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Leiden
The Netherlands

Exploring senescent chondrocytes during aging: sleeper AGEnts of osteoarthritis

Boone, I.

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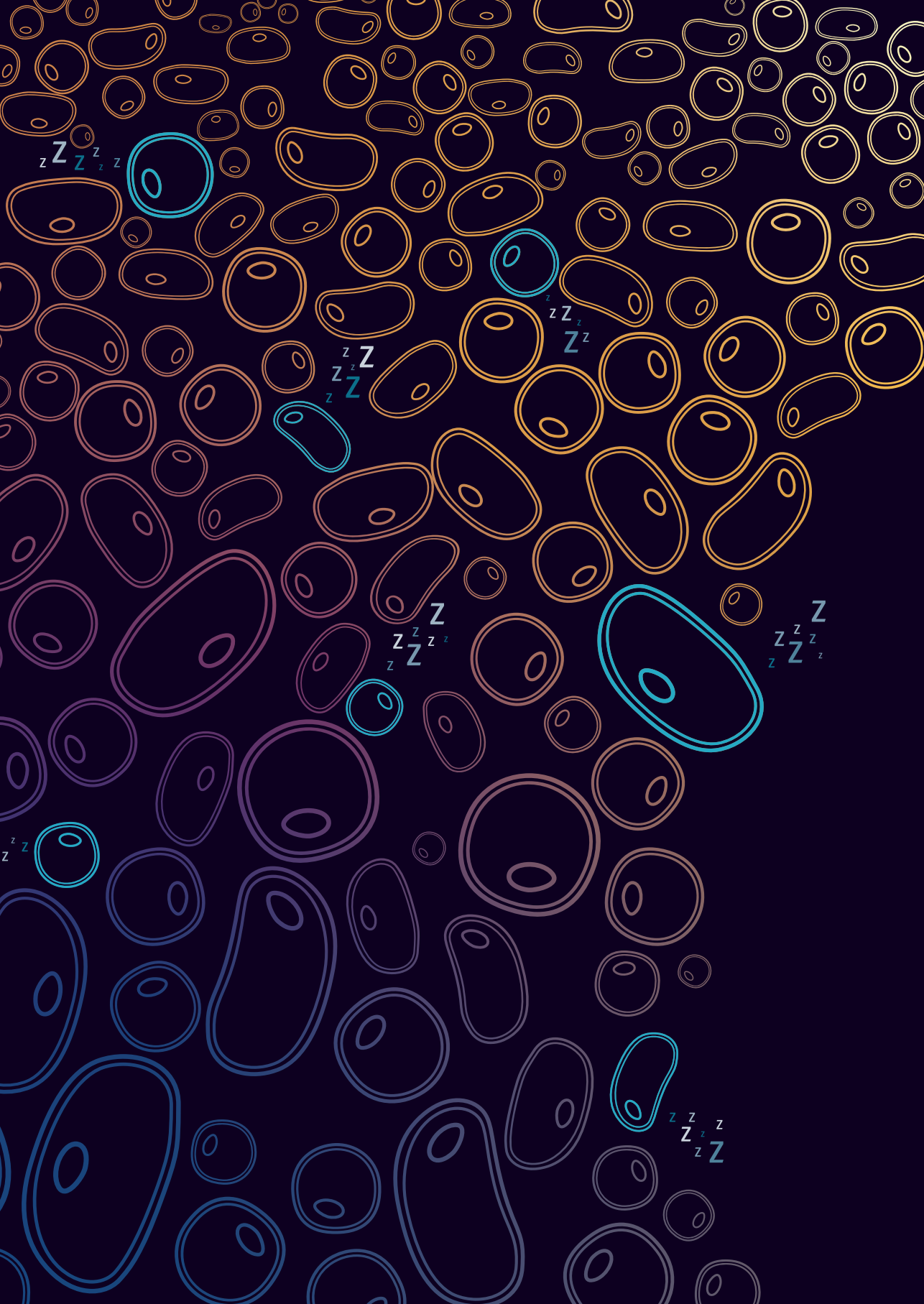
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CHAPTER 8

General discussion and future perspective

Summary

Osteoarthritis (OA) is a distressing age-related degenerative condition. Despite the societal and economic burden that OA puts on society, there are currently no effective evidence based disease-modifying therapies. This strikingly tempered advancement in evidence based treatment development for OA is particularly caused by the complex OA pathophysiology which involves many age-related processes such as cellular senescence.

