Aged human osteochondral explants as biomimetic osteoarthritis model: towards a druggable target in osteoarthritis

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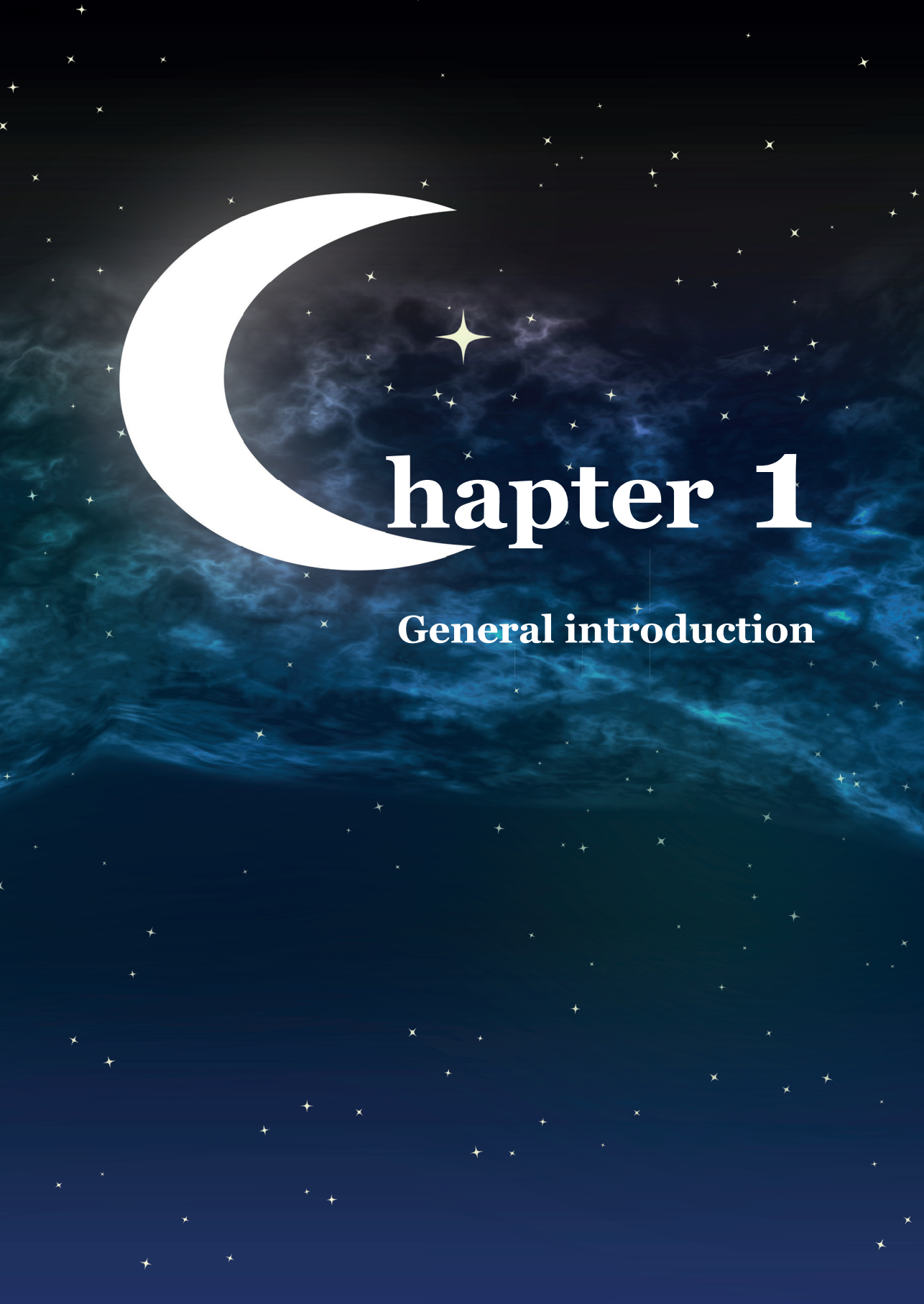

Chapter 1

General introduction

1. Osteoarthritis

Osteoarthritis (OA) is a prevalent chronic age-related joint disease. Proper disease management is amongst others hampered by lack of insight into heterogeneity of disease pathophysiology [1]. As a result, OA is significantly decreasing quality of life while increasing healthcare costs and absenteeism from work [3]. It is estimated that 6.8% of Disability Adjusted Life Years (DALYs) worldwide can be accounted for by the burden of musculoskeletal disorders [4] and OA is ranked as the fifth disease contributing to DALYs in the Netherlands [5]. In 2019, the Netherlands counted almost 1.5 million OA cases (8.6% of population), responsible for 1.2 billion euro (1.4%) of total healthcare costs [2]. Prevalence of OA increases with age and affects more than 21% of men and 34% of women above 70 years (**Figure 1A**) and it is expected that cases of OA will keep rising in the coming years, due to the increasing ageing population (**Figure 1B**) [6].

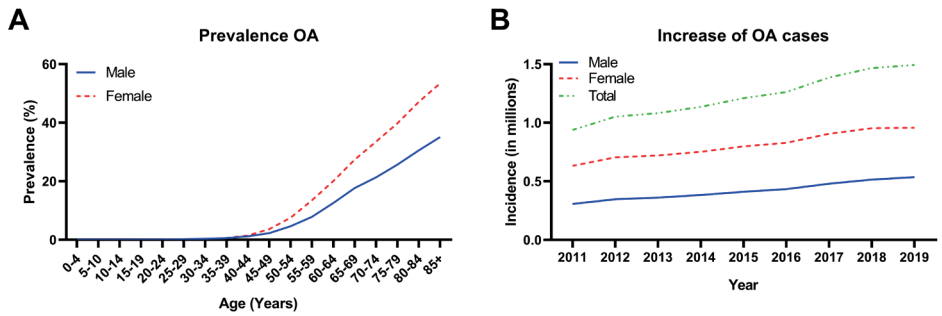

Figure 1 | Prevalence and absolute cases of osteoarthritis reported by GPD in the Netherlands stratified by sex. [A] Prevalence of OA per age groups in 2019. **[B]** Incidence of OA per year. Data from volkgezonheidenzorg.info [2].

The OA disease process itself is characterized by gradual degradation of articular cartilage, thickening of the synovium, formation of bony spurs termed osteophytes and remodeling of the underlying subchondral bone (**Figure 2A**). Clinical symptoms of these processes include chronic pain, stiffness, joint instability, swelling and joint space narrowing [7]. In recent years it has become more apparent that OA is a disease of the whole joint, including the subchondral bone and synovium [8,9]. Pathologic changes in subchondral bone have even been found in some early-OA cases prior to cartilage degradation [9,10]. Currently, no treatment is available that stops disease progression and therefore patients are prescribed pain relief and physiotherapy to reduce symptoms until they are eligible to undergo a joint replacement surgery. While joint replacement are beneficial for patients, with revision rates of only 2-10% after 10 years [11,12], there is a considerable increase in revision rate in the 60 years and younger population [13]. Even more, results after revision surgery are worse than primary implant surgery in this younger population. Therefore a better understanding of OA pathophysiology is necessary to develop therapeutics that preferably target early disease triggers and/or processes and prevent this end-stage of OA necessitating arthroplasty surgery.

