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

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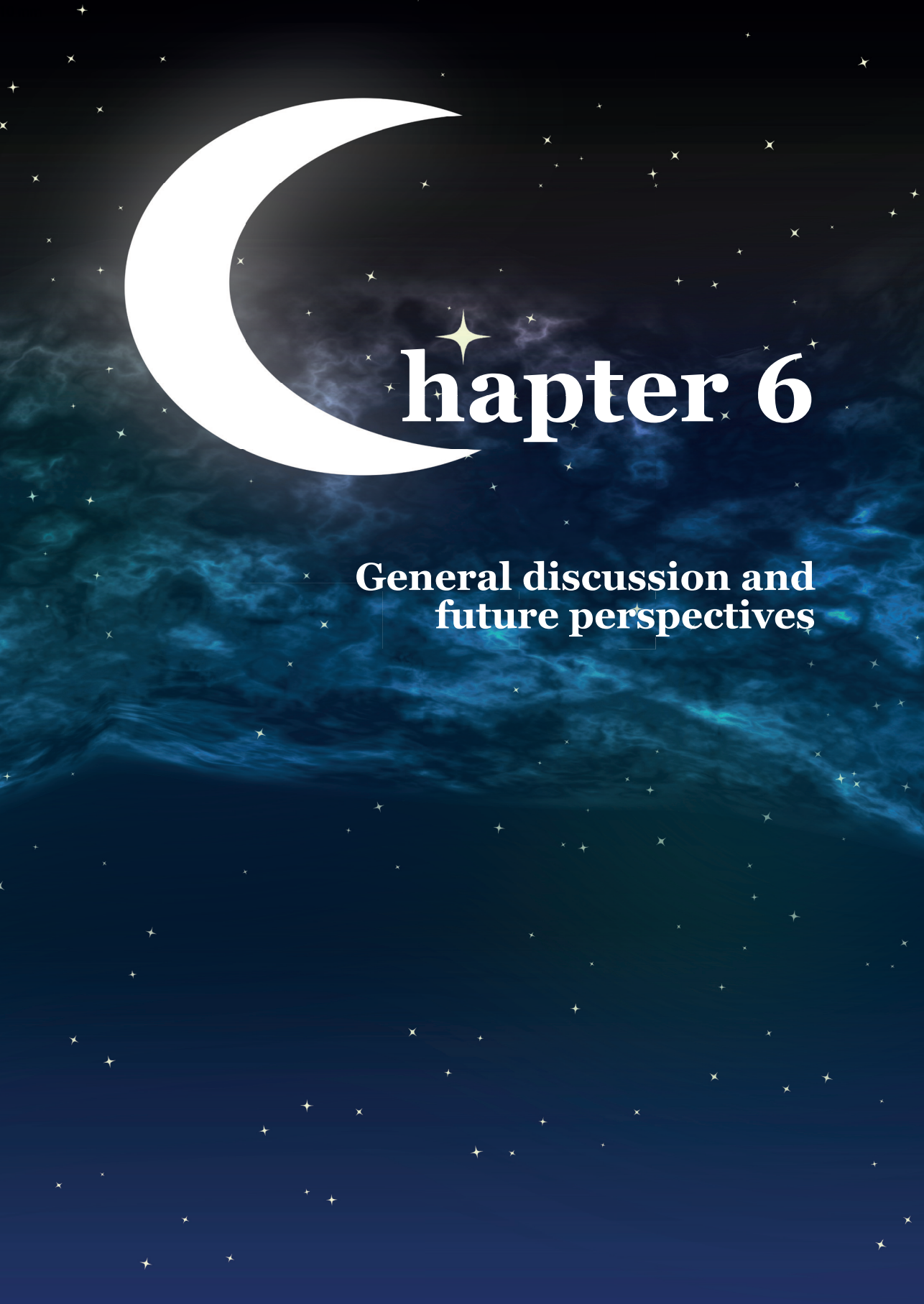

Chapter 6

General discussion and future perspectives

Translating biomedical research from *in vitro* and *in vivo* animal models to clinical applications has critical challenges and shortcomings that need to be addressed in order to advance drug development for osteoarthritis patients. Thus far, pre-clinical models are typically limited to either relatively young animals subjected to hyper-physiological stimuli or to 2D and 3D *in vitro* cell culture models of neo-cartilage from (aged) human primary chondrocytes or stem cells. Nonetheless, these human models do not reliably represent the osteochondral compartment nor do they capture aspects of human aging of articular joint matrix which is prone to initiate OA upon disease relevant triggers. In addition, the use of animals for pre-clinical models is less desirable as they do not support the societal 3R-principle of replacement, reduction and refinement of animal research. Henceforth advancement of clinical development in the OA field requires models reliably mimicking aged human tissue that ideally would allow for transcriptional and biochemical output in response to relevant perturbations initiating OA related tissue damage.

