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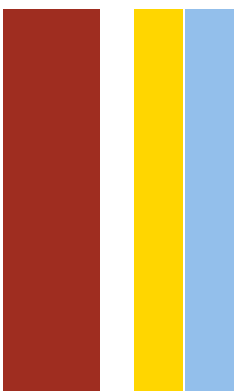

Nanoenabled scaffolds for cartilage repair: the impact of pro-inflammatory mediators

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Abstract

Osteoarthritis is a degenerative joint disease that results in structural and functional failure of synovial joints. It is the most prevalent arthritic disease and a leading cause of disability.

Pro-inflammatory factors such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α or nitric oxide (NO) released upon trauma of articular cartilage or resultant from local joint inflammation, produced by the synovial lining cells stimulates the secretion of catabolic factors (e.g proteinases) leading to cartilage degradation. Since cartilage tissue lacks neural connections and vascular and lymphatic vessels, its ability to self-restoration is highly limited. The repair of damaged cartilage is one of the most challenging issues in the field of regenerative medicine. In cartilage tissue engineering (TE), scaffolds play a vital role providing a three-dimensional (3D) support for chondrocytes to adhere and proliferate.

The introduction of bioactive molecules in these 3D scaffolds that modulate locally tissue/cell response after implantation, represents a promising solution to further improve the TE solutions outcomes. In this project the main aim was to characterize the chondrogenic capacity of the nanoenabled 3D scaffolds-improved through the inclusion of nanocarriers - developed in the framework of the European project, Restore, in presence or absence of the pro-inflammatory cytokine IL-1β.

The chondroprotective effects of the poly(lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) loaded with the anti-inflammatory drug ibuprofen were first evaluated in 3D human articular chondrocyte pellets, submitted to IL-1β (10 or 100 ng/mL) stimulus and treated with the nanocarriers (Ibuprofen-loaded PLGA NPs (PLGA IBU NPs) or Unloaded PLGA NPs (PLGA UNL NPs).

The activity of 3D human chondrocyte pellets was assessed by cytotoxic assays, cytokine production and histological analysis. Under pro-inflammatory conditions, 3D pellets showed a decrease in their size and in matrix deposition (glycosaminoglycans (GAGs)) which is coherent with the effects of IL-1β in the ECM (extracellular matrix) of AC) degradation. Overall, the treatment of 3D pellets stimulated with 100ng/mL of IL-1β with PLGA IBU NPs do not improve GAGs deposition.

On the other hand, the incorporation of these nanoparticles in 3D collagen/poly(lactic acid) scaffolds (nanoenabled scaffolds) appears to induce beneficial chondroprotective effects to primary articular chondrocytes. Here we show that chondrocytes cultured in the nanoenabled scaffolds were able to adhere, migrate and produce abundant ECM proteins. Additionally, under pro-inflammatory conditions (10ng/mL of IL-1β) human chondrocytes seeded in the nanoenabled Col-PLA with PLGA IBU NPs scaffolds express higher levels of the ECM components, aggrecan and type II collagen.

Invasion assays were performed with primary human macrophages stimulated with conditioned media collected from primary articular chondrocytes seeded and

cultured for 7 days in Col-PLA with unloaded PLGA NPs or Col-PLA PLGA IBU NPs scaffolds. The number of macrophages that invaded through the Matrigel coating significantly decreased in the presence of conditioned media from Col-PLA PLGA IBU NPs scaffolds.

Under pro-inflammatory conditions, nanoenabled Col-PLA IBU NPs scaffolds exerts a protective effect on chondrocytes, supported by the production of ECM proteins (aggrecan and type II collagen). Together our data suggest that IL-1β catabolic effect is reduced by nanoenabled Col-PLA IBU NPs, raising this material as promising approach on cartilage repair.

Keywords: osteoarthritis; cartilage degradation; inflammation; tissue engineering

Resumo

A osteoartrite é uma doença articular degenerativa que resulta em falha estrutural e funcional das articulações sinoviais. É a doença artrítica mais prevalente e uma das principais causas de incapacidade.

Fatores pró-inflamatórios como a interleucina (IL)-1β, o fator de necrose tumoral (TNF)-α ou o óxido nítrico (NO) libertados devido a trauma da cartilagem articular ou resultantes de inflamação articular local e produzidos pelas células do revestimento sinovial estimulam a secreção de fatores catabólicos (por exemplo, proteases) que levam à degradação da cartilagem. Como a cartilagem articular (CA) não é inervada nem contém vasos sanguíneos ou linfáticos, a sua capacidade de auto-restauração é altamente limitada. A reparação de lesões de cartilagem é um dos maiores desafios no campo da medicina regenerativa. Na engenharia de tecidos (ET) cartilagíneos, os *scaffolds* desempenham um papel vital, fornecendo um suporte tridimensional (3D) para os condrócitos aderirem e proliferarem.

A introdução de moléculas bioativas nesses *scaffolds* 3D que modulam localmente a resposta tecido/célula após a implantação, representa uma solução promissora para melhorar ainda mais os resultados das soluções de ET. Neste estudo, o principal objetivo foi caracterizar a capacidade condrogénica dos *scaffolds* 3D *nanoenabled* - melhorados através da integração de nanopartículas (NPs), desenvolvidas no âmbito do projeto europeu RESTORE e na presença ou ausência de estímulo pró-inflamatório com IL-1β.

Os efeitos condroprotetores das nanopartículas (NPs) de poli(ácido lático-co-glicólico) (PLGA) carregadas com o fármaco anti-inflamatório ibuprofeno foram primeiramente avaliados em *pellets* de condrócitos articulares humanos 3D, submetidos a IL-1β (10 ou 100 ng/mL) e tratados com as NPs de PLGA carregadas com Ibuprofeno (PLGA IBU NPs) ou com as NPs de PLGA descarregadas (PLGA UNL NPs).

A atividade de *pellets* de condrócitos humanos 3D foi avaliada através de ensaios de citotoxicidade, produção de citocinas e análise histológica. Sob condições pró-inflamatórias, os *pellets* 3D apresentaram diminuição no tamanho e na deposição de matriz (glicosaminoglicanos (GAGs)) o que é coerente com os efeitos da IL-1β na degradação da MEC (matriz extracelular) da CA. No geral, o tratamento de *pellets* 3D estimulados com 100ng/mL de IL-1β com PLGA IBU NPs não melhorou a deposição de GAGs. Por outro lado, a incorporação dessas nanopartículas em *scaffolds* 3D (*scaffolds nanoenabled*) parece induzir efeitos condroprotetores benéficos para os condrócitos articulares primários.

Neste estudo mostramos que os condrócitos cultivados em *scaffolds* de colagénio e poli(ácido lático) (Col-PLA) com PLGA IBU NPs ou PLGA UNL NPs foram capazes de aderir, migrar e produzir proteínas da MEC. Além disso, sob condições pró-inflamatórias

(10ng/mL de IL-1β), os condrócitos humanos cultivados nos Col-PLA com PLGA IBU NPs expressaram níveis mais altos dos componentes da MEC, agrecano e colagénio tipo II.

Ensaios de invasão foram realizados com macrófagos humanos primários estimulados com meios condicionados coletados de condrócitos articulares primários semeados e cultivados por 7 dias em Col-PLA com NPs PLGA descarregadas ou andaimes Col-PLA PLGA IBU NPs. O número de macrófagos que invadiram através do revestimento de Matrigel diminuiu significativamente na presença de meio condicionado de *scaffolds* de NPs Col-PLA PLGA IBU.

Sob condições pró-inflamatórias, os Col-PLA com PLGA IBU NPs exercem um efeito protetor sobre os condrócitos, sugerido pela verificação da produção de proteínas da MEC (agrecano e colágeno tipo II).

Em conjunto, os nossos dados sugerem que o efeito catabólico de IL-1β é reduzido por Col-PLA com PLGA IBU NPs, fazendo este material promissor no reparo da cartilagem.

Palavras-chave: osteoartrite; degradação da cartilagem; inflamação; engenharia de tecidos

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List of Abbreviations

AC	Articular Cartilage
ACL	Anterior Cruciate Ligament
ADAMTS	Aggrecanases
Ang	Angiopoietin
ATP	Adenosine 5'-Triphosphate
bFGF	Basic Fibroblast Growing Factor
BSA	Bovine Serum Albumin
CGRP	Calcitonin Gene-Related Peptide-I
CILP	Cartilage Intermediate-Layer Protein
COL	Collagen
COMP	Cartilage Oligomeric Matrix Protein
COX-2	Cyclo-Oxygenase
CVOCA	Cryopreserved Viable Osteochondral Allograft
DAMPs	Damage-Associated Molecular Patterns
DAPI	4',6-Diamidino-2-Phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Extracellular Matrix
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
GAGs	Glycosaminoglycans
H_2O_2	Hydrogen Peroxide
HA	Hyaluronic Acid
HEPES	(4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid)
HTRA1	High Temperature Requirement A1
IBU	Ibuprofen
IFN	Interferon
IGD	Interglobular Domain
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
JNK	C-Jun N-Terminal Kinase
LAP	Latency-Associated Peptide
LDH	Lactate Dehydrogenase
LIST	Latency-Associated Peptide
LPS	Lipopolysaccharides
M-CSF	Macrophage-Colony Stimulating Factor
MACI	Matrix-Induced Autologous Cartilage Implant
MMP	Matrix Metalloproteinases
NF-ĸB	Nuclear Factor Kappa Light Chain Enhancer of Activated B Cells

NGF	Nerve Growth Factor
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NP	Nanoparticle
NPs	Nanoparticles
NSAID	Non-Steroidal Anti-Inflammatory Drug
OA	Osteoarthritis
$ONOO^-$	Peroxynitrite
PAMP	Pathogen-Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PCM	Pericellular Matrix
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PGA	poly(glycolic acid)
PGE_2	Prostaglandin E2
PIEZO	Piezo Type Mechanosensitive Ion Channel Component
PLA	poly(lactic acid)
PLGA	poly(lactic-co-glycolic acid)
POR	Post-Traumatic Osteoarthritis
PRR	Pattern Recognition Receptors
PTOA	Post-Traumatic Osteoarthritis
RA	Rheumatoid Arthritis
RAGE	Receptor For Advanced Glycation End-Products
RNS	Reactive Nitrogen Species
ROI	Region Of Interest
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute Medium
RT	Room Temperature
SLC	Synovial Lining Cells
Sox	Sry-Box Transcription Factor
SP	Substance P
TE	Tissue Engineering
TGF	Transforming Growth Factor
TGF-β	Transforming Growth Factor-Beta
TIMPs	Tissue Inhibitor of Metalloproteinases
TLR	Toll-Like Receptors
TNF-α	Tumor Necrosis Factor Alpha
TRPV4	Transient Receptor Potential Vanilloid 4
VEGF	Vascular Endothelial Growth Factor

1. Introduction

1.1. Joint Biology

Synovial or diarthrodial joints are freely moving joints in which the articulating bone surfaces are covered in smooth articular cartilage (AC) and separated by a film of viscous synovial fluid [1]. The synovial joint is a complex anatomical assembly that includes several types of structures that interact with each other. These structures include the synovium, muscles, tendons, ligaments, bursae, menisci, AC, and subchondral bone [2, 3].

Of note, the synovium, or the synovial membrane, is a structure of the joint that is responsible for providing nutrients and producing lubricants, such as hyaluronan and lubricin, for the joint. It has two separate layers of cells: the intima, or synovial lining, that is interface between the cavity containing the synovial fluid and the subintimal layer. The intima is composed of two populations of different synovial lining cells (SLC) — type A, or macrophage-like cells; and type B, or fibroblast-like cells [4]. Synovial fibroblasts have intense synthetic capacity producing essential joint lubricants such as hyaluronic acid (HA) and lubricin.

The cells of the intimal and the subintimal layer of the synovium are not completely separated by a basement membrane as would happen in epithelial tissues, however most components of these membrane are present in the extra cellular matrix (ECM) that surrounds the SLCs.

Opposed to AC the subintimal synovial layer is provided of many small blood and lymphatic vessels, shared partly by the joint capsule, epiphyseal bone, and other perisynovial structures, present in both normal and arthritic synovial tissues. In patients with osteoarthritis (OA), lymphatic vessels were detected by Xu, Edwards [5] in all regions of the synovial membrane [6]. The presence of blood and lymphatic vessels is important since the synthetic activity of the cells that compose the synovial membrane requires a supply of nutrients and efflux of cell metabolism products.

1.2. AC

AC is a specialized connective tissue found in the articular ends of bone in synovial joints and is considered to have only one cell type, the chondrocytes. Healthy AC lacks vascularization, lymphatics, and innervation [7, 8]. It provides a weight-bearing, compression resistant, smooth, and low-friction surface for movement between bones [9]. This tissue is composed of an abundant ECM, with chondrocytes only accounting for 3% to 5% of the total cartilage wet weight, with the rest being ECM and water (

Figure 1).

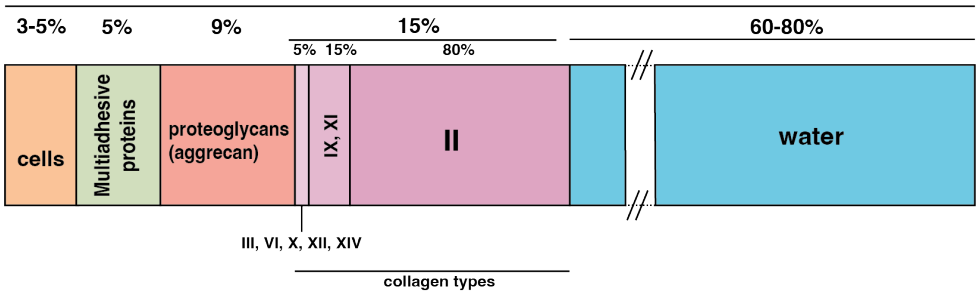

Figure 1. Composition of AC – Composition and weight percentage distribution of the different components of AC. Adapted from Ross and Pawlina [10].

Due to the lack of vascularization in AC and the poor oxygen and nutrient diffusion through the dense ECM, articular chondrocyte metabolism operates at a low oxygen tension with metabolism restricted by low rates of anaerobic glycolysis, with aerobic glycolysis (Warburg effect, usually found in cancer cells [11]) also occurring in normal chondrocytes concomitantly [12].Chondrocytes are not distributed evenly in AC. In fact, in AC we can distinguish different zones according to the distance to the articulating surface (depth), thereby we have the superficial, the intermediate (middle or transitional), the radial (or deep), and the calcified zones. This zonation does not only consider the depth, but also the presence of specific proteins and the arrangement of the ECM collagen fibrils (the ECM composition will be discussed further with more detail) [8, 13]. Additionally, chondrocytes can be found isolated or in isogenous groups. Chondrocytes in isogenous groups have recently divided and disperse with the production of new matrix material around them.

The chondrocytes of the superficial zone produce a specific protein - superficial zone protein (SZP) which is thought to improve the frictional properties of the tissue. This zone is characterized by having densely packed collagen fibers that are parallel to the cartilage surface and the proteoglycans content is low, containing a small number of flattened chondrocytes [10]. Hayes, MacPherson [14] have shown that this zone has progenitor cells that allow for some cartilage growth in layers which does not happen in the deepest layers [15].

The middle zone represents 40% to 60% of all AC weight. This zone's chondrocytes express cartilage intermediate-layer protein (CILP) and are organized in stacks perpendicular to the tissue synovial surface. The ECM is abundant and has all the typical molecules of the ECM of AC being mainly composed by collagen type II, and aggrecan that give the tissue biomechanical resistance and stability. Collagen fibrils are arranged in an oblique fashion. Chondrocytes have a spheroidal shape and do not follow a specific arrangement [16].

Bellow the middle zone, the deep zone has the lowest cell density despite being the bulkiest one. The chondrocytes in this zone tend to be the largest ones and the most active on matrix production. These are likely responsible for the interplay between cartilage and the subchondral bone, that lies beneath it [16, 17]. Collagen fibrils become

thicker, run perpendicular to the articulating surface and the proteoglycans content is high [15].

1.3. The composition of AC's ECM dictates its mechanical features

As shown in

Figure 1 the ECM of AC is composed by several molecules, being rich in collagen, proteoglycans, and other low-abundance components such as fibulin and elastin. It constitutes the physical substrate for the normal cellular and molecular processes of chondrocytes while also providing the mechanical properties that allow for the physiological role of AC [18].

The collagens of AC have a high level of structural organization forming a fibrous structure. The major component of this network is collagen type II that provides tensile strength and stiffness with low resistance to compression. It is the major structural protein of cartilage [19].

Table 1 – Collagens of AC

Key-function collagens present in AC and/or contributing to its homeostasis	
Collagen Type	**Function**
I	Mainly found in healthy subchondral bone [20].
II	Major component of cartilage collagens. Provides tensile strength to cartilage. It is cartilage specific [21].
IX	It is both a proteoglycan and a collagen because it contains a chondroitin sulfate chain attachment site in one of its non-collagen domains. Stabilizes the network of cartilage type II collagen fibers by interaction with proteoglycan molecules at their intersections [10, 21].
VI	Main component of the pericellular matrix and the anchor to other ECM molecules such as hyaluronan, biglycan or decorin (discussed further) [22].

Other key-function collagen present in AC and/or contributing to its homeostasis are type I collagen, which is mainly found in healthy subchondral bone; type VI collagen which is the main component of the pericellular matrix and the anchor to other ECM molecules such as hyaluronan, biglycan or decorin; and type IX that is both a proteoglycan and a collagen because it contains a chondroitin sulfate chain attachment site in one of its non-collagen domains. It stabilizes the network of cartilage type II collagen fibers by interaction with proteoglycan molecules at their intersections. The different collagen types and the main role in AC are explained in more detail in Table 1.

The major proteoglycan found in AC is aggrecan. Like all other proteoglycans, it is composed by a core protein with several strains of glycosaminoglycans (GAGs)

attached to it. However, aggrecan molecules are not found in isolation, instead they are found in aggregates. Each aggregate is composed of a central filament of HA with multiple aggrecan molecules attached to it non-covalently via one end of their core proteins. At high concentrations aggrecans cause a high osmotic swelling pressure due to the negatively charged anionic groups in the GAGs and the mobile ions they carry as counter charges, such as sodium (Na^+). This causes an osmotic imbalance between the cartilage and the surrounding tissues. In response, water is drawn into cartilage, associating with the GAGs making the matrix swell and expand. Upon compression of the cartilage tissue there is displacement of water to the outside which brings the GAG chains closer together. This increases the swelling potential of the aggrecan, and upon removal of the compression the aggrecan will re-swell and restore the original equilibrium [23, 24]. This is the mechanism that allows AC to regain its original shape in physiological conditions, after compression.

The mechanobiology of AC is intimately connected to the physical properties of the molecules that compose the ECM. The collagens and proteoglycans present in AC form important networks between themselves, combining the individual mechanical properties that each contain. Collagen fibrils have a high tensile stiffness and strength but are weak under compression [25] while proteoglycans confer resistance to compression by the mechanism described in the last paragraph and by the drag effect caused by the movement of water through the porous meshwork formed by the aggregates. This drag effect allows cartilage to dissipate de energy created upon compression. Collagen fibers restrain the swelling pressure introduced by proteoglycans and further determine the water amount in the tissue while also resisting the tension that can occur when two AC surfaces slide across one another and pull in a single direction [26].

The study of the mechanics of cartilage ECM is important for the understanding of the different pathologies that affect cartilage, since in diseases like OA and Rheumatoid Arthritis (RA) important functional changes occur in the ECM.

1.3.1 The pericellular matrix and chondrocyte interaction with the ECM

The cartilage ECM is not homogenous, having different relative concentrations of proteoglycans. Based on the staining of proteoglycans, three regions can be described: the pericellular or capsular matrix, which is a ring of densely stained matrix immediately around the chondrocyte; the territorial matrix that that is more removed from the immediate vicinity of the chondrocytes surrounding the isogenous group and contains a randomly arranged network of type II collagen; and the interterritorial matrix which corresponds to the bulk of the ECM. The spatial arrangement of these different ECM zones and the isogenous groups are represented in Figure 2.

