treatment conditions presented higher expression levels of collagen type II, when compared with the inflammatory control. However, this high expression was not observed at day 7 in the conditions with the PLGA nanoparticles loaded with 15 and 30 μg/mL of ibuprofen, although there was still positive staining for collagen type II.

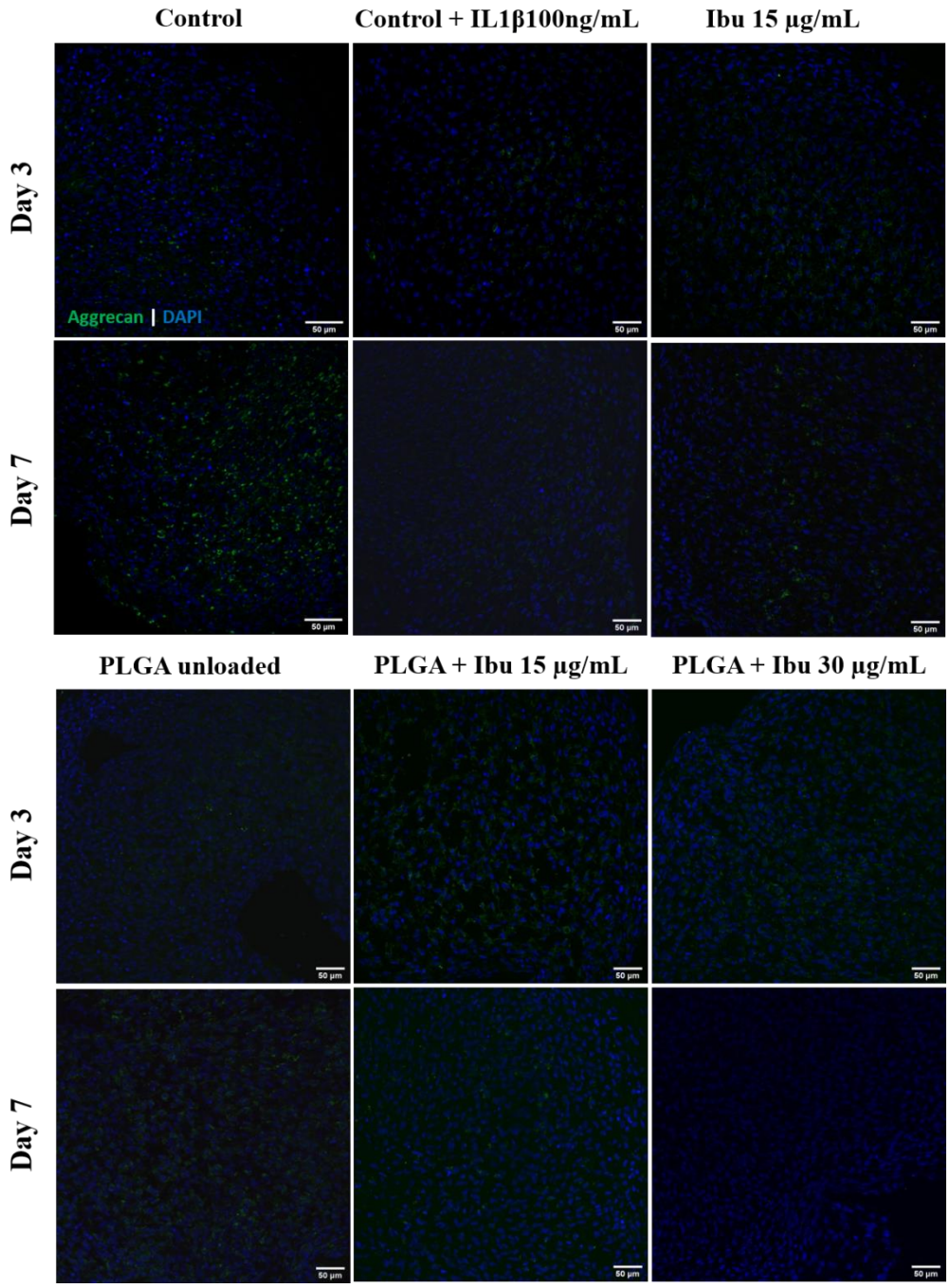

Figure 3.14 - The expression of aggrecan was assessed under inflammatory conditions 3 and 7 days after treatment with (i) ibuprofen (15µg/mL), (ii) 100 µg/mL of unloaded PLGA nanoparticles, (iii) 100 µg/mL of ibuprofen loaded PLGA nanoparticles, and (iv) 200µg/mL of ibuprofen loaded PLGA nanoparticles. The analyses also included a blank control with cells cultured without any proinflammatory cytokines, and with cells cultured with 100ng/mL of IL1ß (inflammatory condition). All the conditions induced, a recovery in the aggrecan expression after exposure to a proinflammatory condition, at day 3. Scale bar = 50µm

