Discussion

8

Parts of this text will be included in an invited review for Current Rheumatology Reports

GENERAL DISCUSSION

Osteoarthritis (OA) is a complex heterogeneous disease with a considerable heritable component. While the incidence of the disease is steadily increasing, up until now no treatment options are available that could cure or stop disease progression. Main impediments in OA management are the lack of biomarkers that detect OA at an early stage when damage is still reversible, or that allow stratification of subsets of OA patients with different disease modes. This considerably hampers development of effective therapies and requires insight into the heterogeneous etiology of OA.

The aim of this thesis was threefold. Firstly, to address OA biomarker impediments by exploring gene expression profiles as biomarkers in osteoarthritic cartilage and blood (chapter 2 and 3), and by investigating whether we could identify genetic factors that influence levels of promising OA biomarkers such as serum COMP and urinary CTX-II in the common population independent of OA status (chapter 4). We hypothesized that sensitivity of such biomarkers could be improved by taking into account these genetic factors. Secondly, to explore the possibility to identify OA susceptibility genes by either applying a GWA study with sibling pairs (familial OA cases) towards the severe end of common/late onset OA phenotype (symptomatic OA at multiple joint sites) of the GARP study (chapter 5) or by applying whole exome sequencing of family members with early-onset symptomatic OA at multiple joint sites (chapter 6). Thirdly, to initiate the necessary shift from genetic towards functional genomic studies in OA research for the deiodinase iodothyronine type-2 (*DIO2*) gene using *in vitro* chondrogenesis models (chapter 7). Together, results will contribute to the selection of druggable targets and the badly needed development of new treatments as well as the identification of biomarkers to early detect ongoing OA.

BIOMARKERS IN OSTEOARTHRITIS

At present, timely diagnosis of OA before joint damage becomes irreversible is still lacking. This is one of the impediments in OA treatment and thus, biomarkers that can ascertain OA at an early stage are urgently needed. In addition, clinical biochemical markers to classify patients with different OA subtypes and to sensitively monitor OA disease activity in an individual over time or to assess quantitative joint tissue remodeling are required. In 2006, Bauer and colleagues proposed the BIPED biomarker classification to be applied in development and analysis of OA biomarkers.[1] BIPED stands for 'Burden of Disease, Investigative, Prognostic, Efficacy of Intervention and Diagnostic' and serves to facilitate research (both independent and collaborative) and to reduce redundancy while specifying and accelerating the validation of potential biomarkers.

Studies thus far have suggested that serum cartilage oligomeric protein (sCOMP) and urinary C-telopeptide of type II collagen (uCTX-II) are promising candidates hence they were listed among a number of biomarkers recommended to focus on in biomarker research.[2] However, due to the high heterogeneity in OA phenotypes and

relatively large inter-individual variation also among healthy subjects, the use of biomarkers is complicated. In chapter 4, we have investigated whether we could identify genetic variants affecting innate levels of sCOMP and uCTX-II that could possibly explain the variance and improve the use of respective biomarkers in the clinic. We found specific loci in association with biomarker levels. Among others, we found a single nucleotide polymorphism (SNP) in close vicinity to the Mannose receptor C type 1 (*MRC1*) gene in association with levels of sCOMP with genome wide significance which was not in association with hip or knee OA. MRC1 is a membrane receptor highly expressed by macrophages and it has been shown that MRC1 mediates specific recognition of sugar residues to allow endocytosis of glycoproteins.[3] Since *MRC1* is only lowly expressed in articular cartilage and carriers of the risk allele have decreased levels of sCOMP, we hypothesize that carriers of the risk allele may have enhanced phagocytosis of serum glycoproteins such as sCOMP which could negatively affect the sensitivity of sCOMP as biomarker of OA in a clinical setting. Our findings are encouraging and incite additional studies to identify genetic loci associated with other biomarkers. However, most important now is to investigate whether taking the genetic variation into account will improve the use of these biomarkers and may allow for the distinction of OA subtypes.

Unconventional biomarkers. Traditionally, biomarkers are thought of as biochemical substances and studies for development of biomarkers are still mainly focused on biochemical components in body fluids that mark either cartilage, bone, or synovial metabolism, or a combination of these.[4] In addition to these more traditional biomarkers, imaging methods such as quantitative magnetic resonance imaging (MRI) to assess cartilage volume and thickness or shape by statistical shape modeling have shown to be suitable as biomarkers for OA.[5-7] Disadvantage of these methods is, however, their relatively high costs. Currently, molecular profiles of genome wide gene expression or epigenetic variation are increasingly considered as optional targets in biomarker development. The fact that organ-specific pathologies can be detected from easily accessible tissues such as blood which was recently confirmed by integration of multiple studies,[8] encourages research for the identification of novel molecular markers. Differentiation of patients based on gene expression profiles in blood has been successfully performed for example for coronary artery disease,[9, 10] and several types of cancer.[11-13] Also for knee OA patients, gene expression profiles in blood were generated successfully and resulted in the identification of different subsets of patients.[14] In this thesis we have used gene expression profiles in blood to identify diagnostic biomarkers that could distinguish patients from the GARP study from healthy controls. Inclusion of the potential molecular biomarkers in the estimated cross-validated algorithm in receiver operating curve (ROC) analysis yielded a 20% increase of the area under the curve (AUC) to 91-95% (chapter 3). This clinically relevant AUC obtained in our analysis is expected to be accomplished by virtue of the combination of body mass index (BMI), also augmenting the AUC with 20% to approximately 72%, with the expression of a specific set of genes. This was

confirmed in an independent selection of the GARP study, however, controls were overlapping and results await replication with population-based controls. Moreover, because our study has a cross-sectional design, further investigation in a longitudinal study is necessary to assess predictive value and putative benefit for early diagnosis as well as disease progression in a population-based setting.