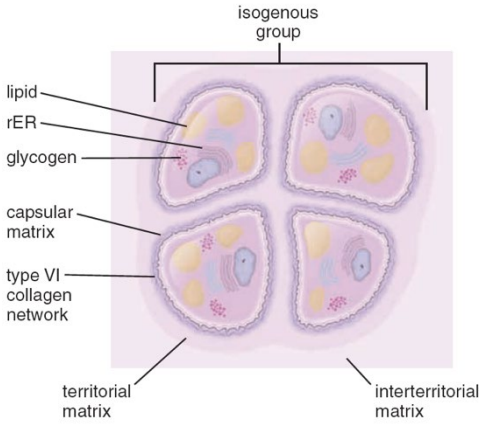

Figure 2. Isogenous groups of cartilage – Representation of the isogenous groups and the different types of AC regions of the ECM. Image from Ross and Pawlina [10]

The pericellular matrix (PCM), has a significantly different composition and structure and a higher matrix turnover, than the other regions of the ECM [21]. The PCM is characterized by the containing almost exclusively type VI collagen, but also other important components, including perlecan, aggrecan, hyaluronan, biglycan, type IX collagen, laminin and fibronectin [27]. The unit composed by the chondrocyte and the pericellular matrix is called the chondron.

The chondrocytes express many integrins that interact with ECM ligands, however these are not specific to this type of cells. Integrins bind specifically to different cartilage matrix components and induce the formation of intra-cellular signaling complexes that regulate cell proliferation, differentiation, survival, and matrix remodeling. Integrins may also serve as mechanoreceptors that mediate responses to normal and abnormal loading of cartilage [28].

The importance of the presence of these integrins as mechanoreceptors lies in the fact that chondrocytes respond to mechanical cues transmitted to them through the ECM. Integrins are the final transducers of these stimuli through the “molecular clutch” mechanism. Since forces applied to cartilage are transmitted to chondrocytes through the ECM, the maintenance of the equilibrium between anabolic and catabolic factors is highly regulated by the forces that chondrocytes experience and the signaling pathways directly controlled by the mechanoreceptors [27].

For example, integrins containing an αV subunit bind a latency-associated peptide (LAP) which binds transforming growth factor-beta (TGF-β) in inactive form that remains in the pericellular matrix after being produced by chondrocytes. Forces >40pN change the conformation of LAP releasing active TGF-β and activating downstream pathways controlled by this growth factor. This change in the distribution of TGF-β activity in AC dysregulates the normal physiology of the latter rendering it mechanically and biochemically unstable, more prone to pathology [29].

Additionally, a part of the chondrocyte’s machinery involved in mechanosignaling are the ionic channels, particularly the different channels found in the diverse chondrocyte channelome [30]. These channels not only participate in signaling,

but also serve diverse functions in the homeostasis of the chondrocyte, namely controlling the flux of ions such as Na^+, calcium (Ca^{2+}), chloride (Cl^-), during water movement to and from the cartilage during normal loading cycles [31].
Calcium plays an important role in mechanotransduction, with transient receptor potential vanilloid 4 (TRPV4) and piezo type mechanosensitive ion channel component (Piezo) 1 and 2 being arguably the most important [32].

TRPV4 can be activated through osmotic changes, which as mentioned previously, are triggered by changes in the load that is applied in cartilage . They play an important role in Ca^{2+} regulation in non-excitable cells [33]. Piezo 1 and Piezo 2 (collectively – PIEZOs) can be directly activated by mechanical forces and contribute to cell uptake of Ca^{2+} when cartilage is stimulated at higher strains or an hyperphysiologic level (injury or high strain). In fact, a study by Du, Li [34] has shown that TRPV4 is associated with transduction via Ca^{2+} signaling of low-strain forces while PIEZOs are associated with higher strains [35].

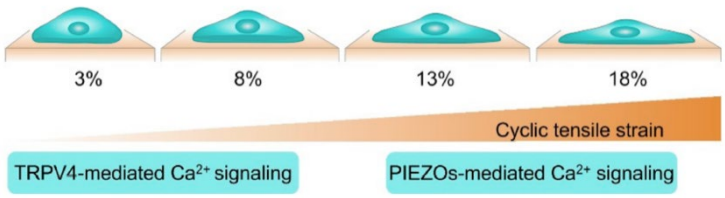

Figure 3. Calcium channels in mechanosignaling - TRPV4 or PIEZOs-mediated Ca^{2+} signaling under different cyclic tensile strengths. Image from Zhang, Meng [35].

Lee and colleagues [36], have found that pro-inflammatory IL-1α upregulated the expression of Piezo1 in porcine cartilage explants leading to an increase in Ca^{2+} influx and higher basal [Ca^{2+}]. This increase in intracellular Ca^{2+} may promote changes in the regulation of different intracellular messenger systems, which are directly regulated by Ca^{2+}. Downstream, this can cause changes in the regulation of chondrocyte's genes (e.g., SRY-Box transcription factor (Sox), nuclear factor kappa light chain enhancer of activated B cells (NF-kB)), altering the phenotype e.g., differentiation, proliferation, anabolic activity to maintain the ECM, or apoptosis.

1.4. AC degeneration and OA

AC homeostasis depends on the chondrocyte to maintain a stable equilibrium between matrix synthesis and degradation. When this equilibrium ceases or when there is damage to AC, these cells try to compensate by proliferating and increasing the synthesis of the several matrix constituents [37]. However, this "activation" is not always effective and usually renders a matrix of inferior quality. Eventually, these repair attempts will be surpassed by the rate of degradation of matrix progressing to pathology [38].

Osteoarthritis is, thus, the degenerative joint disease that results in structural and functional failure of synovial joints [39]. It is characterized by degradation of the AC,

hypertrophy of bone at the margins (i.e., osteophytes), subchondral sclerosis, and a range of biochemical and morphologic alterations of the synovial membrane and joint capsule [21].

1.5. Risk factors associated with OA

The risk of OA is majorly affected by factors such as ageing, obesity (metabolic syndrome), joint malalignment and location, and trauma.

1.5.1 Ageing

Increasing age is considered one of the most prominent risk factors associated with OA. It is associated with a chronic low inflammation state, also called 'inflammaging'. The main hallmarks of ageing, described by Lopez-Otin, Blasco [40] are genomic instability, telomere attrition[1], epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communications.

With ageing, the efficacy of the respiratory chain tends to diminish, thus increasing electron leakage and reducing adenosine 5'-triphospahte (ATP) generation. Progressive mitochondrial dysfunction that occurs with aging results in increased production of reactive oxygen species (ROS). The increase in ROS is associated with the production of pro-inflammatory cytokines such as IL-1β and upregulation of the pathways that lead to the production of matrix metalloproteinases (MMPs), causing matrix destruction. Further, due to the great oxidative capacity of the different ROS, the ECM may also be degraded directly by these compounds [41, 42].

Cellular senescence is defined as the arrest of the cell cycle associated to stereotyped changes in cell phenotype. It is thought of as mechanism that prevents the proliferation of damaged cells that could eventually progress to cancer cells. However, despite not proliferative, these cells are still metabolically active, but with an altered secretome that stems from their propensity to secrete pro-inflammatory cytokines. These cells contain several factors that contribute to the overactivation of the NF-kB and the downstream inflammatory pathways eventually releasing IL-1β and TNF-α. The accumulation of senescent cells in tissues contributes, thus, to the general low-grade inflammatory state mentioned above [43, 44].

1.5.2 Obesity and Metabolic Syndrome

Obesity and overweight are a key feature of metabolic syndrome [45]. In metabolic syndrome there is increase adipose tissue mass due to obesity. The adipocytes and resident macrophages of the adipose tissue secrete pro-inflammatory

[1] Telomere attrition: The shortening and deterioration of the protective caps on the ends of chromosomes that is associated with ageing.

adipokines[2] such as IL-6 and TNF-α which are distributed in the general circulation [46]. Resident macrophages of the adipose tissue generally express markers of an M2 or alternatively activated state however in obesity there is recruitment and accumulation of M1 or classically activated macrophages, contributing to the production of the adipokines [47]. Summing up, obesity and metabolic syndrome provoke a generalized inflammatory state that affect cartilage tissue homeostasis.

1.5.3 Trauma

Trauma to AC or surrounding tissues in the joint may lead to post-traumatic osteoarthritis. Post-traumatic osteoarthritis (PTOA) develops following an injurious event to a joint. Individuals who suffer traumatic injury to a joint have more than a fivefold increased risk of developing the disease which is much higher than the 1.7 fold risk associated with overweight or obesity, which is considered a major risk factor [48]. PTOA accounts for 12% of all cause of symptomatic OA.

Damage to AC resulting from impaction is common after joint injuries. In the case of the knee joint, almost half of the anterior cruciate ligament (ACL) tears present structural damage to the AC of the femoral condyles [48]. Furthermore, ACL injury affects other structures of the knee joint either due to the initial injury or instability secondary to the initial injury. In approximately 65-75% of knees with a ruptured ACL there is also evidence of meniscal damage. By providing stability, lubrication, and adequate load distribution to the joint, the meniscus allows less friction between the femur condyles and the tibial plates and prevents joint space narrowing [49]. Damage to the meniscus reduces the joint stability and correct dissipation of the forces in the joint, leading to additional mechanical stress to AC [50]. In response to damaged, the meniscus may release different pro-inflammatory cytokines soluble enzymes which may accelerate the onset of OA [51]. Partial or complete removal of the meniscus in a procedure called partial or total meniscectomy, respectively, is still preferred for symptomatic relief instead of meniscus repair or other conservative treatments despite the first posing a high risk for OA development [52].

1.5.4 Joint malalignment

Changes in the correct alignment of the joint bones lead to altered load distribution, increased friction, and higher impact loading to cartilage between the bones. The delivery of adequate nutrients to cartilage may also be affected in joint malalignment because of progressive reduction in periarticular blood flow rendering cartilage with an abnormal biochemical composition. These changes increase the risk of damage to AC and progression to OA [53].

[2] Factors that are secreted by the adipose tissue are collectively called adipokines.

1.6. The pathogenesis of OA

Osteoarthritis is, inevitably, a result of the degradation of the ECM of AC. It is marked by an increased secretion of matrix-degrading enzymes and other catabolic factors that shift the balance of cartilage metabolism with loss of homeostasis.

As mentioned previously, after being injured, AC has a poor repair capacity, with chondrocytes initially increasing their synthetic activity in attempt to repair the damage, often failing because of the poor quality of newly produced matrix.

Pro-inflammatory factors such as IL-1, TNF or nitric oxide (NO) released upon trauma to AC, resultant from local joint inflammation and produced by the synovial lining cells or yet present in the circulation due to low-grade inflammation stimulate the secretion of the catabolic factors, which are mainly proteinases [53]. These include MMP-1, MMP-3, MMP-8, MMP-13, MMP-14 and the aggrecanases (ADAMTS)-4 and –5 [54]. The proteinases that mediate the degradation of cartilage matrix and their substrates are presented in more detail in Table 2,below.

Table 2 - Chondrocyte Proteinases that mediate degradation of cartilage matrix. Adapted from Firestein, Budd [21]

PROTEINASE CLASS	CARTILAGE MATRIX SUBSTRATES
Matrix Metalloproteinases	
Collagenases (MMP-1, MMP-8, MMP-13)	Collagens I, Il Aggrecan core protein
Stromelysins (MMP-3, MMP-10)	Aggrecan core protein Collagens IX, XI Link protein, fibronectin, proMMPs, proTNF
Gelatinases (MMP-2, MMP-9)	Collagens II, XI Proteoglycans, link protein
Membrane-type MMPs MT-MMP-1 (MMP-14), MT-MMP-2, MT-MMP-3, MT-MMP-4 (MMP-15, MMP-16, MMP-17)	Collagen Il Fibronectin, aggrecan ProMMP-2 ProMMP-13 ProTNF
Matrilysin (MMP-7)	Link protein
Enamelysin (MMP-20)	COMP, link protein
Aggrecanases	
ADAMTS-1, ADAMTS-4, ADAMTS-5	Aggrecan core protein IGD
Serine	
Plasminogen activators (tPA ,UPA)	Aggrecan, fibronectin, proMMPs
Cathepsin G	Aggrecan, collagen II, proMMPs
High temperature requirement A1 (HTRA1)	Matrilin 3, fibronectin, biglycan, fibromodulin, COMP,collagen VI
Cysteine	
Cathepsins B ,K ,L ,S	Collagens IX ,XI Link protein ,aggrecan
Aspartate	
Cathepsin D	Phagocytosed ECM components

ADAMTS, A disintegrin and metalloproteinase with thrombospondin-1 domains; COMP, cartilage oligomeric matrix protein; ECM, extra-cellular matrix; IGD, interglobular domain; MMP, matrix metallopro-teinase; MT-MMP, membrane-type MMP; proMMP, proenzyme form of MMP; TNF, tumor necrosis factor; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator.

The breakdown products of the ECM by these proteinases constitutes damage-associated molecular patterns (DAMPs) which are released the synovial fluid. These DAMPs then interact with toll-like receptors (TLR), integrins and receptor for advanced glycation end-products (RAGE) in the chondrocytes, further increasing the production of inflammatory and catabolic molecules [55]. Concomitantly, in synovial cells, which express TLR-2 and TLR-4 that bind several types of DAMPs, occurs the activation of different pathways that promote the production of more catabolic molecules such as MMPs; and cytokines like IL-1β and TNF-α which induce the production of other inflammatory mediators like IL-6, IL-8, NO and prostaglandin E_2 (PGE_2) amongst others [56]. The production of these inflammatory mediators is regulated by the activation of the NF-kB by IL-1β and TNF-α both in chondrocytes and synovial cells [57]. The role of the different pro-inflammatory cytokines in the pathogenesis of OA was reviewed by Kapoor, Martel-Pelletier [58].

The production of NO by articular chondrocytes reduced cellularity and synthetic activity of surviving cells, and worse mechanical quality of the ECM generated by old chondrocytes all contribute to an increased risk of OA. Apoptosis in articular chondrocytes is influenced by several factors, including age. NO promotes AC degeneration through inhibition of collagen and proteoglycan synthesis, additional activation of MMPs, bigger susceptibility to injury by other ECM oxidants (e.g., H_2O_2, ROS) and apoptosis. NO production in OA occurs mainly due to the activation of inducible NO synthase (iNOS) by the pro-inflammatory cytokines present in OA. IL-1 and TNF-α are some of the iNOS inducers. NO produced by iNOS rapidly reacts with the superoxide ion ($O_2^{\cdot-}$) forming the peroxynitrite ($ONOO^-$) that leads to the production of prostanoids such as PGE_2 by activating the enzyme cyclo-oxygenase (COX-2), one of the isoforms of the COX enzymes that is induced by inflammatory stimuli [59]. Prostaglandins have a variety of complex actions on chondrocyte metabolism, including increased type II collagen synthesis, activated MMPs, and promoted death. IL-1 stimulates COX-2 expression in cartilage explants, and PGE_2 synthesis works in tandem with proteoglycan breakdown. Proteoglycan breakdown caused by IL-1 is prevented by COX-2 suppression, which can be stopped by adding PGE_2 to cultured cells [60]. Figure 4 shows a schematization of the pathogenesis of OA.

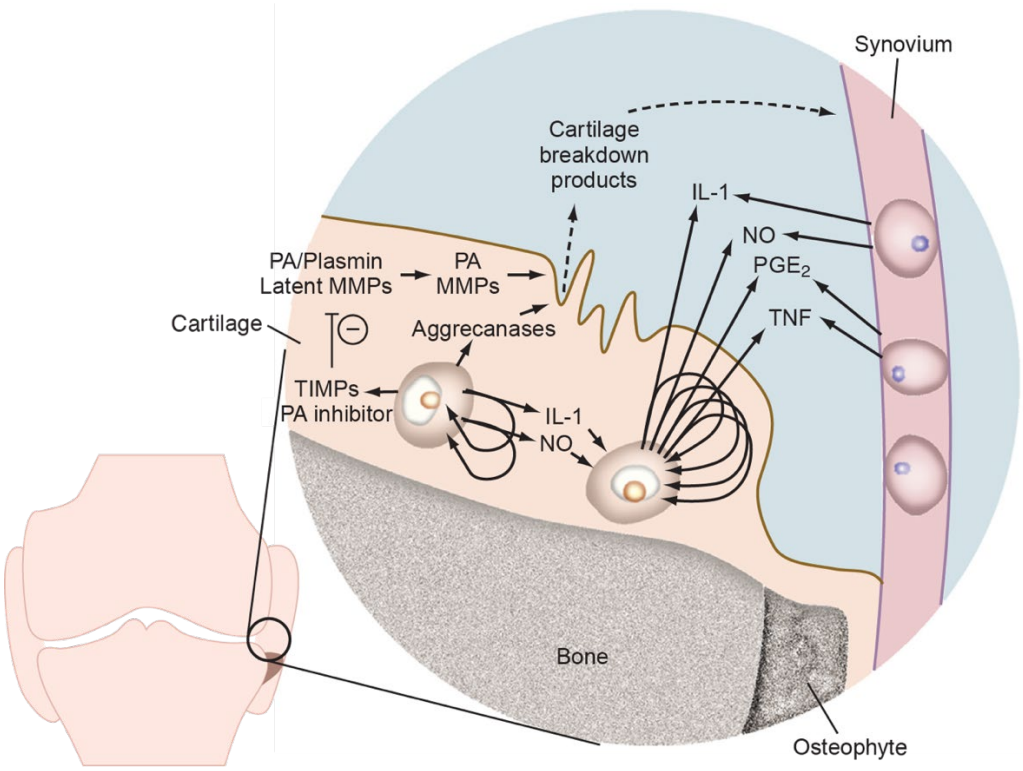

Figure 4. Pathogenesis of Osteoarthritis - Diagram showing the osteoarthritis' pathogenic mechanisms. The release of pro-inflammatory cytokines, MMPs, and mediators like NO and PGE_2 are only a few of the different stimuli that cause changes in metabolism. By encouraging the release of cytokines from synovial lining cells and activating chondrocytes to produce MMP, cartilage breakdown products contribute to the process. IL-1 and TNF generated by chondrocytes act autocrinally and paracrinally accelerating cartilage degradation. Adapted from Di Cesare, Haudenschild [53].

The loss of ECM content due to the actions of the different proteinases presented in Table 2, changes the mechanical properties of cartilage making it susceptible to even more damage when acted upon by forces that would, otherwise, not cause damage.

As mentioned in 1.3.1, the sensing of high strain, injurious loads, is associated with the activation of PIEZOs with higher calcium influx and changes in the regulation of Ca^{2+}-mediated signaling pathways which trigger many catabolic mechanisms, including mitochondrial dysfunction with the consequences already mentioned [61].

1.7. Pain in OA

In the inflamed joint, the changes in chondrocyte phenotype include upregulation of vascular endothelial growth factor (VEGF) expression [62]. This is a potent angiogenic factor that may facilitate the secretion of MMPs while reducing tissue inhibitor of metalloproteinases (TIMPs). The expression of angiopoietin (Ang)-1 and Ang-2, factors that act as vascular stabilizers that also intervene in vessel maintenance and growth after VEGF has triggered the formation of immature vessels, is modulated by pro-inflammatory cytokines IL-1 and TNF-α [63].

