Genes and mediators of inflammation and development in osteoarthritis

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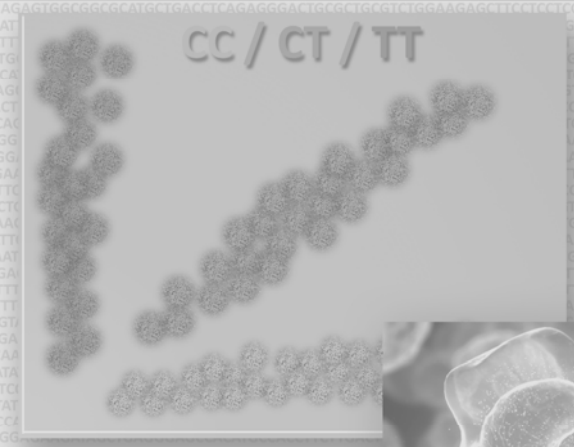

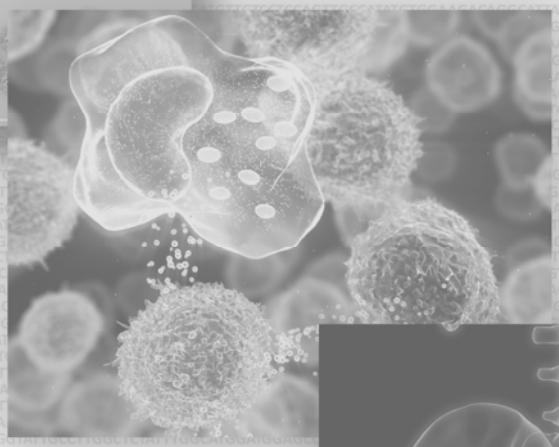

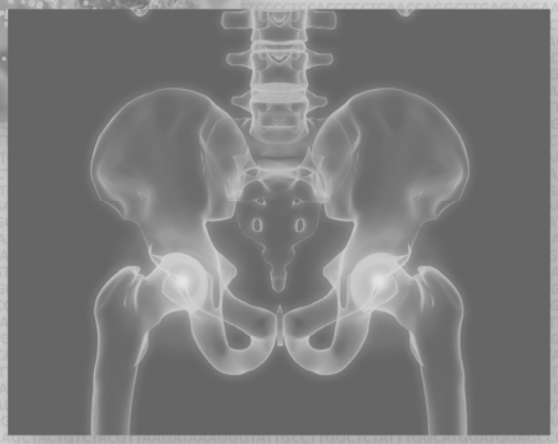

Discussion

4.1 Discussion of results

Osteoarthritis (OA) is a complex disorder with a large heritable component. Research into OA etiology remains a priority to provide adequate therapy for those affected by the disease and to overcome the involved socio-economic burden imposed by the large loss of working force and health care cost. Several risk genes or loci such as *FRZB*, *GDF5, DIO2* and chromosome 7q22 have been identified to date[1,2]; however, these genes only explain a small part of the heritability of the disease. Recent collaborative efforts have proven fruitful in increasing power and should be considered as a positive impulse in OA research[2,3]. These collaborations in addition to previous studies have resulted in the identification of several OA susceptibility genes, in which two pathways appear consistent contributors to the disease. The evidence for genetic contribution to the etiology is most convincing for genes in the inflammatory pathway as well as for genes regulating developmental processes in skeletal formation and maintenance[1,4](Chapter 1). We investigated groups of genes belonging to both pathways; Chapter 2 covered studies where we investigated the relation between genes, inflammatory markers and OA, mainly in the GARP study. The strength of the GARP study is that for these subjects a complete collection of biomaterials such as DNA, serum and urine is available, in addition to extended OA data and demographic data. Subjects are selected for a familial history of OA, thus enriching for the genetic component of OA. This cohort allows investigation of the role of genes in both levels of markers as well as the role of these genes to disease susceptibility. Chapter 3 covered studies that characterize thyroid signaling in general and specifically genetic variation at *DIO2*, which play a role in endochondral ossification during development and for which there is evidence for their involvement in OA[5]. These functional studies have paved a road for functional genomics research for current and future OA susceptibility loci.

