On the relation between genetic variation and osteoarthritis

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GENERAL DISCUSSION

MAIN AIMS

The main aims of this thesis were to address several challenges OA research is currently opposed to. Firstly, given that the apparent link between OA associated genetic variation and gene expression, possibly mediated by CpG methylation, is frequently reported on by candidate gene studies (1–5), we set out to identify novel genetic variation that marks AI in articular cartilage on a transcriptome wide scale. In **chapter 2**, we performed genetic association analyses for OA with SNPs that both marked AI and were differentially expressed between preserved and paired OA affected cartilage. By doing so, we exploited the decreased multiple testing penalty that inherently obstructs canonical GWA studies and reported on novel SNPs that appear to predispose for OA. Further expansion on these *in-silico* derived results by *in-vitro* experiments revealed a potential causally degenerative mechanism by which genetic variation at and upregulation of the *CRLF1* gene predisposes for OA. Furthermore, in **chapter 3** we showed that the generated database of articular cartilage AI SNPs can contribute to canonical GWA studies, delivering increased mechanistic insight of OA susceptibility alleles.

Secondly, whereas it appears eminent that dysregulation of gene expression is linked with OA pathophysiology in articular cartilage, it remains unclear how these altered levels of transcription are brought about. Hence, in light of the described relation between the established OA susceptibility SNP rs225014 (6) and allele dependent transcription of *DIO2* in articular cartilage (5), we explored the regulatory properties of the gene and elaborate on the link between genetic variation, local DNA methylation levels and their influence on *DIO2* expression in an OA context in **chapter 4** (7).

Thirdly, given that DNA methylation appears to be involved in mediating the relation between OA associated genetic variation and gene expression levels (8–10), the methylome has gained increased interest among OA researchers (2,11–14). Additionally, OA associated methylomic profiles might pose an arguable candidate for maintaining the pathological transcriptomic profiles observed in OA affected articular cartilage (15–20). This is reflected by elaborate papers that have reported on extensive numbers of CpGs that mark the ongoing disease process by differential methylation between OA lesioned and preserved or healthy articular cartilage (21–23). Nonetheless, at the beginning of the research project described in this thesis, it remained unclear how to interpret these methylation differences in context of OA related transcriptomic profiles. By integration of (semi) genome wide SNP, methylation and expression data in **chapter 5** we reported on CpGs and SNPs that show presumptive evidence for (dys)regulating gene expression in OA affected articular cartilage. During these academic pursuits we encountered distinctly differing methylomic profiles between hip and knee articular cartilage, irrespective of the tissue's OA affection state. While considerable overlapping methylomic OA related differences were observed among both knee and hip samples, the presence

of marked joint specific profiles might indicate that the actual tissues should be viewed as distinct. These observations and possible implications are described in **chapter 6**.

GENETIC VARIATION AFFECTING GENE EXPRESSION IN ARTICULAR CARTILAGE

Despite our increased mechanistic understanding of established OA susceptibility genes in relation to the disease, a significant challenge that remains unaddressed is the substantial amount of missing heritability (24–27). In part, this can be explained by the existence of considerable phenotypic heterogeneity among OA patients. While this could be addressed by deeper phenotyping of possible OA subtypes, another approach to increase the likelihood of detecting unknown OA associated genetic variation is to adequately reduce the number of statistical tests by *a priori* discarding SNPs that are deemed less likely to contribute to OA pathophysiology, as described in **chapter 2**.

Having acquired whole transcriptome RNA-sequencing data from articular cartilage of primary OA patients (chapter 2 **Supplementary Table S1**), we exploited the mRNA sequence information to identify SNPs that mark imbalanced expression of the respective genes they are located in. Among a total of 13853 transcribed heterozygous SNPs (>25 counts and present in at least two individuals), we identified 2070 located in 1031 unique genes that significantly did so. Next, we analyzed whether these 1031 genes were in addition be subject to AI also significantly differentially expressed between preserved and paired OA affected cartilage. Indeed, 32 genes appeared to do so and we subsequently performed genetic association analysis for OA with the top ten AI SNPs (single most significant SNP per gene). Having substantially reduced the multiple testing correction penalty implied in canonical GWA studies, we were able to identify an otherwise discarded association signal, located in the *CRLF1* gene.