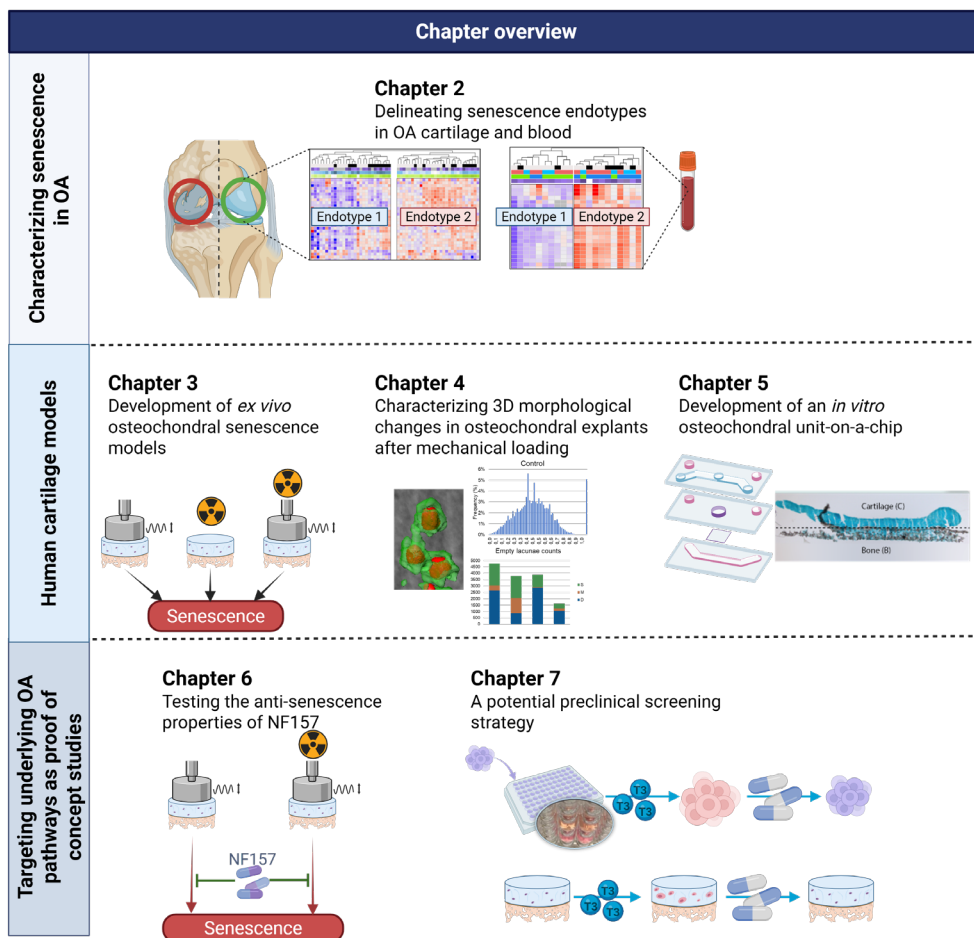


Figure 1 - Thesis overview. This thesis is divided into three parts. In the first part, composing of **chapter 2**, we characterize senescence in post-mitotic chondrocytes using OA cartilage. Here, we hypothesize the presence of two senescence endotypes in OA cartilage and blood. In the second part, composing of **chapters 3-5**, we develop and characterize models for OA. More specifically, in **chapter 3**, models representing different aspects of senescence are created in *ex vivo* osteochondral explants. In **chapter 4**, 3D morphological changes induced by senescence and hyper physiological mechanical loading in these *ex vivo* osteochondral explants are characterized using synchrotron scanning microscopy. In **chapter 5**, a joint-on-a-chip model is developed to study the bone-cartilage interface. In the last part of this thesis, composing of **chapter 6** and **7**, we test targeting underlying OA pathways as proof of concept studies. In **chapter 6**, the anti-senescence properties of NF157 are tested. In **chapter 7**, a preclinical testing strategy for underlying OA processes is proposed utilizing the miniaturized neo-cartilage organoid model and the biomimetic osteochondral explants. Created with Biorender.