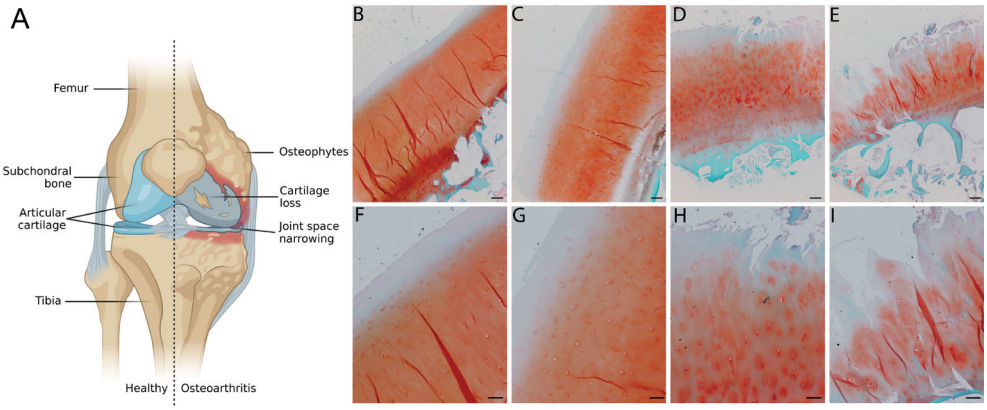

Figure 2 | Schematic overview of OA symptoms and risk factors. [A] Schematic drawing of a healthy knee (left) and a knee undergoing OA (right) created with BioRender.com. **[B-I]** Histological Safranin O staining for proteoglycans in articular cartilage taken from osteochondral explants from different areas of one knee joint shows the general cartilage degradation from mild OA (**A**) to severe OA (**E**) at 4x (**B-E**) and 10x (**F-I**) magnification. Black scale bar are 200μm (**B-E**) or 100 μm (**F-I**).

2. Healthy joint tissues

Although OA is now commonly considered a whole-joint-disease, for long it primarily referred to the degeneration of articular cartilage. Articular cartilage is a highly specialized connective tissue that covers the contact surface of bones in joints and facilitates smooth movement. Cartilage is mainly composed of type 2 collagen, proteoglycans, chondrocytes and water, each with a specific function. The collagen fibres enable resistance to tensile stresses and transmission of mechanical loads, while proteoglycans and water enable osmotic pressure and elasticity to prevent friction. The sole cell type of cartilage, chondrocyte, makes up only 2-5% of cartilage volume and is retained in an extracellular matrix (ECM) environment lacking blood vessels, nerves and lymphatics [14]. Each chondrocyte creates and maintains its own pericellular matrix, preventing migration and limiting direct signal transduction via cell-to-cell interaction while enabling chondrocytes to respond to a variety of stimuli such as mechanical loads, hydrostatic pressures, inflammatory factors and growth factors. Low metabolic activity and limited potential to replicate and migrate contribute to limited intrinsic repair of articular cartilage in response to injury [15,16].

Directly underneath the articular cartilage is the subchondral bone, consisting of a thin cortical layer and a thicker trabecular bone layer. The subchondral bone exerts important shock-absorbing and nutritional functions for cartilage. As the subchondral bone is metabolically very active, structures are dynamically adapted to mechanical forces across the joint by bone remodelling [17,18]. Subchondral bone is formed via endochondral ossification at the secondary ossification centres of bone epiphyses during joint formation (**Figure 3A**). Articular cartilage is replaced by bone during endochondral ossification and starts with chondrocyte proliferation and multicellular cluster formation. Subsequently, these cells become hypertrophic, dramatically increasing their volume while simultaneously secreting ECM, which is eventually mineralized. Finally, hypertrophic chondrocytes undergo apoptosis and their ECM is partially broken down leaving space for entry of blood vessels, osteoclasts and osteoblasts to initiate ossification [19]. Some of the growth plate chondrocytes escape this process and populate the joint contact surface of bones to become and maintain articular

cartilage in a tightly controlled maturational arrested state. Important systemic factors regulating endochondral ossification are growth hormone (GH), Insulin-like growth factor (IGF) and thyroid hormone. GH and IGFs are potent stimulators of bone growth and both stimulate proliferation and initiate chondrocyte hypertrophy [20]. Locally produced IGF-I, induced by GH, is likely to play a more important role in chondrocytes than systemic IGF-I [21,22]. Active thyroid hormone, triiodothyronine (T3), induces expression of hypertrophic markers, such as alkaline phosphatase (ALPL), collagen X (COL10), as well as hypertrophic morphology and cartilage maturation [23-25].

3. OA pathophysiology

3.1. Histopathology

During OA, cartilage undergoes drastic changes that can be observed by light microscopy. **Figure 2 B-I** gives a broad overview of these histological changes occurring from early OA to late OA. In early OA, some chondrocytes cluster and superficial surface fibrillations form (**Figure 2C** and **G**). This damage progresses to fissures and cracks that reach the middle zone, loss of proteoglycans and increased chondrocyte clustering (**Figure 2D** and **H**). In late OA, the fissures and cracks reach the deep zone, there is severe loss of cartilage, apoptosis of chondrocytes, duplication of the calcified layer of cartilage adjacent to the subchondral bone, termed the tidemark, and remodeling of subchondral bone (**Figure 2E** and **I**). This stage is followed by complete loss of cartilage and severe changes to the underlying bone. During OA histopathology chondrocytes start to proliferate, become enlarged and eventually go into apoptosis, resembling growth plate chondrocytes undergoing endochondral ossification (**Figure 3**)[26-28]. To better classify these histological changes several grading systems for OA were developed. For example, Mankin et al [29] developed a histological grading system that scores from 0-14, based on architectural cartilage surface, cellular, proteoglycan content and tidemark changes and is often referred to as Histological-Histochemical Grading System (HHGS). Over the years several other grading systems for *in vitro* or *in vivo* OA were generated, however a modified version of the Mankin score is still one of the most well-known and validated grading systems [30].

3.2 Molecular pathology

Deregulated signalling pathways in OA have been characterized by comparing genome-wide differential expression differences between preserved versus end-stage lesioned OA cartilage [31] and subchondral bone [32]. These studies revealed that OA pathology is marked by recuperation of growth plate signalling, cell adhesion, extracellular matrix organisation and skeletal system development, characterized by deregulated expression of, among others, genes involved in endochondral ossification: *BMP3*, *MGP* and *FRZB*. Similar as during endochondral ossification, OA chondrocytes start proliferating and differentiate into hypertrophic chondrocytes, accompanied by expression of ossification related genes such as alkaline phosphatase (*ALPL*), collagen X (*COL10A1*), runt-related transcription factor 2 (*RUNX2*) and matrix metallopeptidase 13 (*MMP13*), resulting in calcium crystal deposition and apoptotic chondrocyte death in cartilage (**Figure 3B**) [27,28,33-35]. In addition, two genome-wide differential expression studies have highlighted inherent differences in preserved OA cartilage

gene expression patterns between individuals using unsupervised clustering, designating clear subtypes of OA [36,37]. Both studies identified two distinct groups of OA patients, independent of joint site, with a considerable overlap (45%) of significant differentially expressed genes between the two clusters [36]. Strikingly, one group was marked by increased expression of non-chondrogenic genes involved in mechanoreceptors, such as calcium signaling (KCNN3), ion channels (TRPV4) and cytoskeletal organizers (ACTA2). The study by Coutinho et al [36] combined their transcriptomic data with radiographic OA data and determined that the non-chondrogenic group had higher joint space narrowing (JSN) scores and lower osteophyte (OP) scores. These results suggest that with respect to treatment modalities these subgroups of OA patients should be taken into account in the study setup. For example, IL-11 is much more upregulated during OA pathophysiology in one subgroup (FC=60) than in the other OA group (FC=19) and might therefore be a more attractive therapeutical target for the latter group. Likewise, some targets such as CCL2 may be more appropriate for the first OA subgroup.