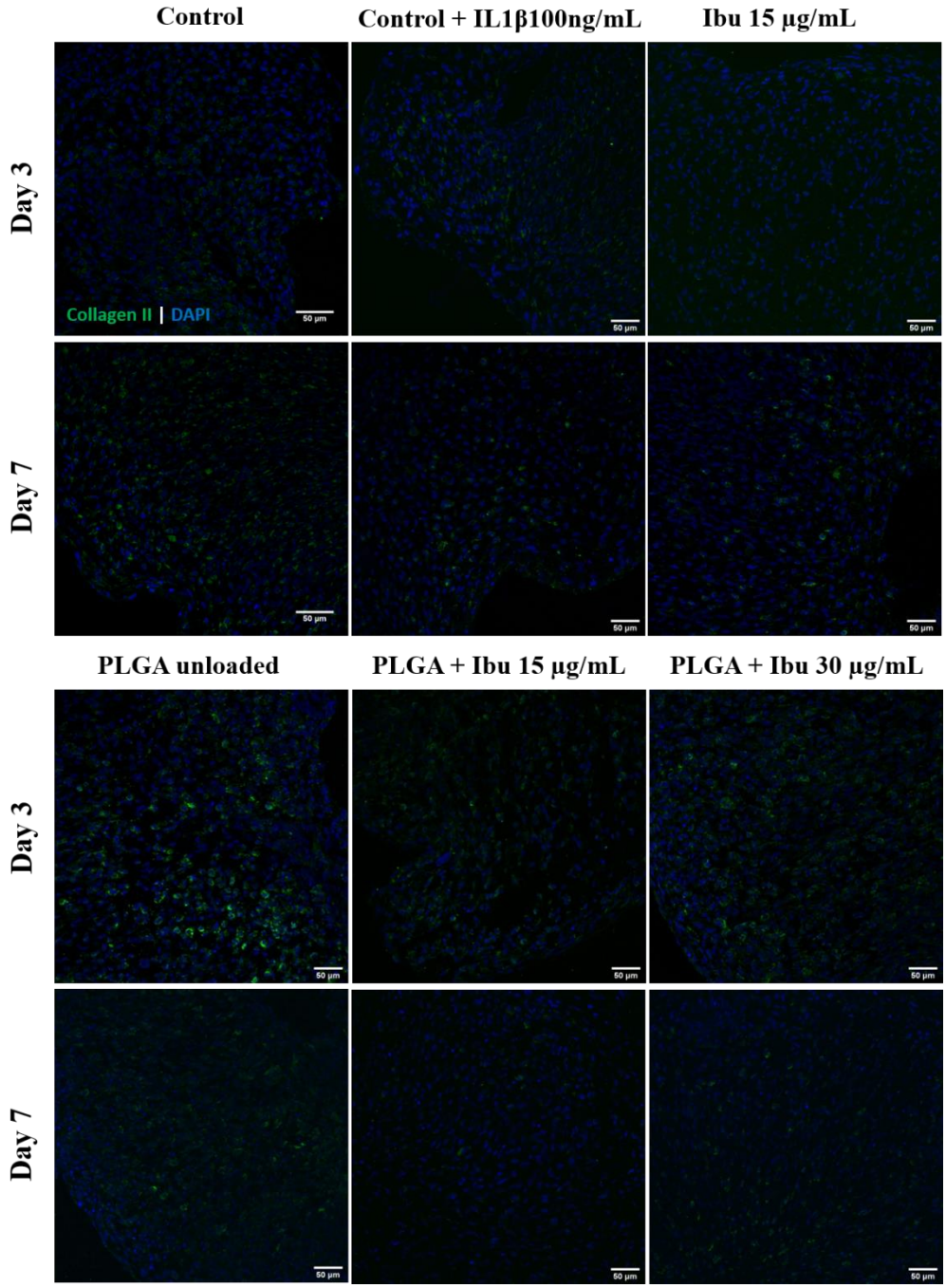

Figure 3.15 - The expression of collagen type II was assessed under inflammatory conditions 3 and 7 days after treatment with (i) ibuprofen (15µg/mL), (ii) 100µg/mL of unloaded PLGA nanoparticles, (iii) 100 µg/mL of ibuprofen loaded PLGA nanoparticles, and (iv) 200µg/mL of ibuprofen loaded PLGA nanoparticles. The analyses also included a blank control with cells cultured without any proinflammatory cytokines, and with cells cultured with 100ng/mL of IL1ß. All the conditions induced, a recovery in the collagen type II expression after exposure to a proinflammatory condition, at day 3. Scale bar = 50µm

Overall, the obtained results suggest that the treatment with the ibuprofen and the PLGA nanoparticles, both together and alone, induces a reduction of the ECM degradation, at day 3. There was also positive staining, for both collagen type II and aggrecan, at day 7 in all the conditions, except for the 200µg/mL of ibuprofen loaded PLGA nanoparticles, where the

presence of aggrecan was not detected. However, the stain intensity did not reach the one presented in the non-inflammatory control.

