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Exploring senescent chondrocytes during aging: sleeper AGEnts of osteoarthritis

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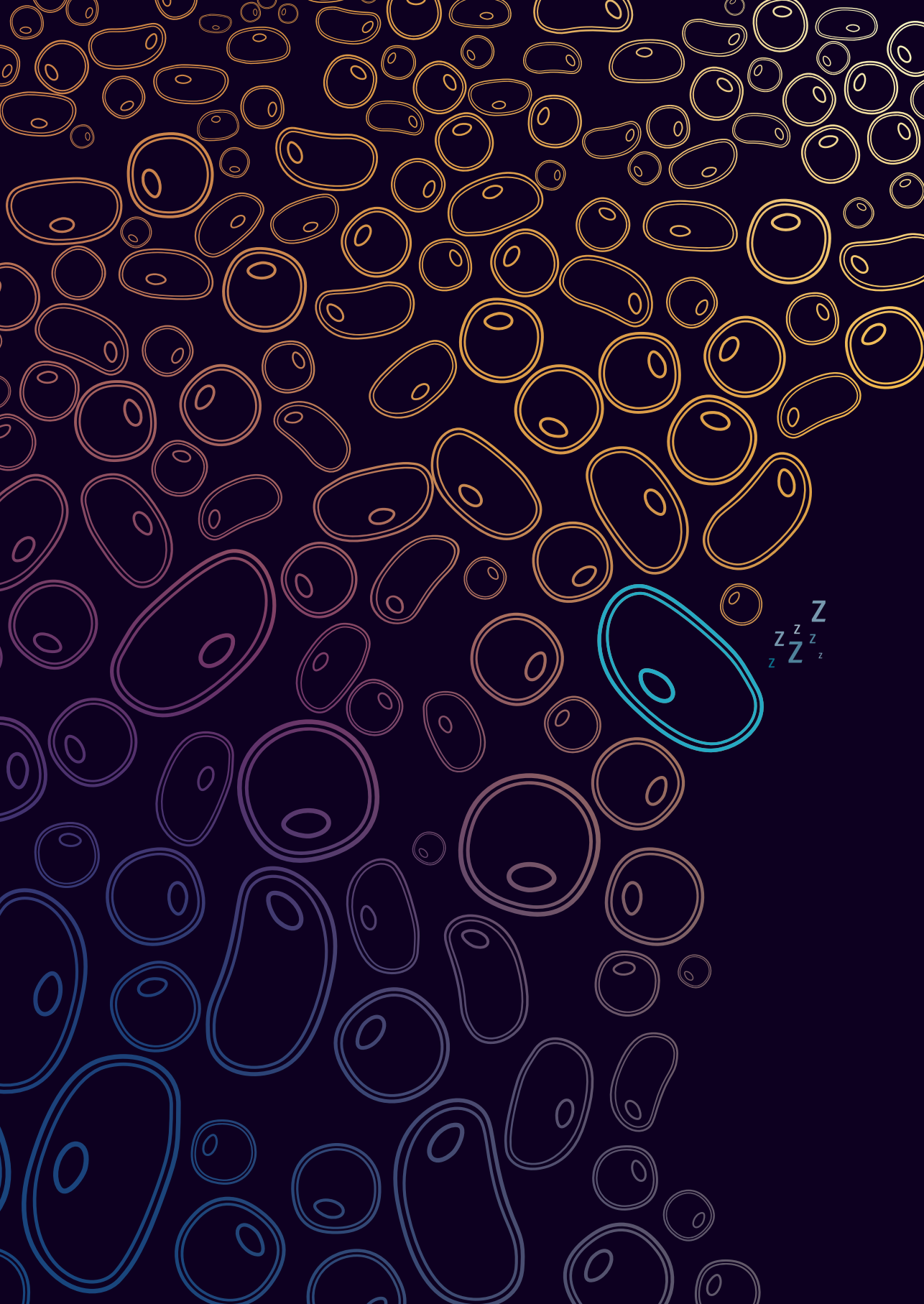

