On the relation between genetic variation and osteoarthritis

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GENERAL INTRODUCTION

OSTEOARTHRITIS

Osteoarthritis (OA) is a prevalent, degenerative muscoskeletal disease that affects all articular joints, although it is most prominent among hip, knee and the phalangeal joints (1). In 2011 it was estimated that over 1,1 million people in the Netherlands (7%) were visiting a general practitioner due to OA related complaints (2). While present across the entire population, OA predominantly affects the elderly, which is reflected by a disease prevalence under 65 years of age of 3.6% and 3.9% for men and women, whereas above 65 years of age these numbers increase to 20.9% and 36.2%, respectively (**Figure 1**) (2). Hence, in light of the increasing proportion of elderly in western civilization, OA's burden on our society is advancing and will likely keep doing so in the near future.

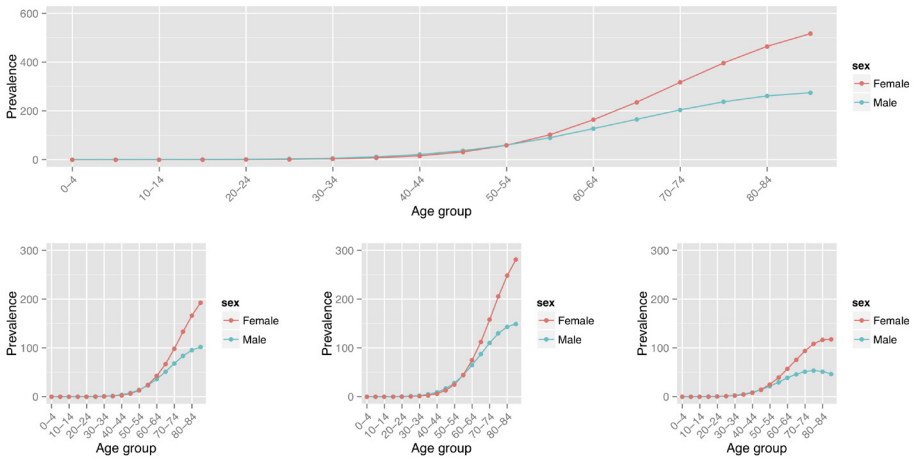

Figure 1. Estimated osteoarthritis prevalence per 1000 inhabitants among the Dutch population in 2011, stratified by gender. (**A**) Overall prevalence of osteoarthritis, measured in all joints. (**B**) Prevalence of hip osteoarthritis. (**C**) Prevalence of knee osteoarthritis. (**D**) Prevalence of hand osteoarthritis. Data from Nationaal Kompas Volksgezondheid 2011 (2).

The disease is principally portrayed by gradual degeneration of cartilage in articular joints, although, in recent years it has become apparent that multiple joint tissues, such as the subchondral bone and synovial membrane, are involved as well (3). Multiple risk factors have been described, and encompass, but are not limited to age, gender (4), body mass index (BMI) (5), joint injuries (6) and genetic predisposition with heritability estimates ranging from 40% to 60% (7). It manifests itself as stiff and painful joints due to joint space narrowing (8), calcified articular cartilage (9), formation of bony outgrowths called osteophytes (10) and remodelling of subchondral bone (11). Presently, no treatment other than pain relief exists and patients are ultimately required to undergo total joint replacement (TJR) surgery to guarantee proper functioning of the joint. While TJR is an effective treatment for end-stage patients, gaining a deeper understanding of the actual biological mechanisms that underlie and/or accompany OA pathophysiology will arguably aid future efforts in developing disease modifying treatments.

Although multiple joint tissues have been implicated in OA pathophysiology (12–14), articular cartilage is allegedly the pivotal tissue affected by the disease. While wear and tear plays a substantial role in cartilage breakdown and thus OA progression, degradation and calcification of the tissue is additionally actively mediated by chondrocytes (15). Chondrocytes, which are thought of to be the single cell type residing in articular cartilage (16), are responsible for maintenance of the extracellular matrix (ECM) and do so by actively breaking down, remodelling and repairing the ECM using a wide variety of both catabolic as well as anabolic proteins and enzymes when they are required to do so (17–21). In order to do so properly throughout life, it is crucial for chondrocytes that after tissue homeostasis is restored upon physical stresses and consequent microtrauma's, they return to their resting state to await reactivation when required. However, chondrocytes residing in OA affected articular cartilage seem to have escaped this perpetual but appropriate cycle of reactivation and resting (15,21,22). Specifically, as OA chondrocytes dedifferentiate, start dividing, form columnar structures and actively start calcifying the ECM. They appear to have lost their articular phenotype and have entered a process that, interestingly, resembles endochondral ossification during fetal development (15,21–26).