This thesis aimed to bridge the gap between biomedical data and clinical translation by developing reliable biomimetic *ex vivo* human osteochondral explant models. These models focussed on the impact of OA relevant triggers in humans and as such allowed for in depth studies of human OA pathophysiology in interaction with genetic factors. For that matter, in **chapter 2** our human aged OA joint tissue model was set up with taking into account different pathophysiological modalities triggering OA related damage being inflammation, hypertrophy of cartilage and injurious mechanical stress. The model was exploited to obtain insight into underlying disease mechanisms in response to injurious mechanical stress in **chapter 3,** whereas in **chapter 4** we explored it in interaction with the strong OA risk gene *MGP*. Finally, in **chapter 5**, a proof of concept pre-clinical study was performed on the chondroprotective effect of iopanoic acid, a pharmacological agent that acts via downregulating thyroid signaling, on mechanically induced OA related cartilage damage.

Development of a reliable human biomimetic joint tissue OA model

In **chapter 2**, a reliable human biomimetic tissue model was set up that captured age-related human articular joint changes prone to initiate OA upon disease relevant triggers. Our established model has several advantages relative to other models. First, to circumvent the need for species translation, we chose to use human tissues in our model. Second, it is extremely difficult to recreate native articular cartilage due to its complexity and presence of multiple dedicated layers. Therefore, to mimic cartilage to the best of our abilities we took macroscopically normal plugs of *ex vivo* tissue from human knee condyles of OA patients undergoing joint replacement surgery. Notable, within these samples several degrees OA changes are present. Third, our model takes ageing into account, given the age related changes that occur in articular cartilage and chondrocytes [1-6]. Finally, an important aspect which makes our model more representative of the human (OA) joint is that we retained the bone-cartilage interface. The rationale for this is that in recent years the perspective from OA as a cartilage disease has shifted towards a multi-tissue disease. This is reflected by the identification of many risk genes (e.g. *TNC*, *MGP, IL11)* in OA that have a function in both bone and cartilage [7]. However, limitation of using aged human osteochondral explants are scalability and dependency on surgery. The latter became more obvious during the COVID19 pandemic when the number of joint replacement surgeries decreased and even came to a hold. With respect to scalability, it should be noted that the number of explants that can be

taken from a joint is dependent on size of joint and degree of OA damage. Moreover, donor differences with respect to age-related changes or extent of OA pathophysiology in the preserved cartilage brings about heterogeneity, that need to be taken into account during analysis. On the other hand, such diversity contributes to a more realistic situation, since the OA patient population is very heterogenous as well, and will not comply to an "one medicine fits all patients" approach. To accommodate higher throughput, an option could be to confirm findings of *ex vivo* models in *in vitro* chondrogenesis models of primary cells such as human chondrocytes. Although, in such a model interaction with the bone compartment is often missing. Even though our aged human biomimetic model closely resembles the human joint, other joint tissues can be added to further complete this model. For that matter, the synovial component could be added by inflammatory stimulation or addition of synovial fluid, synovium explants and/or exosomes from synovial cells. To accommodate throughput as well as multiple tissue interaction, a joint-on-a-chip can be a good alternative model, in which genetic manipulation and co-culture of cartilage and bone is possible. Herein, both cartilage and bone can be cultured separated by a semi-permeable membrane, facilitating crosstalk and allowing for straightforward manipulation. Another advantage of this model is that infinite cell types such as induced pluripotent stem cells (iPSCs) can be used, increasing scalability and reducing heterogeneity of this model. Moreover, such *in vitro* cell models are compatible with genetic engineering technologies such as CRISPR/Cas that allows introduction of additional genetic factors contributing to OA susceptibility.

Relevant triggers for OA like damage in the ex vivo explant model

Inflammation

Inflammation is considered an important trigger to OA related damage and widely applied. Therefore, in **chapter 2** we used the pro-inflammatory IL-1β to induce detrimental catabolic and inflammatory response in human aged articular cartilage. Our observations were in line with many other studies that previously investigated detrimental effects of a similar or higher IL-1β or other inflammatory cytokine stimulation. These studies showed that above a concentration of 10ng/ml, IL-1β consistently increased cartilage degradation, inflammation and MMP13 levels in explants of different species, but lower levels (0.5ng/ml) could not consistently induce cartilage degradation [8-11]. Nonetheless, there are several limitations of this OA model. First, the levels necessary to induce this response are not physiological, in human synovial fluids only very low levels (<1pM) of IL-1 have been measured in some OA patients [12]. Second, this inflammatory response is not typically observed in OA and the inflammatory response is not found as a key pathway in large transcriptomic and genomic studies [13-15]. Evidence from pre-clinical animal studies have determined a conflicting role of IL-1 in OA, with both protection and detrimental effects observed in different species [16-19]. Recently, several large human clinical studies targeting IL-1 have failed to reach the primary endpoint in hand and knee OA, further reducing the fields enthusiasm for IL-1 as target to combat OA [20-23]. Therefore, we advocate that IL-1 and other pro-inflammatory cytokines are not the most promising targets for treatment of OA and focus should shift to other approaches that are preferably based on key pathways involved in OA pathophysiology.