Altogether, the obtained data suggest that both the ibuprofen and the PLGA nanoparticles, either together or alone, are protecting the chondrocytes from the proinflammatory environment induced by the stimulation with IL1β.

Summing up, the treatment with ibuprofen and PLGA nanoparticles, both together and alone, induced a decrease in the release of IL1β. The presence of collagen type II and aggrecan was higher, at day 3, in the conditions with ibuprofen and PLGA nanoparticles (together and alone), when compared to the inflammatory control (100ng/mL of IL1β). Moreover, the obtained results indicate that the treatment with the ibuprofen loaded PLGA nanoparticles (15 or 30 µg/mL of ibuprofen) leads to a decrease in the mRNA expression levels of MMPs by the chondrocyte pellets cultured under a proinflammatory stimulus (100ng/mL of IL1β).

Overall, our results suggest that treatment with ibuprofen loaded PLGA nanoparticles might be mitigating the deleterious effects of the proinflammatory stimulation on the chondrocytes cultured as 3D pellets.

Chapter 4

4. Conclusion

The work developed for this dissertation allowed to effectively implement an isolation protocol of human chondrocytes. Moreover, the isolation of chondrocytes from several donors, allowed to create a cell bank that provided cells for all the performed experiments, and for those to perform in the future. After expansion in monolayer, the isolated chondrocytes were successfully cultured in 3D pellets, as proved by the expression of typical ECM components, such as collagen type II and aggrecan. The establishment of these cultures was crucial to answer the aim of this dissertation: the evaluation of the chondroprotective effects of ibuprofen loaded PLGA nanoparticles in a proinflammatory environment. To achieve this, first, it was defined a robust and reproductive proinflammatory environment in the 3D pellets culture. Between all the tested conditions using the proinflammatory cytokines IL1β and TNFα, the stimulation with 100ng/mL of IL1β was shown not to be cytotoxic, and to induce the highest increase in the chondrocytes proinflammatory response, causing not only an increase in the concentration of other proinflammatory cytokines, such as IL6, but also a trend to the increase in the synthesis of MMPs. Moreover, it promoted a higher degradation of the ECM. The chondroprotective effects of ibuprofen loaded PLGA nanoparticles was determined in the chondrocyte 3D cultures submitted to this proinflammatory stimulus. The obtained data suggest that the treatment with ibuprofen loaded PLGA nanoparticles reduces the ECM degradation induced by the stimulation with IL1β, as indicated by the increased expression of collagen type II and aggrecan observed 3 days after treatment. This is in line with the trend to the decrease in mRNA expression levels of the ECM degrading enzymes MMP-1,2, and 8 and ADAMTS5, observed 3 and 7 days after the treatment. Moreover, the treatment with ibuprofen loaded PLGA nanoparticles showed a trend to the decrease in the release of the proinflammatory cytokine IL1β by the chondrocytes. Overall, these data suggest that the loaded nanoparticles induce a reduction of the

chondrocytes proinflammatory response, a decrease in the MMPs and aggrecanases expression, and a consequent reduction of the ECM degradation.

Altogether, the obtained results highlight the treatment with ibuprofen loaded PLGA nanoparticles as a putative strategy to protect articular cartilage from the deleterious effects of the inflammatory response triggered by injury.

4.1 – Future Work

As future work, motivated by the promising results obtained with this study, the number of independent experiments will be increased to overcome the high donor variability and confirm the results obtained so far. Furthermore, quantitative analyses of the effects on the expression of chondrogenic markers will be performed, and zymography assays will be implemented to complete the analysis of the ECM degrading enzymes. When definitively proved *in vitro* the chondroprotective effects of the ibuprofen loaded PLGA nanoparticles in proinflammatory scenarios, *in vivo* experiments will be developed. Intra-articular injections of the loaded nanoparticles in an animal model of cartilage defect will allow to further confirm the beneficial effects of this approach in cartilage lesions.

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6 Attachments

In attachment there are two abstracts submitted to the TERMIS congress that will take place in 2021 (attachment 1 and 2), and a review article published in Frontiers in Materials (attachment 3).