The goal of this thesis was to investigate the role of senescent chondrocytes in the development of OA and to take a step towards matching senescence therapies for OA patients (Figure 1). Exploring senescence transcriptomic profiles in cartilage in **chapter 2** resulted in two senescence endotypes highlighting heterogeneity upon environmental stressors throughout life. Subsequently, we found two corresponding blood biomarkers metabolic profiles that could function as non-invasive biomarkers, reflecting body wide intrinsic, subject specific, survival strategies. On the road towards utilizing senescence treatments for OA patients, in **chapter 3** we first developed human senescence models using hyper physiological mechanical loading and ionizing radiation, creating a SASP model, a cell cycle arrest model and a complementary senescence model while also optimizing a high-throughput senescence neo-cartilage organoid model. In **chapter 4** we explored the use of high energy imaging to detect subtle 3D structural cellular and matrix changes in the cartilage of osteochondral explants upon senescence inducing perturbations ionizing radiation and/or hyper physiological mechanical loading. Diving further into the development of representative models for OA, in **chapter 5** a joint-on-a-chip model to allow study of the bone cartilage interface was introduced. Further advancing towards proof-of-concept studies, in **chapter 6** we revealed anti-senescence properties of NF157 in our senescent models. Lastly, focusing on drug development strategies for OA, in **chapter 7** we exploited a high-throughput neo cartilage model as a widely applicable preclinical drug screening platform for OA-related terminal maturation. While we exploited the biomimetic human osteochondral models as a validation model for this screening platform. As a proof of concept, we tested three compounds against terminal maturation in these models.

Discovery of senescence-OA endotypes in articular cartilage

Heterogeneity in OA presents a significant challenge for researchers, whether they are focused on drug development, model development, or predictive biomarkers. The consensus is that OA is a complex and heterogeneous disease, and the biggest challenge in OA drug discovery is translating preclinical findings to the clinic.¹ To increase the success of potential OA-reversing drugs, robust molecular endotypes need to be characterized to move beyond the “one treatment fits all” strategy and ensure the right patient receives the right medication.² Tackling this problem requires the development of more effective endotype-directed therapies. An important first step herein is to characterize molecular endotypes. In this respect, in **chapter 2** we characterized two senescence endotypes in relevant OA disease tissue, cartilage. Senescence endotype 1 was characterized by cell proliferating pathways, with possible treatment in FOXO mediated cell cycle processes by targeting *FOXO4*. Senescence endotype 2 was characterized by inflammatory processes with SASP pathways as possible treatment options by targeting *MMPs*. Our results gave insight into the heterogeneity of senescence in aged OA cartilage. To find out the robustness of our senescence OA endotypes, we compared our endotypes with other previously found OA subtypes. Coutinho (2020), previously defined two OA subtypes, where genes enriched in cluster A showed upregulation of small molecular metabolic processes and genes involved in extracellular matrix and cluster B showed an upregulation of an chemokine profile and genes involved in immune system processes.³ We found that patients of our SASP endotype overlaps 100% with cluster B, the chemokine endotype. This implies that these patients indeed express a senescence-orientated intrinsic survival strategy. On a different note, two studies using biochemical markers in serum

and urine both showed the presence of three OA subtypes, a cartilage turnover, inflammatory and a structural damage subtype.^{4,5} While these subtypes are based on serum and urine biochemical markers, they show similarities with our cartilage senescence endotypes and corresponding metabolomic profiles in the circulation. Not only support the serum and urine OA subtypes the robustness of our cartilage-based senescence endotypes, they also support that these endotypes reflect an intrinsic, subject specific, survival strategy of body wide tissues. These results indicate that these OA patients not only show a difference in senescence profile but rather in their complete transcriptome and it is important to stratify for patients transcriptome profiles when looking for possible treatment options.

Senescence is considered a heterogeneous cellular strategy to avoid cell death in response to environmental stressors and is classically defined by permanent cell cycle arrest and a SASP profile.^{6,7} This cell cycle arrest is induced by the expression of cell cycle inhibitors p16, encoded by *CDKN2A* and p21, encoded by *CDKN1A*, both often used to indicate senescence.^{8,9} Although cell cycle arrest is a key marker of senescence, postmitotic cells like chondrocytes also undergo cellular senescence. In postmitotic cells, the specific role of cell cycle arrest induced by *CDKN2A* and *CDKN1A* poorly understood^{7,9-11}, or even simply ignored^{12,13} and the classical phenotype with the cell cycle arrest markers such as *CDKN2A* may not hold. As part of our ongoing efforts to understand the senescence profiles of post-mitotic cells like chondrocytes, we further examined how chondrocytes respond to senescence perturbations in preclinical *ex vivo* osteochondral explant models. Our data in **chapter 3** showed that ionizing radiation in macroscopically normal aged cartilage induced particularly cell-cycle arrest senescence with affected genes e.g. *CDKN1A* and *MKI67*, and hyper physiological mechanical loading induces a SASP profile with affected genes e.g. *IL6* and *IL8*, while *CDKN2A* remained unaffected. When connecting the found senescence regulatory genes of **chapter 3** with the senescence molecular endotypes of **chapter 2**, we found similar senescence patterns. Molecular endotype 1 showed a decrease of differentially expressed *MKI67* while *CDKN2A* remained unaffected. Similar as perturbing the aged osteochondral explants with radiation. Molecular endotype 2, the SASP endotype, showed upregulation of many SASP factors, amongst which *IL6* and *IL8*, similar as perturbing the aged osteochondral explants with hyper physiological mechanical loading (Figure 2). *CDKN1A* however, was slightly higher expressed in endotype 2, indicating that this marker is representative for all senescence endotypes. For patients expressing a SASP endotype, the anti-senescence compound NF157 that targets the SASP profile, tested in **chapter 6** could prove to be a potential therapy.

