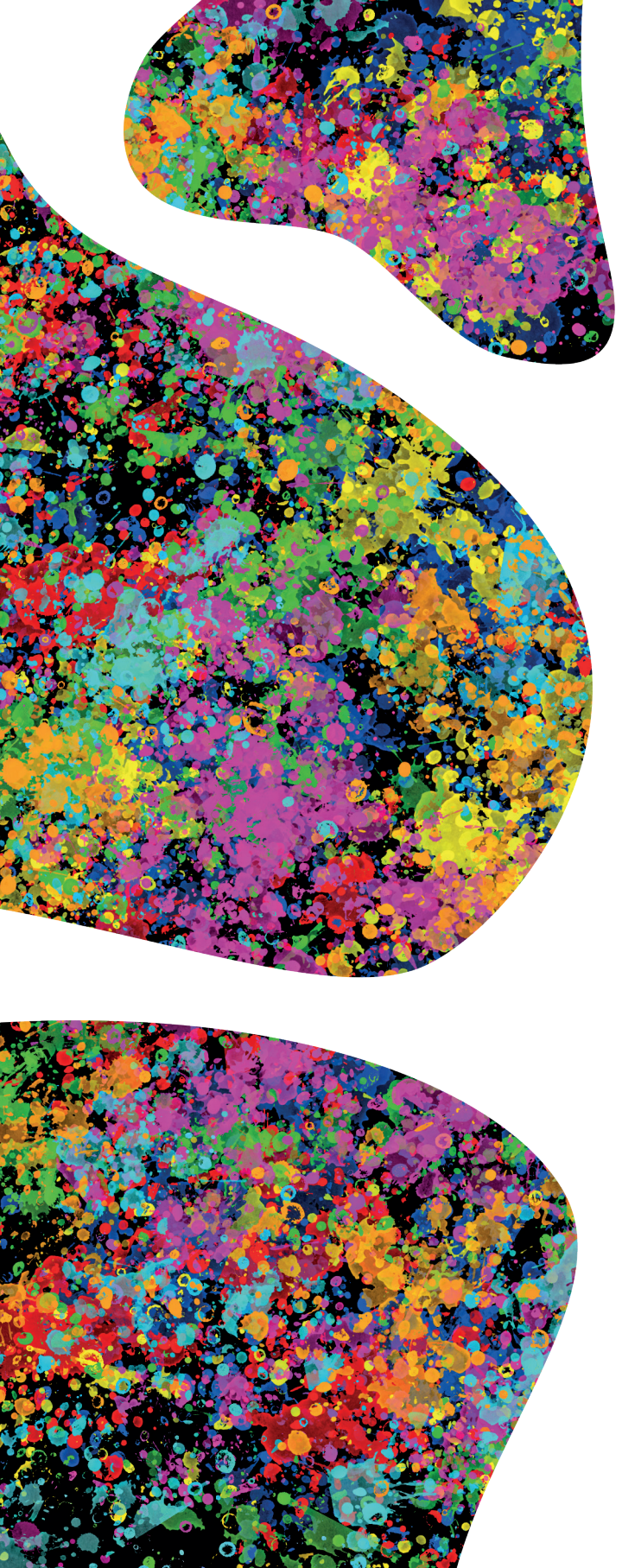
The bone and cartilage interplay in osteoarthritis: key to effective treatment strategy

Tuerlings, M.



CHAPTER 9

General Discussion and future perspectives

Summary

Osteoarthritis (OA) is a prevalent disabling age-related disease with multiple tissues involved. Due to a major backlog in development of disease modifying OA drugs (DMOADs), available treatment options are restricted to pain relief and costly total joint replacement (TJR) surgery at end-stage disease. Despite the societal burden of these large number of TJR surgeries, it provides the OA research society with an invaluable and continues supply of OA disease relevant tissues, such as articular cartilage, subchondral bone, synovium and synovial fluid. In the current thesis the joint tissues collected within the RAAK study were fully exploited to gain insight into the biological mechanisms and the diversity in pathophysiological processes in bone and cartilage [1-3]. Henceforth, three main intertwined strategies were applied 1) study of ongoing OA pathophysiology by molecular characterization of bone and cartilage in interaction, 2) identify non-invasive molecular biomarkers in the circulation that report on these pathophysiological processes, and 3) apply and advance functional genomic studies and *in vitro* disease modelling to study downstream actions of compelling OA risk variants and respective genes on joint tissue homeostasis and chondrocyte function.

Osteoarthritis molecular pathophysiology

The study of ongoing OA pathophysiological processes, thus far, focussed primarily on molecular analyses of articular cartilage, as exemplified by multiple differential -omic studies to date published on healthy, macroscopically preserved and lesioned articular cartilage of an OA joint (**Figure 1**). These studies provided valuable insight in the molecular OA landscapes e.g. mRNA, microRNAs (miRNAs), long non-coding RNAs (lncRNAs) of cartilage as well as the pathways in which these molecules act [3-6]. Nonetheless, insight in the molecular landscape of subchondral bone remained elusive. This despite the fact that multiple OA risk genes identified in genome wide association studies (GWAS) are involved in maintenance processes in both cartilage and bone, hence indicating that unfavorable processes in both tissues could drive OA onset and progression [7-10]. To fill this gap of knowledge, in **chapter 2** and **chapter 3** we applied RNA-sequencing (RNA-seq) of OA subchondral bone to identify mRNA and lncRNAs that mark OA pathophysiology herein. To study the interaction and overlap with OA pathophysiology in articular cartilage, previously assessed transcriptome wide data of matching cartilage [4] was introduced in these analyses. Moreover, the previously identified OA molecular endotypes in cartilage (**Figure 1**) [11-13] were further characterized in subchondral bone in **chapter 5**. Besides the heterogeneity between patients also heterogeneity between joint site is observed (**Figure 1**), which is also further discussed in this thesis (**chapter 2** and **chapter 3**).