In OA, vascular invasion is thought to come from the subchondral bone and the new vessels that cross in AC might be associated with sensory nerves, as might those in developing osteophytes [64]. The nerve fibers that accompany the new blood vessels

secrete neuropeptides like calcitonin gene-related peptide-I (CGRP) and substance P (SP) that primarily act on specific vascular receptors to regulate blood flow and permeability but also to stimulate endothelial cell proliferation, migration, and tube formation. These neuropeptides may then trigger the sprouting of nerve fibers in cartilage, together with nerve growth factor (NGF) and Netrin-1, an osteoclast-secreted protein, which are produced in inflammation conditions by superficial chondrocytes and synovial cells, respectively [65, 66].

Pain is one the most noticeable and incapacitating symptoms of OA [67]. The different inflammatory mediators in the arthritic joint can activate the nociceptive nerve fibers of the joint, since these fibers contain receptors for these mediators [68]. The excitation thresholds of high-threshold neurons are lowered by a variety of inflammatory mediators and molecules like linked to tissue injury, which increases the likelihood that these joint nociceptors may react to both non-noxious and noxious painful stimuli [69].

1.8. The treatment of OA

1.8.1 Global approach to the patient with OA

The first approach to the treatment of osteoarthritis are lifestyle changes which include changes in the diet and the incorporation of an exercise regimen, oftentimes to stimulate weight loss [70].

1.8.2 Pharmacological Treatment

In patients with mild, intermittent pain, analgesics including paracetamol and low-dose orally delivered non-steroidal anti-inflammatory drugs (NSAIDs) (including COX-2 inhibitors) on an as-needed basis, while it is feasible [70, 71]. NSAIDs present several unwanted effects like gastrointestinal disturbances, skin reactions, adverse renal effects and cardiovascular side effects, therefore its chronic use should be avoided and other alternatives must be found [72]. When pain is frequent and it has some impact on function physical methods, the use of oral or topical NSAIDs and centrally acting agents for the treatment of pain are valid [73]. The use of intra-articular injections of corticosteroids, HA or platelet-rich plasma or other formulations are attractive when a single joint is affected and other methods have failed [74].

1.8.3 Surgical treatment

For cartilage repair, several surgical procedures are available. They slow the evolution of cartilage lesions and alleviate related health problems. Each has its own set of advantages and disadvantages. Unfortunately, there are still many barriers to effective tissue healing following damage, and existing therapeutic approaches are insufficient.

Surgical repair strategies include microfracture, osteochondral autograft transfer, matrix-induced autologous chondrocyte implantation, processed allograft cartilage implantation, depicted in Figure 5.

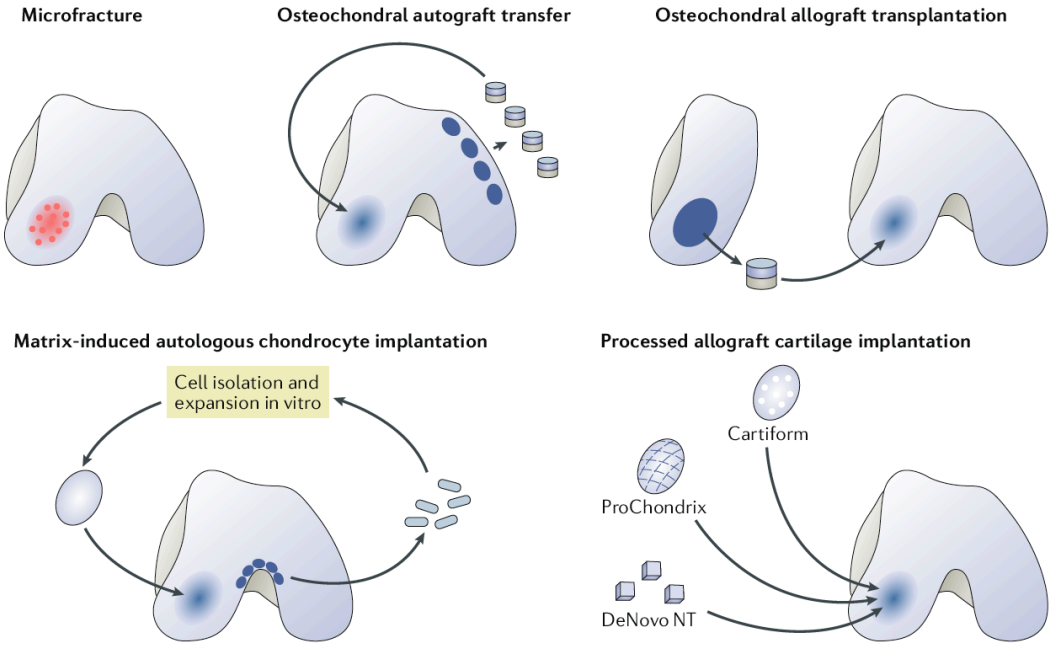

Figure 5. Surgical repair strategies for AC - Microfracture – a small bone puncture is performed to allow bone marrow to cells to invade the defect. Osteochondral allograft – a small osteochondral punch is used to fill the defect. Matrix-Induced autologous chondrocyte injection – the patient's cells are harvested and expanded outside the body seeded in an adequate scaffold that is implanted inside the body. Processed allograft cartilage implantation makes uses of commercially available cartilage explants. Adapted from Kwon, Brown [75].

The microfracture technique makes use of the reparative properties of the bone marrow and marrow stromal cells that infiltrate a lesion and form a stem cell-rich fibrin clot to stimulate intrinsic repair. A microfracture is performed in the subchondral bone to allow the infiltration of these cells eventually leading to the formation of fibrocartilage. Patients who undergo this procedure have an initial improvement followed by a rapid decline in the clinical outcome than those treated with an osteochondral autograft [76].

The osteochondral autograft, or mosaicplasty, involves transplantation of multiple small cylindrical osteochondral plugs to resurface a defected area of the cartilage. These plugs are harvested from less weight-bearing zone of AC. This procedure allows the transfer of native AC into the defect providing high viability of the implanted tissue. Its downsides come from the poor availability of healthy cartilage for autotransplant since patients who require this surgery do not have much healthy cartilage left and the fact that fibrocartilage is formed in the donor sites decreasing overall quality of cartilage in these areas, even if low weight bearing. When the osteochondral plugs are allogeneic, there is no donor site morbidity, however there is the risk of immune rejection of the implants [77].

Autologous cartilage implant (ACI) is a two-surgery procedure. In the first procedure, a small amount of AC is collected via arthroscopy. The patient's cells are then isolated and grown in culture for three to six weeks. The cells are then prepared and inserted in the cartilage defect area and sealed with a periosteal flap. This procedure brought the ability to repair larger cartilage defects and showed superior long-term success. However, despite its upsides, the procedure requires periosteum harvesting and its fixation over the defects. Hypertrophy of the periosteum led to the need of revision arthroscopies and increased the risk of transplant rejection [78].

To overcome this, a new generation of ACI has been devised, in which the isolated and expanded cells are seeded on scaffold prior to surgery, called the matrix-induced autologous cartilage implant (MACI). This biodegradable scaffold is then implanted in defect area eliminating the for the periosteal flap, a better control on the filling of the cartilage defect while also providing a 3D environment for chondrocytes to grow and properly differentiate *in vitro* before implantation [76].

Cartiform and Prochondrix are included in a group of scaffolds called cryopreserved viable osteochondral allograft (CVOCA). A CVOCA is made up of a fine disc with natively structured hyaline cartilage on top of a microscopic layer of bone. The scaffold contains live chondrocytes, ECM, and chondrogenic growth factors. The scaffold is perforated, increasing the surface area for chondrocyte outgrowth from the matrix and making it flexible for shaping along curved surfaces. There is not much patient data on these scaffolds and the literature consists mainly of *in vitro* studies and reports from small cohorts. The long-term success of CVOCA needs further studies to be attested [79]. DeNovo NT is an allograft of juvenile AC that is minced into 1 mm^3 explants. It is delivered with a fibrin adhesive monolayer to which chondrocytes migrate. Young and juvenile chondrocytes gene expression is more favorable to for regeneration than that of adult ones, with upregulation of genes that direct the cartilage growth and expansion [80]. This procedure does not require, therefore, the expansion of cells in vitro, with the ability of being of being a single stage procedure. Like CVOCA it is not a very common procedure and it cannot be chosen as first-line treatment [81].

Arthroplasties, or joint replacement surgeries, have become more common throughout time and have shown to be successful for the knee and hip joints [82, 83].

Only individuals with severe pain and significant functional impairments should be referred for surgery if less invasive treatments have failed to adequately relieve their symptoms. Furthermore, due to the short duration of prosthetics and the restriction on high-impact activities after arthroplasty, joint replacement surgeries are typically postponed in younger, more active patients (less than 60 years old). Younger people are consequently left with fewer therapeutic options and are more vulnerable to possible risks brought on by extended pharmacological treatment [83].

1.8.4 Advanced TE strategies for AC

TE for the repair of osteochondral defects includes different strategies. The use of different types of cells, combined or not with a scaffold or scaffolds with chemical and biological properties that may induce repair are all strategies currently being studied [84].

1.8.5 Scaffolds and Biomaterials for AC

The classic TE approach for the selection and/or design of biomaterials and scaffold is to choose materials with physical and chemical properties that resemble the original tissue [85]. For cartilage, biomaterials may be sourced from natural or synthetic materials. Natural materials include various types of collagen-based materials, chitosan, HA, gelatin, keratin, and fibrin hydrogels. As for synthetic materials, mostly biodegradable polymers such as [86] PGA (poly(glycolic acid)), poly-L-lactic acid, or their composite copolymers poly(lactic-co-glycolic acid) (PLGA), also nonbiodegradable polymers such as polytetrafluoroethylene and polyethylmethacrylate have been studied [85, 87]. Collagen has been widely used for cartilage scaffold production since it is the most abundant protein in mammals [88].

One of the main purposes of scaffold use in cartilage TE is their ability to provide a 3D matrix that can home the cells to be delivered and provide them the physical, chemical, and biological cues for survival, allowing their expansion and differentiation *in vitro* and after implantation. Moreover, they can serve as vehicles for the delivery of drugs to repair tissues. Therefore they must be biocompatible and biodegradable although with a permanence time post-implantation compatible with cells or drugs they carry [89].

1.8.5.1 Natural Scaffolds

Natural scaffolds have strong bioactivity, biocompatibility, and biodegradability to non-toxic components. These scaffolds are comparable to original tissue due to their natural material composition, which means their presence offers an optimal habitat for cells. Thus, the primary value of natural polymers is their resemblance to cartilage's ECM components. Their presence properly enhances chondrogenesis and the preservation of chondrocyte cellular phenotypes. Nevertheless, these scaffolds frequently fail to achieve the required specifications because they quickly lose their structure (sensitivity to an aqueous environment) and convert into a gel-like state. They are also not mechanically robust to maintain the cells and regenerated tissue. As a result, this type of scaffold lacks the necessary characteristics to produce hyaline cartilage.

1.8.5.2 Hydrogels

Hydrogel scaffolds are of particular interest in cartilage regeneration engineering. These scaffolds are produced by cross-linking natural and synthetic (or both) polymers, and they are distinguished by their capacity to absorb water or

biological fluids. All these characteristics make these scaffolds quite comparable to genuine cartilage ECM [90].

Alas, hydrogel scaffolds, like natural scaffolds, have one significant drawback: these scaffolds have reduced mechanical strength due to their solubility in aqueous environments, making them challenging to handle. Intensive research is now being conducted to produce hydrogels using synthetic and hybrid materials [91].

1.8.5.3 Synthetic scaffolds

Numerous articles [92-97] have documented research into the use of synthetic scaffolds for cartilage regeneration. These scaffolds are distinguished by their biocompatibility, biodegradability, and superior mechanical qualities. They may be generated using a variety of techniques due to their superior resistance to physicochemical methods in comparison to natural materials. Currently, few synthetic scaffolds for cartilage TE are being evaluated in clinical studies. Unfortunately, the primary drawback of synthetic polymers is their decomposition, which results in the release of acids. This may result in inflammation throughout the body. In other instances, the deterioration is excessively rapid, causing the membranes to rupture or even dissolve; in addition, they lack appropriate biological characteristics [98]. Therefore, research is being conducted to develop scaffolds composed of both synthetic and natural substances.

1.8.5.4 Hybrid Scaffolds

From the previous sections it is possible to understand that the combination of both natural and synthetic materials to produce scaffolds for cartilage TE may be the most suitable. Natural polymers such as collagen and silk have been combined with PGA and PLA to improve hybrid scaffold biocompatibility while maintaining excellent scaffold mechanical properties.

Indeed, Bellini, Cencetti [99] created a unique collagen-based sponge with superior mechanical characteristics, reduced water absorption, and stronger resistance to enzymatic degradation than natural collagen, to be used as a scaffold for tendon regeneration. The hydrophilic collagen backbone was grafted with PLA chains in a reaction to that preserved the collagen sponge's 3D porosity matrix, which is advantageous in surgical applications and TE, namely for cell seeding. This scaffold was named, for the purposes of the study: Coll-PLA.

Coll-PLA exhibited increased elasticity, as shown by the Young Moduli and E' values obtained in uniaxial tensile and Dynamic mechanical analysis (DMA) small deformation studies, and did not exhibit substantial permanent deformations, as demonstrated by large deformation experiments, when compared to the control Coll-T (collagen-only scaffold that underwent the same chemical reaction).

The Coll-PLA material had a cell proliferation rate equivalent to untreated collagen; it also had a greater cell seeding efficiency, which was likely attributable to its superior mechanical qualities and capacity to keep its form in the swelled condition.

1.9. Delivery to AC

The lack of vascularization and lymphatics in AC makes it more challenging to deliver nutrients and drugs. Furthermore, the electrical charge of the molecules that compose the ECM prevents penetration of many uncharged or countercharged molecules from the synovial fluid to cartilage and the volume of aggrecans sterically hinders the movement of big molecules from the superficial to deepest areas of cartilage e[100].

Due to the vascularization of the synovium, substances present in the general circulation may reach the synovial compartment, being rapidly cleared due to efficient efflux via the capillaries and lymphatics. Not all molecules reach the synovial compartment due to the molecular sieve that is formed by synovial tissue ECM and the fenestrae that are typical of capillaries, making the delivery of molecules to the joint size-dependent [101]. Only after passing the synovium and reaching the synovial space may the substances in the general circulation reach the AC. Further, the delivery of substances to AC must consider the rapid clearance of particles from the synovial fluid [102].

Therefore, there must be a way to increase the time of residence of particles in the synovial space while interacting with cartilage.

The negative electrical charge of cartilage, due to high GAG content, poses another challenge to molecule binding and penetration in AC to which the delivery of cationic molecules particles seems the solution [103]. However the size of aggrecans also impedes the penetration of these particles in AC, with substances like Avidin, with a small hydrodynamic diameter and positive charge, being able to fully penetrate and remain in the cartilage matrix [104].

Although molecules and particles that bind to the superficial layer of cartilage cannot diffuse into it, they overcome the problem of rapid efflux from the synovial fluid. Particles present different types of binding to AC depending on their size, charge and other surface modifications like functionalization [105].

Figure 6 shows the different interactions particles establish with cartilage.

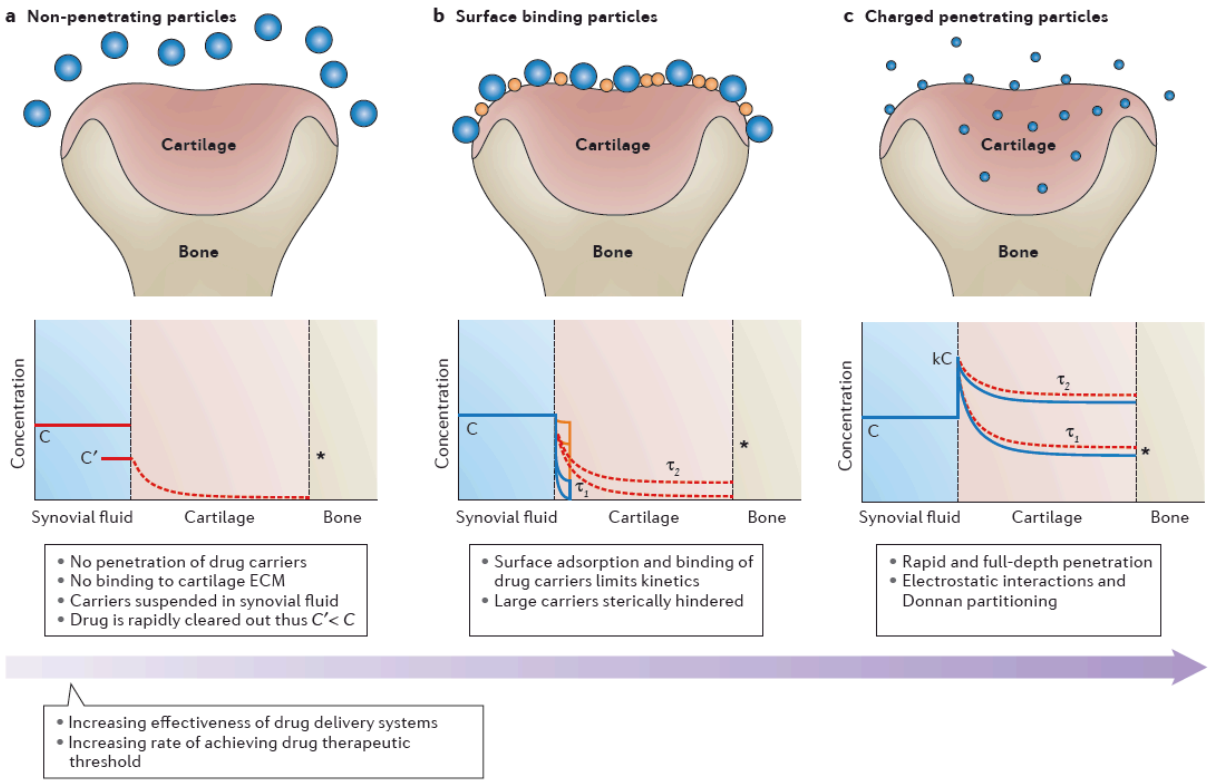

Figure 6. Interactions of particles with AC– a. Large, non-penetrating particles are usually used to target the synovial membrane. These particles are rapidly cleared from the synovial compartment. b. Particles that are positively charged can bind to AC and, if they are used as nanocarriers, may release their contents by diffusion to AC. However, binding kinetics limits their permanence time are eventually cleared. c. Small particles, with a positive charge establish stronger electrostatic and overcome the sterical barrier created by aggrecan, they penetrate cartilage and if used as nanocarriers, may reach the deepest layers and release their contents there. Adapted from Bajpayee and Grodzinsky [106].

It becomes, then, evident that to ensure the delivery of different types of molecules to AC, small positively charged nanocarriers are a good solution for the delivery of otherwise unsuitable substances. Furthermore, strategies to overcome the barrier created by the abovementioned molecular sieve.

1.10. Nanoparticles for local delivery of drugs in cartilage

NPs are composed of biocompatible and biodegradable components such as polymers or solid lipids to allow regulated and sustained medication delivery [107].

The size-controllable nature of NPs allows delivery via intra-articular injections feasible. As carriers, NPs can incorporate drugs in the surface or matrix to protect drugs from enzymatic degradation, improve their penetrations across cartilage matrix, and modulate drug pharmacokinetics, which is advantageous for balancing the efficacy and toxicity of therapeutic compounds [108]. To maximize degradation, toxicity, and mechanical qualities, hybrid NPs composed of two or more components may have greater properties than single-component materials. They may be functionalized to

target cartilage components and/or cells, such as chondrocytes, by modifying their physicochemical features or coating them with moieties [109].

Another advantage is that they can be tracked and adjustments may be made by grafting various imaging-functional components. Further benefits include high stability (e.g., extended shelf life), integration of both hydrophilic and hydrophobic chemicals, and varied delivery methods (intra-articular injection, scaffold, or hydrogel) [110].