CHAPTER 1

Introduction

Osteoarthritis

Osteoarthritis (OA) is a distressing age-related degenerative condition. It is characterized by the degeneration of cartilage and remodeling of subchondral bone (Figure 1). [2] The risk factors for OA are diverse, encompassing older age, obesity, female sex, and genetics (Figure 1). [3-5] Notable is that joint injuries are often the cause of OA in young individuals, while in older adults OA frequently results from the cumulative effects of age-related factors. It puts a significant burden on society and stands as the 15th leading cause of years lived with disability (DALYS) worldwide. [6] In 2020, approximately 595 million individuals globally were diagnosed with OA, and its prevalence has shown a notable increase over recent decades, with projections indicating further escalation. Beyond the personal toll of OA, it also imposes a substantial economic burden on society with increasing direct and indirect healthcare expenses. In the Netherlands alone, direct healthcare costs associated with OA were estimated at around €1.1 billion euros. [7] While the indirect costs, in the form of early retirement, disability, sickness at work or productivity loss accounted for approximately 83% of the total economic burden of OA. [4-8]

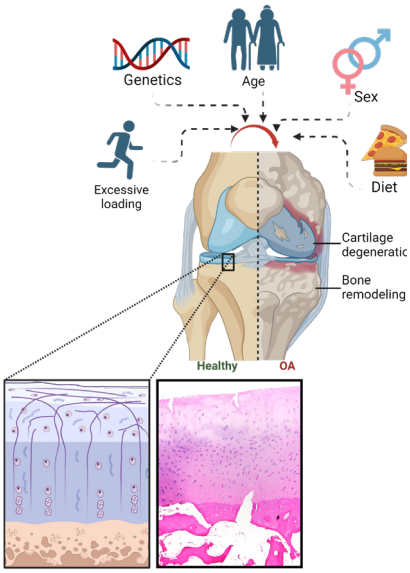

Figure 1 - Risk factors of OA. Schematic overview of a healthy and OA affected knee joint that shows the main characteristics of OA; cartilage degeneration and bone remodeling. Risk factors of OA include excessive loading, genetics, age, sex and diet. The enlarged image shows a schematic inlay of the healthy osteochondral unit, showing the three layers of articular cartilage, from top to bottom; superficial zone, middle zone and deep zone, as well as collagen type 2 fibers, proteoglycans and chondrocytes. This schematic inlay is accompanied with an immunohistochemistry image of this unit. Created with Biorender.

Despite the societal and economic burden that OA puts on society, there are currently no effective evidence based disease-modifying therapies, whereas suboptimal pain management strategies continue to evolve. [9] It is generally acknowledged that the strikingly tempered advancement in evidence based treatment development in OA is particularly caused by the fact that the OA pathophysiology is dynamic and complex, involving many age-related processes such as inflammation, mechano-signaling mitochondrial dysfunction, and cellular senescence. [10-14]

Senescence

Cellular senescence represents a pro-survival strategy of cells to evade death upon environmental stress. It is characterized by a complex process where cells undergo metabolic, morphological and physiological transformations and enter a state of irreversible cell cycle arrest. Despite the cell cycle arrest, senescence cells remain far from dormant, they sustain metabolic activity and exhibit a distinct secretory profile termed the senescence-associated secretory phenotype (SASP).[11] The SASP comprises of cytokines, chemokines, growth factors and proteases that affect matrix degradation and paracrine signaling, driving neighboring cells into senescence. [11,15,16] There is evidence of senescence throughout development, wound healing, tumour suppression, but also during diseases such as, osteoporosis, type 2 diabetes mellitus, neurodegeneration and OA. [15]

Hallmarks of ageing

Senescence is a hallmark of ageing. [1,10] These hallmarks were firstly introduced in 2013, containing 9 aspects of ageing [10] and they were recently updated to contain 12 aspects of ageing (Figure 2). [1] These hallmarks were introduced to stimulate research into identifying different aspects of ageing and druggable targets to improve healthy ageing.

Ageing can be regarded as the accumulation of cellular damage leading to gradual loss of function. It affects most living organisms and is a risk factor for many human pathologies, such as cancer, diabetes, cardiovascular diseases and arthritis. While ageing is currently still not recognized as a target for treatments or drug development, the individual hallmarks of ageing like senescence, are subject of much age-related research. There is much interest into the influence of senescence in age-related diseases and development of anti-senescence treatments. For example, removing senescent cells from the joint of a p16-3MR mouse, that allows tracking of senescent cells by visualizing p16, delayed the onset of OA which improved cartilage remodeling and reduced pain.[17] Treating senescence mice with anti-senescence compound AP20187 resulted in higher bone mass and strength and improved bone microarchitecture, lost during ageing. [18] This shows that anti-senescence compounds could function not only as specific senescence treatment but rather as an intervention aimed at the prevention of age-related diseases.