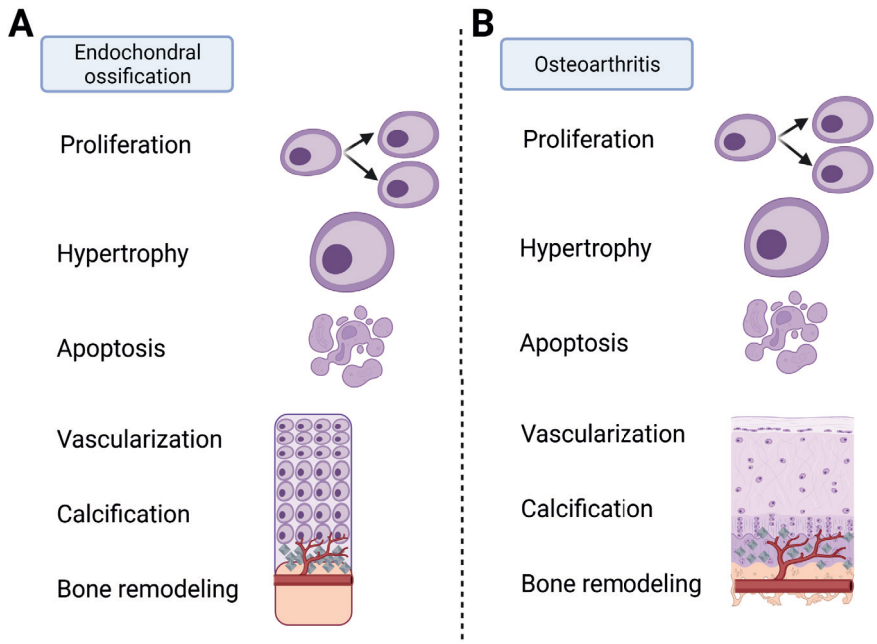

Figure 3 | The overlap between processes occurring in endochondral ossification and osteoarthritis. **[A]** During endochondral ossification stem cells differentiate into proliferating chondrocytes. This is followed by hypertrophy, terminal maturation, mineralization and eventually chondrocyte apoptosis to make space for bone. **[B]** A similar process is reiterated in osteoarthritis, where chondrocytes escape their resting state and start proliferating, become hypertrophic and eventually go into apoptosis. Created with BioRender.com.

As crosstalk between articular cartilage and subchondral bone is likely involved in OA pathophysiology, overlap of differentially expressed genes between cartilage and bone [32] was investigated and was enriched for processes related to the extracellular matrix, characterized by the expression of, among others, *FRZB*, *CCN4* (*WISP1*) and *GDF6*. Nonetheless, the preserved versus lesioned study design by definition captures end-stage pathophysiological OA disease processes and lacks information on early processes triggering cartilage to its diseased state. In contrast, disease-modifying OA drugs should preferably

target early OA disease triggers when irreversible damage of cartilage is not yet occurring. Therefore additional knowledge on the (early) effects of OA relevant stresses should be gathered from an appropriate model in response to OA-relevant triggers, such as mechanical stress, hypertrophy or inflammation.

4. Risk factors for OA

Epidemiology studies have identified that OA has a multifactorial aetiology and results from an interplay between systemic and local risk factors. As shown in **Figure 4**, factors such as obesity, age, gender, repeated mechanical stress and joint injury play a role in OA onset [38]. Importance of these risk factors may vary per joint and stage of diseases. For example, obesity has been associated with both knee and hand OA, indicating that in addition to increased mechanical forces also aberrant metabolism in obesity play a role in OA risk [39,40].

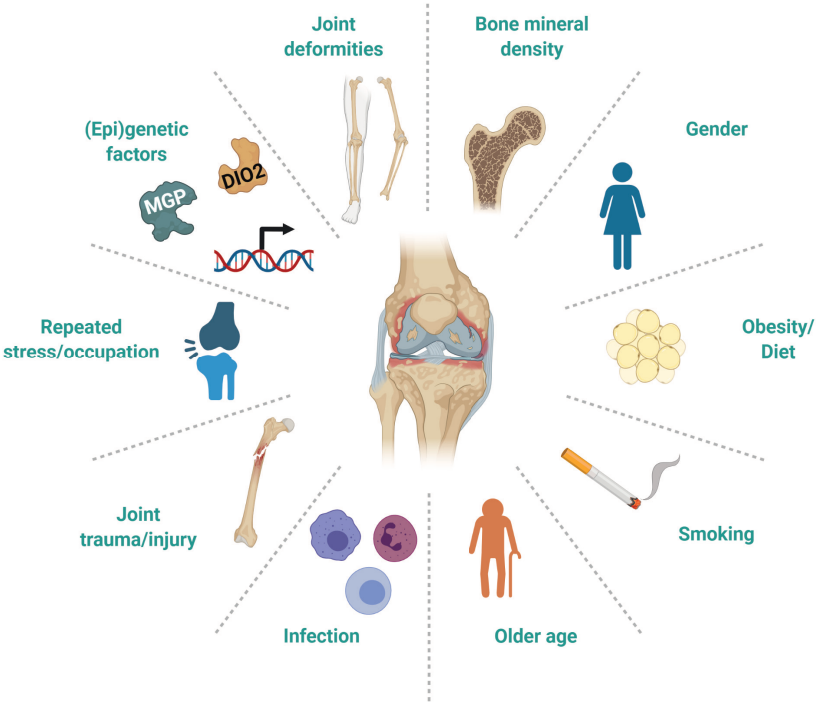

Figure 4 | Risk factor for osteoarthritis. The most common and well-studied risk factors for osteoarthritis. Figure created with BioRender.com.

4.1 Age

The strongest risk factor for OA in all joints is age, likely due to a combination of cumulative exposure to risk factors and the natural changes of cartilage and chondrocytes that occur with ageing [41,42]. With ageing, the articular cartilage matrix changes in amount and composition, resulting in a stiffer environment correlating with biomechanical dysfunctions [43], prone to tensile fatigue [44]. One of the changes is the increased glycation of proteins

(AGEs) [45], likely due to slow turnover of ECM components, increasing cross-linking of collagen molecules resulting in a stiffer matrix susceptible to injury at lower impact loads [46]. Next to collagens, size of proteoglycan aggregates decreases likely due to proteolytic damage, greatly affects the permeability of cartilage [47]. Another highly prevalent change in aged cartilage is the deposition of calcium crystals, such as calcium pyrophosphate (CPP) and basic calcium phosphate (BCP) [48]. Consequently, these calcium crystals stimulate production of inflammatory proteins and matrix degrading enzymes, further contributing to onset and progression of cartilage degradation [49].