6.1 - Attachment 1

IL1β catabolic effect in 3D human chondrocyte pellets is reduced by COPLA® scaffold

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Introduction: Excessive mechanical loading cause inflammation leading to degradation and loss of cartilage (1-2). As cartilage tissue lacks neural connections and vascularization, its ability to self-restoration is highly limited (3). Cartilage repair is one of the most challenging issues in the field of regenerative medicine (4). In cartilage tissue engineering, scaffolds play a vital role providing a three-dimensional (3D) support for chondrocytes cells adhere and proliferate (5). Although diverse materials have been developed for this purpose, cartilage regeneration remains suboptimal. **Objective:** Evaluate 3D human chondrocyte pellets behavior loaded in COPLA® scaffolds, a 3D structure composed of collagen and polylactide (6), in the presence and absence of proinflammatory cytokine IL1β or subjected to excessive mechanical loading (EML) stimulus. **Methods:** The activity of pellets loaded in COPLA®, submitted to IL1β (100ng/mL) stimulus or EML (1Hz) (7), was assessed by cytokine production and immunocytochemistry analysis. **Results:** Here, we show that human chondrocytes where able to adhere, migrate through the COPLA® and produce abundant extracellular matrix (ECM) proteins. In addition, under proinflammatory conditions, 3D pellets seeded in COPLA® retain their size and structure, produce high amounts of anti-inflammatory IL-10 cytokine and express the extracellular anabolic matrix, aggrecan. The effect of EML applied to 3D pellets is currently under investigation. **Conclusions:** Under proinflammatory conditions, COPLA® exerts a protective effect on 3D pellets supported by the maintenance of their structure and production

of ECM. Together our data suggest that IL1β catabolic effect is reduced by COPLA® scaffolds, raising this material as promising approach on cartilage repair.

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6.2 - Attachment 2

Chondroprotective effects of ibuprofen loaded PLGA nanoparticles in 3D human chondrocyte cultures under a proinflammatory stimulus

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Traumatic chondral lesions are common in the young and active population, and if left untreated can evolve towards osteoarthritis. The inflammatory response initiated as a consequence of tissue damage imposes a major challenge in cartilage repair. The released proinflammatory factors induce a shift in chondrocyte activity towards catabolism and promote further tissue degradation (1-2). Several anti-inflammatory drugs have been successfully tested

(2), and we propose ibuprofen-loaded poly(lactic-co-glycolic acid) (PLGA) nanoparticles as a strategy to locally deliver an anti-inflammatory treatment.
Here, we aimed to evaluate the chondroprotective and chondrogenic effects of ibuprofen loaded PLGA nanoparticles. To achieve this, human chondrocytes were cultured as 3D pellets, submitted to a proinflammatory environment (stimulation with 100ng/mL of IL1β), and treated with PLGA nanoparticles loaded with 15 or 30 μg/mL of ibuprofen. Analyses were performed 3 and 7 days after nanoparticles addition.
Our results show that 3 and 7 days treatment with ibuprofen-loaded nanoparticles (15 or 30 μg/mL of ibuprofen) leads to a decrease in both IL1β protein release and metalloproteases mRNA expression (MMP1 and MMP8) by human chondrocyte pellets cultured under a proinflammatory stimulus. Moreover, under similar conditions, 3 days treatment with these nanoparticles induced an increased production of extracellular matrix (ECM) components (collagen type II and aggrecan).
Altogether the obtained data indicate that treatment with ibuprofen loaded PLGA nanoparticles mitigates chondrocytes' proinflammatory response and ECM degradation activity induced in an inflammation scenario. Therefore, this is a promising strategy to overcome catabolic effects of post-traumatic inflammation on cartilage.

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6.3 – Attachment 3

The Mechanisms Underlying the Biological Response to Wear Debris in Periprosthetic Inflammation

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Joint replacement surgery is the gold-standard therapeutic approach to treat patients with end-stage hip and knee arthritis, providing pain relief and joint function recovery. Despite the improvements in implant design and surgical techniques, revisions after total joint replacement are expected to grow. The periprosthetic inflammation, featured by the sustained inflammatory response to the implant debris, elicits the activation of osteoclasts and

consequent periprosthetic osteolysis (PPOL), ultimately leading to implant aseptic loosening, which is the most common cause of long-term implant failure. There are currently no effective strategies to control periprosthetic inflammation, and long-term implant survival remains a major challenge in orthopaedics. A broad knowledge of the mechanisms underlying the biological response to implant debris would support the development of novel and effective pharmacological strategies to manage PPOL and promote implant lifespan. In this review, a detailed description of the cellular and the molecular mechanisms underlying the biological response to implant debris is provided, highlighting the most recent findings. Furthermore, we reviewed novel therapeutic strategies that are being investigated to prevent inflammatory periprosthetic osteolysis.

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