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Genes and Mediators of Inflammation and Development in Osteoarthritis

Steffan D. Bos

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Genes and Mediators of Inflammation and Development in Osteoarthritis

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Contents

Chapter 1	**Introduction**	**7**
Chapter 2.1	**Genetic association of the interleukin-1 gene cluster with innate cytokine production profiles and osteoarthritis in subjects of the GARP study.**	**29**
Chapter 2.2	**A genome wide linkage scan reveals *CD53* as an important regulator of innate TNF-alpha levels.**	**45**
Chapter 2.3	**Allelic variation at the C-reactive protein gene associates to both hand osteoarthritis severity and serum high sensitive CRP levels in the GARP study.**	**61**
Chapter 2.4	**The role of plasma cytokine levels, CRP and selenoprotein S gene variation in OA.**	**73**
Chapter 3	**Functional characterization of type II deiodinase in human OA cartilage; assessment of *DIO2* allelic expression imbalance and immunohistochemistry of thyroid hormone signaling proteins.**	**91**
Chapter 4	**Discussion**	**105**
Chapter 5	**Summary**	**121**
Chapter 6	**Nederlandse samenvatting**	**127**
	List of publications	**137**
	Curriculum vitae	**139**
	Nawoord	**141**

1

General Introduction

1.1 General introduction

The term osteoarthritis (OA) is used to describe a heterogeneous group of common, age related musculoskeletal disorders, characterized by joint pain and limitations in joint laxity[1]. The main affected tissue in OA is the cartilage covering articular bones in joints, however, the disease affects all compartments of these articular joints. In addition to focal loss of cartilage, subchondral bone, ligaments and synovium show signs of involvement in OA[1,2]. Reliable diagnosis and classification of osteoarthritis by a comprehensive set of radiographic criteria was facilitated in 1957 by Kellgren and Lawrence[2], which were illustrated in the "Atlas of standard radiographs of arthritis" in 1963[3]. To date this grading system is typically used by researchers for OA classification. The grading system is based on the decrease of articular cartilage thickness reflected by joint space narrowing and bone remodeling reflected by subchondral sclerosis and osteophyte formation. This score is implemented in an ordinal scaling system using 5 categories representing no OA signs (score 0) to severe OA (score 4). Radiographs are noninvasive and easily obtainable, however, for OA research the collection and reading of the radiographs is relatively expensive and time consuming as compared to collection through clinical assessment or identification of subjects meeting the clinical end point of OA by receiving joint replacements. Furthermore, research on progression of OA using radiographs is subject to high heterogeneity as a result of measurement methods and reading variability[4,5].

Clinically, the OA diagnosis relies largely on manifestations of the disease such as pain and joint deformations. Between the clinical and radiological classification of OA a fair amount of disease heterogeneity is illustrated by poor correlation of these measures. The relation of the disease to age is demonstrated by a study performed in inhabitants of a Rotterdam area where going from mean ages of 55 to 70 years the prevalence of radiographic signs OA was increasing from 80% to 95% for any of the four major joint locations knees, hips, hands and spine[6,7]. Furthermore, a study based on a random population sample of 337 nuclear families from the Framingham area (Massachusetts, USA) shows that at a mean age of 61.2 already 44.1% showed signs of radiographic OA (ROA) of the knee and out of 30 sites scored the mean number of affected joints was 3.4 (SD 5.3). Amongst offspring of these subjects at a mean age 53.9 at the time of radiographic examination 21.6% was affected by ROA at 1 or both knees, scoring a mean number of affected joints of 1.4 (SD 2.9)[8]. Although the correlation between clinical signs and radiological signs is not strong, both features of OA increase with age[9]. The growing number of elderly in the general population will increase the influence of OA on worldwide public health in the future as illustrated by a prospectus from the Dutch institute for health (RIVM) which predicts the incidence of OA to increase by as much as 52% from 2007 to 2040[10].

1.2 Healthy cartilage

Healthy articular cartilage is characterized by sparsely divided chondrocytes in their extracellular matrix (ECM). The ECM is build up by several classes of highly specialized proteins illustrated in Figure 1. The cartilage matrix is laid down during early development and the major proteins present in matured articular cartilage are collagens, a class of fibrillar proteins. Type II collagen is the major constituent, with a lower abundance of type IX and XI present. Collagens form fibrillar networks that mediate the resistance to compressive forces and provide tensile strength. Furthermore, collagens serve as scaffolding for proteoglycans, the second largest class of proteins present in the ECM.

These polyanionic aggregates are formed by sulphated aggrecan monomers linked by linking proteins to hyaluronic acid. The charged acid proteins help to retain fluid within the matrix, thereby contributing to the ECM integrity[11,12]. The surface of the articular cartilage is smooth and provides a gliding surface allowing low friction movements under high loads.

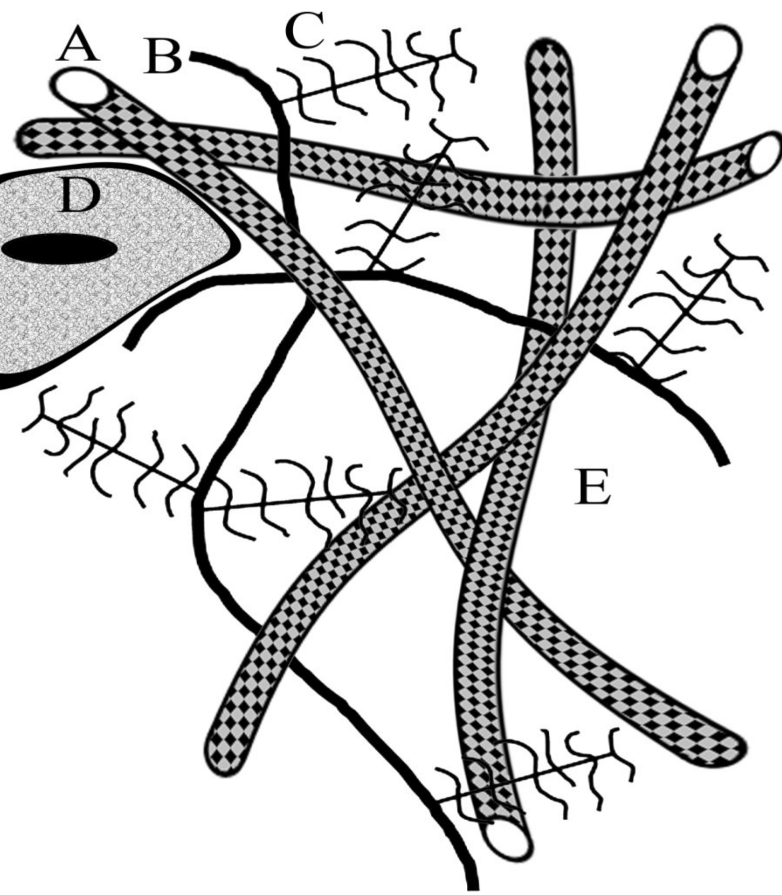

Figure 1. Major extracellular matrix constituents with a collagen fibril network (A), proteoglycans chains (B) and side chains with hyaluronic acids (C). These proteins are laid down during development and maintained by sparsely divided chondrocytes (D). The polyanionic side chains help to retain interstitial fluid in the matrix (E).

During development chondrocytes arise from the mesenchymal stem cell layer in the growth plate. Unlike the chondrocytes in the growth plate which develop into bone, the chondrocytes on the joint surface side enter a state of maturational arrest during their development which maintains these in their differentiated phenotype throughout life under physiologic conditions[13]. The difference between the maturational states of the growth plate chondrocytes and articular chondrocytes is mediated though several pathways and biologic

conditions, in which the Wnt-signaling plays a major role[14]. In addition to molecular signaling to maintain their differentiated phenotype, biomechanical activity and hypoxia are essential. The fluid movement, as a result of biomechanical activity, allows nutrients to enter and waste products to exit the matrix in addition to the diffusion processes[15]. The ECM is not perfused by blood or lymphatic systems, ensuring the hypoxic state with oxygen tension as low as 1%, which is needed to maintain the chondrocytes in their differentiated state[16]. Under healthy conditions the chondrocytes have a low metabolic activity while maintaining the ECM[17]. During live, the quality of articular cartilage may lessen as a result of repeated stress and micro fractures to the collagen matrix, accumulation of advanced glycation end products and changes in the cellular expression patterns as a result of aging in general[18,19]. Although the capacity to resist high peak stresses may be less, aged cartilage may still fulfill its role without any compromises to the joint laxity and mobility as long as the glycosaminoglycans stay entrapped in the collagen network and osmotic potential is retained.

1.3 OA cartilage

Macroscopic features of OA cartilage include an overall decrease of cartilage volume, fibrillation of the surface, vascularisation, calcification and tears. The accompanying chondrocyte features range from increased metabolic activity, cell cluster formation, dedifferentiation, hypertrophy and ultimately apoptosis[12,17]. Although the order in which the changes occur may differ from case to case, in all instances OA results in an overall cartilage loss and is accompanied by pain and lessening of joint laxity. Frequent observation in early OA chondrocytes are increased metabolic activity and proliferation accompanied by production of matrix degrading enzymes such as matrix metalloproteinases (MMPs) and aggrecanases[1]. The enzymatic activity of these proteins breaks up the tight collagen network of the ECM and shortens the aggrecan side chains, jeopardizing the matrix integrity. The weakened matrix is less capable of retaining fluid and loses its elasticity, decreasing the potential to perform its shock absorbing properties. The matrix becomes more prone to tears and micro fractures, which in turn demands more matrix maintenance from the chondrocytes and allows release of hypoxia at fracture sites by the resulting access to the synovial fluid. Unable to respond adequately to this higher demand the chondrocytes proliferate, turn hypertrophic, dedifferentiate and ultimately become apoptotic[12,13]. Once initiated, this cycle may amplify itself by rounds of matrix weakening and a mainly catabolic or apoptotic response from the chondrocytes. A more macroscopic change often observed in OA cartilage is vascularisation of the matrix from the subchondral bone. This loosens the hypoxic state in the articular cartilage as a result of perfusion allowing phenotypic changes of the chondrocytes, which can initiate formation of bone, illustrated by the calcification of matrix surrounding vascularized areas and formation of osteophytes[20]. Bone is also implicated as an endocrine organ which may locally communicate with the articular chondrocytes, possibly resulting in detrimental processes in response to biomechanical overload or excessive local pressures[21]. Immunohistochemistry can be used to identify different OA features and the accompanied changes observed in matrix and chondrocytes are schematically represented in Figure 2. These features are summarized in a microscopic scoring system described by Mankin *et al.*[22], which allows quantification of cartilage damage.

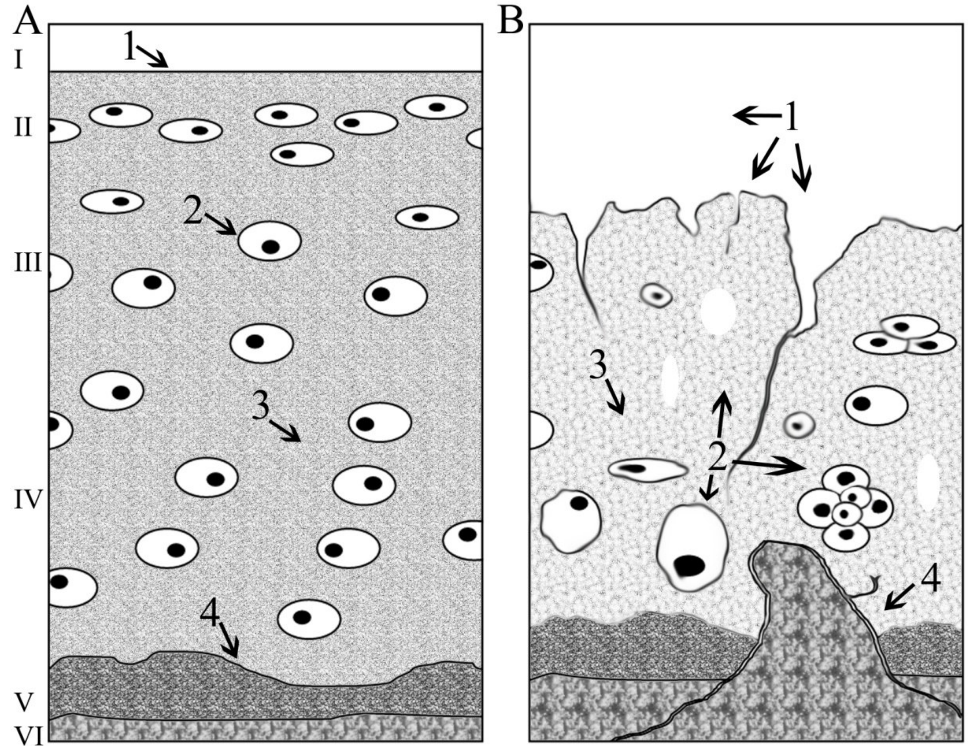

Figure 2. Schematically represented the synovial fluid (I), cartilage of the upper (II), middle (III) and deep (IV) region, a calcified area (V) and cancellous bone (VI). **(A)** In healthy cartilage a smooth running surface lines the boundary of the tissue with the synovial fluid (1). Cells are dispersed evenly over the tissue and are in a quiescent state (2). The proteins of the extracellular matrix, consisting mainly of collagens and glycosaminoglycans retain fluid and provide tensile strength (3). A tidemark separates the cartilage from the calcified area and cancellous bone. **(B)** In OA, the overall thickness of the cartilage is lost, the surface becomes irregular with fractures (1). Cellular features of OA include hypocellularity, clonal expansion of the chondrocytes and chondrocyte hypertrophy (2). The proteins of the extracellular matrix become degraded, losing their elasticity, tensile strength and fluid retaining properties (3). The tidemark becomes less clear and is crossed by blood vessels (4).

1.4 Heritability and genetics of OA

Family studies have shown that a relatively large heritable component exists in OA, with heritability estimates ranging from 40-80%, depending on phenotype and joint site studied[23-25]. Already in the 19th century OA features were recognized as hereditary and a 1941 study reported OA as a dominant trait[26]. Over the years OA has shown to have a complex genetic background. In comparison to other common diseases OA reports a high familial clustering with recurrent risk ratio's, depending on subtype studied, between 1.66 and 8.53[24,27,28]. Given these data it is apparent that genes play a substantial role in OA and although some rare mutations cause a monogenic early onset form of OA[29], the majority of the heritability component is caused by a multitude of genetic factors[30,31]. The complexity of the disease makes the identification of genes involved a meticulous exercise where large well typed cohorts with high genetic resolution are needed to yield enough power to elucidate the underlying genetic background[32]. To date several genome wide approaches, as well as candidate gene studies have delivered multiple genes which show reasonably

consistent contributions to the disease etiology. A list of recent and striking studies in the genetics of OA, which were at least confirmed once or show robust numbers is supplied in Table 1. Identification of genes involved in a trait can be done through several approaches, each of which has advantages and disadvantages over the others and optimal study designs depend entirely on the frequency and penetrance of the genetic variation underlying the disease (Figure 3).

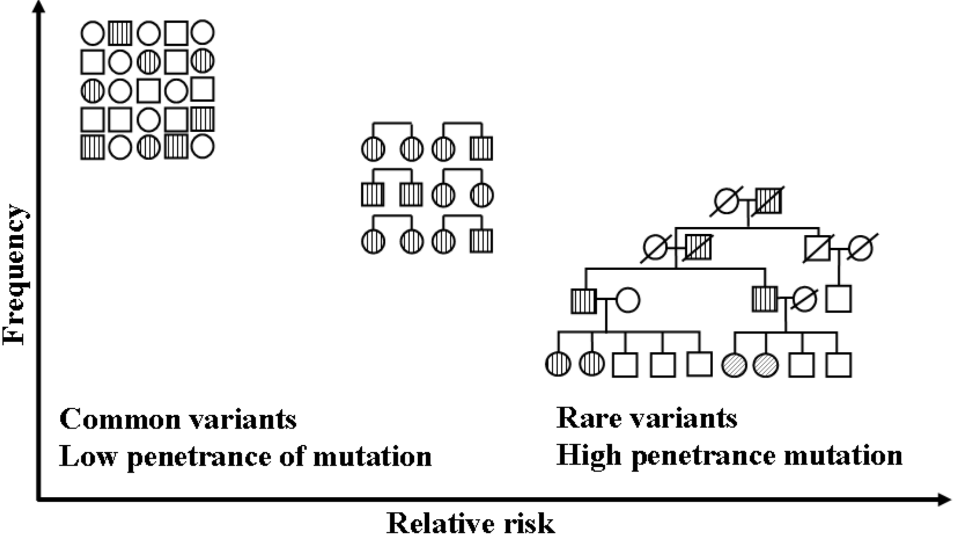

Figure 3 Genetic study designs

Rare mutations with large effect sizes are best identified by model based linkage in extended families where a Mendelian inheritance pattern indicates a single gene to be the major factor in disease onset. Model free linkage studies in affected sibling pairs may be the next approach to detect variants with moderate effects on disease etiology for which the inheritance pattern appears more complex. An advantage over the association studies is the resistance to genetic heterogeneity and the power to identify genetic loci which may carry multiple independent variants which influence disease etiology. Association studies rely on the sharing of ancestral disease associated alleles with lower penetrance and higher allele frequencies amongst cases versus controls, referred to as the common disease, common variant theory[33]. Initially, candidate gene approaches were performed, where based on knowledge of the disease process, the choice to characterize a specific gene used little genotyping resources. Obviously, this only informs the researchers on a specific locus thereby not identifying other genetic loci putatively involved in the disease. Technological advances enabled researchers to type increasingly more variants, up to a point where a genome wide and thereby unbiased approach could be taken and a discovery approach was possible next to the hypothesis based approaches. In genome wide approaches we distinguish a linkage approach, which is based on family studies and association approaches which are population based, identifying cases and controls and comparing their allele frequencies. The genome wide approaches suffer from large numbers of spurious signals, increasing with the number of tests performed. To account for this, a correction for multiple

testing is applied, which requires the signals to reach a 'genome wide significance' level of $P < 1 \cdot 10^{-8}$ before being considered as putative true signals. Although combining several studies in collaborative efforts to accumulate larger numbers in the genome wide studies is generally beneficial, there is an increased chance of heterogeneity of the study population. Especially in the case of a heterogeneous disease such as osteoarthritis such heterogeneity may prevent identification of specific risk genes, cancelling out the beneficial effects of the large combined study sample. Further challenges in the unbiased approaches are the lack of incorporation of putative gene-gene and gene-environment interactions[33]. Once a particular gene is identified as involved in a disease etiology, much work is needed to understand the underlying mechanism. A thorough characterization of the genetic region is needed to identify the (true) causal variant and quantify its contribution to the disease susceptibility and gain understanding of the involved molecular pathways. The recently developed second generation sequencing techniques now allow researchers to sequence a substantial genetic area in large numbers of subjects at a reasonable cost, facilitating the identification of new variants in these areas of interest[34], which is illustrated by the recent study by Johansen *et al,* who showed an accumulation of rare variants at a series of candidate genes in hypetriglyceridemia cases versus controls[35].

Altogether, amongst the results of genetic studies performed in OA (subtypes) two distinct pathways stand out especially with multiple genes implicated. The first group is formed by the inflammatory pathway with inflammatory signaling proteins, their modulators, receptors and the inflammation driven induced degrading enzymes such as matrix metalloproteinases (MMPs) and aggrecanases. Some consistently associating genes from this group are the interleukin (IL)-1 gene cluster[67-69], interleukins[72,77], aggrecanases[37,80] and prostaglandin[36,37,62]. The second group identified consists of genes involved in the developmental processes of chondrogenesis and osteogenesis such as Wnt-signaling proteins and bone morphogenic proteins. Some of the compelling genes in this group are *FRZB*[41,49,94], which codes for an antagonist of the Wnt-signaling, *GDF5*[55] which coordinates formation of bone and joints, *BMP2*[37] and *BMP5*[44,45], regulators of chondrogenesis and articular cartilage formation respectively. Although not all genes are replicated in every study, the overall view is that these developmental genes play a role in the disease etiology.

1.5 The role of inflammatory mediators in OA

Articular chondrocytes respond to specific cytokines by either anabolic or catabolic activity. Pro-inflammatory signaling mediated by e.g. IL-1β has detrimental effects on cartilage since the resident chondrocytes respond by producing MMP's and aggrecanases, effectively degrading the surrounding matrix. On the other hand, signaling by anti-inflammatory cytokines such as IL-10 results in anabolic activity of the chondrocytes which will excrete new ECM components. Under normal, healthy conditions the pro- and anti-inflammatory signals are low and balanced thereby cancelling out their respective effects[95-97]. This balanced mechanism is thought to play a role in repair of small traumas to the cartilage, where catabolic activity in response to pro-inflammatory signaling, resulting from the sensed trauma, clears the damaged matrix[98,99]. Subsequently, the resulting lacuna in the matrix is filled by the newly formed collagen matrix in response to the later stage induced anti-inflammatory signaling[12,95,100]. The cytokine response is, at least in part, under genetic control as is illustrated by several studies showing associations of circulating levels of

Table 1. Recent and confirmed genetic studies for OA susceptibility loci.

Pathway	Gene	Studies +	Studies -	OA subtypes	Gene description and function
Developmental	*AACT*	36,37		Knee	Alpha 1 antiproteinase, extracellular antagonist of the Wnt-signaling pathway
	ASPN	38-41	42,43	Hip, Knee	Asporin, Cartilage extracellular protein that regulates TGF-β signaling
	BMP2	36,37		Knee	Bone morphogenetic protein 2, involved in the chondrogenesis and osteogenesis in development
	BMP5	44,45		Knee	Bone morphogenetic protein 5, regulator of articular chondrocyte development
	CILP	36,37,46		Hip, LDD	Cartilage intermediate layer protein, inhibits TGF-β1-mediated induction of cartilage matrix genes
	DIO2	47		Hip, GOA	Deiodinase 2, intracellular activator of thyroid hormone in target tissues
	DIO3	Meulenbelt *et al.*(in press)			Deiodinase 3, intercellular deactivator of thyroid hormone in target tissues
	FRZB	41,48-51	52	Hip/Knee, GOA	Secreted frizzled related protein 3, Wnt antagonist and modulator of chondrocyte maturation
	GDF5	53-55		Hip	Growth and differentiation factor 5, member of the bone morphogenic family, regulator of growth and differentiation across several tissues including bone and cartilage
	IGF1	43,56		Hip	The protein is similar to insulin in function and structure and is a member of family of proteins involved in mediating growth and development.
	LRP5	57	52	Knee	Low density lipoprotein receptor-related protein 5, receptor involved in the Wnt signaling via the canonical beta-catenin pathway
	RHOB	58	59	Hip, Knee	Ras homologue gene family member B, GTPase in tumor supression and antagonist in the PI3K/Akt pathway
	WISP1	60		Spine	Wnt inducible signaling protein 1, target of the Wnt pathway regulated by beta catenin
Inflammatory and Immunity	*COX2 (PTGS2)*	36,37,61,62		Knee, Spine	Prostaglandin, modulates cartilage proteoglycan degradation through PGE2
	HLA	63-66		Hand/Hip/Kee, GOA	Human leukocyte antigen system, determinant of antigen specificity of the immune response
	IL1 cluster	43,67-70	43,69,71	Hip/Knee	Interleukin 1 alpha, beta and receptor antagonist, regulates metalloproteinase gene expression in chondrocytes and synovium
	IL10	72,73		Knee/Hand	Interleukin 10, anti inflammatory cytokine inhibiting IL1 synthesis
	IL4R	74	43	Hip	Interleukin 4 receptor, Putative role in chondrocyte response to mechanical signals
	IL6	75-77	43	Hip/Knee	Interleukin 6, proinflammatory cytokine, involved in cartilage degradation and IL1Ra induction
Structural/Other & Undefined	*Chr7q22*	78		OA subtypes	Several genes in LD block currently under investigation for further specification of underlying gene(s).
	ADAM12	31,37,79	42	Knee	A Disintegrin and metalloproteinase 12, involved in osteoclast formation and cell-cell fusion
	ADAMTS14	80			A disintegrin and metalloproteinase with thrombospondin motifs 14
	CALM1	81	41,82	Hip	Calmodulin 1, intracellular protein interacting with proteins involved in signal transduction
	COL2A1	41,83-85	43	Knee	Type II collagen, major constituent of articular cartilage
	COMP	41		Knee	Cartilage oligomeric protein, cartilage matrix macromolecule
	EDG2	86		Knee	Lysophosphatidic receptor encoding, EDG receptors mediate diverse biologic functions, including proliferation and chemotaxis.
	ESR1	36,87,88	43	Knee, GOA	Estrogen receptor alpha, chondrocytic modulator of proteoglycan degradation and MMP mRNA expression
	LRCH1	89	90	Hip/Knee	Leucine rich repeats and calpain homology, protein of unknown function
	MATN3	91,92		Hand, Spine	Matrillin 3, extracellular matrix protein
	OPG	36,37		Knee	Osteoprotegerin, regulator of osteogenesis
	TNA	36,37		Knee	Tetranectin, plasminogen-binding protein involved in the degradation of ECM
	TXNDC3	58	59	Knee	Thioredoxin domain containing 3, protein disulfinde reductase
	VDR1	36,41,59	43,93	Knee	Vitamin D receptor, mediates vitamin D signaling which serum levels affect incidence and progression of OA

inflammatory mediators to genetic variation at genes involved in their regulation[101-103]. Cytokines are derived from several cell types, however, one of the main sources of cytokines are the circulating blood lymphocytes. The estimated innate capacity of lymphocytes to produce cytokines has been shown to associate to a range of diseases[104,105] including OA susceptibility and OA progression in sibling pairs affected by OA at multiple joint sites (Genetics of osteoARthritis Progression, or GARP study[106]). In this study, Riyazi *et al.* showed that a high innate IL-1β and low innate IL-10 (together indicating a pro-inflammatory profile) predisposes to OA[107]. Furthermore, data on progression of a subset of these individuals over a 2 year period showed a specific innate tumor necrosis factor(TNF)α and IL-10 profile confers to an increased risk of knee OA progression[108]. The genetic component in variation of the LPS induced innate cytokine response is substantial, underlined by the high heritability of these levels ranging from 57% to 86%[109]. Genetic configuration of innate immunity might confer susceptibility to OA through a lifelong exposure to specific (pro-inflammatory) cytokine profiles[107], as is also observed for several other diseases[110,111]. Polymorphisms in the cytokine genes were shown to explain only a small part of the total heritability estimate of innate immune profiles indicating involvement of additional loci[73,109]. The role of polymorphisms at the *IL-1* gene cluster in the innate levels or OA phenotypes were studied in several smaller cohorts and to date no study combined the polymorphisms, innate levels and OA data in one single study. In addition, the ratio between IL-1β and its biological competitor IL-1 receptor antagonist (Ra) was suggested to be a more suitable representation of the bio-availability of IL-1β signal[112]. The low contribution of the known genetic variation at the respective cytokine genes to the genetic component of innate immunity indicates additional regulating mechanisms are involved. Identification of new loci involved in the regulation of innate immunity may provide new candidate genes to be tested for association to OA. In addition to innate immunity, synovial levels of inflammatory cytokines were reported to be elevated in OA joints[97,113-115], whereas baseline levels of C-reactive protein (CRP) were shown to be associated to erosive hand OA[116]. Furthermore, *in vitro* explants and cell culture experiments show that the chondrocytes are a source of matrix degrading enzymes upon incubation with pro-inflammatory cytokines[117-120]. Taken together the described studies show that inflammatory mediators play a role in OA etiology, although for most reported studies it remains unclear whether the immune system signaling is merely a marker of the ongoing disease process, or whether this may underlie part of the etiology of the disease. Studies into genetics of the immune system may help elucidating cause and effect. As was shown in Table 1, in addition to the genetic component in OA that may be ascribed to genetic variation in inflammatory mediators, recent genome wide linkage and association scans also indicated a substantial role for genes which play a role during early development of the articular joints.

