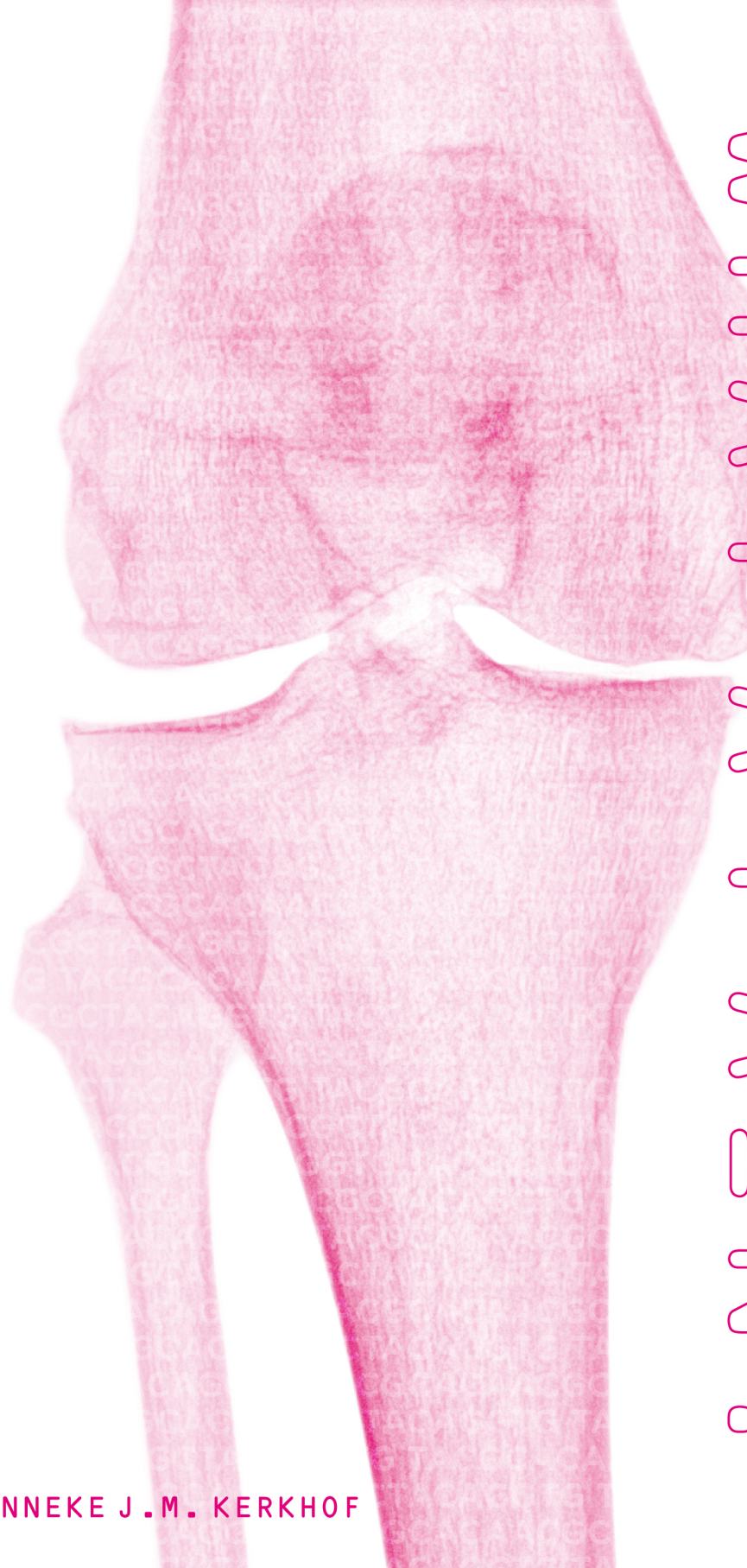

GENETICS OF OSTEOARTHRITIS: WHAT A PAIN!

HANNEKE J.M. KERKHOF

Genetics of osteoarthritis: What a pain!

HANNEKE J.M. KERKHOF

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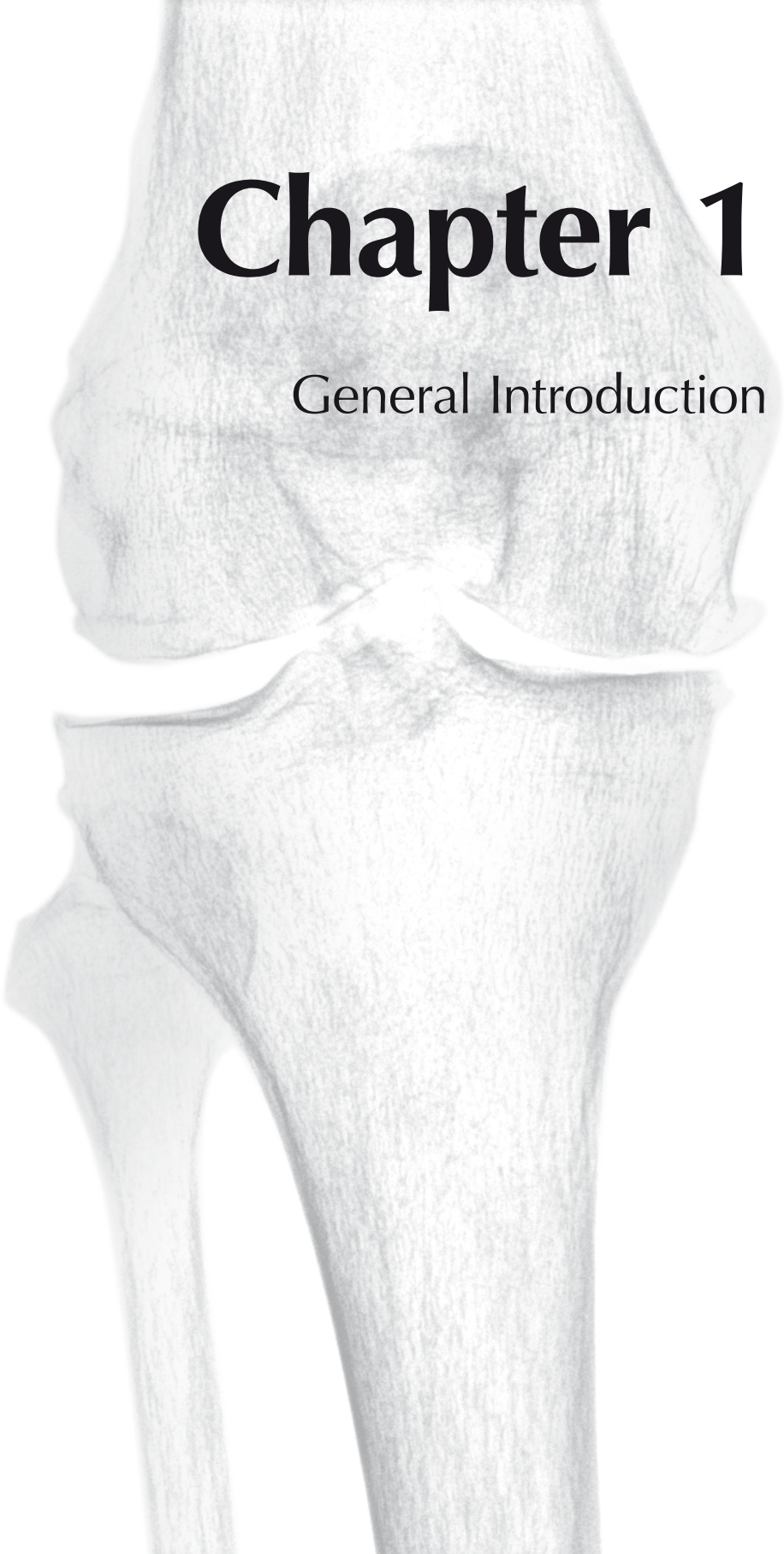
LIST OF ABBREVIATIONS

AUC:	Area under the curve
BMD:	Bone mineral density
BMI:	Body mass index
CI:	Confidence interval
CMC1:	First carpometacarpal
CTX-II:	C-terminal cross-linked telopeptide of type II collagen
DIP:	Distal interphalangeal
DNA:	Deoxyribonucleic acid
GWAS:	Genome-wide association study
HOA:	Hip osteoarthritis
IP:	Interphalangeal
JSN:	Joint space narrowing
K/L:	Kellgren and Lawrence
KOA:	Knee osteoarthritis
LD:	Linkage disequilibrium
MAF:	Minor allele frequency
MCP:	Metacarpohalangeal
OA:	Osteoarthritis
OR:	Odds ratio
PIP:	Proximal interphalangeal
ROA:	Radiographic osteoarthritis
ROC:	Receiving operator characteristic
RS:	Rotterdam Study
SNP:	Single nucleotide polymorphism
SOA:	Symptomatic osteoarthritis
STT:	Scapho-trapezo-trapezoidal
THR:	Total hip replacement
TJR:	Total joint replacement
TKR:	Total knee replacement
TREAT-OA:	Translational Research in Europe Applied Technologies for OsteoArthritis



Chapter 1

General Introduction

Osteoarthritis (OA) is a common, chronic disabling disease characterized by pain and disability, affecting mainly the elderly population. The American College of Rheumatology defined OA as "a heterogeneous group of conditions that lead to joint symptoms and signs which are associated with defective integrity of articular cartilage, in addition to related changes in the underlying bone at the joint margins" (1). Although a disease like OA will not directly lead to a higher mortality, the burden of disease and health care expenditure is enormous. In 2005, healthcare costs for OA in the Netherlands were approximately 540 million euro, which is in women higher than costs for hypertension (2), asthma (2) or hip fracture (2-3). At the same time, the burden of OA is increasing because of aging and the obesity pandemic. In the Netherlands, already 650.000 persons (4% of the total population) had a diagnosis of OA in 2007 (4). Although the incidence of disease stays stable over time, the prevalence is increasing (5) due to the aging population and the population structure in the Netherlands. In addition, the World Health Organization showed in the Global Burden of Disease report that OA is the 4th leading cause of years lived with disability (YLD) at a global level (6). Only scarce symptomatic treatment options are available for OA, such as pain medication, exercise programs and eventually joint replacement. In summary, we can conclude that OA causes a large disease burden to patients and society and a better understanding of the pathophysiology is essential to discover new treatment options.

PATHOPHYSIOLOGY OF OA

Osteoarthritis is a degenerative joint disease affecting the whole joint. In Figure 1 the main structures of the joint affected by osteoarthritis are shown. The main characteristics of the disease process are articular cartilage loss, formation of new bone at the joint margins (osteophytes), increased thickness of the bone (subchondral sclerosis) and cyst formation. However, also soft-tissue structures surrounding the joint are affected, including the synovium, ligaments and muscles, making OA a whole-joint disease.

Whether the bone changes initiate progression of cartilage damage or vice versa is still a matter of debate. OA is a multifactorial disease involving systemic factors, local biomechanical factors and genetics (Figure 2). A complex interplay between all these factors distinguishes persons with and without OA in terms of damage and in terms of pain and/or disability.