Gene expression profiles in blood may either be more systemic and thus independent of OA pathophysiological processes in the joint or reflect ongoing OA processes. Therefore we explored the overlap between expression profiles in blood and in cartilage and found that around 25% of the genes with fold-changes of ≥1.5 coincided. For example, a gene overlapping between our blood and cartilage expression profiles was Serine/Arginine-Rich Splicing Factor 5 (*SFRS5*), highly expressed in cartilage, and in blood contributing most to our prediction model of OA patients. *SFRS5* encodes one of the members of the SR family of proteins that binds to exonic splicing enhancer cis-sequences and it was shown to be involved in alternative splicing of the fibronectin gene specifically in chondrocytes.[15] Another gene overlapping between blood and cartilage profiles was *H3F3B*, encoding the H3 histone family 3B. While in cartilage *H3F3B* was only 1.2-fold decreased, in blood the reduction was more than 2-fold making it particularly attractive to further study its putative application as a biomarker. A recent study showed association of a specific mutation in *H3F3B* with chondroblastoma, while mutations in *H3F3A*, a homolog of *H3F3B*, were exclusively found in association with giant cell tumors of the bone.[16] Interestingly, in *H3F3B* knockout mice reduced H3K4me3 (a marker for active gene transcription) was observed preferentially near genes related to extracellular matrix.[17] Together, this could suggests an essential role for *H3F3B* in chondrocyte proliferation and cartilage homeostasis, and maybe in susceptibility to OA development. However, this needs further investigation.

Remarkably, of the genes overlapping between blood and cartilage expression profiles only half appeared to have the same direction of effects. Possibly, this stems from the differences in the study set-up: while the cartilage expression profiles were generated from joints of patients with end-stage OA, patients from the GARP study have symptomatic OA at multiple joints and blood profiles were compared with those of healthy subjects. Expression differences may therefore relate to the differences in timing and mechanism of development of OA. Alternatively, since expression of genes in blood may rather reflect a systemic profile, this can be different from the more direct pathophysiological processes taking place in the joint tissues. For example, a gene that is increased in blood of OA patients but decreased in OA cartilage is *TGFBR3* (transforming growth factor beta receptor III). As known, involvement of members of the TGF-β superfamily in cell proliferation, differentiation, morphogenesis, tissue homeostasis and regeneration is highly cell- and context-dependent.[18, 19] Therefore, it could very well be that OA patients have increased levels of *TGFBR3* in blood cells while the processes taking place during OA development in articular cartilage result in a decreased expression of the gene. For future studies, combining joint tissue databases with blood expression profiles as well as the inclusion of samples (joint tissues and blood)

from healthy individuals may be of help for the selection of biomarkers that are able to sensitively monitor disease activity.

A field in development in biomarker research is the use of non-coding RNAs as biomarkers. Several types of non-coding RNAs can be distinguished that are generally classified as small RNAs such as miRNA, piRNAs, and long non-coding RNAs or lncRNAs.[20] Micro RNAs can be detected in circulating body fluids such as plasma and serum where they are relatively stable because they are protected from RNAse activity by virtue of their association with secreted membrane vesicles or RNA-binding proteins,[21] and multiple studies now suggest that specific circulating miRNAs sensitively reflect ongoing pathophysiological processes[22] thereby qualifying their use as potential biomarkers, also for OA. Studies investigating expression of OA-specific miRNAs however, are still limited. To our knowledge, the largest study thus far was performed by Beyer *et al* including 816 individuals of the Bruneck cohort.[23] In this longitudinal study, miRNA-let-7e was identified as a negative, dose-dependent predictor for severe knee or hip osteoarthritis. Further research is needed with replication and validation in other cohorts, to obtain data on specificity and sensitivity of miRNAs to monitor dynamic changes in the OA pathophysiological processes over time. In addition, it could be investigated whether miRNAs serve to identify patients prone to fast progression. This would allow for more adequate OA disease management and more efficient design of clinical trials.

IDENTIFYING OA SUSCEPTIBILITY GENES

One of the drawbacks scientists encounter in their research to discover genetic variants causing susceptibility to OA is the so-called 'missing heritability'.[24] In part, the problem might be caused by an overestimation of heritability in the general population based on results of twin and family studies. Alternatively, this may be due to the fact that population-based or case-control studies with GWAS are designed specifically to detect common variants which usually have small effect sizes.[25] As a result, variants with larger effect sizes and/or that associate specifically with more rare and severe OA phenotypes, such as the variants identified for *DIO2* and *FRZB*, are not detected at the genome wide significance level in GWA studies.[26-28] Nevertheless, given the functional impact, these variants are likely contributing significantly to the percentage of heritability. To illustrate, the *DIO2* susceptibility SNP rs225014 was shown to have an odds ratio of OR = 1.79 consequently involving a heritability of 5.3 which is in sharp contrast to the average heritability of established loci as identified by GWAS ($H^2_{average}$ = 0.8). In general, family-based studies are prone to detect variants with moderate to large effect sizes. We investigated whether selection based on cases with familial and relatively severe phenotypes could contribute to the identification of variants with larger effects in GWAS (chapter 5) and in whole exome sequencing (chapter 6). Although identified variants will be rare and frequently private to the respective family their value lies in the expectation that more common variants in the same gene or pathway in which the gene acts will be

present in a larger population.[29] In these studies, the availability of a gene expression dataset of (OA affected and unaffected) articular cartilage (the RAAK study) allowed efficient selection of putative candidates and generalizability of the results.

Whole exome sequencing. In chapter 6 we applied whole exome sequencing to 2 distant members of a family suffering from early-onset OA. A suitable prioritization scheme and analysis of gene expression levels in articular cartilage indicated a mutation in the tumor necrosis factor receptor superfamily member 11B (*TNFRSF11B*) gene encoding osteoprotegerin (OPG) to be likely causal to the phenotype in our family. This was in line with the genome wide significant linkage analysis at this locus (LOD-score of 3.48) and complete segregation of the mutation with early-onset OA in the family.