In addition to cartilage matrix changes, chondrocytes also undergo ageing-associated changes conferring OA risk. These include cell depletion [50,51] and impaired responses to extracellular stimuli [52,53], resulting in a changed gene expression, increased cell differentiation and cellular senescence [54]. This reduced responsiveness to stimuli such as growth factors contributes to an imbalance in cartilage homeostasis. For example, IGF-1, important for chondrocyte survival and matrix synthesis, induces a lower anabolic response [52,53]. This reduced response is likely partially due to increased production of insulin-like growth factor binding proteins (IGFBPs) [52]. Another explanation for the changed response to IGF-1 is the altered signaling observed in aged chondrocytes [55-57]. The increased cellular senescence found in aged cartilage might also contribute to its reduced anabolic response. Reactive oxygen species (ROS) production initiated by mechanical stress could be a large contributor of stress-induced chondrocyte senescence [58,59]. Since senescent cells typically produce pro-inflammatory cytokines and matrix degrading enzymes, they could greatly contribute to cartilage degradation [60]. Taken together, these age related changes in the cartilage matrix lead to a tissue with reduced ability to bear mechanical stress and make it more susceptible for degeneration.

4.2 Mechanical stress

Physiological mechanical loading is necessary to maintain a healthy state and function of articular cartilage and subchondral bone [61,62]. Both the proteoglycan content and collagen patterns are conditioned to local stresses to maintain functionality [63,64]. For example, the patellar surface of femoral condyles, an area regularly subject to high shear stress levels, has a thicker superficial zone and higher collagen content than the tibial plateaus, an area subjected to weightbearing loads and rich in proteoglycans [65,66]. Normal ranges of stresses in joints have been measured to be between 3 and 10 MPa, but maximum forces of up to 18 MPa are reached in the hip joint [67]. The frequency of these stresses during walking is in the magnitude of 1 Hz in humans [68] and cartilage height is displaced between 7% and 23% [69]. Higher peak forces are measured during sport activities, such as running, increasing strains up to 35% [70]. Physiologic levels of cyclical dynamic loading can stimulate anabolic and/or anti-inflammatory functions of chondrocytes [71-73], while hyper-physiologic levels of dynamic loading and injurious loading can induce damage via induction of catabolism in chondrocytes [74-76], and cellular damage, such as apoptosis and necrosis [77,78]. Local biomechanical factors (e.g. amount of joint loading, joint injury/trauma or joint deformity) influence risk of degenerative changes of articular cartilage due to wear and tear, especially when they are repetitive. Approximately 12% of the overall OA burden in hips, knees and ankles arises as a result of previous joint trauma [79]. Depending on the type of injury, OA development was estimated to be between 23% [80] and 44% [81] in people after an injury. In addition,

adequate response of chondrocytes to a load depends on parameters such as frequency, duration, history and age of cartilage. Interesting is also to mention the discrepancy between the degree of radiographic osteoarthritis and clinical symptoms experienced by patients, as was previously observed in a cohort of nearly 7000 Dutch patients [82] and later observed in several other populations [83].

Considering the previously discussed changes occurring during ageing, aged articular cartilage can likely withstand lesser and shorter mechanical compressions when compared to the more flexible younger tissues. However, inactivity of middle-aged joints has also been shown to be unbeneficial for joint health, suggesting that balanced active life style should be initiated from a certain age on [84]. Nonetheless, little knowledge exists on the inherent dysregulation of signaling pathways in human aged articular cartilage upon mechanical stress and there is a knowledge gap on which strains, speed and duration of mechanical stress on aged cartilage is considered beneficial or actually detrimental that needs to be addressed.

4.3 Genetic risk factors

The genetic component of OA is estimated to be around 40%-60% [85,86], dependent on joint site. To gain more knowledge on inherent underlying processes in general OA pathophysiology, research groups have performed candidate and genome wide searches for genetic variants conferring risk of OA. OA has a complex genetic component in which many genetic variants with small effects sizes are expected to play a role in OA onset and progression [87-89]. Therefore, functional follow up is very important to confirm causality and has been performed for several genetic OA risk variants [90-92]. **Table 1** summarizes some of the most robust OA genetic risk variants to date, such as *DIO2*, *MGP* and *IL11*, for which successful and extensive functional follow up has been performed. These risk genes can be associated to one specific joint (*MGP*), or to multiple joints or patients with generalized OA (*DIO2*).

Table 1 | Summary of some of the most interesting OA genetic risk variants for which functional follow up has been performed

Gene	Risk SNP	Risk allele	AEI effect SNP	Expression in OA tissues[$]	In vitro FFU	In vivo FFU	Implication mutation
ALDH1A2	rs3204689 [93]	C	↓ ALDH1A2 [93,94]	↓ AC [94]	RNAi hACs [94]	*Aldh1a2*$^{-/-}$ mice [95]	Acts by decreasing *ALDH1A2*
DIO2	rs225014 [96]	C	↑ DIO2 [97]	↑ SB & AC ↑AC compared to healthy [97]	Lentiviral overexpression in hBMSCs [90]	*Dio2*$^{-/-}$ mice [98], cartilage-specific *DIO2* overexpression in rats [99]	Acts by increasing *DIO2*, increasing active thyroid hormone levels
DOT1L	rs12982744 [100]	C	-	↑in synovial tissue [101]	Inhibition of *DOT1L* [102]	Cartilage-specific *Dot1l*$^{-/-}$ in mice [102]	Abnormal histone modification
GDF5	rs143383 [103]	T	↓GDF5 [104]	↑AC [105]	EMSA, ChIP, RNAi, overexpression in different cell types [106]	Functional null mutation mice[107], heterozygous *Gdf5*$^{Bp-J/+}$ mice [108]	Acts by decreasing *GDF5* expression
IL11	rs4252548 [109,110]	T	↓IL11	↑AC & SB	-	-	Acts by decreasing *IL11* expression
MGP	rs4764133/ rs1800801 [111]	T/T	↓MGP [92,111]	↑AC & SB	RNAi in hACs [92]	*Mgp*$^{-/-}$ mice [112]	Acts by decreasing *MGP*, likely necessary to inhibit mineralization
SMAD3	rs12901071 [113]/ rs12901372[109,110]	A/C	No AEI	↓AC	RNAi and overexpression in ATDC5 [114]	*Col2-Cre;Smad3*$^{fl/fl}$ mice [114], *Smad3*$^{ex8/ex8}$ [115]	Unknown mechanism of risk SNPs
TNC	rs2480930[109]	A	↓TNC	↑AC	Addition of TNC in hACs [116] and explants [117]	TNC antibody and *TNC*$^{-/-}$[118], *TNC*$^{-/-}$ [119]	Acts by decreasing *TNC* expression

[$] Genome wide significant differential gene expression in lesioned compared to preserved articular cartilage (AC) [31] or subchondral bone (SB) [32], unless otherwise indicated. **Legend:** SNP= single nucleotide polymorphisms**;** AC=articular cartilage; SB=subchondral bone; AEI=Allelic expression imbalance; FFU=functional follow up; EMSA=electrophoretic mobility shift assays; ChIP=chromatin immunoprecipitation; RNAi=RNA interference; hACs=human articular chondrocytes; hBMSCs=human bone marrow stem cells.