1.11. The immunology of OA

AC is only populated by chondrocytes. The inflammatory mediators and danger signals produced in cartilage can, however, activate the resident immune cells of the synovium.

During OA development, synovial macrophages lose their stable state and are activated in multiple ways. One way is through the activation of surface pattern recognition receptors (PRRs), which recognize exogenous pathogen-associated molecular patterns (PAMPs) and endogenous DAMPs. Activated PRRs initiate intracellular downstream effects, including the NF-κB pathway, and in turn, secrete inflammatory cytokines and chemokines [111].

Macrophages accumulate and become polarized (M1 or M2) in the synovium and articular cavity during OA development. M1 macrophages are activated by interferon (IFN)-γ, lipopolysaccharide (LPS), or TNF-α. M2 macrophage activation is determined by the Th2 cytokines IL-4 and IL-13, and has been further divided into specific subtypes [112].

Macrophages in the OA synovium secrete cytokines, growth factors, and MMPs. IL-1β and TNF-α drive inflammatory responses in OA. The impact is multilevel and involves not only the induction of aging and apoptosis in chondrocytes but also decreases the synthesis of key components of the ECM. Collectively, those data indicated that synovial macrophages were the key instigators of MMP over-expression and upregulation in the production of inflammatory cytokines with subsequent cartilage degradation. Another mechanism of macrophage activation in OA progression, and in particular, osteophyte formation, is through the mediation of growth factors including TGF-β. Studies to further investigate this could be important for drug discovery and the determination of novel therapeutic targets in OA [113].

Chondrocytes and macrophages establish paracrine interactions that trigger the initiation and development of OA. Specifically. The activated synovial macrophages produce pro-inflammatory cytokines like IL-1β, TNF-α and IL-6 [114]. These cytokines regulate the microenvironment of chondrocytes and as previously explained, upregulate the production of MMPs and aggrecanases, with production of ECM debris. This feeds the ever-growing cycle of ECM destruction triggered by inflammation and the continuous production of pro-inflammatory molecules and catabolic factors due to this destruction [114, 115]. Figure 7 illustrates the interactions between chondrocytes and

macrophages, as well as the molecules that intervene in the activation of the latter and the signaling pathways that are activated.

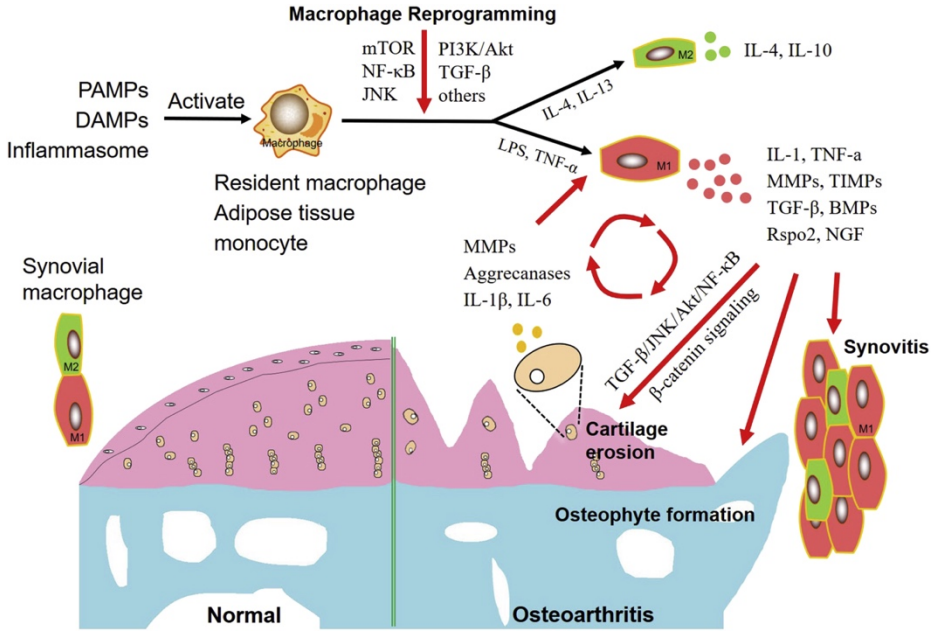

Figure 7. Interactions between chondrocytes and macrophages in OA – PAMPs, DAMPs and the inflammasome promote synovial macrophage activation and polarization, with M1-polarized macrophages secreting factors that lead to inflammation and subsequent cartilage degradation and osteophyte formation. In addition to autocrine connections, polarized macrophages modify the intercellular signaling pathways in chondrocytes, such as TGFβ-, JNK, Akt, NF-kB, and β-catenin, thereby promoting the breakdown of ECM components. ECM functions as DAMPs and further encourages macrophage activation and polarization, culminating in a cycle of inflammation and cartilage breakdown. Adapted from Zhang, Cai [113].

1.12. The COX enzymes and Inflammation

PGE_2 is synthesized by the COX enzymes having as precursor the arachidonic acid. COX-2 is inducible by cytokines like IL-1β and TNF-α then producing PGE_2 [116]. PGE_2 inhibits proteoglycan synthesis induces collagen degradation and but increased MMP-13 secretion by OA in cartilage explant cultures [60, 86].

PCR (polymerase chain reaction) analysis of OA chondrocytes treated with PGE_2 with or without IL-1 revealed that IL-1-induced MMP-13 expression was augmented by PGE_2 and significantly inhibited by the COX-2 selective inhibitor celecoxib, a NSAID [117].

Another NSAID, ibuprofen has shown in vitro anti-inflammatory effects by inhibiting the IL-1β-induced NO and PGE_2 production in articular chondrocytes from New Zealand White rabbits pretreated with ibuprofen then with IL-1β [118]. Ibuprofen, however, presents chronic toxicity due to the inhibition of the production of prostaglandins and thromboxanes that play important roles in various organ systems, including maintaining gastric mucosal integrity, renal blood flow and blood coagulation.

The major challenges of the current cartilage repair approaches based on TE include the repair of tissues with the same biomechanical properties of the original and the integration of TE constructs with the native tissue [119]. Another challenge is to correctly modulate cell behavior when interacting with TE solutions to improve tissue repair either by recruiting other cell types or by escaping interactions with the immune

system that may cause rejection and exacerbated immune responses [120]. To surpass these obstacles, implantable devices mimicking the cartilage components, such as collagens matrices made of random dense interconnected fibers are recognized as a promising approach to promote tissue integration, hence prolonging the functional lifespan of implants. Moreover, the introduction of bioactive molecules in these 3D scaffolds that modulates locally the tissue/cell response after implantation, represents a promising solution to further improve the TE solutions outcomes. In this project the main aim was to characterize the bioactivity of the nanoenabled 3D matrices-improved through the inclusion of nanomedicines developed in the framework of the European project, RESTORE.

2. Motivation and Aim

Osteoarthritis is the most prevalent arthritic disease and a leading cause of disability. According to a systematic analysis performed by Safiri, Kolahi [121], in 2017 there were 303.1 million prevalent cases of knee and hip OA worldwide.

Currently, AC degeneration remains an open challenge for scientists to understand and for clinicians to repair. Once cartilage degeneration has progressed to severe OA the only alternative is total knee replacement [122]. Several contributing factors (e.g. mechanical factors, inflammation, genetic predisposition, age, sex, and obesity) can lead to cartilage degeneration. Over the years, it has become evident that the inflammatory cytokines contribute substantially to the pathogenesis of OA [43].

With this study we aim:

1. **To better understand the influence of some inflammatory cytokines in OA pathogenesis** by
    a. Isolating human articular chondrocytes and growing them in culture;
    b. Setting up three dimensional cultures of human chondrocytes;
    c. Stimulating these chondrocytes with a pro-inflammatory mediator and understanding the changes in cell behavior.

2. **To develop a scaffold system that can have a positive impact in the treatment of OA** by
    a. Studying the interactions chondrocytes establish with this material;
    b. Incorporating the delivery of an anti-inflammatory substance into the scaffold, through a nanocarrier system and studying its impact in inflamed articular chondrocytes.
    c. Testing the immune response to the scaffold to foresee its behavior in vitro.

3. Materials and Methods

3.1. AC biopsies and chondrocyte isolation

The protocol was approved by the Centro Hospitalar Universitário de São João ethics committee (ethical approval ref. 196/19). Written informed consent was obtained from the patients. Cartilage specimens were collected from osteochondral leftlovers from the hip or knee joints of patients undergoing total joint replacement at Centro Hospital Universitário de São João.

The anatomical pieces obtained from these surgeries – either the head of the femur or the proximal part of the tibia – were placed in cold isolation medium (DMEM high glucose (4.5g/L) (ref. 31966-021, Gibco), 5% Penicillin/Streptomycin 5000U/mL (P/S) (Immunotools) and 10% Fungizone (Amphotericin B) 25µg/mL.

3.2. Establishment of primary human chondrocytes cell cultures

The anatomical pieces obtained from these surgeries – either the head of the femur or the proximal part of the tibia – were placed in cold isolation medium (DMEM high glucose (4.5g/L) (ref. 31966-021, Gibco), 1% P/S and 10% Fungizone (Amphotericin B) 25µg/mL. Under sterile conditions these pieces were analyzed by macroscopic observation and the zones considered to still contain healthy AC were identified and carefully scraped. After scraping, the resulting pieces were thinly chopped until they had an area of 4mm^2 or less. These were then transferred to a flask containing digestion medium (DMEM high glucose (4.5g/L) (ref. 31966-021, Gibco), 5% fetal bovine serum (FBS; Biowest) and 0.15% Collagenase B (ref. 1108815001, Roche) that was left incubating overnight at 37°C and shaking at 100 rpm. On the following day, this suspension was filtered through a 100 µm pore sized cell strainer and the filtered solution was pelleted down by centrifugation (1200rpm for 12 min to get a cell pellet and a clear suspension) and the supernatant removed and replaced by expansion medium (EM) (DMEM high glucose (4.5g/L), 10% FBS (Biowest), 1% N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) buffer (1M; Gibco), 1 mM sodium pyruvate (Gibco) and 1% P/S. The cells were resuspended, centrifuged again and after centrifugation the supernatant discarded. Once again, the cells were resuspended in EM and counted. They were then cultured on a cell culture t-flask in EM with freshly added 1ng/mL transforming growth factor-beta-1 (TGF-β1; R&D) and 5ng/mL basic fibroblast growth factor (bFGF, Sigma-Aldrich) until they reached a confluency of 70% to 90%, in a humidified atmosphere with 5% CO_2 at 37°C.

3.3. Establishment of 3D cultures of human chondrocytes

For chondrocyte differentiation, cells at confluent stage, were detached with trypsin (Sigma) and seeded in 15mL polypropylene tubes with a lid with filter (Greiner Bio-One) to allow gas exchanges at density of 1 million cells/tube and 3D pellets were

obtained by centrifugation (5min at 390*g*). These 3D cell pellets were 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 mg/mL insulin (Sigma), 5 μg/mL transferrin (Sigma), 5 ng/mL selenium acid (Sigma), 0.1 mM dexamethasone (Sigma), 0.17mM ascorbic 2-phosphate acid (Sigma), 1mM sodium pyruvate (Gibco), 0.35mM proline (Sigma), 1.25mg/mL bovine serum albumin (BSA) (Sigma), 1% P/S and 10 ng/mL TGF-β3 (R&D)) freshly added that will promote cell differentiation. Medium was changed twice a week.

3.4. 3D pellets treatment with Ibuprofen-loaded PLGA nanoparticles

Poly(lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) loaded with the anti-inflammatory drug ibuprofen (Sigma) were prepared as described elsewhere [123] and delivered to the inflammatory articular chondrocytes. On day 14 of culture and 3 hours after IL-1β stimulus, Ibuprofen-loaded PLGA NPs (PLGA IBU NPs) were added to the 3D chondrocyte pellets to a final concentration of Ibuprofen of 30 μg/ml. Unloaded PLGA NPs (PLGA UNL NPs) at the same concentration were used as a control. On day 17,21 and 28 the supernatants were collected, and 3D pellets were fixed for further analysis.

3.5. Cultivation of freshly isolated chondrocytes onto Col-PLA scaffolds.

Immediately after chondrocyte isolation, cells were seeded at a confluence of 0.5 million cells/scaffold and left to adhere for 10 min. Cells in Col-PLA scaffolds were cultured to assess the cytotoxicity of nano enabled scaffolds throughout the culture time, the release of lactate dehydrogenase (LDH) into the supernatant was determined and live dead assay was performed.

3.6. Preparation of nanoenabled scaffolds for bioactivity assessment

The Col-PLA scaffold was produced at INEB following the COPLA® Scaffold production protocol provided by Askel Healthcare. Nanoenabled Col-PLA preparation details are under a non-disclosure agreement (NDA) between Askel and INEB, and as such no specific information cannot be shared. Throughout this document, we use the follow nomenclatures to designate the hybrid scaffold used in this thesis and in RESTORE project made of collagen fibers (Col) and poly(lactic acid) (PLA): Col-PLA scaffold.

3.7. Stimulation of human chondrocytes with a pro-inflammatory mediator

The proinflammatory condition used in this work was previously established in our group. The pro-inflammatory cytokine, IL-1β (R&D), was used to stimulate cells at a concentration of 10 ng/mL or 100 ng/mL. IL-1β was added to the media: i) at day 14 of differentiation in 3D pellets after cell expansion and ii) three hours after cell seeding in the Col-PLA scaffold. Three days after the addition of IL-1β, the medium was removed and replaced with the same amount of fresh chondrogenic medium in both, 3D pellets

and Col-PLA scaffolds. Afterwards, every three days, the medium was collected and fresh chondrogenic medium was added. As controls, pellets and cells seeded in the Col-PLA scaffold were cultured under chondrogenic medium only. At days 14, 21 and 28 of culture for the pellets and 3, 7 ,14 ,21 and 28 for the Col-PLA scaffolds, the conditioned medium (CM), pellets and scaffolds were collected, stored and/or processed for analysis.

3.8. Evaluation of the size of 3D pellets

To measure the size of 3D pellets, photographs of the pellets were captured under different magnifications, suitable to the size of each 3D pellet. The area of the pellets and the width to height ratio were then measured using FIJI ImageJ2 v2.3.0/1.53q, by selecting the region of interest (ROI) with the Wand Tool.

3.9. Histochemistry of 3D pellets

Pellets were fixed in paraformaldehyde (PFA, 4%, Merck) for 10 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 for 3 min (100%, 96%, 70% and 50%) and finally distilled water for 5 min.

3.9.1 Analysis of glycosaminoglycans deposition in 3D pellets

Alcian Blue staining was used to assess chondrocyte matrix production in 3D pellets. Alcian Blue is a polyvalent basic dye with high affinity for polyanionic substances such as mucopolysaccharides (glycosaminoglycans) found in the cartilage ECM. After rehydration, slides were immersed in Gill Hematoxylin solution during 3 min for nuclei stain, washed in running tap water for 6 min, followed by 30 min in an Alcian Blue solution to stain GAGs. After being washed in running tap water for 2 minutes and rinsed in distilled water and counterstained with PicroSirius Red for 1 hour, followed by a wash in acidified water for 30s and distilled water for 1 minute. After the staining procedure slides were left to air dry and mounted with mounting medium (Entellan).

3.9.2 Cartilage Identification in 3D pellets

Safranin-O was used to identify the formation of cartilage. After rehydration, slides were immersed in Gill Hematoxylin solution during 5 min for nuclei stain, washed in running tap water for 15 min, followed by immersion in fast green for 6 minutes and washed twice for 3 min with acetic acid. They were then stained with Safranin-O for 5 min and rinsed with distilled water. After the staining procedure slides were left to air dry and mounted with mounting medium (Entellan).

All the images were acquired with an Olympus CX31 light microscope equipped with a DP-25 camera (Imaging Software CellˆB, Olympus, PA).

3.9.3 Histological Scoring

After image acquisition, pellets were scored according to Table 3 below compared with native tissue. This scoring system was adapted from O'Driscoll, Keeley [124].

Table 3 – Histological parameters for scoring of 3D pellets

Histological parameter	Score
Nature of predominant tissue Safranin O/Alcian Blue staining of the matrix:	
Normal or nearly normal	3
Moderate	2
Slight	1
None	0

3.10. Immunostaining (Col-PLA scaffolds)

Cells were fixed with PFA 4% in PBS for 15 min. Following fixation, cells were permeabilized, blocked and incubated overnight at 4°C with the primary antibodies: anti-collagen II (1:100, ABCAM, ab34712), anti-aggrecan (1:50; Proteintech Europe, 13880-1-AP). AlexaFluor 488 or 594 Flash Phalloidin (1:100; ThermoFisher) was incubated for 1h at RT, along with the secondary antibodies (1:1000). Nuclei were stained using DAPI 1:10 000 in PBS for 5 min at RT. Images were acquired on the confocal microscopy (Leica TCS SP5) and analyzed using Image J (version 2.1.0/1.53c) software.

3.11. Live Dead assay

Cell viability of human chondrocytes plated immediately after isolation in the Col-PLA scaffolds was qualitatively assessed through fluorescence-based LIVE/DEAD assay. Briefly, cells loaded in the Col-PLA scaffolds at day 7 and 14 were incubated with Calcein AM (Invitrogen) in PBS for 45 min at 37°C. Calcein AM was washed out and Col-PLA scaffolds were incubated with propidium iodide (PI) (Sigma Aldrich) for 5 min at 37°C. Calcein AM (Ex 485 nm/Em 530 nm) stains live cells green, indicating intracellular esterase activity, while PI (Ex 530 nm/Em 645 nm) stains dead cells red, indicating loss of plasma membrane integrity. Images were acquired using confocal microscopy (Leica TCS-SP5) and analyzed, using ImageJ (version 2.1.0/1.53c) software.

3.12. Cytotoxicity Assay

Cytotoxicity throughout the culture time of the cells plated in the Col-PLA scaffolds and 3D pellets was evaluated using the CytoTox®non-radioactive cytotoxicity assay

(Promega), which quantitatively measures LDH following the manufacturer's instructions. LDH is a stable cytosolic enzyme that is released upon cell lysis. Briefly, 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 were also included. Cell death was defined as the percentage of released LDH compared with the maximal LDH activity from cell lysates obtained using 1% Triton X-100 on cells plated on Col-PLA scaffolds or falcon tubes. For each measurement, two replicates per condition were included.

3.13. Macrophages isolation

Human monocytes were isolated by negative selection from healthy donors buffy coats by using RosetteSep™ human monocyte enrichment cocktail (StemCell Technologies), as previously described [125]. The buffy coats were provided by the immunohemotherapy Department Centro Hospitalar Universitário de São João from Porto (Portugal) with the approval of the clinical ethical committee (Protocol Reference 90/19). Cells were resuspended in complete medium RPMI 1640 medium (Corning) supplemented with 10% FBS, 1% P/S and 50 ng/mL of macrophage colony-stimulating factor (M-CSF) (R&D). Monocytes were cultured for 6 days in complete medium and supplemented with 50 ng/mL of M-CSF. After 6 days of differentiation the medium was changed, cells were washed with PBS 1X and fresh RPMI without FBS and M-CSF was added for 24 hours. At day 7, cells were detached and counted for the invasion assay.