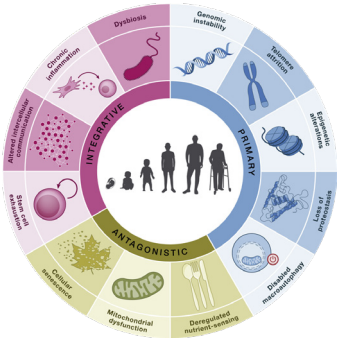

Figure 2 - Hallmarks of ageing. The 12 hallmarks of ageing divided into 3 groups, primary, antagonistic and integrative characteristics. [1]

Senescence biomarkers

The identification of reliable markers for senescent cells is essential for advancing the understanding and therapeutic targeting of senescent cells. However, because senescence varies across cell types, tissues, and inducers, a definitive universal marker has yet to be established. This makes the identification of senescence-specific markers particularly challenging. Nevertheless, certain characteristics of senescent cells may serve as potential biomarkers (Figure 3). [19]

Cell cycle arrest is considered as one of the most important features of senescence cells. Factors and pathways mediating the cell cycle are therefore often used to indicate presence of senescence. Important pathways regulating the cell cycle are the P53/p21$^{WAF1/CIP1}$ and p16^{INK4A}/pRB pathways. As visible in Figure 3, p16 is an inhibitor of CDK4/6 kinases and thereby a regulator of the cell cycle. [11] These kinases are vital for the progression of the cell cycle. [20] Additionally, p16 ensures prolonged cell cycle arrest by preventing phosphorylation of pRB. Increased expression of p16 is often seen in aged tissues and during senescence. [20] The P53/p21$^{WAF1/CIP1}$ pathway regulates senescence when p53 activates p21, which also prevents phosphorylation of pRB and inhibits the active cell cycle (Figure 3). [20] While these two pathways are well known and often used to indicate senescence, these markers are not exclusive for senescence, for example in post-mitotic or quiescent cells proliferation markers are not expressed. [21] This makes cell cycle mediators unsuitable as robust markers for senescence.

Another characteristic of senescent cells is increased DNA damage. Especially double-strand breaks are often seen in senescent cells and cause increase of the ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and Rad3-related (ATR) protein kinases to the site of the double-strand breaks. These kinases are known to phosphorylate many substrates, amongst which cell cycle mediators, and activate the p53/p21 signaling pathway, leading to cell cycle arrest. [20]

A widely used marker for senescence in tissues is the staining of senescence-associated β-galactosidase (SA-β-gal). [11,20] SA-β-gal reflects metabolic changes in the cell and the increased number and size of lysosomes, as a result of the cells failing defense mechanism (Figure 3). Because SA-β-gal is not detected in post-mitotic and differentiated cells, it is a useful biomarker for senescence. Notable however, SA-β-gal is also present in cells with high lysosomal B-galactosidase activity like macrophages and osteoclasts. [22] Therefore, SA-β-gal should be considered as a general indicator of cellular senescence that lacks specificity and sensitivity to discriminate the different aspects of the diverse senescent phenotype.

A senescent cell undergoes several morphological changes. The most well-known changes are flattening and enlargement of the cells, in particular enlargement of the nucleus caused by a reduction in laminin B1 (Figure 3). [23] As a nuclear membrane protein, Laminin B stabilizes the nucleus. Loss of this protein therefore results in an instable nucleus, reassembly of the chromatin organization and loosening of the heterochromatin. [20] Also the mitochondria appear enlarged in senescent cells, as the result of an attempt to compensate for dysfunction. Additionally, malfunctioning mitochondria are retained due to reduced autophagy. This accumulation of mitochondria leads to a decrease in the mitochondrial membrane potential, accelerating reactive oxygen species (ROS) production (Figure 3). [20]

The SASP contains many factors that can function as biomarkers. Soluble growth factors like insulin-like growth factor binding proteins (IGFBPs), vascular endothelial growth factors (VEGFs) and interleukins (IL) are part of the SASP profile. [23] IL6 has been marked as an important mediator of the SASP and appears directly driven by prolonged DNA damage. Other interleukins, for example IL8, are general members of the SASP. ECM remodeling enzymes are also important members of the SASP profile, for example matrix metalloproteinases (MMPs), particularly MMP1/3, and serine/cysteine proteinase inhibitors (SERPINs), particularly SERPINE1. [23] Although SASP factors are considered markers of senescence (Figure 3), the composition of the SASP is heterogeneous, depending on cell type, tissue type, senescence inducer and strength of the inducer. [20] The SASP should therefore be better characterized, depending on tissue source and inducer before being applicable as a robust senescence marker.