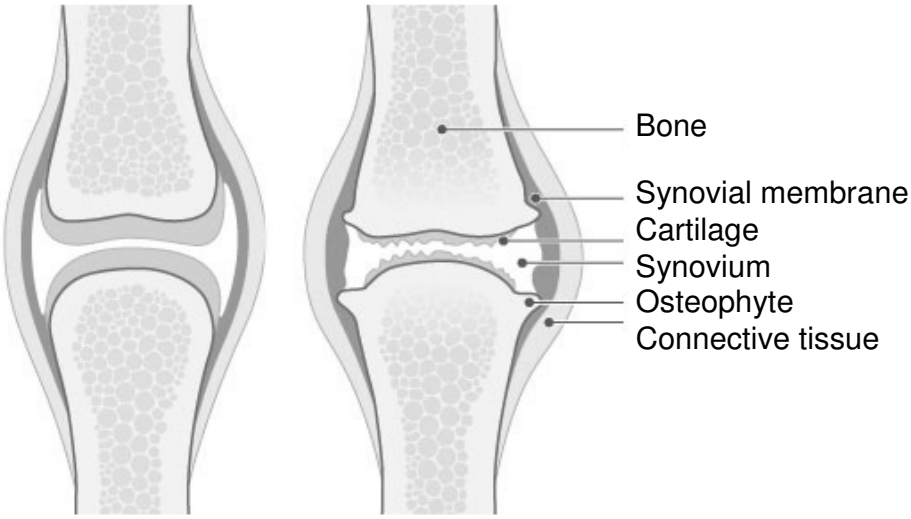


Figure 1. Structures of the joint affected by osteoarthritis (adapted from www.reumafonds.nl).

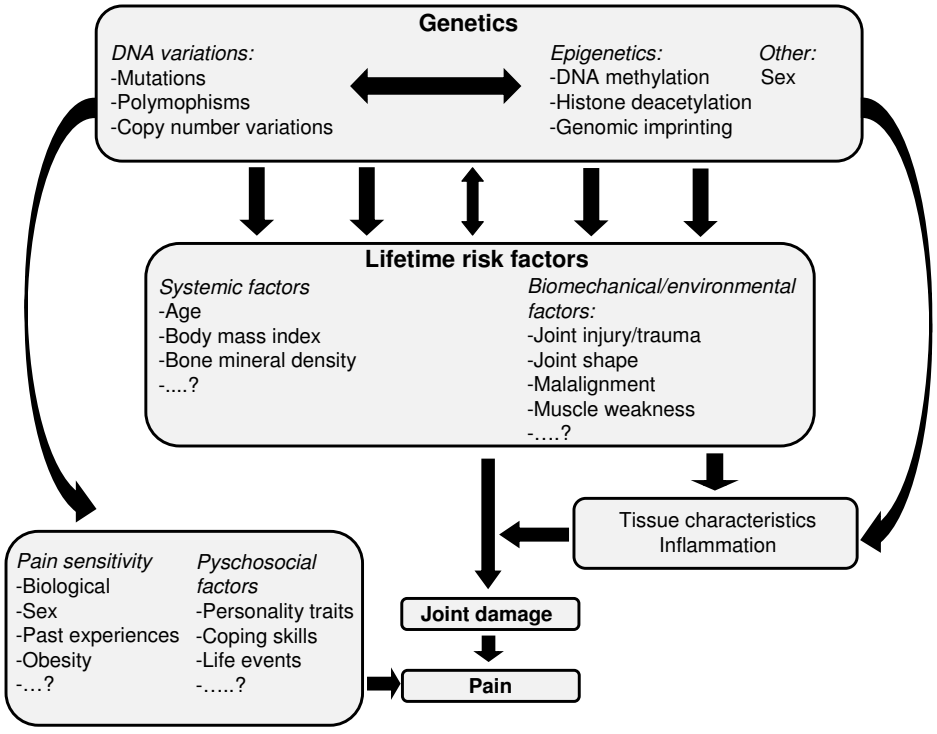


Figure 2. A complex interplay between risk factors involved in osteoarthritis.

DEFINITION OF OSTEOARTHRITIS

A major problem in OA research is the definition of OA. A distinction can be made between symptomatic OA (SOA) and radiographic osteoarthritis (ROA). However, even within these 2 categories many different definitions have been used in literature. As mentioned, different bone and cartilage abnormalities are seen in OA, but also soft-tissues structures are affected. A definition of OA can therefore be based on any (or a combination) of these structures. There is no consensus on defining hip, knee or hand OA for epidemiological or clinical studies. One of the most important disadvantages of this lack of consensus is that in research combining data from different studies, for example large-scale meta-analyses, it will be difficult to compare results due to heterogeneous definitions resulting often in heterogeneous results. Variation in disease definition among different studies reduces power to find consistent associations in any disease (7). In Figure 3, the 4 grades of the Kellgren and Lawrence (K/L) scale are shown for the knee. As this seems a straightforward way to define ROA, it is known that different interpretations and modified versions of the K/L score have been used, again leading to a lack of consensus how to define OA (8). The need for a consensus is high, not only in genetic studies, but in all epidemiological studies on OA, and will be discussed in *Chapter 3.1* of this thesis.

WHY STUDY GENETICS OF OA?

Osteoarthritis is a complex disease in which both genetic and environmental factors play an important role. Two twin studies conducted in women aged 50 years and over showed that the heritability for knee OA is ~40%, hand OA ~65% and hip OA ~60% (9-10). This means that a substantial proportion of variation in risk for OA can be attributed to genetic variation, i.e., DNA variation in or near genes involved in the aetiology of OA. Knowledge about the genetic factors that contribute to the disease is important, because this can give us new insights in the pathogenesis and may identify new targets for prevention and treatment.

GENETIC VARIATION

The human genome consists of four nucleotides; Adenine (A), Thymine (T), Guanine (G) and Cytosine (C). Deoxyribonucleic acid or better known as DNA is a nucleic acid that contains the genetic instructions used in the development and functioning of humans. Only a part (~1.5%) of the human DNA encodes the approximately

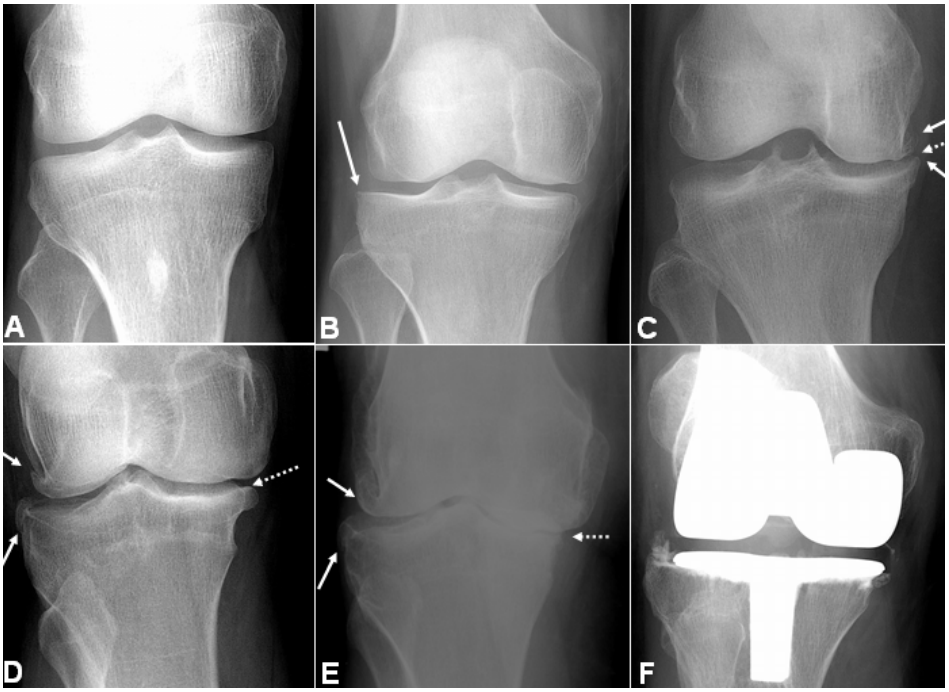


Figure 3. Kellgren and Lawrence grading scale for knee osteoarthritis. Dashed arrows show joint space narrowing (possible, definite, severe) and bold arrows show osteophytes (small/lipping, moderate and large). (**A**) K/L 0: no signs of OA, (**B**) K/L 1: osteophytic lipping/doubtful osteophytes, (**C**) K/L 2: mild osteoarthritis with definite osteophytes and possible joint space narrowing, (**D**) K/L 3: moderate osteoarthritis with definite osteophytes, definite joint space narrowing and some sclerosis, (**E**) K/L 4: severe osteoarthritis with gross loss of the joint space and deformation of the femoral head, (**F**) total knee replacement.

24,000 genes which are transcribed in human cells. The rest of the DNA consists of non-coding RNA genes, regulatory sequences, introns, and DNA sequences with unknown function. Between human subjects differences exist in for example hair color, risk for a disease and height. This is partly caused by variations in the DNA sequence, so-called genetic variation. Although the DNA sequence of any two persons is for >99% identical, on average 1 in every 100-300 base pairs differs between unrelated individuals in a population.

There are different types of variants present in the DNA, such as copy number variants (CNVs) and a change of a single base pair in the DNA sequence. Of these last mentioned DNA variants, the most common are the single nucleotide polymorphisms (SNPs). A SNP is a site in the genome where only one base pair of the chromosome varies and this must occur in >1% of the population (otherwise it is called rare variation or a mutation). For example, 40% of the chromosomes in a population have an A at a certain location of the DNA (GTGCCT**A**AGCTCG), whereas the other 60% have

a C at this location (GTGCCT**C**AGCTCG). Subjects can be homozygous for the A (AA) allele or the C (CC) allele or heterozygous (AC). SNPs can occur in coding (genes) or non-coding regions of the human genome. Copy number variations (CNVs), another form of structural variation, are alterations (deletions or duplications) of the DNA such that the cells have a variable number of copies of one or more sections of the DNA. For example, a chromosome that usually has sections like A-B-C-D can have A-B-B-C-D in case of a duplication of "B" (for clarification see Figure 4). Many of such CNVs are tagged by SNPs. The focus in this thesis will be on examination of the relationship between SNPs and OA rather than CNVs or mutations.

HAPLOTYPES AND THE HAPMAP PROJECT

Genetic variants are often inherited together in segments of DNA called haplotypes. These ancestral genomic segments are inherited as discrete units with little genetic shuffling across generations. Haplotypes are formed by novel mutations and recombination, a process by which there is exchange of genetic material either between chromosomes or between different regions of the same chromosome. Alleles of these haplotypes are co-inherited and lead to specific correlation patterns between nearby variants, better known as linkage disequilibrium (LD). LD refers to the non-random association of alleles at two or more loci. In other words, if alleles A and B at two loci tend to co-occur on haplotypes in proportions different than expected if they would be assumed independent, we speak of LD.

The 2 most popular statistics to describe LD are D' and r^2. D'=1, also called complete LD, if two sites have not experienced recurrent mutation or gene conversion and if there has been no recombination between the two sites. However, the correlation (r^2) between alleles can only be high if their minor allele frequencies (MAFs) are the

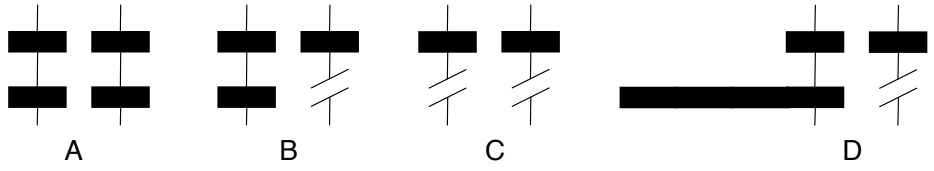


Figure 4. Different types of copy number variants. (**A**) normal copy number (copy number 2), (**B**) hemizygous deletion (copy number 1), (**C**) homozygous deletion (copy number 0), (**D**) double copy duplication (copy number 4).