The decoy receptor OPG antagonizes receptor activator of NF-κB (RANK) in the process of osteoclastogenesis. Functional *in vitro* analysis now showed that the mutation in *TNFRSF11B* results in enhanced OPG-mediated RANK antagonism and the significant upregulation of *TNFRSF11B* in articular cartilage suggests that this is a phenomenon in general OA pathology. Our results that the mutation in OPG is causal to the FOA phenotype in our family are highly convincing, nevertheless, further research is required. The phenotype of the family has to be studied in more detail and final proof for the causal role of OPG in OA should be provided by the performance of *in vivo* experiments in transgenic mice bearing the identified mutation to confirm the role of the identified mutation in development of OA. OPG transgenic and knockout mice have been generated previously and are viable,[30, 31] nonetheless, they were never challenged with OA-inducing trials such as mechanical loading through forced running. Interestingly, OPG transgenic mice are osteopetrotic which is caused by the impaired bone remodeling due to failure of the osteoclasts to resorb bone, but their phenotype is different from other mouse strains with osteopetrosis. The phenotype, also characteristic for RANKL knockout mice,[32] is impaired tooth eruption and shortened, 'club-shaped' long bones due to complete lack of mature osteoclasts. It was suggested that, depending on the differences in bone surfaces (e.g. endosteal and periosteal surfaces), the capacity of systemic OPG to inhibit RANKL varies. These circumstances make the application of experimentally induced OA in OPG transgenic mice even more interesting since results are likely to lead to deeper insight into the role of the bone-cartilage interaction in OA. Further *in vitro* analyses which can be performed among others by comparing chondrogenic and osteogenic potential of iPS cells derived from affected and unaffected family members can give more insight into the underlying mechanisms of the mutated OPG in OA.

Increased RANK antagonism leads to decreased osteoclastogenesis and it may thus result in concurrent increased bone mineral density (BMD). The variation in BMD towards higher levels would be in line with standing epidemiological studies showing increased BMD as a potential risk factor for OA.[33-37] On the other hand, 2 clinical studies,[38, 39] although subject of debate,[40-42] suggested that OA patients benefit from treatment with strontium renalate, an anti-osteoporotic drug that increases bone formation while decreasing bone resorption. Also *in vitro*[43] and *in vivo*[44-46] experiments indicated putative beneficial effects of strontium renalate. In this respect it

should be noted that the methods applied in the *in vivo* studies are broadly used in animal studies, nevertheless, it has been questioned whether these methods are the best to investigate pathophysiological processes of primary OA as it occurs in humans (see '*In vivo animal models in OA*'). Possibly, subtypes of OA exist that will benefit from bone forming therapies while others will not. For example, among patients suffering from hip OA those with atrophic OA had systemically lower BMD as compared to those with normotrophic OA and as compared to the controls, while those with hypertrophic (osteophytic) OA had higher BMD.[47] Given that gene expression profiles of human osteoarthritic cartilage indicated enhanced OPG-mediated RANK antagonism as a more general phenomenon in the OA pathophysiological process (chapter 2 of this thesis), we advocate that careful investigation of the long term effects of these bone forming therapies in osteoarthritis are required and that, in contrast to the use of bone-forming therapies such as strontium renalate, (at least for some patients) agents counteracting OPG function could contribute to the development of new disease modifying treatments in osteoarthritis.

GWAS using familial phenotype towards the severe end of the OA spectrum. By performing a genome wide association study in a very specific cohort consisting of familial cases with severe symptomatic and radiographic OA (KL>2) in at least two joints (the GARP study) we aimed at the identification of loci that may be less common but with larger effect sizes.[25] Several loci annotated to interesting genes (e.g. the calcium sensing receptor or *CASR*) were identified with larger effect sizes (mean odds ratio 1.8 with range: 1.5-2.1). However, none of these loci were genome wide significant (smallest p-value: 1.2×10^{-7}) which may indicate a lack of power despite the predictions of power calculation programs. Besides lack of power, a problem when performing GWAS with a specific phenotype is subsequent replication of the results obtained in the discovery stage. The more specific a cohort is, the smaller the chance to find another study with the same phenotype. We have not been able to find other cohorts with genotype data of cases with familial generalized OA comparable with the GARP study. Furthermore, effect sizes of a study with more severe phenotype (deCODE), albeit not selected for familial affiliations, were again much smaller indicating either heterogeneity in the phenotype or false positive findings in the discovery cohort. Since GWAS platforms are generally based on common variants the causal (less common) SNP may not be included or removed by selection for allele frequencies of at least 5%. Replication in cohorts with more common phenotypes did not result in robust associations. This was also demonstrated by a GWA study from Evans *et al* for hip OA.[48] For the discovery analysis with genome wide significant results, cases were carefully examined and strictly selected to acquire a more homogeneous cohort. However, results of the combined analyses of discovery and replication were not genome wide significant ($p=1\times10^{-6}$). In conclusion, genome wide significant results in GWAS derive from a trade-off between large sample sizes and homogeneous cohorts. Confirmation of our results in a cohort similar to the GARP study could ascertain whether selection of samples towards the severe end of the spectrum of OA and with a familial phenotype may indeed be a promising approach to find loci with larger effects (average odds ratio of the

Table 1 Top 25 of gene-based association analysis. Analysis was performed by joining all SNPs within known druggable genes (N= 4,290) and subsequently testing each of the genes for association with OA.

Gene Symbol	# SNPs	*p*	FDR
SLCO2B1	12	7.17E-05	2.79E-01
CXCL11	3	1.62E-04	3.15E-01
KCNQ4	14	2.72E-04	3.53E-01
ABCB11	26	5.82E-04	5.66E-01
SLC12A6	22	6.45E-04	5.02E-01
SELP	24	7.07E-04	4.59E-01
FTL	3	7.44E-04	4.14E-01
SLCO5A1	49	8.46E-04	4.12E-01
KITLG	12	1.02E-03	4.43E-01
STRADA	1	1.36E-03	5.30E-01
DPYD	117	1.50E-03	5.30E-01
RHBDL1	6	1.53E-03	4.96E-01
NCOA3	20	1.86E-03	5.58E-01
BAI3	148	2.08E-03	5.79E-01
GRIN2B	154	2.12E-03	5.50E-01
ADAMTS12	108	2.14E-03	5.20E-01
RPP25	1	2.29E-03	5.25E-01
ASPRV1	1	3.08E-03	6.66E-01
KCNK4	1	3.24E-03	6.64E-01
BNIP3	1	3.33E-03	6.47E-01
CD44	47	3.34E-03	6.20E-01
CTRB2	7	3.45E-03	6.10E-01
IGFBP3	7	3.59E-03	6.07E-01
CRABP2	9	3.92E-03	6.36E-01
PCDHB9	3	4.02E-03	6.26E-01

established OA susceptibility loci detected in large meta-analyses with genome wide significance is 1.2 with range 1.1-2.0 compared to 1.8 in our study).