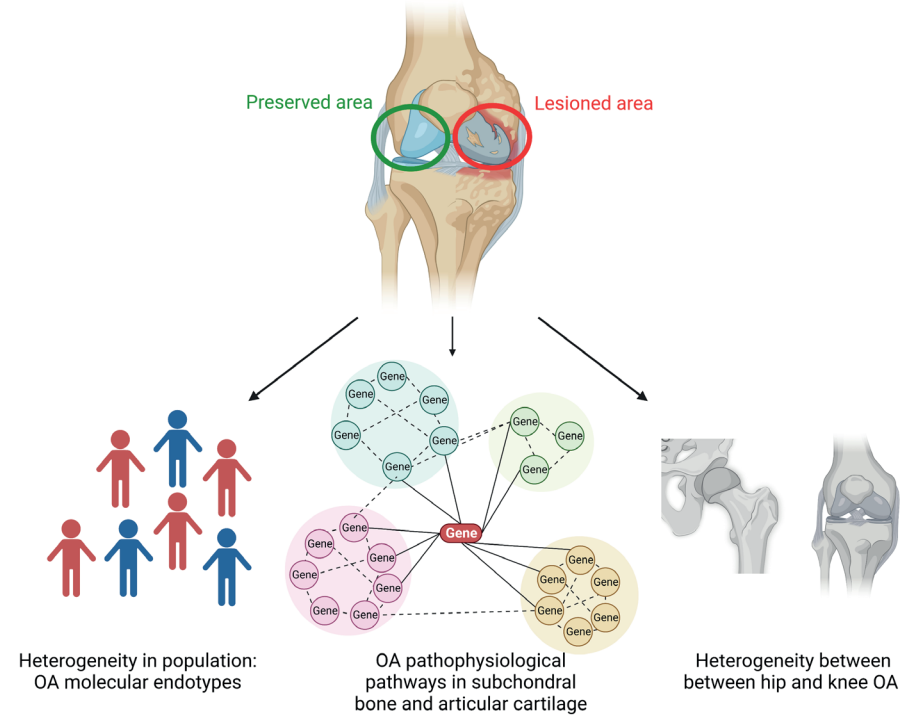


Figure 1 – Transcriptomic data analysis to characterize OA pathophysiology.
Transcriptomic data can be used to characterize pathophysiological pathways. In addition, transcriptomic data can be used to investigate OA heterogeneity either between patients or between joint site. (created with Biorender.com)

Non-invasive biomarkers for osteoarthritis

Thus far, classification and/or diagnosis of OA is solely based on radiography and clinical symptoms, such as pain and stiffness. This indicates that there is an unmet need for reliable biomarkers that reflect ongoing processes in joint tissues in general [14]. Most studied biomarkers for OA are biochemical markers in serum or urine, such as sCOMP and uCTX-II, which are often degradation products of joint tissues [15-17]. Nonetheless, only a few of these biochemical markers are tested for clinical use, as these markers often are a-specific with regard to OA pathophysiological tissue progression, they do not mark early OA, and they are not highly predictive [18-20]. For that matter, studies implicate circulating microRNAs as novel promising biomarkers, as they are stable in plasma and serum and could report on (patho-)physiological processes [21-23]. Recently, we showed for the first time that miRNAs in plasma were indeed able to reflect early OA related mRNA expression patterns in articular cartilage [24]. As proof-of-principle, in this thesis, we exploited plasma miRNA dataset for identification of miRNAs that could serve as biomarkers for classification of patients based on their