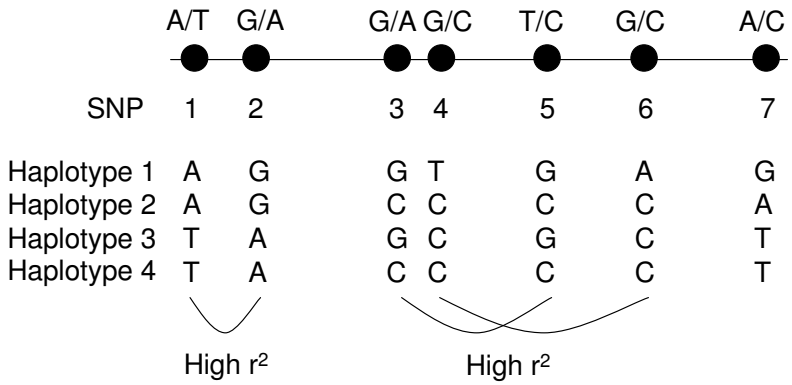


Figure 5. SNPs, haplotypes and r^2. A hypothetical short stretch of DNA is shown with 7 SNPs. Each SNP has 2 possible alleles. Each row represents a haplotype and each column represents a SNP. As shown, the haplotypes are particular combinations of alleles from nearby SNPs. High r^2 exists between SNP 1 and 2, SNP 3 and 5 and SNP 4 and 6. As for example for SNP 1 and 2 an A allele at position 1 always co-exists with a G allele at position 2. Therefore, these SNPs are in perfect LD and each one of those SNPs can be used as a so-called tag SNP. By genotyping a tag SNP information on the SNPs in perfect LD with that SNP is also known and therefore only 1 SNP needs to be genotyped. Adapted from a figure by Carlson *et al.* (11).

same. If genotypes perfectly correlate, r^2=1 and there is perfect LD. So if r^2 is high, D' is always high, but it is possible that D' is high, whilst r^2 is low. In Figure 5, the concept of SNPs, haplotypes and LD are explained visually.

Because haplotypes are shared by the majority of the human population, they can be used to decipher the genetic differences that make some people more susceptible to disease than others.

In many cases, the SNPs in a given haplotype are redundant, such that only a few (so-called tag SNPs) are needed to characterize the genetic diversity of the entire block (Figure 5). The International HapMap project, set up to determine the common patterns of DNA sequence variation in the human genome (12), created a catalogue of the by now known ~10 million SNPs with allele frequencies and the correlation patterns between nearby variants. The original DNA-sample set for HapMap comprised a total of 270 people. There were 30 sets of samples of two parents and an adult child from an Yoruban population, 45 unrelated individuals from Japan, 45 unrelated individuals from China and thirty trios from U.S. residents with northern and western European ancestry. In addition, an extended set of 1,184 subjects from 11 populations were studied to provide more data from each of the continental regions as described above as well as data from some more admixed populations residing in the United States (13).

GENETICS OF COMPLEX DISEASES

Within the field of disease genetics there are three main approaches to identify relevant genes for complex diseases in epidemiological studies: 1) genome-wide linkage analysis, 2) a candidate gene approach and 3) genome-wide association (GWA) analysis. The (dis)advantages and characteristics of these methods are shown in Table 1. Genome-wide linkage and association studies both try to find genes involved in a disease without any *a priori* knowledge on the underlying biology or risk genes/alleles, in other words hypothesis-free testing.

Linkage analysis

Linkage analysis exploits the fact that genes and other genetic markers have the tendency to be inherited together because of their location near one another. There is linkage if two alleles co-segregate more often than expected, which is usually expressed as a Logarithm of the ODds (LOD) score. A LOD score of 3 or more is generally considered as evidence of linkage. After identification of linkage, all genes in the linked region need to be identified and the candidate gene for the disease of interest needs to be found. Subsequently, a search for mutations can be conducted. Genome-wide linkage analyses, which identify stretches of DNA which are relatively long, have been very successful for rare Mendelian disorders, caused by a defect in a single gene. However, results are disappointing for complex traits such as OA. There are several reasons for the limited success of this approach in complex diseases. First, linkage analysis has low power for discovering genes with modest effects (14). The power of linkage analysis is proportional to the square of heritability (15), which can result in a large impact of environmental influences which is likely in studies of common diseases especially in elderly populations. A genome-wide linkage analysis

Table 1. Characteristics of genetic studies for complex diseases

Type of approach	Study population	Resolution[1]	Effectiveness	Advantage	Disadvantage
Genome-wide linkage study	Family-based	5-20 million bp	-	Family-based + identification loci for single gene disorders	Low resolution and low power
Genome-wide association study	Population-based	5-50 thousand bp	+	Hypothesis-free and high resolution	Large sample sizes needed
Association analyses of candidate genes	Population-based	1 bp	+/-	High resolution	Up-front hypothesis + large sample size needed

Bp:base pair; [1]the genomic area analysed

requires related subjects from a multigenerational family or an isolated population with inbreeding, with and without the disease. In complex diseases, results across different linkage analyses are often very different, meaning that different chromosomal regions are identified. This lack of reproducibility probably reflects low power, but it might also be caused due to the fact that perhaps rare mutations with large effect sizes (which you detect with linkage) are less important in most complex diseases such as OA.

Using linkage analysis, one gene was identified to be possibly involved in hip OA in Caucasians. The Iodothyronine-Deiodinase enzyme type 2 (*DIO2*) gene, which is associated with severe hip OA in women (20).

Candidate gene association studies

A more powerful approach for complex diseases is association analysis in which the frequency of the minor allele of a SNP is compared between cases and controls. Candidate gene association studies are hypothesis based-studies, and since our knowledge of the pathogenesis of OA is limited, candidate gene studies alone will be inadequate to completely explain the genetic basis of OA.

There have been several hundreds of candidate gene studies conducted in the past decade with limited success (for reviews see (16-17). Genes in many different pathways have been studied, but often with conflicting results. Examples of such genes are the *FRZB* gene, an antagonist of Wnt signalling, and the *CALM1* gene, an intracellular protein that interacts with a number of proteins involved in signal transduction. So far, using the candidate gene approach, only the growth differentiation factor 5 (*GDF5*) gene has been consistently identified to play a role in knee OA (21-22).

Was all the time and money spent on these projects then needless? Probably not, because we have to note that a P-value threshold of $5x10^{-8}$ is now considered statistically significant in GWAS studies and this is a rather stringent threshold. It is very likely that many associations with very small effects with P-values in the range of 10^{-4} or 10^{-5} in meta-analyses are also true positive, but that lack of power or heterogeneity are responsible for this relatively high P-value. So, although candidate gene studies have not been very effective in the past in discovering genes involved in complex diseases, there remains a place for these kind of studies and we will show results on candidate gene studies in this thesis in *Chapter 2*.

Genome-wide association study (GWAS)

More recently, a novel and revolutionary approach was developed to search for important risk genes involved in a complex trait: a hypothesis-free genome-wide association study (GWAS). In a GWAS a dense set of Single Nucleotide Polymorphisms (SNPs) across the genome is genotyped to survey the most common genetic variation

for a role in disease or to identify heritable quantitative traits that are risk factors for the disease. Compared to candidate gene association studies, the number of SNPs studied is much larger and *a priori* knowledge on the biology of the disease is not needed. When combining data of multiple studies in a GWAS meta-analysis, power can be increased and the chances of success in discovering new loci (involved in OA), is higher. The current dense SNP arrays cover approximately 90% of the human genome (18). As individual studies often use genotyping platforms with different SNP contents, a method to combine these platforms is necessary. Imputation, the substitution of missing SNP genotypes based on haplotype information, can bridge the gap in coverage between genome-wide SNP platforms. Using haplotype information from the HapMap project (19) this can be achieved.

In 2008, the start of this thesis, there were no loci discovered for OA by means of GWAS, but success was already achieved using GWAS for another common arthritic disease, rheumatoid arthritis (RA). In 2007, three GWAS studies reported the 6q23 locus to be involved in RA (23-25).

In the past many claims of genes involved in complex diseases were criticized because they appeared to be false-positive. However, this was in the pre-GWAS area where often individual, low-powered, studies were performed. This thesis will show that by combining data of different studies using a GWAS meta-analysis and achieving a stringent level of statistical significance of $P<5x10^{-8}$, true positive results can be discovered (*Chapter 3.3-3.5*).

PREDICTION OF OA

Although many factors involved in OA are identified (22, 26-33), it is unknown if we can identify those subjects who are at high risk of OA. In *Chapter 4* different prediction models for OA including clinical, genetic and biochemical risk factors are shown. To build a prediction model, first risk factors which are believed to be involved in the disease are selected. Second, univariate analyses are performed to assess the effect size of these risk factors in OA. Third, multivariate models are tested for association with OA including multiple risk factors.

The risk prediction model created in the study of interest needs to be tested for calibration and discrimination in that same study (internal validation) and in an independent study (external validation). Calibration assesses how closely the predicted probabilities reflect the actual observed risk. The Hosmer-Lemeshow χ^2 statistic for goodness-of-fit is frequently used for calibration, where small χ^2 values and large P-values indicate good calibration. Discrimination examines the probability that an individual with the disease will be assigned a higher risk than an individual without

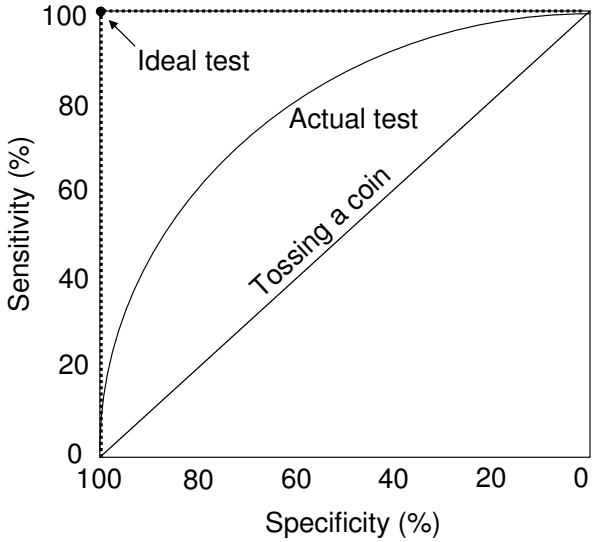


Figure 6. Example of a receiver operating characteristic (ROC) curve.

disease. It says therefore something about the ability to correctly classify cases and controls. The area-under-the-curve (AUC) of a receiving operator characteristic (ROC) curve is a measure of discrimination between cases and controls with a range from 0.50 (tossing a coin) to 1.00 (perfect prediction).