4.2 Inflammation in OA

In our studies described in Chapter 2 we tried to investigate the highly complex interaction, illustrated in Figure 1, between inflammatory mediators and OA. In particular, we aimed to distinguish the putative causal (genetic) inflammatory associations influencing OA susceptibility and associations to inflammatory mediators that may mark the ongoing disease process. The general working hypothesis investigated was based on the role of cytokines in normal cartilage metabolism. These pro- and anti-inflammatory cytokines act on the chondrocyte and thereby regulate ongoing catabolic and anabolic processes during both the maintenance and repair of cartilage (Figure 1). Subtle changes in the delicate balance between these mediators might confer a risk to OA and identification of cause and effect beyond an established association is one of the challenges when OA and inflammation are studied. We approached this by measuring levels of inflammatory mediators, testing for genetic variation that might be associated to these mediators and establishing the relation of these variants and OA, touching Mendelian randomization[6] at a small scale. *Ex vivo* cytokine production profiles of LPS stimulated lymphocytes were measured which represent an innate marker of an individual's inflammatory potential to respond to inflammatory challenges throughout life. In addition, circulating levels were used as a measure of current inflammatory status and ongoing disease processes. Genetic configuration of the innate and circulating levels was used to identify possible underlying mechanisms of these inflammatory mediators which might influence OA onset or disease

characteristics. We expected to find high levels of pro-inflammatory cytokines and genetic variants associated to such inflammatory profiles to be predisposing to OA.

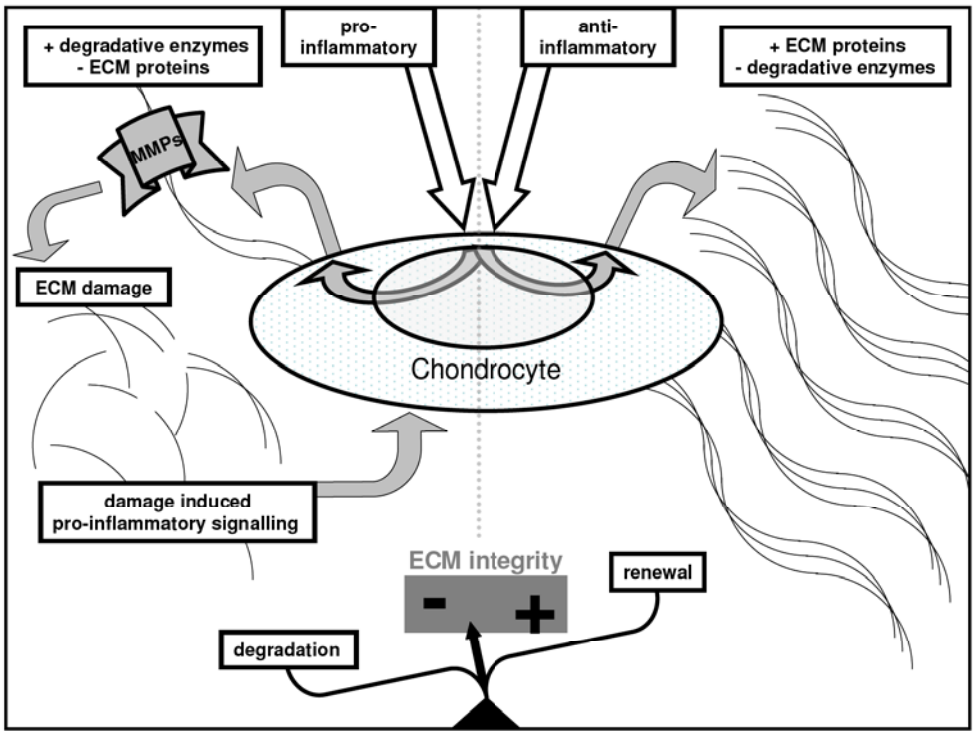

Figure 1. Processes of pro- and anti-inflammatory signaling on the chondrocyte and resulting extracellular matrix (ECM) turnover.