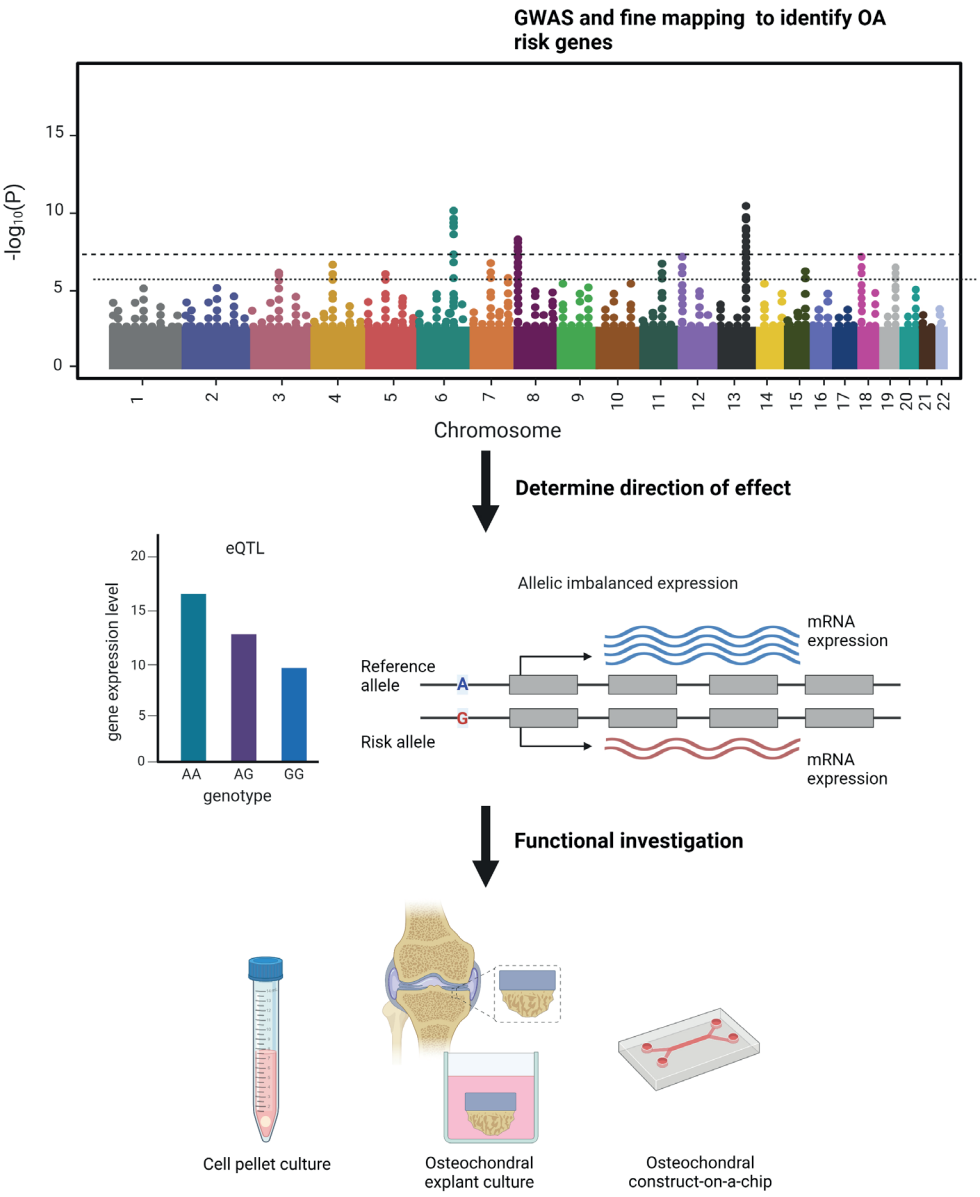


Figure 2 – Concept workflow for translation of OA risk genes towards development of DMOADs. OA susceptibility genes are identified by performing genome-wide association studies and fine mapping. Subsequently, the direction of effect should be determined by either eQTL or allelic imbalanced expression analysis. Finally, the hypothesis on the direction of effect needs to be tested in an in vitro or ex vivo disease model system. (Created with Biorender.com)

previously identified OA molecular endotypes of preserved articular cartilage (**chapter 4**).

Genetic predisposition

Genetic predisposition is found to be a strong risk factor in many age-related chronic diseases [25], including OA [26]. The function of genes conferring this risk, that are identified by genome wide association studies (GWAs), provide valuable information on the biological pathways involved in disease aetiology [27]. For that matter, multiple comprehensive GWAS studies for OA have been performed with over 100 robust association signals [28-30]. The function of identified OA genes highlighted that OA aetiology is driven by dysfunctional maintenance processes in cartilage and bone. Nevertheless, strikingly little progress has been made in translating OA risk SNPs to underlying biological mechanisms, drug targets, and development of DMOADs [31]. In this thesis we, therefore, applied a functional genomic approach (**Figure 2**) to study two compelling OA risk alleles. Rs1052429-A, located in the 3'UTR of *WWP2* gene, was previously shown to be associated with increased expression of *WWP2* [32, 33] and rs4252548-T, located in *IL11* gene, that was shown to decrease stability of IL11. Hereto, we studied the downstream effects of *WWP2* upregulation in 3D chondrocyte pellet cultures in **chapter 6** and studied whether hrIL11 protein addition could rescue the OA state in osteochondral explant cultures in **chapter 7**. Finally, in **chapter 8** we developed a novel biomimetic *in vitro* model system representing functional articular cartilage and subchondral bone in interaction to study OA-related perturbations and/or OA susceptibility genes.

Together in this thesis, we tried to make a step forward in transition from bench-to-bedside in OA by combining previously reported GWAS and allelic imbalance results, with molecular profiling of subchondral bone and articular cartilage and functional investigation of OA risk genes (**Figure 2**).

Molecular characterization of subchondral bone osteoarthritis pathophysiology in comparison to articular cartilage

To gain insight in OA pathophysiology of subchondral bone, in interaction with articular cartilage, in this thesis we have compared gene and lncRNA expression levels between macroscopically preserved and lesioned OA subchondral bone. Subsequently, we have compared these results with previously reported results on (differentially) expressed genes and lncRNAs in OA articular cartilage [4, 6]. Moreover, we have integrated these results with genetic findings [30] and allelic imbalanced expression [32, 33].

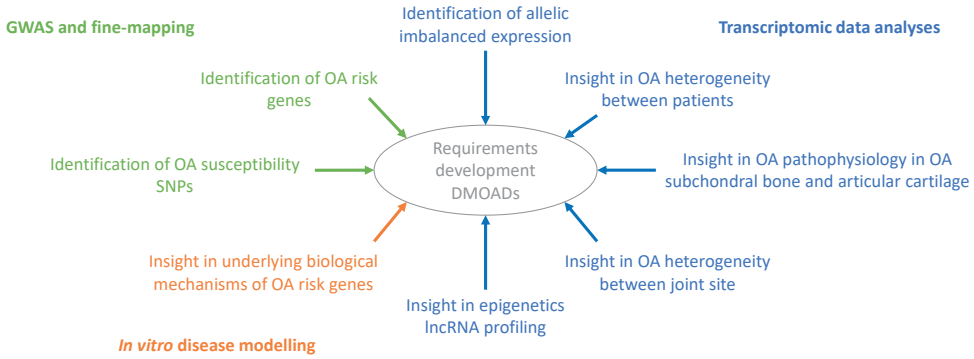


Figure 3 – overview of different requirements in the development of DMOADs.
Development of DMOADs requires combining GWAS, transcriptomic data analyses, and in vitro or ex vivo disease modeling.