In Figure 6 an example of a ROC curve is shown. In this ROC curve the sensitivity is plotted against the specificity. Sensitivity is the proportion of true-positives which are correctly identified as such (i.e., the percentage of people who have OA and are correctly identified as having OA). Specificity measures the proportion of negatives which are correctly identified (i.e., the percentage of healthy people who are correctly identified as not having OA).

THE ROTTERDAM STUDY

The majority of the studies described in this thesis are performed within a large population based cohort study, the Rotterdam Study (RS). In the Netherlands this study is also known as the Erasmus Rotterdam Gezondheid Onderzoek (ERGO) Study. RS is a population-based prospective cohort study ongoing since 1990 to study determinants of chronic disabling disease (34). It consists of three sub-populations. The Rotterdam Study-I (RS-I), ERGO baseline, is the first cohort of 7,983 persons, aged 55 years and over living in Ommoord, Rotterdam in the Netherlands. This cohort was extended in 1999 with 3,011 participants using the same inclusion criteria (the Rotterdam Study-II (RS-II), ERGO plus). A further extension, the Rotterdam Study-III (RS-III), ERGO jong,

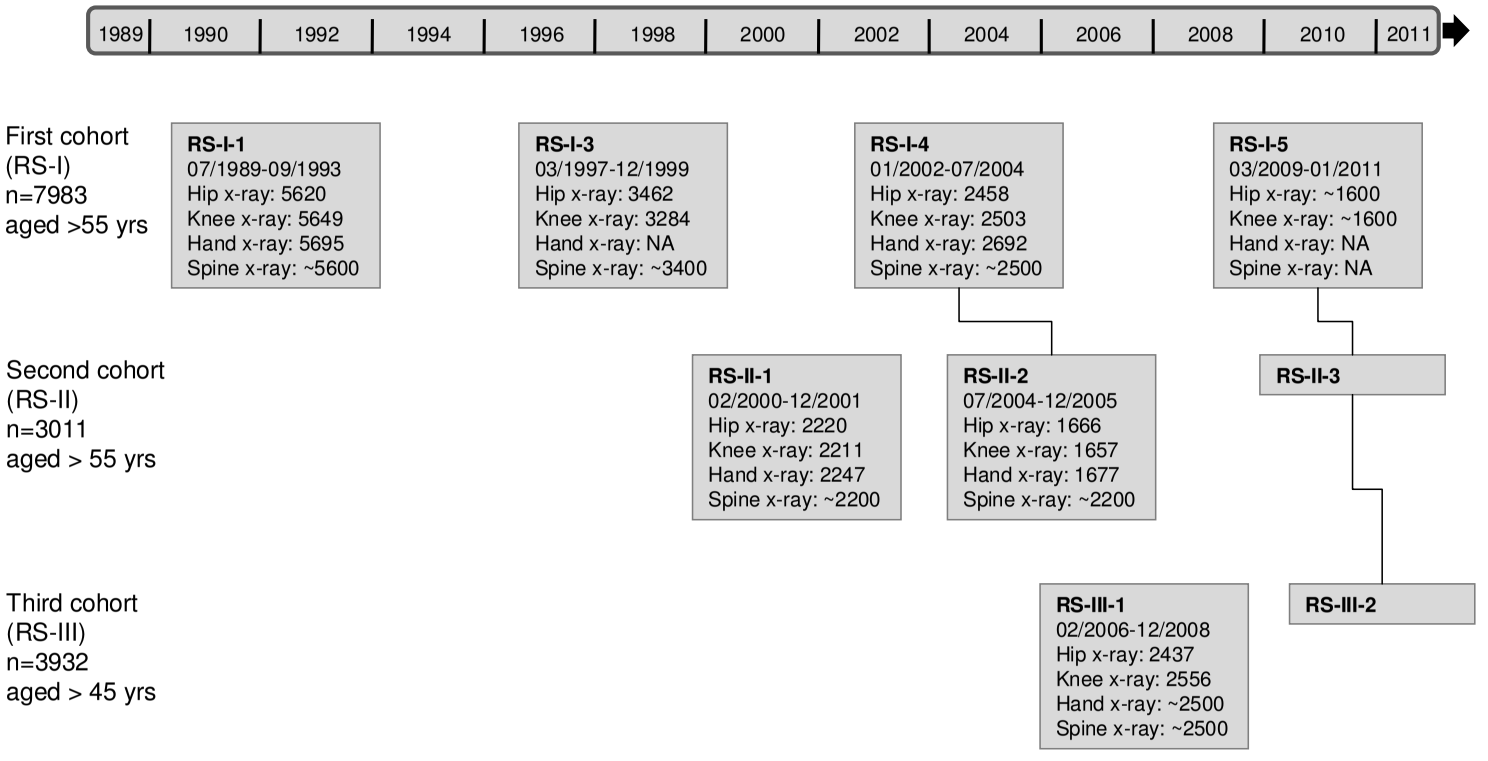


Figure 7. Overview of the Rotterdam Study and availability of X-rays. NA: not available; RS: Rotterdam Study.

was initiated in 2006. Since the start of the study there have been several follow-up visits to the research centre. All participants were examined at baseline in detail (34). In summary, a home interview was conducted (~2 hours) and subjects had an extensive set of approximately 1,500 examinations at the research centre (~5 hours). Amongst others, blood and urine is collected and x-rays of the knees, hips, hands and spine are taken. All these examinations were repeated every 3-4 years. An overview of baseline and follow-up visits and the availability of x-rays within the Rotterdam Study is given in Figure 7. The study has been approved by the institutional review board (Medical Ethics Committee) of the Erasmus Medical Center and by the review board of The Netherlands Ministry of Health, Welfare and Sports and written informed consent was obtained from each participant.

AIM OF THIS THESIS

The overall objective of this thesis is to identify genes involved in osteoarthritis. In *Chapter 2*, candidate genes involved in signaling pathways (Wnt, estrogen) and inflammation (*CRP* and *IL-1*) are studied in relation to OA using a candidate gene approach. In *Chapter 3,* hypothesis-free genome-wide association studies, both individual and large-scale meta-analyses, are presented with the goal to discover novel genes and/or pathways involved in hip, knee and hand OA. The predictive value of clinical risk factors and biochemical and genetic markers will be tested in both the general population as well as in certain high risk groups (*Chapter 4*).

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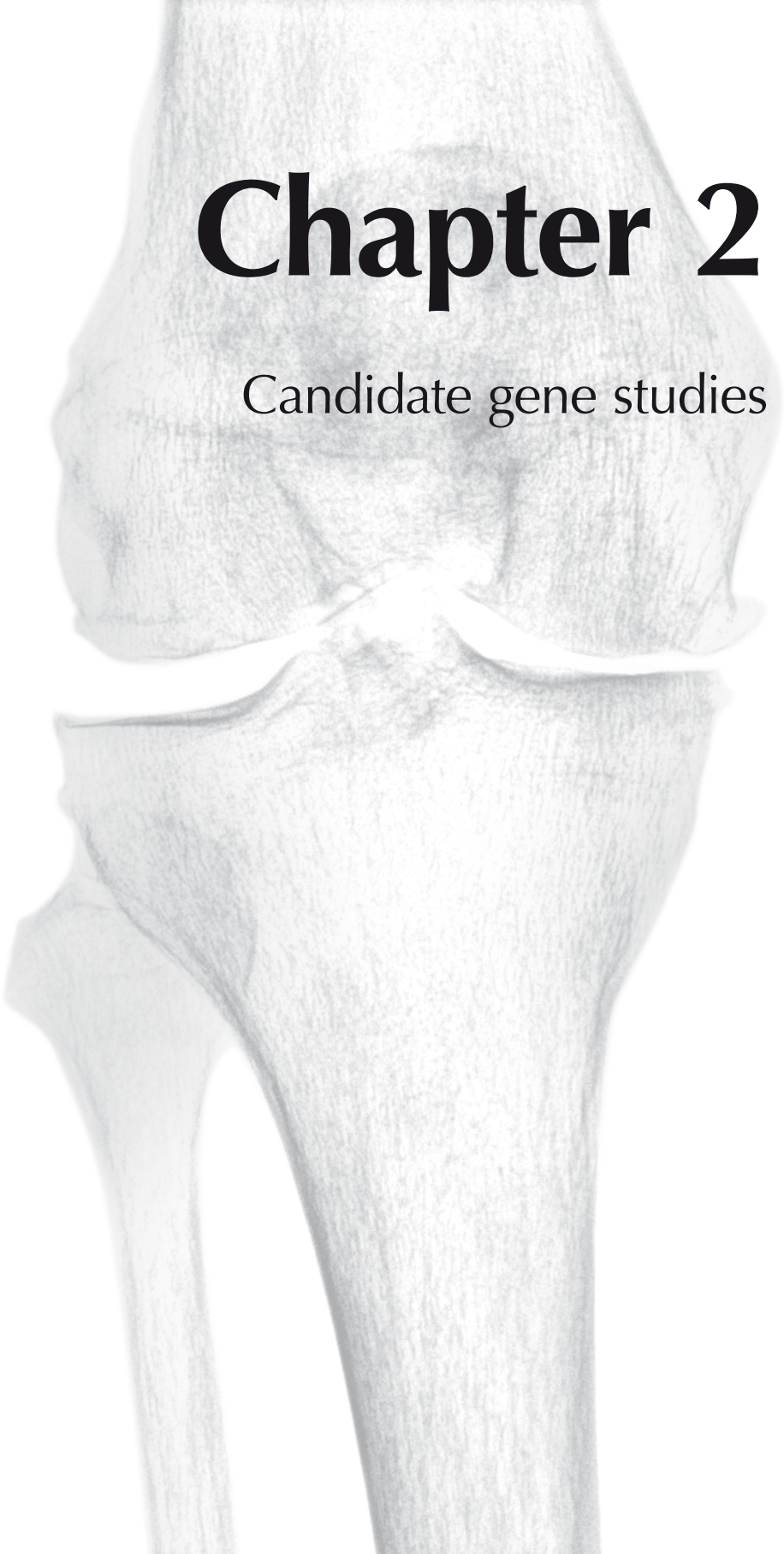

Chapter 2

Candidate gene studies

Chapter 2.1

Radiographic osteoarthritis at three joint sites and *FRZB*, *LRP5* and *LRP6* polymorphisms in two population-based cohort studies

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ABSTRACT

Background: The objective of this study was to examine the association of genetic variation in key players in the Wnt signaling pathway with aspects of osteoarthritis (OA) in two population-based cohort studies: the Rotterdam Study and the Chingford Study.

Methods: Radiographic OA (ROA) was defined as a Kellgren/Lawrence score (K/L score) ≥ 2 for the knee and hip. Total hip replacement was scored. Hand OA was defined as presence of ROA (K/L≥2) in 2 out of 3 hand joint groups (DIPs, PIPs, CMC1/STT) of each hand. The concentration of urinary C-terminal cross-linked Telopeptide of type II collagen (CTX-II) was standardized to the total urine creatinine. Genotypes for the amino acid variants, Arg200Trp and Arg324Gly of *FRZB*, Ala1330Val of *LRP5* and Ile1062Val of *LRP6*, were obtained using the Taqman allelic discrimination assay. A meta-analysis was performed for the *FRZB* Arg324Gly polymorphism and hip and knee OA using RevMan version 4.3.