1.6 The role of developmental characteristics in OA

The role of skeletal development in OA was first illustrated by the onset of OA as a result of severe chondrodysplasia or defects in skeletal morphogenesis[121,122]. The joint shape is determined early in life, where severe malformations ultimately lead to OA. The endochondral ossification process plays a key role in determining joint shape during development and is responsible for the growth and shape of the long bones, including the articular joints[14]. In this process chondrocytes arising from a mesenchymal stem cell layer

subsequently proliferate, differentiate and turn hypertrophic. During these phases respectively, the chondrocytes increase in number, lay down a cartilage matrix and eventually start excreting cartilage degrading enzymes, and ultimately the initial cartilage layer is replaced by bone by blood vessel invasion and settlement of osteoblasts. The chondrocyte phases are tightly controlled by distinct gene expression patterns in which the Wnt-signaling is intimately involved[123]. Contrary to the chondrocytes in the growth plate, the chondrocytes of the articular layers on the long bones escape this sequence and enter a state of maturational arrest maintaining their differentiated phenotype. It can be hypothesized that less pronounced joint shape variations can predispose to OA as a result of a lifelong slightly altered mechanical loading of the joint. Several studies are ongoing in an attempt to define joint shape by the use of radiographs or MRI and relating this to the incidence of OA. Although confirmation is needed, initial studies show joint shape modeling may be used to predict OA onset or progression[124,125]. Through a genome wide linkage scan in the GARP study, a new OA susceptibility locus was identified at 14q32.11[47]. Subsequent association analysis identified the local thyroid hormone regulator *DIO2* coding for type II deiodinase (D2), which is also active in the growth plate during development as the most likely gene in the linkage region involved in OA etiology. Genetic variation at *DIO2* was confirmed to associate to OA in multiple other studies across different geographic areas and ethnicities. *DIO2* is the first gene identified which indicated that thyroid signaling may be involved in OA and secondly that such signaling in the growth plate during endochondral ossification may be involved in OA etiology[126]. Several other genetic studies likewise identified genes in the osteogenesis and chondrogenesis pathways that associate to OA[41,55], indicating a broader involvement of genes which orchestrate the process of endochondral ossification. In addition to developmental differences originating from developmental stages these genes may contribute to OA etiology later in life; the processes observed in the articular chondrocytes and ECM resemble processes observed in the growth plate in osteogenesis[95,127], possibly the (aging) articular chondrocytes lose their maturational arrested phenotype. Loss of this arrest allows articular chondrocytes to re-enter the cycle of their counterparts in the growth plate, thereby mimicking the downstream events and ultimately turning apoptotic and causing the cartilage integrity to fail. We have discussed the features of ageing and the role of developmental genes in OA in a review on this subject[126].

1.7 Outline of the thesis

In the current thesis one of the main study populations that is being investigated is the GARP study, consisting of middle aged sibling pairs (total N=382) affected with OA at multiple joint sites. This study is characterized for OA features at four joint sites; OA at the hips, knees and hand and degeneration of spinal discs were assessed by radiographs as well as by clinical assessment[106]. A high level of familial clustering of these features was observed in the GARP study, which led us to identify the genes underlying this high heritable component in OA. In addition to radiological and clinical data for these joint sites, extensive demographic questionnaires, blood samples and urine were collected at different time points (0, 6, 12, 24 and 60 months). The collection of multiple time points allows studies aimed at identifying markers which can either show or predict OA progression. Genetic studies in the GARP study by Min at al. have thus far shown associations to *FRZB* and *MATN3* for hand and hip OA respectively[51,91]. The associations of the described genes

in the GARP study together with the results of genetic studies in other OA cohorts explain only part of the heritability of OA, indicating that additional genes are involved in OA etiology.
In this thesis we set off to gain more insight in the role of inflammatory mediators and their genetic variation in OA. The question to be answered is whether (innate) cytokine profiles are merely a reflection of ongoing OA processes and genetic configuration, or whether the genetic configuration of genes orchestrating these cytokines plays a role in OA etiology. We characterized several genes and inflammatory markers and investigated their respective roles in OA in order to elucidate whether the observed associations follow Mendelian randomization[128], or whether an etiologic relation exists between genetics of cytokine regulating genes and OA. Chapter 2 describes the research performed in the GARP study to substantiate the causal role of inflammatory mediators and inflammation modulating genes in OA.
In Chapter 2.1 we studied the interaction between genetic variation at the IL-1 gene cluster (*IL1A*, *IL1B* and *IL1RN* gene) and innate *ex vivo* production upon LPS stimulation of the IL-1β and IL-1Ra (together expressing the IL-1β bio-availability) as well as the interaction of these characteristics with OA. Although genetic variations associated to innate cytokine levels were found as described Chapter in 2.1 of this thesis and in literature, together these genes explain only a small part of the heritability of these levels and elucidation of quantitative trait loci for innate immunity might reveal new insights in the regulation of the cytokine response. Follow up on these putative innate modulating genes may provide more insights in the role of innate immunity in OA etiology. Chapter 2.2 describes a genome wide linkage study where we set off to identify new quantitative trait loci for innate IL-1β, IL-1Ra, IL-10 and TNFα and whether these associate to OA. In Chapter 2.3 we explored the association between *CRP* haplotypes and serum high sensitive CRP levels as shown by Carlson *et al.*[102] and used these haplotypes and CRP levels in an association analysis with OA subtypes. Finally in Chapter 2.4 we investigated whether serum CRP and plasma cytokine and/or chemokines levels marked the ongoing OA disease process in the GARP study. Furthermore, using these levels we investigated whether the previously identified association between genetic variation at the *SELS* gene and pro-inflammatory cytokines[101] in plasma could also be identified in the inflammatory profiles of GARP subjects and their OA subtypes.
As indicated, previous studies identified the *DIO2* gene as the most likely gene explaining a significant linkage peak in OA sibling pairs of the GARP study[47]. A hypothesis on the role of genes involved in endochondral ossification was generated during the research of this thesis[126]. We characterized a *DIO2* risk allele tagged by polymorphism rs225014 for possible *cis* regulatory elements through analyses of putative differential allelic expression and we sought further evidence for the involvement of D2 in OA by characterization of OA and non-OA cartilage for the presence of D2 including other thyroid hormone regulatory proteins by use of immunohistochemistry described in Chapter 3.

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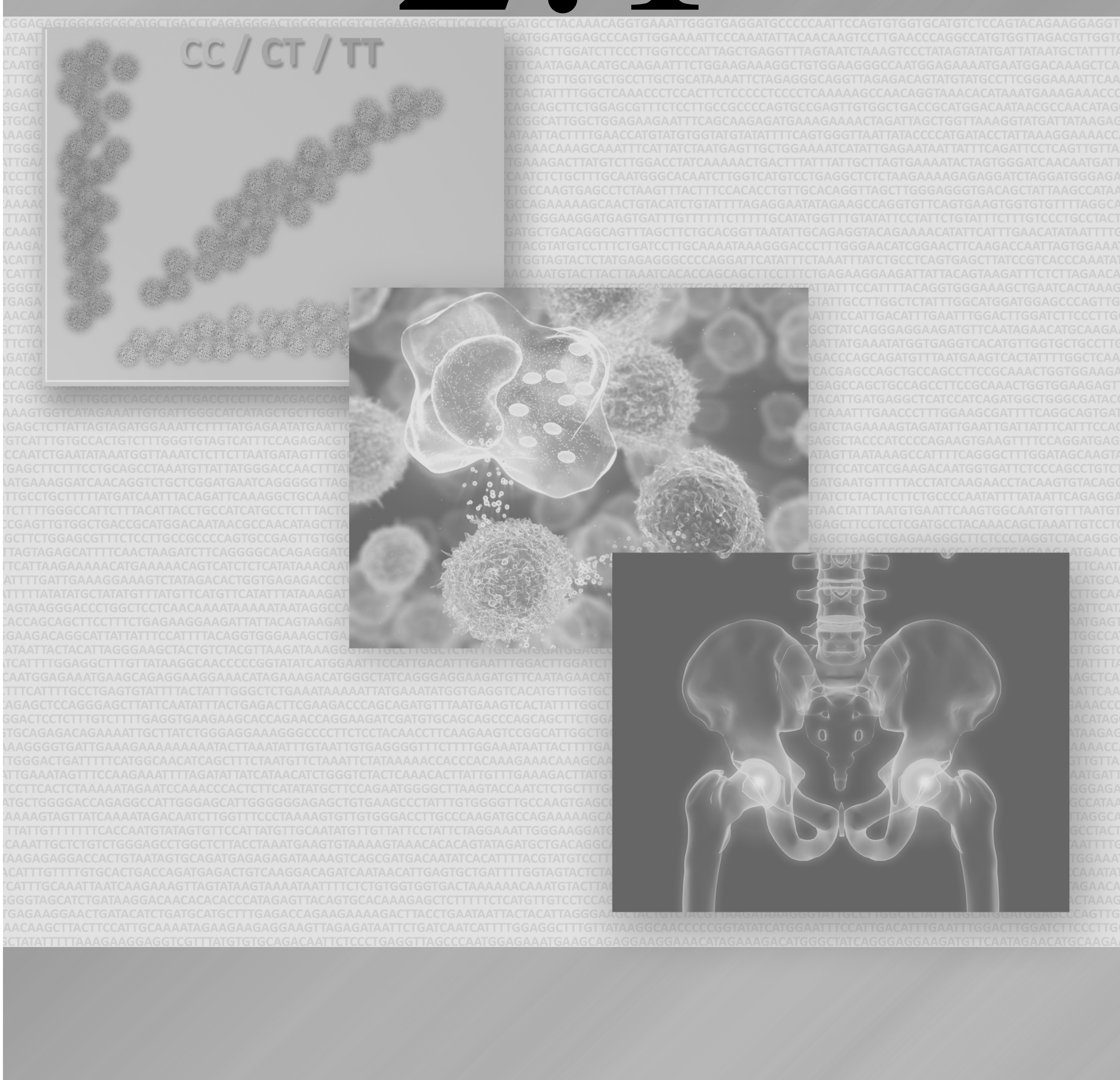

Genetic association of the interleukin-1 gene cluster with innate cytokine production profiles and osteoarthritis in subjects of the GARP study.

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Abstract

Objective: To assess whether genetic variation in the interleukin (IL)-1 gene cluster contributes to familial osteoarthritis (OA) by influencing the innate *ex vivo* IL-1ß or IL-1Ra cytokine production.
Methods: Innate *ex vivo* IL-1β and IL-1Ra production upon LPS stimulation (10 ng/ml) of whole blood cells was measured in the GARP study which consists of sibling pairs predominantly with symptomatic OA at multiple sites. Radiographic characteristics of OA (ROA) were assessed by Kellgren and Lawrence score. Subjects of GARP and controls of the Rotterdam study were genotyped for 7 single nucleotide polymorphisms (SNPs) encompassing the IL-1 gene cluster on chromosome 2q13. Linkage disequilibrium (LD) analysis, genotype and haplotype association analysis were performed in order to assess the relationship between the IL-1 gene cluster SNPs, innate *ex vivo* cytokine production and OA.
Results: Haplotype VNTR, +8006 and +11100 2-2-1 of the *IL1RN* gene was within the GARP study significantly associated to lower innate *ex vivo* bio-availability of IL-1ß upon LPS stimulation (P-value = 0.026) and to subjects with ROA at the highest number of joint locations.
Conclusion: We show that genetic variation at the IL-1 gene cluster associates to lower IL1ß bio-availability and to OA at a large number of joint locations. Furthermore, our data also indicates that among subjects with OA at the highest number of joints the innate immune system may be activated thereby obscuring possible underlying mechanisms.

Introduction

Osteoarthritis (OA) is a common joint disease and is an important cause of pain and disability in the general population. Genetic factors play an important role in the etiology of (various subtypes) of OA[1-5]. There has been a large interest in the role of cytokines as mediators of joint damage and inflammation in the pathogenesis of OA. Chondrocytes are known to respond to interleukin (IL)–1β by decreasing synthesis of matrix components and increasing the synthesis of metalloproteinases (MMPs)[6]. MMPs degrade extracellular matrix (ECM) components in articular cartilage. IL-1Ra is the natural competitive inhibitor of IL1ß, occupying the cell surface IL-1 receptor without triggering signal transduction and its levels might be considered critical in determining the IL-1β bioavailability[6].
One way of investigating the influence of cytokine profiles on disease is by measuring innate *ex vivo* cytokine production upon lipopolysaccharide (LPS) stimulation of whole blood samples. Studies of twins have shown that *ex vivo* production of cytokines IL-1β, IL-1Ra, TNFα, IL-10 varies by 60-70% based on heritability alone[7]. Subjects can thus be characterized as high (pro-inflammatory) or low (anti-inflammatory) producers based on these cytokines profiles[8,9]. Such a characteristic may influence susceptibility to diseases with an inflammatory component[10-12]. Data supporting this hypothesis in OA comes from our previous studies[13] in which we demonstrated that a pro-inflammatory profile, high innate *ex vivo* cytokine IL-1β and IL-1Ra and low IL-10, occurs among subjects with familial OA at multiple sites of the GARP study as compared to controls. The innate *ex vivo* production of TNFα which did not associate to the onset of OA, was the only cytokine that predisposed to knee OA progression[14]. In recent years the concept that inflammation in OA

contributes to symptoms and augments many pathological changes has become generally accepted[15,16], however, it is unclear whether this is a causal association or marks the ongoing disease process. Furthermore, the interplay between secreted IL-1ß and IL1-Ra levels should be taken into account since together they influence the IL-1ß bio-availability[17].

The genes encoding IL-1α, IL-1β and IL-1Ra (*IL1A*, *IL1B* and *IL1RN,* respectively) reside within a 430 kb region on chromosome 2q13. Although not always consistently and determined in relative small studies, it has been shown that several DNA variants within the genes of the IL-1 gene cluster may be responsible for the variation in the heritable innate *ex vivo* cytokine production upon LPS stimulation[12]. Furthermore, *in vitro* experiments have shown functional ability of IL1B promoter SNPs to enhance IL-1ß production upon LPS stimulation[18]. The role of the *IL1RN* VNTR allele 2 appears most consistent in affecting of cytokine production *in vivo*[19] and may be considered most important for the fine tuning of the IL-1ß bio-availability as determined by the ratio of the innate *ex vivo* cytokine production upon LPS stimulation of IL-1ß and IL-1Ra[17]. Multiple genetic association studies, tried to investigate whether these potential functional aspects of the IL-1 gene cluster polymorphisms may in part explain genetic susceptibility of OA. Previously, we and others have reported associations of the IL-1 gene cluster for knee, hip and hand OA[20-25], although others failed to confirm these associations[26,27]. Together the effects of the IL-1 gene cluster SNPs on innate *ex vivo* cytokine production and OA may be complex and involve interactions among different polymorphic sites and should therefore be investigated by means of independent haplotypes.

Combining *ex vivo* IL-1ß bio-availability measures upon LPS stimulation, genetic variation at the IL-1 gene cluster and OA disease status in a single study population allows to assess possible underlying relationships[28]. We have tested for the influence and interaction of the IL-1 gene cluster polymorphisms and haplotypes on the IL-1ß bio-availability in a relatively large number of individuals of the GARP study. In the same study it was investigated whether the haplotypes relevant for IL-1ß bio-availability correlate to a proportioned score of the number of ROA affected joint locations.

Patients and methods

The GARP study (Genetics, osteoARthritis and Progression)

The ongoing GARP study, which consists of 191 Caucasian sibling pairs of Dutch ancestry affected with symptomatic OA at multiple sites. Probands (aged 40-70 year) and their siblings were included in the GARP study with OA at multiple joint sites of the hand according to the American College of Rheumatology criteria or with symptomatic OA in two or more of the following joint sites: hand, spine (cervical or lumbar), knee or hip[29]. In the spine, knee or hip symptomatic OA was defined as having symptoms of OA in addition to radiographic signs[30-33].

Conventional radiographs of the hands (dorso-volar), knees (Posterior-Anterior in weight bearing / semi flexed and lateral), hips (AP), lumbar (AP and lateral) and cervical spine (AP, lateral and transoral) were obtained of all participants. This was performed in a standard manner with a fixed film-focus distance and a fixed joint position. Radiographic characteristics of OA were defined according to Kellgren and Lawrence[34] by a single, experienced and trained radiographer according to an agreed protocol as described in detail

elsewhere[29]. In the current paper we used (the highest quartile of) the total ROA score. The total ROA score (0-10) represents a summed score proportional to radiological cartilage abnormalities at each joint location in knee (0-2), hip (0-2), hand (0-2), facet joints (0-2), and spinal discus degeneration DD (0-2) as described previously in detail[35]. The highest quartile of the total ROA score represents GARP subjects with the highest number of joint locations with radiographic abnormalities which were compared to other subjects of the GARP study and/or random subjects of the Rotterdam study. We compared affected sibling pairs from the GARP study to a random sample of unrelated subjects aged 55-65 years (n = 809) of the Rotterdam study as reference group representing the general population[36]. Both studies comprise of Caucasian subjects from the western areas of the Netherlands with a mean age of 60.3 years and may represent the same genetic background. In this sample symptomatic OA has not been assessed.

Whole-blood stimulation system

Whole-blood sample stimulation was performed as previously described[37]. In short, blood samples were collected in pyrogen-free heparinized tubes (Endotube®, Chromogenix, Mölndal, Sweden). Eight-millilitre whole-blood samples were diluted 1:1 with RPMI 1640 (Gibco Life Technologies, Paisely, United Kingdom) and stimulated with 10 ng/mL *Escheria coli* LPS (Difco Laboratories, Detroit, Mich). To minimize the influence of circadian rhythms and measurement errors, blood samples were taken between 8 AM and 11 AM, the time frame between blood collection and stimulation was less than one hour and a half, and all stimuli were performed with the same endotoxin batch. One medium-diluted blood without LPS was used as negative control. After 24-hour incubation, samples were centrifuged twice (600g) and the supernatants stored at -70ºC. IL-1β and IL-1Ra production were measured in one batch by enzyme-linked immunosorbent assays (ELISA) according to manufacturer's guidelines (Central Laboratory of the Blood Transfusion Service, Amsterdam, the Netherlands). Nine patients were excluded from the analyses because either whole-blood samples had not been obtained (N = 5) or levels of IL-1Ra or IL-1β were missing (N=4).

Genotyping measurements

Genomic DNA was isolated from blood samples from the GARP study, for 1 subject DNA was missing. In total 809 subjects of Rotterdam and 381 subjects of the GARP study were genotyped for 7 SNPs encompassing the IL-1 gene cluster on chromosome 2q13; 1 SNP located in the *IL1A* gene (*C-889T* rs1800587), 3 SNPs in the *IL1B* gene C3953T (rs1143634), T-31C (rs1143627) and C–511T (rs16944) and 3 SNPs in the *IL1RN* gene VNTR in intron 2, T8006C (rs419598) and T+11100C (rs315952). The genotypes of the C3953T C–511T and VNTR in the Rotterdam study were assessed previously[22]. The genotypes of the SNP were determined by mass spectrometry (homogeneous Mass ARRAY system; Sequenom Inc., San Diego, CA), using standard conditions. Genotypes were analyzed by using Genotyper 3.0 software (Sequenom Inc.). Throughout the paper the common alleles of the SNPs are designated 1 and the rare alleles as 2. For the controls genotypes from the Rotterdam study were available of 788 subjects.

Statistical analysis

The contribution of the individual genotypes of the SNPs of the IL-1 gene cluster to the innate *ex vivo* cytokine production upon LPS stimulation was estimated using a mixed model regression analyses performed with the logarithmically transformed cytokine levels as dependent variable and as co-variable the genotypes of the SNPs and sex. In the mixed model analyses, random effects modeled the familial dependencies that might occur for the cytokine levels. These analyses were carried out with SPSS version 14 software (SPSS, Chicago, Illinois, USA). Haplotypic effects on the quantitative innate *ex vivo* cytokine production and/or total ROA scores were assessed by the THESIAS 3.1 program[38] and adjusted for sex and/or BMI where indicated. When interpreting the THESIAS results it should be taken into account that in these analysis siblings of the GARP study were used as independent individuals. To assess the strength (odds ratio) of the haplotypic effect in the GARP subjects with the highest number of joints logistic regression with robust standard errors to adjust for family relationship[39] was used in Stata SE8 software (Stata Corporation, USA). In this case the haplotypes of individuals were estimated by the expectation maximization algorithm implemented in SNPHAP version 1.3 and posterior haplotype probabilities were used as sampling weight in the analysis. Instead of adjusting *P* values a priori for multiple testing, nominal *P* values are provided in order to allow the reader to interpret the level of significance.

Results

In Table 1 the characteristics of the 382 patients with symptomatic OA at multiple sites who were included in the GARP study are provided. The study consists predominantly of women (82%). Whole blood innate *ex vivo* productions of IL-1β and IL-1Ra upon LPS stimulation were previously measured in all subjects of the GARP study[13]. As the IL-1ß and IL-1Ra levels were significantly lower in females as compared to males (P-value = 1.6×10^{-5} and P-value = 0.002, respectively) and the IL-1Ra levels were significantly associated to body mass index (BMI, P-value = 0.01), all analyses concerning these levels were adjusted for sex and BMI. We could not detect an effect of age to these levels. To take into account the interaction between IL-1ß and IL-1Ra levels, we examined the effect of the IL-1ß bio-availability as expressed by the ratio between *ex-vivo* IL-1ß and IL-1Ra production upon stimulation with LPS. In total 7 SNPs encompassing the IL-1 cluster on chromosome 2q13 were measured in both the GARP study and controls. All SNPs were in Hardy Weinberg equilibrium. In Figure 1, the linkage disequilibrium (LD) pattern of the SNPs in the case and control group together across the region is visualized by the HAPLOVIEW program of the Hapmap project[40]. Notably a low LD occurs between the -511 *IL1B* and VNTR *IL1RN* SNPs with D' = 0.41 and r^2 = 0.1 dividing the region in two separate blocks, the first block consisting of *IL1A* –889, and *IL1B* +3953, -31, and –511 and the second block consisting of *IL1RN* gene VNTR, +8006 and +11100 which were used for the haplotype association analysis.

Association analysis of IL-1 cluster haplotypes and IL-1ß bio-availability based on *ex vivo* production levels

As shown in Table 2 there were 2 haplotypes in the second block (VNTR, +8006 and +11100) covering the IL1RN gene that associated significantly to the cytokine production

levels. Haplotype 2-2-1 (frequency 0.22) was associated to lower IL-1β production levels (P = 0.002) whereas haplotype 2-2-2 (frequency 0.02) to higher IL-1Ra production level (P = 1.1×10^{-4}).

Table 1: Characteristics of study populations

	GARP study
Total number of subjects[1]	382
Number of females (%)	311 (82)
ROA scores (> 0) of subjects within (N=382):	
Hip (%)	107 (28)
Knee (%)	150 (39)
Hand (%)	213 (56)
Facet (%)	235 (62)
DD (%)	256 (67)
Mean total ROA score (range)	3.45 (0-9)
Mean age in years (range)	60.3 (43-79)
Mean body mass index in kg/m^2 (SD)	27.0 (4.7)
Mean IL1-ß /IL-1Ra ratio, IL1ß bio-availability (SE)	0.798 (0.003)

[1]Numbers represent GARP subjects with symptomatic OA at multiple joint locations including subjects with uni and/or bilateral joint replacement (N=38 for hip and N = 8 for knee). DD = spinal disc degeneration SD = standard deviation IQR = interquartile range.

These haplotypes are not tagged by one of the individual SNPs and the associations appear more significant but are in agreement with the results obtained for the genotype analysis (Supplementary Table 1).
To take into account the interaction between IL-1ß and IL-1Ra levels we examined the effect of the haplotypes to the bio-availability as expressed by the ratio between IL-1ß and IL-1Ra (Table 2). As can be seen only *IL1RN* haplotype VNTR, +8006 and +11100 2-2-1 showed significant lower bio-availability of IL-1ß upon LPS stimulation (P-value = 0.026). Next we assessed whether these haplotypes also contributed to the degree of cartilage abnormalities in the GARP subjects expressed by the summed ROA score of all joint locations.