Hypertrophy

Next we investigated the effect on the induction of hypertrophy by perturbation with the active thyroid hormone, triiodothyronine (T3). In this aged human osteochondral explant model we observed mainly upregulation of hypertrophic and mineralization markers such as *COL10A1*, *MMP13, COL1A1* and *ALPL,* similarly to upregulation of these markers in the process of OA [24-28]. In addition, two critical and detrimental transcription factors, *RUNX2* and *EPAS1*, were slightly upregulated after perturbation with T3, indicating that they are possibly downstream of T3. We also measured upregulation of *COL2A1* expression after T3 treatment, possibly as a response to initiate cartilage repair or remodeling. Similar studies into the effects of T4 and/or T3 have measured increased collagen production in the absence of hypertrophic markers [29,30]. Conversely, other studies did observe that T3 induced terminal differentiation by initiating hypertrophic morphology in chondrocytes and expression of molecular hypertrophy markers without proliferation [31-33]. Chen-An et al [34] determined effects of treatment with T3 in (relative young) bovine articular cartilage explants and observed increased expression of hypertrophic markers, such as *ALPL* and *IHH* and increased size of lacunas indicating hypertrophy without affecting cell viability. These difference between the above mentioned studies and what our study found could be due to differences in cell type, model, species or T3 concentrations used. These diverse results are not unexpected as the effects of T3 are known to be time and tissue specific partially due to expression of the different deiodinases (D1, D2 and D3) that control intracellular concentrations [35]. Taken together, our results indicated that treatment of aged human osteochondral explants with T3 induced hypertrophy and that this is not necessarily detrimental to cartilage matrix in our timeframe as cartilage matrix integrity was not affected. Nonetheless, this model can be used to investigate the potential of hypertrophy inhibitors in the treatment of OA.

Mechanical loading

Overloading conditions in a joint are considered a major trigger in the initiation of OA. Nonetheless, little knowledge exists on the chondrocyte signaling response in aged human cartilage triggering OA onset. Therefore in **chapter 2** we demonstrated that mechanical stress at a strain of 65% of cartilage height induced detrimental changes that affected cartilage integrity of aged human osteochondral explants whereas in **chapter 3** we performed genome-wide differentially expressed mRNAs in articular cartilage following repeated exposure to 65% mechanical stress using our human *ex vivo* osteochondral explant model. Our results gave insights into how injurious mechanical strain on the short term affects signalling in aged human articular chondrocytes that could enlighten how these cells lose their maturated state and converse towards the OA disease state. Our data indicated that the short term response to injurious mechanical stress of aged human cartilage involves pathways such as IGF I and II binding, cellular senescence and focal adhesion. In addition, amongst the highly upregulated genes we identified *MMP13* and *IGFBP5* as early markers of injurious stress. Strikingly, both genes are not found to be responsive in OA pathophysiology, i.e. differentially expressed between preserved and lesioned OA cartilage [13], and might therefore reflect the initial unbeneficial response to injurious loading rather than the ongoing OA disease process. Though other collagenases exist and are involved in cartilage degradation, MMP13 has been observed to have the highest affinity for cleaving collagen II and to a lesser extent other collagens, aggrecan, osteonectin and perlecan [36,37]. In addition, knockout of *MMP13* in

mice reduced cartilage damage [38]. Conversely, cartilage specific overexpression of *MMP13* induced OA-related joint pathologies and increased collagen II breakdown [39]. Knockout of other MMPs, such as *MMP3*, actually increased cartilage degradation and catabolic enzyme production instead of protecting the joints [18]. This suggests that MMP3 might play a more essential role in healthy cartilage remodeling while MMP13 is more involved in the (early) pathophysiological OA processes and therefore a good marker of detrimental underlying processes.

Next to providing knowledge to facilitate clinical development of counteracting unbeneficial chondrocyte signalling upon injurious stress, we advocate that a set of the here identified response key genes, such as *MMP13*, *IGFBP4/5* and *TNC*, can be used to distinguish between beneficial and unbeneficial mechanical stress in different age categories. Such experiments could entail submitting explants from different age categories to different strains and velocity of loading, while subsequently measuring upon which type of loading these unbeneficial markers start responding. Given the known age related changes occurring in cartilage [1,40,41] and the fact that immobilisation is unbeneficial for joint health [42], increasing our understanding of which personal circumstances, for example weight, movement speed or age, exercise is preventive or even curative can develop scientifically founded OA therapies in elderly. This need for more knowledge on physical activity and exercise therapy was recently reviewed by Nissen et al [43]. Many clinicians have limited knowledge into exercise and movement as a therapy and they recommend a multitude of exercises based on their "experience of feeling knees", basically based on the presence of effusion and synovitis [43]. In addition, biomarkers could be used in human *in vivo* studies to determine if injurious stress has occurred and thus the long term and almost irreversible degradation of cartilage could be prevented by for example changing to a lighter exercise regime. The most direct biomarkers are those excreted by chondrocytes or other joint tissues into the synovial space. However, insufficient accessibility and invasiveness limits utility for (early) diagnostic biomarker development based on synovial fluid. Blood plasma, serum and urine overcome this obstacle and hold great potential as a biomarker to reflect joint tissue status.