Results: No consistent associations were observed between the *FRZB*, *LRP5* and *LRP6* amino acid variants and radiographic hip, knee, or hand osteoarthritis or total hip replacement, in either study population. While power was limited for most studies published to date, a meta-analysis of all published studies regarding the *FRZB* Arg-324Gly polymorphism was performed for hip and knee OA seperately. This showed no significant associations between the Gly^{324} allele and risk for hip or knee osteoarthritis, although there was large heterogeneity between studies for hip OA in females.

Conclusions: No association was seen between *FRZB*, *LRP5* and *LRP6* variants with radiographic osteoarthritic outcomes in two population based-cohorts. In future studies, increased power and standardization of OA phenotypes is highly recommended for replication studies and to allow meta-analysis.

INTRODUCTION

Osteoarthritis (OA) is a chronic, age-related, degenerative disease of the synovial joints. It is characterized by cartilage degradation, formation of osteophytes, subchondral sclerosis and synovitis (1). The etiology of OA is multifactorial, i.e., environmental and genetic factors play an important role in the development of OA. Primary OA has an estimated heritability of 40% for the knee, 60% for the hip and 65% for the hand (2). This means that a substantial proportion of variation in risk for OA can be attributed to genetic variation, i.e., polymorphisms in genes involved in the etiology of OA. The vast majority of the genes are unknown and their identification could explain much of the pathogenesis of OA, which at the moment remains unclear.

Evidence is accumulating, showing that the Wnt signaling pathway is involved in cartilage degeneration and OA (3-11). Several Wnt signaling proteins were shown to play an important role in regulating many aspects of chondrogenesis and skeletal development, including limb formation and osteoblast maturation[4]. Genes encoding proteins of the Wnt signaling pathway are therefore interesting candidates to search for common genetic variants that could contribute to risk for OA.

Frizzled-Related protein gene (*FRZB*) is a key player in the Wnt signaling pathway with regard to cartilage metabolism and OA (4,6,7,10-12). Several studies have investigated the relationship between OA and two polymorphisms in the *FRZB* gene: the *FRZB* Arg200Trp and Arg324Gly variants. Loughlin *et al*. observed in female carriers of the *FRZB* Gly^{324} allele an increased risk for total hip replacement (6). However, subsequent studies have yielded conflicting results (7,11,13-15). This could partially be explained by the variety of OA definitions across studies. In general, many different phenotypes are used as endpoints in genetic studies of osteoarthritis and those on *FRZB* are no exception. Such phenotypes may vary from clinical endpoints such as total hip replacement to biochemical assessments (such as with urinary C-terminal cross-linked telopeptide of type II collagen (CTX-II)), and include radiographic definitions of osteoarthritis as defined by the Kellgren/Lawrence (K/L) score at several possible sites (knee, hip or hand), and be even based on composites and/or subphenotypes from this score. Furthermore, individual studies might be underpowered, which could be a reason why true associations might not be observed. A meta-analysis could help to overcome such limitations and document the true effect (if any) of such genetic variants on the risk of osteoarthritis.

In three papers, the relationship between the rare *FRZB* "Trp^{200}-Gly^{324}" haplotype (frequency of 0.6-5.0%) and OA has been studied (6,11,13). They found that female carriers of this haplotype have an increased risk of THR (6), severe JSN of the hip (11) and clinical knee OA (13) further supporting a role for *FRZB* variants in osteoarthritis. In addition, Gordon et al.(16) showed recently that the *FRZB* Trp^{200} allele was as-

sociated with a decreased risk of osteolysis after total hip arthroplasty. In this study, we examined the relationship between combined genotypes (*FRZB* Arg200Trp and Arg324Gly variant) and hip and knee osteoarthritis.

Low-Density Lipoprotein Receptor-Related Protein 5 and 6 (*LRP5* and *LRP6*) are two known co-receptors for Wnt proteins (17). Since Wnt signaling is involved in cartilage degeneration and OA (3-11) and *LRP5* and *LRP6* are key players in the Wnt signaling pathway (17,18), these genes are logical candidate genes to study in relation to OA. Several studies have shown that genetic variation in the *LRP5* and *LRP6* genes is associated with bone-related outcomes (19-26). With respect to osteoarthritis, studies of *LRP5* and *LRP6* polymorphisms are scarce. One study observed a common haplotype of the *LRP5* gene, to be associated with an increased risk of knee OA (27).

In the present study, we examined the association of the Arg200Trp and Arg324Gly variants of *FRZB*, the *LRP5* Ala1330Val variant and the *LRP6* Ile1062Val variant with the risk of radiographic hip, knee and hand osteoarthritis, total hip replacement, and CTX-II levels. We investigated these associations in two population-based cohort studies: the Rotterdam Study and the Chingford Study. In addition, a meta-analysis was performed for the *FRZB* Arg324Gly polymorphism and hip and knee OA.

MATERIALS & METHODS

The Rotterdam Study (RS-I) and the Chingford Study

A description of the study populations is given in Appendix 1. Depending on the OA outcome studied, there were 2,685-3,001 subjects with data available for OA outcomes in the Rotterdam Study. For example for total hip replacement we had data available for 2,998 subjects.

Assessment of radiographic osteoarthritis

Radiographs were scored for the presence of a total hip replacement and radiographic OA (ROA) of the hip, knee and hand according to the K/L score (38). Knee and hip ROA were defined as a Kellgren-Lawrence score ≥2 of one or both joints (29,39). Also, a JSW in one or both hips ≤1.5 mm was defined as ROA. Hand OA was defined as presence of a K/L score ≥2 in 2 out of 3 hand joint groups (DIPs, PIPs, CMC1/STT) of each or both hands (30). Furthermore, in a subgroup of RS-I, we used a K/L score ≥3 of the knee and K/L score ≥2 of the MCP joints of the hand as additional outcomes for severe osteoarthritis. Clinical OA was defined as having a K/L ≥2 plus joint complaints in that specific joint.

Biochemical measurement

Urinary CTX-II was measured as described before (29,40). The concentration of CTX-II (ng/liter) was standardized to the total urine creatinine (mmol/liter).

Genotyping

Genotyping of both RS-I and the Chingford Study was done by the Genetic Laboratory of the Department of Internal Medicine in Rotterdam. Genomic DNA was extracted from peripheral venous blood samples according to standard procedures. Genotypes were determined using the Taqman allelic discrimination assay. The Assay-by-Design service (www.appliedbiosystems.com) was used to set up a Taqman allelic discrimination assay for the *FRZB* Arg324Gly, the *FRZB* Arg200Trp, the *LRP5* Ala1330Val and the *LRP6* Ile1062Val polymorphisms. The primers and probes used are available on request and conditions of the assay were as previously described (26).

Statistical analysis

Allele frequencies were estimated by allele counting and Hardy-Weinberg equilibrium was tested using a χ^2 test. For reasons of power, homozygous and heterozygous carriers of the risk alleles were pooled for estimation of the odds ratio for all SNPs studied. Differences in baseline characteristics and CTX-II levels were evaluated by analysis of co-variance (ANCOVA). In order to compare the CTX-II levels in the two cohorts, sex-specific standard deviation scores were calculated separately for each subject in each cohort as described elsewhere in detail (41). Odds ratios (ORs), crude and adjusted for age and BMI, were estimated from logistic regression for cross-sectional analysis of all the binary osteoarthritis variables. Linkage Disequilibrium (LD) between the Arg200Trp and Arg324Gly *FRZB* SNPs was estimated by D' and r^2 and was calculated using Haploview. SPSS version 11.0 (SPSS INC., Chicago, USA) was used to construct combined genotypes. Interaction between the different SNPs was tested using the multi-locus model implemented in FAMHAP and is described elsewhere in detail (42). Power calculations were performed with the program PS (http://biostat.mc.vanderbilt.edu/twiki/bin/view). Stratification according to gender was performed. A P-value ≤ 0.05 was considered significant. If not stated otherwise, we used SPSS version 11.0 software for all analyses.

Power considerations: For *FRZB* Arg324Gly, *LRP5* Ala1330Val and *LRP6* Ile1062Val polymorphisms, we performed power calculations for detectable effect sizes in our study at $\beta=0.80$ and $\alpha=0.05$.

Meta-analysis: We conducted a meta-analysis of data from all published studies regarding the *FRZB* Arg324Gly polymorphism, in order to assess whether the Gly^{324} allele is associated with increased risk for hip or knee OA. ORs for the individual studies were estimated using the number of subjects and allele frequencies presented

in the papers. Forest plots were created using RevMan Analyses version 1.0 (43). By eyeballing and using the Q-statistic (Mantel-Haenszel chi-square) and I^2 statistic, heterogeneity between studies was assessed. A P-value ≤0.10 was considered significant for the Q-statistic. The DerSimonian and Laird test was used in RevMan Analyses version 1.0 (43) to estimate an odds ratio in case of heterogeneity (random-effects model), otherwise a Mantel-Haenszel test was performed (fixed-effect model). Analyses were performed separately for males and females, since positive studies only found an association in females. In the case more than one definition of hip or knee OA occurred in one study, only the definition for which most power was observed, was included in the meta-analysis.

RESULTS

Genotyping

In RS-I, all genotypes were in HWE. However, in the Chingford Study we observed a HWE deviation for *LRP5* Ala1330Val and *LRP6* Ile1062Val genotype distributions, but this was borderline significant (*P*=0.03 and 0.05, respectively). To exclude genotyping errors we re-genotyped all homozygote carriers of the *LRP5* Val^{1330} allele and the *LRP6* Val^{1062} allele, but no discrepancies were detected. While all genotyping was done at the same laboratory, it is unlikely that genotyping errors are the cause of the slight imbalance in HWE in the Chingford Study. Allele frequencies for all polymorphisms where similar to the ones found in previous studies on Caucasians (6,7,11,14,27).

Baseline characteristics

Characteristics of RS-I and the Chingford Study are shown in Table 1. In women, there were no associations between these characteristics and the two studied polymorphisms in the *FRZB* gene, neither in RS-I nor in the Chingford Study (data not shown). For males in RS-I, carriers of the *FRZB* Gly^{324} allele were on average one year older as the non-carriers (data not shown).

Osteoarthritic outcomes

Table 2 shows no consistent significant associations between the *FRZB* Gly^{324} variant and hip, knee, or hand osteoarthritis outcome measures in the Rotterdam, nor in the Chingford Study.

Adjusting for co-morbidity factors such as lower limb disability, coronary heart disease and diabetes did not change these results.