3.14. Invasion assay

Studies on invasion of primary human macrophages were performed using a transwell chamber system. Membrane filters with a pore size of 8 µm previously coated with Matrigel™ (BD Biosciences) were used. The lower compartments of the invasion chamber were filled with 750 µL DMEM medium (without FBS), as a negative control, chondrogenic medium, as the experimental control or the conditioned medium of primary human chondrocytes seeded and cultured on Col-PLA scaffolds (Col-PLA PLGA UNL NPs or Col-PLA (Col-PLA PLGA IBU NPs)) for 7 days. Then, Matrigel-coated inserts that had been pre-incubated for 1h with serum-free DMEM were placed in the wells, forming the upper compartment. Macrophages (1×10^3 cells/insert) in 500µL serum-free DMEM medium were seeded into the upper compartment. The invasion chambers were incubated for 24 h at 37°C/5% CO_2 . After incubation, inserts were washed with PBS 1X and cells were fixed in PFA 4% for 15 min at RT. Inserts were washed with PBS and kept at 4°C until analysis. Cells on the top surfaces of filters were wiped off with cotton swabs, stained with DAPI for 10 min and washed with PBS 1X. Then the membrane was carefully cut and mounted in a slide with Fluoromount™ mounting media (Sigma). Cells that invaded into the lower compartment and attached to the lower surface of the filter were counted in an inverted fluorescence microscope (Zeiss Axiovert). Cell nuclei were

counted in ten 200× fields of view for each membrane. The number of migrated cells was estimated by considering the area of a field of view.

Figure 8 summarizes the project methodology.

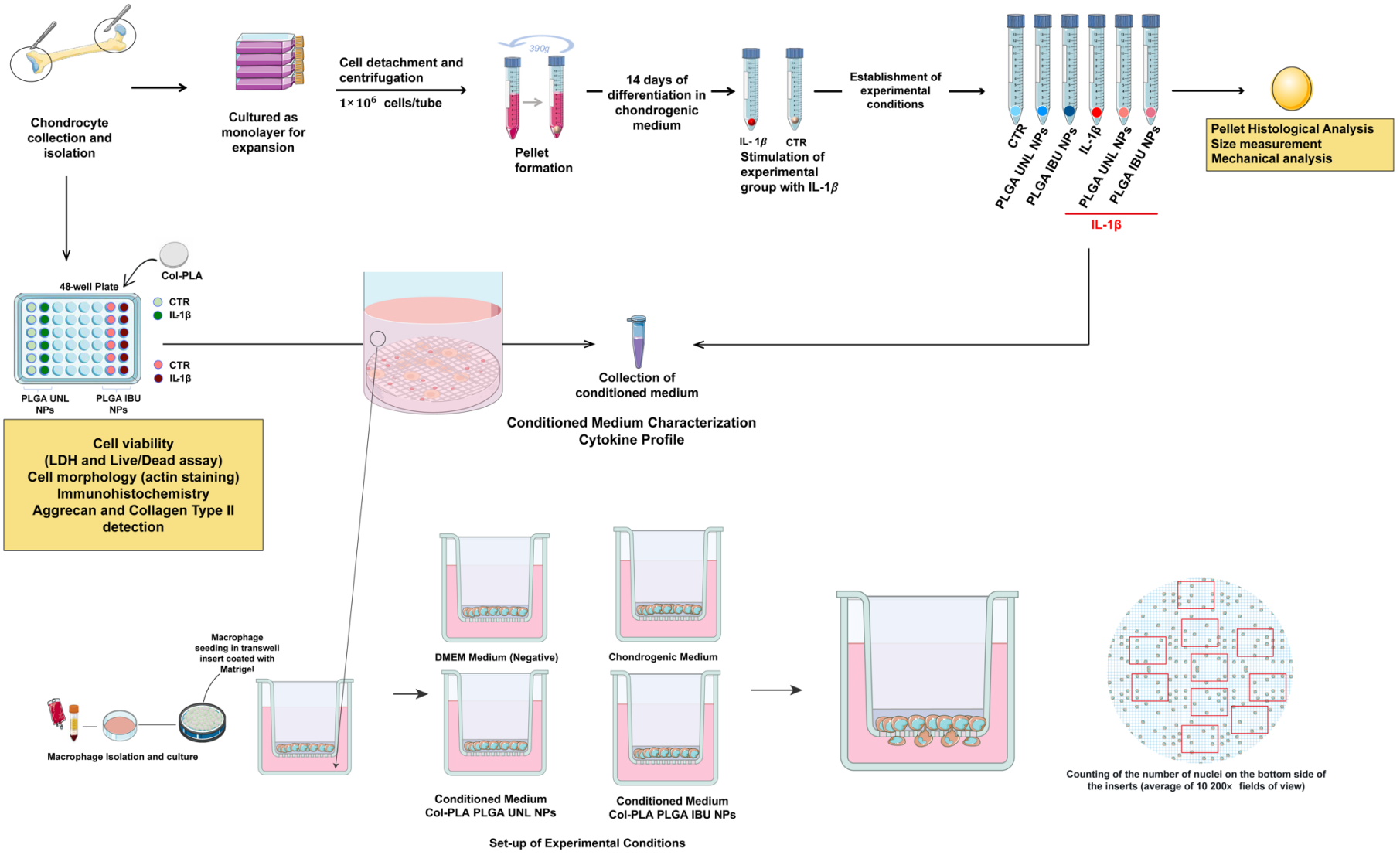

Figure 8 – Schematic methodology of this work.

4. – Results and Discussion

4.1. Human chondrocyte pellets size evaluation

A proteolytic environment that recapitulates OA in the 3D cultures of chondrocytes was instigated by the stimulation with 10 ng/mL or 100 ng/mL of the pro-inflammatory cytokine, IL-1β is a main player in the inflammatory processes occurring in cartilage associated diseases, promoting cartilage degradation by stimulating the production and secretion of MMPs [86]. To evaluate the chondroprotective effects of the nanocarriers, 3D pellets were formed and left to differentiate for 14 days. At this time-point, 3D pellets were exposed to 10 or 100 ng/mL of IL-1β and 3 h later different nanocarriers were added to the culture, as previously optimized for the RESTORE project. The area of the human articular chondrocyte pellets, with and without IL-1β stimulus and treated with the nanocarriers (PLGA IBU NPs) and PLGA UNL NPs) was analyzed to evaluate ECM deposition on the different conditions to which pellets were exposed [126]. Pellets without IL-1β stimulus and treated with PLGA Ibuprofen NPs (loaded and unloaded) were used as control to verify the cytotoxicity effect of the nanocarriers.

The graph in Figure 9 shows the areas of pellets for the different experimental conditions.

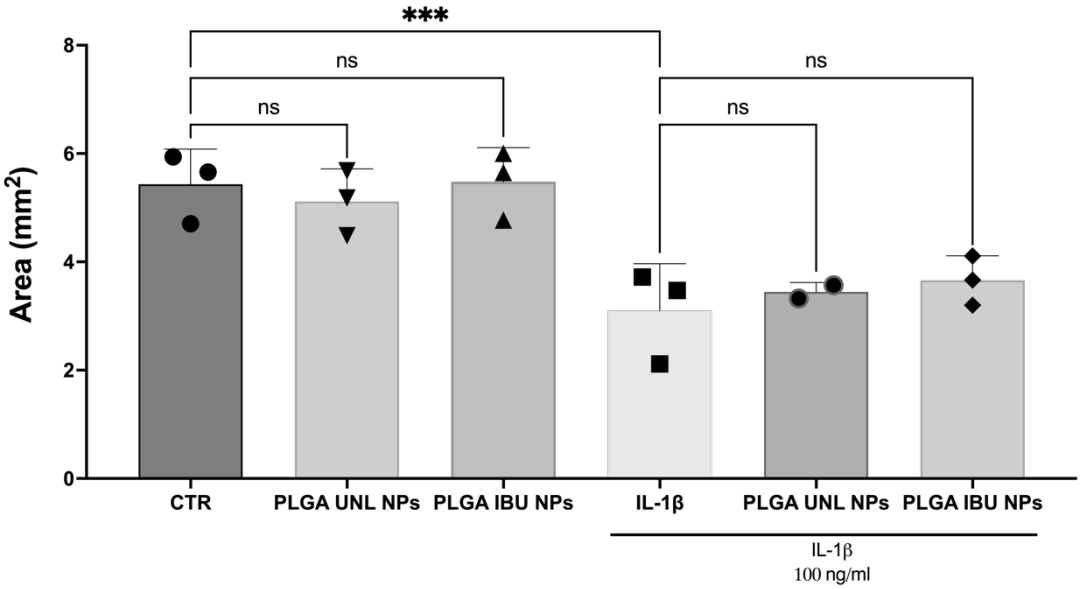

Figure 9 . Area of pellets cultivated after 100ng/ml IL-1β stimulus – The area of pellets cultured under the indicated conditions: CTR- Control, IL-1β - pellets that were exposed to IL-1β at day 14 of culture and treated 3h after with the nanocarriers: PLGA Unloaded IBU NPs (PLGA UNL NPs) and PLGA IBU 30µg/mL NPs (PLGA IBU NPs).

The area in each control condition (CTR, PLGA UNL NPs or PLGA IBU NPs) and the area of the pellets that were exposed to IL-1β (100ng/mL) was statistically different, with pellets that received the pro-inflammatory stimulus being smaller than their non-stimulated correspondents. This shows that there was a decrease in matrix deposition

with the pro-inflammatory stimulus which is coherent with the effects of IL-1β in the deposition of ECM in AC. No differences were observed in pellets stimulated with IL-1β and treated with nanocarriers.

Pellets that were exposed to lower concentration of IL-1β (10ng/mL) had a similar size to the control. This could show that the stimulus with a lower concentration of IL-1β that is later removed from the pellet's medium can be counteracted by cells, regarding matrix deposition. A larger area (Figure 10) in the PLGA IBU NPs condition shows that delivery of ibuprofen to pellets may not only have counteracted the effect of the IL-1β stimulus but may have also shown a chondroprotective effect with higher matrix deposition.

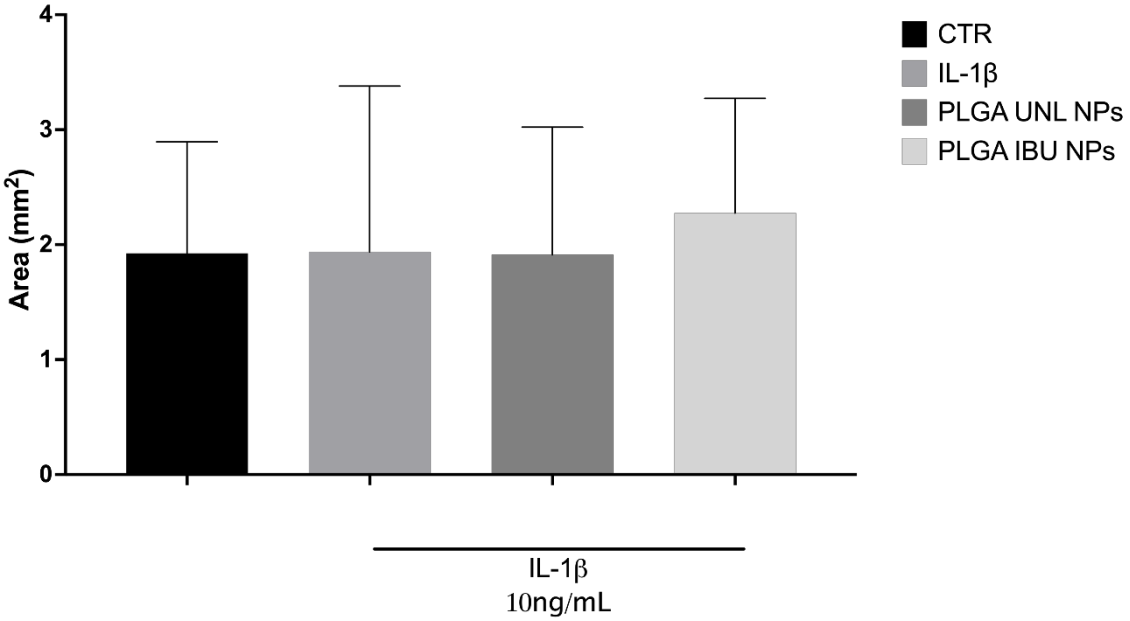

Figure 10. Area of pellets cultivated after 10ng/ml IL-1β stimulus – The area of pellets cultured under the indicated conditions: CTR- Control, IL-1β - pellets that were exposed to IL-1β at day 14 of culture and treated 3 h later with the nanocarriers: PLGA UNL NPs and PLGA IBU NPs.

Figure 11 shows the aspect ratio (AR) of pellets under the different experimental conditions. Ideally pellets should have an aspect ratio of 1, meaning that their cross sections are perfect circumferences, with matrix deposition occurring radially.

The IL-1β condition presents the higher AR. The higher AR of IL-1β stimulated pellets demonstrates that it promotes physical changes and irregularities in the ECM of the pellets that lose their spherical shape. The stimulated pellets treated with PLGA UNL NPs seem to have been able to either regain or maintain their spherical shape. As for the pellets treated with PLGA IBU NPs, the NPs seem to have counteracted the effect of IL-1β in pellet shape, given that heir aspect ratio is almost the same of those that did not receive the IL-1β stimulus.

Overall, the effects of IL-1β on the shape of pellets seems to have been counteracted by both the unloaded (PLGA UNL NPs) and loaded (PLGA IBU NPs) nanoparticles. It would be interesting to verify the pattern of deposition of NPs in the

pellets to understand if they are position in the pellet mass could be related to the aspect ratio.

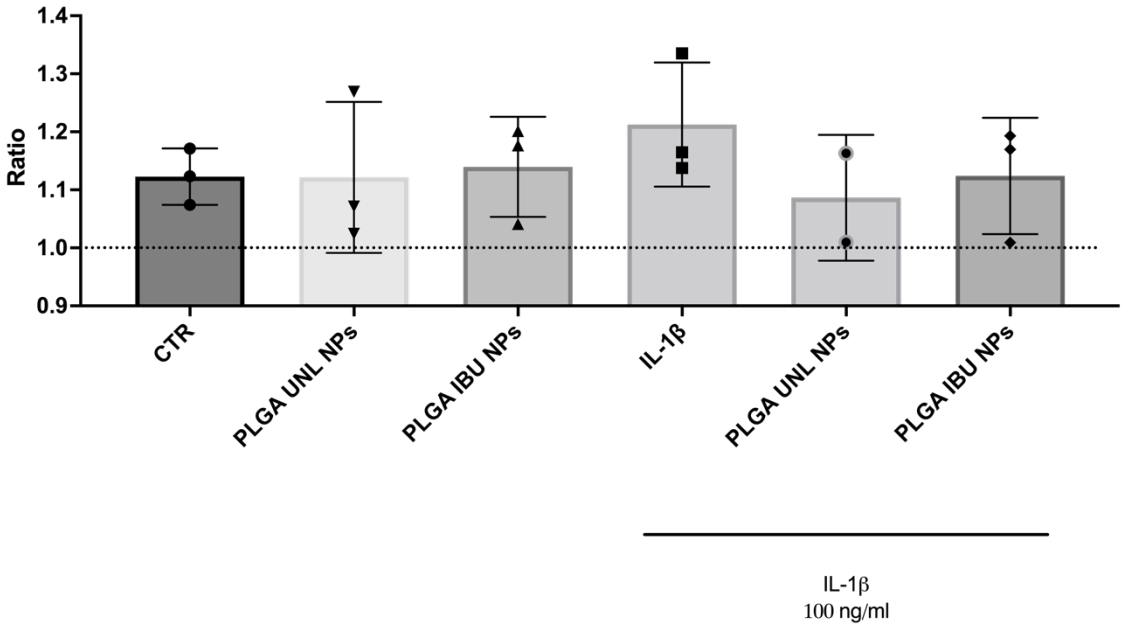

Figure 11. Aspect ratio of the pellets - The AR of pellets as the ratio between the biggest and the smaller side of the smallest rectangle that virtually encloses the area of the pellet.

4.3. Alcian Blue and Safranin-O Stains

The ECM protective effects of nanocarriers under pro-inflammatory stimulation were assessed through the evaluation of their impact on the GAGs levels. The analysis was performed by histochemical stains (Alcian Blue and Safranin O). To detect ECM deposition in the 3D pellets microsections of these pellets were stained with either Safranin-O or Alcian Blue. They were then scored as described in section 3.9.3 using as control the sections human AC in panel A and D of Figure 12. These pellets were made with cells from three different donors (DA, DB and DC). The presence of cartilage was confirmed with the staining of Safranin-O in all pellets. Overall, the samples that were stimulated with IL-1β had lower histological scores which could indicate the influence of IL-1β in cartilage degradation.

Our results show that the stimulation with IL-1β decreases the GAGs deposition, when compared with the control, mounting evidence that the PLGA IBU NPs do not affect the deposition of matrix substance. It is also possible to verify that in the conditions stimulated with IL-1β the Alcian Blue stains were not so homogenous, which evidences the catabolic effect of IL-1β on ECM. The IL-1β stimulus clearly creates an inflammatory process that cannot be countered by the delivery of PLGA IBU NPs. In fact pellets that were stimulated with IL-1β and were treated with PLGA IBU NPs do not improve GAGs deposition. PLGA UNL NPs showed the lowest histological score in the case of Safranin-O and the second lowest in the case of Alcian Blue. Panels B and E of Figure 12 show evident differences in the color intensity of stains between donors for the same conditions. In fact, donor variability influences the overall behavior of cells in culture. Collectively, these results suggest that the stimulus with 100 ng/mL of IL-1β induced an extremely catabolic effect that does not allow the anti-inflammatory effect of PLGA IBU NPs. Taking this into account we decided to perform the same experiments, using a lower concentration of IL-1β (10ng/mL) to induce an inflammatory environment more coherent with the normal serum circulating values of IL-1β in healthy adults, ranging from 0.5 to 12 pg/mL [127].

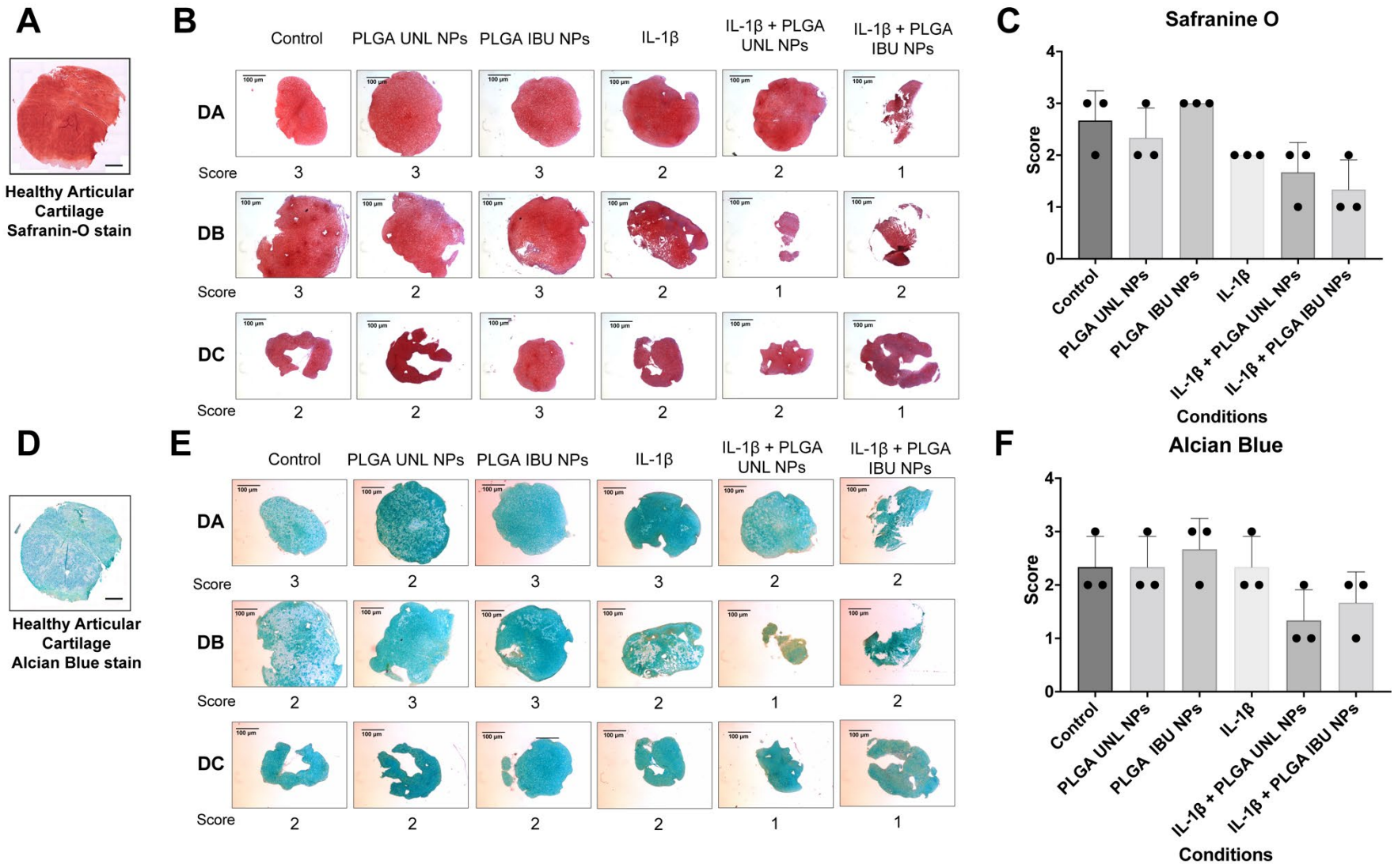

*Figure 12. Histology of Pellets – **A and D** – Healthy AC stained for Safranin-O and Alcian Blue, respectively. Used as "normal" histological parameter. **B and E** - Photographs of the pellets with score attributed to each below, Safranin-O and Alcian Blue, respectively. (Scale bar=1 mm). **C and F –** graphical representation of the scores attributed to each pellet, organized per condition, Safranin-O and Alcian Blue, respectively.*

4.4. Cytotoxicity of different concentrations of IL-1β on 3D pellets and effect of ibuprofen

To evaluate the cytotoxicity of IL-1β and the influence of the PLGA NPs on cell viability the quantification of LDH release into the supernatant was measured.