Insight into pathophysiological aspects of the OA risk gene MGP

A reliable human biomimetic model capturing age-related OA development in human articular joints is useful in gathering knowledge on disease mechanisms as well as interactions with OA risk genes. In **chapter 4** we exploited a RNA sequencing dataset of preserved and lesioned articular cartilage and subchondral bone, or 3D *in vitro* cartilage model of primary human chondrocytes as well as our ex vivo explant model with mechanical loading as trigger to induce OA related damage. As such we gather data to strengthen causality of risk SNPs and their effector gene and clarify the direction of effects. For this purpose, *MGP* expression as function of the OA risk allele rs1800801-T was measured. We determined that carriers of the risk allele had inherent lower expression of *MGP* in articular cartilage and subchondral bone and that its expression is upregulated during OA pathophysiology, specifically in carriers of the rs1800801-T risk allele. Our results suggest that to counteract the low inherent MGP levels in OA tissues, chondrocytes increase *MGP* expression, but this is still not enough to reach the same level as those measured in reference allele carriers. In addition, by measuring *MGP* expression as function of rs1800801 in our biomimetic model, we determined that risk allele carriers do not have a dynamic response. This paints a complex picture of action and

response of *MGP* expression that is different in carriers of the OA risk allele rs1800801-T when compared to reference allele carriers, suggesting that besides lifelong lower expression of *MGP* also its inability to change expression in response to a trigger are likely responsible for increased OA risk (**Figure 1**).

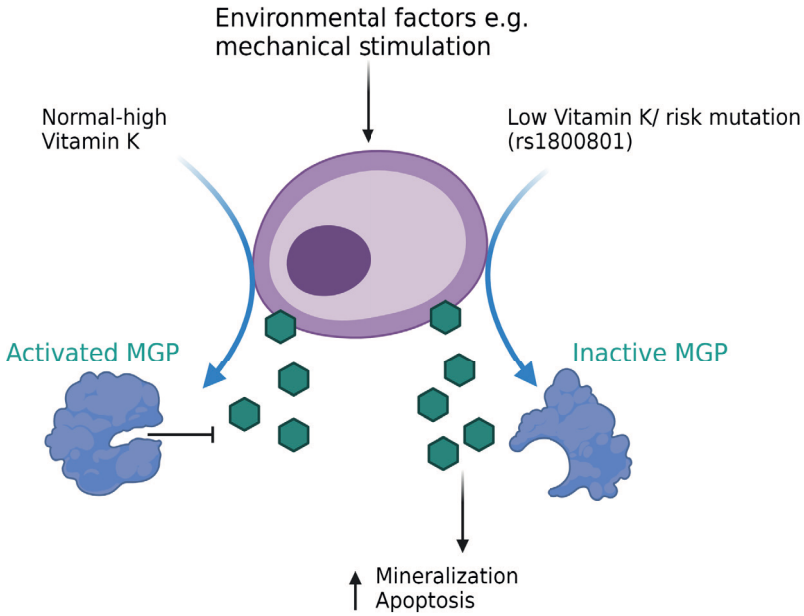

Figure 1 | Suggested mechanism of low vitamin K or risk mutations on decreased activated MGP levels. In response to an external trigger, upon sufficient vitamin K levels, MGP excreted by cells can be carboxylated into its active form and inhibit mineralization. When there are low vitamin K levels or reduced MGP expression, there are insufficient activated MGP levels to completely inhibit mineralization, eventually resulting in apoptosis of cells, induced by an external trigger.

Upon confirming *MGP*, encoding an inhibitor of ectopic calcifications, as strong OA risk gene, it was hypothesized that the OA risk was conferred via calcification of cartilage tissue [10, 11]. Moreover, as MGP protein is activated by vitamin K dependent □-carboxylation (c-MGP) our findings underscored the relevance of previous found associations between OA and low vitamin K status for OA prevention and treatment [44,45]. We demonstrated that exposure of human osteochondral explants to the vitamin K inhibitor warfarin, provoked unbeneficial chondrocyte signaling towards hypertrophy, reduced bone formation and altered bone remodeling together likely resulting in bone loss [46,47]. Future studies should further explore the influence of rs1800801 genotype on the response of cells to warfarin. Two recent studies, one from the Rotterdam study [48] and one case-control study from the UK [49], showed a strong association between vitamin K dependent blood anticoagulant use and incidence and progression of knee and hip OA. In light of our combined result, we advocate that non-vitamin K antagonist should be preferred as anticoagulants to reduce the risk of evoking OA [50], especially in carriers of the rs1800801-T *MGP* risk allele. In addition, to overcome reduced active MGP levels, supplementation with vitamin K could be a potential novel OA-modifying treatment option in an appropriate subset of patients. Until now, only one clinical trial on vitamin K supplementation was performed. Although the study contained a relatively small

number of patients with low vitamin K levels at baseline, beneficial effects on OA progression in individuals were observed in these individuals [51]. Another reasons to consider vitamin K supplementation as OA treatment, is the correlation between low dietary vitamin K and increased progression of knee OA [52] and the increased uncarboxylated MGP and GRP proteins levels in OA cartilage [53,54]. Altogether and given the evidence from genetic risk genes and clinical patient data, there is a strong rationale to undertake further clinical trials to address treatment of vitamin K supplementation in a subgroup of OA patients with low vitamin K levels and/or carriers of the rs1800801-T OA risk allele.