There were no significant associations between the three other studied variants, *FRZB* Trp^{200}, *LRP5* Ala^{1330}, *LRP6* Ala^{1062}, and hip, knee or hand OA (Supplementary

Table 1. Characteristics of the Rotterdam Study and the Chingford Study for the *FRZB* Arg324Gly polymorphism

	Rotterdam Study		Chingford Study
n Males/n Females	n = 1765 males	n = 2448 females	n = 780 females
Age (years)[1]	67.1 ± 7.3	68.0 ± 7.9	64.2 ± 6.2
Height (cm)[1]	175.1 ± 6.7	161.9 ± 6.5	160.6 ± 6.1
Weight (kg)[1]	79.1 ± 10.5	70.1 ± 11.1	69.1 ± 12.4
BMI (kg/m²)[1]	25.8 ± 2.9	26.8 ± 4.1	26.9 ± 4.7
No. THR cases/total (%)	18/1276 (1.4)	62/1722 (3.6)	NA
No. Hip OA cases/total (%)	113/1276 (8.9)	168/1722 (9.8)	245/750 (32.7)
No. Knee OA cases/total (%)	164/1063 (15.4)	479/1622 (29.5)	291/773 (37.6)
No. Hand OA cases/total (%)	267/1329 (20.1)	600/1672 (35.9)	100/636 (15.7)
CTX-II levels (ng/mmol)	142.8	209.4	227.9

[1]Values are averages with standard deviations; OA: osteoarthritis; NA: not applicable; THR: total hip replacement

Table 2. Hip, knee, and hand OA risk by *FRZB* Arg324Gly genotype in both study cohorts

Phenotype	Rotterdam Study					Chingford Study				
	Allele frequency		OA by genotypes			Allele frequency		OA by genotypes		
Females	*Controls*	*Cases*	*Arg/Arg*	*Arg/Gly + Gly/Gly*	*OR (95% CI)*[1]	*Controls*	*Cases*	*Arg/Arg*	*Arg/Gly + Gly/Gly*	*OR (95% CI)*[1]
THR	0.09	0.06	56/1433 (3.9)[2]	6/289 (2.1)	0.55 (0.23-1.29)	NA	NA	NA	NA	NA
K/L hip	0.09	0.09	141/1433 (9.8)	27/289 (9.3)	0.99 (0.64-1.53)	0.09	0.05	224/648 (34.6)	21/102 (20.6)	0.56 (0.34-0.91)
K/L knee	0.08	0.09	399/1363 (29.3)	80/259 (30.9)	1.09 (0.80-1.48)	0.08	0.07	257/689 (37.3)	42/116 (36.2)	1.10 (0.71-1.71)
Hand OA	0.09	0.08	503/1387 (36.3)	97/285 (34.0)	0.94 (0.71-1.25)	0.08	0.06	89/560 (15.9)	12/96 (12.5)	0.82 (0.41-1.63)
Males										
THR	0.09	0.06	16/1044 (1.5)	2/232 (0.9)	0.53 (0.12-2.37)	NA	NA	NA	NA	NA
K/L hip	0.09	0.11	90/1044 (8.6)	23/232 (9.9)	1.14 (0.70-1.86)	NA	NA	NA	NA	NA
K/L knee	0.09	0.09	137/882 (15.5)	27/181 (14.9)	0.81 (0.50-1.30)	NA	NA	NA	NA	NA
Hand OA	0.10	0.09	223/1089 (20.5)	44/240 (18.3)	0.78 (0.54-1.14)	NA	NA	NA	NA	NA

[1]ORs are adjusted for age and BMI; [2]Number of subjects defined as case/total number of subjects with data on this OA phenotype (%); NA: not applicable; THR: total hip replacement; K/L: Kellgren/Lawrence

Tables 1, 2 and 3 (Appendix 2)) and there were no associations between the *FRZB*, *LRP5* and *LRP6* variants and other osteoarthritic outcomes such as joint space width of the hip, clinical OA, OA in the MCP joints of the hand and a K/L ≥3 for the knee as a proxy for severe OA. Similarly, no associations were observed between the genetic variants and total hip replacement, although power was limited.

We next examined CTX-II levels as a measure of generalized cartilage degradation. As shown in Figure 1, in the Rotterdam Study, female carriers of the *FRZB* Gly^{324} allele had 0.32 SD lower levels of CTX-II (*P*=0.002), while a similar non-significant trend was seen in males with 0.13 SD lower levels for Gly^{324} carriers. In the Chingford study, we also observed a similar -though not significant- trend with 0.14 SD lower CTX-II levels in *FRZB* Gly^{324} carriers. When we combined males and females from the Rotterdam Study, and females from the Chingford Study according to standard deviation scores, carriers of the *FRZB* Gly^{324} allele had 0.29 SD lower CTX-II levels (*P*=0.001).

For alleles of the *FRZB* Arg200Trp and Arg324Gly polymorphisms we could not estimate haplotypes because LD was too low (D'=0.06; r^2=0.003 in the Rotterdam Study). We therefore analysed combined genotypes and compared the combination of Trp^{200} and Gly^{324} carriers with non-carriers of these two variants. There were no significant associations for hip or knee OA in the Rotterdam Study or the Chingford Study. In females of the Rotterdam Study, we observed an OR of 0.94 for hip OA

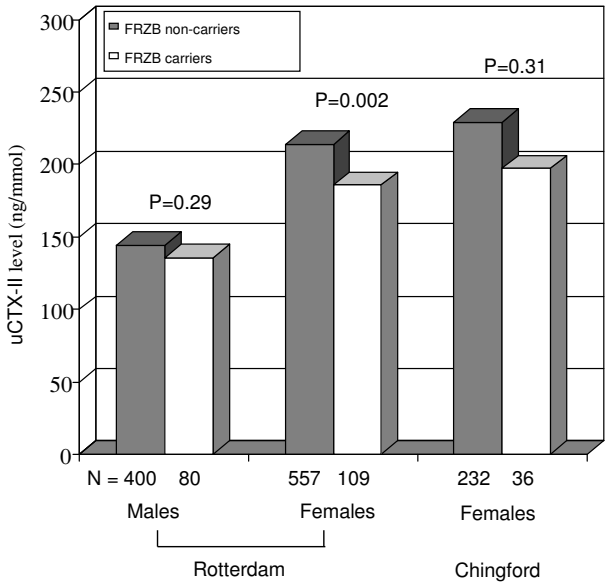


Figure 1. uCTX-II levels by *FRZB* Arg324Gly genotype of men and women of the Rotterdam Study and women of the Chingford Study. Values are adjusted for age and BMI.

Table 3. Overview of published studies on the relationship of the *FRZB* Arg324Gly polymorphism with OA outcomes

	OA Phenotypes	Hip OA						Knee OA						Hand OA				Generalized OA		CTX-II levels	
		THR		*ROA*		*Power*[3]		*COA*		*ROA*		*Power*[3]		*COA*		*ROA*		*ROA*			
Author and study population (n)	**Case-control/ cohort**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Loughlin *et al.* Oxford (n=1696) (6)	Case-control	■	□			1.9	1.8					NA	NA								
Min *et al.* Rotterdam Study (n=1369) (7)	Cohort			■	■	NA	NA					NA	NA					□	□		
Min *et al.* GARP (n=382) (7)	Family study[1]					NA	NA					NA	NA					□	□		
Lories *et al.* Leuven (n=314) (14)	Case-control		■			NA	2.8					NA	NA								
Lane *et al.* SOF (n=4706) (11)	Cohort				□	NA	1.5					NA	NA								
Valdes *et al.* Nottingham/ Oxford (n=1202) (13)	Case-control[2]					NA	NA	■	■			2.0	2.2								
Rodriguez *et al.* Spanish patients (n=1123) (15)	Case-control	■	■			2.5	2.2	■	■			3.1	2.1	■	■						
Kerkhof *et al.* Rotterdam Study (n=4472)	Cohort	■	■	■	■	2.2	2.0			■	■	2.0	1.6			■	■			■	▒
Kerkhof *et al.* Chingford Study (n=814)	Cohort				▒	NA	1.9			■		NA	1.8				■				■

SOF: Study of Osteoporotic Fractures; M: males; F: females; ROA: radiographic OA; COA: clinical OA; THR: total hip replacement; TKR: total knee replacement; CTX-II: urinary CTX-II levels; [1]1082 Control subjects from the Rotterdam Study are shared between that study and the Rotterdam Study described in this paper.;[2]185 female control subjects from Oxford are shared between that study and the original study by Loughlin et al. [3]Power is expressed as ORs that can be detected with beta=80% and alpha=0.05

■ No association found ▒ Significantly increased risk Gly^{324} allele ▒ Significantly decreased risk Gly^{324} allele □ Not studied

(95%CI 0.44-2.01) and 1.33 for knee OA (95%CI 0.79-2.22) and in the Chingford Study an OR of 0.46 (95%CI 0.17-1.24) for hip OA and 1.29 for knee OA (95%CI 0.58-2.96), but for both studies power was limited.

In the Rotterdam Study, we constructed a multiple locus association model incorporating all four polymorphisms in the three genes of the Wnt signaling pathway to test for possible interactions. No significant interactions between the polymorphisms in relation to OA endpoints were observed (data not shown).

Power considerations

We calculate power for all published studies and our own study (Table 3). For the *FRZB* Arg324Gly polymorphism we had 80% power to detect risks of at least 1.6 for knee OA in females of the Rotterdam Study and a risk of 1.9 in the Chingford Study. For females in the Rotterdam Study, we had 80% power to detect risks of 1.9 for hip OA and 1.6 for hand OA. For total hip replacement we had 80% power to detect a risk of 2.6, indicating very limited power in our study to detect the originally reported OR of 1.5 for THR by *FRZB* genotype (6).

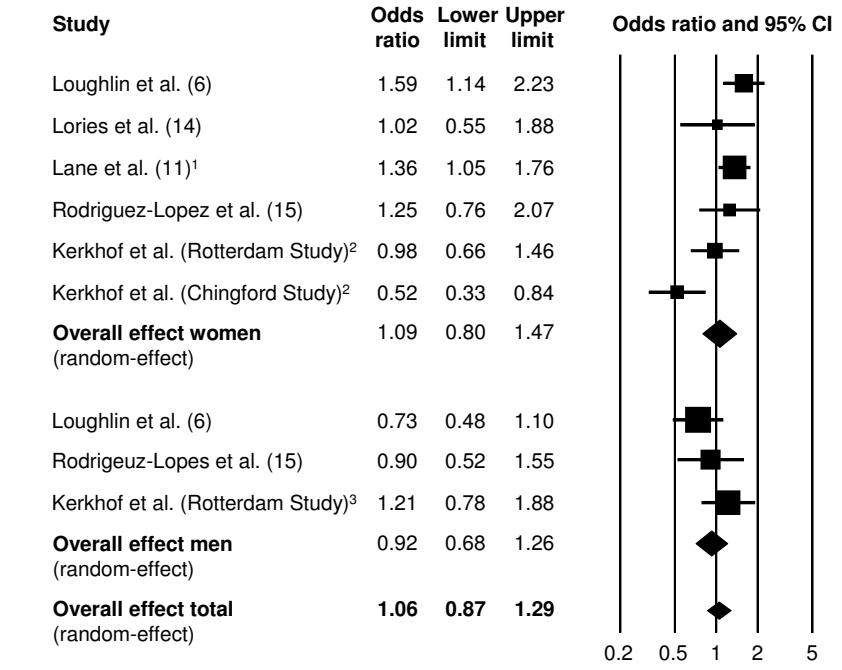
For the *LRP5* and *LRP6* variants we had higher power, given the higher allele frequencies. For example, for knee OA in females in the Rotterdam Study, we could detect (with 80% power) an OR of 1.5 for the *LRP5* variant and an OR of 1.4 for the *LRP6* variant (data not shown). In the Rotterdam Study and the Chingford Study we had very low power to investigate the role of the rare *FRZB* combined genotype and OA. In the Rotterdam Study, we had 80% power to detect a OR of 2.3 in females for hip OA, in the Chingford Study this was a OR of 2.5.