4.2.1 OA, innate IL-1β bio-availability and haplotypes of the IL-1 gene cluster

In line with the general hypothesis, it was previously shown that on average the subjects of the GARP study had a higher innate IL-1β and lower innate IL-10 levels than controls[7,8]. Although not always consistent and determined in relatively small studies, it was also shown that several genetic variants within the IL-1 gene cluster and more consistently the *IL1RN* gene may be responsible for part of the variation in this heritable innate *ex vivo* cytokine production upon LPS stimulation[9,10]. Subsequently, association studies investigated whether these potential functional aspects of the IL-1 gene cluster polymorphisms may explain part of the genetic susceptibility to OA. Compelling associations of the IL-1 gene cluster are reported for knee, hip and hand OA[11-16], however others failed to confirm these associations[17,18]. We have combined *ex vivo* IL-1β bio-availability measures upon LPS stimulation, a large part of the genetic variation at the IL-1 gene cluster and OA disease status in one single study population (the GARP study). In this study, haplotype 2–2–1 (frequency 0.22) covering the *IL1RN* block showed a significant lower bio-availability calculated by the ratio of IL-1β and IL-1Ra as compared to the other *IL1RN* haplotypes and this haplotype associated to subjects (25%) with the highest number of ROA affected joints. Surprisingly, this result would imply that low innate IL-1β bio-

availability predisposes to OA, or that an OA protective effect is associated to high innate IL-1β bio-availability. We hypothesize that when repair of minor damage to the cartilage is needed, a sufficient IL-1β induced release of ECM degrading enzymes is needed for proper clearing of the damaged cartilage. A lower IL-1β bio-availability might leave damaged cartilage strands thereby compromising matrix integrity, whilst the inflammatory signals arising from the sensed cartilage damage remain[19]. Alternatively, feedback loops in IL-1β orchestrated pathways need a high enough stimulus to initiate an anti-inflammatory feedback with subsequent anabolic ECM producing processes[20,21]. The finding of association of lower innate IL-1β bio-availability to OA severity among subjects of the GARP study seems contrasting with respect to a previous observation which showed that subjects of the GARP study as compared to healthy controls had higher innate IL-1β levels[7]. We consider it likely that *ex vivo* cytokine production of lymphocytes is not fully independent of the disease status of the donor, however the fact that the observed association of the haplotype block to lower innate IL-1β bio-availability is observed mainly in subjects with lower ROA scores indicates that this association is not a result of bias introduced through OA severity. These findings are schematically represented in Figure 2. The LPS provoked immune reaction of the lymphocytes in OA affected subjects however, may be sensitized towards pro-inflammatory reactions as a result of the donors' ongoing OA processes for example through upregulation of Toll-like receptors (TLR) on lymphocytes. To further substantiate the value of this finding, the associations need to be explored in a substantial cohort of healthy individuals.

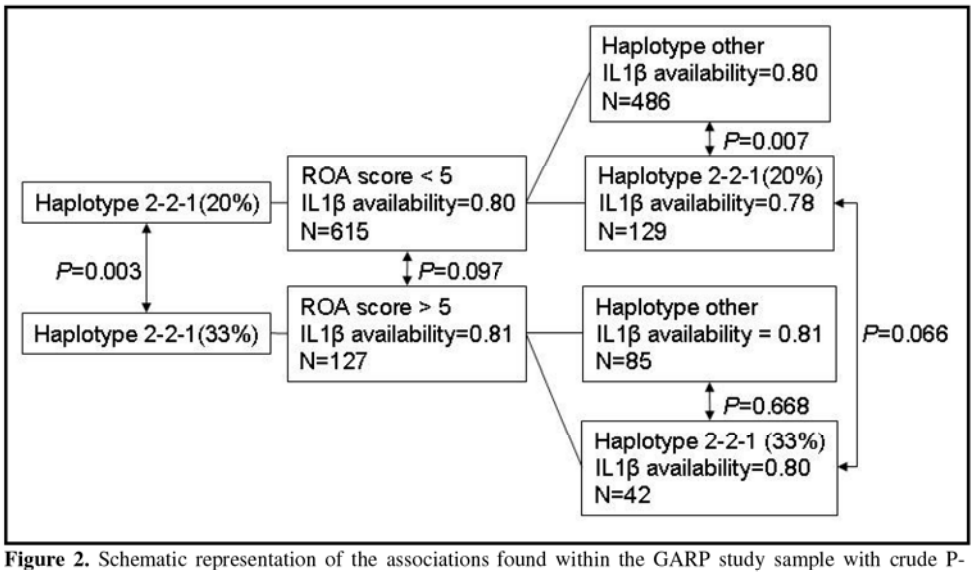

Figure 2. Schematic representation of the associations found within the GARP study sample with crude P-values provided. It is shown that the association between the haplotype and the lower IL-1β availability is mainly due to the effect observed in the subjects that do not have OA at the highest number of joint locations (P value = 0.007) whereas the risk haplotype occurs more frequently among cases with the highest number of joint location with OA (P=0.003). The IL-1β availability in this patient group is, however, higher than expected both in the 2-2-1 haplotype group (0.78 as compared to 0.80, P = 0.066) and in the total group (0.80 as compared 0.81, P =0.097).