Opportunities to address effects of OA risk genes with the ex vivo osteochondral model

To explore the direction of effect of high potential risk SNPs such as rs34195470 (*WWP2*), or rs4252548 (*IL11*) in OA, a similar strategy as performed in **chapter 4** could be undertaken. Expression of its effector gene as function of its OA risk allele in articular cartilage, subchondral bone and our human biomimetic models can help determine their therapeutic potential. Exploration of risk SNP's like rs34195470 (associated with effector gene *WWP2* involved in skeletal development) can be performed. Styrkarsdottir et al. [55] reported on reduced *WWP2* expression in carriers of the risk allele of a proxy SNP rs4985453 (R^2=0.79) in adipose tissue. Conversely in GTEx[56], rs34195470-G was associated to increased *WWP2* expression in arteries while another proxy SNP (rs1052429 (R^2=0.77)) increased *WWP2* expression in four different tissues. This underscores that further research is necessary, preferably in OA relevant tissues, to investigate expression levels of OA risk genes and alleles prior to determining direction of effects.

Another example of an interesting gene which can be further validated in our biomimetic model is *IL11*. Multiple independent GWAS studies have found the risk allele rs4252548-T [15,55,57] in *IL11* and it was one of the highest upregulated genes in both articular cartilage [13] and subchondral bone [7] with OA pathophysiology. The IL-11 protein has a well-established role in osteoclast development and bone turnover and mice lacking this protein had impaired bone formation [58]. One study showed that rs4252548-T reduced IL-11 stability, however changes implied by this low frequency SNP on gene expression level have not been identified [59]. Since a large response is observed in both cartilage and subchondral bone and it is likely a genetic risk factor for OA, modulating IL-11 levels is a promising therapeutical target in OA. More investigation on the effects of decreased and increased IL11 levels could be performed in our relevant human aged biomimetic model to unravel its role in OA and the implications of rs4252548 in both tissues.

Proof of concept pre-clinical study to explore effects of IOP as chondroprotective agent against mechanical induced OA related damage

IOP, a chondroprotective pharmacological agent

In **chapter 5**, a drug based clinical target in an OA relevant model was investigated to determine if this could be an effective treatment strategy. For the OA risk gene *DIO2* [60], encoding for the type II iodothyronine deiodinase D2 enzyme, previous *in vitro* and *in vivo* research

demonstrated causality in OA and beneficial effects were observed in the absence of *Dio2* or with *DIO2* inhibition using iopanoic acid (IOP) [61,62]. However, evidence of efficacy of IOP in a relevant human aged model subjected to a relevant trigger was essential to complement the line of evidence. The *ex vivo* aged human osteochondral explant model was chosen for this purpose because they retain aged chondrocytes in their native extracellular matrix, they are derived from human tissue, and they can be subjected to OA relevant perturbations such as mechanical stress. Our results show that the deiodinase inhibitor IOP reduced mechanical induced detrimental chondrocyte signaling, likely by reducing metabolic activity of cells, thereby confirming potential for treatment with IOP. However to advance IOP towards clinical trials, appropriate *in vivo* mice studies are deemed necessary as evidence. If the research field wants to increase complying to the 3Rs (replacement, reduction and refinement) of laboratory animals, an option could be to perform efficacy studies first in relevant *in vitro* human models prior to human clinical studies.

The road from translation to clinical application

The European Medicines Agency (EMA) states that a variety of toxicity and safety testing of a drug *in vivo* animal studies are required prior to the initiation of human clinical trials. Therefore, animal and human clinical trials are often performed concurrently, which gives the impression that many clinical trials are not based on efficacy animal study results and also suggests that animal studies do not give required information and are even often ignored [63-65]. For example, only recently a study reported that the anti-ADAMTS5 drug GLPG1972 reduced cartilage damage and bone sclerosis in mice and rat OA models [66], while recruitment for a clinical phase II trial on efficacy and safety already started in 2018 and finished in 2020 [67]. The additional rationale and benefits of this animal study are unclear and unfortunately several additional examples of studies simultaneously performed in animals and humans exist [63-65]. In addition, translational success rates from animal-to-human range from 0 to 100%, suggesting that success is partially unpredictable and these could not be explained by species, study size, field or year of publication [68]. However, as unfortunately a lot of animal studies are still not of sufficient quality, partially due to study design, and animal to human translation is unpredictable, pre-clinical animal studies are unable to completely predict safety and efficacy in humans. This erratic safety and efficacy translation is still the main reason why many phase I-III trials fail [69-71]. Another review compared methodology of trials in animal and human studies of methotrexate, a rheumatoid arthritis drug, and found large differences dependent on sex and how power calculations and statistics were performed [72]. This misalignment of designs is problematic as it decreases the animal to human translation and validity. Therefore, to improve translatable conclusions, more often relevant *in vitro* models should be taken into consideration for drug efficacy testing. In addition, with the increasing computational modeling power, more reliable predictions can be made with respect to expected toxicity of drugs *in vivo* [73]. Both *in vitro* and *in silico* models can further reduce the need for extensive *in vivo* animal studies.