Association analysis of IL-1 cluster haplotype and OA

For haplotype *IL1RN* VNTR, +8006 and +11100 2-2-1 of the second block, which associated significantly and consistently to lower IL-1ß availability, we could not detect an association among GARP subjects as compared to random controls of the Rotterdam study. However, when we explored the quantitative association with the total ROA score for all joint locations among subjects of the GARP study in THESIAS, a trend towards a higher mean summed ROA score was observed for haplotype 2-2-1 (P = 0.07) as compared to the other haplotypes. Upon further investigation it was shown that subjects that reside within the highest quartile of the total ROA score (ROA score > 5, N = 64) showed significant

association with this *IL1RN* haplotype with an OR of 1.76, 95%CI 1.14-2.76, P-value = 0.011 when compared to the subjects of the Rotterdam study (N = 788) and an OR of 1.91, 95% CI 1.21-3.02, P-value = 0.006 when compared to the remaining GARP subjects (N = 317). Adjusting for age, sex or BMI did not considerably affect this haplotypic association. None of the other haplotypes was associated to OA (subtypes).

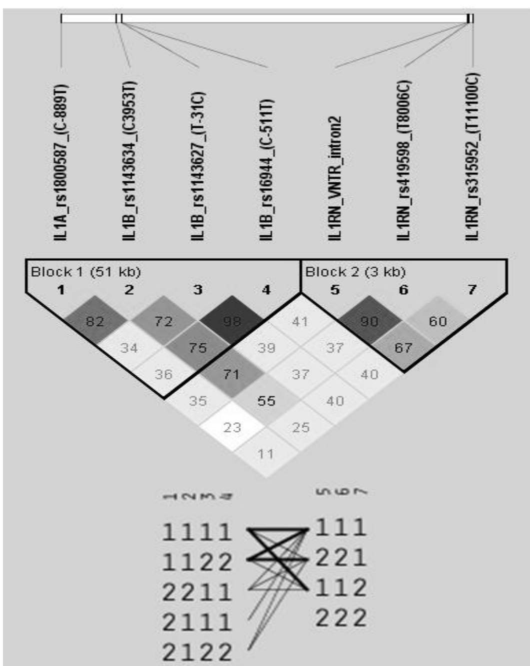

Figure 1. Pairwise linkage disequilibrium across the IL-1 cluster single nucleotide polymorphisms (SNPs) as visualized by the Haploview program and expressed by the linkage disequilibrium coefficient D'.

When combining the effect of haplotype *IL1RN* VNTR, +8006 and +11100 2-2-1 to both IL-1ß availability and the total ROA score within GARP subjects, the haplotype was independently and significantly associated to lower IL-1ß availability (P-value = 0.007) and to subjects (25%) with highest number of joint locations with ROA (P-value = 0.006). In contrast, we did not observe lower innate *ex vivo* IL-1ß availability among the subjects with the highest number of joint locations with ROA within the GARP study.

Discussion

It was investigated whether genetic variation at the IL-1 cluster contributes to innate *ex vivo* cytokine production upon LPS stimulation and whether the relevant haplotypes contribute to symptomatic OA at multiple joint sites as assessed in the GARP study.
Haplotype 2-2-1 (frequency 0.22) of the *IL1RN* block showed significant lower bio-availability calculated by the ratio of IL-1ß and IL-1Ra as compared to the other *IL1RN*

Table 2. Haplotype association analysis of block 1 consisting of the *IL1A* (–889) and *IL1B* (3953, -31, and -511) gene and block 2 consisting of 3 the *IL1RN* gene;VNTR_T8006C_T11100C with the innate *ex vivo* IL-1β and IL-1Ra production upon LPS stimulation of whole blood cells.

Haplotypes IL1 block 1	%	Haplotypic mean (95% CIL)[1]		
		Log IL-1β[1]	Log IL-1Ra	Log IL-1ß / IL-1Ra
1-1-1-1	40	1.72 (1.69-1.75)	2.14 (2.08-2.20)	0.39 (0.36-0.41)
1-1-2-2	27	1.73 (1.69-1.77)	2.12 (2.07-2.17)	0.40 (0.37-0.42)
2-2-1-1	22	1.77 (1.72-1.81)	2.14 (2.09-2.20)	0.40 (0.37-0.43)
2-1-2-2	4	1.66 (1.57-1.75)	2.17 (2.08-2.26)	0.38 (0.35-0.41)
2-1-1-1	4	1.71 (1.61-1.81)	2.11 (1.99-2.22)	0.38 (0.35-0.42)
others	3			
Haplotypes IL1 block 2	**%**	**Haplotypic mean (95% CIL)[1]**		
		Log IL-1β	Log IL-1Ra	Log IL-1ß / IL-1Ra
1-1-1	44	1.75 (1.72-1.78)	2.14 (2.09-2.19)	0.40 (0.37-0.42)
1-1-2	31	1.74 (1.71-1.78)	2.14 (2.09-2.19)	0.39 (0.36-0.42)
2-2-1	22	1.66 (1.62-1.71)**	2.11 (2.06-2.16)	0.38 (0.35-0.40)*
2-2-2	2	1.83 (1.58-2.08)	2.27 (2.19-2.34)***	0.40 (0.35-0.46)
others	1			

Data was analyzed using the THESIAS program for quantitative phenotypes for IL1β and IL1Ra levels logarithmically transformed. P-value is determined by comparing the specific haplotypic mean as compared to the haplotypic mean of all other haplotypes. [1]Values for IL1ß were adjusted for sex whereas values for IL1Ra and IL1ß/IL1Ra were adjusted for sex and BMI. HAP = haplotype allele frequency. *P<0.05, **P<0.005 and ***P<0.0002

haplotypes (P-value = 0.026) and to subjects (25%) with highest number of joint locations with ROA (P-value = 0.006). Our result of this *IL1RN* haplotype with IL-1ß bio-availability are in line with the results of Vamvakopoulos *et al.*[17] who showed that the *IL1RN* VNTR allele 2 was significantly associated to lower IL-1β production levels upon stimulation with LPS. Together our results confirm that genetic variation within the *IL1RN* gene exert their influence on the IL-1β bio-availability possibly via a functional difference of the IL-1Ra protein. As antagonist of IL-1β to the IL-1 receptor, aberrant IL-1Ra may hamper a correct regulation of the biological IL-1β level. In normal cartilage lower IL-1ß bio-availability, as result of genetic variation at the IL-1 gene cluster, may cause an inefficient repair of damaged cartilage and thereby influence the propensity to develop OA at various joint sites. To this end the association with the *IL1RN* VNTR, +8006 and +11100 haplotype 2-2-1 to lower IL-1ß availability and to ROA at the highest number of joint locations in our dataset appear consistent. The subsequent absence of low IL-1ß availability among this severe ROA subtype is, however, more difficult (final sentence of the result section). Moreover, we previously showed higher IL-1ß, IL-1Ra and lower IL-10 cytokine production levels upon LPS stimulation occur among subjects of the GARP study as compared to controls[13] whereas these levels did not predispose to knee OA progression[14]. A possible explanation

could be that the actual whole blood *ex vivo* cytokine production measurement is not entirely independent on disease status, but brings about increased sensitivity to LPS activation in subjects with severe OA disease pathology. In our own dataset, this explanation is substantiated by the observation that although the *IL1RN* 2-2-1 haplotype is more frequent among subjects with OA at the highest number of joints, the association of the haplotype with low IL-1ß availability gets lost in this particular group (results not shown) possibly due to an activated innate immune system. Similar to the relation between plasma CRP and ischemic events as discussed by others[41], the association observed in epidemiological studies between high innate *ex vivo* cytokine IL-1ß and IL-1Ra with OA[13] may not reflect causality but rather a marker of the ongoing disease process that affects an individual's sensitivity for LPS stimulation. As elegantly outlined in a review of Scanzello *et al.*[42] OA may indeed be considered as a chronic wound in which the innate immune response (via up regulation of Toll like receptors) may be activated by molecular signals of tissue damage. The fact that we observe such an effect mainly in subjects with a high number of OA affected joints may indicate that this is particularly true in subjects with advanced disease. To validate these effects further it should be investigated whether indeed an individuals cytokine production capacity upon LPS stimulation changes in the course of OA onset or progression and/or whether healthy subjects with a specific inflammatory cytokine production profile are prone to develop OA (at multiple joint locations).
Our data show a common *IL1RN* haplotype that is significantly associated to lower IL-1ß availability and to subjects with highest number of joint locations with ROA. The fact that this association is counter intuitive to the concept that inflammation in OA contributes to symptoms and augments many pathological changes underlines the complex interplay between cytokines and the OA disease process.

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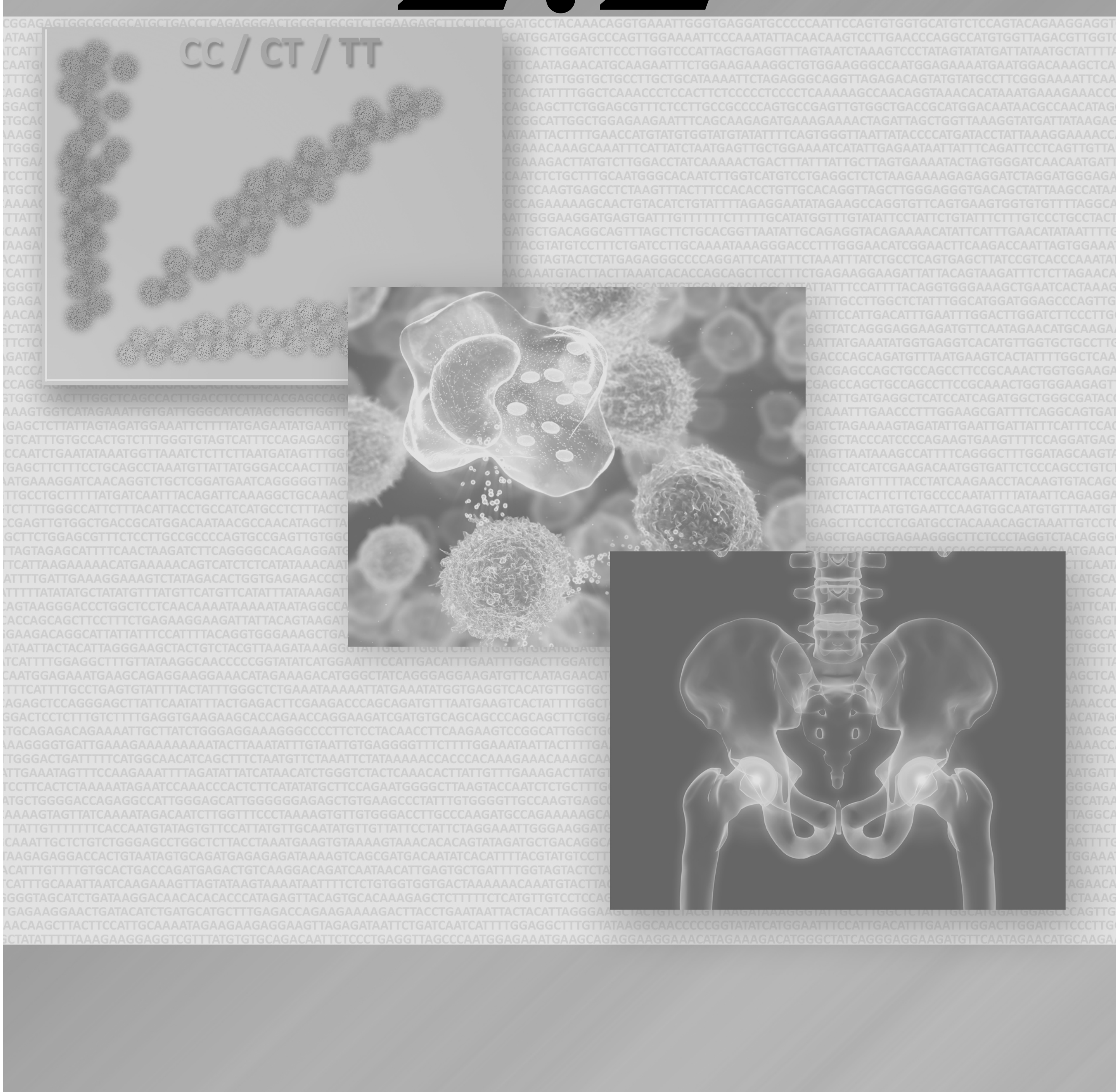
Supplementary Table 1. Genotype association analysis of logarithmically transformed innate *ex vivo* cytokine production upon LPS stimulation measured in the GARP study.

Genotypes (N)	Log IL-1β		Log IL-1Ra	
	Mean	P-value[1]	Mean	P-value[1]
Overall (368)	3.49		4.37	
*IL1A*_-889 0 (172)	3.47		4.37	
*IL1A*_-889 1 (147)	3.50		3.36	
*IL1A*_-889 2 (38)	3.50		4.40	
*IL1B*_3953 0 (203)	3.46		4.37	
*IL1B*_3953 1 (144)	3.51		4.36	
*IL1B*_3953 2 (18)	3.58		4.44	
*IL1B*_-31 0 (160)	3.49		4.39	
*IL1B*_-31 1 (169)	3.49		4.35	
*IL1B*_-31 2 (34)	3.45		4.36	
*IL1B*_-511 0 (162)	3.50		4.39	
*IL1B*_-511 1 (154)	3.48		4.35	0.047
*IL1B*_-511 2 (29)	3.42		4.36	
*IL1RN*_VNT 0 (205)	3.53		4.38	
*IL1RN*_VNT 1 (137)	3.43	0.004	4.37	
*IL1RN*_VNT 2 (20)	3.44		4.32	
*IL1RN*_8006 0 (201)	3.53		4.37	
*IL1RN*_8006 1 (127)	3.42	0.005	4.38	
*IL1RN*_8006 2 (24)	3.47		4.33	
*IL1RN*_11000 (166)	3.47		4.35	
*IL1RN*_11100 1 (161)	3.49		4.39	0.056
*IL1RN*_11100 2 (39)	3.55		4.39	

[1]Data was analyzed using mixed model regression analyses with IL1β, and IL1Ra levels logarithmically transformed as dependent variable and as co-variables the genotypes coded as 0, 1, 2 carriers of the rare allele and for IL1ß adjusted for sex and for IL1Ra adjusted for sex and BMI. Family numbers were used as random effect variables to adjust for the family relationship between siblings.

2.2



A genome wide linkage scan reveals *CD53* as an important regulator of innate TNF-alpha levels.

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Abstract

Cytokines are major immune system regulators. Previously, innate cytokine profiles determined by LPS stimulation were shown to be highly heritable. To identify regulating genes in innate immunity we analyzed data from a genome wide linkage scan using microsatellites in osteoarthritis patients (The GARP study) and their innate cytokine data on IL-1β, IL-1Ra, IL-10 and TNFα. A confirmation cohort consisted of the Leiden 85-Plus study.
In this study, a linkage analysis was followed by manual selection of candidate genes in linkage regions showing LOD scores over 2.5. A SNP gene tagging method was applied to select SNPs on the basis of highest level of gene tagging and possible functional effects. QTDT was used to identify the SNPs associated to innate cytokine production. Initial association signals were modelled by a linear mixed model. Through these analyses we identified 10 putative genes involved in the regulation of TNFα. SNP rs6679497 in gene *CD53* showed significant association to TNFα levels (P = 0.001). No association of this SNP was observed to osteoarthritis. A novel gene involved in the innate immune response of TNFα is identified. Genetic variation in this gene may play a role in diseases and disorders in which TNFα is intimately involved.

Introduction

The immune system is a complex network of interacting pathways and signaling proteins which enables organisms to respond to pathogens as well as to many other events that challenge homeostasis. The immune system is regulated through cytokines, which are mainly secreted by lymphocytes. The ability of lymphocytes to produce cytokines can be characterized by *ex vivo* stimulation with, for example, the bacterial surface molecule lipopolysaccharide (LPS). This ligand triggers *ex vivo* lymphocytes to produce a maximal pro-inflammatory cytokine response, subsequently followed by an anti-inflammatory response[1]. The as such determined maximal cytokine production profile has been shown to be stable[2] and to contain a significant heritable component estimated between 53% and 86%, indicating a strong genetic control[3].
Based on innate *ex vivo* cytokine profiles, individuals can be characterized as pro- or anti-inflammatory and it has been shown that these profiles can be predisposing to diseases with an inflammatory component such as multiple sclerosis and cardiovascular events[4,5]. In osteoarthritis (OA), a pro-inflammatory profile may affect the articular cartilage homeostasis, which depends on a delicate balance of catabolic respectively anabolic activity induced by pro- (tumor necrosis factor (TNF)α, interleukin(IL)-1ß) and anti-inflammatory (IL-10 and IL-1 receptor antagonist(Ra)) cytokines[6,7]. In line with this hypothesis, Riyazi *et al.* showed that patients of the Genetics osteoARthritis and Progression (GARP) study with OA at multiple joints sites simultaneously, have high innate IL-1β and IL-1Ra and low innate IL-10 production as compared to controls[8]. Although a later study indicated that the mechanism underlying this association may be more complex, it confirmed the association of genetic variation associated to the innate cytokine levels to OA features[9]. We and others have shown that genetic variation of genes involved in the regulation of the immune system may be reflected by a specific profile of circulating plasma inflammatory markers[10-12]. Furthermore, it was shown that DNA variants

within the *IL10* gene and genes of the *IL-1* cluster may be responsible for a part of the variation in the heritable innate *ex vivo* cytokine production upon LPS stimulation[13-16]. A large part of the heritability however, cannot be explained by the currently known genes.
Characterisation of the genes that explain a considerable part of the individual variation in the innate cytokine profiles may shed more light on the regulatory elements designed to obtain or maintain proper balance of these cytokines. Through a better understanding of these elements more insight in underlying disease processes in diseases with an inflammatory component such as OA can be obtained, thereby enabling identification of putative therapeutic targets. In the present study we set off to discover such putative quantitative trait loci for innate cytokine levels by use of the available genome wide linkage data of subjects of the GARP study[17], as well as data on their *ex vivo* LPS stimulated production of IL-1β, IL-1Ra, IL-10 and TNFα[8]. Confirmation by association analysis of innate cytokine levels was performed using 563 unrelated individuals of the Leiden 85-Plus study[18]. Identified genetic variation influencing the innate immunity profile was tested for association to OA and radiographic OA subtypes as assessed in the GARP study.

Materials & methods

Study subjects

The GARP study consists of 191 Caucasian sibling pairs affected predominantly by symptomatic OA at multiple joint sites. Characteristics of the GARP study are listed in Table 1. Details on description of the phenotype and data collection are described by Riyazi *et al.*[19]. As a confirmation cohort, we used the Leiden 85-Plus study which consists of inhabitants of Leiden (Netherlands), who were asked to participate in this study upon reaching the age of 85 years between September 1, 1997 and September 1, 1999. The response rate was 87% and in total 599 individuals were included in this study. OA data are not available for the Leiden 85-Plus study[20].

Phenotyping

In the GARP study and Leiden 85-Plus study, for most participants (N=370 and N=563 respectively) an *ex vivo* whole blood sample was stimulated with 10 $ngml^{-1}$ LPS and after a 4 hours incubation the sample was centrifuged and the TNFα levels were determined in the supernatant by use of an enzyme linked immunosorbent assay. In a second sample a similar protocol was performed with a 24 hour incubation after which the plasma levels of IL-1β, IL-1Ra and IL-10 were determined[1,19]. In concordance with previous studies[3,4,5], the *ex vivo* LPS stimulated cytokine levels were not normally distributed and influenced by gender. In our analyses log transformed cytokine levels were used and analyses were adjusted for sex.

Genotyping

Previously, a genome wide microsatellite scan was performed in the GARP study to identify new OA susceptibility loci, a detailed description of the genotyping methods and control policy has been described by Meulenbelt *et al.*[17]. In short, 417 microsatellite markers on an average spacing of 10 cM across the genome were measured. Initial linkage peaks were identified and two peaks were fine mapped by typing 3 additional microsatellite markers for each in the region of linkage (Supplementary Table 1). SNP multiplex genotyping assays were designed using Assay Designer software 3.1. iPlex assays were

measured on the Sequenom MassARRAY system (Sequenom, San Diego, CA). PCR's were carried out in a final volume of 5 μl and contained standard reagents and 5 ng of dried genomic DNA. Genotypes were called using the Genotyper v3.1 software (Sequenom, San Diego, CA). All SNPs were checked for deviations from Hardy-Weinberg equilibrium and approximately 8% of the subjects were genotyped twice as a check for genotyping and calling consistency. Of the 47 genotyped SNPs in this study, 3 SNPs failed quality check due to low amplification, bad cluster separation or low confidence in called genotypes and were excluded from further analysis.

Linkage analysis and candidate gene selection

The GARP microsatellite genotype data and log transformed LPS stimulated levels were analyzed using the variance components option implemented in Merlin to assess linkage of the levels to genetic loci[21]. Merlin output files were modified to tab delimited files with LOD score per marker to facilitate uploading to a custom track in the UCSC genome browser genome graph function. The significance level of linkage peaks was assessed by use of random gene dropping simulations in Merlin using 5000 reruns. The regions showing LOD scores over 2.5 were explored for candidate genes by use of the UCSC genome browser[22], where the individual markers' LOD scores were uploaded on a custom track. All UCSC annotated genes in the 1-LOD-drop region within the flanking areas of a linkage peak over 2.5 were considered for possible involvement in the cytokine response. This manual selection of genes was based on location within the linkage region, GO terms and Swissprot description provided in the UCSC genome graph function. Genes selected were genes which are described as being involved in immune system communication, antigen recognition and immune response. We selected 10 positional candidate genes (three linkage areas). Candidate genes were subsequently tagged using SNPs selected from the International HapMap Project genome browser[23]. SNP selection was based on genetic position and function as well as potential to tag genetic variation present within these genes. Tagger software implemented in the Haploview program with settings "$r^2 > 0.8$" and "pair wise tagging" was used to optimize tagging SNP selection[24]. In the selection process we included only SNPs with minor allele frequencies over 0.05 in CEPH data. A prioritization was applied to SNPs in coding regions by forced inclusion of non-synonymous SNPs and lowest priority given to downstream SNPs. In total, 47 SNPs were selected for genotyping.

Association analysis of quantitative innate cytokine levels

The GARP data were analyzed for association of LPS levels to SNPs in the candidate genes using QTDT[25]. Initially, the -WEGA and –WEGD commands were applied to test for association given linkage on a specific locus under an additive and dominant model respectively. To test for possible population stratification we used the –AP –WEGA or –AP -WEGD command. Furthermore, a linear mixed model was used to model the association with the SNP in the GARP and Leiden 85-Plus data including sex, age, BMI as covariates. Here, the random family effect models correlation between siblings of the GARP study due to shared genetic and environmental effects[26]. In the combined GARP and Leiden 85-Plus analysis, in addition to family numbers and covariates sex, age and BMI, we included study identifiers in the model to correct for putative batch differences. Genotypes were coded as 0 (homozygote common allele), 1 (heterozygote) and 2 (homozygote rare allele) to test an additive model and 0, 1 and 1 respectively for testing a dominant model with one degree of

freedom. All reported p-values are nominal p-values uncorrected for multiple testing for the reader's interpretation of the results, unless mentioned otherwise.

Qualitative association analysis of OA status and SNPs

To asses association of SNPs to OA at multiple joint sites as defined in the GARP study, a logistic regression was performed in STATA/SE 8.0, using the Leiden 85-Plus sample as a reference sample, whereby we used family numbers in the GARP study as a random effect variable to model familial dependencies. Dominant effects for the rare allele were tested by pooling heterozygotes with homozygotes for the rare allele.

Results

Characteristics

The characteristics of GARP and the study sample of the Leiden 85-Plus study where both innate cytokine production levels and genotypes were available are shown in Table 1. The participants of the Leiden 85-Plus study were significantly older as compared to the GARP participants (P < 0.01) and we observed significant differences in the transformed LPS stimulated levels between the GARP and Leiden 85-Plus participants for IL-1β (P = 0.034), IL-1Ra (P <0.01) and TNFα (P <0.01). To check for age dependencies of the LPS stimulated cytokine profiles the correlation with age was analyzed. We observed no significant correlations of age and IL-1β, Il-10 or IL-1Ra. However, TNFα showed a significant (P=0.037) correlation with age with a Pearson correlation coefficient of 0.11 (data not shown). The older subjects of the GARP study had on average a higher LPS stimulated TNFα level.

Table 1. Characteristics GARP and Leiden 85-Plus study

	N	The GARP Study	N	Leiden 85-Plus Study
No.	-	382	-	563
Age, mean ± SD*	-	60.4 ± 7.6	-	85[1]
No. women (frequency)*	-	301 (0.81)	-	375 (0.67)
Mean Log(IL1β) ± SD* (variance)	370	3.49 ± 0.30 (0.088)	559	3.54 ± 0.38 (0.142)
Mean Log(IL1Ra) ± SD* (variance)	369	4.37 ± 0.15 (0.023)	560	4.55 ± 0.20 (0.041)
Mean Log(IL10) ± SD (variance)	369	2.87 ± 0.19 (0.035)	560	2.85 ± 0.30 (0.090)
Mean Log(TNFα) ± SD* (variance)	368	3.87 ± 0.18 (0.032)	561	3.98 ± 0.21 (0.045)

* Significant difference between studies (t-test) [1]All subjects of the Leiden 85-Plus study were 85 years old at sampling.

IL-1β, IL-1Ra and IL-10 linkage and association analysis

The genome wide linkage analysis to find quantitative trait loci involved in innate IL-1β, IL-1Ra and IL-10 analyses using variance components did not reveal any evidence for linkage above a LOD score of 2.5 (Figure 1A, 1B and 1C respectively). We did not select candidate genes and single nucleotide polymorphism(SNP)s for follow up analysis of these traits. No substantial evidence for linkage was observed at the loci encoding cytokines IL-1β and IL-1Ra (2q13), IL-10 (1q32.1) or TNFα (6p21.33) (Figure 1 A-D).