In development of DMOADs (**Figure 3**) targeting genes or pathways that are active with OA pathophysiology in both articular cartilage and subchondral bone could be an attractive strategy, as targeting these genes or pathways would have therapeutic effects in these two most important joint tissues affected by OA. Since using transcriptomic data to identify genes and/or pathways based on differential expression analysis with ongoing OA per definition does not provide insight in cause or consequence, integration of these data with genetic studies is required to identify common genes in articular cartilage and subchondral bone that are most likely causal to OA. In this respect and given that drug targets founded by genetic evidence have at least two times higher success rates [34, 35], in **chapter 2** we have searched for genes that were responsive to OA pathophysiology in both articular cartilage and subchondral bone with similar directions of effects and filtered these genes for OA risk genes identified in previously reported comprehensive GWAS [30, 36]. In doing so, we have identified 305 genes marking the OA pathophysiological process in both articular cartilage and subchondral bone, of which *IL11* and *CHADL* were previously identified as OA risk gene. To make firm hypothesis on the direction of effect of *IL11* and *CHADL,* we have explored previous findings on these genes. Given that the *IL11* risk variant is a missense variant resulting in a thermally unstable protein [37], we and others hypothesized that decreased expression of IL11 confers risk to OA. As such, increasing IL11 protein levels was previously proposed as OA treatment strategy [36]. Remarkable is that *IL11* gene expression levels are highly upregulated in lesioned compared to preserved OA articular cartilage and subchondral bone, reflecting that there is not necessarily a lack of potency to produce IL11 or signal via IL11, unless translation of the protein or binding of IL11 to its receptors is hampered. Founded by expression quantitative trait loci (eQTL) in adipose and skeletal muscle tissue, the OA risk variant located in an intron of *CHADL* acts

via increased expression of *CHADL* [38]. It has previously been shown that recombinant CHADL binds to collagen and inhibits collage fibril formation *in vitro* [39]. Moreover, *CHADL* knockdown in a chondrogenic mice cell line was stated to increase chondrogenic differentiation as shown by increased deposition of *COL2* and *ACAN* [40]. Based on these findings, we hypothesize that increased levels of *CHADL* have negative effects in both articular cartilage and subchondral bone, by decreasing the extracellular matrix (ECM) stability in both tissues and thereby predisposing to OA. Hence, we propose inhibition of *CHADL* as a potential therapeutic strategy for OA. Thus far, no approved drugs are available to inhibit *CHADL* [41] and further functional investigation is required to better understand the mode-of-action of *CHADL* in predisposing to OA.

More recent, the largest GWAS meta-analysis so far was reported, identifying 100 independent SNPs being associated with OA [28]. Upon intersecting the nearest genes of these 100 SNPs with the 305 genes showing similar directions of effect in articular cartilage and subchondral bone, we additionally found *GLIS3, DGKI,* and *SLC44A2* as potential druggable targets (**Table 1**). The functions of *GLIS3, DGKI*, and *SLC44A2* in articular cartilage and/or subchondral bone are still unknown. Nonetheless, risk allele rs10405617-A, located in an intron of *SLC44A2,* marks higher expression of *SLC44A2* compared to rs10405617-G in skeletal muscle tissue according to GTEx project [38], suggesting that increased *SLC44A2* expression confers risk to OA. Functional *in vitro* or *ex vivo* studies on these potential therapeutic targets are still necessary to understand their mode-of-action and confirm their effects on articular cartilage and subchondral bone causing predisposal to OA.

LncRNAs are relatively new molecules being investigated in the OA field as they are poorly evolutionarily conserved and are generally less abundantly expressed, making them more difficult to study compared to for example mRNA or miRNAs [42]. Nonetheless, lncRNAs could serve as attractive potential druggable targets since they are known to be highly tissue specific and have regulatory roles in various transcriptional and (post-)translational processes [43, 44]. Given these unique features, targeting lncRNAs as therapeutic strategy for OA might be superior to targeting proteins in terms of potential unbeneficial side-effects associated with their targeting [45]. Henceforth, in **chapter 3** we have characterized lncRNAs that are (differentially) expressed in OA subchondral bone in comparison to OA articular cartilage to identify potential therapeutic targets. We found a relatively large number of lncRNAs (N=1090 lncRNAs) that were robustly expressed in both articular cartilage and subchondral bone. This large overlap in expressed lncRNAs might be due to the common mesodermal origin of these tissues. Among differentially expressed lncRNAs between preserved and lesioned OA tissue we have identified five lncRNAs overlapping between articular cartilage

and subchondral bone, including *AC005165.1* being downregulated in both lesioned tissues. In subchondral bone *AC005165.1* was even identified as the most significantly downregulated lncRNA. To explore which genes or pathways are targeted by *AC005165.1* in subchondral bone, we have performed spearman correlations between *AC005165.1* expression levels and all differentially expressed protein-coding genes in subchondral bone. The highest (positive) correlation was found between *AC005165.1* and *FRZB* and this potential interaction was confirmed by downregulation of *AC005165.1* in primary osteogenic cells using LNA GAPmeRs resulting in consistent downregulation of *FRZB*. Interestingly, *FRZB* is a well-known OA gene and it is known to inhibit canonical WNT-signaling [46]. Moreover, decreased expression of *FRZB* was previously shown to confer risk to OA [47-49]. These data, together with the fact that *AC005165.1* and *FRZB* are both significantly downregulated with OA in both articular cartilage and subchondral bone, suggest that dysregulated *AC005165.1* directly or indirectly causes dysregulation of *FRZB*. Therefore, upregulation of *AC005165.1* might be an attractive therapeutic strategy to maintain *FRZB* levels in both tissues. Nonetheless, the mode-of-action of how *AC005165.1* affects *FRZB* gene expression remains still unknown and additional functional investigation in both articular cartilage and subchondral bone is required. A first follow-up experiment could be generating a lentiviral mediated upregulation of *AC005165.1* in an advanced *in vitro* OA model representing cartilage and bone and perform RNA-seq to identify all direct and indirect targets of *AC005165.1* in both tissues. Compared to 2387 differentially expressed genes previously identified between preserved and lesioned articular cartilage [4], we have identified only 1569 genes differentially expressed in subchondral bone (**chapter 2**). Similar results were seen for differentially expressed lncRNAs, as we previously have identified 191 differentially expressed lncRNAs between preserved and lesioned articular cartilage [6] while only 21 lncRNAs have been identified in subchondral bone (**chapter 3**). These differences in number of differentially expressed genes and lncRNAs might reflect the fact that bone as multicellular tissue is more heterogeneous in terms of expression levels, while articular cartilage only resides one cell-type. The advantage of using bulk RNA-seq data of subchondral bone was that we were able to almost directly compare our findings with previous findings on articular cartilage. However, a disadvantage was that the identified genes and lncRNAs represent overall average expression changes, confounded by variation in cell type proportions and ignoring cell-specific changes present in subchondral bone. To overcome this issue of multicellular tissues, cellular deconvolution methods are computational methods that can be applied to correct for cell type proportions present in bulk data [50]. However, these methods require sensitive markers for each cell type expected to be present in the bulk data, which were not yet available for subchondral bone. Moreover, these cellular deconvolution methods are affected by normalization and transformation of the data. Alternative and more