In the genomic region around rs347842, locus with strongest evidence for association with OA, another strong signal was identified which may be independent but which may also be (partially) shared: rs17236749, located close to *C15orf29*. Therefore, we reasoned that analysis for burden of association per gene could possibly increase the power to identify genetic regions or genes involved in development of OA. Results of our GWAS were used to perform gene-based association analysis by joining all SNPs within known druggable genes (N= 4,290). Subsequently, each of these genes was tested for association with OA. Table 1 shows the 25 most significant genes [YFMR & S Tsonaka, unpublished results]. Unfortunately, none was significant after correction for multiple testing. However, besides *SLC12A6* several other genes previously identified in association with OA were present such as *NCOA3*[28] and *IGFBP3*.[48] This observation may add to the credibility of our GWAS results and validate selection of loci close to druggable targets for further analyses despite the absence of genome wide significant loci.

Identifying strong candidate genes. United efforts of large consortia have ensured the discovery of loci within or close to compelling OA candidate genes by applying GWAS (see Table 1 of the Introduction). However, different from whole exome sequencing where high impact mutations are identified that are damaging for protein function, OA susceptibility loci identified by GWAS generally represent intergenic loci, in regions with multiple genes that have not yet been implicated in OA etiology, or within gene deserts and establishment of the causal gene has been proven difficult. Even when a SNP is localized within a plausible gene, such as the SNP identified within the *CASR* gene that was identified with the GARP GWAS

(chapter 5), after confirmatory genetic replication consecutive verification at multiple levels (molecular, cellular, whole organism) is required before definitive conclusions can be drawn. This was emphasized by the study of Smemo *et al*[49] for a locus associated with obesity in a very large GWAS and localized within the *FTO* (fat mass and obesity associated) gene that recently appeared to mainly target the *IRX3* gene, localized mega bases further away from the SNP. The finding stresses the observation of Freedman and colleagues[50] that 'even if a transcript is associated with a risk allele, it does not necessarily mean that the annotated gene is definitively involved in the trait of interest and that functional follow-up with assays relevant to the trait are needed to actually show that a gene is directly involved with disease development'.

Risk alleles likely act by affecting joint tissue specific gene expression.[51, 52] Therefore, a starting point to come to the gene affected by the genetic variant is analysis of expressed quantitative trait loci (eQTL) which should be performed on a more regular base in GWAS.[53] Challenge for studying eQTLs is the availability and selection of disease relevant tissues beyond the collection of blood. Although online databases exist (http://biogps.org) cartilage is not a tissue commonly included and in this respect comprehensive biobanking in OA research is of enormous importance. In chapter 2, we have shown how comparison of a relatively large number of samples from diseased and unaffected areas of cartilage from the same joint hint at generic pathological processes causing OA. Genome wide eQTL analysis was not performed within the RAAK study, but the generated dataset has been extensively used to prioritize genes in subsequent studies within the scope of this thesis (chapter 3-6) and follow-up research.[28, 54] A typical example for prioritization in regions with multiple genes could be the SNP identified within the *ASTN2* gene, rs4836732, associated with hip OA with genome wide significance in females and thus put forward as novel likely OA susceptibility gene.[27] Although we do not exclude the possibility that *ASTN2* affects OA susceptibility, the gene was not detected well in our microarray expression analysis. Having said this, we advocate that *PAPPA*, a protease for IGF binding proteins localized 100 kb upstream of *ASTN2* which increases osteoblast proliferation[55] and is 2-fold up-regulated in OA affected cartilage, should be considered as the OA susceptibility gene.

Taken together, when the correct datasets are available these can be used to identify, in high throughput manner, strong positional candidates that subsequently can be employed to cell models to address the specific effects of the genetic variation.

MOLECULAR PROCESSES IN OA

During OA, several processes take place in the joint such as cartilage degradation, bone remodeling, and inflammation. At the molecular level, each of these processes is reflected by specific gene expression networks, however, these networks are strongly intertwined among each other. Figure 1 shows a schematic representation of the different processes and some of the genes involved.