In conclusion, it is tempting to speculate that patients expressing senescence endotype 2 exhibit SASP responses primarily as a consequence of excessive mechanical loading. The prominent role of inflammatory markers in senescence endotypes, particularly molecular endotype 2, raises the question whether the observed inflammation is attributable to OA pathogenesis or to the SASP. Notably, several SASP components analyzed in this study, including *IL6* and *IL8*, are not differentially expressed in OA tissues during disease progression but are notably responsive to senescence perturbations.^{14,15} This suggests that the observed inflammatory responses are more likely driven by the SASP rather than by OA-associated inflammation. The characterization of body-wide senescence endotypes of OA patients in **chapter 2**, gives us more insight into the disease pathogenesis and molecular endotypes that will allow for more effective endotype-directed therapies.

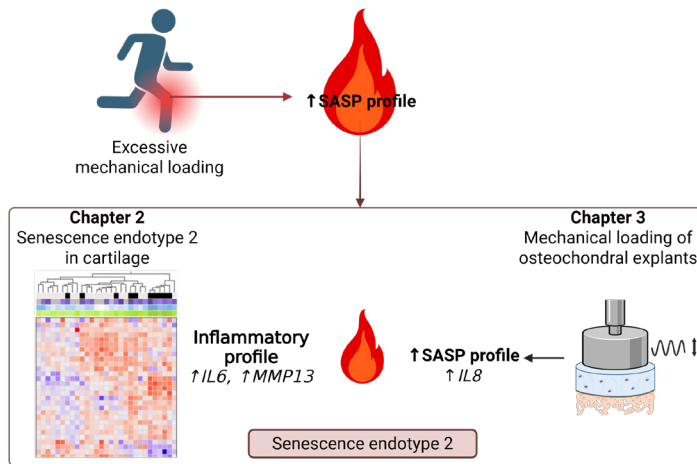


Figure 2 – Schematic depiction that excessive mechanical loading leads to an increase of SASP in the cartilage. Identified using gene expression profiles of OA cartilage in **chapter 2** combined with the experimental data of the osteochondral model perturbed with hyper physiological mechanical loading in **chapter 3**. Created with Biorender.

Biomarkers delineating senescence in OA

Besides characterization of molecular endotypes in OA, important is also the development of non-invasive biomarkers that can delineate these molecular endotypes in a clinical setting. However, using biomarkers as clinical predictors, preferably non-invasive, requires accuracy, sensitivity, and specificity, which is currently lacking. Therefore, in **chapter 2** we set out to explore the use of metabolites as blood-based biomarkers to identify specific cartilage transcriptomic patterns. Using a similar unsupervised hierarchical method, we found two corresponding metabolomic profiles in blood. Since these two metabolomic profiles correspond to the two senescence endotypes in cartilage, it is tempting to speculate that these senescence endotypes are body wide represented, suggesting the importance of senescence during aging. These metabolite profile measurements were part of the large DuSRA-VOILA consortium. This consortium focuses on improving health and vitality during aging rather than increasing longevity. One of its goals is to understand individual risks of accelerated aging by predicting responses based on metabolite health, which is linked to the risk of cardiovascular events, type 2 diabetes mellitus, and vascular mortality.^{16,17} When examining the senescence endotypes with two different blood-metabolite profiles, we do not observe a difference in these metabolite health scores, indicating no overall difference in metabolite health (results not shown).¹⁷

Nonetheless, to use these metabolite markers as clinical diagnostic biomarkers, we considered it essential to first identify the metabolites that define the two endotypes using methods such as LASSO regression. To enhance the robustness of diagnostic biomarkers, they should encompass multiple omics levels, including genomics, transcriptomics, proteomics, and metabolomics. There are many advantages of biomarkers that span multiple omics levels, for example they give a fuller picture of the disease state, can be cross-validated across multiple omics levels, decreases background noise by increasing the signal to noise ratio. This multi-omics approach provides a robust framework for early detection, comprehensive disease profiling, and the advancement of precision medicine. Previously, circulating miRNAs were used to delineate the OA disease progress,

revealing the interplay of circulating miRNAs and mRNA expression in OA cartilage.¹⁸ Therefore, we set out to explore circulating miRNAs as potential biomarkers in addition to the metabolites. Our work in **chapter 2** shows the possibility of using blood biomarkers for senescence subtypes present in OA tissue.