Panel A - Figure 13 shows that cell viability of the pellets under different conditions was high (above 80% in all conditions) after 21 days of culture (7 days after stimulus with 10 ng/mL of IL-1β). Control pellets showed the lowest cell viability of all the four conditions with IL-1β.

For the IL-1β concentration of 100 ng/ml there is an overall decrease in cell viability when comparing the conditions that were common in the study of each stimulus (CTR, IL-1β, PLGA UNL NPs, PLGA IBU NPs). Interestingly, cell viability was higher in the pellets stimulated with both concentrations of IL-1β and even higher in the PLGA IBU NPs. There are, however, no studies on the influence of IL-1β on LDH activity. To further assess cytotoxicity LIVE/DEAD assays should be performed. Other explanations for LDH activity measured from supernatants are provided with the analysis and discussion of the results in the next section.

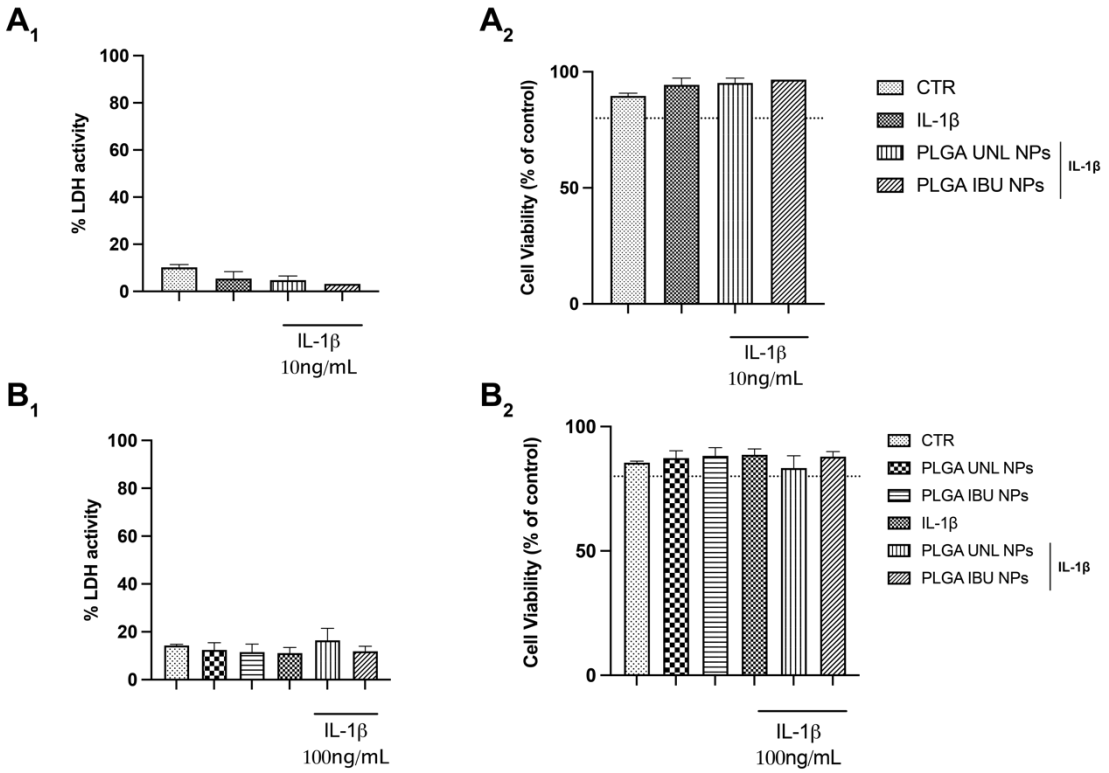

Figure 13. Cell viability of cells in 3D pellets – LDH activity was measured in pellets subjected to different culture conditions after stimulus with A – 10ng/mL and B – 100 mg/mL of IL-1β. 1 and 2 subscripts refer to LDH activity and cell viability (Cell viability=1-((LDH activity)), respectively.

4.5. Chondrogenic capacity of the Col-PLA scaffold with Ibuprofen loaded PLGA nanoparticles

Cytotoxicity and morphological assessment

The effect of Col-PLA scaffold with Ibuprofen loaded PLGA nanoparticles (Col-PLA PLGA IBU NPs) on cell viability was analyzed through the LIVE/DEAD cytotoxic/viability assay and LDH activity. Col-PLA scaffold with unloaded Ibuprofen PLGA nanoparticles (Col-PLA PLGA UNL NPs) were used as control. The LIVE/DEAD assay is a quick and easy two-colour assay, used to determine viability of cells based on plasma membrane integrity and esterase activity. Calcein stains live cells green, indicating intracellular esterase activity, while propidium iodine (Ex 530 nm/Em 645 nm) stains dead cells red, indicating loss of plasma membrane integrity. The microscopic evaluation of cell viability by the LIVE/DEAD assay showed the presence of a low number of dead cells in the Col-PLA PLGA UNL NPs and on Col-PLA PLGA IBU NPs scaffolds (Panel A - Figure 14), with no apparent differences between these two conditions. The stimulation with IL-1β (100ng/ml) increased the cell death in all conditions.

The quantification of the release of LDH into the supernatant was applied also to determine the scaffolds' toxicity towards cells due to the evidence that LDH is a cytosolic enzyme that is released into the culture medium by damaged cells. As demonstrated in Panels B_1, B_2 C_1 and C_2 - Figure 14 cells seeded onto Col-PLA PLGA IBU NPs scaffolds and Col-PLA PLGA UNL NPs scaffolds maintained their viability during the first 7 days in culture. Further, the concentration of IL-1β, one of the first triggers of cartilage degradation in OA, does not seem to interfere with LDH release and consequent cell death of chondrocytes when these are seeded in both types of scaffolds, with results showing similar cell viability in unstimulated and stimulated conditions for both concentrations. However, the results of Live Dead assays showed the opposite, this disparity could be explained with the fact that the supernatant for the analysis of LDH activity was collected 7 days after IL-1β stimulus and probably the high levels of LDH was released few hours after stimulus. In addition, the half-life of LDH released from cells into the surrounding medium is approximately 9 hours. With these results we concluded that the anti-inflammatory nanoenabled Col-PLAs (Col-PLA PLGA IBU NPs scaffolds), allowed human chondrocyte culture without cytotoxicity effects (resting conditions).

Regarding cell morphology In Panel C - Figure 14 it can be observed that cells seeded immediately after isolation presented the round shape typical of the differentiated chondrocytes with no differences can be noticed between Col-PLA PLGA UNL NPs scaffold and Col-PLA PLGA IBU NPs scaffold.

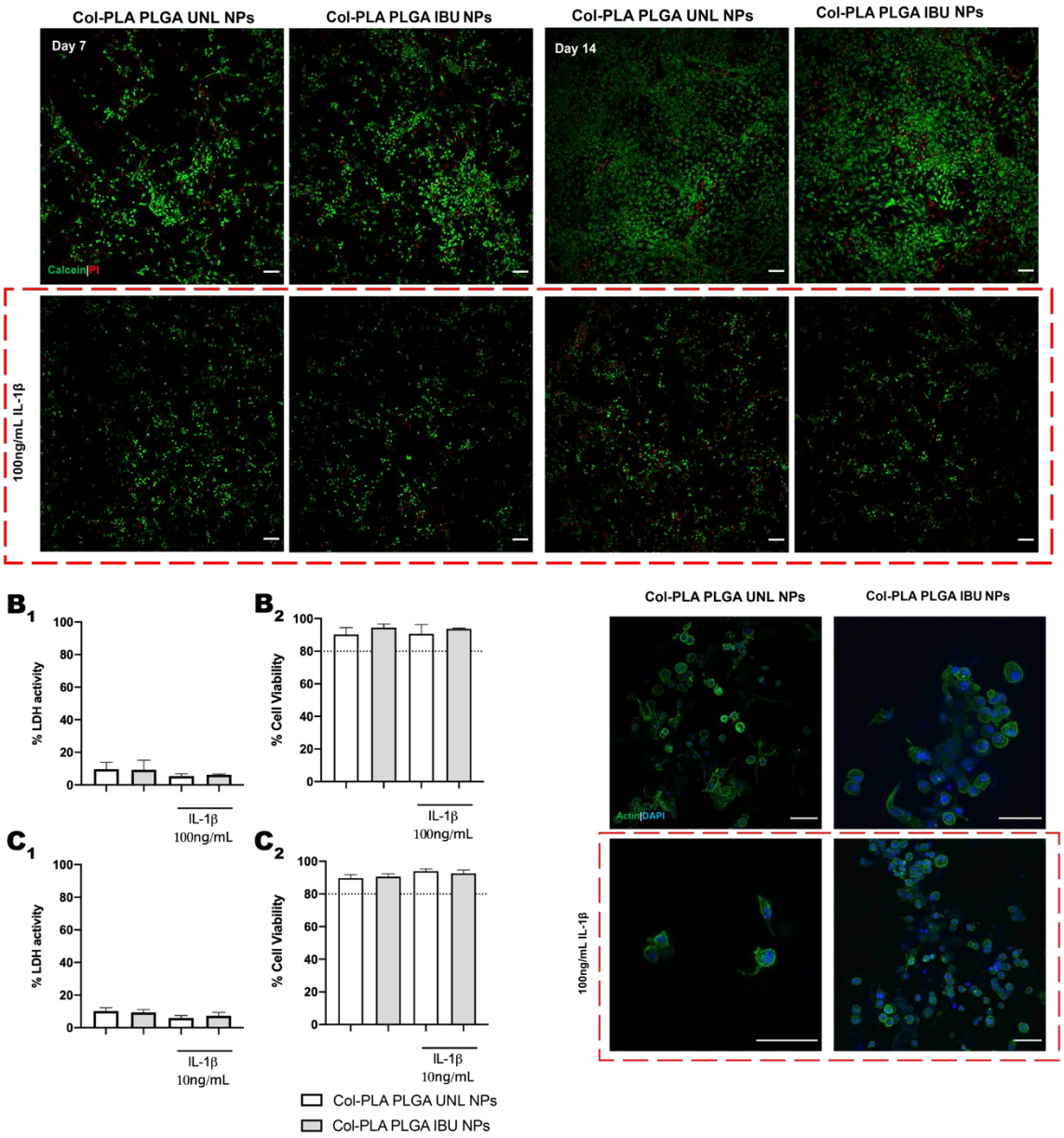

*Figure 14. Cell viability– **A** - LIVE/DEAD assay of chondrocytes seeded in Col-PLA PLGA UNL NPs and Col-PLA PLGA IBU NPs scaffolds immediately after cell isolation and culture for 14 days. Without or with stimulation with 100ng/ml of IL-1β. Propidium iodide stains dead cells in red and calcein AM stains live cells in green (Scale bar: 50µm). n = 3. **B and C** - LDH activity of human chondrocytes seeded on the Col-PLA and cell viability after 7 days for scaffolds unstimulated vs stimulated with 100ng/ml IL-1β (**B_1** and **B_2** , respectively) or 10ng/mL IL-1β (**C_1** and **C_2** , respectively). **C** - Actin staining of chondrocytes seeded in the Col-PLA scaffold immediately after isolation. With and without pro-inflammatory stimulus of 100ng/ml of IL-1β. Analysis performed at day 7 of cell culture. (Scale bar represents: 50µm). n= 3*

4.6. Cytokine profile

4.6.1 IL-1β and IL-6

In OA, IL-1β induces the chondrocytes to produce IL-6. In fact, OA induced cartilage releases significant amounts of IL-6. Its production is linked to activation of transcription factor NF-kB, which further increases the expression of IL-1β and other proteins like NO synthase and COX-2 creating the previously mentioned cycle of cartilage auto-destruction [86].

This would explain why IL-1β stimulation of Col-PLA scaffolds greatly increases the expression of IL-6 (Figure 15). The anti-inflammatory effect of ibuprofen appears to be insufficient to stop cycle of production of inflammatory cytokines, even after 7 days post removal of IL-1β from the medium, with Col-PLA PLGA IBU NPs scaffolds only showing a slight reduction of IL-6 concentration

As for the stimulation of pellets, even after 7 days post-stimulation, IL-6 is very high, although there is a reduction of IL-6 in the PLGA IBU NPs condition. In this case, similarly to scaffolds, after the initial IL-1β trigger.

The reversion of the pro-inflammatory cytokine cycle would be difficult since ibuprofen inhibits the COX-2 enzyme only stopping the production of prostaglandins, which are downstream in the inflammatory pathways. However, the small reduction associated to PLGA IBU NPs may be linked to inhibition of the self-amplification loop of COX-2-PGE_2-EP2. In this positive feedback process PGE_2 induces PGE_2 receptor 2 activation. This receptor induces NF-kB contributing to the perpetuation of inflammatory processes, including the induction of COX-2 [128].

Regarding the evaluation of IL-1β levels after stimulation with 100ng/ml of exogenous IL-1β, we observed an increase in both conditions (Col-PLA PLGA UNL NPs and Col-PLA PLGA IBU NPs scaffolds), as expected, being more pronounced in the Col-PLA PLGA IBU NPs scaffolds, 7 days after stimulation. In the case of 3D pellets this stimulus may lead to an acute inflammatory response, perhaps not accompanying in vivo conditions. This acute inflammatory response leads to a great activation of catabolic pathways and perpetuation of the cytokine production cascade, as previous showed with the decrease in pellets size and in the ECM content (Figure 12) which is why new experiments with a lower concentration of IL-1β (10 ng/mL) were set up and are under analysis.

Under these new conditions, preliminary results regarding IL-1β production after the stimulus with a lower concentration (10ng/mL of IL-1β) was lower in the Col-PLA PLGA IBU NPs scaffolds than in the Col-PLA PLGA UNL NPs. Concomitantly, also 3D pellets treated with PLGA IBU NPs also showed a reduction in the production of IL-1β.

From these data we can infer that a stimulus not as acute as the one delivered by 100ng/ml IL-1β may provide cells with time to repair from inflammation. The positive results with PLGA IBU NPs may show that ibuprofen can be effective in the treatment/ or symptom reduction of mild or low-grade inflammation. The results with the scaffolds also bring forward the importance of providing the cells with a biocompatible matrix for optimization of drug delivery.

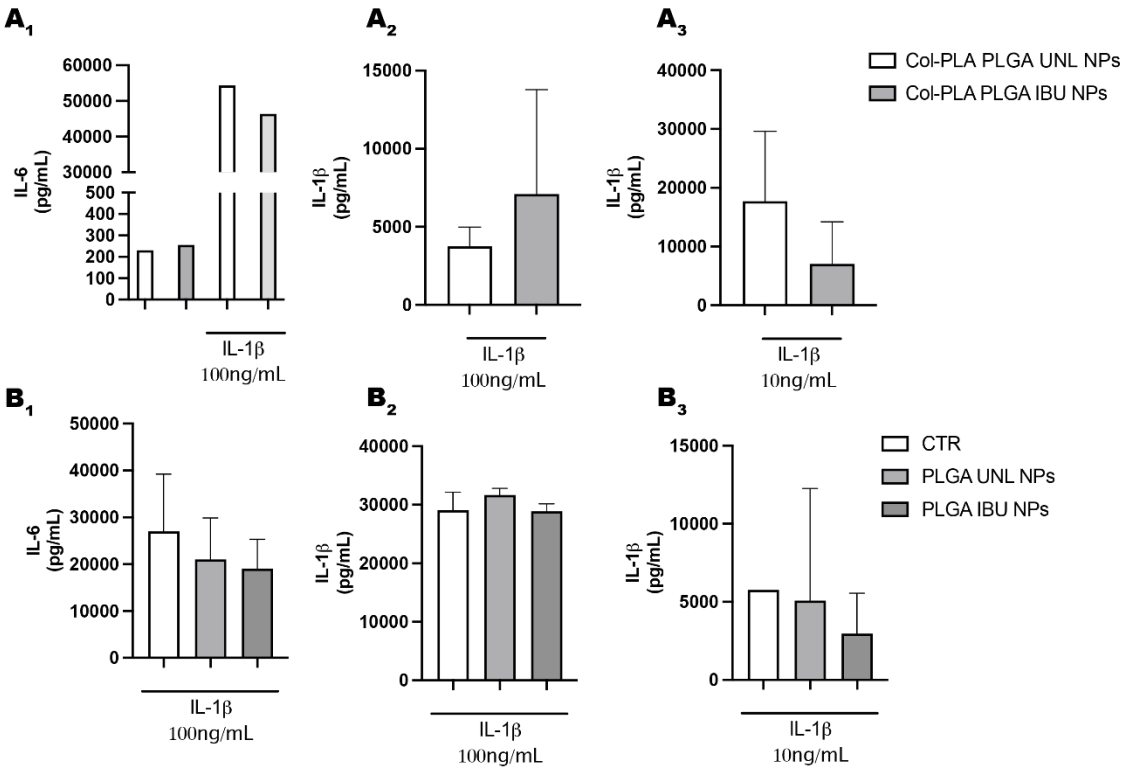

*Figure 15. Cytokine Profile – **A** – cytokines present in the medium of Col-PLA scaffolds, 7 days after stimulation with IL-1β (100 ng/ml and 10 ng/ml) (**A_1** – IL-6, **A_2** and **A_3** endogenous IL-1β); **B** - cytokines present in the medium of 3D pellets, 21 days after stimulation with IL-1β (100 ng/ml and 10 ng/ml) (**B_1** – IL-6, **B_2** and **B_3** endogenous IL-1β).*

4.6.2 PGE_2

Ibuprofen is an NSAID that inhibits, with different powers, the COX-1 and 2 enzymes. The inhibition of these enzymes, especially the COX-2 leads to anti-inflammatory action by reducing PGE_2 and prostacyclin, while providing analgesia at the same time. The measurement of cytokine levels released by chondrocytes when cultured in Col-PLA PLGA UNL NPs scaffolds with and in Col-PLA PLGA IBU NPs scaffolds was performed by ELISA. This analysis was carried out for pro-inflammatory mediators PGE_2 with commercially available kits, following the manufacturer's instructions. In Figure 16 are presented the experimental conditions in which IL-1β (100ng/mL) was added. The results showed a slightly decrease in the release of PGE_2 at day 14 when cells were seeded in Col-PLA PLGA IBU NPs scaffolds versus those seeded in Col-PLA PLGA UNL NPs scaffolds. This result reveals a direct effect of ibuprofen in the inhibition of COX2, and that Col-PLA PLGA IBU NPs scaffolds seem to be effective to control the inflammatory response.