5. Functional follow up

The investigation of an OA risk single nucleotide polymorphism (SNP) does not stop at its identification, but (functional) follow up is necessary to demonstrate causality. Freedman et al [120] have suggested a systematic strategy for the post-genome wide association studies (GWAS) functional follow-up to identify causality and the additional hurdles. For complex diseases such as OA, many SNPs from non-protein coding regions have been associated with disease risk. These trait-associated alleles likely exert their effects by changing transcription through different mechanisms. Often, multiple independently associated risk SNPs in a locus may be functionally linked to the disease and therefore it is important to first identify the causal allele. After the causal allele has been identified, knowledge on the regulatory landscape of the risk region can elude how risk alleles affect transcription. For example, if it is in a regulatory area such as a promotor, enhancer or silencer, altered transcription factor binding could change efficiency of transcriptional induction. As regulatory sequences are often very tissue-specific [121], this could explain why common susceptibility alleles often associate with a specific trait or disease. To identify which transcription factor binds to which DNA region, chromatin immunoprecipitation followed by sequencing (CHIP-Seq) can be performed. In addition, reporter gene assays, such as luciferase assays, can be used to provide evidence whether a SNP is localized in such a regulatory region. To connect SNPs to their target gene, the association between genotype and local and distant gene expression can be determined. After causality of an allele has been determined, a next step is to investigate if the SNP and/or gene affect tissues in appropriate *in vitro* or *in vivo* OA models. Examples of some well-studied OA risk SNPs for which functional follow up was performed are summarized in **Table 1**. Another approach for which these OA risks SNPs can be used for is to identify common pathways or mechanisms underlying OA pathophysiology. This knowledge can further increase our understanding of the onset and progression of OA.

5.1 Common underlying mechanisms in OA based on genomics

Large-scale GWASes have identified reproducible and highly significant OA risk SNPs in genes involved in OA aetiology. Functional follow-up studies have demonstrated that risk SNPs frequently modulate pathology by altering transcription of genes in *cis* in both bone and cartilage [91,94,97,122]. A striking overlap between many of these OA risk genes is their involvement in different processes vital in endochondral ossification (**Figure 5**). For example, *DOT1L*, *FRZB* and *TNC* are involved in the differentiation of stem cells to chondrocytes; *GDF5* and *BMP3* initiate hypertrophy in proliferating chondrocytes; *DIO2* and *MGP* are involved in terminal maturation and mineralization.

5.2 Follow up studies on SNPs function

With the increase of OA tissues being sequenced, generating large mRNA, miRNA and methylation datasets in combination with freely online expression databases, such as the Genotype-Tissue Expression (GTEx) Project [123], *in silico* functional follow up has become more readily available and allows for investigation of functional effects of intergenic and intronic variants. In addition, studies have already used such datasets to investigate the genome wide allelic expression imbalance of SNPs [124] and the epigenetic landscape [125] in

articular cartilage. Using these tools generates more insight into how OA risk SNPs could induce a life-long altered expression of a gene. To acquire knowledge on influences of the SNP on expression or stability of a transcript, researchers have measure expression and methylation fraction of genes in the vicinity of the SNP per genotype to determine expression quantitative trait loci (eQTL; **Figure 6A**), methylation quantitative trait loci (mQTL; **Figure 6B**) and allelic expression imbalance (AEI; **Figure 6C**). These SNPs can affect gene expression by, for example, influencing binding of transcription factors or influencing methylation fraction of a region.

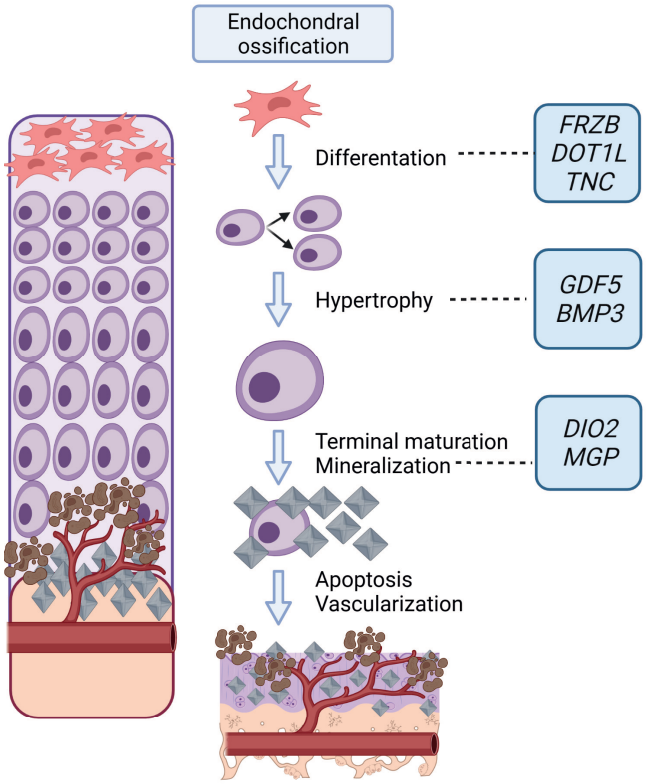

Figure 5 | The overlap between processes occurring in osteoarthritis and endochondral ossification. Many of the OA risk genes are involved in the different endochondral ossification steps. Some examples and the processes these genes are involved in are given. Figure created with BioRender.com.

A notable OA risk gene with strong evidence for allelic imbalance is Matrix Gla protein (MGP). A SNP in this gene was identified as a strong OA risk SNP for hand OA in a genome wide association study (GWAS) via rs4764133 [111] with proxy SNPs rs1800801 and rs4236 (**Table 1**) [126]. Identification was followed up by measuring AEI of rs1800801, showing its mechanism to be decreased expression of the OA conferring rs1800801-T allele relative to the rs1800801-C non-risk allele in a range of joint tissues [92,111]. The MGP protein regulates extracellular calcium levels via high affinity to its γ-carboxyglutamic acid (Gla) residues and inhibits calcification. Prior to identification of *MGP* as OA risk gene, *mgp* deficient mice were shown to have severe and lethal vascular calcifications in combination with abnormal calcification of growth place cartilage increasing premature bone mineralization resulting in

reduced bone mass [112,127]. As the OA risk allele leads to a reduced *MGP* gene expression [128] and increased vascular calcification [129], this would suggest simultaneous increased cartilage calcification and a reduced bone mineral density in carriers of the OA risk allele [112,128,130].