Table 1 – OA risk genes identified in large GWAS that are differentially expressed between preserved and lesioned OA subchondral bone and articular cartilage, showing similar directions of effect.

			Subchondral bone		Articular cartilage		
SNP	**Nearest gene**	**Suggested gene expression risk**	**FC gene**	**FDR gene**	**FC gene**	**FDR gene**	**GWAS reference**
rs4252548	IL11	decreased expression	4,16	2,44E-03	22,80	1,53E-20	[28, 30, 36]
rs117018441	CHADL	increased expression	0,63	2,33E-02	0,63	1,29E-02	[30, 40]
rs10974438	GLIS3	Unknown	1,45	5,95E-03	1,88	1,02E-05	[28]
rs571734653	DGKI	Unknown	1,44	1,50E-02	2,07	5,49E-05	[28]
rs10405617	SLC44A2	increased expression	0,85	1,91E-02	0,80	2,85E-02	[28]

advanced methods to take into account multiple cell types present in bone are single-cell RNA-seq (scRNA-seq) or spatial transcriptomics [51, 52]. Next to providing valuable insight in cell types present subchondral bone or OA-disease state, scRNA-seq could also provide insight into cell-type specific alterations between preserved and lesioned tissue [53]. In spatial transcriptomics, this single-cell level expression pattern is linked to cellular location within the tissue [54]. Both methods could provide additional insight in OA pathogenesis and should be implemented in future studies.

Heterogeneity in osteoarthritis: molecular endotypes and non-invasive biomarkers

Failure of DMOADs development is partly caused by the fact that it has followed a "one-drug-fits-all-patients" approach, in which heterogeneity, such as OA molecular endotypes, is ignored [17, 55]. Previous studies have identified two consistent and robust OA endotypes based on unique molecular landscapes of OA preserved articular cartilage [12, 13]. Molecular endotype A and B described a hypertrophy-driven and inflammatory-driven OA pathophysiological process, respectively. Moreover, endotype B OA patients showed significantly more joint space narrowing compared to endotype A. Given this intrinsic difference in OA pathophysiology between endotype A and B patients, we evaluated whether the genes we put forward as potential druggable targets in this thesis are specific to one of the endotypes. As shown in **Table 2**, *CHADL, GLIS3, DGKI, SLC44A2, MAP2K6* and *WWP2* expression levels were only responsive to OA pathophysiology in endotype A patients, suggesting these targets may be particularly or only of interest for treatment of endotype A relative to endotype B patients. Vice versa, *HLA-DPA1* expression levels were only responsive to OA pathophysiology in endotype B patients, suggesting targeting *HLA-DPA1* may be particularly of interest for treatment of endotype B relative to A patients. These data indicate that different OA therapeutic strategies between these patients are necessary and endotype-based stratification of patients before starting clinical trials, for example, could potentially result in higher success rates [17, 55]. Nonetheless, since these potential druggable targets play a role in both articular cartilage and subchondral bone, knowledge on whether these OA molecular endotypes exist in subchondral bone is required to enable treatment of the osteochondral unit as a whole. Therefore, in this thesis we have identified blood-based biomarkers to enable OA endotype stratification before starting treatment and we have characterized these OA molecular endotypes in subchondral bone.

To enable molecular endotype-based stratification of patients before treatment starts, non-invasive biomarkers that reflect ongoing processes in articular cartilage are required. Based on our recent work showing that circulating miRNAs are able to reflect ongoing processes in articular cartilage of OA joints [24], in **chapter 4** we have

searched for circulating miRNAs that mark OA molecular endotypes. In doing so, we have identified four miRNAs that together with sex and age predicted OA molecular endotype with 86% accuracy in a dataset different from the training dataset. These miRNAs could potentially be used in the clinic to stratify patients on their OA molecular endotype before treatment starts. Of note is that the datasets used to identify and validate these circulating miRNAs were small and replication in a large dataset would be required to confirm these results. Nonetheless, circulating miRNAs as biomarker for OA molecular endotypes and OA pathophysiology in general could provide a new window of opportunities for effective personalized OA treatment strategies and might result in more successful clinical trials.

To gain knowledge on whether similar molecular differences are seen in subchondral bone between OA molecular endotypes identified in articular cartilage, in **chapter 5** we focused on characterization of these endotypes in subchondral bone of 14 patients. We have shown that endotype A and B patients indeed exhibit distinct transcriptomic profiles in preserved OA subchondral bone. This difference between endotypes was shown to be enriched for similar processes in articular cartilage, such as immune response and positive regulation of IL6 production, with higher expression of these markers in both tissues in endotype B relative to endotype A patients. Moreover, differential expression analysis between preserved and lesioned OA subchondral bone suggested that particularly endotype B patients showed excessive bone formation in response to OA pathophysiology, characterized by expression of *COL1A1, COL1A2, GDF6*, and *CXCL9,* which is in line with observed increased joint space narrowing. Altogether, these data indicate that endotype B patients exhibit an atypical OA disease process, with detrimental inflammation in both articular cartilage and subchondral bone, resulting in excessive cartilage degeneration and bone formation. Related to the potential druggable targets we put forward in this thesis (**Table 2**), we found *CHADL* and *FRZB* being responsive to OA pathophysiology in subchondral bone specifically in endotype A patients, while *GLIS3* was responsive specifically in endotype B patients. More extensive characterization of these OA molecular endotypes in subchondral bone in a larger dataset could provide additional information on the other potential druggable targets, as larger datasets are more sensitive to identify smaller gene expression differences.