In light of the acquired regulatory, transcriptional and genetic evidence as well as having these technically and/or biologically replicated, we initiated mechanistic *in-vitro* studies addressing the suspected relation between *CRLF1* and OA. The respective protein products of *CRLF1* and its reported binding partner *CLCF1* assert their function through the ciliary neurotrophic factor receptor, encoded by the *CNTFR* gene. We treated primary human chondrocyte cultures with the heterodimerized protein complex and observed a marked upregulation of anabolic genes, while catabolic genes remained unaffected. Arguably paradoxical, Tsuritani *et al.* (2010) reported downregulation of anabolic and no effect on catabolic genes in similar experiments using a chondrogenic mouse cancer cell line (ATDC5) (28). Whether these apparent opposing results are due to species differences and/or a consequence of utilizing distinct cell types remains subject of future research, but it appears *CRLF1* is involved in establishing and/or maintaining OA associated, aberrant cartilage homeostasis.

Apart from the exploratory nature of chapter 2, the RNA sequencing dataset generated therein also complements ongoing GWA approaches, as we describe in **chapter 3**. In a GWA study and combined meta-analyses for replication on bilateral hand OA, we identified several coding variants within the *MGP* gene. By lack of hand articular cartilage samples, we interrogated the knee and hip articular cartilage RNA sequencing dataset and found significant AI of the *MGP* gene, marked by alleles of the OA associated SNP. Custom targeted assays were performed and validated this observation, not only in articular cartilage, but also in subchondral bone. Given that *MGP* is reported to be an inhibitor of cartilage calcification (29–32), high expression in articular cartilage needs to be warranted in order to maintain healthy tissue homeostasis. In concordance with the OA predisposing property of the identified risk allele, we found lower expression of *MGP* marked by this allele among heterozygotes, suggesting that carriers are more prone to develop OA due to their inability to halt cartilage calcification, a well described pathological manifestation of ongoing OA. Of note, in light of the observed imbalanced *MGP* expression marked by OA risk alleles in articular cartilage of knee and hip joints, one could hypothesize that these alleles are therefor likely to harbor a genetic association with other types of OA as well, in addition to the initial quantitative association with bilateral hand OA. While a lookup in hip and knee OA GWA data did not satisfy these expectations, one could argue that the observed AI is systemically present among multiple tissues, but exerts its downstream effects only in those that are susceptible to it. In parallel, the phenotypic heterogeneity among hip and knee OA patients might be larger when compared to patients in which bilateral hand OA was measured quantitatively.

In addition to the RNA sequencing dataset's successful applications described above, we were unable to replicate some other reported AI SNPs from literature in the context of OA affected articular cartilage. While we were able to verify AI of rs3204689 (risk allele C marked significant lower expression of *ALDH1A2*, Styrkarsdottir *et al*. (2015) (45)) as well as suggestive AI of rs225014 (3/4 heterozygotes revealed higher *DIO2* expression of the risk allele C, Bos *et al.* (2012) (5)), other genes reported to be subject to AI in articular cartilage such as *COL11A1* (rs1676486) (1), *GNL3* (rs11177) (3) and *SPCS1* (rs6617) (3) were not observed as such in our dataset. The absence of those SNPs among our results might have arisen due multiple reasons. Firstly, we were bound to address AI with the amount of respective heterozygotes present in our data, whereas the initial reports had gathered larger samples sizes. Secondly, the alleles of AI SNPs are not by definition the mechanistically driving source leading towards the observed transcriptional imbalance. In other words, the actual genetic variation that interferes with the transcription machinery might be positioned in relatively high LD elsewhere, for example in close or distal regulatory elements. If LD between the addressed SNP and respective regulatory element is low, however, one could expect inconsistent results and consequentially face difficulty in replicating earlier observations.

OA ASSOCIATED DNA METHYLATION PATTERNS

Given the large number of OA associated SNPs that seem to exert predisposition for OA through AI in cartilage, as well as the marked number if differentially expressed genes in OA affected cartilage when compared to control, we set out to interrogate possible regulatory mechanisms that might mediate these observations.