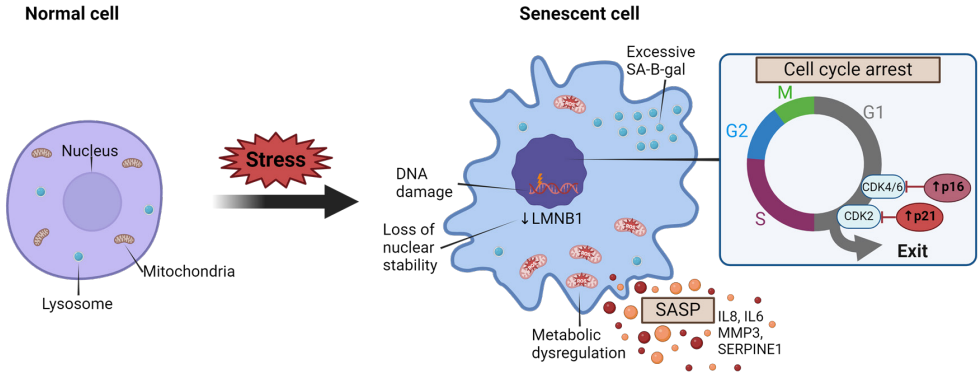

Figure 3 - Characteristics of a senescent cell. Particularly cell cycle arrest and presence of a senescence-associated secretory phenotype are considered hallmarks of senescence cells. Increase of cell cycle inhibitors p16 and p21, decrease of nuclear stability, as well as the increase of SA-B-gal activity are considered as biomarkers of senescence cells. Created with Biorender.

Senescence treatments

There are numerous treatments available and under development that target senescent cells. There are two classes of senescence treatments, senolytic agents target that eliminate senescence cells and senomorphic agents that dampen the SASP profile while unaffecting the viability of the cells.

Senolytics

Senolytic agents eliminate senescent cells by disrupting their pro-survival pathways, after which immune mechanisms clear the resulting debris. Key mediators of apoptosis, including Bcl-2, p53, p21, and FOXO4, have been identified as targets of senolytic interventions. The selectivity of these agents arises from their ability to exploit the distinct gene expression profiles of senescent cells. However, despite extensive evidence supporting the beneficial effects of senolytics, the heterogeneity of senescence profiles presents a critical challenge to their specificity and therapeutic efficacy. For example, promising senolytic agent UBX0101, targeting the MDM2/p53 interaction, showed improvement of OA symptoms when administered to a OA mouse model. [17] This drug proved safety in a phase I clinical trial. [24] However, when UBX0101 was tested in a phase II clinical trial on 180 OA patients in 2020, it showed no significant improvement with treatment and was preliminary terminated. [25] As mentioned above, senescent cells lack a body-wide homogeneous

profile, which contributes to adverse off-target effects. This could be prevented through localized delivery strategies, such as nanoparticle-based targeting, rather than systemic administration.[26] Additionally, because senescence profiles vary among osteoarthritic patients, stratification based on these individual profiles will be essential for effective therapy.

Senomorphics

Senomorphic agents directly inhibit or neutralize SASP factors, silencing the SASP profile. [27] For example by using specific antibodies against SASP members like interleukins, or disrupting pro-inflammatory pathways activated by the SASP, for example the NF-κB pathway.