Attractive druggable targets identified in this thesis: potential treatment strategies

Based on genetics, differential expression between macroscopically preserved and lesioned OA articular cartilage and subchondral bone, and/or molecular endotypes, in this thesis we have put forward nine genes (**Table 2**) as attractive potential targets for OA treatment strategies. Of these nine genes, *FRZB* and *IL11* gene expression levels were

Table 2 – Potential druggable targets put forward in this thesis and their expression differences stratified for molecular endotype in articular cartilage and subchondral bone

	Articular cartilage			Subchondral bone		
Gene	**Fold change OA vs P endotype A**	**Fold change OA vs P endotype B**	**Fold difference B vs A**	**Fold change OA vs P endotype A**	**Fold change OA vs P endotype B**	**Fold difference B vs A**
***CHADL* (chapter 2)**	0.56	n.s.	n.s.	0.51	n.s.	n.s.
***IL11* (chapter 2 and 7)**	18.87	59.64	n.s.	n.s.	n.s.	n.s.
***FRZB* (chapter 3)**	0.25	0.26	0.34	0.53	n.s.	n.s.
***MAP2K6* (chapter 5)**	0.70	n.s.	0.38	n.s.	n.s.	n.s.
***HLA-DPA1* (chapter 5)**	n.s.	0.33	5.89	n.s.	n.s.	n.s.
***WWP2* (chapter 6)**	0.74	n.s.	n.s.	n.s.	n.s.	n.s.
***GLIS3* (chapter 9)**	2.24	n.s.	n.s.	n.s.	2.46	n.s.
***DGKI* (chapter 9)**	2.39	n.s.	2.39	n.s.	n.s.	n.s.
***SLC44A2* (chapter 9)**	0.71	n.s.	n.s.	n.s.	n.s.	n.s.

responsive to OA pathophysiology in both articular cartilage and subchondral bone and in both OA molecular endotypes, suggesting that targeting these genes could be a therapeutic strategy for all OA patients. The other genes were either more specific to OA molecular endotype A or molecular endotype B, stressing the importance of personalized medicine in OA treatment strategies. Because development of therapeutics is a time-consuming and expensive process with high failure rate, last decade drug repurposing strategies gained interest for various diseases [56, 57]. Drug repurposing is a strategy that involves application of approved drugs outside the scope of the original use [58]. Valuable tools in drug repurposing are publicly available online databases, such as drug-gene interaction database (DGIdb 4.0), in which drug-gene interactions are predicted [41]. For that matter, we have screened our genes of interest for interactions with FDA approved drugs (**Table 3**). We only have found drug-gene interactions for *IL11* and *MAP2K6.* Drug-gene interactions for *IL11* include bisphosphonates (alendronic acid and etidronic acid), usually prescribed for treatment of osteoporosis, anti-depressants (escitalopram and citalopram), and cancer therapeutics (azacytidine, fluorouracil, and doxorubicin). The directions of effect of these drugs on *IL11* remains unknown. Drug-gene interactions for *MAP2K6* were all three cancer therapeutics and are predicted to have inhibitory effects on *MAP2K6*, matching the direction of desired effect. Nevertheless, predicted drug-gene interactions does not necessarily reflect effectiveness of the drug with OA. Therefore, functional studies are required to first understand how these genes affect articular cartilage and subchondral bone homeostasis and second to understand what the effect of the drug is. Moreover, application of these drugs in the clinic require additional studies on administration methods, side-effects, and doses [59].

Proof-of-concept: in vitro investigation of OA risk genes *WWP2* and *IL11*

To enable translation from genetic and genomic studies towards the development of DMOADs, insight in underlying biological mechanisms of OA risk genes is essential. As proof-of-concept (**Figure 2**), the next step in this thesis was to functionally investigate OA risk genes *WWP2* and *IL11* using two models that we have set up previously: 3D primary chondrocyte pellet cultures [60, 61] and human osteochondral explant cultures [62-65].

One of the top findings in a previously performed screen for allelic imbalanced expression in articular cartilage was rs1052429, located in *WWP2* gene. This SNP showed highly significant allelic imbalanced expression and allele rs1051429-A was associated to minimal joint space width, a characteristic of OA, and marked increased expression of *WWP2*. Moreover, rs34195470, a proxy of rs1052429, was identified conferring genome-wide significant risk to OA in large comprehensive genome-wide meta-analyses [28, 30]. Comparison of macroscopically preserved and lesioned OA articular cartilage previously