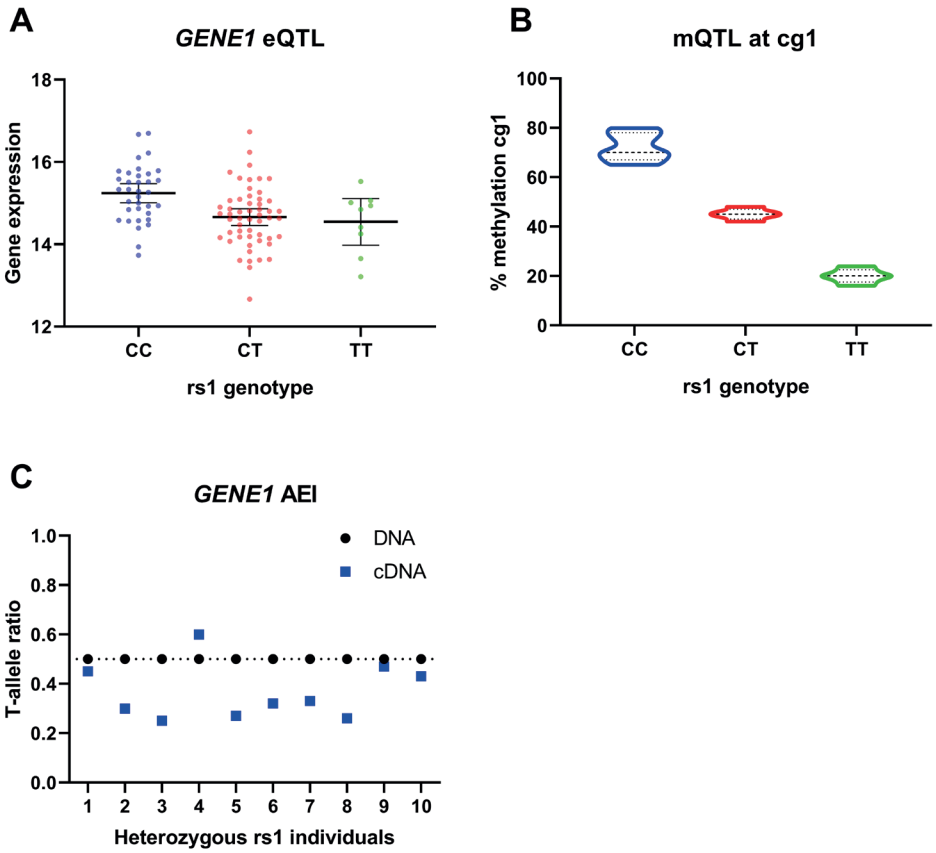

Figure 6 | Functional follow up of the SNP Methods to investigate how risk SNP affects transcription. **[A]** Comparing gene expression for the genotypes of a risk SNP. **[B]** Comparing methylation fraction of a cg site for the genotypes of a risk SNP. **[C]** T-allele ratio of cDNA for the risk SNP in comparison to the reference allele in heterozygous individuals. **[D]** Schematic representation of a cis allelic expression imbalance. Legend: eQTL=expression quantitative trait loci; mQTL=methylation quantitative trait loci, AEI=allelic expression imbalance; UTR=untranslated region.

5.3 Functional follow up in OA models

A major problem in the field of OA is the lack of appropriate *in vitro* and *in vivo* models for functional follow up of genetic risk variants and drug screening. In the current models used to investigate OA, the choice of cell type, species and culture method can greatly influence the results. Nevertheless, these models are crucial to advance research into the different aspects of OA pathophysiology and subsequent design and testing for safe drug development. **Table 2** summarizes the main advantages and disadvantages of the most commonly used OA models (**Figure 7**).

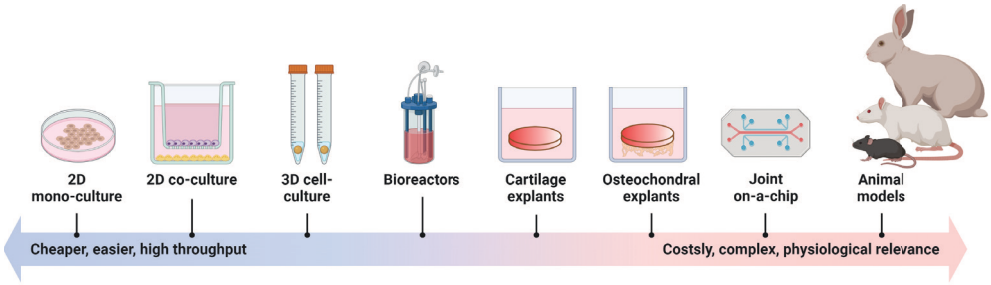

Figure 7 | Most commonly used OA models. Several models can be applied to investigate OA, ranging from more easy and cheap models such as 2D cell culture to more complex and expensive models such as animal models. Table 1 summarizes the advantages, disadvantages and applications of these models. Figure created with BioRender.com.

5.3.1 In vitro models

Established cell lines, easy and cheap to obtain and culture, have extensively been used to perform short term experiments. However, these cell lines are likely to have accumulated mutations and other stable modifications, increasing the possibility that they might not reflect a 'normal' chondrocyte environment. Therefore, the preferred cell source are primary chondrocytes that can be isolated from cartilage while maintaining their methylation profile [131] and can be used to investigate genetic alterations, such as overexpression or point mutations. To understand the consequences of an OA risk gene, overexpression or silencing of a gene can help determine if changes in its expression are vital for cartilage formation or maintenance. For example, AEI determined decreased *MGP* expression associated with the OA risk SNP rs1800801. To understand consequences of decreased *MGP* expression, small interfering RNA (siRNA) targeting *MGP* were transfected in monolayer primary human articular chondrocytes [92]. After 48 hours, *MGP* depletion resulted in altered expression of several cartilage markers, including the cartilage degrading enzymes *MMP13* and *ADAMTS4* and the ossification markers *COL1A1*, *ALPL*, *COL10A1* and *VEGFA*. However, a major downside of experiments with primary chondrocytes in monolayer is that they have limited proliferation capacity and are prone to change their phenotype into a fibroblastic-like cell type [132]. Therefore, such short-term experiments in an environment that does not completely encompass the cartilage environment and thus (expression) changes should be interpreted carefully.

Another component to consider in OA models is their highly specialized ECM. This ECM is likely very important in maintaining primary chondrocytes, as studies have observed a hypertrophic phenotype when cultured in monolayer that is resolved by 3D culture [132,133]. For 3D culture, cells can be pelleted by centrifugation or cultured in a biomimetic environment, such as a scaffold. Subsequently, stimulating chondrogenesis will enable cells to produce their own ECM. Some major advantages of these models are the cell-ECM interaction and the provided structural support. However, as the ECM needs to be produced, this model can be time consuming and only allows culture of one cell type. For example, the OA associated risk SNP rs225014 located near *DIO2,* for which increased expression was the likely culprit resulting from AEI and gene and protein levels investigation[97], was investigated in a 3D *in vitro* chondrogenesis model with human bone marrow derived mesenchymal stem cell (hBMSC). Lentiviral overexpression of D2 in this model confirmed increased *DIO2* to

be unbeneficial for cartilage homeostasis, as observed by greatly reduced expression of articular cartilage genes (*COL2A1*, *ACAN* and *COL10A1*) and upregulation of hypertrophic and breakdown markers (*MMP13*, *ADAMTS5*, *RUNX2* and *EPAS1*) [90]. In addition, such (mature) 3D pellet cultures can be used to investigate the effects of stimulation or inhibition of a target. For example, 14 day old 3D pellets were treated with active thyroid hormone (T3) or iopanoic acid (IOP), to respectively simulate increased and decreased D2 enzyme functionality [90]. Another OA risk gene for which functional follow up was performed in 3D cultures is increased *WISP1* expression associated with decreased methylation via rs6982341 [134,135]. Addition of recombinant human WISP1 to 3D *in vitro* pellet cultures from primary aged human chondrocytes resulted in reduced proteoglycan content, pellet size and matrix component production, suggesting that indeed increased WISP1 levels are detrimental for cartilage [134].