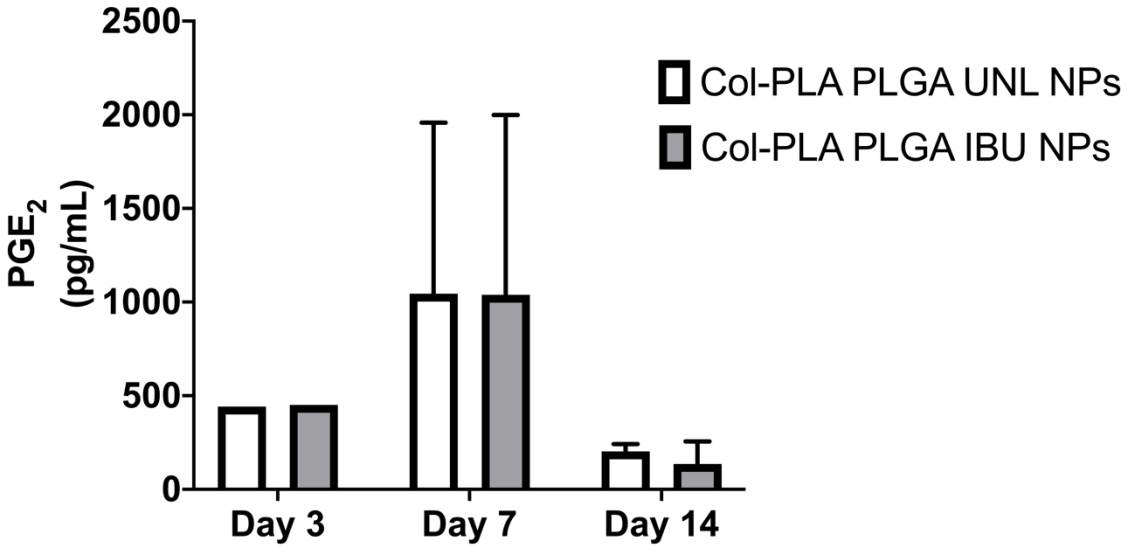

Figure 16 - Levels of PGE_2 measured in the CM 3, 7 and 14 days after stimulation under pro-inflammatory stimulus (IL-1β (100ng/ml)).

4.7. Expression of ECM markers type II collagen and aggrecan

To evaluate how the Col-PLA PLGA IBU NPs scaffolds could protect chondrocytes ECM production after pro-inflammatory stimulation, the expression of the chondrogenic markers aggrecan and type II collagen were assessed in Col-PLA PLGA UNL NPs scaffolds and Col-PLA PLGA IBU NPs scaffolds by immunofluorescence staining analysis. As seen in Figure 17, aggrecan production is impacted by the addition of IL-1β. However, the Col-PLA PLGA IBU NPs scaffold experiments showed higher aggrecan production than the Col-PLA PLGA UNL NPs scaffold (Panel A - Figure 17). The same tendency, as described above for aggrecan production, was observed for type II collagen production (Panel B - Figure 17). The number of experiments were increased to conclude on the possible protective effect of Col-PLA PLGA IBU NPs scaffolds regarding the ECM degradation induced by IL-1β. These experiments are under analysis.

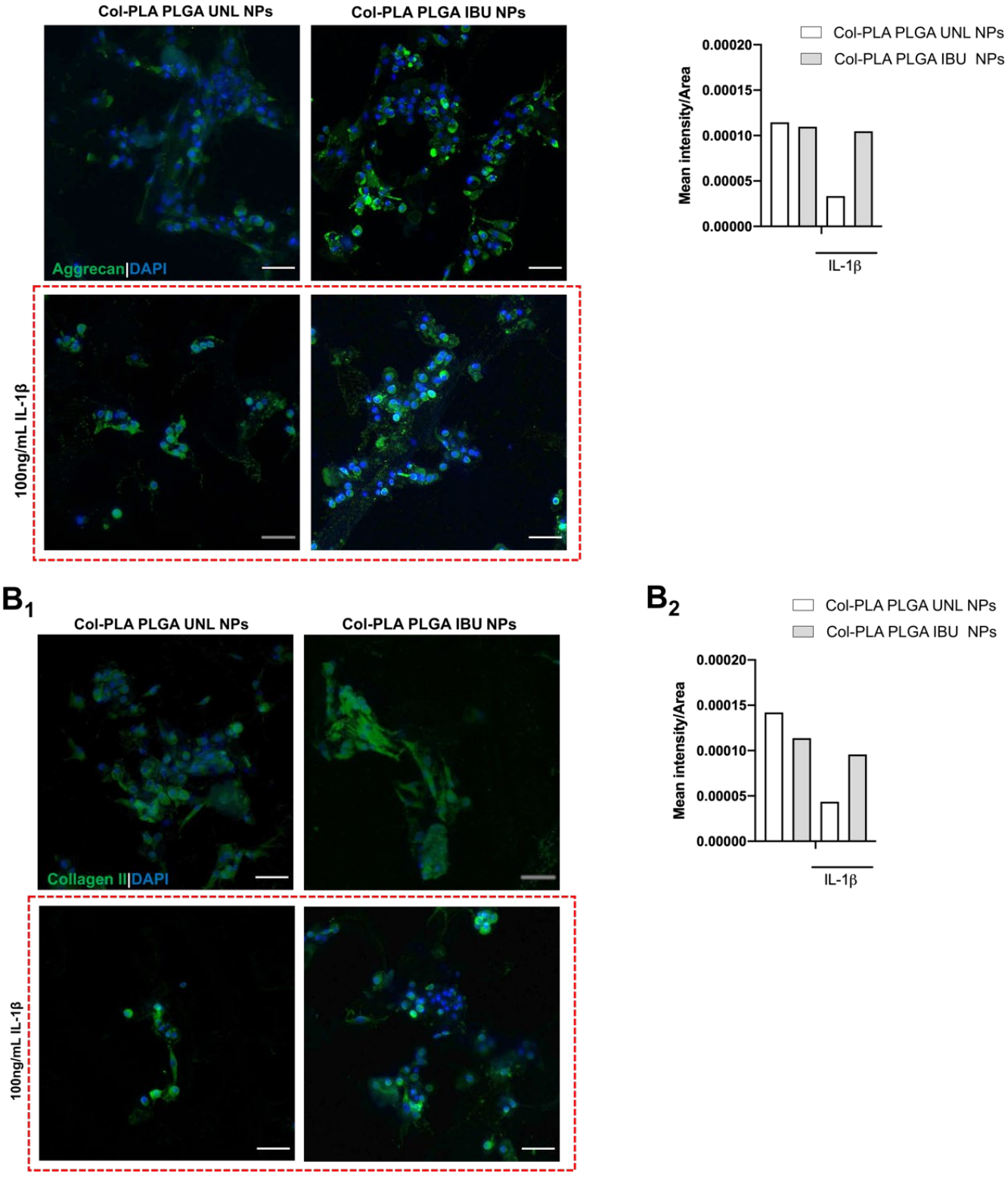

Figure 17. Aggrecan and Collagen type II expression - ***A_1 and B_1 -*** Immunohistochemical staining against aggrecan and type II collagen, respectively, at day 7 of culture of chondrocytes in Col-PLA PLGA UNL NPs scaffolds and Col-PLA PLGA IBU NPs scaffolds. With and without stimulation with 100ng/ml of IL-1β. ***A_2 and B_2 –*** the graphs presented are the mean intensity per area. (Scale bar: 50 μm).

4.8. Invasion assays

In OA, besides paracrine interactions between macrophages and chondrocytes, ECM degradation products and cell debris can activate and recruit macrophages. Invasion assays were performed with primary human macrophages stimulated with conditioned media collected from primary articular chondrocytes seeded and cultured for 7 days in Col-PLA PLGA UNL NPs or Col-PLA PLGA IBU NPs scaffolds. The number of macrophages that invaded through the Matrigel coating significantly decreased in the presence of conditioned media from Col-PLA PLGA IBU NPs scaffolds (Figure 18). This indicates that chondrocytes seeded and cultured in the Col-PLA PLGA UNL NPs scaffolds produce soluble factors that promote macrophage invasion. In these invasion assays cells must remodel the matrix to cross the transwells. The presence of PLGA IBU NPs seems to reduce recruitment of macrophages to the conditioned medium produced by chondrocytes. This is essential in scaffolds since low immunogenicity improves the chance of scaffold integration *in vivo.*

The conditioned medium of chondrocytes should be analyzed to understand what type of molecules could interact with macrophages in the context of this Col-PLA-nanoparticles-chondrocyte microenvironment.

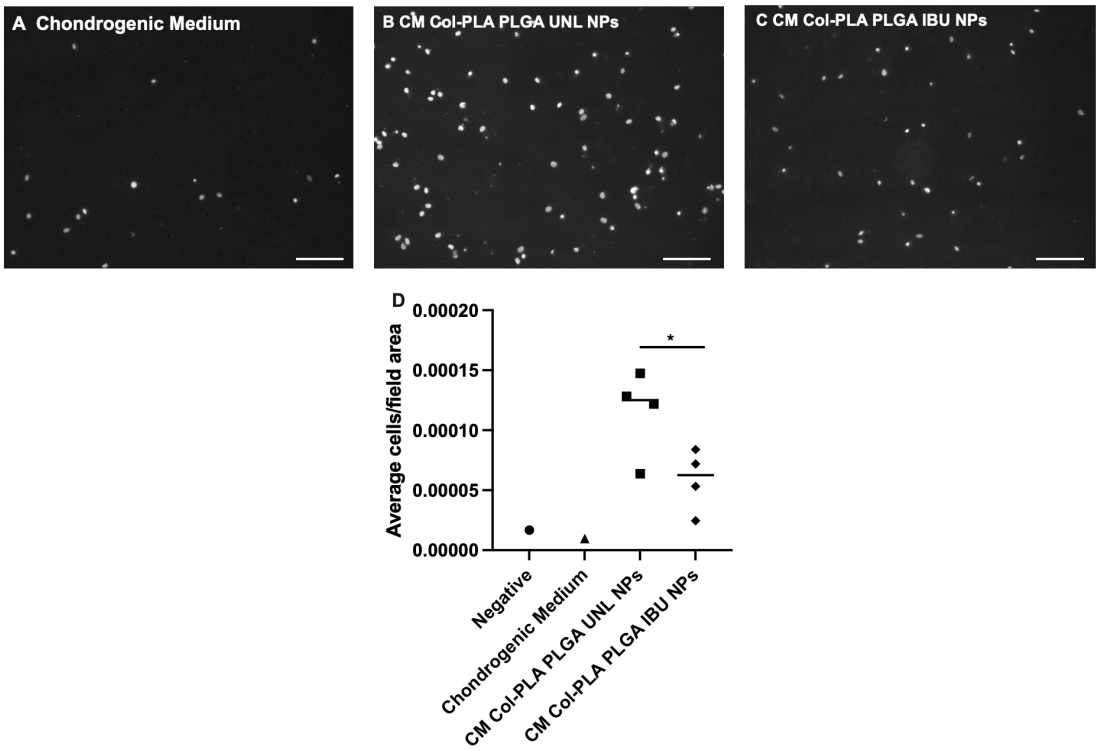

Figure 18. Invasion Assay - Macrophage's invasion through filters with 8 µm pores and coated with Matrigel was determined after 24 h of incubation. The lower compartment of the invasion chamber was filled with serum-free medium, as a negative control, or complete chondrogenic medium with TGF-β3 (as experimental control) or with CM of chondrocytes seeded and cultured for 7 days in Col-PLA PLGA UNL NPs or Col-PLA PLGA IBU NPs scaffolds. Kruskal-Wallis test. Each dot represents 1 independent donor.

5. Conclusions and future work

Damage to AC rapidly progresses is highly influenced by the inflammatory status. Throughout time many strategies were developed to treat OA and the pain and discomfort associated to it. Starting with classical pharmacological treatment, then with the first TE strategies and surgeries. TE has opened space to develop new types of treatments and to study the cell interactions with biomaterials with potentially healing capacities.

Throughout this work, we successfully established primary human chondrocytes cultures first expanding them and then using these expanded chondrocytes in chondrogenic conditions. We were able to document the effects of IL-1β in the ECM of AC deposition and understand that is has detrimental effects in AC. We also established that once this inflammatory stimulus has been added it is difficult to overturn it, as shown by the high concentrations of IL-1β , IL-6, and PGE_2 even after this exogenous stimulus was removed. Nanoparticles loaded with ibuprofen were partially effective in counteracting the effects of inflammation in AC.

The use of a novel nanoenabled Col-PLA scaffold for the seeding and culture of articular chondrocytes was successful, with low cell death when seeded on them. Articular chondrocytes continued to produce and deposit type II collagen and ECM on these scaffolds even after an inflammatory stimulus. These results were even more positive when the scaffolds contained PLGA IBU NPs where there was even more ECM deposition.

Preliminary results have shown that the scaffold with ibuprofen loaded NPs seems to be less immunogenic than the one with unloaded NPs.

In the future, other drugs should be used to load in the NPs like corticosteroids or other inhibitors of molecules that participate upstream in the catabolic pathways.

6. References

1. Standring, S., *Gray's anatomy : the anatomical basis of clinical practice*. Forty-second edition. ed. 2021, New York: Elsevier,.
2. Scanzelo, C.R. and S.R. Goldring, *Biology of the Normal Joint*, in *Firestein & Kelley's textbook of rheumatology*, G.S. Firestein, et al., Editors. 2020, Elsevier, Inc.: Philadelphia. p. volumes cm.
3. Standring, S., *Functional anatomy of the musculoskeletal system*, in *Gray's anatomy : the anatomical basis of clinical practice*. 2016, Elsevier Limited: New York. p. 81-122.
4. Goldring, S.R. and M.B. Goldring, *Chapter 1 - Biology of the Normal Joint*, in *Kelley and Firestein's Textbook of Rheumatology (Tenth Edition)*, G.S. Firestein, et al., Editors. 2017, Elsevier. p. 1-19.e4.
5. Xu, H., et al., *Distribution of lymphatic vessels in normal and arthritic human synovial tissues.* Ann Rheum Dis, 2003. **62**(12): p. 1227-9.
6. Hui, A.Y., et al., *A systems biology approach to synovial joint lubrication in health, injury, and disease.* Wiley Interdiscip Rev Syst Biol Med, 2012. **4**(1): p. 15-37.
7. Decker, R.S., E. Koyama, and M. Pacifici, *Articular Cartilage: Structural and Developmental Intricacies and Questions.* Curr Osteoporos Rep, 2015. **13**(6): p. 407-14.
8. Sophia Fox, A.J., A. Bedi, and S.A. Rodeo, *The basic science of articular cartilage: structure, composition, and function.* Sports Health, 2009. **1**(6): p. 461-8.
9. Sanchez-Adams, J., et al., *The mechanobiology of articular cartilage: bearing the burden of osteoarthritis.* Curr Rheumatol Rep, 2014. **16**(10): p. 451.
10. Ross, M.H. and W. Pawlina, *Cartilage*, in *Histology : a text and atlas : with correlated cell and molecular biology*. 2016, Wolters Kluwer Health: Philadelphia. p. 194 - 213.
11. Vander Heiden, M.G., L.C. Cantley, and C.B. Thompson, *Understanding the Warburg effect: the metabolic requirements of cell proliferation.* Science (New York, N.Y.), 2009. **324**(5930): p. 1029-1033.
12. Zheng, L., et al., *The role of metabolism in chondrocyte dysfunction and the progression of osteoarthritis.* Ageing Res Rev, 2021. **66**: p. 101249.
13. Poole, A.R., et al., *Composition and structure of articular cartilage: a template for tissue repair.* Clin Orthop Relat Res, 2001(391 Suppl): p. S26-33.
14. Hayes, A.J., et al., *The development of articular cartilage: evidence for an appositional growth mechanism.* Anat Embryol (Berl), 2001. **203**(6): p. 469-79.
15. Bi, X., et al., *Fourier transform infrared imaging spectroscopy investigations in the pathogenesis and repair of cartilage.* Biochim Biophys Acta, 2006. **1758**(7): p. 934-41.
16. Decker, R.S., E. Koyama, and M. Pacifici, *Articular Cartilage: Structural and Developmental Intricacies and Questions.* Current Osteoporosis Reports, 2015. **13**(6): p. 407-14.
17. Killen, M.-C. and C.P. Charalambous, *Advances in cartilage restoration techniques*, in *Advances in Medical and Surgical Engineering*, W. Ahmed, et al., Editors. 2020, Academic Press. p. 71-83.
18. Peng, Z., et al., *The regulation of cartilage extracellular matrix homeostasis in joint cartilage degeneration and regeneration.* Biomaterials, 2021. **268**: p. 120555.

19. Myers, E.R., W.M. Lai, and V.C. Mow, *A continuum theory and an experiment for the ion-induced swelling behavior of articular cartilage.* J Biomech Eng, 1984. **106**(2): p. 151-8.
20. Eyre, D.R., *The collagens of articular cartilage.* Seminars in Arthritis and Rheumatism, 1991. **21**(3, Supplement 2): p. 2-11.
21. Firestein, G.S., et al., *Firestein & Kelley's textbook of rheumatology*. 11. ed. 2020, Philadelphia: Elsevier, Inc. volumes cm.
22. Guilak, F., et al., *Osteoarthritis as a disease of the cartilage pericellular matrix.* Matrix Biol, 2018. **71-72**: p. 40-50.
23. Roughley, P.J. and J.S. Mort, *The role of aggrecan in normal and osteoarthritic cartilage.* J Exp Orthop, 2014. **1**(1): p. 8.
24. Kiani, C., et al., *Structure and function of aggrecan.* Cell Res, 2002. **12**(1): p. 19-32.
25. Myers, E.R., W.M. Lai, and V.C. Mow, *A Continuum Theory and an Experiment for the Ion-Induced Swelling Behavior of Articular Cartilage.* Journal of Biomechanical Engineering, 1984. **106**(2): p. 151-158.
26. Athanasiou, K.A., et al., *Articular cartilage*. Second edition. ed. 2016, Boca Raton: Taylor & Francis. p.
27. Hodgkinson, T., et al., *Mechanosignalling in cartilage: an emerging target for the treatment of osteoarthritis.* Nat Rev Rheumatol, 2022. **18**(2): p. 67-84.
28. Loeser, R.F., *Integrins and chondrocyte-matrix interactions in articular cartilage.* Matrix Biol, 2014. **39**: p. 11-6.
29. Zhen, G., et al., *Mechanical stress determines the configuration of TGFbeta activation in articular cartilage.* Nat Commun, 2021. **12**(1): p. 1706.
30. Barrett-Jolley, R., et al., *The emerging chondrocyte channelome.* Front Physiol, 2010. **1**: p. 135.
31. Mow, V.C., C.C. Wang, and C.T. Hung, *The extracellular matrix, interstitial fluid and ions as a mechanical signal transducer in articular cartilage.* Osteoarthritis and Cartilage, 1999. **7**(1): p. 41-58.
32. Agarwal, P., et al., *A dysfunctional TRPV4-GSK3beta pathway prevents osteoarthritic chondrocytes from sensing changes in extracellular matrix viscoelasticity.* Nat Biomed Eng, 2021. **5**(12): p. 1472-1484.
33. Liedtke, W., *TRPV4 plays an evolutionary conserved role in the transduction of osmotic and mechanical stimuli in live animals.* J Physiol, 2005. **567**(Pt 1): p. 53-8.
34. Du, G., et al., *Roles of TRPV4 and piezo channels in stretch-evoked Ca(2+) response in chondrocytes.* Exp Biol Med (Maywood), 2020. **245**(3): p. 180-189.
35. Zhang, M., et al., *TRPV4 and PIEZO Channels Mediate the Mechanosensing of Chondrocytes to the Biomechanical Microenvironment.* Membranes (Basel), 2022. **12**(2).
36. Lee, W., et al., *Inflammatory signaling sensitizes Piezo1 mechanotransduction in articular chondrocytes as a pathogenic feed-forward mechanism in osteoarthritis.* Proceedings of the National Academy of Sciences, 2021. **118**(13): p. e2001611118.
37. Ryu, J., B.V. Treadwell, and H.J. Mankin, *Biochemical and metabolic abnormalities in normal and osteoarthritic human articular cartilage.* Arthritis Rheum, 1984. **27**(1): p. 49-57.