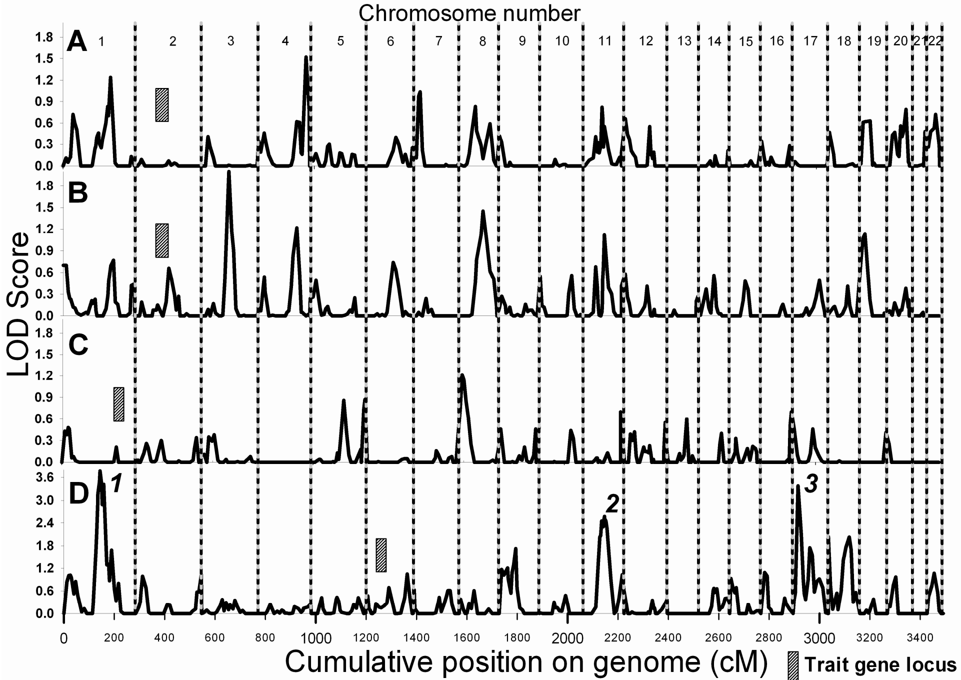

Figure 1. LOD scores for genome wide linkage analyses for QTL's of (A) IL-1β, (B) IL-1Ra, (C) IL-10 and (D) TNFα.

TNFα linkage and association analysis

Genome wide linkage analysis of innate TNFα levels revealed three regions with positive evidence for linkage with LOD scores over a LOD score of over 2.5 (Figure 1D), of which 1 peak reached a genome wide linkage significance level. The linkage peak on chromosome 11q12.1 (Figure 1D, peak 2) was fine mapped using three microsatellite markers and after fine mapping showed a maximum LOD score of 2.57 (marker D11S1314 P = $3.0•10^{-4}$, genome wide p-value 0.497) between markers D11S935 and D11S901 (width 53 megabases (Mb), Figure 2A). Furthermore on chromosome 17p13.2 (Figure 1D, peak 3) we observed a narrow linkage peak with maximum a LOD score of 3.38 (marker D17S938 P = $4.0•10^{-5}$, genome wide p-value 0.064) between markers D17S831 and D17S799 (width 5Mb, Figure 2B) which was not fine mapped. The largest peak on chromosome 1p13.3 (Figure 1D, peak 1) was fine mapped using three additional microsatellite markers and after fine mapping showed a maximum LOD score of 3.77 (marker D1S2726 P = $3.0•10^{-4}$, genome wide p-value 0.018) between markers D1S2868 and D1S484 (width 52Mb, Figure 2C). Within these linkage peaks we selected 10 candidate genes (Table 2). On chromosome 11 we selected candidate genes *MADD, SELH, CD6* and *CD5*, on chromosome 17 *GPS2, TNFA-SF12/13* and *CD68* and on chromosome 1 *CSF1, CD53* and *FAM19A3*. The genes were tagged using 44 haplotype tagging SNPs, thereby tagging from 30-100% of the genotypic variation recorded in the HapMap database (Table 2). QTDT analysis indicated significant associations to TNFα levels for SNPs in *CD53* and *FAM19A3* (Table 2). We were unable to model the observed associations of *FAM19A3* in a linear mixed model,

however, when a dominant linear mixed model was fitted for *CD53* rs6679497 we again observed a significant association in both the GARP and Leiden 85-Plus separately (*P* = 0.013 and 0.032 respectively, Table 3). When we combined the data of both studies in a linear mixed model using a dominant model we observed a highly significant association of rs6679497 (*P*=0.001, Table 3). The association remains significant after a Bonferroni correction to account for the 11 SNPs tested on the locus (corrected p-value = 0.012), or accounting for all 44 SNPs selected upon the linkage analysis (corrected p-value = 0.047). The minor allele of this intron SNP (frequency 0.48) associated with significantly lower innate TNFα levels.

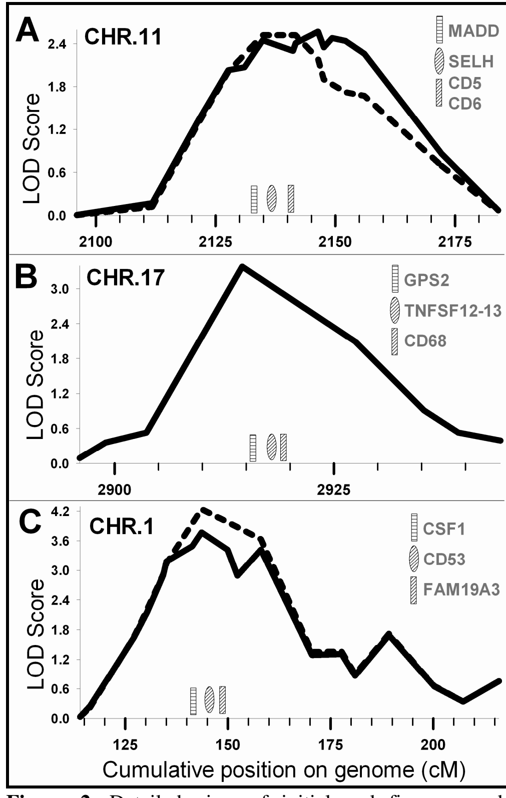

Figure 2. Detailed view of initial and fine mapped linkage peaks identified on chromosome 11 (Pane A, peak 2), chromosome 17 (Pane B, peak 3) and chromosome 1 (Pane C, peak 1). Schematically represented are the tested genes positions in the linkage area. The dotted lines represent the initial linkage signal, whereas the solid lines represent the finemapped linkage signal.

Table 2. Genes and selected SNPs in Linkage Peak, TNFα

Chr	Gene (coverage[1])	Short description[2]	SNP	Position	QTDT add[3]	QTDT dom[4]
1	CSF1 (58%)	Granulocyte/macrophage colony-stimulating factors are cytokines that act in hematopoiesis by controlling the production, differentiation, and function of 2 related white cell populations of the blood, the granulocytes and the monocytes-macrophages	rs915357 rs333968 rs333970 rs3738760	Intron Intron Intron Coding Exon	0.055^{+}	0.060^{+}
	CD53 (52%)	cell surface glycoprotein that is known to complex with integrins.. Familial deficiency of this gene has been linked to an immunodeficiency associated with recurrent infectious diseases caused by bacteria, fungi and viruses. Alternative splicing results in multiple transcript variants encoding the same protein	rs10494122 rs10857833 rs6679497 rs4839581 rs3790722	Intron Intron Intron Intron Intron	0.012*	0.009**
	FAM19A3 (100%)	Contains conserved cysteine residues at fixed positions, and are distantly related to MIP-1alpha, a member of the CC-chemokine family.	rs4450019 rs11102524	Intron Intron	0.019*	0.033*
11	MADD (68%)	Tumor necrosis factor alpha (TNF-alpha) is a signaling molecule that interacts with one of two receptors on cells targeted for apoptosis. The protein encoded by this gene is a death domain-containing adaptor protein that interacts with the death domain of TNF-alpha receptor 1 to activate mitogen-activated protein kinase (MAPK) and propagate the apoptotic signal	rs7114704 rs10501320 rs10501321 rs10838689 rs2290149 rs11039183 rs753993	Intron Intron Intron Intron Intron Intron Intron		
	SELH (100%)	This gene encodes a selenoprotein, which contains a selenocysteine (Sec) residue at its active site.	rs9420 rs3017889	Intron bound. Downstream		
	CD6 (30%)	CD6 is a monomeric 105- or 130-kD membrane glycoprotein that is involved in T-cell activation.	rs2905504 rs11230550 rs11230553 rs2283263 rs11230559 rs11230563 rs2074225 rs1050922	Intron Intron Intron Intron Intron Coding exon* Coding exon* Coding exon		
	CD5 (68%)	Human T-cell surface glycoprotein of relative molecular mass (Mr) 67,000, has been implicated in the proliferative response of activated T cells and in T-cell helper function.	rs3862667 rs572350 rs671444 rs12364244 rs637186	Intron Intron Intron Intron Coding exon*		
17	GPS2 (100%)	This gene encodes a protein involved in G protein-mitogen-activated protein kinase (MAPK) signaling cascades.	rs2270981 rs2292064	Coding Exon Coding Exon		
	TNFA-SF (80%)	This gene encodes a member of the tumor necrosis factor superfamily. It encodes a hybrid protein composed of the cytoplasmic and transmembrane domains of family member 12 fused to the C-terminal domain of family member 13. The hybrid protein is membrane anchored and presents the receptor-binding domain of family member 13 at the cell surface. It stimulates cycling in T- and B-lymphoma cell lines.	rs9899183 rs12937543 rs4968211 rs11552708 rs3803800 rs6608	Intron Promoter Promoter Coding Exon* Coding Exon* 3'UTR		
	CD68 (50%)	This gene encodes a 110-kD transmembrane glycoprotein that is highly expressed by human monocytes and tissue macrophagesThe protein is also a member of the scavenger receptor family. Scavenger receptors typically function to clear cellular debris, promote phagocytosis, and mediate the recruitment and activation of macrophages. Alternative splicing results in multiple transcripts encoding different isoforms.	rs9896688 rs9901673 rs9901675	Intron bound. Coding Exon* Coding Exon*		

$^{+}$ $P < 0.1$; * $P < 0.05$; ** $P < 0.01$

[1] Gene coverage was based on genetic variation present in the HapMap database build 18.

[2] Short description adapted from gene ontology description provided in the UCSC genome graph tool.

[3] *P* values reported by QTDT using command WEGA (additive model), tests for association given linkage on a specific locus.

[4] *P* values reported by QTDT using command WEGD (dominant model), tests for association given linkage on a specific locus.

Table 3. Genotype analysis and linear mixed model for CD53 SNP rs6679497, assuming a dominant model of association

Study	N (11)[1]	Log(TNFα)	N (12)[1]	Log(TNFα)	N(22)[1]	Log(TNFα)	P LMM[2]	P LMM[3]
GARP	86	3.90	178	3.86	99	3.85	0.013*	0.001**
85-Plus	312	4.01	564	3.97	238	3.97	0.032*	

+ P < 0.1; * P <0.05; ** P < 0.01
[1] 11=homozygote common allele; 12=heterozygote; 22=homozygote rare allele
[2] Modelling dominant effect, corrected for familial relationship, age, sex and BMI
[3] Modelling dominant effect combining studies, corrected for study differences in level, familial relationship, age, sex and BMI

Association analysis of rs6679497 to OA

No significant association was observed for *CD53* rs6679497 when GARP subjects (cases) were compared to subjects of the Leiden 85-Plus study as controls using the dominant model (adjusted for age, sex and BMI, *P*=0.142), indicating that TNFα QTL locus did not confer susceptibility to OA.

Discussion and conclusion

Through a genome wide linkage scan we were able to identify SNP rs6679497 in *CD53* of which the minor allele associates to lower innate TNFα levels. It can be hypothesized that the specific genotype of rs6679497 predisposes or protect its carriers from diseases and disorders in which TNFα plays a substantial role. Previously, it was shown that TNFα does not play a major role in the onset of OA[8] and in line with this hypothesis we were unable to show associations of rs6679497 to OA as defined in the GARP study.

CD53 codes for cluster of differentiation 53, a leukocyte surface antigen. The protein family which this cell surface glycoprotein belongs to is known to complex with integrins, cellular components involved in cell-cell and cell-matrix interactions. CD53 deficiency has been linked to recurring infectious diseases caused by bacteria, fungi and viruses[27], susceptibility to these might be increased for carriers of the minor allele of rs6679497. The protein is implicated in elevated cellular glutathione in response to LPS activation and may increase cell survival under UV-B and oxidative conditions[28]. Furthermore, treatment of neutrophils with TNFα down-regulates the presence of the CD53 antigens on the cell surface through a proteolytic mechanism[29]. This indicates that the protein may play a substantial role in cellular stability and the inflammatory response to adverse conditions. Furthermore, the protective effect of ligated CD53 on the cellular surface may help specific tumors to escape from programmed cell death[30]. Although rs6679497 or any of the SNPs in its LD block are investigated for effects on the expression levels or protein function, the gene is under strong genetic control[31] and genetic variation might have a role in tumor biology or other diseases. Such a relation can readily be elucidated through investigation of this marker SNP in cancer cohorts. Furthermore, in a study which characterizes leukocytes from normal and rheumatoid arthritis (RA) patients, CD53 was found to be elevated on the RA lymphocytes surfaces[32]. Investigating the role if this gene in relation to this and other TNFα driven diseases later in life may show protective effects of the rs6679497 minor allele.

The SNP *CD53* rs6679497 resides within the intron of the gene, which shows low levels of conservation across species, and it is in an LD-block across several introns and exons

encompassing at least 23 other intron SNPs(recorded in the HapMap phase 1 & 2 data). Several transcripts are known as recorded in the UCSC database (accession number ENSG00000143119), however, only a proportion of these will actually be translated into a protein. Given the current level of total variation tagging (52%) it is likely the SNP is only a proxy marker in LD with a causal (functional) polymorphism which could have a more obvious implication to the gene regulation or protein stability and functioning. This is substantiated by the fact that the *CD53* SNP explains only part of the linkage as determined in QTDT analyses (results not shown). To find the true functional variant, a more detailed analysis of this gene by sequencing or SNP saturation is necessary. Alternatively, in our candidate gene approach we may have missed additional genetic variation at the loci of interest because of a knowledge bias on both the presence and role of genes at these loci.
Although it was shown previously that the *IL1* cluster haplotypes were associated to IL-1ß bio-availability[33,34], we observed no evidence of linkage on the genetic loci for the respective cytokines' genes, the *IL-1* gene cluster on chromosome 2, *IL-10* on chromosome 1 or *TNF* on chromosome 6. Possibly, the genetic variation in LPS stimulated cytokine levels explained by these loci is not readily detected by linkage analysis, which is known to be most suitable to detect loci that explain a major part of genetic variation. Furthermore, our linkage analyses of innate IL-1β, IL-1Ra and IL-10 levels in general revealed only moderate linkage peaks up to a LOD score of 2.5. More likely as was shown in a previous study[33], particularly the estimate of innate IL-1ß production upon LPS stimulation may not be entirely independent of the OA disease status, possibly by sensitization of the Toll-like receptor pathways as a result of disease activity[35] or otherwise sensitization of the response by lymphocytes, which may have interfered with the current linkage analysis by introducing cohort heterogeneity or bias. In the linkage analyses, we checked whether the levels in the GARP study sample were normally distributed to facilitate powerful linkage analysis using the variance component option. The innate levels of IL-1β, IL-10 and TNFα were normally distributed, whereas innate IL-1Ra levels were normally distributed after removal of 1 extreme value, which did not alter the linkage analysis results (data not shown). The use of Merlin-Regress[21] which may be more appropriate for use in highly selected samples showed a similar pattern of LOD scores for all traits, with slightly lower maximum LOD scores. It remains possible that the observed association only occurs in middle aged and elderly, therefore, further searches for genetic loci that influence the *ex vivo* innate cytokine profiles may benefit from the use of healthy young subjects in these searches.
Following a genome wide linkage analysis, association analysis of positional candidate gene SNPs within the 1-LOD-drop interval of a linkage peak, we show a consistent association of SNP rs6679497 in *CD53* to innate TNFα levels in both the GARP study (P = 0.013) and the confirmation cohort consisting of Leiden 85-Plus participants (P= 0.032). A dominant linear mixed model analysis on combined data from the GARP and Leiden 85-Plus study showed that the minor allele of this SNP associated to a highly significantly lower innate TNFα level independent of age and sex effects. In diseases with a large TNFα component such as inflammatory bowel disease or rheumatoid disorders, the minor allele of rs6679497 might exert a protective effect in susceptibility or severity.

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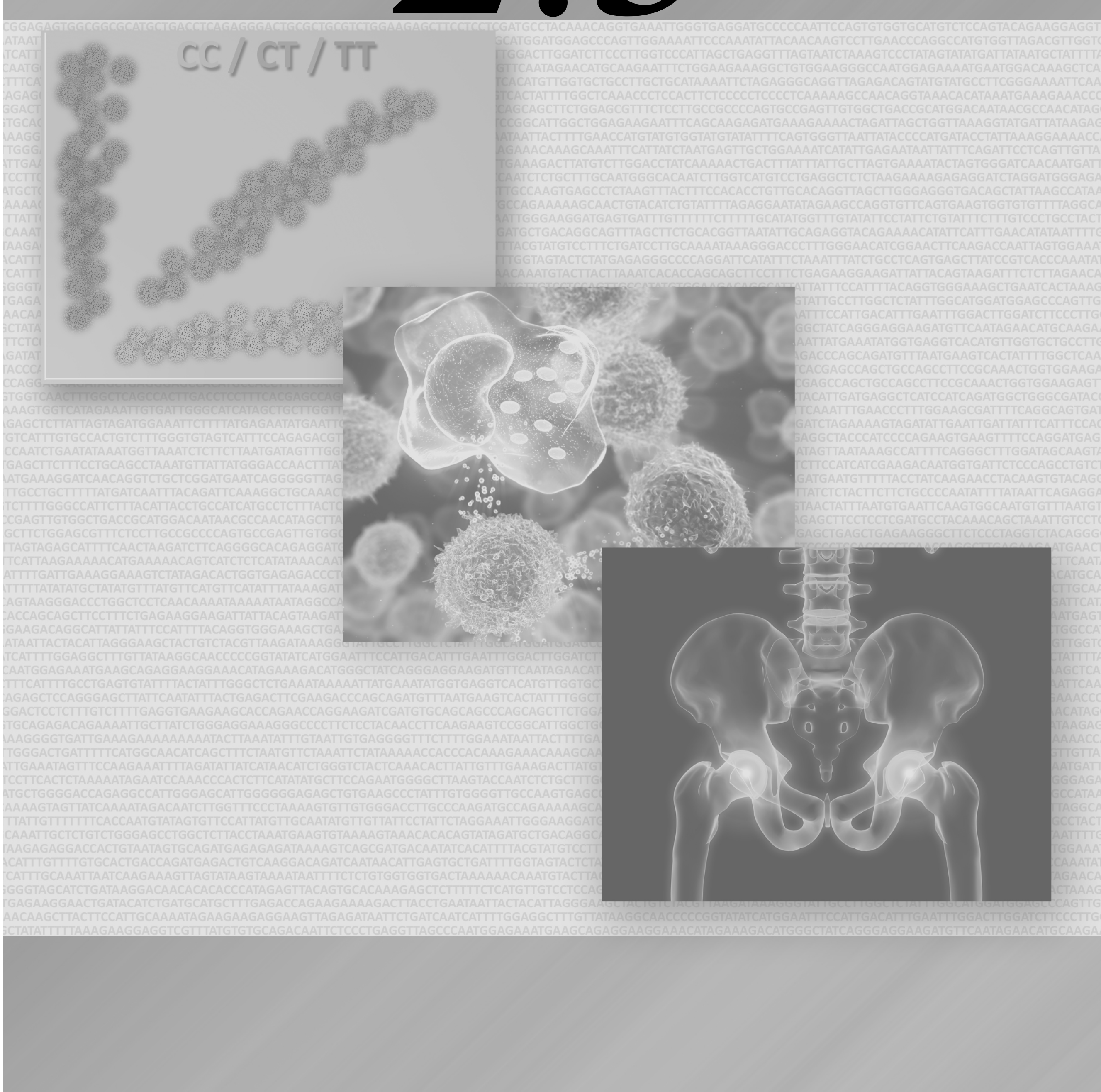
Supplementary Table 1. Markers used in fine mapping of initial linkage peaks.

Marker	Position (cM)
D1S2626	135.42
D1S2778	141.321
D1S2696	152.317
D11S986	68.014
D11S1889	78.155
D11S4081	86.331

2.3



Allelic variation at the C-reactive protein gene associates to both hand osteoarthritis severity and serum high sensitive CRP levels in the GARP study.

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Abstract

Objective: To gain more insight into the role of genetic variation of the C-reactive protein (*CRP*) gene in serum CRP levels and osteoarthritis (OA).
Methods: Serum high sensitive CRP (S-HsCRP) levels were measured in the Genetics of osteoARthritis and Progression (GARP) study. Furthermore, to assess genetic variation of the *CRP* gene, genotypes of five tagging single nucleotide polymorphisms were assessed in the GARP study and a random control sample.
Results: A significant and consistent relation between S-HsCRP levels and observed haplotypes was identified. Additionally, a *CRP* haplotype, which also associated to a significantly higher expected phenotypic mean S-HsCRP level, was associated to severe hand OA. This haplotype was tagged by a single nucleotide polymorphism (rs3091244). Carriers of this allele have an increased risk for the presence of severe hand OA with an OR of 2.3 (95% confidence interval 1.2 to 4.3, p=0.009).
Conclusions: A haplotype of the *CRP* gene, associated to high basal S-HsCRP level, is also associated to severity of hand OA, indicating that innate high basal S-HsCRP levels may influence OA onset.

Introduction

Osteoarthritis (OA) is characterised by degeneration of articular cartilage and remodelling of bone. Heritability estimates range from 30% to approximately 80%, depending on the specific joint affected or the number of joint sites involved. Although OA pathophysiology lacks a large-scale inflammatory process, there may be a low-grade systemic inflammatory component[1,2]. Chondrocytes are known to respond to pro-inflammatory stimuli by decreasing synthesis of extracellular matrix components and increasing synthesis of metalloproteinases. As such, an innate low-grade pro-inflammatory state of the body may affect susceptibility to the onset of OA[2], or may exacerbate progression once the OA disease process is initiated[3].
S-HsCRP is a sensitive marker of both low-grade[4] and acute phase systemic inflammation[5]. Previously, *CRP* haplotypes (locus 1q23.2) were identified that may partly explain the heritability of S-HsCRP levels (52%)[6,7]. Furthermore, S-HsCRP level, as a marker of low-grade inflammation, has been associated to a range of OA features[4,8-10]. In the current study we would like to investigate whether the innate inflammatory state, as expressed by the S-HsCRP level and variation at the *CRP* gene, contributes to the presence of OA in the Genetics of osteoARthritis and Progression (GARP) study.

Material and methods

The GARP study

The ongoing GARP study consists of 191 (n=382) Caucasian sibling pairs affected with symptomatic OA at multiple sites. For the current paper genotypic information was available for 381 individuals and S-HsCRP levels for 353 individuals. Detailed descriptions of the phenotypes and inclusion criteria can be found elsewhere[11]. In the current paper "quantitative hand OA" was defined by the number of hand joints (out of 20 scored) with radiographic OA (ROA). "Severe hand OA", as a qualitative measure, was defined by

presence of seven or more ROA affected hand joints, equalling 27% of subjects. Partners of the offspring in the Leiden longevity study were used as a random control population (n=739)[12].

Statistical analysis

Haplotypic means were assessed using Thesias V3.1[13]. Haplotypic associations were analysed by testing the particular haplotype to the remaining haplotypes. To assess the strength of association to severe hand OA a logistic regression analysis was performed in STATA. In this analysis robust standard errors were estimated from the variance between sibling pairs to compensate for familial relationships within GARP[14]. A linear mixed model was tested to assess association between S-HsCRP levels and OA phenotypes, with family numbers included as random variables to model possible familial effects. Differences in allele frequencies between subjects with and without severe hand ROA were calculated by Pearson's χ^2. Analyses were done in SPSS14.0 unless mentioned otherwise.

Results

Study characteristics

For 381 GARP subjects and 739 controls genotypes were completed. Baseline characteristics of these are shown in table 1.

Table 1. Characteristics of the GARP study and the random control population.

	GARP	Control
Total, no.	382[1]	739
Women, no. (%)	311 (81.4)	429 (58.1)
Age, median (range) years	59.7 (42.7-79.4)	58.3 (30.0-79.0)
BMI[2], median (range)	26.0 (19.1-46.5)	
S-HsCRP[3], mean (SE of mean)	3.63 (0.29)	
S-HsCRP[3], median (range)	1.83 (0.21-56.8)	
Mean number of affected hand joints (range)	4.62 (0-20)	
Subjects with severe hand OA (%)	103 (27)	

[1] GARP study sample consists 191 sibling pairs, for 381 subjects DNA was available.
[2] BMI stands for Body Mass Index in kgm^{-2}
[3] S-HsCRP stands for Serum High Sensitive CRP level in mgl^{-1}, numbers are calculated for subjects with S-HsCRP levels available (N=353). In all analysis logarithmic transformed values of S-HsCRP were used.

C-reactive protein gene haplotype frequencies

As is shown in table 2, six common haplotypes were resolved with frequencies ranging from 0.01 to 0.33. The frequencies in GARP and the control population were comparable with the frequencies observed by Carlson *et al*[6]. No significant differences in haplotype frequencies were found between the GARP study and the control population.

Association of C-reactive protein haplotypes with high sensitive C-reactive protein serum levels

Figure 1A shows the mean log(S-HsCRP) level for each haplotype within the GARP sample (n=353). Haplotype 1 (H1) has a significant lower (p=0.009), whereas haplotype 7/8 (H7/8) has a significant higher (p=0.02) contribution to the mean log(S-HsCRP) level.