A common feature of OA cartilage is that

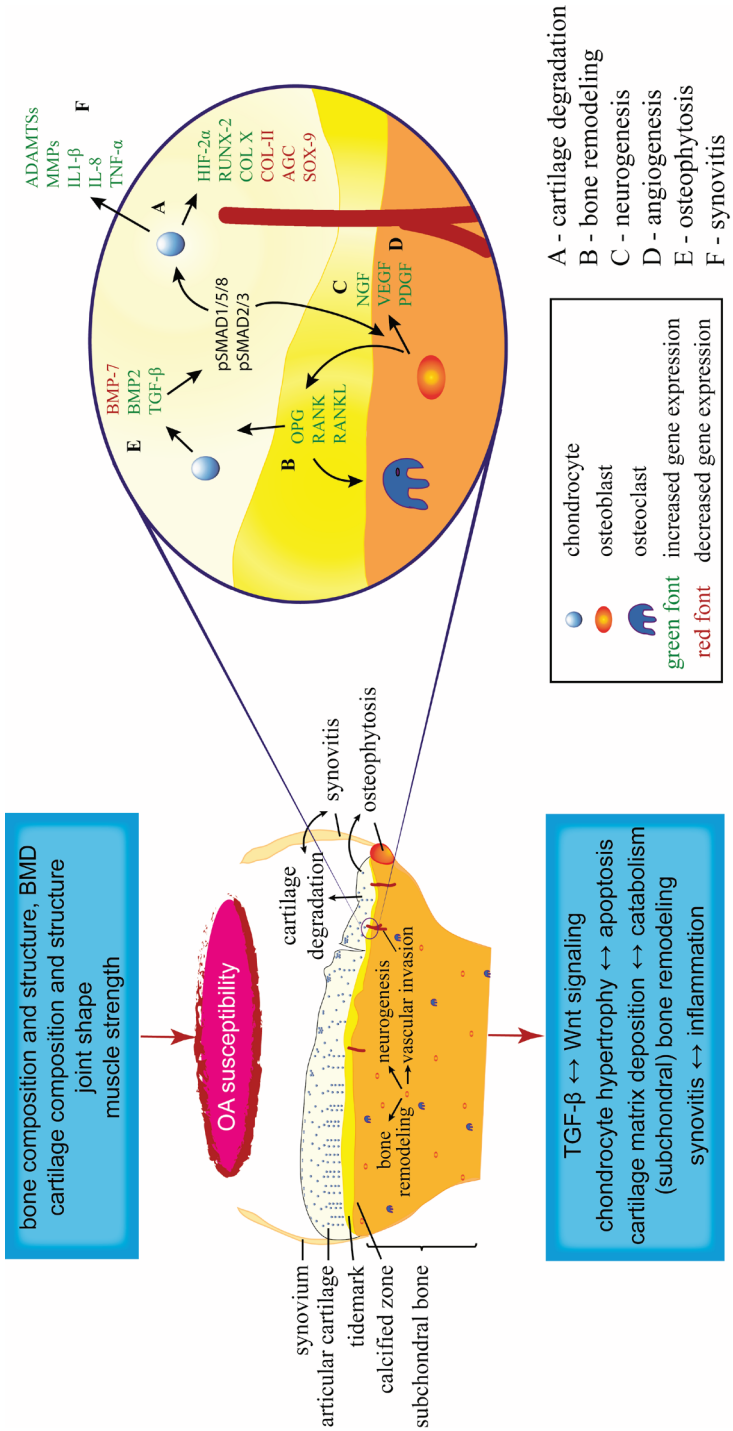

Figure 1 Schematic representation of molecular processes involved in OA.

the chondrocytes have lost their specific maturational arrested phenotype and are characterized by hypertrophy. This results in various degrees from detrimental genetic variation, cellular responses required for matrix-repair processes in damaged cartilage, and deregulated gene expression following loss of epigenetic control due to pathophysiological processes and/or aging. Chondrocyte hypertrophy results in mineralization of the cartilage due to increased expression of *RUNX2* and concurrent increase of *COL10* making the cartilage more prone to damage following mechanical stresses. In addition, hypertrophic chondrocytes express higher levels of secretion factors such as cytokines (*IL1B*, *TNF-a*), catabolic enzymes (*MMPs*, *ADAMTSs*) and members of the transforming growth factor-beta superfamily (reviewed in [56] and [57]). These are responsible for the inflammatory aspects of the arthritic joint.

One of the genetic loci underlying susceptibility to OA is annotated to the *DIO2* gene, and our studies showed that indeed impaired thyroid signaling predisposes articular chondrocytes towards hypertrophy with concurrent increased expression of *COL10*, and catabolic genes such as *MMP13*, resulting in mineralization of the articular cartilage (chapter 7). Possibly, aberrant thyroid signaling involves one of the first episodes on the way to OA. It has been shown that the *EPAS1* promoter encoding Hypoxia-inducible factor-2α (HIF-2α) contains a thyroid binding element both in mice[58] and man.[59] While HIF-1α contributes to cartilage extracellular matrix preservation via induction of *SOX9*, *COL2A1*, and *AGC*,[60] expression of HIF-2α results in cartilage hypertrophy due to up-regulation of *RUNX2* and catabolic factors (*ADAMTS5*, *MMP13*, etc.).[61, 62]

During ongoing OA, expression of *TNFRSF11B* is increased in cartilage (chapter 2) which may contribute to the mineralized phenotype of arthritic cartilage. This increased expression during OA pathophysiology is in line with the identification of a gain of function mutation as probably causal in a family with early onset OA. It has been shown that the promoter of *TNFRSF11B* contains a thyroid binding element.[59] This could be underlying the increased expression of *TNFRSF11B* in arthritic cartilage since also *DIO2* expression is increased and thus the thyroid signaling. In addition, it could be speculated that augmented thyroid signaling not only predisposes to OA via mechanisms that are detrimental to the cartilage, but also via increased bone remodeling. In fact, arthritic bone was also found to express increased levels of *TNFRSF11B*.[63]

The increased bone remodeling in OA triggers formation of channels which extend from the subchondral bone into the articular cartilage thereby breaching the tidemark. These channels allow for increased vascularization which is frequently accompanied by innervation, both of sympathetic and of sensory nerves.[64] Among others, this results in increased nerve growth factor expression within vascular channels and may explain that, besides genes involved in bone development, GWAS and gene expression analyses also have identified multiple genes involved in neurogenesis. Alternatively, it could link to aberrant neurogenesis during development with a concurrent irregular locomotor system. This would predispose to OA during aging as a result from an accumulation of joint damage.

In line with this, in mice targeted deletion of *SLC12A6*, identified in our GWAS as a putative OA susceptibility gene (chapter 5), leads to a locomotor and a sensorimotor gating deficit, suggesting a critical role for *SLC12A6* in development of the nervous system.[65, 66]

Blood expression profiles of OA patients from the GARP study were found to be enriched for genes involved in apoptosis (chapter 3). This is striking given previous observations of increased apoptosis in OA cartilage at early as well as at late stages[67] despite the fact that OA cartilage expression profiles were not enriched for apoptosis-related genes (chapter 2). However, it should be noted that the study had a cross sectional design. Consequently, gene expression differences between cases and controls can be both, the result of OA pathophysiological processes or innate processes. Until further studies have been performed the blood expression profiles should only be used for diagnosis but not for prediction or prognosis. In addition, it is known that multiple interactions exist between apoptotic genes and cytokines (e.g. *IL1B* and *IL8*, also present in the blood expression profiles). Therefore, it is also possible that the apoptotic genes rather reflect an inflammatory state which is also recognized in OA patients.[14]

COMING FROM, GOING TO: FUNCTIONAL GENOMICS

Although the number of published genetic studies that lack experimental data showing the biological relevance of identified genetic variation is still large, in the field of OA the step from genetics to biological function, also named 'functional genomics', has currently started to be implemented.[48, 54, 68, 69] To proceed and improve the approaches taken we propose a combination of those suggested by Freedman *et al*[50] and by Sunyaev[70] (Figure 2), which are similar to those applied in this thesis for OA.

Knowledge of the direction of effects of the susceptibility locus and understanding the functional consequences of the genetic variation of candidate genes is required before translation to the clinic can be considered. Alterations in gene expression most likely originate from changes in epigenetic control mechanisms[51, 71] or from changes in transcription factor binding sites[72] as generally acknowledged and outlined previously.[50, 73] Therefore, a first approach especially interesting for intronic or intergenic SNPs is to investigate whether genetic variation at the identified locus results in differential allelic expression (DAE) of the susceptibility gene as was done for example for *GDF5*,[74] *ALDH1A2*,[54] and *DIO2* by our own group.[52] Alternatively, for high impact protein variants, the effect of the variant needs to be established starting from *in silico* analysis of the predicted impact of the variant (more specifically for protein mutations), to proceed via *in vitro* cell models to the *in vivo* effects.