Although (semi-)genome wide technologies and respective analysis tools are rapidly emerging as affordable and accessible (33–37), the OA research field has also gained substantial knowledge from gene targeted measurements of regulatory mechanisms and expression in recent years. In this regard, the rs225014 T>C SNP located within the *DIO2* gene might confer susceptibility towards OA by multiple mechanisms, as it both marks an amino change (threonine to alanine), as well as AI of *DIO2* in articular cartilage as shown by Bos *et al*. (2012) (5). Given that the resulting amino acid change induced by the rs225014 OA susceptibility allele C did not directly result in altered turnover of inactive (T4) into active thyroid hormone (T3) (29), susceptibility seems mediated through increased *DIO2* expression, presumably leading to higher protein levels, marked by the rs225014 risk allele. Additionally, it was reported that *DIO2* is significantly higher expressed in articular cartilage among OA patients when compared to that of healthy controls (5). Hence, in **chapter 4** we set out to elucidate how *DIO2* expression is regulated in articular cartilage. Publically available CHiP-Seq data showed that CTCF, a DNA binding protein and regulator of gene expression, binds at the rs225014 locus in multiple cell types (ENCODE consortium) (38). Hence, we assessed whether the rs225014 alleles influence binding of CTCF in cultured primary human chondrocytes. We observed that CTCF protein does bind the DNA at the rs225014 locus in chondrocytes, however no relation between the respective alleles and the amount of bound CTCF was observed. Nonetheless, while allele dependent CTCF binding did not appear to explain the observed *DIO2* AI in articular cartilage, we did observe that three CpG dinucleotides were significantly differentially methylated between macroscopically preserved and paired lesioned articular cartilage of 52 OA patients (29 knee, 22 hip), independent of gender, age and affected joint. Among these, a single CpG dinucleotide >2kb upstream of the *DIO2* transcription start site associated significantly with *DIO2* expression. Interestingly, whereas overall this CpG is hypermethylated in OA affected compared to paired preserved cartilage, OA associated hypermethylation among carriers of the rs225014 risk allele C is significantly more pronounced when compared to homozygous wildtype carriers. Accordingly, *DIO2* expression was expressed higher in OA articular cartilage in general, but even more so among rs225014 risk allele carriers. These observations imply that both genetic and epigenetic variation at OA susceptibility loci, as well as the apparent interaction between the two, are mechanisms worthwhile to assess in order to deepen our understanding of OA associated gene expression in articular cartilage. This statement is further strengthened by efforts from other academic research groups studying these apparent regulatory

relationships. For example, Roach *et al.* (2005) reported that OA associated upregulation of multiple ECM degrading MMPs was marked by demethylation of CpGs located in respective promotor regions (39), Reynard *et al*. (2013, 2015) showed that DNA methylation associated with both basal expression as well as AI of *GDF5* marked by alleles of the OA susceptibility rs143383 (C>T) SNP (2,11) and Takahashi *et al*. (2015) has revealed that OA associated upregulation of *IL8* is accompanied by differences in DNA methylation at the gene locus (40). As such, probing the relation between genetic, epigenetic and transcriptional features, has delivered valuable insight into mechanisms of pathological gene expression observed in OA affected articular cartilage.

It deservers mentioning that when descriptive measurements are performed as described above it is inherently challenging to assert causality towards DNA methylation in terms of it leading to differences in expression. In addition, it remains unclear as to whether upregulation of *DIO2*, or other disease responsive genes for that matter, is causing OA associated cartilage degradation as opposed to *vice versa*. Due these matters, which are inherent to paired cross-sectional study designs, we will also be unable to detect genes involved in the disease's onset. This could arguably be addressed to certain extent by comparing articular cartilage transcriptional and/or methylation profiles across affected and unaffected individuals. This would, however, oppose another challenge by means of the distinct genetic backgrounds of the respective individuals. Mendelian randomization might additionally offer insight here into, but unfortunately requires substantially larger samples sizes than we were able to address here.

METHYLOMIC PROFILING OF OA AFFECTED ARTICULAR CARTILAGE

The development of affordable omics technologies (*e.g.* microarrays and next generation sequencing) has substantially influenced the rate at which new molecular biology discoveries are being reported. The OA research field is hereto no exception, and in **chapter 5** we have measured and integrated DNA methylation and gene expression data on semi genome wide scales among preserved and paired OA lesioned cartilage samples, followed by inspection of the respective genetic environment.

Given that the majority of CpGs located at a gene locus do not appear to be associated with expression of the respective gene, we first set out to identify CpGs in articular cartilage that do correlate with gene expression levels. Although a large number of statistical tests were performed, possibly leading to increased false positive results, we allowed ourselves to lessen the canonical multiple testing correction penalty (Bonferroni) when testing for correlations between methylation and expression (41). As opposed to reduce the significance threshold **α** by the total number of statistical tests, we reduced **α** separately for each gene, depending on the number of CpGs annotated to it. As such, we observed 3748 CpGs of which the extent of methylation correlated with expression of 2324 genes