Table 2. Assigned haplotype frequencies, expected phenotypic means of log(S-HsCRP) for GARP and the control sample haplotype frequencies.

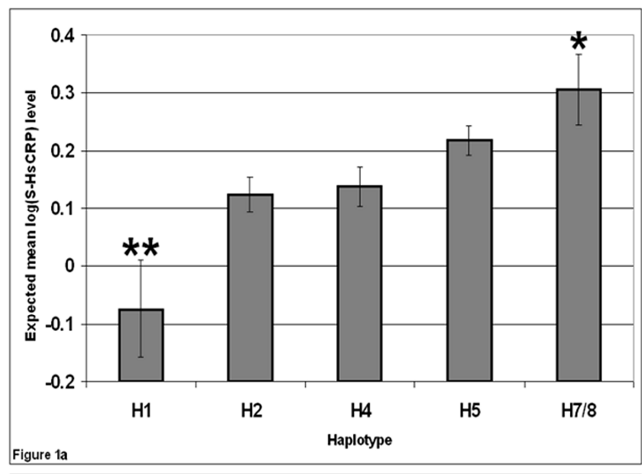
Haplotype[1]	*Study*	*N*	*frequency*	*Log(HsCRP)*[2]	*Se(log(HsCRP))*[2]
Other[3]	GARP	7	0.01	-	-
	Control	7	0	-	-
Haplotype 1 CACAA	GARP	46	0.07	-0.074	0.084
	Control	97	0.07	-	-
Haplotype 2 CAGAA	GARP	199	0.28	0.123	0.030
	Control	389	0.26	-	-
Haplotype 3 CAGGA	GARP	7	0.01	0.	0.10
	Control	10	0.01	-	-
Haplotype 4 CAGGG	GARP	188	0.27	0.137	0.034
	Control	423	0.29	-	-
Haplotype 5 TTGGA	GARP	217	0.31	0.217	0.026
	Control	482	0.33	-	-
Haplotype 7/8 AAGGA	GARP	42	0.06	0.306	0.062
	Control	70	0.05	-	-
TOTAL	GARP	706	1	-	-
	Control	1478	1	-	-

[1] Genotyping was done on a Sequenom™ platform with slightly modified protocols. SNPs used to resolve haplotypes with gene positions relative to AFF449713 and minor allele frequencies were rs3091244 , 1440 (C>T>A, 0.315/0.057), rs1417938 1919 (A>T, 0.248), rs1800947 2667 (G>C, 0.063), rs2808630 5237 (A>G, 0.268) and rs2808628 (A>G, 0.336). The latter SNP is in close LD to SNP rs1205 used in the original study by Carlson *et al.*[6], of which the haplotype nomenclature used was adapted.
[2] Levels displayed are the expected haplotypic contribution to the mean log(S-HsCRP) level of carriers as calculated by the Thesias program. In individuals the expected S-HsCRP level is determined by the contribution of the 2 carried haplotypes. The Thesias program does not allow correction for familial relationship.
[3] Rare haplotypes with frequencies below 0.01 were pooled as "other".

Association of C-reactive protein haplotypes with osteoarthritis subtypes

A significant positive association of H7/8 was observed for increasing number of ROA affected hand joints. As shown in fig 1(B), quantitatively a significantly higher (p=0.04) expected mean number of affected hand joints was observed for H7/8 (mean 3.88, SE 0.66) as compared with remaining haplotypes (mean 2.23, SE 0.13).

Subsequently it was investigated whether H7/8 associated to GARP subjects with severe hand ROA (n=103). The frequency of H7/8 in severe hand ROA cases (frequency 0.096) was significantly higher as compared with the other subjects of GARP (frequency 0.04, p=0.038) and as compared with a random control sample (n=739, frequency 0.046, p=0.016). H7/8 is discriminated by the rarer allele of single nucleotide polymorphism rs3091244. Carriers of the A allele have an increased risk (p=0.009) of severe hand OA as compared with the random controls with a crude OR of 2.3, 95% CI 1.2 to 4.3. The frequency of the A allele does not allow robust recessive model testing. Adjusting for age and/or body mass index in the logistic regression did not change the extent or significance of the genotypic risk.



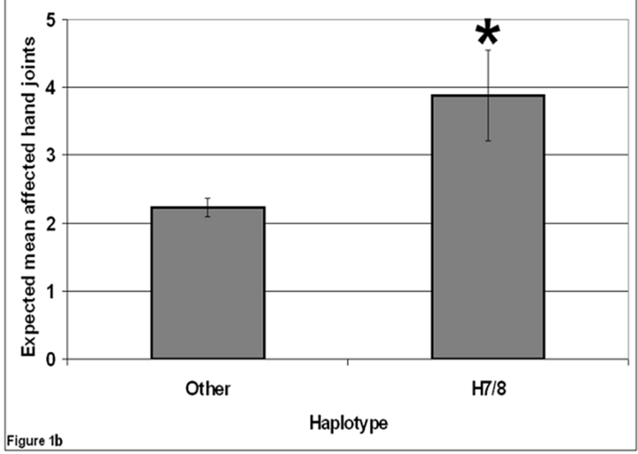


Figure 1 (**a**) Expected mean log(S-HsCRP) levels of common (frequency >0.05) *CRP* haplotypes in the GARP sample for which both genotype and S-HsCRP levels were available (N=353). H1 to H7/8 represent clades of the phylogenetic tree of the SNPs in the *CRP* gene, where H1 is the most recent and H7/8 represent the most ancient split (See Carlson *et al.*[6]). (**b**) Expected mean number of affected hand joints of common (frequency >0.05) *CRP* haplotypes in the GARP study (N=381).
Error bars represent standard error of the mean, * $P < 0.05$ ** $P < 0.01$

There were no significant differences in *CRP* haplotype frequencies between subjects with and without involvement of knee, hip, spine or extent of clinical features of OA expressed by WOMAC (Western Ontario MacMaster osteoarthritis questionnaire) scores.

Association of serum high sensitive C-reactive protein levels and osteoarthritis

Moderate positive associations were observed between S-HsCRP levels and both knee ROA (p=0.06) and WOMAC scores for pain and stiffness (p=0.08). Both these associations, however, were merely due to their association with high body mass index. We could not asses direct association between S-HsCRP levels and hand OA.

Discussion

S-HsCRP serum levels and *CRP* gene haplotypes were assessed in the GARP study to investigate the role and extent of low inflammatory processes in the development of symptomatic OA at multiple joint sites. We show that mean and median basal S-HsCRP levels observed in the GARP study as a whole are not within acute phase ranges[6], confirming that OA is not a large-scale inflammatory disorder.
Furthermore, *CRP* haplotypes, with frequencies ranging from 0.01 to 0.31, showed a specific pattern of mean S-HsCRP level. An increasing S-HsCRP level from H1 to H7/8 was observed, which coincides with the phylogenetic clades of the *CRP* gene[6]. This may indicate an evolutionary development towards low innate S-HsCRP levels. Although the mean S-HsCRP level in GARP was slightly higher (approximately 1 mg/l) the specific haplotypic pattern was strikingly similar to the one identified in the study of Carlson *et al.* in healthy individuals[6]. Of these haplotypes, H1 had a significantly lower and H7/8 had a significant higher expected mean S-HsCRP level as compared with other haplotypes. Furthermore, an allele that discriminates H7/8 associated to the mean number of affected hand joints with an OR of 2.3 for the presence of severe hand OA. The low-grade pro-inflammatory profile brought about by this single nucleotide polymorphism may affect cartilage homeostasis and may ultimately lead to a systemic form of OA. As in many genetic studies we cannot exclude the possibility of false positive findings due to multiple testing; however, this is the first report of an association of a *CRP* gene polymorphism to OA. Punzi *et al*[9] showed an association of erosive hand OA and high serum CRP levels. Despite the association between H7/8 of both S-HsCRP levels and hand OA, no direct association between S-HsCRP levels and hand ROA could be established in this study. Initial associations observed between, S-HsCRP levels and knee ROA and WOMAC scores in the GARP study were merely confounded by body mass index[15]. Our study may either not provide enough power to show associations between S-HsCRP profiles and other OA features, or acute phase responses, by, for example, obesity, may obscure association of disease and innate ongoing low-grade inflammatory effects. Furthermore, it is known that S-HsCRP may not cover the whole spectrum of inflammatory processes, therefore, future studies may focus also on other inflammatory mediators in relation to OA. To show absence of familial effects in our data the analyses were repeated in unrelated individuals of the GARP study yielding similar results (supplemental figure 1).
Together the current study confirms that genetic contribution of the low-grade basal CRP levels may be attributed to haplotypes of the *CRP* gene. Furthermore, it is shown that a specific systemic low-grade pro inflammatory profile may predispose to severe hand ROA among subjects of the GARP study as compared with healthy individuals. To investigate further the role of CRP in OA of the hand, upcoming progression data in this study may provide more insights into the prognostic effect of *CRP* haplotypes and in baseline CRP levels.

Acknowledgments

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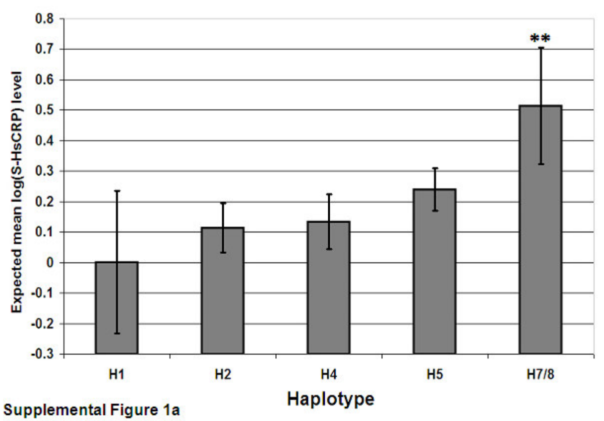
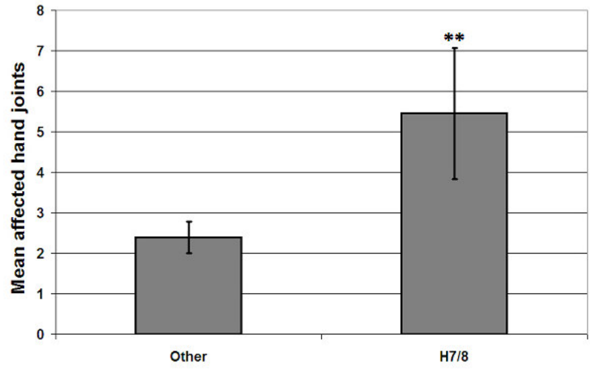
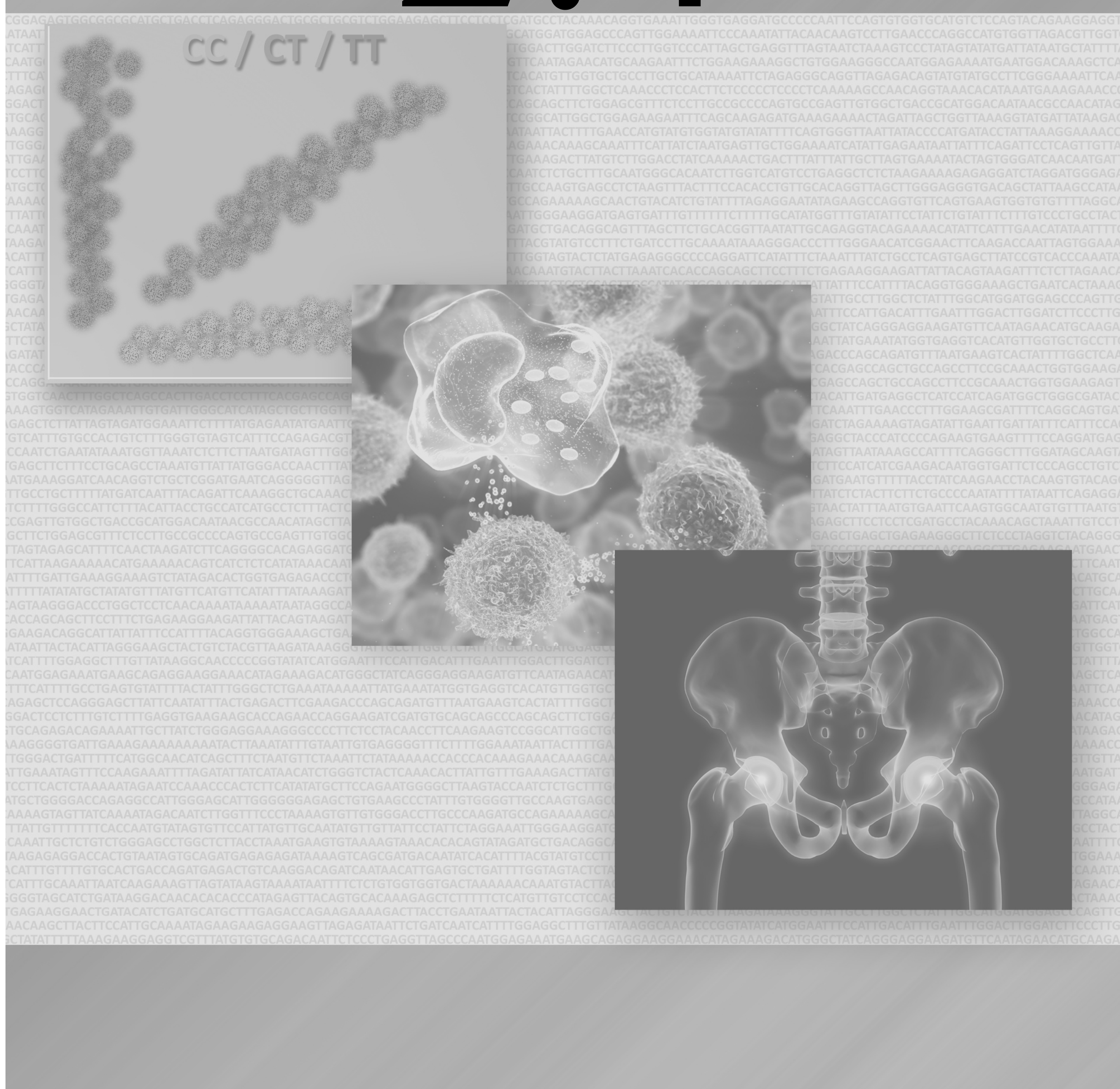




Supplemental Figure 1. (**a**) Expected mean log(S-HsCRP) levels of common (frequency >0.05) *CRP* haplotypes in the unrelated GARP sample for which genotype and S-HsCRP levels were available (N=187). (**b**) Expected mean number of affected hand joints of common (frequency >0.05) *CRP* haplotypes in the unrelated GARP sample (N=191).
Error bars represent standard error of the mean, ** $P < 0.01$

2.4



The role of plasma cytokine levels, CRP and selenoprotein S gene variation in OA.

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Osteoarthritis & Cartilage. 2009 May;17(5):621-6.

Abstract

Objective: Investigating the association between plasma levels of cytokines and chemokines, Selenoprotein S (*SELS*) gene variation and osteoarthritis (OA) subtypes.
Methods: The GARP study consists of 191 sibling pairs with symptomatic OA at multiple joint sites. We have measured plasma levels of 17 cytokines and chemokines and genetic variation at the *SELS* gene.
Results: Nine out of 17 serum markers could be assessed quantitatively, whereas eight markers were assessed qualitatively. Principal component analysis (PCA) on the quantitatively assessed markers and serum high sensitive C-reactive protein (S-HsCRP) revealed that three components underlie 61% of the total plasma variation. Three single nucleotide polymorphisms (SNPs) in the *SELS* gene revealed four common haplotypes, one of which, GAG (frequency 3.5%) showed significant association to an anti-inflammatory (P = 0.019) and acute phase related (P = 0.036) component. OA subtype analysis showed that one component (mainly representing chemokine variation) was significantly associated to hand OA and disc degeneration (P = 0.029 and P = 0.010 respectively) as well as a physical component score (PCS) (P = 0.042). The CRP related component also showed a strong association to the PCS (P = 0.007). *SELS* haplotypes showed no association to OA subtypes in the GARP study.
Conclusion: Genetic variation in the *SELS* gene associates to components representing inflammatory signaling. Another component, representing chemokine variation, showed association to hand OA and disc degeneration in the GARP study indicating chemokines may contribute to OA pathogenesis.

Introduction

Osteoarthritis (OA) is a common joint disease and an important cause of pain and disability in the general population. Elucidation of common pathways that are involved in the onset and progression of the disease will assist in the development of new drug targets and provide a better management of this disabling condition in the future. Several studies have shown that genetic factors play an important role in OA etiology[1,2]. Although OA is not regarded to be an inflammatory disease, there is increasing evidence for the involvement of an innate low grade systemic inflammatory component which may partly explain the genetic susceptibility[3-7]. More specifically, support for the hypothesis that local variation in cytokine levels in the joint may influence OA onset and progression is found in the catabolic effects of pro-inflammatory cytokines, and the protective effects of anti-inflammatory cytokines in articular cartilage[8,9]. Gene expression studies have shown that in OA cartilage several cytokines and chemokines are highly expressed[10-12]. The recent introduction of multiplexed cytokine assays facilitates sensitive measurements of different cytokines using small amounts of valuable sample material from different origins. This sensitive technique allows investigation of the basal levels of chemokines and cytokines in plasma in relation to the ongoing disease processes for diseases where, as in OA, no pronounced and obvious inflammatory component is present.
In a previous study, Curran *et al.* showed that subjects can be characterized by a high (proinflammatory) or low (noninflammatory) plasma cytokine profile depending on a common promoter single nucleotide polymorphism (SNP) -105G > A (rs28665122) in the

Selenoprotein S (*SELS*) gene. A significant association of this *SELS* SNP was observed specifically with higher plasma levels of tumor necrosis factor alpha (TNFα), interleukin beta (IL1b) and interleukin 6 (IL6)[13]. SELS is a widely expressed protein involved in maintaining the functional integrity of the endoplasmatic reticulum (ER) by participating in the removal of misfolded proteins and regulating the cellular redox balance. SELS inhibition by siRNA revealed that a functionally impaired ER leads to activation of numerous pro-inflammatory cytokines mediated by nuclear factor kb (NF-kb) activation[13]. In a cohort of patients with intestinal inflammation, there was no association to the *SELS* locus, however, the pro-inflammatory allele associated to a high serum CRP levels in Crohn Disease patients with active disease[14]. Finally, *SELS* polymorphisms associated with coronary heart disease and ischemic stroke[15]. These studies trigger the question whether *SELS* gene variation also influences inflammatory responses and the etiology of OA. Hence, we set off to map the plasma levels of 17 cytokines and chemokines by use of a multiplexed bead array system in subjects with symptomatic OA at multiple joint sites of the GARP study. We tested whether *SELS* gene variation influenced these inflammatory plasma mediators and OA, and whether these markers associated to OA subtypes and severity.

Materials and methods

The GARP study

The GARP study consists of 191 sibling pairs. All participants have symptomatic OA at multiple sites in the hand or OA at two or more joint sites of four joint sites examined[16]. Symptomatic OA was determined following the American College of Rheumatology recommendations[17-19] whereas radiographic OA (ROA) was scored according to Kellgren/Lawrence[20]. Details on the GARP OA phenotype and inclusion criteria can be found in previous publications[16]. Physical functioning was assessed with the physical component score (PCS), a subscore of the Dutch validated RAND 36-item Health Survey. This questionnaire covers health related aspects including social functioning, role limitations, mental health and vitality[21,22]. A higher score on the PCS indicates a better physical functioning. For the current study we used the proportionate ROA score based on the presence of ROA at each joint location and on the number of joints with ROA identical as described previously. In short, scores 0, 1 and 2 represent respectively no, uni- and bilateral hip and knee OA for these joint sites. The hand ROA score (0-2) represents subjects with, respectively, 0-2, 3-6, and ≥7 hand joints affected out of 20 scored. For spinal discus degeneration (DD) score (0-2) represents subjects with DD at respectively 0-2, 3-5 and ≥6 levels out of 11 levels scored[23]. Hand OA following the ACR criteria[17] was analyzed in addition to the ROA criteria. Genomic DNA was extracted from peripheral blood leukocytes using standard protocols. Written informed consent as approved by the ethical committee was obtained from all subjects in the GARP study.

Serum and plasma collection and measurements

For each participant of the GARP study a morning serum and EDTA plasma sample was collected. Samples were processed within 4 h upon collection and stored at -80˚C until measurement. Serum high sensitive C-reactive protein (S-HsCRP) was assayed earlier[24]. A 17-plex bead assay provided by Bio-RAD was used to measure plasma levels of cytokines

and chemokines on a Luminex platform. Intra assay variation was estimated at 6.2%. The standard protocol was adjusted using twice the indicated amounts of plasma sample in half the amount of sample buffer to increase signal in the measurements. On each plate 10-15% of samples were in duplicate, in which no inconsistencies were observed. For calculation of z-scores the average value of duplos was used as a single value. For cytokines and chemokines with less than 60% of the fluorescence levels above background noise, a qualitative measure of detectable vs not detectable level was used (qualitative analysis, QL). For cytokines and chemokines with more than 60% fluorescence levels above background noise, z-scores of fluorescence levels were calculated per plate (quantitative analysis, QT). In the principal component analysis (PCA) individuals with missing values in five or more out of nine cytokine/chemokines measurements were excluded (N = 13). For the analyzed individuals remaining missing values were given the specific marker mean score. Sample distribution was random per plate on a total of eight plates.

Genotyping

In the current study three SNPs of the *SELS* were genotypes selected from the original paper of Curran *et al.*[13]. SNP positions relative to translation start of the *SELS* gene are -105 (G > A, rs28665122), +3705 (G > A, rs4965814), and +6218 (A > G, rs9874). SNPs were measured using hME™ chemistry on a matrix assisted laser adsorption/ionization time-of-flight mass spectrometry (MALDI-TOF) Mass Spectrometer (Sequenom Inc., San Diego, CA, USA). Assays were designed using the Sequenom MassARRAY Assay Design software (version 3.1). Assay conditions were standard conditions as described earlier[25]. In addition to the genotypes obtained by the Sequenom for rs28665122, a Taqman assay (Applied Biosystems, Foster City, California, United States) was performed to deal with low genotyping success rates this SNP using the Sequenom technique. Primers used for the ABI-genotyping were: forward primer 5'GGGTCGGCCTGCGA and reverse primer 5'CTTCCGGTGCGCTCCTA, probes were 5'TGGCCGGGACCAC labeled with VIC and 5'TTGGCCA GGACCAC labeled with FAM. Assays were run on a 7900HT (Applied Biosystems) according to the manufacturers specifications. Genotypes of both techniques were used in addition to each other where necessary and were used as a control where both techniques provided reliable genotypes. No inconsistencies were observed between techniques for the reliable genotypes.

Statistical analysis

PCA was applied to reduce the correlated data of the cytokines and chemokines plasma levels. Subjects (N = 341) with available levels of S-HsCRP and with >4 out of nine quantifiable markers were entered in the PCA analysis. In PCA analyses random missing data (see Table 2) was replaced by mean values. Both empirical criteria (percentage of variance explained by factors and Eigen values > 1) and interpretability were used to determine the number of factors. We explored the interpretability of these factors after applying a Varimax rotation with Kaiser Normalization. The loading score of each variable onto the individual factors represents the contribution of that variable to the variance observed in the resulting factor. For analysis only individual variable contributions of >0.4 qualified for loading a component[26]. A factor loading represents the linear relationship (Pearson correlation under Varimax rotation) between a variable and a factor.

In order to assess the relationships between OA characteristics, genetic variation at the *SELS* gene and the clusters of cytokines and chemokines, a mixed model regression analyses was performed. To investigate the individual associations, subject specific regression scores of each extracted cluster were used as dependent variable and age, sex, BMI (Body Mass Index) and all OA subtypes as co-variables. OA features and subtypes tested were specific ROA scores as defined previously; knee (0-2), hip (0-2), hand (0-2) and spinal DD (0-2)[24] and the PCS derived from the SF36 questionnaire. Furthermore, for each linear mixed model analysis family identity numbers (representing family relations) as random variables in order to model the familial dependencies that might occur for the levels.
Genotype distributions of *SELS* SNPs were checked by use of the HWE program available at http://linkage.rockefeller.edu. Thesias V3.1 was used to assess linkage disequilibrium between the SNPs and to assess the expected haplotypic contribution to a mean quantitative measure in carriers of a specific haplotype[27]. Quantitative measures analyzed by use of Thesias are e.g., the subject specific regression scores as determined by the PCA analyses. In individuals the expected quantitative measure is determined by the contribution of the two carried haplotypes. The Thesias program allows for adjustments of co-variables but not for familial relationship. Chi square analyses were performed to test for association of individual SNPs or proportionate ROA scores to the qualitatively analyzed cytokines and chemokines. P-values are unadjusted to multiple testing. Analyses were done in SPSS version 14 (SPSS, Chicago, IL, USA) unless mentioned otherwise.

Results

Cytokine and chemokine measurements

Characteristics of the GARP study are displayed in Table 1. For all participants of the GARP study cytokine and chemokine levels were analyzed in a plasma sample by use of a 17-plex bead array system. Table 2 shows cytokines and chemokines measured and the percentage of samples with levels above detection limit. In nine instances (see Table 2) we were able to assess a semi quantitative measure by the use of z-scores reflecting relative plasma levels. For the remaining eight instances (see Table 2), plasma levels were analyzed in a qualitative matter (detectable vs not detectable).