Articular cartilage

Healthy cartilage

Articular cartilage, a thin layer of connective tissue, covers the ends of bone and is responsible for smooth movement and force distribution across the joint. [28,29] It composes of a dense extracellular matrix (ECM) with a total thickness of 2-4 mm. To enable painless movement and force distribution, there is no vascular nor nerve system present. [30] Due to this non-perfused state of the tissue, the flow of water is important to allow transport of nutrients and waste material. 60% of cartilage dry weight consist of collagen II fibers, other components of the ECM are water and proteoglycans. [28] These strong fibers consist of a triple helix, providing cartilage with high shear and tensile properties. The second largest group of ECM proteins, proteoglycans, are glycosylated proteins and consist of a protein core with glycosaminoglycans side chains. [31] These proteoglycan structures are negatively charged, attracting water. [29] The main glycosaminoglycan in cartilage is aggrecan. The unique composition of these materials causes a high swelling pressure in the tissue, allowing it to be able to withstand large forces. The sole cell type in cartilage are post-mitotic chondrocytes, which are surrounded by a pericellular matrix (PCM). [32-34] By connecting directly to the cells, the PCM is important in cell-matrix interactions and force transduction. [29] Chondrocytes detect environmental stresses and subsequently maintain the cartilage ECM. Due to the limited regenerative capacity of cartilage, preservation of ECM integrity is essential.

Cartilage ECM structure can be divided into three zones, the superficial zone, the middle zone and the deep zone (Figure 4). [28,35] In the superficial zone, the collagen type II fibers are oriented parallel to the articular surface, and chondrocytes are flattened and aligned along these fibers. [30] This tightly organized surface matrix provides resistance to sheer, tensile and compressive forces.[36] The middle zone follows beneath the superficial zone where collagen fibers adopt an oblique orientation and chondrocytes are round, sparsely distributed, and randomly arranged. The deep zone is characterized by collagen fibers oriented perpendicular to the surface, with chondrocytes organized into columns that follow the fiber alignment. This zone contains the highest content of proteoglycans and provides the most resistance against compressive forces. This layer contains a low cell number and most cells are hypertrophic. The tidemark follows the deep layer and connects the cartilage to the subchondral bone. This calcified region anchors collagen fibers into the subchondral bone, thereby integrating cartilage with the underlying bone.

Age-related changes articular cartilage

In tissues with poor regenerative capacities like cartilage, accumulation of environmental stressors throughout life gives rise to cellular damages, making it prone to enter a disease state. Research has therefore focused on age-related changes in cartilage that contribute to the development of OA. [12,37]

During ageing, collagen type II fibers undergo increased degradation and crosslinking largely driven by the accumulation of advanced glycation end products (AGEs). [38,39] Especially the low turnover rate of collagen type II fibers in cartilage allows accumulation of AGEs, which in turn decreases the anabolic activity. [37] These age-related changes in collagen type II fibers, particularly the increased rate of crosslinking, alter the biomechanical propertie and leads to increased stiffening of the cartilage (Figure 4). [37]

On macroscopic level the effects of ageing can be seen by increased cartilage thinning, which is correlated to a loss of cartilage matrix and a decrease in cartilage hydration and cellularity (Figure 4). [37,40] The decrease in cartilage hydration is particularly caused by a loss of hydrophilic glycosaminoglycans. As seen in Figure 4, not only the glycosaminoglycan content is decreased during ageing, there is also an increased degradation and reduction in size, affecting the overal tissue hydration and resilience. [41,42]

The maintenance of cartilage ECM depends entirely on chondrocyte activity. [40] Between the ages of 20 and 90, the density of chondrocytes decreases in the superficial zone by 50%. [37] With the absence of phagocytic cells in cartilage, the increase in cell death causes cell debris can produce pyrophosphate and can precipitate calcium, contributing to the pathologic cartilage calcification.[38] Chondrocytes also become less responsive to growth factors, leading to an altered cell signaling and results in an imbalance in anabolic and catabolic activity. [38] Elevated ROS production, known to occur during ageing, causes oxidative damage that eventually also leads to an altered cell signaling or deregulated nutrient-sensing. [12,38] Due to the limited regenerative capacity of cartilage, age-related changes makes the tissue prone to diseases such as OA.

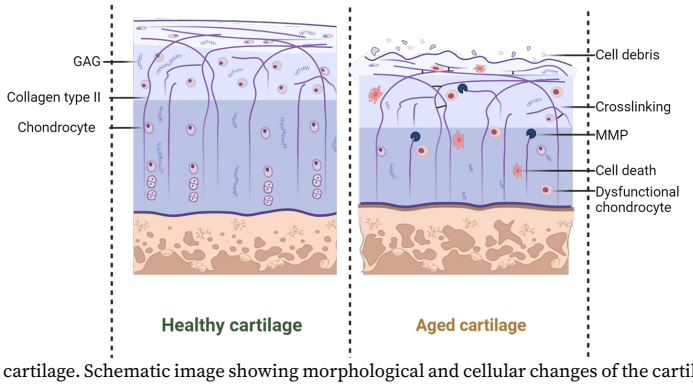

Figure 4 - Ageing cartilage. Schematic image showing morphological and cellular changes of the cartilage due to ageing. GAG= glycosaminoglycan, MMP= matrix metalloproteinase. Created with Biorender.