38. Umlauf, D., et al., *Cartilage biology, pathology, and repair.* Cell Mol Life Sci, 2010. **67**(24): p. 4197-211.
39. Kumar, V., et al., *Robbins & Cotran pathologic basis of disease*. 2021, Elsevier,: Philadelphia, Pa. p. 1 online resource.
40. Lopez-Otin, C., et al., *The hallmarks of aging.* Cell, 2013. **153**(6): p. 1194-217.
41. Henrotin, Y.E., P. Bruckner, and J.P.L. Pujol, *The role of reactive oxygen species in homeostasis and degradation of cartilage.* Osteoarthritis and Cartilage, 2003. **11**(10): p. 747-755.
42. Bolduc, J.A., J.A. Collins, and R.F. Loeser, *Reactive oxygen species, aging and articular cartilage homeostasis.* Free Radic Biol Med, 2019. **132**: p. 73-82.
43. Loeser, R.F., J.A. Collins, and B.O. Diekman, *Ageing and the pathogenesis of osteoarthritis.* Nat Rev Rheumatol, 2016. **12**(7): p. 412-20.
44. Shmulevich, R. and V. Krizhanovsky, *Cell Senescence, DNA Damage, and Metabolism.* Antioxid Redox Signal, 2021. **34**(4): p. 324-334.
45. Eckel, R.H., *The Metabolic Syndrome*, in *Harrison's principles of internal medicine*, J. Loscalzo, et al., Editors. 2022, McGraw Hill: New York. p. 3150-3157.
46. Eckel, R.H., S.M. Grundy, and P.Z. Zimmet, *The metabolic syndrome.* The Lancet, 2005. **365**(9468): p. 1415-1428.
47. Ouchi, N., et al., *Adipokines in inflammation and metabolic disease.* Nat Rev Immunol, 2011. **11**(2): p. 85-97.
48. Little, C.B. and D.J. Hunter, *Post-traumatic osteoarthritis: from mouse models to clinical trials.* Nat Rev Rheumatol, 2013. **9**(8): p. 485-97.
49. Fox, A.J., et al., *The human meniscus: a review of anatomy, function, injury, and advances in treatment.* Clin Anat, 2015. **28**(2): p. 269-87.
50. Englund, M., et al., *Meniscus pathology, osteoarthritis and the treatment controversy.* Nat Rev Rheumatol, 2012. **8**(7): p. 412-9.
51. Wang, L.J., et al., *Post-traumatic osteoarthritis following ACL injury.* Arthritis Res Ther, 2020. **22**(1): p. 57.
52. Bansal, S., et al., *Meniscal repair: The current state and recent advances in augmentation.* J Orthop Res, 2021. **39**(7): p. 1368-1382.
53. Di Cesare, P.E., et al., *Pathogenesis of Osteoarthritis*, in *Kelley and Firestein's Textbook of Rheumatology*, G.S. Firestein, et al., Editors. 2017, Elsevier. p. 1685-1704.e8.
54. Goldring, M.B., et al., *Defining the roles of inflammatory and anabolic cytokines in cartilage metabolism.* Ann Rheum Dis, 2008. **67 Suppl 3**: p. iii75-82.
55. Martel-Pelletier, J., et al., *Osteoarthritis.* Nat Rev Dis Primers, 2016. **2**: p. 16072.
56. Lambert, C., et al., *The Damage-Associated Molecular Patterns (DAMPs) as Potential Targets to Treat Osteoarthritis: Perspectives From a Review of the Literature.* Front Med (Lausanne), 2020. **7**: p. 607186.
57. Rigoglou, S. and A.G. Papavassiliou, *The NF-kappaB signalling pathway in osteoarthritis.* Int J Biochem Cell Biol, 2013. **45**(11): p. 2580-4.
58. Kapoor, M., et al., *Role of proinflammatory cytokines in the pathophysiology of osteoarthritis.* Nat Rev Rheumatol, 2011. **7**(1): p. 33-42.
59. Kim, S.F., *The role of nitric oxide in prostaglandin biology; update.* Nitric Oxide, 2011. **25**(3): p. 255-64.
60. Hardy, M.M., et al., *Cyclooxygenase 2-dependent prostaglandin E2 modulates cartilage proteoglycan degradation in human osteoarthritis explants.* Arthritis Rheum, 2002. **46**(7): p. 1789-803.

61. Gong, X., et al., *Altered spontaneous calcium signaling of in situ chondrocytes in human osteoarthritic cartilage.* Sci Rep, 2017. **7**(1): p. 17093.
62. Murata, M., K. Yudoh, and K. Masuko, *The potential role of vascular endothelial growth factor (VEGF) in cartilage: how the angiogenic factor could be involved in the pathogenesis of osteoarthritis?* Osteoarthritis Cartilage, 2008. **16**(3): p. 279-86.
63. Ashraf, S. and D.A. Walsh, *Angiogenesis in osteoarthritis.* Curr Opin Rheumatol, 2008. **20**(5): p. 573-80.
64. Suri, S., et al., *Neurovascular invasion at the osteochondral junction and in osteophytes in osteoarthritis.* Annals of the rheumatic diseases, 2007. **66**(11): p. 1423-1428.
65. Mapp, P.I. and D.A. Walsh, *Mechanisms and targets of angiogenesis and nerve growth in osteoarthritis.* Nat Rev Rheumatol, 2012. **8**(7): p. 390-8.
66. Kahraman, E., et al., *Cutting-Edge Technologies for Inflamed Joints on Chip: How Close Are We?* Front Immunol, 2022. **13**: p. 802440.
67. Hunter, D.J., J.J. McDougall, and F.J. Keefe, *The Symptoms of Osteoarthritis and the Genesis of Pain.* Medical Clinics of North America, 2009. **93**(1): p. 83-100.
68. Schaible, H.-G., *Nociceptive neurons detect cytokines in arthritis.* Arthritis Research & Therapy, 2014. **16**(5): p. 470.
69. Fu, K., S.R. Robbins, and J.J. McDougall, *Osteoarthritis: the genesis of pain.* Rheumatology (Oxford), 2018. **57**(suppl_4): p. iv43-iv50.
70. Firestein, G.S., et al., *Kelley and Firestein's textbook of rheumatology*. Tenth edition. ed. 2017, Philadelphia, PA: Elsevier. 1 volume (pagings).
71. da Costa, B.R., et al., *Effectiveness of non-steroidal anti-inflammatory drugs for the treatment of pain in knee and hip osteoarthritis: a network meta-analysis.* Lancet, 2017. **390**(10090): p. e21-e33.
72. Rang, H.P., et al., *Rang and Dale's pharmacology*. Eighth edition. ed. 2016, Edinburgh?: Elsevier Churchill Livingstone. xv, 760 pages.
73. Abramoff, B. and F.E. Caldera, *Osteoarthritis: Pathology, Diagnosis, and Treatment Options.* Med Clin North Am, 2020. **104**(2): p. 293-311.
74. Ayhan, E., H. Kesmezacar, and I. Akgun, *Intraarticular injections (corticosteroid, hyaluronic acid, platelet rich plasma) for the knee osteoarthritis.* World J Orthop, 2014. **5**(3): p. 351-61.
75. Kwon, H., et al., *Surgical and tissue engineering strategies for articular cartilage and meniscus repair.* Nat Rev Rheumatol, 2019. **15**(9): p. 550-570.
76. Gudas, R., et al., *Ten-year follow-up of a prospective, randomized clinical study of mosaic osteochondral autologous transplantation versus microfracture for the treatment of osteochondral defects in the knee joint of athletes.* Am J Sports Med, 2012. **40**(11): p. 2499-508.
77. Bedi, A., B.T. Feeley, and R.J. Williams, 3rd, *Management of articular cartilage defects of the knee.* J Bone Joint Surg Am, 2010. **92**(4): p. 994-1009.
78. Zellner, J. and P. Angele, *Cell-Based Cartilage Regeneration*, in *Cartilage: Volume 3: Repair Strategies and Regeneration*, S. Grässel and A. Aszódi, Editors. 2017, Springer International Publishing: Cham. p. 95-107.
79. Hinckel, B.B., et al., *Algorithm for Treatment of Focal Cartilage Defects of the Knee: Classic and New Procedures.* Cartilage, 2021. **13**(1_suppl): p. 473s-495s.
80. Adkisson, H.D.t., et al., *The potential of human allogeneic juvenile chondrocytes for restoration of articular cartilage.* Am J Sports Med, 2010. **38**(7): p. 1324-33.

81. Yanke, A.B., et al., *DeNovo NT Particulated Juvenile Cartilage Implant.* Sports Med Arthrosc Rev, 2015. **23**(3): p. 125-9.
82. Hunter, D.J., D. Schofield, and E. Callander, *The individual and socioeconomic impact of osteoarthritis.* Nat Rev Rheumatol, 2014. **10**(7): p. 437-41.
83. Wainwright, C., et al., *Age at hip or knee joint replacement surgery predicts likelihood of revision surgery.* J Bone Joint Surg Br, 2011. **93**(10): p. 1411-5.
84. Makris, E.A., et al., *Repair and tissue engineering techniques for articular cartilage.* Nat Rev Rheumatol, 2015. **11**(1): p. 21-34.
85. Armiento, A.R., et al., *Biomaterials for articular cartilage tissue engineering: Learning from biology.* Acta Biomater, 2018. **65**: p. 1-20.
86. Attur, M.G., et al., *Autocrine production of IL-1 beta by human osteoarthritis-affected cartilage and differential regulation of endogenous nitric oxide, IL-6, prostaglandin E2, and IL-8.* Proc Assoc Am Physicians, 1998. **110**(1): p. 65-72.
87. Byambaa, B. and J.P. Vacanti, *Chapter 53 - Engineering cartilage and other structural tissues: principals of bone and cartilage reconstruction*, in *Principles of Tissue Engineering (Fifth Edition)*, R. Lanza, et al., Editors. 2020, Academic Press. p. 979-987.
88. Funayama, A., et al., *Repair of full-thickness articular cartilage defects using injectable type II collagen gel embedded with cultured chondrocytes in a rabbit model.* Journal of Orthopaedic Science, 2008. **13**(3): p. 225-232.
89. Pearce, H.A., et al., *2.6.3 - Tissue Engineering Scaffolds*, in *Biomaterials Science (Fourth Edition)*, W.R. Wagner, et al., Editors. 2020, Academic Press. p. 1317-1334.
90. Hutmacher, D.W., *Scaffold design and fabrication technologies for engineering tissues--state of the art and future perspectives.* J Biomater Sci Polym Ed, 2001. **12**(1): p. 107-24.
91. Lee, K.Y. and D.J. Mooney, *Hydrogels for Tissue Engineering.* Chemical Reviews, 2001. **101**(7): p. 1869-1880.
92. Conoscenti, G., et al., *PLLA scaffolds produced by thermally induced phase separation (TIPS) allow human chondrocyte growth and extracellular matrix formation dependent on pore size.* Materials Science and Engineering: C, 2017. **80**: p. 449-459.
93. Rosenzweig, D.H., et al., *3D-Printed ABS and PLA Scaffolds for Cartilage and Nucleus Pulposus Tissue Regeneration.* International Journal of Molecular Sciences, 2015. **16**(7): p. 15118-15135.
94. Singhvi, M.S., S.S. Zinjarde, and D.V. Gokhale, *Polylactic acid: synthesis and biomedical applications.* J Appl Microbiol, 2019. **127**(6): p. 1612-1626.
95. Christensen, B.B., et al., *A novel nano-structured porous polycaprolactone scaffold improves hyaline cartilage repair in a rabbit model compared to a collagen type I/III scaffold: in vitro and in vivo studies.* Knee Surgery, Sports Traumatology, Arthroscopy, 2012. **20**(6): p. 1192-1204.
96. Theodoridis, K., et al., *Hyaline cartilage next generation implants from adipose-tissue–derived mesenchymal stem cells: Comparative study on 3D-printed polycaprolactone scaffold patterns.* Journal of Tissue Engineering and Regenerative Medicine, 2019. **13**(2): p. 342-355.
97. Sonomoto, K., et al., *Spontaneous Differentiation of Human Mesenchymal Stem Cells on Poly-Lactic-Co-Glycolic Acid Nano-Fiber Scaffold.* PLoS One, 2016. **11**(4): p. e0153231.

98. Wasyleczko, M., W. Sikorska, and A. Chwojnowski, *Review of Synthetic and Hybrid Scaffolds in Cartilage Tissue Engineering.* Membranes (Basel), 2020. **10**(11).
99. Bellini, D., et al., *PLA-grafting of collagen chains leading to a biomaterial with mechanical performances useful in tendon regeneration.* Journal of the Mechanical Behavior of Biomedical Materials, 2016. **64**: p. 151-160.
100. Chandran, P.L. and F. Horkay, *Aggrecan, an unusual polyelectrolyte: Review of solution behavior and physiological implications.* Acta Biomaterialia, 2012. **8**(1): p. 3-12.
101. Evans, C.H., V.B. Kraus, and L.A. Setton, *Progress in intra-articular therapy.* Nat Rev Rheumatol, 2014. **10**(1): p. 11-22.
102. Owen, S.G., H.W. Francis, and M.S. Roberts, *Disappearance kinetics of solutes from synovial fluid after intra-articular injection.* Br J Clin Pharmacol, 1994. **38**(4): p. 349-55.
103. Vedadghavami, A., C. Zhang, and A.G. Bajpayee, *Overcoming negatively charged tissue barriers: Drug delivery using cationic peptides and proteins.* Nano Today, 2020. **34**.
104. Bajpayee, A.G., et al., *Avidin as a model for charge driven transport into cartilage and drug delivery for treating early stage post-traumatic osteoarthritis.* Biomaterials, 2014. **35**(1): p. 538-549.
105. Li, X., et al., *Nanoparticle-Cartilage Interaction: Pathology-Based Intra-articular Drug Delivery for Osteoarthritis Therapy.* Nanomicro Lett, 2021. **13**(1): p. 149.
106. Bajpayee, A.G. and A.J. Grodzinsky, *Cartilage-targeting drug delivery: can electrostatic interactions help?* Nat Rev Rheumatol, 2017. **13**(3): p. 183-193.
107. Murthy, S.K., *Nanoparticles in modern medicine: state of the art and future challenges.* Int J Nanomedicine, 2007. **2**(2): p. 129-41.
108. Li, S.-D. and L. Huang, *Pharmacokinetics and Biodistribution of Nanoparticles.* Molecular Pharmaceutics, 2008. **5**(4): p. 496-504.
109. Sanita, G., B. Carrese, and A. Lamberti, *Nanoparticle Surface Functionalization: How to Improve Biocompatibility and Cellular Internalization.* Front Mol Biosci, 2020. **7**: p. 587012.
110. Gelperina, S., et al., *The potential advantages of nanoparticle drug delivery systems in chemotherapy of tuberculosis.* Am J Respir Crit Care Med, 2005. **172**(12): p. 1487-90.
111. Amos, N., et al., *Adenoviral gene transfer into osteoarthritis synovial cells using the endogenous inhibitor IκBα reveals that most, but not all, inflammatory and destructive mediators are NFκB dependent.* Rheumatology, 2006. **45**(10): p. 1201-1209.
112. Sica, A. and A. Mantovani, *Macrophage plasticity and polarization: in vivo veritas.* The Journal of clinical investigation, 2012. **122**(3): p. 787-795.
113. Zhang, H., D. Cai, and X. Bai, *Macrophages regulate the progression of osteoarthritis.* Osteoarthritis and Cartilage, 2020. **28**(5): p. 555-561.
114. Samavedi, S., et al., *A Three-Dimensional Chondrocyte-Macrophage Coculture System to Probe Inflammation in Experimental Osteoarthritis.* Tissue Eng Part A, 2017. **23**(3-4): p. 101-114.
115. Bondeson, J., et al., *The role of synovial macrophages and macrophage-produced cytokines in driving aggrecanases, matrix metalloproteinases, and other*

destructive and inflammatory responses in osteoarthritis. Arthritis research & therapy, 2006. **8**(6): p. 1-12.
116. Neeb, L., et al., *IL-1β Stimulates COX-2 Dependent PGE2 Synthesis and CGRP Release in Rat Trigeminal Ganglia Cells.* PLOS ONE, 2011. **6**(3): p. e17360.
117. Attur, M., et al., *Prostaglandin E2 exerts catabolic effects in osteoarthritis cartilage: evidence for signaling via the EP4 receptor.* J Immunol, 2008. **181**(7): p. 5082-8.
118. Li, R., et al., *Ibuprofen attenuates interleukin-1β-induced inflammation and actin reorganization via modulation of RhoA signaling in rabbit chondrocytes.* Acta Biochim Biophys Sin (Shanghai), 2019. **51**(10): p. 1026-1033.
119. Liebschner, M., B. Bucklen, and M. Wettergreen, *Mechanical Aspects of Tissue Engineering.* Semin Plast Surg, 2005. **19**(3): p. 217-28.
120. Sadtler, K., et al., *The Scaffold Immune Microenvironment: Biomaterial-Mediated Immune Polarization in Traumatic and Nontraumatic Applications.* Tissue Eng Part A, 2017. **23**(19-20): p. 1044-1053.
121. Safiri, S., et al., *Global, regional and national burden of osteoarthritis 1990-2017: a systematic analysis of the Global Burden of Disease Study 2017.* Ann Rheum Dis, 2020. **79**(6): p. 819-828.
122. Roos, E.M. and N.K. Arden, *Strategies for the prevention of knee osteoarthritis.* Nat Rev Rheumatol, 2016. **12**(2): p. 92-101.
123. Neto, E., et al., *Micropathological Chip Modeling the Neurovascular Unit Response to Inflammatory Bone Condition.* Adv Healthc Mater, 2022. **11**(11): p. e2102305.
124. O'Driscoll, S.W., F.W. Keeley, and R.B. Salter, *The chondrogenic potential of free autogenous periosteal grafts for biological resurfacing of major full-thickness defects in joint surfaces under the influence of continuous passive motion. An experimental investigation in the rabbit.* JBJS, 1986. **68**(7): p. 1017-1035.
125. Vasconcelos, D.P., et al., *3D chitosan scaffolds impair NLRP3 inflammasome response in macrophages.* Acta Biomater, 2019. **91**: p. 123-134.
126. Zhang, Z., et al., *Hyaline cartilage engineered by chondrocytes in pellet culture: histological, immunohistochemical and ultrastructural analysis in comparison with cartilage explants.* Journal of Anatomy, 2004. **205**(3): p. 229-237.
127. O'Neill, C.M., et al., *Circulating levels of IL-1B+IL-6 cause ER stress and dysfunction in islets from prediabetic male mice.* Endocrinology, 2013. **154**(9): p. 3077-88.
128. Narumiya, S., T. Yokomizo, and J. Aoki, *Lipid Mediators in Inflammation*, in *Inflammation*. 2017. p. 651-694.