Meta-analysis

In Table 3 some characteristics of all published studies are shown that were considered for the meta-analysis, including our own data from the current study. For the *FRZB* Arg324Gly polymorphism, the forest plots for hip OA and knee OA are depicted in Figure 2a and 2b, respectively. Large heterogeneity was observed between the studies on hip OA in females reflected by a Q-statistic of 16.69 (df=5), with a P-value of 0.005 and a I^2 of 70%. Therefore, a random-effects analysis was performed for this phenotype. This analysis showed no evidence of association between the *FRZB* Gly^{324} allele and hip OA in females (combined effect estimate OR 1.09 95% CI 0.80-1.47, *P*=0.58).

For knee OA no large heterogeneity between studies was observed, and so both fixed- and random-effect analysis were performed. Both analyses showed no evidence of association between the Gly^{324} allele and knee OA in males and females (combined effect estimate OR 1.04 95% CI 0.89-1.20, *P*=0.63.

A



B

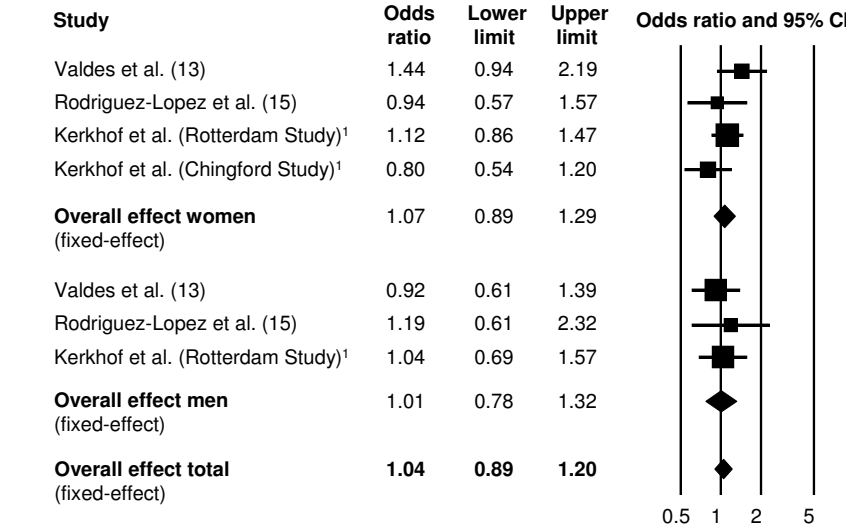


Figure 2. Forest plot of studies regarding (**A**) *FRZB* Arg324Gly polymorphism and hip osteoarthritis in men and women. [1]Joint space narrowing is used as definition of hip OA. Other hip OA phenotypes are excluded from the analysis. [2]A K/L score ≥ 2 is used as definition of hip OA. The phenotype total hip replacement is excluded from the analysis. (**B**) *FRZB* Arg324Gly polymorphism and knee osteoarthritis in males and females. [1]A K/L score ≥ 2 is used as definition of knee OA. The phenotype total knee replacement is excluded from the analysis.

DISCUSSION

The present study showed that in two population-based cohort studies no consistent associations were observed between selected polymorphisms in the *FRZB*, *LRP5* and *LRP6* genes and several measures of OA including, radiographic hip, knee and hand osteoarthritis and total hip replacement. A meta-analysis of all published studies and including our own data indicated no evidence of association between the *FRZB* Gly^{324} allele and hip or knee OA, although large heterogeneity was observed between studies concerning hip OA in females.

Recently, Loughlin *et al*. observed in 957 females the Gly^{324} variant of the *FRZB* gene to be associated with a 1.5 times higher risk of total hip replacement (6). In our study, however, we did not observe an increased risk for total hip replacement by genotype for the *FRZB* Arg324Gly or *FRZB* Arg200Trp polymorphisms, although power in our population was very limited for purposes of replication of this phenotype. For other hip-related OA outcome measures such as the joint space width and the Kellgren/Lawrence score, we had somewhat higher power, but we could not detect an association or a trend in the predicted direction. Within the Rotterdam Study we can only exclude risks of 2.0 for hip ROA based on power calculations, while Loughlin *et al*. found an odds ratio of 1.5 for the *FRZB* Gly^{324} allele (and then for THR, rather than hip OA defined by radiography). Therefore, the lack of power, especially for hip OA, could be one of the reasons for our negative findings. Alternatively, the difference in severity of OA (hip replacement cases versus radiological defined milder OA in our study) could be another reason for the negative findings. Lastly, negative findings could be based on confounding due to co-morbidity factors. However, in the Rotterdam Study no differences in risk for OA were observed after adjusting our results for co-morbidity factors such as lower limb disability, coronary heart disease and diabetes.

We also explored the association with more clinical/severe definitions of OA in a subset of our subjects. We identified a subgroup with a JSW <=1.5 mm of the hip, a K/L score ≥3 of the knee, current pain in the hip/knee/hand coinciding with ROA of that joint and K/L score ≥2 of the MCP joints of the hand as a proxy for severe hand OA (30). However, again there were no associations observed between these more severe and clinical phenotypes and the four polymorphisms.

In line with our observation, also three other reports found conflicting results for the role of the *FRZB* Arg324Gly polymorphism in osteoarthritis, although each study used a different definition of OA (7,11,14). This highlights a general difficulty in genetic studies of OA: the lack of consensus on OA definitions or outcomes to use and how to compare different joint sites for replication (44). In Table 3, we compared OA endpoints used in different studies on the *FRZB* Arg324Gly polymorphism. This

table shows: a) that different studies used different outcomes, b) only very few studies analyzed the same phenotypic OA outcome, and c) that there is no consistency in the results for this association. Considering that results are conflicting and power of individual studies is limited, a meta-analysis may help in determining the true effect sizes if any. The meta-analysis of all published studies including our results showed no evidence of association between the *FRZB* Gly^{324} allele and hip or knee OA. In the process of the meta-analysis, we had difficulty with handling the different OA phenotypes used in the published studies. For example, for hip OA it was necessary to combine both clinical en radiographic OA outcomes, resulting in a large heterogeneity between studies. Therefore, the power becomes lower to observe an association. Recently, general recommendations have been published by working groups of HuGEnet and NCI-NHGRI (45-47) for replication studies in genetic epidemiology studies. One of the recommendations was to preferably investigate the same or a very similar phenotype in replication studies and for genetic studies in OA this represents a substantial challenge as is discussed above in some more detail.

Previously, Min *et al.* (7) published a paper regarding the *FRZB* Arg324Gly polymorphism and OA using a random sample of 1369 subjects of the Rotterdam Study. They found a significant association of the *FRZB* Arg324Gly polymorphism with generalized OA. In the present study, apart from testing additional polymorphisms, we focused more on the many different joint sites of osteoarthritis, including hip, knee and hand OA and CTX-II measurement. Furthermore, we used more subjects of the Rotterdam Study (4,472 instead of 1,369, average overlap between the study of Min *et al.* and the Rotterdam Study for hip, knee and hand OA is 79%), thereby increasing power.

In the Chingford Study and in the Rotterdam Study we used a cut-off point for the K/L score of the hip of grade 2, which is considered the golden standard for hip OA (48). However, the prevalence of hip OA was three times higher in the Chingford Study compared with the Rotterdam Study when using grade 2+. This is highly likely due to the fact that agreement between observers for the grading of osteoarthritis is rather low, particularly for hip OA (48).

This is a problem we cannot overcome at this moment and is a general problem for all OA studies. The differences in K/L scoring between different cohorts is becoming apparent because of the population-based design of our study. It was picked up due to frequency differences between the two cohorts. These frequency differences cannot be observed in a case-control study.

Cartilage degradation is one of the main characteristics of osteoarthritis and is commonly assessed by the joint space width (JSW) measurement. However, significant cartilage degradation has to occur in order to visualize a reduction in JSW on radiographs (49). Biochemical markers may be more sensitive than radiographs to pick

up early changes in the cartilage (50,51). Urinary concentrations of C-telopeptide fragments of type II collagen (CTX-II levels/cartilaps) are a biochemical marker of cartilage degradation (29). CTX-II levels are elevated in diseases that are characterized by increased cartilage turnover such as rheumatoid arthritis and osteoarthritis (49,50,52). We observed a highly significant association with the *FRZB* Arg324Gly polymorphism and CTX-II levels in females of the Rotterdam Study, which suggests a protective effect for OA (assessed by CTX-II levels) for carriers of the *FRZB* Gly^{324} allele. The Chingford study showed a non-significant trend in the same direction. This finding is counter-intuitive since previous research lead to the hypothesis that carriers of the FRZB Gly^{324} allele are less capable of antagonizing Wnt signaling (6), which would lead to a higher Wnt-signaling in cartilage and hence, in an increased risk of OA (3,8-10). Although a false positive result due to multiple testing is still possible, the partial confirmation in Chingford and overall significance of the two combined study populations makes this a less likely explanation. It therefore suggests a true effect of *FRZB* genotype on CTX-II levels but this observation still warrants further validation in other cohorts.

One earlier report examined the relationship between polymorphisms in the *LRP5* gene and OA and found an association between a certain haplotype of the *LRP5* gene and knee OA (27). In this study, we examined the relationship between the *LRP5* Ala1330Val variant and osteoarthritis. This is not a direct replication of the study of Smith *et al* (27). Previous research showed that genetic variation in exon 18 of *LRP5*, in particular the Ala1330Val variant, modulates Wnt signaling (53), and therefore the Ala1330Val variant is a promising variant to study in relation to osteoarthritis. In our study, we did not observe any relation between the *LRP5* Ala1130Val variant with knee, hip and hand OA or CTX-II levels in either cohort. Considering the earlier results of Smith *et al*. who studied other polymorphisms in this gene as we did, we cannot exclude that the *LRP5* gene does play a role in the pathogenesis of OA, but if so perhaps through other variants than the Ala1330Val variant. A tagging approach of the large *LRP5* gene would be most appropriate to investigate the role of this gene in the pathogenesis of OA, however we did not use this approach in our study, which is a limitation of this study. To our knowledge, we are the first to study the relationship between the *LRP6* amino acid variant and OA. de Ferrari *et al*. showed that the *LRP6* Val^{1062} allele leads to a decreased beta-catenin signalling (54) and could therefore decrease the risk of OA. However, we did not observe significant associations between this variant and osteoarthritic outcomes.

Lastly, we would like to discuss power issues. For females in the Rotterdam Study, we had 80% power for the *FRZB* Trp324Gly variant to detect risks of 2.0 for hip OA and 1.6 for knee OA as defined by the K/L score, while for THR power was 2.6. For the *LRP5* and *LRP6* variants and other osteoarthritic endpoints we had overall some-

what higher power, given the higher allele frequencies. Therefore, it is possible that in our study some true associations were not detected due to lack of power, especially for total hip replacement. Yet, we note that most previously published studies on this association had very limited power and so a meta-analysis of all published studies including our own results could have overcome this problem, if this was the only reason for missing the association in our study. However, the meta-analysis failed to provide convincing evidence to support an association of the *FRZB* Arg324Gly polymorphism with several OA endpoints, although for hip OA in females, large heterogeneity existed between studies.