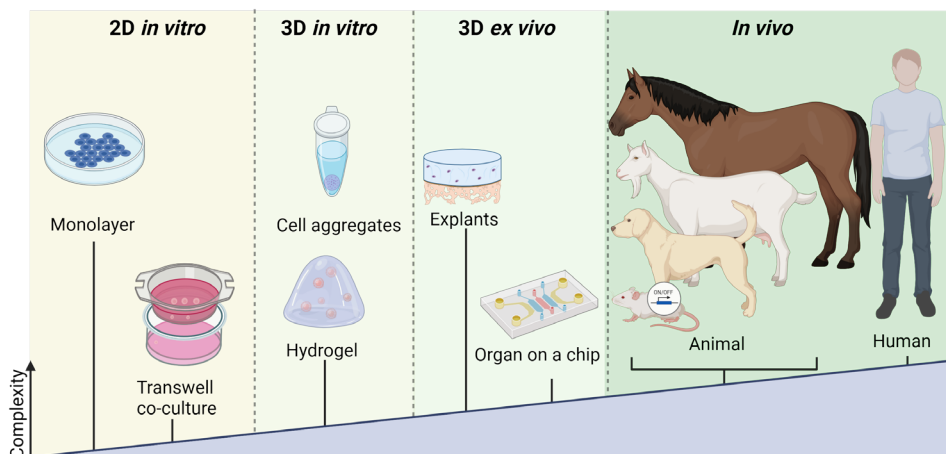


Figure 3 - Schematic depiction of the different models available for drug discovery. Showing increased complexity of the models from left to right. Created with Biorender.

Human cartilage models for drug discovery

Discovery and generation of effective therapeutic treatments are crucial, where preclinical models provide the foundation and are key for successful and impactful outcomes. Irrespective of the fact that there is an increasing societal wish to reduce, refine and replace animal studies, the animal model is still considered the golden standard and is legally required for entering the phase I clinical trial stage. Partially due to the limited number of human measurement models that have been validated and accepted in healthcare research.¹⁹ These human measurement models provide a controlled and reproducible environment to study changes due to senescence. However, they often entail proliferation of monolayer chondrocytes in *in vitro* cell culture models using primary cells or even cell lines (Figure 3) and do not necessary reflect the senescence situation *in vivo*. There are two ways to induce senescence in these *in vitro* experiments, by excess proliferation or by excess doses of environmental stressors like irradiation, oxidative stress or oncogenic stress.²⁰ Although these models can provide basic information into cellular responses, they lack physiological relevance at the tissue or patient level.²¹ In this thesis we therefore built on our previously established human *ex vivo* explant models²² and neo-cartilage organoid model of primary joint tissue cells²³. These models were already equipped with different OA-related perturbations²⁴ and OA risk genes²⁵. Therefore, we here set out on one hand to exploit the human *ex vivo* explant models to study in depth cellular senescence of the postmitotic chondrocytes and subchondral bone. While on the other hand we miniaturized the neo-cartilage organoid model of primary joint tissue cells accommodate scalable preclinical screening of multiple conditions.

Exploring senescence in human *ex vivo* osteochondral models

In order to reduce animal-based research in senescence and OA, we explored the previously established OA *ex vivo* osteochondral explant model (Figure 3).²² This model maintains the native complexity of the cartilage and bone interface and biomechanical and biochemical properties of the tissues, which is needed for accurate predictability for humans. By applying OA and senescence-relevant triggers, hyper physiological mechanical loading and ionizing radiation, to osteochondral explants, in **chapter 3** we created three biomimetic senescence models that can function as senescence-OA models. This *ex vivo* model is known to have a high donor variability, which could also be an advantage since it represents the heterogeneity within OA, highlighting that osteochondral explants closely mimic the *in vivo* situation. Since much OA research is focused on cartilage development, changes in bone are often neglected. Advantage of this *ex vivo* model is the inclusion of the subchondral bone, allowing bone-cartilage crosstalk. In this respect, we observed the presence of consistent SASP markers, *IL6* and *SERPINE1*, as well as tissue specific SASP markers, where *IL8* was specific for cartilage and *MMP13* for bone. Additionally, our data showed that bone responds stronger with a cell cycle arrest profile to the senescence perturbations with upregulation of *CDKN2A*, probably because bone contains mitotic cells in contrast to cartilage. Therefore it is of interest to further explore how bone influences senescence in cartilage during OA, preferably in the osteochondral explants.