in articular cartilage, independent of age, gender, affected joint and OA status. Interestingly, where increased methylation was canonically associated with decreased expression, we observed CpGs to harbor transcriptional associations in both directions with expression of proximal genes. Specifically we reported on 1741 negative (i.e. decreased expression alongside increased methylation) and 2007 positive correlations (i.e. increased expression alongside increased methylation). These observations indicate that, albeit challenging to assert definite causality, DNA methylation appears involved in general in mediating gene expression levels in articular cartilage. In parallel, however, we additionally observed that less than 3% (N=87) of these CpGs were also differentially methylated between preserved and paired OA affected samples. Interestingly, the genes near these specific CpGs are enriched for developmental pathways, not unlike those observed during endochondral ossification during fetal development. While this might still be a mere reflection of the ongoing pathophysiology, it does support the proposition that in an OA context, chondrocytes appear to dedifferentiate and start dividing, form columnar structures and actively start calcifying the ECM. Whereas chondrocytes are presumably required to actively remodel the ECM upon everyday stresses and potential microtraumas, this remodeling requires dynamic and timely regulation of both catabolic genes as well as ECM anabolic genes, to remain homeostasis. However, perpetual adjustment of DNA methylation is possibly prone to errors and might therefore affect gene expression inappropriately, leading to pathological remodeling of the ECM. Nonetheless, even if we are to refrain from interpreting these results in a directional context, we can state which actively expressed genes and/or pathways seem over- or underrepresented among chondrocytes residing in OA affected articular cartilage. This insight might aid in dissecting possible future therapeutic strategies that aim to revert chondrocytes residing OA affected cartilage back towards a healthy state.

Other groups have also reported on extensive numbers of CpGs that reflect the ongoing OA process in articular cartilage. Fernández-Tajes *et al.* (2013) were among the first to utilize microarrays (Illumina Infinium HumanMethylation27 BeadChip) to address DNA methylation differences in a non-targeted fashion in articular cartilage (22). They observed 91 CpGs that were significantly differentially methylated between knee OA cartilage and articular cartilage derived from fractured hip joints. Interestingly, upon principal component analysis (PCA) knee samples grouped together in two distinct clusters, which appear to be driven by 1357 CpGs that were enriched for inflammatory pathways. Interestingly, 450 genes located in close proximity of those CpGs revealed differential expression between the observed clusters, albeit by utilizing a partially overlapping microarray dataset in terms of samples. Also previous to our efforts, Rushton *et al.* (2014) considerably expanded on the work of Fernández-Tajes *et al.* by addressing over 450.000 CpGs (Illumina Infinium HumanMethylation450 BeadChip) in cartilage from both OA knee and OA hip joints, and also compared the acquired methylation levels to cartilage from fractured hip joints (21). While Rushton *et al.* did not

integrate their findings with gene expression data, they were able to replicate inflammatory enriched clusters, however, only among hip and not among knee OA patients. Furthermore, they observed that independent of the pathological state, hip and knee cartilage seem to harbor joint specific methylation profiles, reflected by 5547 differentially methylated CpGs between the two. Finally, methylation levels of 5322 CpGs comprised a distinct OA hip cartilage profile, when compared to control hip cartilage. These initial reports by Fernández-Tajes *et al.* and Rushton *et al.* have assigned a prominent role to DNA methylation in OA pathophysiology. Nonetheless, these elaborate studies did leave us challenges that we have aimed to address in the current thesis. Firstly, as Rushton *et al.* uncovered the presence of joint specific profiles independent of pathological status, the observed differentially methylated CpGs by Fernández-Tajes *et al.* might partially be driven by inherent joint related differences. Secondly, the presence of mQTLs will interfere when DNA methylation levels of OA cartilage are compared to separate, unrelated controls. And thirdly, although Fernández-Tajes *et al.* did intersect their results with gene expression data, it remained unclear whether there existed a direct correlation between the reported OA associated methylation profiles and respective *in-cis* expression.

Lastly, we integrated our methylation and transcription data with SNP data in order to identify to what extent the local genetic context contributes hereto. By doing so, we identified SNPs of which the alleles correlated with DNA methylation (N=36), transcription (N=26) or both (N=3). Given that mQTL and eQTL analyses generally require ample sample sizes, we cannot rule out the presence of possible false positives, but do propose that these SNPs are enriched for association signals that confer OA susceptibility. Perhaps more surprisingly, we were unable to observe alleles of SNPs that associated with DNA methylation and/or transcription levels as described in chapter 5, as well as additionally mark AI of the respective genes as described in chapter 2. While the lack of overlap could have arisen by a multitude of technical, analytical or even biological causes, and most likely has so due to a combination of them, a clear impediment in comparing the results from these two chapters is the lack of overlapping samples.