OSTEOARTHRITIS ASSOCIATED GENETIC VARIATION

Due to the substantial genetic component of OA, marked by high heritability estimates (7), in recent years, significant efforts have been made to elucidate the complex genetic architecture of the disease (27–46). However, the search for putative genetic variation that might predispose for or protect against the disease has emerged as a considerable challenge. The hereditary nature of OA is therefor still incompletely understood. Association analysis of genetic variation, traditionally performed among candidate genes and more recently by linkage analysis and genome wide association (GWA) studies, has identified a multitude of loci that are involved in OA pathophysiology. Specifically, the presence of common point mutations or single nucleotide polymorphisms (SNPs) in genes such as *GDF5* (31,39,41–43), *FRZB* (39,44,45), *ALDH1A2* (36), *DOT1L* (40,46), *GNL3* (47) and *DIO2* (32) has been associated with in- or decreased prevalence of OA among carriers. As a result, OA is designated a so-called genetically complex disease, i.e. there is no single, common genetic variant that determines whether respective carriers develop generalized OA (48–50). In that regard, uncommon genetic variants, often segregating by Mendelian nature within affected families, have been linked directly with the development of specific OA subtypes (e.g. chondrocalcinosic OA caused by a read-through mutation in the *TNFRSF11B* gene (51)). Whereas these monogenetic forms of OA are generally due to high-impact mutations, the associated effect sizes of SNPs associated with generalized forms of OA are relatively small (50), and as such leading to missed heritability for OA in general.

The nature by which these relatively small genetic effects arise seems to be due modest downstream effects of the associated genes. While these OA associated genetic variations rarely induce structural differences in the resulting protein product, alleles of common OA susceptibility SNPs do frequently mark transcriptional differences of genes in close proximity. Traditionally, SNPs that affect gene expression levels have been addressed by so-called expression quantitative trait locus (eQTL) analysis (52–54), which identified a magnitude of SNPs that affect gene expression of proximal genes marked by allele correlated expression levels. Albeit successful in doing so, eQTL studies require substantial sample sizes to reach statistical significance. In that regard, it has been shown that assessing the extent of imbalance of expressed alleles within heterozygous individuals was shown to be an effective alternative to identify genetic variation that affects gene expression levels using significantly less samples (36,42,55–59). Imbalanced expression of alleles of heterozygous carriers, generally referred to as an allelic imbalance (AI), allele specific expression (ASE) and/or allelic expression (AE), occurs when two alleles of a certain SNP are not expressed to equal extent. Because eQTLs frequently are tissue specific (60–62) and the fact that articular cartilage biobanks are generally of limited size, measuring AI of OA susceptibility SNPs in articular cartilage has gained increasing interest in recent years (36,42,55–59). Multiple common SNP alleles associated with OA have been reported to mark AI in articular cartilage and as such provided mechanistic insight into the putative biological mechanism that underlies the statistical genetic association. For example, it was shown that the risk allele C of rs225014, located in exon 3 of OA susceptibility gene *DIO2*, is expressed to higher extent compared to the wild type allele T in articular cartilage of heterozygous carriers of the SNP (57). Similar results have been reported for other robust OA susceptibility genes such as *GDF5* (42,56) and *ALDH1A2* (36), as well as for genes proximal to SNPs of which it is currently not completely certain which genes are respectively affected (*SPCS1* (55), *GNL3* (55), *COL11A1* (58)).

Nonetheless, as a consequence of these relative small effects governed by generalized OA associated SNPs, attempts to reduce missing heritability for OA is to some extent obstructed in even the largest GWA studies. In parallel, generalized OA arguably comprises a spectrum of subtypes (e.g. affected joint, osteophyte formation and/or presence of joint space narrowing) which might have just partly overlapping genetic determinants. As subtypes are generally combined in GWA studies, true association signals will be diluted into the noisy background. And although testing millions of alleles for association with larger sample sizes and/or deeper phenotypes will presumably yield novel OA associated loci, another worthwhile approach might be reducing the vast number of statistical tests, and thus lowering the multiple testing correction penalty. Selecting for SNPs that are more likely to confer susceptibility, for example by exclusion of SNPs that do not reside in proximity of genes that are involved in articular joint tissue homeostasis, might prove beneficial in this sense.