Association analysis of cytokine/chemokine levels with OA features

Nine cytokines and chemokines and S-HsCRP were analyzed in a quantitative manner, the remaining eight cytokines and chemokines were qualitatively analyzed. In the quantitatively analyzed cytokine and chemokine z-scores we observed substantial correlations (Supplementary Table 1). In addition to the association between S-HsCRP and BMI shown previously24, Supplementary Table 1 also shows the frequently observed association between BMI and plasma IL6 levels. GARP subjects in the highest BMI quartile (BMI > 29.1 kg/m^2) had an OR of 1.7 (95% C.I. 1.01-2.82) to reside in the highest quartile of IL6 plasma levels (P = 0.042) when corrected for age, sex and familial relationship. To reduce the redundancy between the markers to more independent components in which these variables cluster, a PCA including all inflammatory markers for which a quantitative measure was available (see Table 2) and S-HsCRP was performed. Table 3 shows the three components that were extracted. The coefficients depicted in Table

3 explain how well each individual marker is represented within the clusters. The marker levels of IL2, IL6, Granulocyte CSF Q3 (GCSF) and IL10 loaded together on the first component, explaining 34.1% of the total variation in the GARP study.

Table 1. Study characteristics of the 382 patients with OA at multiple joint sites (GARP study).

	N (%)	***Mean (sd)***	***range***
Women	312 (81.7)	-	-
Age (years)	382 (100)	60.27 (7.54)	42.66 – 79.44
BMI (kgm^{-2})	379 (99.2)	27.00 (4.67)	19.10 – 46.48
S-HsCRP (mgl^{-1})	354 (92.7)	3.63 (5.43)	0.21 – 56.80
Clinical hand OA	271 (70.7)	-	-
PCS SF36	375 (98.2)	54.02 (21.10)	8.75-98.75
ROA Score[1]	**0**	**1**	**2**
Knee	232	90	60
Hip	275	56	51
DD	125	181	76
Hand	169	110	103

BMI body mass index, S-HsCRP serum high sensitive C-reactive protein, ROA radiographic osteoarthritis, DD discus degeneration, PCS physical component scale

[1]All subjects were affected by OA at multiple joint sites. The scores 0, 1 and 2 represent a proportionate OA score, as described earlier[24]. In genetic analysis hip and knee replacements (respectively 38 and 8) were considered as OA, numbers indicated are patients with diseased joints at sampling, or had replacements within the year prior to sampling)

Table 2. Cytokines and chemokines in the 17-plex bead assay with levels above background noise per cytokine and chemokine.

Cytokine / Chemokine	*N*	*Detectable (%)*	*Analysis*
Interleukin 1β	107	28.3	QL
Interleukin 4	26	6.9	QL
Interleukin 12	90	23.8	QL
Interleukin 13	172	45.5	QL
Interleukin 17	25	7.1	QL
Interferon γ	179	47.4	QL
Tumor Necrosis Factor α	165	43.4	QL
Granulocyte Monocyte CSF	144	37.8	QL
Interleukin 2	234	61.6	QT
Interleukin 5	222	58.4	QT
Interleukin 6	374	98.4	QT
Interleukin 7	274	72.1	QT
Interleukin 8	359	94.5	QT
Interleukin 10	327	86.1	QT
Granulocyte CSF	248	65.3	QT
MCP-1	377	98.9	QT
MIP-1β	375	98.4	QT

QL stands for a qualitative analysis, QT stands for a quantitative analysis, CSF stands for colony stimulating factor., MCP stands for Monocyte Chemotactic Protein, MIP stands for Macrophage Inflammatory Protein.

Table 3. Individual factor cytokine scores extracted by principal component analysis of 10 inflammatory markers measured in blood.

Component[1]	1	2	3
IL2	0.839		
IL6	0.815		
Granulocyte CSF	0.764		
IL10	0.594		
IL7	0.561		0.474
MCP-1		0.799	
IL8		0.799	
MIP-1β		0.765	
S-HsCRP			-0.747
IL5			0.585
Total variation explained	34.1%	15.7%	11.6%

[1]Extraction Method: Principal Component Analysis in which missing values were replaced by mean levels.
IL stands for Interleukin, CSF stands for colony stimulating factor, MCP stands for Monocyte Chemotactic Protein, MIP stands for Macrophage Inflammatory Protein, S-HsCRP stands for serum high sensitive C-reactive protein.

Component 2 is determined by three chemokines MCP (Monocyte Chemotactic Protein), IL8 and MIP (Macrophage Inflammatory Protein), explaining 15.7% of the variation, whereas the third component is determined by S-HsCRP, IL5 and IL7 explaining 11.6% of the variation. It should be noted that S-HsCRP has a negative value in the third component (Table 3), indicating that on average, within subjects there is an inverse relation between S-HsCRP levels and IL7 and IL5.
Subsequently the relationship between the three components as dependant variables and the presence of OA characteristics (Table 1) as co-variables was investigated by mixed model regression analysis. The upper section of Table 4 shows that component 2, consisting of chemokines IL8, MIP and MCP, has significant negative associations to hand ROA score (beta = -0.14 P = 0.039) and to disc degeneration ROA score (beta = -0.22 P = 0.005), independent of age, sex and BMI. This implies that subjects with high chemokine levels have lower hand ROA and DD scores. In addition, component 2 showed a similar association to subjects that had hand OA according to the ACR criteria (beta= -0.26; P = 0.024, data not shown). When analyzing the relationship of the components to the PCS derived from the SF36 a significant negative association of component 2 (P = 0.035) and a positive association to component 3 (P = 0.004) was observed, independent of sex, age and BMI (Table 4, lower section). This indicates that subjects with high chemokine levels experience more functional impairment whereas subjects with high IL7, IL5 and low S-HsCRP levels experience less functional impairment. The association of PCS to component 2, however, appeared not independent of the hand ROA scores (data not shown). In each of the mixed models significant associations with BMI were observed for component 1 (positive, P = 0.049), for component 2 (negative, $P < 1 \times 10^{-5}$) and component 3 (negative, $P < 1 \times 10^{-5}$). In the qualitatively analysis of the cytokines no significant associations were observed for OA subtypes (data not shown).

Table 4. Effect sizes (β) of the linear relationships between the extracted principal components (1-3) reflecting variation at plasma chemokines / cytokine levels and OA characteristics (ROA score, clinical symptoms) of the subjects of the GARP study sample.

OA characteristics	Components[1]		
Radiographic OA	**1**	**2**	**3**
Hip ROA score	0.079	0.098	0.035
Knee ROA score	-0.079	0.043	-0.088
Hand ROA score	0.030	-0.140*	-0.042
DD ROA score	0.002	-0.223*	-0.060
SF36 outcome	**1**	**2**	**3**
PCS	0.001	-0.006*	0.007**

[1]Component 1 contains IL2, GCSF, IL6, IL10 and IL7. Component 2 contains MCP, IL8, MIP and component 3 contains S-HsCRP (negative), IL5 and IL7. Data was analyzed using mixed model regression analyses with the components as dependent variable and as co-variables the joint specific ROA scores (top) or the PCS (bottom) in addition to age, sex and BMI. PCS stands for physical component score.
* $P < 0.05$ ** $P < 0.01$.

SELS gene variation, inflammatory parameters and OA

In the GARP study we could not confirm the previously reported association of rs28665122 with TNFα, IL1-b and IL6 plasma levels by Curran *et al.*[13].

The influence of the *SELS* SNPs on the cytokine and/or chemokine levels, as expressed by the three components, was investigated by haplotype analysis since high linkage disequilibrium was observed between the three *SELS* SNPs (D' > 0.8). As shown in Table 5, four common haplotypes with frequencies over one percent were observed similar to a Finnish population[15]. A significant association was observed between haplotype GAG (frequency 3.5%) and component 1 ($P = 0.019$). Since component 1 reflects variation in IL2, IL6, GCSF and IL10 levels this association indicates that carriers of this haplotype have higher levels of the cytokines in this component. Upon further investigation, this association appeared to be mainly driven by IL10 variation (univariate analysis $P = 0.001$). In addition, the GAG haplotype shows association to component 3 ($P = 0.036$) containing IL5, IL7 and inversely S-HsCRP. This association appeared to be mainly attributable to S-HsCRP levels in the component (univariate analysis $P = 0.002$). These associations were independent of BMI, age and sex. The fact that both components associate to the GAG haplotype indicates some interrelation between the haplotype and these components. We were not able to asses association between *SELS* SNPs or haplotypes and OA subtypes.

Table 5. Haplotype frequencies within the GARP study with their mean haplotypic contribution to the component scores as extracted from the data of 9 cytokines/chemokines and CRP.

Haplotype	Number (%)	C1[1,2] mean (95% C.I.)	C2[1,2] mean (95% C.I.)	C3[1,2] mean (95% C.I.)
GGA	638 (83.5)	-0.25 (-0.61 - 0.11)	-0.27 (-0.58 - -0.05)	0.85 (0.56 - 1.15)
AAG	81 (10.6)	-0.22 (-0.65 - 0.20)	-0.39 (-0.79 - 0.00)	0.83 (0.48 - 1.18)
GAG	27 (3.5)	0.68 (0.24 - 1.10)*	-0.23 (-1.06 - 0.61)	1.98 (1.35 - 2.61)*
GAA	10 (1.3)	-0.68 (-2.57 - 1.21)	-0.37 (-1.38 - 0.64)	0.92 (0.29 - 1.54)
other	8 (1.1)	-	-	-
Total	764 (100)			

* BMI adjusted *P*-value <0.05 for contribution of the haplotype GAG to the component score as compared to the other haplotypes as determined by THESIAS. C1 component 1, C2 component 2, C3 component 3. [1]Scores displayed are the expected haplotypic contribution (independent of the BMI effects) to the mean "principle component regression score" of subjects calculated by the Thesias program. In individuals the expected level is determined by the contribution of the 2 carried haplotypes. The THESIAS program does not allow correction for familial relationship. Alleles are in the following order of SNPs in the *SELS* gene -105 (rs28665122G>A), +3705 (rs4965814G>A), and +6218 (rs9874A>G). [2]Component 1 reflects variation of IL2, IL6, GCSF and IL10 levels, component 2 reflects variation of chemokines MCP, IL8 and MIP and component 3 reflects variation of HsCRP (negative), IL5 and IL7.

Discussion and conclusion

In inflammation driven diseases high circulating plasma levels of pro-inflammatory cytokines and S-HsCRP are present well above detection limits of current methods and readily used for diagnostic and prognostic purposes[28]. Cytokines, however, are known to exert their wide ranged actions also in very low concentrations. The recent introduction of multiplexed cytokine assays facilitates simultaneous measurements of multiple cytokines. However, the described absence of a large scale upregulation or strong association of any of the measured cytokines or chemokines in the plasma of subjects with familial OA at multiple joint sites indicates that, in blood plasma, these markers are not sufficiently suitable to monitor the ongoing OA process. Synovial fluid measurements might better reflect the ongoing disease process since it better reflects the cytokine activities near the site where the disease is mainly active[29]. PCA analyses of cytokine/chemokines measurements revealed 3 components. component 1 reflects variation at IL2, IL6, GCSF and IL10 levels, component 2 reflects variation at chemokines MCP, IL8 and MIP and component 3 SHsCRP (negative), IL5 and IL7. The components seem to reflect different ongoing (patho) physiological processes identified by subjects underlying the components. Component 1 may be classified as a marker of ongoing anti-inflammatory signaling based on the strong involvement of IL10, whereas component 2 shows chemokine signaling and component 3 reflects more acute phase related signaling. The observed (Supplementary Table 1) and known correlation of IL6 to S-HsCRP is, with the current setting in the PCA (Eigen values > 1), not reflected in the components since together they do not explain sufficient amount of the variation.
We found a significant negative association for component 2 to hand OA and DD as well as to the SF36 derived PCS. This indicated that especially the subjects that exhibit high

functional impairment and have low hand ROA scores have high chemokine levels. It should be noted that, by definition of the GARP selection criteria, subjects of the GARP study with low hand or disc ROA scores have OA at other joint sites. We could not, however, attribute the negative association of hand and disc ROA to positive effects caused by these other joint sites. Given these results it appears the higher levels of chemokines act protective in hand and disc OA among subjects of the GARP study. The strong association of the PCS to component 3 including S-HsCRP might reflect impairment of physical functioning mediated or reflected by the individual markers in this component, the observed effects are independent of BMI and ROA status. Due to the relative large amount of missing values in the IL5, IL7 and GCSF data we may have missed specific associations with these markers.

Previously, it has been shown that there is a major upregulation of chemokines in human OA affected cartilage[12]. Our analysis show that, amongst subjects of the GARP study, this upregulation might be less pronounced in subjects with hand OA and disc degeneration of the spine as compared to the other subjects, when correcting for all involved joint sites. This may reflect a different pathophysiological process underlying hand OA and disc degeneration as compared to knee and hip OA. This needs to be further explored in other cohorts of OA patients and especially using control samples.

Given the earlier found associations of *SELS* SNPs to inflammatory factors measured in blood[13], we expected to find associations of cytokines and chemokines especially to rs28665122, however, no direct associations of these levels or presence of these cytokines and chemokines were observed for variation at the *SELS* gene. A recent paper of Seiderer *et al.*[14] also showed no confirmation of the association for rs28665122 to cytokine levels in a study including patients with intestinal inflammation. As compared to the study in intestinal inflammation patients, however, Curran *et al.* used a more sensitive method of measuring cytokines. Seiderer *et al.* did observe an association of the pro-inflammatory allele to higher serum CRP levels in a subgroup of Crohn disease patients with higher signs of disease activity[14]. In our study, possibly the association to either cytokines or S-HsCRP for rs28665122 is not observed due to a smaller sample size or upregulation of these cytokines by the ongoing disease processes is not sufficient to show the genotype effect as observed by Seiderer *et al.*. However, haplotype association analyses revealed a specific *SELS* haplotype (GAG, 3.5%) significantly associated to components of increased IL10 blood levels and decreased S-HsCRP levels, confirming that the *SELS* gene variation may interfere with or affect the homeostasis of the inflammatory pathways.

In the PCA individuals with missing values in five or more out of nine cytokine/chemokines measurements were excluded (N = 13). For the analyzed individuals remaining missing values were given the specific marker mean score. Although we could not readily detect the cause of values being missing most likely these occurred due to bad sample quality or assay errors and less likely due to individuals being out of range. Performing PCA using a list wise case selection (using only cases with all markers available), replacing missing values by the lowest observed value, imputation of the missing values by use of regression analysis or by use of multiple imputation using a winMICE implemented EM algorithm[30] did not affect formation of components or subsequent associations (data not shown). The components reported should be considered robust. The effect of the familial dependencies on the component formation is considered to be minimal as the total number of pairs in the dataset cancels out possible intra sibling pair

correlations. This is strengthened by the results of an analysis of the data using only one member per sibling pair (data not shown) which shows highly similar component and scores.

Moreover, by excluding individuals with over four out of nine missing values we may have excluded individuals with a particular low-inflammatory profile. Finally, missing values in the qualitatively analyzed cytokines may likewise have been subject to possible misclassification of individuals due to bad sample quality, assay errors or measurement problems.

Since OA patients are likely to use drugs which alter the immune system we explored whether the use of NSAIDs significantly influenced our results. In a split analysis for use vs no use of NSAIDs the formation of the components in the PCA showed no major changes, except for the third component in NSAID users where IL5 and IL7 disappeared. The described subsequent associations remained present in both separate datasets (data not shown).

In earlier investigations of the GARP study, we have found that S-HsCRP levels are independently associated to a *CRP* haplotype[7,13] and by the occurrence of the closely related factors knee ROA, BMI and high WOMAC scores[24]. In the current study we show that a *SELS* haplotype is additionally influencing the S-HsCRP level. When we fitted a mixed model with S-HsCRP as dependent variable and as co-variables the *CRP* haplotype H7/8, *SELS* haplotype GAG, and the factor representing knee ROA, BMI and WOMAC24, it was shown that all three consistently and independently influence the S-HsCRP level. In this model, the *SELS* haplotype (GAG) significantly decreased (beta = -0.42; $P = 1.9 \times 10^{-5}$) S-HsCRP levels in carriers whereas the S-HsCRP haplotype H7/8 (beta = 0.14; P = 0.08) and the PCA component (beta = 0.14; $P = 1.6 \times 10^{-9}$) increased S-HsCRP level.

The strength of the GARP study lies in the availability of extended clinical and radiological data of OA features for four joint sites. Furthermore, demographic data for the participants is available, as well as additional familial information, a range of biological fluids and DNA. A downside for this study is, however, that the sample size is relatively small for genetic studies and no synovial fluid samples and control samples of healthy individuals are available for the cytokine measurements in this study.

The analysis of a range of cytokines as measured in blood has not shown any strong associations of one single cytokine to OA features; however, a component comprised of several chemokines did show association to OA in smaller joints and PCS. Another component which has strong involvement of S-HsCRP shows a highly significant association to this PCS, indicating physical impairment might be reflected or mediated by the markers in this component, independent of presence of specific ROA subtypes. Future measurements of chemokines and related signaling proteins in synovial fluid may shed more light on the origin of this association. Long term effects of lower circulating SHsCRP, cytokine or chemokine levels might be reflected by OA progression. Upcoming follow up data in the GARP study will reveal whether these inflammatory parameters associate to further active progression of OA.

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Supplementary Table 1. Spearman's rank correlations with P-values between chemokine, cytokines, HsCRP and BMI among subjects of the GARP study.

P-values

	HsCRP	IL2	IL5	IL6	IL7	IL8	IL10	GCSF	MCP	MIP	BMI
HsCRP		$7.1x10^{-1}$	$2.3x10^{-1}$	$1.7x10^{-4}$	$3.8x10^{-1}$	$1.4x10^{-2}$	$6.9x10^{-1}$	$1.5x10^{-2}$	$4.2x10^{-1}$	$6.0x10^{-1}$	$1.0x10^{-6}$
IL2	-0.03		$9.1x10^{-1}$	$1.0x10^{-6}$	$1.0x10^{-6}$	$2.1x10^{-2}$	$1.0x10^{-6}$	$1.0x10^{-6}$	0.09	0.01	$4.3x10^{-1}$
IL5	-0.08	0.01		$1.3x10^{-2}$	$4.0x10^{-4}$	$5.8x10^{-2}$	$3.2x10^{-5}$	$1.5x10^{-1}$	$1.3x10^{-2}$	$3.1x10^{-3}$	$4.5x10^{-1}$
IL6	0.20	0.58	0.17		$1.0x10^{-6}$	$1.0x10^{-6}$	$1.0x10^{-6}$	$1.0x10^{-6}$	$1.0x10^{-6}$	$1.0x10^{-6}$	$2.0x10^{-3}$
IL7	-0.05	0.38	0.27	0.32		$7.9x10^{-3}$	$1.0x10^{-6}$	$3.8x10^{-4}$	$1.2x10^{-4}$	$1.4x10^{-6}$	$4.4x10^{-1}$
IL8	0.13	0.16	0.13	0.33	0.17		$1.0x10^{-6}$	$2.7x10^{-2}$	$1.0x10^{-6}$	$1.0x10^{-6}$	$6.6x10^{-1}$
I10	0.02	0.36	0.29	0.47	0.33	0.42		$1.3x10^{-5}$	$1.0x10^{-6}$	$1.0x10^{-6}$	$6.8x10^{-1}$
GCSF	0.16	0.42	0.11	0.38	0.26	0.14	0.28		$5.0x10^{-2}$	$9.0x10^{-2}$	$3.7x10^{-1}$
MCP	0.04	0.11	0.17	0.27	0.23	0.49	0.37	0.12		$1.0x10^{-6}$	$2.4x10^{-1}$
MIP	-0.03	0.17	0.20	0.28	0.29	0.49	0.38	0.11	0.50		$1.5x10^{-1}$
BMI	0.36	0.05	-0.05	0.16	-0.05	0.02	-0.02	0.06	0.06	0.08	

Spearman's correlations

Functional characterization of type II deiodinase in human OA cartilage; assessment of *DIO2* allelic expression imbalance and immunohistochemistry of thyroid hormone signaling proteins.

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Submitted

Abstract

Objective. Functional characterization of OA risk polymorphism rs225014 at *DIO2* and respective thyroid signaling in OA and non-OA cartilage
Design. Alleles of the OA risk single nucleotide polymorphism rs225014, a T to C transition coding for a Thr92Ala substitution in type II deiodinase (D2) at *DIO2* were analyzed for differential allelic expression in mRNA extracted from OA cartilage. Immunohistochemical staining of D2 and associated thyroid signaling proteins was performed on OA and non-OA cartilage.
Results. We assessed allelic expression imbalance of *DIO2* using rs225014 in cartilage of OA patients heterozygous for the polymorphism. A significantly higher amount of expression was observed for the OA-associated C allele relative to the T allele in 17 out of 20 donors, indicating that *cis*-acting regulatory effects may underlie the association of this polymorphism to OA. Furthermore, to assess the ongoing thyroid signaling in cartilage we have used immunohistochemistry to stain anatomically "healthy" non-OA and OA affected human cartilage for D2, type III deiodinase (D3) and thyroid hormone receptors alpha and beta. We show that in OA affected cartilage thyroid hormone signaling is substantially increased and that this increase overlaps with an increasing Mankin score, indicative of increasing thyroid hormone activity with increasing cartilage damage.
Conclusion. Our analyses show activated thyroid signaling in OA cartilage, which should be considered detrimental to cartilage homeostasis. In addition, disruptions of cartilage homeostasis may be augmented by the increased expression in cartilage of the OA risk C allele of *DIO2* SNP rs225014.

Introduction

Osteoarthritis (OA) is a common, degenerative disease of the articulating joint that causes pain and disability. Currently, treatment of the disease is limited to pain suppression with no drug yet available that can effectively slow down or reverse the disease process. Ultimately, affected joints need replacement thereby imposing a considerable burden on patients and on health care systems[1]. Previously, using genome wide linkage and association approaches in multiple centers across different ethnic groups, we identified *DIO2* as a gene harboring susceptibility for OA. A *DIO2* haplotype consisting of SNPs rs225014 and rs12885300 showed consistent association to OA[2]. *DIO2* codes for type II deiodinase (D2), which is expressed in specific tissues where it has a vital role in the regulation of intracellular thyroid hormone 3,5,3'-triiodo-L-thyronine (T_3) levels through deiodination of inactive thyroid hormone thyroxine (T_4)[3,4]. In the growth plate, chondrocytes express D2 in the transition to terminal differentiation which is controlled at least in part through T_3 levels[5-7]. Functional differences of *DIO2* in this process might lead to subtle differences in joint shape or bone composition, which could predispose to OA as a result of a lifelong exposure to overt biomechanical factors on the articular cartilage. Furthermore, later in life functional differences of *DIO2* might enhance the predisposition for articular chondrocytes to turn hypertrophic, thereby initiating or augmenting their release from the maturational arrest that sustains chondrocytes in articular cartilage[8]. No *in vivo* cartilage studies specifically aimed at D2 have been performed, however *DIO2* RNA transcripts were shown to be up regulated in OA cartilage as compared to healthy

cartilage[9,10]. Here we investigated the role of D2 in articular cartilage and OA by functional genomic research of patient and control cartilage.

We assessed putative differential allelic expression (DAE) of rs225014 alleles by allelic discrimination assays in OA cartilage RNA. Although the OA risk allele of *DIO2* rs225014 codes for a Thr92Ala substitution in the protein, to date no compelling evidence has been reported showing that this substitution has a major influence on protein function[11-14]. Possibly, the polymorphism acts as a *cis* regulatory element or is in linkage disequilibrium with such an element; earlier studies of OA cartilage samples have indicated the presence of polymorphic *cis* regulatory elements on alleles of OA associated genes such as *FRZB*, *GDF5* and *BMP5*[15-17]. DAE effects are present throughout the genome[18] and potentially contribute to the observed genetic associations seen for many common complex phenotypes, including OA. It could be that for *DIO2* such modest but persistent imbalances of specific allelic expression differences throughout life renders subjects more susceptible to OA.

To explore ongoing thyroid hormone signaling in OA affected and non-OA cartilage, immunohistochemical (IHC) staining of proteins involved in thyroid signaling was performed. In addition to D2 staining we studied the thyroid hormone receptors alpha (THRA) and beta (THRB), which bind activated T_3 and which can subsequently alter gene expression through activation of thyroid responsive elements on DNA. Furthermore, D3 was included in our IHC analyses since this protein is responsible for inactivation of thyroid signaling through the conversion of T_4 into inactive reverse T_3 as well as active T_3 into inactive T_2[3]. Altogether these proteins may indicate the activity of the ongoing thyroid hormone signaling.

Materials and methods

Subjects.

For DAE assessment, subjects undergoing a joint replacement as a result of primary OA were recruited at the Nuffield Orthopaedic Centre (Oxford, UK; Nuffield samples, N=7) and at the Leiden University Medical Centre (Leiden, the Netherlands; LUMC samples, N=13). Ethical approval for the study was obtained from appropriate ethics committees. Immediately upon joint replacement the affected articular cartilage was collected, frozen in liquid nitrogen and subsequently transferred to -80°C for storage. For the immunohistochemical assessment of thyroid hormone signaling proteins 6 non-OA and 11 OA affected hip cartilage tissues embedded in paraffin were retrieved from the archives of the department of pathology, LUMC (Leiden, the Netherlands). Non-OA samples originated from subjects receiving a replacement after a hip fracture.

Nucleic acid isolation and rs225014 genotyping.

To enable isolation of RNA and DNA the frozen cartilage samples were powderised using a Retsch Mixer Mill 200 with continuous liquid nitrogen cooling. RNA isolation was performed using Qiagen RNAeasy Midi kits as described earlier[19], the first wash flowthrough in this isolation was used for subsequent DNA isolation. RNA was stored in precipitated state after addition of 1/10th volume of sodium acetate (3M, pH 5.2), linear acrylamide to an end concentration of 10 $\mu g l^{-1}$ and 2 volumes of ethanol. cDNA was synthesized by use of random hexamer primers as described earlier[17]. The wash flow-

through collected during RNA isolation was used to isolate genomic DNA by addition of 1/10th volume of sodium acetate (3M, pH 5.2) and 3 volumes of ethanol. The precipitated DNA was washed and resuspended in 60 µl of milliQ water. To assess rs225014 genotypes, genomic DNA was PCR amplified using forward primer 5'-TACCACACTCTATTAGAGCC-3' and reverse primer 5'-CACACACGTTCAAAGGCTAC-3' targeted at a 586 basepair *DIO2* fragment encompassing rs225014. The PCR products were incubated with restriction enzyme RsaI at 37˚C for 3 hours and genotypes were visually called by running the digested PCR products on a 3% agarose gel showing cut (TT), uncut (CC), or both (TC) fragments of DNA. Only heterozygous subjects were included in this study.