5.3.2 In vivo models

The most accurate reflection of the whole-joint are *in vivo* animal models. Animals, especially small animals such as mice and rats, have been extensively used for genetic manipulation and subsequently to investigate the effects of knockout or knock-in to model gene expression changes from conception to birth and during ageing. There are also techniques creating tissue-specific overexpression in animals, which can be very useful to investigate diseases. For example, in a forced running OA model, *Dio2*$^{-/-}$ mice did not show a different phenotype but these mice were protected from cartilage degradation when compared to their wild-type littermates [98]. In line with this, Nagase et al [99] observed that transgenic rats with cartilage-specific overexpression of human DIO2 (hD2Tg) had no articular cartilage defects, however, upon increasing the biomechanical burden by applying an injury-induced OA model, hD2Tg rats had increased cartilage damage when compared to their wild-type littermates.

It should be taken into account, however, that animal models can be difficult to manipulate and the shift towards the 3Rs on refining, reducing and replacing makes them less desirable. On another note, small animals such as mice are used because they are cheaper and easier to house, but due to their smaller size contain less material for biochemical assays. Another factor to consider is that animal joints are not fully translatable to the human situation given the different structure and biomechanical loading [136], and spontaneous OA is often absent. Currently, most experiments are performed in relative young animals subjected to a hyper-physiological trigger such as collagenase or DMM to initiate OA pathophysiology, likely not completely representing the slow progressive OA occurring in aged human tissues. Larger animals, such as bovine are likely more suited to study OA due to their larger joints and longer life-span, however they are also a lot more expensive and come with more ethical considerations. Therefore, careful conclusions should be taken from results obtained in OA animal studies and species, animal strains and OA triggers used should be critically reviewed prior to initiating investigation in human clinical studies.

5.3.3 Ex vivo models

As increasing evidence show crosstalk between multiple tissues to be involved in OA, systems such as co-cultures or osteochondral explants might be more suitable to study treatment modalities. The advantage that explants have over co-culture systems is that it retains chondrocytes in their natural 'aged' ECM, likely representing age-related joint tissues prone to enter the OA process upon disease-initiating cues. However, genetic manipulation studies cannot be performed in this model, limiting its purpose to investigation of OA relevant triggers and treatment modalities. The osteochondral explant model typically encompasses both the cartilage and bone compartments and therefore allows a readily investigation of the interplay between articular cartilage and the underlying subchondral bone. The finding that IL-1β treatment induced TNF-α production only in cartilage explants and not in osteochondral explants is an example highlighting that this interplay between tissues is important to take into account when investigating OA pathophysiology [137]. Most commonly used explant-based models thus far were often derived of bovine origin and applied a hyper-physiological perturbing factor of either a fierce inflammatory cytokines treatment [138-140] or mechanical loading [74,75,141] to induce detrimental signaling. Next to inflammation and mechanical loading, recapitulation of endochondral ossification and thereby hypertrophy is also thought to be one of the major mechanisms driving the processes in OA [142]. In cartilage explants, active thyroid hormone triiodothyronine (T3) induced expression of hypertrophic markers (ALPL, COL10), hypertrophic morphological changes and cartilage degradation and formation [143]. Even though the closest human OA-like model would be the use of human osteochondral explants obtained from macroscopically preserved areas of OA joints or cadavers, some limitations are that their number is limited, with high dependency on surgeries, ethical issues, heterogeneity between patients and difficulty to maintain tissues in long-term culture. Nevertheless, once the experimental set-up is achieved, these models can greatly benefit knowledge of OA pathophysiology and treatment modalities in the OA field. In addition, since ageing is the largest risk factor partially due to ECM and chondrocyte changes, using older tissues for research could be an important extra step to predict if a drug can be used to treat OA, thereby reducing the number of failing clinical studies. For example, treatment of IGF-1 greatly increased cartilage synthesis in calf explants [144]. Contrarily, in an aged human explant model, IGF-1 only slightly increased *COL2A1* and cell viability, and failed to abolish trauma-induced MMP13 secretion and type II collagen breakdown, likely due to desensitization to IGF-1 in aged tissue [145].

5.3.4 DIO2

An example of an OA susceptibility gene following many of these functional follow up steps is the previously mentioned deiodinase iodothyronine type II (D2) gene (*DIO2*). Genetic linkage studies identified an association of rs225014 (**Table 1**) located in the *DIO2* gene (*DIO2*), with generalized OA [96]. D2 activates thyroid hormone intracellularly by converting the prohormone thyroxine (T4) into active triiodothyronine (T3). To determine the direction of effect, AEI was measured and a 30% increased expression of the OA risk associated rs225014-C allele, likely due to loss of epigenetic silencing, was identified as the underlying risk mechanism [90]. This was followed up by investigating the role of *DIO2* and D2 in OA tissues. In human lesioned OA articular cartilage, a marked higher amount of *DIO2* expression and D2 staining was observed relative to healthy cartilage [90,146]. However, it should be noted that the

still macroscopically healthy looking preserved OA cartilage also has these increased *DIO2* levels. Therefore it was hypothesised that high D2 was not enough for cartilage breakdown, but that an additional trigger, such as mechanical stress, is necessary to initiate OA [142]. *In vitro* functional follow up in a 3D *in vitro* chondrogenesis model of hBMCs confirmed that increased expression of *DIO2* was an OA risk by determining detrimental effects of lentiviral overexpression of D2 [90]. Furthermore, in the same 3D model after ECM was established, pellets were treated with T3 or IOP, to simulate increased and decreased D2 activity, respectively. The results found herein confirmed the hypothesis that increased D2 activity was detrimental for cartilage homeostasis, while reducing its activity was beneficial [90]. *In vivo* animal functional follow up experiments were performed in a forced running OA mouse model and an injury-induced OA rat model. *Dio2*$^{-/-}$ mice did not show a different phenotype but were protected from running induced cartilage damage when compared to their wild-type littermates [98]. In rats with cartilage-specific overexpression of human DIO2 also no phenotypical differences were observed. However, upon increasing the biomechanical burden by applying an injury-induced OA model, hD2Tg rats had increased cartilage damage when compared to their wild-type littermates [99]. Before clinical studies should be initiated there is still a missing step in this line of work. Since species and age are such important factor in the mechanisms of cartilage response, a logical follow up step is to investigate if inhibition of D2 activity can prevent mechanical stress induced detrimental signaling in an aged human osteochondral explant model.

Table 2 | Advantages and disadvantages of the most commonly used models of osteoarthritis.