4.2.2 Genome wide linkage search to identify putative regulatory genes in immune responses.

As an example, the data discussed in Chapter 2.1 show that although associated to the innate IL-1β level, the genetic variation at *IL1RN* can not explain the full heritability component of this level, indicating that additional regulating genetic variation exists. In Chapter 2.2 we aimed to find additional genes influencing innate immunity of several innate cytokine levels by a genome wide linkage analysis of the GARP sibling pairs. For this analysis we used the assessed LPS stimulated profiles of cytokines (IL-1β, IL-1Ra, IL-10 and TNFα) and data on microsatellite repeats across the genome. Confirmation of initial linkage signals ranging from 2.57 to 3.77 was done by combined linkage-association analysis of genetic variation within new candidate genes using the GARP data and replication was performed using the Leiden 85-Plus study, aiming to find genetic variation influencing innate immunity which was not only affecting OA subjects as present in the GARP study. We identified a SNP (rs6679497) in the *CD53* gene which associated to innate TNFα levels (combined studies P-value < 0.01). The *CD53* gene codes for a cell surface signaling protein, known to be expressed by lymphocytes and involved in the regulation of immune response. Previously, this protein was shown to be downregulated upon activation of neutrophils in response to activating stimuli by e.g. pro-inflammatory cytokines[22] and *CD53* deficiency has been linked to recurrent infectious diseases[23]. We tested whether the SNP was associated to OA in the GARP study as compared to the Leiden 85-Plus study subjects and to rheumatoid arthritis (RA) in a cohort of RA subjects with matched controls (personal communication, Prof. Dr. R. Toes *et al.*, department of Rheumatology, LUMC) however no association to either disease was observed. It should be noted that the SNPs measured at *CD53* tag 52% of the genetic variation recorded in the Hapmap database for this gene. Furthermore, innate TNFα was also not a major factor in OA onset as was shown by Riyazi *et al.* in the GARP study by comparing the GARP subjects to controls[7]. OA progression data in the subsequent studies in GARP subjects indicate a role for TNFα in progression of knee OA[24], however no association to progression (at 2 years) was observed for the *CD53* SNP either (personal communication, Dr. Kloppenburg *et al.*, department of Rheumatology, LUMC). It should be noted that the number of progressing subjects in knee OA was small for genetic association analysis, thereby possibly giving false negative results. Other diseases and disorders, in which the TLR4 pathway that is mainly triggered through the LPS stimulation plays a prominent role might be affected by *CD53* genotypic variation. In addition, no functional role for the identified SNP is known, and it is likely that this SNP is in partial LD to the true functional polymorphism, which might better show possible associations to OA or progression. Alternatively, we might have missed additional genetic variation on this locus of other genes as a result of a knowledge bias for known genes and their function. It is remarkable that in the linkage analysis we detected no linkage signals for innate IL-1β, IL-1Ra and IL-10. Underlying to this may be the use of a patient population, where distribution of levels of at least innate IL-1β and IL-10 might be abnormal as compared to healthy controls, as indicated in Chapter 2.2. However, the use of Merlin-regress[25], which deals with potentially skewed population traits, yielded similar results in our analyses. Most likely, the severe disease status of the most severely affected GARP subjects may have biased the innate response of the lymphocytes to the stimuli, thereby compromising the power and robustness

of the linkage analysis. Preferably, to identify putative regulatory loci of these possibly confounded levels healthy subjects should be used.