In vitro cell models in OA. *In vitro* cell models can be broadly applied and are especially suitable to investigate transcriptional regulation of a gene of interest and the consequences of genetic variation[69, 71, 72] which eventually may serve to interfere with its regulation in a clinical setting. Cell models can also be used to determine the gene targeted by the susceptibility locus: Chromosome Confor-

Table 2 Comparison of different cell models

	cell lines	primary cells	iPS cells
lifespan	Unlimited	Limited	Unlimited
transformation	Yes	No	No
donor variation	No	Yes	No
phenotypic stable	Yes	No	Yes
3D pellet model doable	No	Yes	Yes
experiment duration	3 weeks	3 months	3 months

mation Capture (3C), Circularized Chromosome Conformation Capture (4C), or Carbon-Copy Chromosome Conformation Capture (5C) can be applied to explore chromosome folding and regions of the genome interacting with the site of interest. In fact, the use of 4C sequencing has been of valuable help in the identification of *IRX3* as the gene genetically targeted by the obesity associated locus within the *FTO* gene.[49]

Besides the study of gene and protein perturbations to obtain mechanistic insight of OA risk genes, cell models can be used to select drugs or biologicals that *in vitro* counteract the detrimental effect of the mutations and may therefore also be selected for *in vivo* studies. In chapter 7 this was done to investigate the effects of counteracting the thyroid hormone pathway on deposition of cartilage extracellular matrix. Iopanoic acid (IOP), a pharmacological inhibitor of deiodinases, was added to our 3-dimensional (3D) pellet cultures and was found to be beneficial: upregulation of catabolic enzymes was attenuated, while the COL2A1/COL1A1 ratio was more stable and cartilage matrix structure denser leading to prolonged 'healthy' cartilage homeostasis. Although encouraging, results await confirmation of the effects of IOP on articular cartilage *in vivo*. IOP belongs to the so-called 'oral cholecystographic agents', originally used as alternative contrast agent to identify gallbladder pathology.[75] The drug is primarily a potent inhibitor of the conversion of peripheral serum inactive thyroid (T4) to active thyroid (T3) making it an effective medication for the control of hyperthyroidism and thyrotoxicosis. IOP appears to have few side effects within the window of its application, however, long-term effects and consequences for joint health following oral use remain to be established. This should first be tested in mice and can be combined with methods used to induce OA to investigate whether intake of IOP reduces development of the disease. In addition, for the long-term use it could be an advantage to screen for small molecules that act more specifically to inhibit DIO2 but not the other deiodeninases.

Different cell models can also be employed to investigate the effect of strong OA candidates. In these studies, the most important choice is which cells to use. The easy way out is to use established cell lines such as the mouse ATDC5 (chondrogenic) or MC3T3 (osteogenic) cells which, typically, are easy to obtain and maintain promoting that studies can be performed within a relatively short period of time (around 3 weeks). However, within the scope of this thesis, we have used primary human cells since established cell lines are usually transformed and gene expression is likely

modified during the transformation process. Furthermore, in cell lines the association between growth and differentiation may be altered and they have frequently accumulated mutations in the course of the extended proliferation thereby changing the responses such that they do not necessarily reflect the effect of gene variations *in vivo* in humans. Challenges of primary cells such as their phenotypic instability and limited lifespan thereby restricting study possibilities can be circumvented by using induced pluripotent stem cells (iPS) that provides a stable source of cells and which can also be differentiated towards multiple lineages including chondrogenesis and osteogenesis,[76] reflecting more likely the effects of genetic variants in men. Table 2 summarizes properties if the different types of cells.

For models of chondrogenesis it was shown previously that monolayer chondrocyte cultures mainly result in a hypertrophic phenotype,[77] thereby hampering the investigation of the effects of variable expression of the gene of interest. We observed the same for the ATDC5 cells, and attempts to form 3D pellets with ATDC5 cells similar to those formed with human bone marrow derived mesenchymal stem cells (hBMSCs) failed (data not shown). Therefore, we think that to study chondrogenesis the best option is to perform 3D pellet cultures with hBMSCs (chapter 7) or with primary human chondrocytes. To study the effect of the OPG mutation (chapter 6) we also used primary human cells for the generation of mature osteoclasts: commercially available pre-osteoclasts. An alternative for the use of the commercial pre-osteoclasts could be blood mononuclear cells that also can be differentiated towards osteoclasts, but in these cells we observed enormous donor variation and therefore this option was discarded [YFMR & AE Oostlander, unpublished data].

Cell lines are less well fitted to learn about the organic consequences on the joint as a whole since the different tissues actively interact with each other during development and disease. Therefore, likely more suitable although not applied in this thesis is the performance of cocultures. Indirect cocultures can be applied by exchange of conditioned medium as well as by culturing cells in transwell chambers that allow the diffusion of secreted factors to investigate effects on cell function, extracellular matrix deposition, and survival. Direct cocultures can be performed by using different combinations of mesenchymal stem cells, chondrocytes, osteoblasts, explants or synoviocytes. Frequently applied in studies for development of tissue regeneration and replacements suitable for implantation is the combination of mesenchymal stem cells and chondrocytes.[78-81] Of note are some studies in which even multiple-lineage coculture models were employed. Recently, in the lab of R. Tuan a 3-dimensional 'microtissue' was developed suitable to study OA pathogenesis.[82] The model system can also be used to evaluate the effects of different reagents and compounds on osteochondral health.[83] He *et al*[84] established cultures of osteoblasts, mesenchymal stem cells, and fibroblasts on a hybrid silk scaffold to investigate whether BMSCs cocultured in the presence of ligament and bone cells would differentiate into fibrocartilage, and Leyh *et al*[85] showed that mechanical and biochemical properties of extracellular matrix deposited by mesenchymal stem cells and chondrocytes is impaired in the presence of OA cartilage