Type of model	Examples of applications	Advantages	Disadvantages	Examples of the application of the model
Monolayer culture	Cytokine/protein stimulation, osmotic pressure, genetic manipulation	Inexpensive, high throughput, equal exposure of cells, allows for preliminary investigation of mechanisms and compounds, easy to manipulate gene/protein expression	Not representative of normal chondrocyte environment, dedifferentiation, one cell type at a time, cell lines may have modifications	Mgp depletion [92], Aldh1a2 depletion [94], T3 or IGF-1 stimulation [147], osmotic pressure [148]
Co-culture	Cytokine/protein stimulation, osmotic pressure, genetic manipulation	Crosstalk between different cell types, easy to manipulate	Different conditions for each cell type, can results in altered phenotype when isolated, does not allow the natural 3D environment.	MSCs and ACs static [149,150] or with mechanical stress [151]
3D cell culture	Cytokine/protein stimulation, osmotic pressure, genetic manipulation, physical injury and loading regimens	Cell-ECM interaction, provides structural strength, easy to manipulate	One cell type at a time, slow proliferation rate, structural strength differs between material cultured in (Scaffold/hydrogel)	DIO2 overexpression, stimulation with T3 or IOP [90], WISP1 addition [134], osmotic pressure [152]
Explant based models	Cytokine/protein stimulation, osmotic pressure, physical injury and loading regimens	Cell-ECM interaction, natural ECM, simple, cheap and easy to produce	Difficult to culture long term, cell death at edge, limited number of cells, limited tissue available, inter-experimental variabil-ity	IGF-1 addition [144], TNC addition [117], mechanical injury [74,75,141,153], inflammatory [138-140], hypertrophy [143]
Animal models	Cytokine/protein stimulation, osmotic pressure, genetic manipulation, physical injury and loading regimens	Whole joint is investigated, possibility to investigate synovial	Costly, many animals necessary, ethical issues, huge variation between strains and species, anatomy: biomechanics and histol-ogy of joints differ from humans, natural occurrence of OA is uncommon in most species	Dio2-/- forced running [98], hD2 overexpression injury-induced OA model [99], destabilization of the medial meniscus (DMM), repeated tibial compression [154]

Different type of models used in OA are shown together with the variables that can be applied, their advantages, disadvantages and examples of some applications in the OA field. Legend: ECM=extracellular matrix; OA=osteoarthritis; Mgp=matrix gla protein; aldh1a2=Aldehyde dehydrogenase 1 family member a2; T3=triiodothyronine; DIO2=Type II iodothyronine deiodinase; IOP=iopanoic acid; WISP1=WNT1-Inducible-Signaling Pathway Protein 1; IGF-1=insulin-like growth factor 1; TNC=tenascin; MSC=Mesenchymal stem cell; AC=articular chondrocyte

6. Current clinical trials

Another problem in the OA field is incorrect usage of OA models for drug testing prior to clinical trials. Next to the arising evidence of subtypes between OA patients that is often not taken into account when starting a clinical trial, drugs are often tested in non-human non-aged models subjected to a hyper-physiological trigger. Examples of trials that likely failed partially due to being based on unrepresentative models are given in **Table 3**. The most recent example is the failed phase II clinical trial of the ADAMTS5-inhibitor GLPG1972. Their evidence of functionality was mainly based on mouse cartilage explants subjected to high levels of IL-1 treatment [155], while IL-1 OA synovial fluid levels are variable between patients but much lower in comparison to the experimental condition [156,157]. As mentioned earlier, it is already known that aged chondrocytes respond differently to certain stimuli [52,53], showing the importance of including older tissues in the pre-clinical development. In addition, changing the focus of drug targets in OA towards those based on functional data of OA risk genes and their pathways could increase chances of developing effective disease modifying OA drugs, given that genetically supported drug targets have been shown to double clinical success rate [158,159]. Therefore we advocate that for clinical trials to have a higher chance of success, OA models that represent the human aged-chondrocyte environment should also be included and may even be prioritized in the pre-clinical screening and clinical trials should target drugs based on genetically supported data.

Table 3 | Examples of clinical trials for OA therapies with discouraging findings that had promising results in pre-clinical *in vitro* and/or *in vivo* studies.

Drug Name	Druggable target	Evidence underlying drug	Outcome clinical trial
GLPG1972/S201086	ADAMTS5-Inhibitor	Reduction of glycosaminoglycan release after interleukin-1 stimulation in mouse cartilage explants [155]. Reduced cartilage structural damage and bone sclerosis in mice and rat OA models [160]	Phase II clinical trial: No difference of cartilage thickness with placebo [NCT03595618]
PG-116800	MMP inhibitor	Reduced joint damage induced by iodoacetate injection into rat knees [161]	Terminated in phase II trial due to musculoskeletal toxicity [162]
Risedronate	Bisphosphonates	Reduced cartilage degeneration and no osteophyte formation in a rat anterior cruciate ligament transection (ACLT) model [163]; Inhibited bone resorption and some chondroprotective effects in a papain rabbit model [164]	phase III trial: No reduction of radiographic progression compared to placebo [164]
Anakinra	IL-1 receptor antagonist	Protected from surgery induced OA in rabbits [165] and dogs [166]	No clinical effect [167]
Adalimumab	Tumor necrosis factor-alpha	Inhibits progression in a number of arthritic diseases, including rheumatoid arthritis and psoriatic arthritis [168]	No effect in erosive hand OA patients on pain, synovitis or bone marrow lesions [169]
Tocilizumab	IL-6 receptor	Slowed the progression of experimental OA in mice [170,171]	No effect on pain relief in patients with hand OA and more adverse events than placebo [172]

Legend: ADAMTS5=A disintegrin and metalloproteinase with thrombospondin motifs 5; MMP=Matrix metalloproteinase; IL-1=Interleukin 1; IL-6=Interleukin 6; OA=osteoarthritis.

7. Outline of this thesis

This thesis aims to increase the understanding of human OA pathophysiology by developing reliable biomimetic *ex vivo* human osteochondral explant models and focussing on the role of injurious mechanical stress and interacting genetic factors for developing increasingly necessary treatment targets in these models. Human aged joint tissues used for the studies performed in this thesis were collected as part of the Research in Articular Osteoarthritis Cartilage (RAAK) biobank [173], containing patients that undergo a joint replacement surgery for symptomatic end-stage OA.

To add to existing knowledge of OA pathophysiological processes, in **chapter 2** aged human *ex vivo* osteochondral explants were subject to three OA relevant triggers, being inflammation, hypertrophy and injurious mechanical stress. Subsequently, a range of output measures were investigated to determine specific mechanisms of the different OA triggers.

In **chapter 3**, knowledge on early initiating processes occurring in mechano-pathology was generated by applied RNA-sequencing to cartilage of aged human osteochondral explants subjected to injurious mechanical stress.

To show that the human osteochondral explant model could also be used for genetic interaction studies, we investigated expression of the OA risk gene *MGP* in relation to rs1800801 genotypes in **chapter 4**. By combining information from RNA-sequencing datasets of cartilage and bone with OA-relevant triggers in cartilage and bone explants we investigated the role of *MGP* and vitamin K in OA.

Lastly, the *ex vivo* osteochondral explant model was exploited in **chapter 5** to determine the efficiency and effectivity of inhibition of the OA risk gene DIO2 produced protein D2 by iopanoic acid (IOP) treatment either by burst or prolonged release by PLGA-PEG nanoparticles in preventing injurious mechanical induced stress.

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