Osteoarthritic tissue development

While OA is considered a multi-tissue disease, a prominent feature is cartilage degeneration. One of the earliest changes during OA is the increase of water in and swelling of the cartilage matrix, associated with the loss of glycosaminoglycans and degradation of the cartilage in the surface zone (Figure 5). [43] This is macroscopically visible by surface fibrillations. [2] During the onset of OA, the chondroprotective ECM is disrupted. Chondrocytes attempt to repair the damage by increasing their synthetic activity, particularly for proteoglycans.

As visible in Figure 5, during the progression of OA, cartilage degradation is also visible in the deeper zones and the integrity of the collagen network is disrupted by increased activity of matrix metalloproteinases. The cartilage matrix shows fissures into the cartilage which are no longer restricted to the superficial layer. In an attempt to repair the damages, chondrocytes re-enter the cell cycle, forming cell clusters or go into senescence, preventing tissue regeneration. [11]

In end-stage OA, the layer of calcified cartilage expands into the articular cartilage. Macroscopically, fissures reach into the deep zone with fragments disrupting the tidemark, exposing the calcified cartilage and subchondral bone (Figure 5). There is ingrowth of vascular vessels, containing blood vessels and nerves, causing joint pain and changing hypoxic conditions. At this point, there is a decrease in chondrocyte density and presence of empty lacunae, indicating chondrocyte death. Remaining chondrocytes express a hypertrophic phenotype with increased expression of collagen type X, while also increasing the synthesis of matrix degrading enzymes like MMP13. Changing chondrocyte phenotypes eventually trigger inflammatory processes in the synovial fluid on top of the cartilage. [32]

The effects of OA are not limited to the cartilage, particularly the underlying bone is affected and plays a role in cartilage degradation. In the initial stages of OA, the bone undergoes remodeling due to changes in the force distribution over the joint, increasing bone porosity (Figure 5). [43] During the progression of OA, there is thickening of the cortical bone plate and formation of osteophytes. In end-stage OA, the subchondral bone shows drastic remodeling, with an increase in vascular channels, deformation of the cortical bone plate, increased osteophyte formation and cell death. The vascular channels that contain blood vessels and nerves also penetrate into the cartilage, altering the crosstalk between subchondral bone and cartilage. Resulting in further aggravating cartilage breakdown.

Senescence in OA

Cellular senescence is increasingly recognized as a significant factor in the pathophysiology of OA. [17,44-46] While less thoroughly studied in post-mitotic cells, it is generally acknowledged that chondrocytes also undergo cellular senescence. Though with a less pronounced role for the p16 and p21 cell cycle arrest markers [3,11,17,47] and a more prominent role for the harmful SASP profile, which negatively impacts chondrocyte health and potentially promoting a OA progression. [3,48,49] Nonetheless, irreversible cell cycle arrest has been observed *in vitro* in chondrocytes [12] Although these cells typically exist in a proliferative resting state while retaining their proliferative potential, they are susceptible to senescence, especially upon re-entering the cell cycle. [11,50]

Additional evidence implicates cellular senescence in OA pathophysiology, such as the presence of senescence markers in OA tissue, age-related increases in SA-β-gal activity, and elevated levels of SASP markers in OA cartilage. [3,49,51] In mice, the implantation of senescent cells into cartilage induced an OA-like environment characterized by cartilage degeneration and osteophyte formation. [52] Importantly, the clearance of senescent cells has shown promise in preventing or delaying OA onset. [11,53,54, 27,55] As noted above, ageing is accompanied by an increase in cellular senescence, which heightens the body's vulnerability to damage. In cartilage, the accumulation of senescent cells with age makes the tissue more susceptible to harmful environmental factors, thereby increasing the risk of OA development. [3]

To study the role of senescence and the use of anti-senescence compounds in OA pathophysiology, a variety of models are employed. However, these models frequently fall short in accurately representing the complete senescence cartilage profile *in vivo*, underscoring the need for the development of more representative models.