4.2.3 Association analysis *CRP* genetic variation, serum CRP levels and OA

In chapter 2.3 we explored the association of *CRP* haplotypes to the measured baseline serum HsCRP levels and to OA phenotypes in the GARP study. In the GARP study, Meulenbelt *et al.* showed that higher levels of HsCRP associated to increased BMI and knee OA[26]. Shortly after this publication a compelling study by Carlson *et al.* showed common haplotypes of the *CRP* gene associated to basal serum HsCRP levels in healthy individuals[27]. This publication triggered the obvious question whether these haplotypes underlie the association of HsCRP levels to OA as mentioned above. Upon determination of the *CRP* haplotypes in the GARP study the haplotypic pattern and its association to serum HsCRP was strikingly similar to the study by Carlson *et al.*[27]. Overall the HsCRP levels of the subjects of GARP study were not in the acute phase range and only subtle differences were observed between the haplotype mean levels. We could not find an association of any of these haplotypes to knee OA or BMI indicating that the previously observed associations are not likely causally related, but that the high CRP levels reflect ongoing processes of OA and/or high BMI. In the current data we cannot distinguish BMI driven effects on the serum HsCRP levels from effects mediated through knee OA because these are highly correlated features. However, haplotype 7/8(H7/8), which associated to higher serum CRP levels, associated to GARP subjects within the highest quartile of number of OA affected hand joints (frequency cases 0.096 whereas control frequency was 0.046). The association of the *CRP* haplotype to both high serum HsCRP level and severe hand OA may indicate that H7/8 may causally contribute to OA etiology in hand joints. A lifelong exposure to subtle increased circulating CRP levels may have tipped the balance to pro-inflammatory responses upon damage, affected the integrity of the cartilage with age and ultimately leading to the onset of the OA process. It should be noted that the frequency of H7/8 was relatively low (frequency overall in the GARP study 0.06) and in the GARP study no direct association of serum HsCRP to hand OA was shown. The fact that we do not observe higher circulating serum HsCRP levels among subjects with hand OA might be attributable to the influence of confounding factors such as the effects of BMI and/or knee OA among subjects of the GARP study[26], that easily affect the HsCRP levels and obscure possible OA subtype associations to circulatory levels. In a study of erosive hand OA patients Punzi *et al.* have shown increased levels of serum HsCRP[28], however, this study has no genotype information available which could help to elucidate whether the haplotype association is observed in these subjects as well. In the GARP study 42 individuals scored positive for signs of erosive hand OA out of 260 individuals scored. In the GARP subjects, no association of the high serum HsCRP haplotype H7/8 was observed to the erosive hand OA phenotype (personal communication, Dr. Kloppenburg *et al.*, department of Rheumatology, LUMC). Alternatively, serum HsCRP levels may act in OA through flares of disease activity, which can explain why we do not consistently observe increased serum HsCRP associations to hand OA in this cross sectional study design. It is remarkable that only the hand joints appear to be affected by the *CRP* haplotype in OA, the smaller joints of the hand might be more susceptible to subtle changes of circulatory serum levels of immunity signaling proteins, whereas the bigger joints have a more stable synovial level of inflammatory markers through a smaller surface to volume ratio. The reported associations

between the *CRP* haplotypes, levels and OA would benefit from confirmation in other larger study populations with severe hand OA as defined by ≥7 of 20 scored joint sites affected.

4.2.4 Association analysis of SELS genetic variation, baseline inflammatory mediators and OA subtypes

To further explore the role of circulating levels of inflammatory mediators we measured 17 multiplexed cytokines and chemokines in plasma obtained from GARP subjects. Simultaneously, Curran *et al.* reported a promoter polymorphism -105 G/A of the *SELS* gene which was associated to higher levels of circulating pro-inflammatory cytokines[29]. In line with the working hypothesis this might increase susceptibility to OA. In addition to this promoter polymorphism, we genotyped 2 SNPs in the gene to increase the coverage of genetic variation present within the *SELS* gene. In this study we aimed to identify plasma markers of the ongoing OA process, as well as to characterize the subjects of GARP for possible predisposition to OA through genetic variation of the *SELS* gene. We observed no large scale up regulation of any of the measured cytokines and chemokines in relation to total ROA score or OA subtypes, indicating that none of the measured cytokines or chemokines is suitable as a marker for OA severity in the OA stages as present in the GARP study. The reported -105G/A promoter polymorphism association to higher circulating levels of IL-6, IL-1β and TNFα[29] was not confirmed in the GARP study, nor could we detect any association between genetic variation at the *SELS* gene and OA.