Combining hyper physiological mechanical loading and ionizing radiation

The radiation and hyper physiological mechanical loading perturbed models separately show distinct features of senescence, while combining the perturbations by subjecting the explants firstly to radiation followed by hyper physiological mechanical loading showed a complementary senescence response with aspects of both cell cycle arrest and SASP. Many studies report an important cell cycle arrest role of *CDKN2A*, however these studies report on chondrocyte behavior in monolayer cell culture and mice models.^{9,26,27} This highlights the importance to study senescence in the right models. When looking at the effect of the disease relevant triggers, we observed a central role for *CDKN1A* in chondrocyte senescence while *CDKN2A* remained unresponsive. We hypothesize that while chondrocytes are post-mitotic cells, they retain the ability to re-enter into the cell cycle. This ability is no longer available when a chondrocyte becomes senescent, controlled particularly by *CDKN1A*. Henceforth, the cells are no longer cope with environmental stressors, giving rise to age-related diseases like OA. In this respect, it is vital to explore senescence during OA in disease-relevant models like the human aged osteochondral explant model.

High-energy 3D cartilage imaging as tool to quantify damage in cartilage

With the development of the human explant models to study cellular senescence of joint tissues, we observed difficulties in characterizing morphological changes of senescent chondrocytes. It remained elusive how senescence affects the 3D morphological structures of chondrocytes because conventional histology is limited to 2D imaging while CT and MRI lack the required resolution to image cellular changes.^{28,29}

As an answer, in **chapter 4**, we explored 3D morphological changes of chondrocytes in respect to mechanical loading and senescence by performing synchrotron imaging on our human explant models. Our data showed that this technique is sensitive enough to distinguish cellular and matrix changes evoked by environmental stressors such as hyper physiological mechanical loading. Moreover, this technique allowed for study of individual cells and matrix components as well as general changes in the zonal structure of cartilage. We showed that applying disease-relevant hyper physiological mechanical loading to the osteochondral explants resulted in an increase of empty lacunae and a drop in cell numbers, indicating apoptosis. While inducing senescence-associated cell cycle arrest by firstly applying radiation, as shown in **chapter 3**, followed by hyper physiological mechanical loading prevented these changes. Simultaneously, we observed an increase of chondrocyte cell size, a marker of senescence. This could mean that senescent chondrocytes can no longer respond to environmental stressors. Since senescent chondrocytes accumulate with age, this could lead to age-related diseases like OA.

While this data shows the potential of high-energy synchrotron imaging to visualize 3D morphological changes in cartilage, in order to increase statistical power and limit the donor-dependent effect, multiple donors need to be analyzed. Additionally, to gain a full image of the changes in cartilage and bone during OA (-related senescence), analysis of this imaging technique should be extended to allow study of the bone tissue of the osteochondral explants.

Targeting senescence processes underlying OA as proof of concept studies

To assess the ability of our biomimetic senescence *ex vivo* models for validation of senescence compounds as OA treatment, in **chapter 6**, we tested the anti-senescence properties of NF157. Our results showed that incubation with NF157 resulted in a diminished SASP profile in the senescence models and an overall reduction of senescent cells as visualized by the senescence staining. We highlighted the importance of calcium homeostasis in chondrocyte health and during loading by suggesting a potential mode of action of NF157 (Figure 4), indicating an important role of mitochondria in senescent chondrocytes. Therefore, further research could entail the knockout of dysfunctional mitochondria.

The large role of dampening the SASP profile in the working mechanisms of NF157, together with the fact that mechanical loading increases the SASP profile in the osteochondral explant found in **chapter 3** and the presence of a SASP-endotype among OA patients in **chapter 2**, highlights the important role of the SASP profile in chondrocyte senescence and as a potential treatable target.

Human in vitro neo-cartilage organoid model of primary joint tissue cells

While the human *ex vivo* osteochondral explant models are suitable to reliably study complex OA-related processes, they have a limited throughput, are time consuming, and require much material. As an answer to these issues, we showed the potential of miniaturizing the *in vitro* neo-cartilage organoid model to allow fast study for many OA-related processes. In **chapter 3**, we demonstrated the use of this high-throughput model for senescence by applying ionizing radiation, and in **chapter 7** for terminal maturation by incubating with T3. However, before applying this model for multiple underlying OA processes, it needs to be further optimized, by including for example mechanical