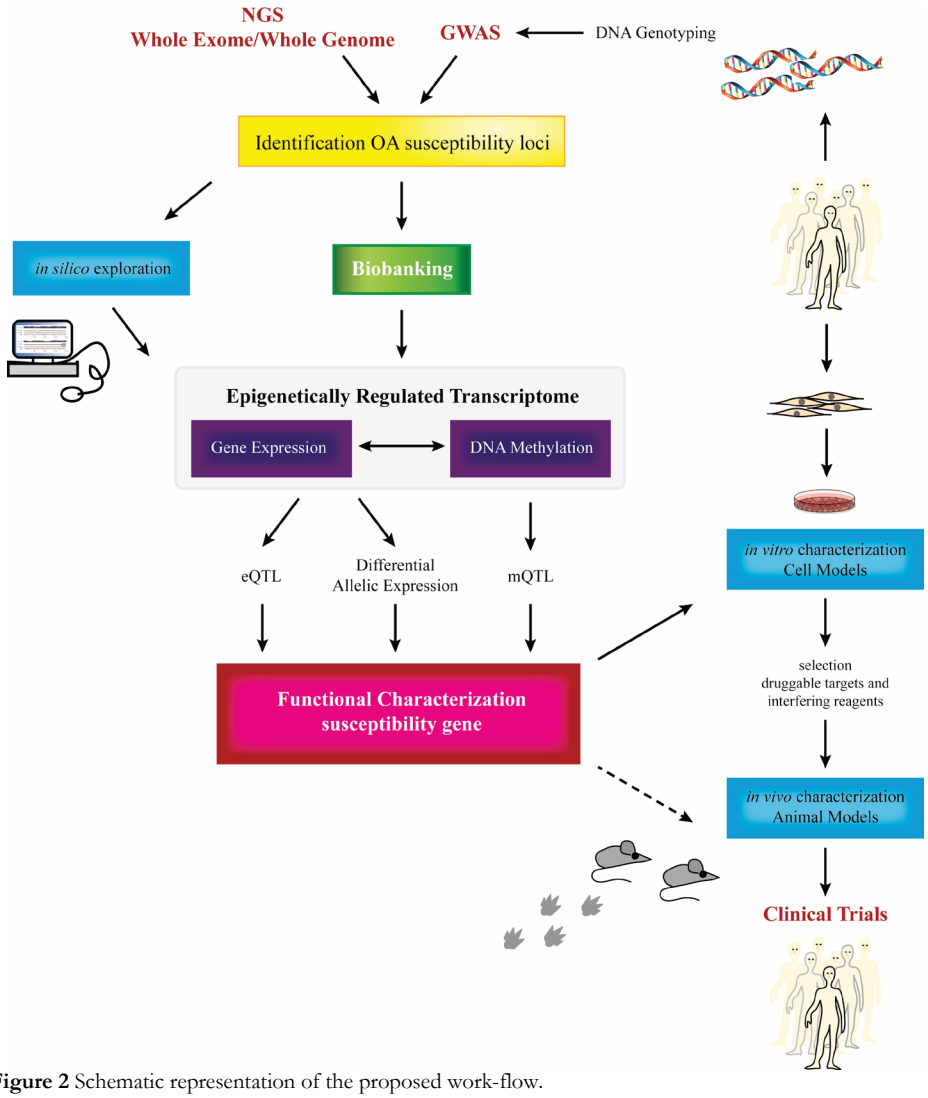

Figure 2 Schematic representation of the proposed work-flow.

explants due to altered fibrillar network. To study the interaction between cartilage and bone, chondrocytes-osteoblasts-osteoclasts, isolation of explants from patients undergoing joint replacement surgery may also be a good alternative.[86] These multi-lineage 3D *in vitro* models are the 'last best' option before getting to animal models.[87]

In vivo animal models in OA. As stated by Sunyaev *et al*[70] genetic manipulation of organisms (mouse, rat, rabbit, horse, zebrafish) provide a possibility to test the phenotypic rather than molecular consequences of human allelic variants, which is by some considered the ultimate proof for the causal effect of a susceptibility locus. Animal models can also be used to investigate new treatment options and to test

compounds selected because of their positive effect in *in vitro* assays as discussed before for IOP. Use of drugs with encouraging outcome, *i.e.* inhibited or reversed disease severity, can subsequently be put forward in clinical trials.

Of the different types of laboratory animals, mice are used more frequently because they are fairly cheap, and relatively easy to handle and to breed. Although multiple different mouse strains have been generated for candidate genes, known to be involved in chondrogenesis or endochondral ossification, this has hardly been done for discovered OA susceptibility genes. An exception has been the generation of *FRZB* knockout mice of which its characteristics were studied using established OA models.[88, 89] *GDF5* knockout mice were generated long before the identification of the *GDF5* susceptibility locus and were post-hoc used to study development of OA following different established methods (reviewed by Cornelis *et al*, 2011[90]). Also *DIO2* knockout mice were generated before discovery of the association with OA to investigate the intracellular effect of thyroid hormone bio-availability with respect to brain function.[91] So far, no studies applying OA models on mice have been published but transgenic rats overexpressing *DIO2* in articular cartilage were shown to be more prone to cartilage damage,[92] thereby confirming that the detected overexpression of *DIO2* in OA cartilage[52] is most likely not only the result of OA pathophysiology but may underlie disease development. Results presented in chapter 7 suggest that inhibition of the deiodinases is protective in cartilage, however, this awaits confirmation in *in vivo* studies to determine whether counteracting the thyroid signaling pathway may be a novel therapeutic approach.

Although not identified as an OA susceptibility gene, in 2005, a study was published reporting for the first time on a single gene deletion, *ADAMTS5*, that resulted in the abrogation of cartilage degeneration in an animal model of osteoarthritis.[93] Many other mouse models for OA have followed since, such as for HIF-2α,[61, 62] and for TGF-β,[94] and most of the recently developed mouse models are inducible and chondrocyte-specific such as the transgenic BMP2 mice[95] or the mTOR knockout mice.[96] This allows controlled expression of the gene of interest: during embryogenesis or post-natal, prior to injury or after the induction of OA. Most transgenic or knockout mice do not spontaneously develop OA but its development is provoked by means of surgery (destabilization of the medial meniscus or DMM; cruciate ligament transection), with enzymes or chemical substances (intra-articular collagenase injections), or by mechanical loading (Figure 2). An exception are STR/Ort mice that upon aging spontaneously develop OA with unknown cause,[97] and the recently generated cartilage-specific double knockout mice Nfatc1/Nfatc2-/- which exhibit early-onset, aggressive OA affecting multiple joints.[98] According to the authors this is highly favorable in OA research since it reduces the time-cost ratio of the model and does not require further surgical or chemical intervention. However, the fact that these mice are extremely prone to develop OA shortly after birth points out that it is a very specific model system, more resembling cases of early-onset OA during development than the slowly progressing 'normal' primary