DISEASE ASSOCIATED GENE EXPRESSION LEVELS

A well described hallmark of ongoing OA is the substantial amount of differentially expressed genes in OA affected cartilage when compared to control cartilage (18,63–72). And while identification of these has traditionally been performed in a gene targeted fashion, advances in transcriptome wide measurements, such as microarray and sequencing technologies, have markedly extended the number of genes that seem to partake in OA pathophysiology. For example, genes involved in extra cellular matrix (ECM) anabolism (e.g. *COL11*, *AGC*, *CILP*, *PRG4*) (17,18), catabolism (e.g. *MMP3*, *MMP9*, *ADAMTS5*) (73), inflammation (e.g. *IL11*, *IL1*, *TREM1*) (73) and development (e.g. *DKK3*, *DIO2*, *GDF5*, *FRZB*) (73,74) have repeatedly been observed to be expressed at different levels in OA affected cartilage. Dynamic expression and subsequent silencing of these genes is arguably required during normal life to cope with everyday physical stresses, consequent microtraumas and to maintain articular cartilage homeostasis in general. Seen with OA pathophysiology, however, it seems as if gene transcription is no longer balanced and tends to favour cartilage catabolism, consequently leading to gradual degradation and calcification of the tissue. In his regard, chondrocytes residing in OA affected articular cartilage appear to be constituently active and no longer able to return to their proposed resting state. Worth mentioning in this context, are the apparent differences between gene expression profiles of distinct joints. Nonetheless, irrespective of the joint of interest, from a pathway point of view OA associated expression profiles reflect the aforementioned shift towards catabolism when compared to unaffected cartilage.

Nevertheless, despite the valuable observations that have resulted from (semi) transcriptome wide profiling of OA affected articular cartilage, it is at the current time not possible to designate the apparent transcriptomic disbalance as either pathologically causal and putatively driving the disease, or consequential and merely constituting a biomarker. While it is eminent that changes in gene expression are compulsory to effectuate degradative remodelling of the ECM, it can be expected that the resulting consequences will in turn affect gene transcription rates in the respective chondrocytes. In human OA research this proposed feedback mechanism opposes a challenge when interpreting results generated from transcriptomic experiments, whereas it is not encountered as such in the context of genetic association analyses. In an attempt to bridge this gap (and while doing so refrain from complex clinical translation of animal and *in-vitro* experiments), studying the underlying mechanisms that might regulate gene expression in human articular cartilage *in-vivo* might just help us in understanding the complexity at hand.

EPIGENETICS IN OSTEOARTHRITIS

Epigenetic mechanisms are traditionally described as heritable modifications of the genome without affecting the actual nucleotide sequence (75,76), although in recent years they have emerged as cellular mechanisms that serve as dynamic regulators of gene expression (77–79). Nonetheless, despite the ambiguous inheritance of epigenetic modifications, its strong correlation with the proximal nucleotide sequence, gene expression levels as well as tissue specificity (80) make it highly applicable for OA research. While a broad spectrum of epigenetic modifiers and levels exist, such as histone modifications (81) and small non-coding RNA expression (82–84), DNA methylation appears studied most intensively (68,85–89). Addition of a methyl group to the 5th carbon atom of cytosines in cytosine-guanine residues (CpGs) within the genomic DNA is known to correlate with gene expression levels, likely due to interference with binding of DNA binding proteins (DBPs) to the genomic DNA (79). Although gene expression is regulated by a vast number of mechanisms, binding of DBPs such as transcription factors is pivotal herein. Additionally, DNA methylation levels at specific positions along the genome are known to be highly tissue specific, partly due to mitotic inheritance of the respective somatic layers during development, but presumably also to maintain cellular differentiation in adult tissues (80). Hence, in light of the strong relation with gene expression as well as constituting distinct tissue profiles, studying DNA methylation seems an appealing endeavour to further dissect the molecular genetic facets of OA (3,90).