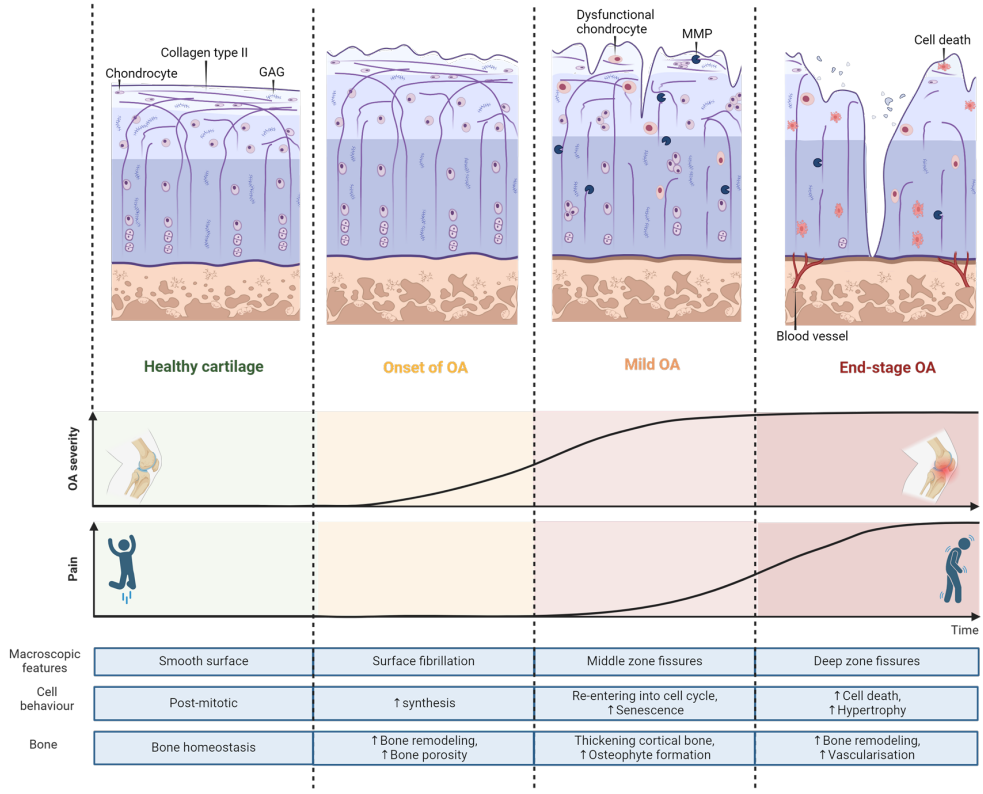

Figure 5 - Osteoarthritis development. Schematic depiction of the tissue changes during the OA pathophysiology. Depicting the development of OA from healthy cartilage to end-stage OA. GAG= glycosaminoglycan, MMP= matrix metalloproteinases. Created with Biorender.

Outline of this thesis

In this thesis, we aim to gain insight into the role of senescence in the OA pathophysiology by characterizing and defining biomarkers, introducing new models and testing pre-clinical models.

To characterize senescence in OA, in **chapter 2**, we explored senescence transcriptomic profiles in cartilage, using unsupervised hierarchical clustering. We used the same unbiased approach to unravel corresponding blood biomarkers metabolic profiles.

In **chapter 3**, we introduce novel model biomimetic and high-throughput models to study senescence during OA by investigating the possibility of perturbating aged human *ex vivo* osteochondral explants with hyper physiological mechanical loading and ionizing radiation. Furthermore, in **chapter 4**, we explored the use of synchrotron imageing to visualize structural cartilage changes in our aged human *ex vivo* osteochondral explants, providing more insight into new imageing techniques for cartilage. Diving further into model development for OA, in **chapter 5**, we developed a new joint-on-a-chip model system, creating an *in vitro* bone-cartilage interface and we explored the possibility to induce terminal maturation in this system as a possible OA-related perturbation.

Subsequently, to explore the possibility to use the biomimetic *ex vivo* senescence models established in **chapter 3** for testing of anti-senescence compounds, in **chapter 6** we tested the potential senomorphic NF157 in this model. Finally, in **chapter 7**, we explored the possibility to use the high-throughput neo-cartilage organoid model as a screening platform for potential terminal maturation therapeutics.

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