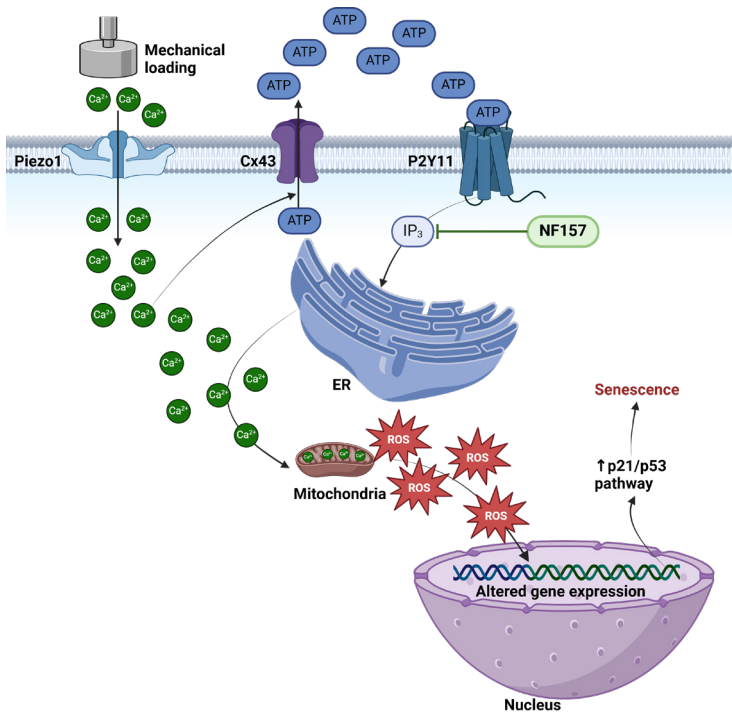


Figure 4 - Schematic depiction how P2Y11 purinergic receptor can lead to chondrocyte senescence and the NF157 target in this pathway. Mechanical loading activates Piezo1, increasing the intracellular calcium concentration. This in turn activates the release of ATP content via Connexin 43, which causes an upregulation of purinergic receptors, among which P2Y11. These receptors trigger a calcium release from the endoplasmic reticulum that accumulates in the mitochondria. This calcium excess in the mitochondria triggers the activation of ROS that affects gene expression and upregulates the p21/p53 pathway leading to senescence. NF157 is a P2Y11 receptor antagonist and prevents calcium release from the endoplasmic reticulum. ATP: adenosine triphosphate, Cx43: Connexin 43, P2Y11: P2Y purinoceptor 11, PLC: phospholipase C, IP₃: inositol-1,4,5-trisphosphate, ER: Endoplasmic reticulum, Ca^{2+} : Calcium, ROS: reactive oxygen species. Created with Biorender.

loading to be able to study the complementary senescence model. However, the bone-cartilage interface as well as the underlying bone play an important role during OA. Nonetheless, these *in vitro* organoid models do not accommodate interaction processes. Therefore, the high-throughput 3D model should also be developed for bone tissues to allow screening in a high-throughput bone model.

Targeting underlying processes of OA as proof of concept studies

In **chapter 7** we presented a potential drug testing strategy for compounds targeting thyroid-associated OA. We explored the role of thyroid signaling in OA-related terminal maturation which was previously identified as important process during OA pathophysiology³⁰ and tested potential drugs targeting terminal maturation in two model systems. The initial findings of the high-throughput screening model were validated in the biomimetic explant model. Our results suggest that targeting the thyroid hormone receptor reverses the detrimental hypertrophic effects during OA.

However, more importantly, we showed the workings of a drug testing strategy that can be applied for processes underlying OA, like senescence. Before application for multiple OA processes the high-throughput model needs to be further optimized, by including for example mechanical loading to be able to study the complementary senescence model and by using hiPSCs as a stable cell source.

Modelling the bone-cartilage interface using joint-on-a-chip platform with primary joint tissue cells

While this (high-throughput) neo-cartilage organoid model represents a relatively simple 3D model, which allows for real-time high-throughput readout and imaging, it lacks complexity needed for accurate human predictions. An answer to this are joint-on-a-chip platforms, which allows the interaction of multiple tissues present in the joint (Figure 3). In **chapter 5**, we showed the development of a joint-on-a-chip model which allows study of the hydrogel-free bone-cartilage interface. We showed the possibility of using multiple read-outs, on RNA and protein level for this model. This model should be further optimized to allow study of underlying processes, by applying OA-related perturbations like mechanical stimulation or induce senescence using ionizing radiation.

All these models have their advantages and disadvantages and which model to use depends on the specific research questions and objectives. Therefore, rather than choosing one specific research model, in **chapter 7**, we propose a pre-clinical treatment strategy for potential drug candidates by using the high-throughput *in vitro* neo-cartilage organoid model, composing thus far of primary human chondrocytes, as pre-screening model and the biomimetic osteochondral *ex vivo* model as validation model.