Early studies assessing DNA methylation in OA affected articular cartilage have revealed that DNA methylation levels of specific CpGs in or near known OA associated genes often reflect the disease status of the respective tissue. For example, DNA methylation levels of CpGs near multiple matrix metalloproteinases (*MMP3*, *MMP9* and *MMP13*) (91–93), *GDF5* (86,87), *SOX9* (94,95), *IL1* (93,96), *NOS2* (97) and *COL9* (98) were shown to be significantly different between OA affected and unaffected articular cartilage. Notably, these differences in methylation levels coincided with a significant difference in expression levels of the respective genes as well. More recently, multiple studies have reported on (semi-)genome wide DNA methylation profiles of articular cartilage in the context of OA, in part due to the development of affordable genome wide DNA methylation arrays (85,88). By comparing DNA methylation at the methylome level between OA affected and unaffected articular cartilage, numerous differentially methylated CpGs have been reported and confirmed (85,88). However, it is presently still unclear if, and to what extent these CpGs correlate with gene expression levels in articular cartilage. While it was generally accepted that increased methylation or hypermethylation marks decreased transcription of respective genes, an increasing number of CpGs have been reported of which hypermethylation correlates with increased expression. Interestingly, the canonical view of transcriptional downregulation alongside hypermethylation is often observed for CpGs that reside in transcriptional promotors or enhancers, whereas CpGs that do so in opposite

direction are generally located between the transcription start and ending sites of genes (77,79,81). Consequently, given that interpreting cross-sectional DNA methylation differences is challenging on its own, doing so without simultaneous and quantitative knowledge of the transcriptome appears to considerably impede our ability to do so accurately.

SUPERIMPOSING CAUSALITY AND/OR DIRECTIONAL EFFECT

In light of the descriptive nature of the aforementioned transcriptomic and methylomic experiments (i.e. cross-sectional data points), we are inherently refrained from implying causality. This statistical impediment affects *in-vivo* human OA research in multiple ways. Firstly, when we study OA transcriptomic and/or methylomic profiles as described earlier, we cannot differentiate between drivers and markers of the ongoing pathophysiological processes. Secondly, if our interest goes out to understanding the relationship between DNA methylation and gene expression in articular cartilage, we encounter a proposed regulatory feedback loop that prevents us from stating a directional relationship between the two. Within these boundaries, however, identification of CpGs for which their methylation levels quantitatively correlate both with the presence of particular alleles as well as with proximal gene expression, would be indicative of a regulatory relation that propagates from genome to methylome to transcriptome.

AIM AND CONTENT OF THIS THESIS

The here presented thesis aims to address some of the challenges and concerns outlined in the previous sections. As such, the following chapters will elaborate on a number of specific scientific challenges to which the OA research field is currently opposed to.

As candidate gene approaches have resulted in the successful identification of multiple OA predisposing SNPs that exert their disease association through AI in articular cartilage, we have assessed AI on a genome wide scale in articular cartilage. By simultaneously utilizing both transcript sequence as well as abundancy information derived in RNA sequencing data generated from human articular cartilage, **chapter 2** describes a transcriptome wide approach to detect novel SNPs that mark AI in articular cartilage. Subsequently in **chapter 3**, we reveal that the generated data and results in chapter 2 can be used to support ongoing OA GWA studies.

Multiple reports on OA susceptibility SNPs that mark imbalanced expression in articular cartilage of their respective genes have been published, as our group has done so for rs225014 (*DIO2*). Given these observations, we were interested in the regulatory mechanisms that might underlie the reported AI and investigated the role of epigenetic regulation of *DIO2* expression by DNA methylation in knee and hip articular cartilage. **Chapter 4** describes the relation between the alleles of rs225014,

proximal DNA methylation and expression of *DIO2* in articular cartilage. Given the insightful results obtained in chapter 4, we next addressed DNA methylation in articular on a semi-methylome wide scale. In **chapter 5** we integrate methylomic and transcriptomic data derived from preserved and paired OA lesioned articular cartilage and discuss the associations in the context of the genetic background. As part of quality control we observed, to some extent by serendipity, distinct clustering of samples upon dimension reduction of the DNA methylation data described in chapter 5, independent of gender and affection status. **Chapter 6** expands hereon and describes apparent joint specific epigenomic profiles. Consequently, motivated by both the increasing body of literature on DNA methylation research in OA, as well as our own efforts, **chapter 7** summarizes the current status and the putative future perspectives of DNA methylation research in OA.

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