revealed that *WWP2* gene expression was significantly downregulated in lesioned tissue [4]. Nevertheless, differential expression analysis reflects the tissue response to OA pathophysiological process and gene expression differences identified in such analysis are not necessarily causal to OA pathophysiology. Therefore, we advocate that is essential to formulate hypotheses on direction of effects of OA genes based on genetics, such as allelic imbalanced expression or eQTL (**Figure 2**). Based on allelic imbalanced expression [32], we made the firm hypothesis that rs1052429-A acts via increased expression levels of *WWP2* conferring risk to OA. Henceforth, to mimic this effect we have generated lentiviral particle-mediated upregulation of full length *WWP2* in 3D primary chondrocyte pellet cultures (**chapter 6**). After seven days of pellet culture, we have observed detrimental effects on cartilage matrix deposition in terms of gene expression levels upon *WWP2* upregulation. These detrimental effects were reflected by significant downregulation of *ACAN* and *COL2A1,* marking cartilage anabolism, and changed gene expression of *EPAS1, GJA1, GDF10,* and *STC2,* all four genes involved in chondrocyte dedifferentiation to bone [66-71]. Chondrocyte dedifferentiation results in a shift from collagen type 2 to collage type 1 production, resulting in more fibrotic cartilage [72]. Since *WWP2* is known to be involved in (post-)translational modifications [73], we have performed proteomics analysis on our 3D chondrocyte pellet cultures to gain insight in protein expression differences upon *WWP2* upregulation. As articular cartilage ECM consists of dense insoluble collagen networks, extracting proteins can be challenging [74]. Consequently, we have performed proteomics on day three (less ECM expected) and day seven (more ECM expected) of chondrocyte pellet cultures. Protein extraction of both days was successful and cartilage markers, such as COL2A1, ACAN, COMP, and FN1, were already abundantly expressed after three days of pellet culture. Based on these results we have pooled proteomic data of day three and seven (to increase power) to evaluate the effects of WWP2 upregulation. In total, 42 proteins were identified being differentially expressed, which were enriched for ubiquitin conjugating enzyme activity. Altogether, our hypothesis based on genetics was confirmed by functional investigation of *WWP2* in cartilage. Nonetheless, this study did not yet include cartilage in interaction with subchondral bone. Moreover, multiple differentially methylated CpGs located in *WWP2* are identified [75], of which the effects are not addressed in this thesis. Since WWP2 is a E3 ubiquitin ligase and differentially expressed proteins were enriched for ubiquitination-related processes, upregulation of WWP2 could also affect proteins cellular location, activity, and protein-protein interactions without changing expression levels itself [76, 77], which was not captured by our read-outs. Finally, in this thesis we suggested that miRNA-140 could potentially have a regulatory effect on *WWP2,* which should be further investigated. In conclusion, to get full understanding of the underlying mechanisms of how *WWP2* confers risk to OA, integration of multi-omics data and functional experiments, preferable in a model system representing functional

Table 3 – Predicted drug-gene interactions for genes of interest using DGIdb [41], showing interactions for IL11 and MAP2K6.

Gene	Gene expression risk	Reported drug-gene interactions (approved)[41]	Direction of drugs
***CHADL* (chapter 2)**	Increased	none	-
***IL11* (chapter 2 and 7)**	*Decreased*	ALENDRONIC ACID, ETIDRONIC ACID, ESCITALOPRAM, CITALOPRAM, AZACITIDINE, FLUOROURACIL, DOXORUBICIN	unknown
***FRZB* (chapter 3)**	*Decreased*	none	-
***MAP2K6* (chapter 5)**	*Increased*	COBIMETINIB, BINIMETINIB, TRAMETINIB	inhibitory
***HLA-DPA1* (chapter 5)**	*Increased*	none	-
***WWP2* (chapter 6)**	*Increased*	none	-
***GLIS3* (chapter 9)**	*Unkown*	none	-
***DGKI* (chapter 9)**	*Unkown*	none	-
***SLC44A2* (chapter 9)**	*Increased*	none	-

cartilage and bone, should be performed in future studies.

Multiple GWAS meta-analysis have identified rs4252548, a missense mutation in *IL11*, conferring risk to OA [28, 30]. As the missense mutation results in a thermally unstable protein, it was hypothesized that this SNP confers risk to OA via reduced function of IL11 protein. Therefore, addition of hrIL11 protein was previously suggested as therapeutic strategy for OA [36]. Nonetheless, functional investigation is required to confirm these potential beneficial effects of hrIL11 on articular cartilage and subchondral bone. The importance of including subchondral bone in functional investigation of *IL11* is stressed by the fact that gene expression levels of *IL11* are among the top 25 genes being responsive to OA pathophysiology showing the highest foldchange in both articular cartilage and subchondral bone (**chapter 2**). Moreover, IL11 is known for its role in bone homeostasis and metabolism, as it regulates osteoclastogenesis via RANKL expression by osteoblasts [78-81]. To study the effects of hrIL11 on articular cartilage and subchondral bone with OA, we had available our human *ex vivo* osteochondral explant cultures (**chapter 7**) [62]. These osteochondral explants are isolated from preserved areas of a human OA joints and can be exposed to OA-related perturbations, such as mechanical loading, inflammation, and hypertrophy. Previously it was shown that excessive mechanical loading of 3D chondrocyte pellet cultures resulted in increased levels of *IL11*, comparable as seen between preserved and lesioned OA cartilage [61]. Based on these findings, we have applied mechanical loading on these osteochondral explant to model OA, with or without addition of hrIL11. Mechanical loading of explants

resulted in similar effects on articular cartilage as reported previously [64]. However, we did not observe any effects in articular cartilage and subchondral bone gene expression levels upon hrIL11 addition (data not shown). Since mechanical loading represents only a portion of OA pathophysiology, we decided to isolate osteochondral explants from the lesioned areas of the joint to fully recapitulate OA. Nonetheless, exposing lesioned osteochondral explants to hrIL11 had again minimal effects on both tissues. Based on the significant upregulation of *SPP1* and downregulation of *WNT16* upon hrIL11 exposure resulted in unbeneficial response of lesioned articular cartilage, while significant downregulation of *PTGES* in subchondral bone might potentially be beneficial. The latter might suggest rs4252548-T confers risk to OA via subchondral bone, however, more research is needed to confirm. Together, these results indicate the importance of functional investigation of OA risk genes in both articular cartilage and subchondral bone, as we showed that treating the whole joint with hrIL11 does not necessarily have beneficial outcomes. Additional studies including higher sample size are necessary to confirm our findings. Moreover, gene expression of IL11 receptor *IL11RA* was previously also shown to be dysregulated with OA in articular cartilage [4]. Hence, it might be valuable to include *IL11RA* in future functional investigations.