Differential allelic expression assessment.
cDNA and genomic DNA from heterozygous samples were subjected to a 15 µl PCR amplification using 20 and 5 replicates respectively using forward primer 5'-ATGCTGACCTCAGAGGGACT-3' (cDNA) or 5'-AGTGGCAATGTGTTTAATGTGA-3' (genomic DNA) and reverse primer for both PCR reactions 5'-CACACACGTTCAAAGGCTAC-3'. Amplification products were treated with exonuclease and shrimp alkaline phosphatase to an end volume of 18 µl prior to further processing. For the Nuffield samples 1.8 µl of SNaPshot Multiplex Ready Reaction Mix (Applied Biosystems), 0.1 µl of HPLC-purified extension primer (5'-CACTGTTGTCACCTCCTTCTG-3') and 0.2 µl milliQ water was added to 1.2 µl of PCR product in an extension reaction. The samples were subjected to 25 cycles of extension consisting of 10 seconds at 96˚C, 5 seconds at 50˚C and 30 seconds at 60˚C, upon which the sample was cooled to 4˚C. 1 µl of extended product was added to 10 µl of Hi-Di formamide containing 120LIZ size standard (Applied Biosystems). Analysis of the fluorescence for C and T alleles (TAMRA and ROX label respectively) in each sample was performed on an ABI 3130xl Genetic Analyzer (Applied Biosystems) and data was loaded in Genemapper 3.1 for quality control, genotype calling and exporting fluorescence peak height values. Samples which failed to amplify in the assays or cDNA samples which were outliers in the respective samples replicate group of normalized log transformed peak height ratios were omitted from further analysis. For the LUMC samples cleaned PCR products were diluted 500 times and 1 ul was used as template in a Taqman realtime assay (C__15819951, Applied Biosystems) in a final volume of 5 µl. The dilution was aimed to reach a cT threshold after 15-20 cycles of amplification. Samples were subjected to 10 minutes of denaturation at 95°C, and 40 cycles of 92°C for 15 seconds and 1.25 minutes at 60°C on an ABI Prism 9700HT (Applied Biosystems). Reactions were followed real-time and after cycling an end measurement of fluorescence levels was performed.

Statistics.
Peak height ratio of allele C over T from each individual cDNA sample was normalized using the pooled genomic DNA samples (1:1 ratio of allelic presence) to account for technical variation in fluorescence of the labels. Per centre, the log transformed normalized peak height ratio per cDNA sample replicate series were analyzed for differential allelic expression by a two-tailed Mann-Whitney non parametric test (SPSS Version 16.0) against all genomic DNA samples.

Histological assessment.

HE and toluidine blue staining were used to score all samples according to Mankin *et al.*[20] by 3 observers blinded towards the clinicopathological data. In this scoring system ranging from 0 (no signs of OA) to 15 (total destruction of cartilage layer) samples are scored for microscopic OA features such as decreased proteoglycan content reflected by decreased toluidine blue staining, hypo- or hypercellularity, clonal expansion of chondrocytes, tidemark crossing by blood vessels and cartilage (micro)fractures.

Immunohistochemistry.

Antibodies and protocols used for antigen retrieval and blocking are listed in Table 1. Immunohistochemistry (IHC) was performed according to standard procedures as described previously[21]. Visualization was performed by Powervision incubation followed by incubation in 0.05% diaminobenzidine tetrahydrochloride (Sigma) with 0.01% hydrogen peroxide and hematoxylin was used to counterstain the slides.

Table 1. Characteristics of antibodies used in the IHC analysis of thyroid signaling proteins.

Antibody	Manufacturer	Type	Target protein	Positive control	Antigen retrieval	Block	Dilution
COLX	Quartett (X53)	Monoclonal	Collagen Type X	Growth plate	prot-K & hyaluronidase	none	1:100
D2	Custom[1]	Polyclonal	type II deiodinase	Thyroid Gland	citrate	none	1:8000
D3	Custom[1]	Polyclonal	type III deiodinase	Placenta	proteinase-K	10% nonspecific goat serum	1:5000
THRA	Genetex (GTX16846)	Monoclonal	Thyroid Receptor α	Stomach	Tris-EDTA	none	1:50
THRB	Genetex (GTX17898)	Monoclonal	Thyroid Receptor β	Colon	none	10% nonspecific goat serum	1:400

[1]Antibodies were kindly provided by Prof. Dr. T.J. Visser[22] (Department of Endocrinology, Erasmus University MC, Rotterdam, the Netherlands).

Immunohistochemical staining for D2, THRA and THRB in the superficial, middle and deep cartilage layers was scored for nuclear and cytoplasmic localization whereas D3 was scored for cytoplasmic and extracellular staining by scoring 0 (no staining), 1 (weak or moderate staining) or 2 (strong staining).

Results

Differential allelic expression analysis

Characteristics of the Nuffield and LUMC OA cartilage samples are listed in table 2, all individuals included in this study were heterozygous for rs225014. The relative abundance of rs225014 alleles was analyzed by use of a SNaPshot extension reaction (Nuffield samples) or Taqman realtime PCR assay (LUMC samples) on the amplified target region encompassing rs225014. Table 2 lists the relative ratios of the T and C alleles for rs225014 for each cDNA sample, with the pooled genomic DNA ratios serving as the 1:1 allelic reference. Six of the 7 Nuffield samples demonstrated a higher expression of the C allele and this was a significant observation ($P \leq 0.01$) for 4 of these 6 samples. In an overall analysis of all 7 Nuffield samples allele C was significantly more abundantly present in the

cDNA samples (P<0.01). All 13 LUMC samples demonstrated a significantly higher expression of the C allele (Table 2, Figure 1). Overall therefore, 17 of the 20 samples studied (85%) demonstrated a significantly higher expression of the C allele of SNP rs225014 relative to the T allele. The mean percentage of relative difference was 17% and 36% for the Nuffield and LUMC samples, respectively. The DAE observations were consistent between different skeletal sites and between the two genders. None of the tested genomic DNA samples showed significant deviations from allelic balance when tested against the remaining genomic DNA samples (data not shown).

Table 2. Characteristics of the 20 OA cartilage samples studied and the results of the allelic expression analysis.

	Donor	Gender	Age[1]	Joint	cDNA[2] (%)	Genomic[3] (%)	Relative ratio C/T	P-value[4]
Nuffield samples	1	Female	71	Left Knee	11 (55)	3 (60)	0.97	0.269
	2	Female	72	Left Knee	11 (55)	5 (100)	1.28	<0.01
	3	Female	65	Right Knee	20 (100)	5 (100)	1.14	0.052
	4	Female	75	Right Knee	20 (100)	4 (80)	1.24	<0.01
	5	Female	53	Left Knee	19 (95)	2 (40)	1.07	0.268
	6	Male	88	Right Hip	17 (85)	5 (100)	1.29	<0.01
	7	Male	61	Right Knee	19 (95)	5 (100)	1.18	<0.01
LUMC samples	8	Female	70	Right Shoulder	18 (90)	N/A	1.30	<0.01
	9	Female	79	Left Hip	19 (95)	5 (100)	1.26	<0.01
	10	Male	61	Right Hip	20 (100)	5 (100)	1.29	<0.01
	11	Female	59	Right Hip	16 (80)	4 (80)	1.34	<0.01
	12	Male	71	Right Hip	19 (95)	4 (80)	1.32	<0.01
	13	Female	75	Left Knee	20 (100)	5 (100)	1.33	<0.01
	14	Female	78	Right Hip	19 (95)	5 (100)	1.25	<0.01
	15	Female	75	Left Hip	20 (100)	5 (100)	1.60	<0.01
	16	Female	79	Right Knee	20 (100)	5 (100)	1.38	<0.01
	17	Male	56	Left Hip	17 (85)	5 (100)	1.36	<0.01
	18	Female	62	Right Hip	18 (90)	5 (100)	1.41	<0.01
	19	Female	79	Right Hip	17 (85)	5 (100)	1.42	<0.01
	20	Female	62	Shoulder	17 (85)	5 (100)	1.42	<0.01

[1] *Age at time of joint replacement*
[2] *cDNA PCR measurements passing quality control (max. 20)*
[3] *Genomic PCR measurements passing quality control (max. 5)*
[4] *Mann-Whitney non parametric test (cDNA's per individual versus genomic DNA samples per centre)*

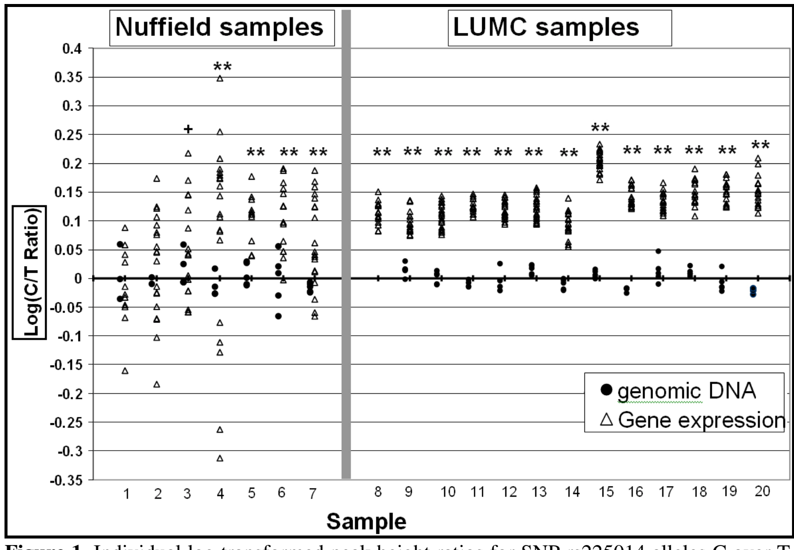

Figure 1. Individual log transformed peak height ratios for SNP rs225014 alleles C over T. Genomic ratios are indicated by black circles whilst mRNA ratios are indicated by triangles. + P < 0.1, ** P < 0.01

Immunohistochemical assessment of thyroid hormone signaling

To assess ongoing thyroid hormone signaling in OA cartilage as compared to non-OA cartilage we stained hip cartilage sections for D2 as well as for D3, THRA and THRB. Characteristics of the donors used are shown in Table 3. The non-OA subjects who received a joint replacement as a result of a fracture were on average significantly older than subjects who received a joint replacement as a result of OA. Using the Hematoxilin and Eosin (HE) staining and toluidine-blue staining (Figure 2A, A' and B, B' respectively) a Mankin score was assessed for each sample to quantify the OA damage. Four out of five non-OA samples had both HE and toluidine blue slides available and a mean Mankin score of 2.4 ranging from 0 to 7 whereas 11 OA samples had a statistically significant higher mean Mankin score of 9.5, ranging from 5 to 12 (Table 3). For the OA samples we observed increased collagen type X staining in all layers of the cartilage, indicating that chondrocytes in the OA samples have turned hypertrophic (Figure 2C, C'). Immunohistochemical stainings for THRA stained nuclear and equally throughout all samples and was not included in further analyses. Most nuclei and cytoplasm of chondrocytes throughout the different layers of cartilage in OA samples stained strongly for D2 whereas only some of the nuclei and cytoplasm in the non-OA cartilage stained positive for D2 (Figure 2D, D'). D3 staining showed increased numbers of positive cells in the OA affected areas of samples as compared to non-OA samples (Figure 2E, E'). For THRB most samples stained positive, however, the OA affected samples showed a more pronounced staining of nuclei and cells throughout all layers of the cartilage (Figure 2F, F').

Table 3. Characteristics of samples used in immunohistochemical analysis of thyroid signaling proteins.

Donor (OA)	Sex	Age	Mankin score	D2[1] nuc./cyto	D3[1]	THRB[1] nuc./cyto
1	F	62	5	1/0	2	1/1
2	F	68	7	2/2	2	2/2
3	M	51	9	2/1	2	1/1
4	M	54	9	1/0	0	1/1
5	F	61	9	2/2	2	2/2
6	F	59	10	2/1	2	2/1
7	M	39	10	2/2	2	2/2
8	F	72	11	1/2	1	1/1
9	F	38	11	2/2	2	2/2
10	M	80	11	2/1	2	2/2
11	F	79	12	2/2	2	2/2
Mean (SD)	-	***60.3 (14.2)***	***9.5 (2.0)***			

Donor (noOA)	Sex	Age	Mankin score	D2[1] nuc./cyto	D3[1]	THRB[1] nuc./cyto
12	M	93	0	1/0	1	0/1
13	F	79	1	1/2	2	1/1
14	F	79	2	1/1	1	1/1
15	F	79	2	2/2	2	1/1
16	F	71	7	1/1	1	2/2
17	F	82	n/a	1/1	2	1/1
Mean (SD)	-	***80.5 (7.1)****	***2.4 (2.7)****			

*t-test p-value < 0.01

[1] nuc: nuclear staining score cyto: cytoplasmic staining score; 0 no staining, 1 weak to moderate staining, 2 strong staining.

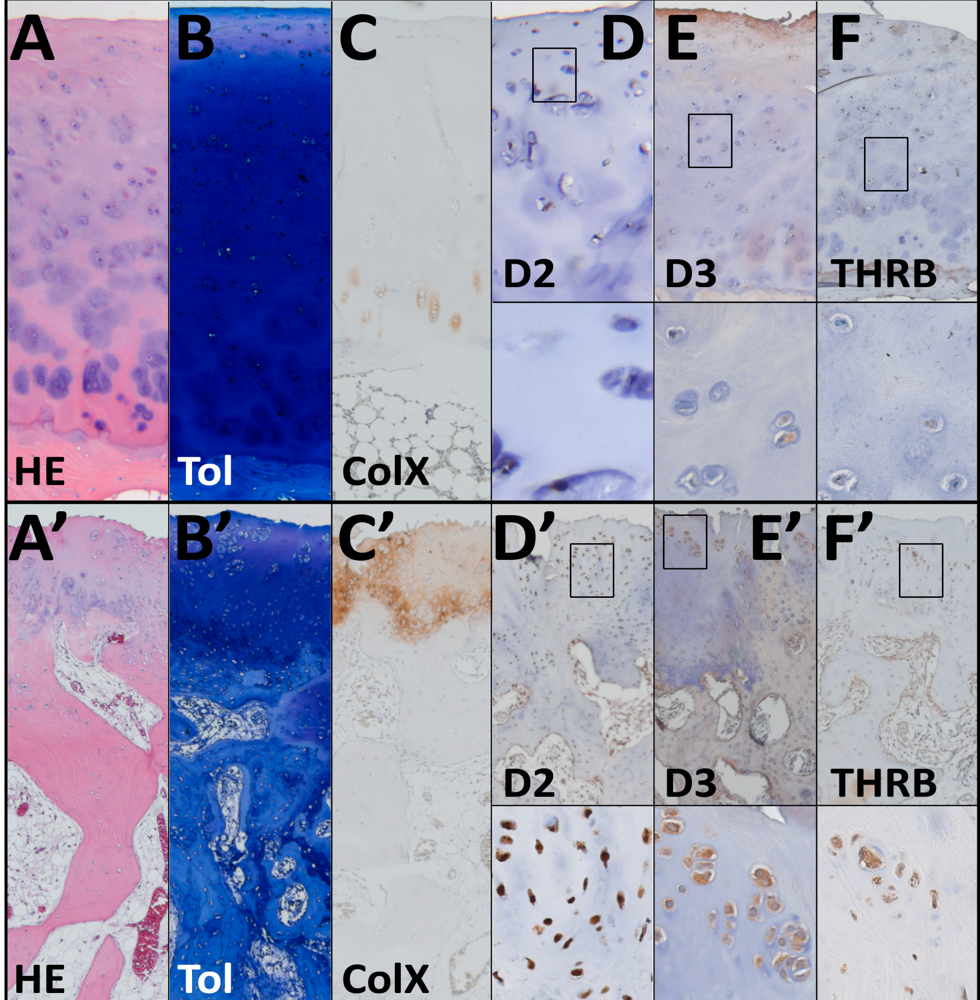

Figure 2 A-F non-OA cartilage samples & **A'-F'** OA cartilage samples**. A/A'** HE staining of non-OA (A) and OA (A') sample. **B/B'** Toluidine-blue staining of non-OA (B) and OA (B') sample. **C/C'** Collagen type X staining of non-OA (C) and OA (C') sample. **D/D'** D2 staining of non-OA (D) and OA (D') sample. **E/E'** D3 staining of non-OA (E) and OA (E') sample **F/F'** THRB staining of healthy(**F**) and OA (**F'**) sample. Tol-toluidine blue ColX-Collagen Type X. Magnifications 50x, insets 200x.

Together the increased presence of thyroid signaling proteins throughout all layers of the cartilage indicates that in OA cartilage thyroid signaling is upregulated. We semi-quantitatively assessed the staining presence and intensity for these three proteins throughout the different cartilage layers (Table 3) and observed that samples with higher Mankin scores were on average inclined to stain more abundantly and at higher intensity for these proteins and that, as opposed to the non-OA group in which mainly superficial staining was observed, the stainings in the OA group were present in all cartilage layers. The age of the subjects had no obvious relation to the Mankin grade or to the staining intensities within the groups of OA and non-OA subjects.

Discussion

Using transcript SNP rs225014 we were able to demonstrate that *DIO2* is subject to highly significant and consistent differential allelic expression (DAE) in OA cartilage with the OA risk C allele being more abundantly expressed in OA cartilage than the T allele. A large majority of the individuals studied demonstrated deviation from allelic balance indicating that the polymorphism itself or a polymorphism in strong linkage disequilibrium (LD) with it is the *cis*-acting regulatory polymorphism. This may act by influencing the transcription rate of *DIO2* or the stability of the *DIO2* mRNA. Previously, in smaller samples sizes the RNA expression of *DIO2* was shown to be up regulated in OA cartilage[9,10] and it is possible that this is partly accounted for by homozygote carriers of the OA risk C allele who, from our previous genetic studies, would be expected to be more prevalent in an OA cohort. It is possible that homeostatic feedback mechanisms ensure that the expression of the *DIO2* encoded protein D2 is balanced in response to different genotypes, and that challenges to this homeostasis mean that the response generated by the OA risk allele C is relatively strong and causes an aberrant thyroid signal in the cartilage. Investigation of *DIO2* DAE in non-OA cartilage samples may elucidate whether the observed allelic imbalance depends on the conditional use of *cis* regulatory elements in response to OA cartilage disease activity or whether it is independent of this and therefore functions as a conventional risk factor. Putatively underlying the observed association of the risk allele to OA is the possibility that during early development the increased expression of the C allele might lead to subtle changes in joint morphology and aberrant joint loading, thereby predisposing to OA. Alternatively, the maturational arrest of the chondrocytes may diminish as a function of age and allow for activation of genes not active in healthy cartilage. A greater expression of the risk allele C might increase the speed of cartilage degradation towards clinical outcomes as described earlier[2,23]. The fact that in the Netherland's LUMC study all samples showed significant DAE, whereas in the UK Nuffield study 4 of 7 samples showed significant DAE might be ascribed to the more sensitive technique used in the LUMC study. It is reassuring to note however that the use of two different techniques to measure allelic expression imbalance both highlighted relative increased expression of the C allele of rs225014. Since both studies comprised an analysis of Caucasian individuals from Northern Europe it is unlikely that genetic differences would account for the fact that not all of the UK samples demonstrated DAE. Having demonstrated DAE at *DIO2,* deep sequencing of this gene and of its proximal regulatory elements is now merited to identify additional variants that might also regulate *DIO2* expression and contribute to OA susceptibility.

Our DAE results suggest that the increased expression of the risk C allele of rs225014 might underlie the association of the polymorphism to OA. Relevant to the increased signaling of the allele is whether in OA the presence of the D2 protein in cartilage is aberrant. To assess D2 protein levels in OA cartilage as compared to non-OA cartilage we performed immunohistochemical analysis of the protein in cartilage specimens obtained from OA and non-OA joints. To get a more complete overview of ongoing thyroid hormone signaling we also stained these sections for the thyroid hormone inactivating protein D3 and THRA as well as THRB, which are responsible for activation of gene transcription through thyroid responsive elements on the DNA. THRA was present in all samples and cartilage layers, indicating at least a baseline thyroid signaling activity; however we did not observe

differences between the OA and non-OA samples. In bone, THRA and THRB isoforms appear to have different properties[24], however little is known about their roles in cartilage. To establish the roles of these specific receptors in cartilage additional research is warranted. We observed increased protein expression of D2, D3 and THRB through all layers of osteoarthritic cartilage, indicative of increased thyroid hormone signaling in OA cartilage as compared to non-OA, "healthy" cartilage from hip fracture patients. This increased signaling may preferentially act through the THRB receptor. Although it is unclear whether the observed changes in thyroid signaling are causal or merely a marker of the ongoing OA disease process, the up-regulation of thyroid signaling would be detrimental to cartilage homeostasis: the increased presence of thyroid hormone related proteins in cartilage could antagonise the maturational arrest of chondrocytes and promote phenotypic changes resembling those observed in the growth plate[23], contributing to cartilage loss. The increase in presence of the thyroid hormone inactivating protein D3 may be considered as a response to the D2 induced increased levels of active thyroid hormone T_3. In some of the superficial layers of the cartilage from the non-OA patients moderate D2, D3 and THRB staining is observed, indicating that random variation of thyroid hormone proteins is present, possibly as a result of the aging process or as a reflection of the higher availability of oxygen in this layer that may predispose it to dedifferentiation. The OA samples show a more abundant staining in all layers of the cartilage and in cellular components. One of the non-OA samples that stained positive for D2 had a Mankin score of 7, which is indicative of moderate cartilage damage, although OA was not recorded in the medical history of this subject. We were unable to stain the thyroid hormones T_4 and T_3 in the cartilage sections to confirm the ongoing thyroid signaling by these hormones directly. This may be attributable to the fact that the concentration for effective signaling of thyroid hormones can be very low. Future analysis of additional samples with both affected and preserved cartilage regions as well as non-OA, "healthy" cartilage samples at different ages may help to elucidate whether the observed increased thyroid signaling precedes cartilage damage or whether this occurs once the damage is present.
Overall, our analyses showed that activated thyroid signaling in OA cartilage may play a role in OA etiology, and that this may be augmented by the increased expression in cartilage of the OA risk C allele of *DIO2* SNP rs225014. Given the role of activated thyroid hormone T_3 during endochondral ossification, where it enhances the terminal maturation of chondrocytes, the described increased presence of D2 protein and increased expression of the *DIO2* risk allele in OA cartilage should be considered detrimental to cartilage homeostasis.

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4

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.

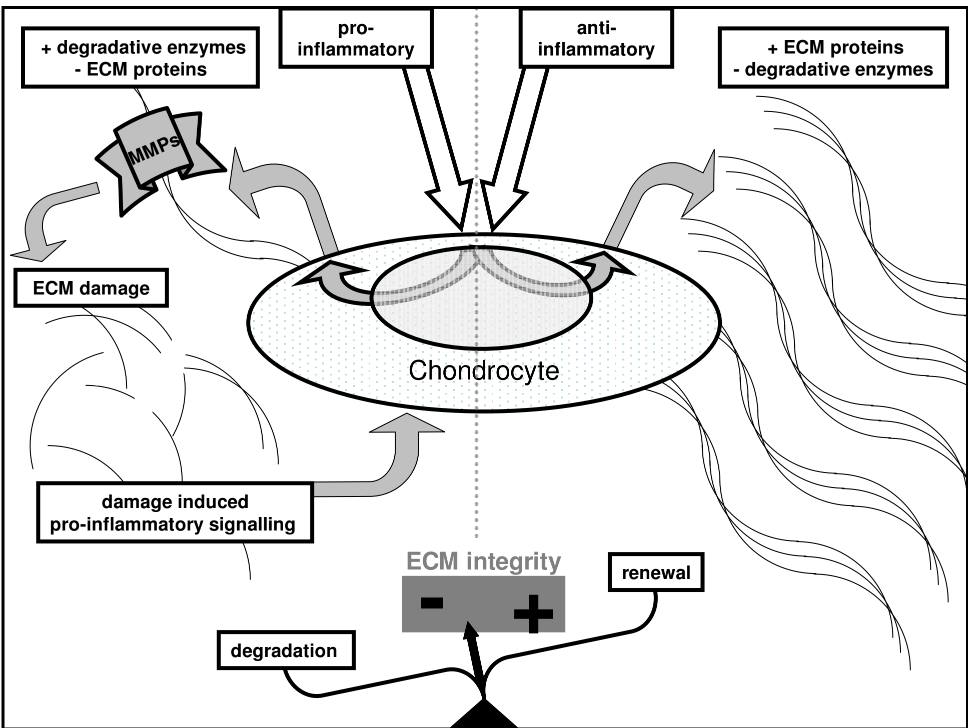

Figure 1. Processes of pro- and anti-inflammatory signaling on the chondrocyte and resulting extracellular matrix (ECM) turnover.

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.