Since some of the 17 measured serum levels of cytokines and chemokines were highly correlated we performed a principal component analysis on 9 of these levels and included serum HsCRP levels in this analysis. We extracted 3 components representing 61.4% of the total variation in these markers. One haplotype ('GAG' frequency 0.04) of the *SELS* gene associated to 2 components, one of which depended mainly on serum IL-10 levels, whereas the other was mainly driven by serum HsCRP levels, indicating that genetic variation at *SELS* may indeed influence baseline inflammatory mediators. A third component which mainly represented variation of chemokines MIP-1β, MCP-1 and IL-8, was significantly associated to hand OA and disc degeneration. Contrary to expectation of increased pro-inflammatory signaling, low levels of these chemokines captured in this component were associated to higher ROA scores. In the interpretation of this, it should be noted that subjects of the GARP study are selected on OA at multiple joint sites meaning that subjects with no hand OA had OA at other joint sites, however, we could not assess positive association between the chemokine levels and OA at other joint sites among GARP subjects. Previously, synovial chemokine levels were shown to be upregulated in OA affected joints as compared to controls[30,31], our study shows that this up regulation might not be reflected in circulating serum levels, or this up regulation may be less pronounced in hand OA and disc degeneration as compared to hip OA and knee OA. Alternatively, the observed association is spurious, which calls for confirmation in other cohorts which have both data on hand OA and circulating chemokine levels available. Given the current results, it might be that the underlying pathophysiological processes of hand and spine OA as opposed to knee and hip OA are different, reflected in the associations found for the individual joint sites studied in the GARP subjects.

4.2.5 Inflammatory mediators' levels and genes in OA

In summary, our studies on mediators of inflammation and candidate genes in the inflammatory pathways, in addition to earlier publications on this subject, show that although significant associations were found between inflammatory mediators and OA, the underlying mechanism is highly complex and cannot be easily elucidated. The relation between cause and effect may be obscured once the process of cartilage damage is initiated and causal factors may inversely become markers of the ongoing disease processes. The identification of loci coding important immune modulating genes in healthy individuals may assist in the identification of cause and effect in OA research. Once the OA process is initiated a redundant cycle of cartilage damage and mainly catabolic activity in response to the damage may occur (Figure 1), which would be amplified through genetic predisposition towards pro-inflammatory states. The presence of these OA predisposing alleles may be a result of an advantageous effect in early development and life, where a fast chondrocyte cycle may be preferable in growth and repair of minor cartilage defects, however, may underlie OA onset and progression later in life after the reproductive phase.

Individuals with OA in the GARP study show higher innate levels of IL-1β as compared to controls, however, the only observed genetic predisposition present in our data is to a low IL-1β bio-availability haplotype. This contrast illustrates the complexity of interactions of levels with disease status and predisposition through genetic variation reflected in levels. We aimed to find additional candidate genes for association analysis to OA and by a genome wide linkage scan we identified a *CD53* SNP which associated to innate TNFα levels, however, we were unable to detect association of this SNP to either OA or RA. The estimates of the innate immunity were obtained through a stimulation using LPS which acts on the Toll-like receptor 4 (TLR4). It can be argued that for a better or more complete overview of innate immunity in relation to osteoarthritis stimulation through the TLR2 pathway may provide additional information. Both TLR pathways are implicated in autoimmunity, however, TLR4 is more intimately involved in the pathogen response whereas TLR2 is more involved in allergies and autoimmunity protection. In addition to innate immunity estimates, we investigated circulating levels of immune signaling proteins aiming to identify mediators or markers of the disease process. We showed that a *CRP* haplotype H7/8 associated to high serum HsCRP levels as well as to OA at multiple sites of the hand. In this case we were not able to show higher serum HsCRP levels for individuals of GARP who had OA of the hand as compared to subjects without involvement of the hand, nor did we find evidence for association of this haplotype to erosive hand OA, which is reported to associate to higher serum HsCRP levels. In this respect, confounding by other joint sites and traits might obscure possible associations of serum HsCRP levels to OA, as is shown especially in the case of knee OA which is confounded by the correlation of BMI and knee OA[26]. We were unable to show any relation between *SELS* haplotypes associated to specific inflammatory components and features of OA. By use of prospective or early OA cohorts, such as the Cohort Hip and Cohort Knee (CHECK) study, which is currently at a 10 year follow up of a thousand subjects with early clinical signs putatively caused by OA, the causal role of levels of inflammatory mediators in the onset of OA might be better studied since the disease status may not yet evoke a strong reaction of these inflammatory system.