Novel biomimetic *in vitro* model system of osteochondral tissue: osteochondral-unit-on-a-chip

Since 3D chondrocyte pellet and osteochondral explant cultures both have their limitations (**Table 4**), development of a novel state-of-the-art model system based on microfluidic tissue-on-chip principles would be preferred. Therefore, in the last part of this thesis, we developed a novel dual-tissue microfluidic model system in which we cultured interacting neo-cartilage and neo-bone deposited by primary chondrocytes and osteogenic cells, respectively (**chapter 8**).

Table 4 – Advantages and limitations of 2D cell cultures, 3D chondrocyte or osteogenic cell pellet cultures, and ex vivo osteochondral explants.

Model	Advantages	Limitations
2D cell culture	• Easy to use • Easy to increase sample size	• Lack complexity • Lack interaction with ECM • Chondrocytes are prone to lose phenotype
3D chondrocyte/ osteogenic cell pellet culture	• Allow gene expression alterations by e.g. lentiviral transduction • Easy to increase sample size	• Neo- cartilage and bone are produced, being less prone to develop OA • Interaction between cartilage and bone is lacking
***Ex vivo* osteochondral explants**	• Consist of aged ECM that is prone to OA onset • Represent interaction between cartilage and bone	• Sample size is dependent on joint replacement surgery and joint size/OA state

The main advantage of our developed osteochondral-unit-on-a-chip compared to available microfluidic systems is that cells deposit biological ECM instead of matrix island withing hydrogels [82, 83]. As proof-of-principle, we have implemented OA-related perturbation to our system to mimic a pathophysiological process. In our previous study on osteochondral explant cultures [62], we have showed that OA-related perturbations could be used to mimic inflammatory OA (IL1β), post-traumatic OA (mechanical loading), and age-related OA (hypertrophy-inducing thyroid hormone T3). Inducing inflammation using IL1β was shown to be very detrimental for cartilage tissue in osteochondral explants and application of mechanical loading in our microfluidic chip would require adaptations in chip design. Consequently, we choose to expose the system to hypertrophy-inducing thyroid hormone T3. Upon T3 exposure the chondrogenic compartment changed its gene expression pattern towards an osteogenic phenotype, similar to OA pathophysiology and to the effects observed in osteochondral explants. In the osteogenic compartment expression levels of hypertrophic markers were also increased. Together, this shows that our model system could serve as a platform to perform in depth investigations of underlying mechanisms of OA risk genes or OA-related stimuli in both cartilage and bone. Subsequently, our system could be used for the identification of druggable targets and eventually drug testing, which will contribute to cost-efficient preclinical research and reduce, refine, and replace animal experiments.

Enhancements to our developed osteo-chondral-unit-on-a-chip could still be accomplished. To completely overcome the dependency of patients that undergo total joint replacement surgery the use of a more stable cell line, such as induced pluripotent stem cells (iPSCs), could be an attractive alternative to produce neo-cartilage and neo-bone [84]. Another advantage of using iPSCs is that it allows genome editing techniques, such as CRISPR/Cas9, to knock-in or knock-out complete genes or to insert or repair specific mutations, after which these conditions can be compared to their isogenic control. Finally, CRISPR/Cas9 can also be used to create reporter iPSC lines that can provide real-time read-out upon perturbation and/or drug candidate testing [85-88]. Therefore, it would be of added value to optimize the iPSC culturing protocol in terms of differentiation towards chondrocytes and osteoblasts, seeding process and culturing time for the use in our microfluidic model system. This would allow in-depth investigation of specific mutations in interacting cartilage and bone tissue. Furthermore, implementation of other cell types, such as synoviocytes, adipocytes, osteoclasts, and immune cells would advance the system even further. The most simple way to add different cell types is by placing multiple microfluidic chips in parallel. Synoviocytes, for example, could be implemented to the system by placing a synoviocyte containing microfluidic chip in front of our osteochondral-unit-on-a-chip (**Figure 4**).

By doing so, chondrocytes will be exposed to synoviocyte-conditioned media. To allow complete remodeling of the ECM by cells present in the osteogenic compartment the polycaprolactone (PCL) matrix could be replaced by, for instance, a collagen type I or silk fibroin matrix [89]. Mechanical loading has been shown to be an important trigger in OA [64, 90-92]. To apply mechanical loading to neo-cartilage and neo-bone in our chip, we need to incorporate an actuation chamber or for example a controllable micro-piston to the design [93]. Finally, in order to use our system as part of pre-clinical studies screening for newly developed potential OA drugs, it would be necessary to further miniaturize our system and make it more compatible with high-throughput screens. In this respect, using iPSC reporter cell lines might provide faster and real-time read-out [85].

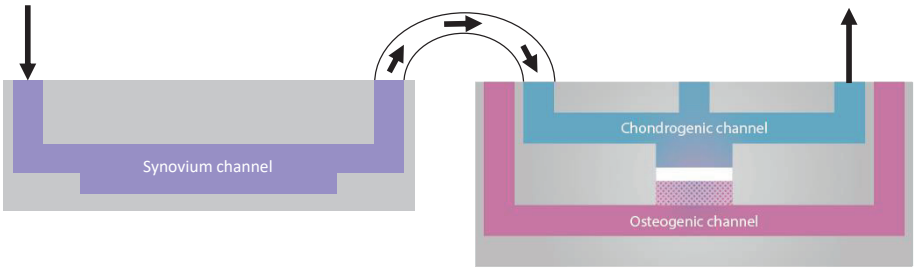


Figure 4 – Schematic overview of a relatively simple way to implement additional cell types, such as fibroblast to mimic the synovium, to our osteochondral-unit-on-a-chip system.

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