JOINT SPECIFIC ARTICULAR CARTILAGE DNA METHYLATION PROFILES

In our efforts to describe general OA related methylomic and associated transcriptomic profiles, we have corrected our analyses performed in chapter 5 for a number of variables, among them the joint from which articular cartilage was sampled. Correcting for joint was deemed necessary, as GWA studies increasingly report on SNPs that predispose for joint specific OA or related phenotypes (42–51). Even more so, in **chapter 6** we describe how hip and knee articular cartilage appear to harbor highly joint specific DNA methylation profiles. Exploratory analysis by means of dimension reduction (principal component analysis, PCA) of generated DNA methylation data revealed that

knee and hip articular cartilage samples formed tight clusters, independent of the tissues' disease state. As such, we applied a sliding window algorithm developed by Slieker *et al.* (52), to determine robust tissue specific DNA methylation profiles.

While stretches of differentially methylated CpGs were found near 245 genes, most strikingly were the substantial differences in homeobox (HOX) clusters and respective co-factors. HOX genes are well known for their role in development and specifically for controlling segmentation of the body plan along the head-tail axis (53,54). Expression of HOX genes during development is regulated in a spatiotemporal pattern, *i.e.* along the head-tail axis the four different HOX clusters express a different number of HOX genes in a linear pattern when development eventuates (55). While in adult tissues this spatiotemporal expression pattern has been described to remain to some extent (56), we found that in adult articular cartilage there is little to no linear correlation between the four HOX clusters. In other words, it appears as though somewhere during development and/or life, specific HOX genes have been activated differently between knee and hip articular cartilage and remain as such. Despite generic processes in knee and hip OA, it thus appears that alongside joint specific OA predisposing SNPs, joint specific DNA methylation and associated gene expression profiles might substantially contribute to OA pathophysiology differences between joints. In this regard, it might be a worthwhile pursuit to address these patterns in articular cartilage from other joints that are frequently affected by OA, such as the spine and hands. Also, these apparent joint related differences in methylation and expression of key developmental genes, raises the question whether efforts to generate cartilage *in-vitro* need to be tailored for the joint they aim to address.

FUTURE PERSPECTIVES

In the last years, we have seen considerable improvements within the biomedical scientific community to scale up experimental setups, both in terms of sample size as well as in the number of measured features, and it can be expected that this will continue on in the near future. It is eminent that these developments will not only aid researchers in directly identifying new disease associated genes, whether they do so by interrogating and/or integrating (epi)genetic variation, altered transcription or other type of omics platforms, but will also allow more complex analyses. Although complex analyses as such might not be the point by which the OA research field will benefit from the most, it is clear that the enormous amount of data that is currently produced across numerous research groups does contain information that is currently not exploited. Consequently, further research into developing appropriate bioinformatic tools to integrate multiple layers of data and sources is required to accurately interpret the descriptive data the field are currently generating, and will additionally open up the possibility to address other (epigenetic) layers of information as well. When able to do so, we might finally be able to thoroughly start investigating the proposed combined

effect of putative common genetic variants that are associated to OA by only relatively small extent. It should be noted, however, that if that is indeed the direction the field is heading towards, we need to accept that mechanistic follow-up experiments will become exceedingly complicated. Both *in-vitro* as well as *in-vivo* studies aimed at elucidating the combinatorial effect of multiple genetic variants or genes will be prone to noise, due to the fact that the background in which the proposed experiment will be performed (e.g. cell type, mouse strain, genetic background, etc.) will have a relatively large impact on the outcome, when compared to single, but large-effect perturbations.

However, seen from the patient's perspective, these proposed developments might not be the most obvious goals to pursue. Increasing our understanding of the complex pathological process of OA will undoubtedly help generate novel and better treatments on the middle to long term, and is a very worthwhile academic pursuit in its own. Nonetheless, dissecting the molecular genetic signals that are associated with OA might just not be the most opportunistic approach at the current time, in the context of developing treatments in the short term. In recent years, we have seen a substantial increase in our ability to generate cell cultures *in-vitro* that resemble the actual tissue *in-vivo*, using patient derived differentiated cells and/or induced pluripotent stem cells (iPSCs) (57,58). From a highly pragmatic point of view, if we are able to (re-)generate articular cartilage, using cells from the actual patient, we might not need a thorough understanding of the complex interplay between environment, genetics and molecular changes that appear to underlie and/or drive OA. Of course, given that we are currently unable to do so, efforts into generating *in-vitro* cell cultures that harbor the desired chondrocytic phenotype will benefit from descriptive studies that report on the dynamic states of articular chondrocytes *in-vivo*, not entirely unlike the chapters in this thesis.

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Nederlandse Samenvatting

Dankwoord

Curriculum vitae

Publicatielijst