Altogether, our investigation of patterns between genetic variation of markers, the levels of the markers and disease status has not revealed a clear relationship between inflammatory

mediators, genetic variation and OA. None of the associations found for genetic or circulatory levels was present in all subjects of the GARP study, underlining that OA is a multifactorial disorder, in which several aspects influence the disease onset and progression. Identifying each of the associating genetic and circulating factors might together comprise a risk profile for OA and can help to better identify early OA and provide patient prognosis. For better pattern recognition analyses may benefit from an approach using the Mendelian randomization model[6]. In this approach, a sufficiently large sample of healthy individuals is used to identify which genetic variants influence which levels. Subsequently, these patterns can be investigated in diseased cohorts to identify possible associations of variants to the disease, or whether levels are confounded by the disease process itself. In particular for diseases in which the marker levels may not be independent from the disease status this approach may be very beneficial. Furthermore, the OA process mainly acts in the synovial compartment, which may not necessarily be reflected in the circulating levels of inflammatory mediators. More insights in the relation between circulating levels of inflammatory mediators and local signaling in the joints are needed to reliably identify putative disease monitoring markers. In addition, our analyses were aimed at specific inflammatory mediators, whereas the immune system is highly complex with more factors involved. In our analysis we may have omitted markers which are intimately involved in the disease process, however, not identified as OA markers.

4.3 Developmental characteristics of OA

In addition to genes of the inflammatory pathway, recent candidate gene studies and genome wide linkage and association scans indicate that genes which act during the early stages of osteogenesis and chondrogenesis should be considered as regulators of an important pathway in the etiology of OA[1,3,32,33](see Chapter 1 for a complete overview). Variants of developmental genes may affect joint morphology of the bone during early development, where subtle changes in shape might create a lifelong exposure to aberrant joint loading, ultimately wearing out the cartilage and initiating OA. Currently, in collaboration with the Erasmus University of Rotterdam (Prof. Dr. H. Weinans and Dr. J.H. Waarsing *et al.* department of Orthopedics, Erasmus MC), the shape of hips is being characterized using statistical shape models. These can subsequently be used to identify putative predisposing shape aspects, as well as identify whether genes influence the shape of joints, possibly contributing to the OA etiology through these shape aspects. Secondly, loss of maturational arrest in articular chondrocytes and initiation of chondrocyte hypertrophy might be one of the events which set off a cycle of processes where the chondrocytes follow a path resembling that observed in the growth plate which is debilitating for the articular cartilage[21,34,35]. Genetic variation at the genes which regulate these processes might exert their effects later in life when the cartilage matrix ages and age related expression changes in cells occur[36-38]. *DIO2* is one of the genes which is active in the growth plate during endochondral ossification, and was identified in the GARP study as candidate OA susceptibility gene and confirmed by several additional OA cohorts[5]. *DIO2* codes for type II deiodinase (D2) which regulates the availability of active thyroid hormone T_3 in the growth plate during endochondral ossification[39]. Active T_3 signals in the growth plate direct the chondrocytes towards terminal differentiation, eventually resulting in the formation of bone. Recent findings using mouse models show the reactivation of endochondral ossification genes in a mouse model of mechanically induced OA, supporting

the possibility that reactivation of these genes plays a role in OA etiology[35,40]. In addition to these data, chondrocytes from aged donors are less responsive to anabolic signaling and have decreased mitotic ability, thereby increasing the susceptibility to OA onset[41,42]. In the growth plate, developmental genes are known to regulate the ongoing process of endochondral ossification; the breakdown of cartilage and formation of bone. These features are also major characteristics of the ongoing OA process. This hypothesis was recently reviewed by us, where *DIO2*, *GDF5* and *FRZB* in particular were discussed[1]. In this thesis we performed two studies further characterizing D2 in OA cartilage as compared to healthy cartilage and characterizing the *DIO2* risk allele of rs225014 in OA cartilage. These studies can be considered the first efforts to follow up a gene identified through a genome wide approach in OA. Depending on the genes and polymorphisms identified in several ongoing genome wide association studies and meta analyses of these, a similar path of follow up experiments can be expected to further investigate the involvement in OA etiology of these and other discovered OA susceptibility genes.

In Chapter 3 we showed that the OA risk allele of *DIO2* SNP rs225014 is more abundantly transcribed in OA cartilage at the mRNA level as compared to the reference allele. Though a higher rate of transcription of the risk allele a subtle imbalance of D2 protein production may be present which in turn activates more thyroid hormone T_3 during life. The fact that this SNP resides in an exon, which generally do not harbor the majority of the regulatory elements of genes, may indicate that this SNP is merely a marker for variation in *cis*-acting regulatory elements such as putative methylation sites or promoter variation which form the true underlying cause. Moreover, it should be noted that the polymorphism is very close to a CTCF binding site, which are known to be involved in gene activity regulation through DNA methylation, possibly, the methylation status of this site is altered under the influence of the OA risk allele. The identified risk SNP rs225014 codes for an amino acid change in the D2 protein, however, no conclusive evidence is found that this amino acid change has major effects on the enzyme efficacy and stability[43]. The absence of changes in enzyme activity or stability[44] further points towards a regulatory *cis*-acting element underlying the observed association.