The potential of OA therapies based on genetics underlying development of OA pathophysiology would be an important improvement in the treatment of OA. Many of the current clinical trials are hypothesis driven, however similar to candidate gene studies such hypothesis driven targets may not cover risk factors that have the highest impact on development or progression of OA. Two examples of recently failed clinical trials based on hypothesis driven targets are

the ADAMTS5-inhibitor GLPG1972 and the IL-1 targeting Anakinra and Lutikizumab [20-23]. In both cases, clinical trials were based mainly on hypothesis driven OA pathways, but the selected targets are not present as OA risk alleles in large GWAS studies. Neither could be substantiated that they have a large impact on OA pathophysiology, i.e. these genes are not among those highly differentially expressed between healthy, preserved and lesioned OA cartilage or subchondral bone [7,13]. In retrospect, AstraZeneca published a report that variables such as genetically supported data or a stronger evidence of the target in the disease etiology contribute to success rate of clinical trials and increase efficacy [69]. In addition, they concluded that targeting molecules with a genetic link to the disease increased successfulness of projects in Phase II to 70%. These findings are supported by other studies showing a doubling in clinical success rate when genetically supported drug targets are chosen [74,75].

Opportunities for (pre-)clinical studies based on OA risk SNPs and their direction of effect

Recently, a concise list of potential OA therapeutical drugs targeting established risk genes was published in Cell [15]. For a portion of these drugs experimental research in OA has been performed while others are investigated in other diseases. Some notable examples of genes on the list that have experimentally been investigated in relation to OA are: vitamin D receptor (*VDR*), insulin-like growth factor 1 receptor (*IGF1R*) and carbohydrate sulfotransferase 3 (*CHST3*). Supplement with vitamin D has given mixed results on OA symptoms and progression, but suggests a small benefit in individuals with insufficient vitamin D levels [76-80]. Another example is Mecasermin, an agonist of the OA risk gene *IGF1R*, reducing apoptosis and increasing matrix production of OA chondrocytes [81,82]. Finally, thalidomide, an agonist of *CHST3,* was effective in early OA development in a DMM mouse model likely via a *VEGF* dependent mechanism [83]. Interestingly, this list also mentions the widely used hyperthyroidism anti-thyroid agent carbimazole, to target thyroid peroxidase (*TPO*), as potential drug. Its active component, methimazole, is a competitive inhibitor of TPO, preventing iodination of thyroglobulin and thereby thyroid hormone (T4 and T3) production. Though its mode of action is different from the in **Chapter 5** used pharmacological anti-thyroid agent IOP, it supports the potential of anti-thyroid hormone drugs in the treatment of OA.

Information often omitted in describing OA risk SNPs and their effector genes is the expected direction of effect on the gene expression, while knowledge on this is important when identifying therapeutical targets. Boer et al [15] recently reported a list of genes identified in a large GWAS of over 800,000 subjects with different OA phenotypes. For some genes on this list the direction of effect has been determined previously by expression quantitative trait loci (eQTL) or allelic expression imbalance (AEI) analyses and is also available in a large eQTL database GTEx, though this database lacks joint tissues [56]. In **Table 1**, direction of effects based on previous research or online databases has been added to the list OA risk genes.

Table 1 | Leading risk SNPS with their effector gene adapted from Boer et al [15].