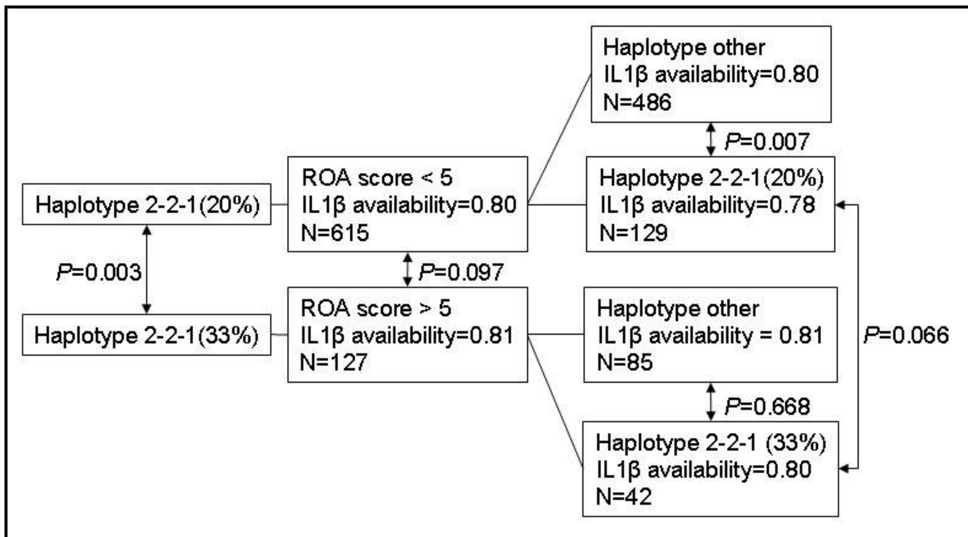

Figure 2. Schematic representation of the associations found within the GARP study sample with crude P-values provided. It is shown that the association between the haplotype and the lower IL-1β availability is mainly due to the effect observed in the subjects that do not have OA at the highest number 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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Summary

Summary

We have investigated associations of levels of inflammatory mediators, genetic variation and features of osteoarthritis (OA). A summary of our findings is provided in Table 1. Overall, we were only able to find fitting associations for the *CRP* gene. We identified a haplotype of the *CRP* gene which associated to higher circulating levels of serum HsCRP as well as to increased numbers of affected joints of the hand in the GARP study (Chapter 2.3), which follows the generally accepted hypothesis that a high inflammatory status is detrimental to the cartilage. The association is most likely reflecting a causal relation between high basal CRP levels and the onset of hand OA. It is contradictory that in spite of the identified association the levels of the GARP subjects with high amounts of hand OA are not different from the other GARP participants, possibly due to the fact that all participants in this study are affected by OA at multiple joint sites.
A more complex association and interaction described in this thesis regards the haplotypes of the IL-1 gene cluster which previously were shown to be associated with the innate levels of cytokines and OA features thereby verifying the generally accepted hypothesis that high innate IL-1β production capacity is detrimental to the cartilage. However, as described in this thesis we identified a haplotype associated to innate lower IL-1β bio-availability which was also associated to subjects with the highest overall ROA scores, indicative of more complex mechanisms underlying the associations found (Chapter 2.1).
Furthermore, for the *SELS* gene we found evidence for a role of Selenoprotein S genetic variation in the circulating levels of components representing pro- and anti-inflammatory signaling in GARP subjects. In spite of these associations to cytokine levels we were unable to show that these genetic variations associated to OA features, whereas we did observe association of a third component mainly representing chemokine variation to features of hand OA and degeneration of the spinal discs (Chapter 2.4).
In these analyses for each answer found, we raised new questions towards the role of genetic variation of genes encoding regulators of the immune system. In search of new loci associated to innate immunity and thereby possibly involved in OA etiology, we showed the association of *CD53* to innate TNF alpha levels, however, the innate levels of TNF alpha appear to have no substantial role in OA etiology. As we expected based on this knowledge, the *CD53* genetic variation which was associated to TNF alpha did not show associations to OA or any subtypes thereof (Chapter 2.2).

Table 1. Summary of associations between genetic variation, inflammatory mediators and OA.

	Associations		
Gene	**Haplotypes to levels**	**Haplotypes to OA**	**Levels to OA**
CRP	**Yes** (Serum HsCRP)	**Yes** (Hand ROA)	**No**
IL-1 gene cluster	**Yes** (Innate IL-1β bio-availability)	**Yes** (ROA)	**Yes** (Innate IL-1β bio-availability)*
SELS	**Yes** (Cytokines and serum HsCRP)	**No**	**Yes** (Chemokines)

* Innate IL-1β bio-availability hold an inverse relation for haplotype association and levels association to OA, i.e. the associated haplotype mediates lower Innate IL-1β bio-availability whereas the associated levels appears to be high innate IL-1β bio-availability

Aided by the various genetic findings in the OA research, including larger genome wide approaches, the developing view in the OA research field is that in addition to the long investigated inflammatory genes there is mounting evidence that genetic variation at genes involved in the processes of skeletal development and maintenance, and the endochondral

ossification in particular may contribute to the heritabile component of OA. The identification of *DIO2* genetic variation which associated to OA in a large, multicenter and multiethnic study triggered us to investigate the coding polymorphism rs225014. By use of a differential allelic expression assay we showed that the risk allele is transcribed at a higher rate in the affected cartilage of heterozygous carriers of this polymorphism (Chapter 3). This leads us to hypothesize that a *cis* acting regulatory element, such as a differential methylation or promoter polymorphism may underlie the *DIO2* association to OA. These data are corroborated by the immunohistochemical analyses of OA and non OA cartilage, where we show higher protein presence of *DIO2*'s gene product type II deiodinase (D2), as well as for the other thyroid signaling related proteins type III deiodinase and thyroid receptor beta (Chapter 3). Through a higher protein presence of D2 which may be at least in part caused by the higher activity of the risk polymorphism, the inactive thyroid hormone T_4 is converted to active thyroid hormone T_3. During the endochondral ossification, D2 activity increases T_3 levels in the growth plate, and this hormone triggers the chondrocytes to enter their terminal differentiation, ultimately forming the bone and facilitating the longitudinal growth of bones. This process has a striking resemblance to the processes observed in the articular cartilage where the chondrocytes display similar features. Through the identification of *DIO2* genetic variation associated to OA, we may have found additional proof that a loosening of the maturational arrest of chondrocytes during life may be lost, ultimately leading to the loss of articular cartilage and formation of osteophytes.

Nederlandse samenvatting

6.1 Introductie

Artrose is de meest voorkomende aandoening van het bewegingsapparaat. Het proces wordt gekenmerkt door de afbraak van het kraakbeen op de gewrichtsdelen en de vorming van bot op de randen van de gewrichten (osteofyten). Op röntgenfoto's zijn deze 2 kenmerken zichtbaar als een vernauwing van de gewrichtsspleet en verbreding van de gewrichtsoppervlakten. Klinische symptomen van artrose zijn pijn in de gewrichten en beperking van de bewegingsvrijheid. De meest voorkomende aangedane gewrichten zijn de handen, knieën, heupen en wervelkolom. De oorzaak van de ziekte is nog grotendeels onbekend, hoewel duidelijk is dat de balans van aanmaak en afbraak van de componenten van het kraakbeen verstoord is.

Artrose heeft een grote erfelijke component, wat verklaard waarom de ziekte vaak in families voorkomt. Naast de erfelijke component spelen ook andere factoren zoals leeftijd, geslacht, overgewicht en beroep een rol wat het onderzoeken van de erfelijke component moeilijker maakt. De recente bevindingen die door genetisch onderzoek zijn gedaan duiden erop dat in het ontstaan van artrose twee processen een grote rol spelen. Het eerste proces is het ontstekingsmechanisme en het tweede proces is de endochondrale verbening die plaatsvindt tijdens de lengtegroei van botten en formatie van gewrichten.

6.2 Kraakbeen en onderzoeksdoelen

Het kraakbeen wordt gevormd gedurende de ontwikkeling van de gewrichten. Het ontstaat uit een groep gespecialiseerde cellen die tijdens de vorming van de botten als kraakbeen cellen een matrix om zich heen leggen die het articulaire kraakbeen genoemd wordt. Eenmaal gevormd blijft het kraakbeen levenslang haar functie van soepele beweging en demping van schokken verrichten. De kraakbeencellen zijn maar spaarzaam aanwezig in het kraakbeen, delen zich niet en hun activiteit is heel laag (Zie ook Hoofdstuk 1 blz. 12, Figuur 2A). Gedurende het leven gaan kleine hoeveelheden van de bouwstenen van het kraakbeen kapot en de kraakbeencellen zijn net actief genoeg om dit op te ruimen en weer aan te vullen. Dit proces is zorgvuldig gebalanceerd, onder andere doordat de kraakbeen cellen gevoelig zijn voor ontstekingsparameters die onder invloed van schade aan het kraakbeen vrijkomen. Het is bekend dat teveel van deze ontstekingparameters deze balans van opbouw en afbraak kunnen verstoren, waardoor teveel kraakbeen verloren gaat. Een van de algemeen aanvaarde hypothesen in artrose is dat deze balans verstoord is in de richting van de afbraak van het kraakbeen. In dit proefschrift hebben wij voor meerdere ontstekingsparameters onderzocht of we deze hypothese konden bevestigen. Daarnaast zijn in de recente wereldwijde onderzoeken naar artrose genen gevonden die coderen voor eiwitten die in de vroege ontwikkeling van bot en kraakbeen een rol hebben. Een van dergelijke genen, die door ons werd geïdentificeerd is het *DIO2*. Verder onderzoek moet nu uitwijzen wat mogelijke achterliggende oorzaken zijn voor de betrokkenheid van dit gen bij artrose. We onderzochten mogelijke verschillen in de snelheid van het afschrijven van varianten van dit gen. Daarnaast werd onderzocht of het eiwit waar *DIO2* voor codeert, samen met enkele aanverwante eiwitten daadwerkelijk aanwezig is in kraakbeen met en zonder artrose.

6.3 Studie populaties

Om te onderzoeken welke processen een rol spelen in artrose zijn in dit proefschrift verschillende studies gebruikt, waarvan de GARP (**G**enetica **AR**trose en **P**rogressie) en RAAK (**R**esearch **A**rtrose en **A**rticulair **K**raakbeen) de belangrijkste twee zijn.

De GARP studie, een samenwerking met de afdeling Reumatologie, bestaat uit een grote groep van broer- en/of zus-paren tussen de 40 en 70 jaar met klinische klachten en radiologisch aangetoonde artrose in twee of meer gewrichtsgroepen (hand, heup, knie en wervelkolom). Van deze patiënten hebben wij ook DNA, RNA, bloed en urine afgenomen zodat we relevante metingen kunnen doen. In het kader van dit proefschrift hebben we vooral ontstekingsparameters gemeten en deze, met relevante genen voor deze parameters in associatiestudies* met artrose onderzocht. Daarnaast is in de GARP studie met behulp van een koppelingsonderzoek** gezocht naar nieuwe varianten van genen die de maximale productiecapaciteit van bloedcellen voor ontstekingsparameters beïnvloeden.

De RAAK studie is een lopende samenwerking met afdeling Orthopedie in het Leids Universitair Medisch Centrum in Leiden en het Rijnland Ziekenhuis in Leiderdorp waarbij de chirurgen bij operaties van patiënten die een gewrichtsvervanging nodig hebben het verwijderde gewrichtsmateriaal voor onderzoek insturen. In dit materiaal kunnen we onderzoek doen in de voor artrose meest relevante weefsels zoals kraakbeen, bot en de ligamenten.

***Associatiestudies** *vergelijken twee groepen personen, waarvan één groep aangedaan is met de ziekte, en de andere groep de 'normale' bevolking representeert. Voor een kandidaatgen, dat ervan verdacht wordt een rol te spelen bij de ziekte kan worden bekeken of varianten van dit gen vaker voorkomen bij de patiënten dan bij de controle personen; er is dan sprake van associatie van een variant met de ziekte. De oorzaak van deze associatie kan dan liggen in een eigenschap van deze variant zelf, of een variant die hier dichtbij ligt op dezelfde DNA streng. Bij het overerven van het DNA worden grote stukken met hierop meerdere varianten vaak geheel overgedragen van ouder op kind. Zo'n stuk DNA heet een haplotype, en in dit proefschrift is voor meerdere genen onderzoek gedaan naar dergelijke haplotypen. Wij onderzochten niet alleen of haplotypen van genen vaker bij aangedane personen te vinden waren, maar ook of deze haplotypen bloedwaarden van relevante ontstekingsparameters beïnvloeden.*

****Koppelingsstudies** *maken gebruik van het gelijktijdig overerven van grote stukken DNA binnen families waarin een ziekte vaker voorkomt. Broers en zussen delen onderling gemiddeld 50% van hun DNA, de overige 50% is verschillend. Door in kaart te brengen welke delen van het DNA gedeeld worden en welke delen uniek zijn, kunnen we bij aangedane familieleden nagaan op welke 50% van het DNA de genen liggen die betrokken zijn bij het ontstaan van de ziekte. Door het verzamelen van heel veel van dit soort paren uit verschillende families is het mogelijk om nauwkeurig vast te stellen welk deel van het DNA door al deze paren vaker gedeeld wordt dan wat op basis van toeval verwacht wordt. Door deze 'koppeling' van ziekte aan een stukje van het DNA kunnen we in dit stukje DNA verder zoeken naar de mogelijke genvarianten die het risico op de ziekte verhogen. In dit proefschrift gebruikten wij deze aanpak om binnen een groep broer-zus paren te onderzoeken welke gebieden op het DNA genen kunnen bevatten die de bloedwaarden van relevante ontstekingsparameters kunnen beïnvloeden.*

6.4 Interleukine-1 gen cluster onderzoek

Interleukine-1 is een sterk pro-inflammatoire ontstekingsparameter, die de kraakbeencellen kan aanzetten tot de productie van enzymen die de kraakbeen matrix afbreken (Zie ook Hoofdstuk 4 blz. 108, Figuur 1). Door bloedcellen te stimuleren met een stof die lijkt op een bacterie produceren deze bloedcellen op maximale snelheid ontstekingsparameters, zoals de verschillende vormen van het interleukine(IL)-1. In bloed van de GARP patiënten hebben we onderzocht of de genen die coderen voor IL-1 (*IL1RN, IL1A* en *IL1B*) associëren met de maximale capaciteit van bloedcellen om IL-1 bèta en IL-1 receptor antagonist(Ra) te produceren. We vonden dat een haplotype van het *IL1RN* gen associeert met de biologische beschikbaarheid van IL-1 bèta (de verhouding tussen IL-1 bèta en de IL-1 receptor antagonist). Dragers van dit haplotype hebben een lagere biologische beschikbaarheid van IL-1 bèta, maar meer radiologische handartrose. Gegeven dat pro-inflammatoire signalen het kraakbeen aanzetten tot productie van afbraakenzymen, verwachtte wij bij een lage biologische beschikbaarheid van IL-1 bèta juist minder aanleg voor artrose. Een mogelijke verklaring voor onze bevinding is dat gedurende het leven het kraakbeen onderhouden wordt door een balans tussen de pro- en anti-inflammatoire eiwitten, waarbij bij kleine beschadigingen mogelijk een voldoende sterke actie van IL-1 bèta nodig hebben om de reparatie op gang te krijgen, of de kleine schade voldoende op te ruimen voordat de reparatie van de kraakbeenmatrix kan plaatsvinden.

6.5 Koppelings analyse met maximale productiecapaciteit van ontstekingsparameters

De genetische variatie waarvan bekend is dat het ontstekingsparameters beïnvloed verklaart niet de volledige erfelijkheid hiervan. Met behulp van een genoom wijde koppelings analyse in de GARP studie zochten we naar genen die ook aan de genetische component van deze bloedwaarden bijdragen. In deze analyse ontdekten wij een variant in het *CD53* gen, die associeert met de maximale productie capaciteit van bloedcellen voor tumor necrose factor (TNF) alpha, een sterk pro-inflammatoir eiwit. Uit eerdere onderzoeken blijkt dat maximale capaciteit van de bloedcellen om TNF alpha te produceren niet geassocieerd is met de aanwezigheid van artrose, en deze genetische variant heeft dan ook geen relatie met het ontstaan van artrose in de GARP studie. Mogelijk is de door ons geïdentificeerde variant wel van belang bij andere aandoeningen waarbij de maximale TNF productiecapaciteit van de bloedcellen wel een grote factor is bij het ontstaan of voortgang van de aandoening, een hypothese die nader onderzocht moet worden.

6.6 CRP haplotypen, CRP levels en artrose

CRP is een eiwit wat een breed scala aan processen reguleert en sterk pro-inflammatoire ontstekingsparameters aanzet. Een eerder onderzoek wees uit dat genetische variatie in het *CRP* gen de basale bloedwaarden van dit eiwit beïnvloed. Wij onderzochten of wij deze relatie van gen en bloedwaarden in de GARP studie konden bevestigen, en of de genetische variatie mogelijk een invloed heeft op de mate van artrose. Het haplotype 7/8 van dit gen, wat geassocieerd is met hoge bloedwaarden voor het CRP associeert ook met ernstige handartrose (gedefinieerd als 7 of meer aangedane gewrichten van 20 gescoorde gewrichten). Dit zou kunnen duiden op een relatie tussen de hoge CRP waarden onder invloed van dit haplotype en de ernstige schade aan het kraakbeen van de handen. De bloedwaarden van de mensen die ernstige handartrose hebben zijn echter niet hoger in

vergelijking met de rest van de GARP studie. Dit mogelijk is te wijten aan het feit dat alle deelnemers in de GARP studie artrose op meerdere gewrichtslocaties hebben.

6.7 Selenoprotein S gen variatie, ontstekingsparameters en artrose

In een associatie studie, uitgevoerd door een buitenlandse onderzoeksgroep werd aangetoond dat het selenoprotein S gen (*SELS)* associeert met bloedwaarden van IL-6 , IL-1 bèta en TNF. In de GARP studie onderzochten wij of we deze relatie konden verifiëren in onze artrose patiënten. Hoewel de gerapporteerde associatie niet werd gereproduceerd was de genetische variatie in het gen wel met verschillende cytokines geassocieerd, wat duidt op een rol van dit gen in de bloedwaarden van ontstekingsparameters. De genetische variatie in het gen associeerde echter niet met artrose wat erop duidt dat de rol van dit gen niet direct met artrose heeft te maken.

6.8 Samenvatting ontstekingsparameters en artrose

In Tabel 1 geven we de gevonden relaties tussen genen, ontstekingsparameters en artrose weer welke in dit proefschrift beschreven zijn.

Tabel 1. Samenvatting van gevonden relaties tussen genen en ontstekingsparameters in artrose

	Associaties		
Gen	**Haplotype met bloedwaarden**	**Haplotypen met artrose**	**Bloedwaarden met artrose**
CRP	**Ja** (Serum HsCRP)	**Ja** (Hand ROA)	**Nee**
IL-1 gen cluster	**Ja** (Max. biol. beschik. IL-1β)	**Ja** (ROA)	**Ja** (Max. biol. beschik. IL-1β)*
SELS	**Ja** (Cytokines en serum HsCRP)	**Nee**	**Ja** (Chemokines)

* De biologische beschikbaarheid van IL-1 bèta heeft een tegengestelde relatie voor de haplotype associatie en bloedwaarden associatie met artrose; het haplotype wat associeert geeft lage bloedwaarden, waar de bloedwaarden die met artrose associëren juist de hogere waarden zijn.

Max. biol. beschik. IL-1β staat voor de maximale biologische beschikbaarheid van IL-1 bèta, ROA staat voor radiologisch bepaalde artrose.

Samenvattend kunnen we stellen dat artrose en ontstekingsparameters een intieme relatie hebben waarbij het moeilijk is om de oorzaak en gevolg te onderscheiden. De met artrose gepaard gaande afbraakprocessen van het kraakbeen zorgen mogelijk voor een subtiele verhoging van de bloedwaarden van de patiënten waardoor het moeilijk is om te achterhalen of de genen die met artrose associëren zorgen voor deze hoge waarden, of juist de bloedwaarden hoog zijn als het gevolg van het artroseproces. Het opstellen van studies van gezonde personen of personen met vroege artrose zou een goede stap zijn om deze relatie nader te onderzoeken. Zulke onderzoeken kunnen op verschillende tijdspunten de relatie tussen het ontstaan van artrose en bloedwaarden van ontstekingsparameters waarnemen en hierdoor een richting aan de associaties geven die worden waargenomen.

6.9 Karakterisatie van het DIO2 gen in artrotisch kraakbeen

In de GARP studie toonde een eerder onderzoek van onze groep aan dat de rs225014 T>C variant in het gen *DIO2* het risico op het krijgen van artrose verhoogt. *DIO2* codeert voor type II deiodinase(D2), een eiwit wat inactief thyroid hormoon T_4 omzet in actief thyroid hormoon T_3. Tijdens de lengtegroei van botten en de formatie van gewrichten (endochondrale verbening) is D2 en daarmee actief T_3 aanwezig wat signaal geeft aan de chondrocyten in de richting van terminale differentiatie en de uiteindelijke formatie van bot stimuleert.

In de RAAK studie is artrotisch kraakbeen van patiënten verzameld, en in deze studie zijn de kraakbeen biopten van personen die zowel een risicovariant als een normale variant van rs225014 dragen onderzocht om mogelijke verschillen in de genactiviteit te onderzoeken. We hebben met dit onderzoek aangetoond dat de risico variant een veel hogere genactiviteit geeft in vergelijking met de normale variant van het gen. Een dergelijk verschil in activiteit kan worden veroorzaakt doordat de regulatie van de twee varianten verschilt. Dit verschil in activiteit duidt aan dat dit mogelijk onderliggend is aan het hogere risico op het ontstaan van artrose in personen die drager zijn van de C-variant.
In samenwerking met de afdeling Pathologie van het LUMC hebben we ook artrotisch en niet artrotisch kraakbeen materiaal microscopisch onderzocht voor de aanwezigheid van het *DIO2* genproduct D2, evenals aanverwante eiwitten type III deiodinase en de thyroid hormoon receptor bèta. In het kraakbeen van artrosepatiënten werd met toenemende mate van kraakbeenschade een hogere activiteit van deze eiwitten waargenomen, in vergelijking met de niet artrotische coupes. De hogere aanwezigheid van het eiwit duidt op meer activiteit van de thyroid hormoon as, wat de overgang van de chondrocyten naar de terminale differentiatie zal bevorderen. Deze terminale differentiatie zal de kraakbeenkwaliteit verslechteren en nog verder doen afbreken.

6.10 Conclusie

Het onderzoek naar artrose krijgt, onder invloed van de wereldwijde vergrijzing en gepaarde toename van de gevolgen van deze aandoening onder de bevolking meer en meer aandacht. In dit proefschrift hebben we de rol van ontstekingsparameters en de genen die deze reguleren in artrose onderzocht. De complexiteit van de communicatie over en weer tussen de genen, ontstekingsparameters en artrose ontstaat doordat de ziekteactiviteit ook de ontstekingsparameters beïnvloed. We toonden aan dat een haplotype van het *CRP* gen dat codeert voor hogere CRP bloedwaarden bij handartrose betrokken is. Daarnaast identificeerden we een component van chemokines die ook invloed heeft op de mate van handartrose in de GARP studie, de richting van deze laatste associatie anders is dan verwacht, wat de complexiteit van de rol van ontstekingsparameters in artrose onderstreept of een gevolg is van de manier waarop de GARP studie verzameld is. Een nog ingewikkeldere associatie vonden wij voor de rol van de maximale biologische beschikbaarheid van IL-1 bèta in artrose. Een haplotype dat associeert met lage biologische beschikbaarheid geeft meer handartrose, mogelijk door onvoldoende activatie van matrix reparatiemechanismen of opruiming van de beschadigde matrix bij kleinere schades aan het kraakbeen. Naast de ontstekingsparameters komen de genen betrokken bij de ontwikkeling van bot en gewrichten meer en meer onder aandacht als gevolg van bevindingen in het genetische onderzoek. Deze genen kunnen zowel vroeg in het leven, bij de ontwikkeling en vorming van gewrichten als later in het leven, bij het onderhoud van het articulaire kraakbeen een rol spelen. Subtiele verschillen in gewrichtsvorm of botsamenstelling vergroten mogelijk het risico op artrose door lokaal grotere stress op het kraakbeen bij normaal gebruik. De genen die actief zijn in de groeiplaat gedurende de lengtegroei zijn in het gezonde articulaire kraakbeen niet actief, maar mogelijk wordt met het ouder worden deze onderdrukking van activiteit verloren onder de invloed van activiteit van genen zoals *DIO2*. Het onderzoek beschreven in dit proefschrift toont aan dat de genetische variatie die associeert met artrose een grotere genactiviteit zou kunnen veroorzaken in kraakbeen. Daarnaast werd tussen gezond kraakbeen en artrotisch kraakbeen een verhoging van de

aanwezigheid van dit eiwit waargenomen, wat de hypothese van verhoging van de genactiviteit en daarmee gepaard gaande afbraak van het kraakbeen ondersteunt.
Toekomstige genetische bevindingen kunnen op soortgelijke wijze op functionele activiteit getoetst worden, waarbij de nieuwe technieken zoals sequencing en DNA-methylatie metingen kunnen bijdragen aan respectievelijk de identificatie van nieuwe functionele varianten en bepaling van de achterliggende oorzaak achter toegenomen genactiviteit.

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Curriculum vitae

Steffan Daniël Bos was born January 17th, 1979 in Beverwijk, the Netherlands. He attended secondary school in Alkmaar at the Jan Arentsz, where he passed his exams in 1998. The same year he started his study Biopharmaceutical Sciences at the University of Leiden, the Netherlands. During this study he did an internship at the department of Biofarmaceutics of the University of Leiden. He received his degree in 2004 and after working for the research institute Pharmo for a short period het started working as a technician in December 2004 at the department of Molecular Epidemiology of the Leiden University Medical Center, headed by Prof. dr. P.E. Slagboom. In February 2005 he was appointed as a PhD student on a project funded by the Dutch Arthritis Association, supervised by Prof. dr. P.E. Slagboom and Dr. I. Meulenbelt at the department of Molecular Epidemiology, Leiden University Medical Center. During this research period he spend two months in Newcastle, UK, where he was supervised by Prof. dr. J. Loughlin, upon which the techniques learned in this laboratory were applied at the laboratory in Leiden. The results of the research performed during the project are described and discussed in this thesis. In April 2009 he started working as a post-doctoral researcher at the department of Molecular Epidemiology at the Leiden University Medical Center where he works on a project funded by the Netherlands Consortium for Healthy Ageing, which focuses on the healthy ageing and the locomotor apparatus in particular.

Nawoord

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