Furthermore, in the IHC analyses the observation that in OA cartilage as compared to non OA cartilage D2 is both more abundantly expressed throughout all the cartilage layers as well as at higher staining intensity, together with similar pattern of expression differences of the thyroid hormone receptor beta and DIO3 indicates that in OA cartilage increased thyroid signaling is present. Possibly, the OA risk allele may contribute to this higher expression of D2 through the described allelic imbalance. Overall, it is unclear whether age related dedifferentiation of the chondrocytes contribute to the observed increase in expression of thyroid signaling proteins or whether the differences observed in expression level is merely a marker of the ongoing disease process. To overcome this a study cohort which allows investigation of the influence of aging on the expression of these proteins in healthy cartilage is needed.

4.4 Future perspectives

Recently, candidate gene and genome wide approaches have shown consistent associations for several genes in osteoarthritis. In a follow up of these initial findings, these genes now need to be characterized in relevant tissues and through *in vitro* and *in vivo* models in order to further elucidate the mechanisms behind the associations. In designing new experiments,

researchers should take into consideration that focus should be on identifying common genetic variation which cumulatively add up to a composite risk for OA development, as well as on the identification of rare, large effect genetic variation which has in itself a stronger effect on the OA predisposition. International collaborative GWAS and linkage approaches significantly increase the power of studies into OA, however, also bring about a new challenge regarding the phenotypic heterogeneity of the combined cohorts. In order to cope with these challenges, additional research will be needed to further homogenize the OA phenotyping of already assessed cohorts and new OA research initiatives. Through collaborations researchers in the genetics of OA may lay down a path towards new therapeutic approaches for disease modification and treatment[45]. Once genetic variation contributing to OA is identified, the research enters a new phase of characterizing the specific variation identified. Through promoter activity assays, sequencing, animal models, expression analysis and many other emerging genomics and proteomics techniques a more detailed insight can be gained, where each specific gene may have an obvious next step to take. One of these arising genomics fields allows high throughput assessment of epigenetic configuration of genes as determined by methylation status. Especially in silencing of e.g. developmental and catabolic genes, methylation is a potent genetic regulatory mechanism, which may play a role in OA etiology. Use of DNA extracted from OA and healthy joints can readily test hypothesis regarding methylation of specific genetic areas and is currently ongoing. Furthermore, as no functional roles for the *DIO2* OA risk polymorphisms are identified we aim to use high throughput sequencing techniques to find putative new rare variants with large impact, which may give us more insight in the molecular mechanisms behind the associations. The possible link between the processes of the endochondral ossification and osteoarthritis calls for a new range of experiments where this link can be further characterized. By challenging cartilage explants of donors of different ages with signaling similar to that found in the growth plate during endochondral ossification we may better understand the observed similarities between OA and endochondral ossification and the relation of the disease to aging. Characterizing the differentiation processes of mesenchymal stem cells or pluripotent cells derived from (more easily obtainable) fibroblasts using donors of different genotypes under similar conditions may tell us more about why associations of these alleles occur. Furthermore, allelic imbalance of transcripts may be mediated through copy number variation, which is one of the advantages in the next generation sequencing techniques, which are highly reliable in identifying copy number variations. Further experiments using healthy cartilage to determine the reported rs225014 allelic imbalance might show whether in OA cartilage aberrant signaling as compared to healthy samples occurs at the level of ongoing thyroid signaling. Such differences might indicate presence of transcription factors not normally active in the cartilage, or differential epigenetic regulation of *cis*-regulatory elements in OA etiology.

Ultimately, new techniques and insights will allow better distinction of ongoing disease processes, thereby creating more understanding of the origin of the observed disease heterogeneity. Osteoarthritis may be the end stage of several distinct pathways; through identification of markers reflecting the ongoing processes possibly these pathways are better understood, monitored and targeted. Furthermore, to provide clinicians with effective disease modifying drugs, these experiments may unravel the complex mechanisms involved, thereby identifying potent drugable targets in OA.

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