Lead OA SNP	EA	EAF	eQTL dir GTEx (dn/up)	eQTL (GTEx/OA tissue)	eQTL/AI OA tissues	OA pathophysiology Cartilage		Bone	Expected dir effect
						Gene	*Protein*	*Gene*	
rs3740129	A	0.46	*CHST3* (2/6)	*CHST3* (8/1)	AC(-)	*CHST3*(+)			*CHST3* (-)
rs12908498	C	0.54	*SMAD3* (1/1)	*SMAD3* (2/0)	AC(0), FP(0), SY(0)	*SMAD3*(-)			Unclear
rs143384	A	0.59	*GDF5* (10/1)	*GDF5* (11/2)	AC(-),SY(-)	*GDF5*(+)			*GDF5*(-)
rs67924081	A	0.74	*LTBP3* (0/8)	*LTBP3* (8/0)			LTBP3(+)		*LTBP3*(+)
rs7294636	A	0.37	*MGP* (5/0)	*MGP* (5/4)	AC(-), SB(-), FP(-), SY(-)	*MGP*(+)		*MGP*(+)	*MGP*(-)
rs1530586	T	0.8	*FGFR3* (16/0)	*FGFR3* (16/0)		*FGFR3*(-)			*FGFR3*(-)
rs17615906	T	0.84	*FBN2* (5/1)	*FBN2* (6/0)				*FBN2*(+)	*FBN2* (-)
rs62578126	T	0.37	*LMX1B* (5/0)	*LMX1B* (5/0)					*LMX1B*(-)
rs1149620	A	0.44	*TSKU* (9/0)	*TSKU* (9/0)		*TSKU*(-)			*TSKU*(-)
rs7967762	T	0.16	*COL2A1* (2/0)	*COL2A1* (2/1)	AC(-)	*COL2A1*(-)			*COL2A1*(-)
rs7967762	T	0.16	*PFKM* (0/5)	*PFKM* (5/0)			PFKM(-)		*PFKM*(+)

The column 'eQTL dir GTEx(up/dn)' describes in how many tissues and in which direction the lead OA SNP is associated with gene expression in GTEx. The column 'eQTL (GTEx/OA tissue)' describes the number of different tissues in GTEx and OA relevant tissues with eQTL findings for this SNP. The column 'eQTL/AI OA tissues' describes associations between SNP and eQTL and/or AI in OA relevant joint tissues. The column 'OA pathophysiology' describes if a changed gene or protein expression was found between preserved vs lesioned OA cartilage or bone. In columns 'eQTL OA tissues', 'cartilage', 'bone' and 'expected direction of effect': – represents the SNP associates with a downregulation of expression of corresponding gene, + represents the SNP associates with an upregulation of expression of corresponding gene, 0 represents that the SNP was associated with no change of expression of corresponding gene. Legend: Lead OA SNP, rs number of the lead variant; EA, effect allele; EAF, effect allele frequency; eQTL, expression quantitative trait loci; dir, direction; GTEx, The Genotype-Tissue Expression project; OA, osteoarthritis; AC, articular cartilage; SB, subchondral bone; FP, Fat pad; SY, synovium.

Future perspectives in OA research

To further improve OA research the work performed in this thesis is to emphasize that there is a need for good biomimetic models of OA in the aged population. The latter should be a prerequisite for showing treatment modalities and can give a solid genetic basis for druggable targets. The use of pharmacological agents based on genetic risk genes or pathways in combination with testing of these drugs in appropriate relevant disease models will greatly benefit clinical drug development in OA. Another important factor to be taken into account in clinical trials of OA targets is patient selection. Due to the heterogeneity of the disease it is extremely unlikely that there will be a 'one drug fits all' treatment option.

In 2011, Freedman et al [84] proposed a good systematic strategy to follow up on risk loci, however there is more to it. For future purpose we propose to follow the path that could be

taken for a druggable target stemming from genetic risk alleles in **Figure 2**. A first early step when the causal risk SNP has been identified is to investigate if it affects expression of a nearby gene. This can be done *in silico* by looking up the risk SNP and/or its proxy SNPs in an online database, such as GTEx[56], or in published papers describing OA relevant tissues, such as articular cartilage [85]. If an effect of the SNP on gene expression is identified, additional *in silico* databases (UCSC Genome Browser, HaploReg, ENCODE, etc.) can be explored to identify if the risk SNP is in a regulatory region such as a promotor or enhancer. Another factor that increases rational of a risk SNP is if the associated gene is involved in OA pathophysiology, i.e. differentially expressed between healthy, preserved and/or lesioned OA relevant tissues. A final *in silico* step that strengthens causality is if the gene has been linked to a disorder or a musculoskeletal phenotype in humans or mice databases (OMIM, Mouse Genome Informatics (MGI), Knockout mouse project (KOMP), etc.).