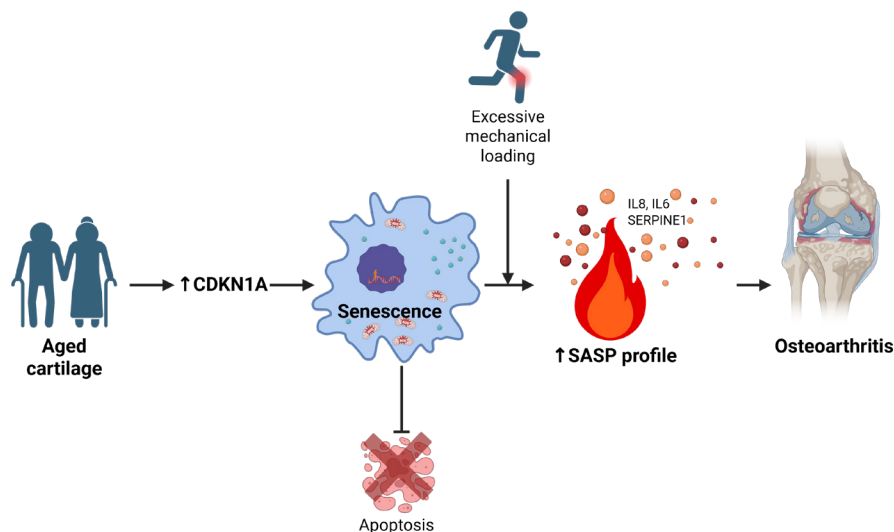


Figure 5 – Overview of the interaction between senescence and osteoarthritis. Aging leads to increased DNA damage in post-mitotic chondrocytes, which accumulates over time and promotes senescence via the *CDKN1A* pathway. Upon exposure to environmental stressors such as excessive mechanical loading, these senescent cells respond with increased SASP secretion, making the cartilage prone to age-related diseases like OA. Created with Biorender.

Future perspectives for senescence in cartilage during OA

In this thesis we highlighted the importance of senescence during OA and in aged tissue. We show the importance of using the right models to define and to test compounds against senescence, especially among post-mitotic cells. From our exploration in the molecular profiles of OA cartilage and the testing in aged human models, we propose the importance of p21, encoded by *CDKN1A*, rather than p16 in senescent chondrocytes (Figure 5). This stresses the importance of using tissue specific markers and representative models to indicate senescence, especially in the case of post-mitotic cells, and not to use generic markers to indicate body wide senescence.

Senescent cells accumulate with age.^{31,32} This is also visible in aged cartilage with a high level of senescence staining in unaffected aged cartilage of OA patients, as seen in **chapter 3**. When we look at the influence of senescence later in the OA development, we observed that both cell cycle arrest markers as SASP markers are mostly unaffected and other processes, like apoptosis and vascular ingrowth³³, play a vital role in the pathophysiology. This stresses the vital role of senescence during the onset of OA rather than the end-stage development of OA. Following this, we hypothesize senescence plays a vital role during the onset of OA rather than at the end-stage of OA. As shown in **chapter 3** and **4**, these dysfunctional senescent chondrocytes present in aged cartilage are no longer able to respond to environmental stressors, such as mechanical loading, and express a harmful SASP phenotype, making the cartilage prone age-related diseases like OA (Figure 5). However, more research is needed into the role of senescence as inducer of OA, starting with assessing *in vivo* markers of senescence, particularly *CDKN1A*. The human biomimetic *ex vivo* senescence models that also represent the molecular endotypes could be the answer to understanding and targeting chondrocyte senescence in OA. These biomimetic models will allow for better translational of pre-clinical studies to clinical output and less animal testing.

The initial role of senescence in OA and the fact that there are many therapeutic options available for senescent cells, makes it a promising therapeutic target. However, previous failed clinical trials, like the failed phase II clinical trial of UBX010134, teaches us that it is important to use the right models for the right application. Therefore, to facilitate successful development and testing of OA drugs, firstly, the heterogeneous OA populations needs to be taken into account and stratification is needed during drug discovery work and treatments. Secondly, the right human models needs to be used instead of animal models, to accurate study the mechanisms of processes underlying the OA pathophysiology in humans. Since there is currently not one specific model that can replace animal models, in this thesis we propose a drug testing strategy, starting with a relatively simple but fast model which should be followed up with validation in a complex model. These developments stimulate and highlight the value in using a personalized medicine approach rather than the ‘one drug fits all’ approach that is currently being used.

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