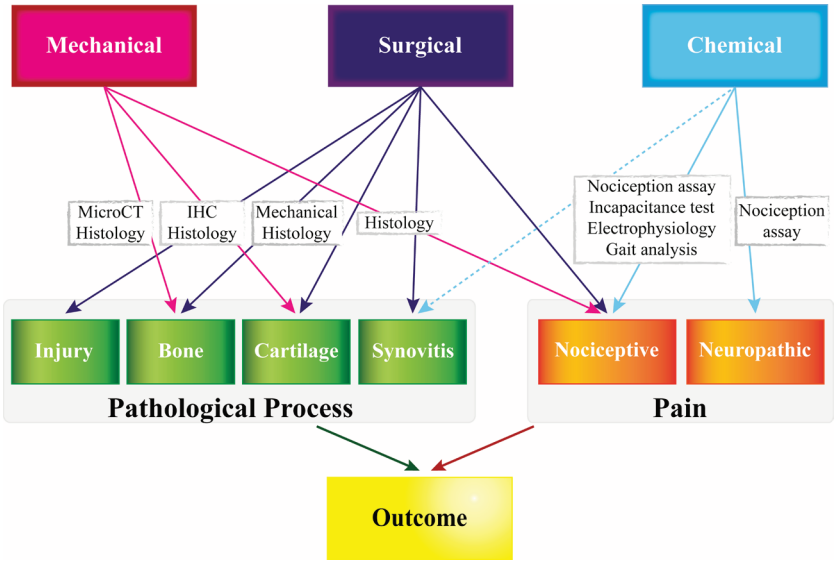

Figure 3. Animal models of OA and methods of assessment (adapted from Vincent et al 2012[89]).

OA at older age. We should be more aware of the susceptibility loci that are identified in genetic studies and generate mice with these variants to functionally characterize the effects of this 'natural' variation as it occurs in humans.

It is of note that, although destabilization of the medial meniscus was called 'minimally traumatic'[99] and here development of OA is less 'aggressive' than when using enzymes, it is yet more similar to the type of osteoarthritis developing in humans after an injurious event better known as secondary or post-traumatic OA.[100, 101] In addition, as reviewed by Aigner *et al*,[102] the pathophysiology and severity of the different methods used to induce OA are diverse and thus far no paradigm has been formulated that encompasses all of its aspects in particular its relation to aging which is an important risk factor in OA. This is not surprising since we know that OA itself is a heterogeneous disease. We should therefore take advantage of the differences between the different methods and, in case economic possibilities permit, try to include aging in the mouse models. Personally, I think mechanical loading (running) is the most appropriate stimulus since it provides a subtle way to induce OA and in particular when performed at different ages (young versus old mice) it may largely resemble the human situation.

FINAL REMARKS AND SUGGESTIONS FOR FUTURE STUDIES

To improve the clinical application of biochemical markers in OA, the genetic factors identified for sCOMP and for uCTX-II should be taken into account. In addition, it should be considered to perform further meta-analyses of GWAS for other promising biomarkers such as serum hyaluronan and serum Procollagen type II N-terminal propeptide (sPIIANP). Additionally, these efforts could identify genetic loci that

influence the OA biomarker levels as well as OA (endo)phenotypes thereby actually reflecting OA status as we have found for the *COMP* gene and sCOMP levels (chapter 4). The use of gene expression profiles which in our hands seemed very promising to distinguish patients from controls could be further analyzed. These profiles should be examined in prospective studies to establish whether the biomarkers are predictive for OA. Alternatively, in case the expression profiles in blood correlate to expression of respective genes in cartilage its change over time could be investigated which would learn whether the profiles are useful as markers to monitor the ongoing disease process.

With respect to genetic studies, given the fact that scientists have been able to collect large numbers of samples by collaborations towards large consortia, we have been able to discover several compelling OA susceptibility loci. To come to clinical applications and druggable targets, the next step in these analyses should be taken. The most recent large-scale analyses were done upon stratification for joint resulting in additional, joint-specific, OA susceptibility loci.[26, 28] We could move forward by performing rigorous phenotyping to identify more homogeneous subtypes for selection of samples as suggested previously by Kerkhof and colleagues.[103] It would be very interesting to investigate whether performing GWAS for the different OA phenotypes as distinguished by Castaño-Betancourt *et al* (atrophic, normotrophic, and hypertrophic[47]) would point towards variation in different genetic loci. Nevertheless, it is likely that the problem will be to replicate the findings since few groups have collected or characterized such particular phenotypes. Depending on the effect sizes of such less heterogenic groups, this may result in lack of power and thus genome wide significant loci may not be found. In fact, this occurred with the GWAS analysis performed within the GARP study (chapter 5). It could be questioned how many of the suggestive loci contain genes relevant for the trait. And instead of only focusing on p-values for 'genome wide significance' we may as well investigate potential susceptibility loci in the neighborhood of compelling genes at multiple biological levels before considering these loci false positives. This approach could very well open doors to interesting new targets in drug research. Functional studies are highly time consuming, however, hence applying balanced selection criteria based on both statistical and biological evidence may be more optimal.

In the course of the studies described in this thesis we have tried to make a step forward in functional genomics. In particular, this was performed by setting up *in vitro* model systems to functionally characterize identified mutations in the *TNFRSF11B* (chapter 6) and the *DIO2* gene (chapter 7), and these can be used in future studies to characterize other genes and variants such as *NCOA3*[28] and *ALDH1A2*.[54] Besides the models used in the course of this thesis, for further investigations I suggest investment in the development of co-culture models in which the interaction between different cell lineages can be studied in more detail since the interactions between the different joint tissues have a high impact on development of OA. This may also be very helpful in drug discovery research, since it can be investigated whether particular drugs have opposite direction of effects on different types of cells which is very important before initializing *in vivo* experiments.

In conclusion, the doors are open towards improvement of genetic studies and their interpretation and towards development of new strategies for the application of functional genomics in OA.

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