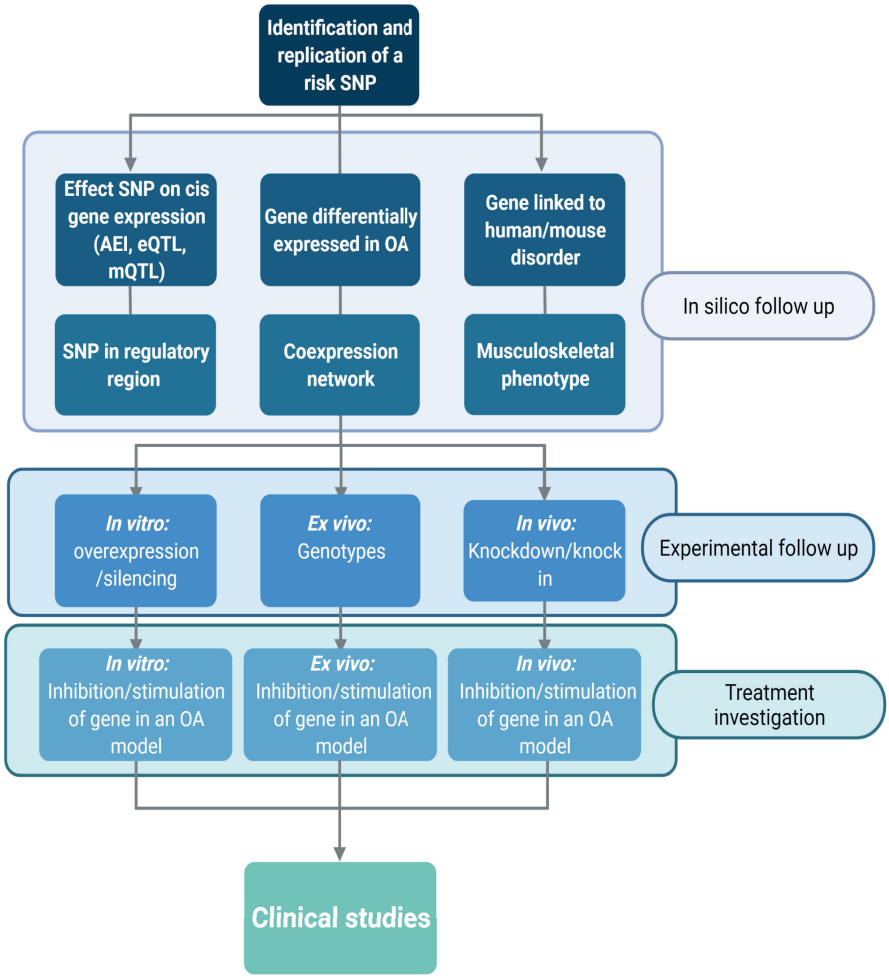

Figure 2 | Proposed example of an investigation scheme to go from identification of a risk SNP to treatment.

When involvement of a gene is shown *in silico*, follow up experiments can consist of either *in vitro* silencing/overexpression in human aged relevant cells, stratified *ex vivo* human tissues based on genotypes or *in vivo* knockdown/knock-in in animals. This can increase causality of the risk SNP and/or gene and gives a rational to start investigating it as a possible treatment. To investigate toxicity and efficacy of treatments, inhibition or stimulation of a gene or its pathway in *in vivo* OA animal models can be performed. In addition, to improve translation to human, efficacy of this treatment should also be proven in a relevant human aged *in vitro* and/or *ex vivo* OA model. Hopefully by using such a strategy, the OA field can reduce the number of animal studies and failing clinical trials that are based on ineffective targets.

The aged human biomimetic model described in this thesis mimics the human joint quite well, however addition of other joint components, such as the synovium, could further add to its completion. In addition, other *in vitro* models such as the joint-on-a-chip are taking of in the OA field. These models can simulate the joint environment plus enable genetic manipulation, are more scalable and have reduced heterogeneity. As mentioned in this thesis, the biomimetic human OA models can be exploited for several applications. One of them is the development of biomarkers for what is “healthy physical activity” in elderly, as current guidelines are not based on empirical data, while “healthy physical activity” could be of great benefit for managing healthy joints in OA patients. Another example our model can be used for is to (further) clarify or confirm the direction of effect of other OA risk SNPs (**Table 1**).

With the increase of reliable models for OA it is important that the legislation on when human clinical trials are approved should be revisited. With the high number of clinical trials failing, we should reconsider giving less credit to results from *in vivo* (small) animal studies. With this thesis we show an alternative to animal models and demonstrate different applications of this biomimetic *ex vivo* human aged osteochondral model. Our research group has the benefit of access to end-stage osteoarthritic human joints coming from joint replacement surgeries in the Research in Articular Osteoarthritis Cartilage (RAAK) biobank [86]. However, when this is not possible, other human models such as iPSCs and joint-on-a-chip have further increased research possibilities in the OA field in recent years. Finally, an important side note for clinical application of many drugs is the need to further develop safe and effective methods for local intra-articular delivery in patients, thereby reducing the need for repeated injections and hopefully increasing drug retention and efficacy.

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PEBP1
MBIP
KAT2B
CKB
RCC2
TET1
TYRO3
SOCS5
LPIN1
CDKN1C
RCAN2
KLF2
GADD45
SOCS3
HTRA1
IGFBP4
CD9
CDA
SES
IGFBP6
MYC
STC2
FOXO1
GAPDH
ENG
IGFBP7
IGFBP5
SERPINE1
AK1
PRKAG2
ADAM12
NFKBIA
CAV1
PDK4
CYR61
EHD3
MMP13
FKBP5
FRZB
CMBL
PTGS
TSC22D3
WARS
MXRA5
AMFR
PSAT1
LRRC16A
EDEM1
GSN
SCUBE1
OS9
ALDH1L1
IARS
TNC
ITM2A
SLC25A5
ITGA10
RPL10A
TLN2
RPS12
RGCC
RIOK3
DPP2
NEK6
DNHD1
CNTFR
CRLF2