In conclusion, we studied polymorphisms in several key players of the Wnt signaling pathway but did not observe any association between the *FRZB*, *LRP5* and *LRP6* amino acid variants with osteoarthritic outcomes in two population based cohorts. A meta-analysis for the *FRZB* Gly^{324} allele showed no evidence of association with hip or knee OA. This study underscores that more consensus is needed to standardize phenotypes of interest in future genetic studies of OA as well as increasing sample sizes. The recently formed European Consortium to explore the genetics of OA called Treat-OA (www.TreatOA.eu) has access to up to 30.000 DNA samples and hopefully should be able to overcome some of these issues in the near future.

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Chapter 2.2

Common genetic variation in the Estrogen Receptor Beta (*ESR2*) gene and osteoarthritis: results of a meta-analysis

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ABSTRACT

Background: The objective of this study was to examine the relationship between common genetic variation of the *ESR2* gene and osteoarthritis.

Methods: In the discovery study, the Rotterdam Study-I, 7 single nucleotide polymorphisms (SNPs) were genotyped and tested for association with hip (284 cases, 2,772 controls), knee (665 cases, 2,075 controls), and hand OA (874 cases, 2,184 controls) using an additive model. In the replication stage one SNP (rs1256031) was tested in an additional 2,080 hip, 1,318 knee and 557 hand OA cases and 4,001, 2,631 and 1,699 controls, respectively. Fixed- and random-effects meta-analyses were performed over the complete dataset including 2,364 hip, 1,983 knee and 1,431 hand OA cases and approximately 6,000 controls.

Results: The C allele of rs1256031 was associated with a 36% increased odds of hip OA in women of the Rotterdam Study-I (OR 1.36, 95%CI 1.08-1.70, *P*=0.009). Haplotype analysis and analysis of knee and hand OA did not give additional information. With the replication studies, the meta-analysis did not show a significant effect of this SNP on hip OA in the total population (OR 1.06, 95%CI 0.99-1.15, *P*=0.10). Stratification according to gender did not change the results. In this study, we had 80% power to detect an odds ratio of at least 1.14 for hip OA (α=0.05).

Conclusions: This study showed that common genetic variation in the *ESR2* gene is not likely to influence the risk of osteoarthritis with effects smaller than a 13% increase.

INTRODUCTION

Epidemiological observations show sex-specific differences in the prevalence and incidence of osteoarthritis (OA) (1): the prevalence of OA among women increases rapidly after the menopause. In addition, men have a higher prevalence of OA before the age of 50 compared to women. This has led to the hypothesis that sex hormones may be involved in the etiology of osteoarthritis (2). Estrogen receptors α and ß are present in chondrocytes (3) and several *in vitro* and *in vivo* animal experiments showed a chondro-protective effect of estrogens (4-5). The estrogen receptors α (*ESR1* gene) and β (*ESR2* gene) are nuclear proteins. Both function as ligand-regulated transcription factors and show tissue specific expression.

Previously, three studies (in total 577 cases and 1,837 controls) reported an association between two Single Nucleotide Polymorphisms (SNPs) (rs2234693 and rs9340799) of the *ESR1* gene and radiographic knee and generalized OA (6-8). However, currently, only one small study (158 cases, 193 controls) investigated the role of variation in the *ESR2* gene in relation to OA. A 4.5-fold increased risk of knee OA was observed in individuals carrying long alleles of the c.1092+3607(CA)n repeat polymorphism of the *ESR2* gene (9).

In this study, we examined the relationship between common genetic variation of the *ESR2* gene and radiographic hip, knee and hand osteoarthritis in a large population-based cohort study (the Rotterdam Study-I). For replication purposes, 6 additional studies were genotyped for common genetic variation in the *ESR2* gene and a meta-analysis was performed combining all 7 studies with in total 2,364 hip-, 1,983 knee-, and 1,431 hand OA cases and, respectively 6,773, 4,706 and 3,883 controls.

MATERIALS & METHODS

Selection of study populations

We searched PubMed to identify articles which could be included on this meta-analysis on common genetic variation in the *ESR2* gene and OA. One study (9) performed an association study on common genetic variation in the *ESR2* gene and OA in Caucasians, but this variant was not of interest to our study. Therefore, only novel, and therefore unbiased data, is included in this meta-analysis. Study populations with both DNA and at least hip OA data available were approached to join this meta-analysis.

Table 1. Baseline characteristics of all studies

	Rotterdam Study-I	Oxford Study	SOF Study	Chingford Study	GARP Study	Greek cases	Spanish cases
N (hip OA cases/controls)	284/2772	1065/727	366/1365	247/511	107/724	49/258	246/416
N (knee OA cases/controls)	665/2075	361/727	NA	302/506	148/724	258/258	249/416
N (hand OA cases/controls)	874/2184	NA	NA	99/559	244/724	NA	214/416
OA definition	ROA	COA	ROA	ROA	CROA	COA	COA
% women	58%	55%	100%	100%	70%	71%	76%
Mean age (range)	67 (55-94)	67 (55-89)	78 (72-98)	64 (54-100)	60 (43-79)	64 (18-90)	68 (55-94)
Mean BMI (range)	26 (15-59)	NA	27 (15-56)	27 (17-50)	27 (19-46)	28 (17-64)	NA

NA: not applicable; ROA: radiographic OA; COA: clinical OA; CROA: clinical and radiographic OA; SOF: Study of Osteoporotic Fractures

Study populations

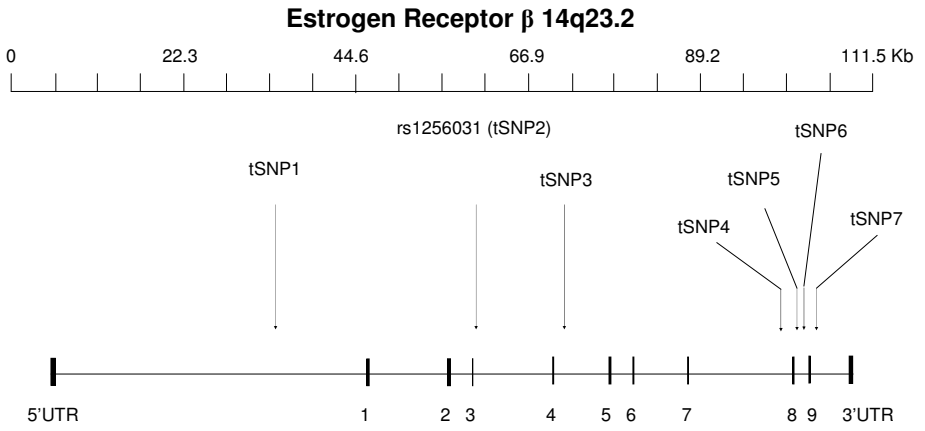
Details of all study populations are given in Appendix 1 and Table 1.

Osteoarthritis

In studies with radiographic OA (ROA), radiographs were scored for the presence of ROA of the hip and knee according to the Kellgren/Lawrence (K/L) score (10). Hip ROA was defined as at least definite JSN and a definite osteophyte and knee ROA was defined as at least 2 definite osteophytes and possible joint space narrowing. Hand OA was defined as presence of at least one definite osteophyte in 2 out of 3 hand joint groups (DIPs, PIPs, CMC1/STT) of each or both hands. Clinical studies defined hip, hand and/or knee clinical OA (COA) as symptomatic OA (i.e., pain and ROA) or a TJR, which is described in the supplementary material in Appendix 1 for each study individually. In addition, one study (GARP) selected cases on the basis of both clinical and radiographic OA (CROA) at two or more joint sites among hand, spine (cervical or lumbar), knee or hip.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes according to standard procedures. In RS-I, we genotyped seven tagging SNPs (tSNPs): rs3020450, rs1256031, rs1256044, rs1256061, rs1109056, rs1256064 and rs4986938 (tSNP1 until tSNP7, respectively). These SNPs were selected using the program Tagger, with force include of rs1256031 and rs4986938, incorporated in Haploview. 80% of all common genetic variation in the *ESR2* gene is covered by these 7 SNPs. In Figure 1 the genetic variation in the *ESR2* gene is depicted together with the D'and r^2 values for the 7 SNPs. We used genotype data of each of the 7 SNPs to infer frequency of



		D'						
	SNP	*tSNP1*	*tSNP2*	*tSNP3*	*tSNP4*	*tSNP5*	*tSNP6*	*tSNP7*
r^2	*tSNP1*		0.98	0.98	0.61	0.58	0.20	0.74
	tSNP2	0.41		0.99	0.98	0.44	0.60	0.95
	tSNP3	0.38	**0.91**		0.99	0.97	0.96	0.95
	tSNP4	0.21	**0.72**	**0.68**		1.00	0.28	0.89
	tSNP5	0.05	0.01	0.05	0.07		1.00	0.99
	tSNP6	0.01	0.03	0.08	0.01	0.64		0.99
	tSNP7	0.45	0.46	0.43	0.52	0.04	0.07	

Figure 1. The *ESR2* gene Linkage Disequilibrium (D') and correlation (r^2) between the tagging SNPs. Each box in the table represents D' or r^2 for the two SNPs indicated.

the haplotype alleles using the program PHASE version 2.1 (11). Haplotypes with an estimated probability <95% were excluded from analysis (387 individuals=5.9%).

The rs1256031 SNP was genotyped for the replication studies using a Taqman allelic discrimination assay (Chingford Study, SOF, GARP Study, Oxford Study, Greek cases and Spanish cases) (assay-on-demand service: www.appliedbiosystems.com) or by mass spectrometry (homogeneous Mass ARRAY system; Sequenom Inc., San Diego, CA), using standard conditions with genotypes analyzed by Genotyper 3.0 software (Sequenom Inc.)

Quality control

The allele and genotype frequencies for rs1256064 deviated slightly from HWE proportions in RS-I (P=0.04), all other SNPs were in HWE proportions (data not shown). Genotyping was repeated for a random selection of subjects (5%) to check the accuracy of the genotyping. No discrepancies were detected. In addition, the allele frequency of rs1256064 is not significantly different from that reported in the CEU

Hapmap population. No statistically significant deviations from HWE proportions could be detected for the rs1256031 SNP in the replication studies.

Statistical analysis

For the individual SNPs, allele frequencies were estimated by allele counting and Hardy-Weinberg Equilibrium (HWE) was tested using Haploview. Differences in baseline characteristics were evaluated by analysis of co-variance (ANCOVA). Odds ratios (ORs) with 95% confidence intervals (CI) were estimated with logistic regression for all the OA outcomes and were subsequently adjusted for gender, age and BMI (additive model). In RS-I, the discovery study, additive models as well as dominant and recessive models were tested. In the Oxford Study and for the Spanish cases it was not possible to adjust for age and BMI since only part of the subjects had data available for these covariates. For the GARP study P-values were adjusted for family relationships by using robust standard error analyses, using Stata SE8 software (Stata corporation, College Station, TX) (12). The meta-analysis was performed using the program Comprehensive Meta-analysis by Biostat (www.meta-analysis.com) using fixed-effects and random-effects models. Odds ratios and 95% confidence intervals of each study were used to estimate the overall effect size for the association between SNP rs1256031 and hip OA. If the heterogeneity metric I^2 exceeded 25% a random-effects model (DerSimonian and Laird) was also used for the analysis, otherwise only a fixed effects model (inverse variance method) was applied. The analyses were performed on the total population of all studies and were subsequently stratified for gender to reveal, if any, gender-specific associations. A P-value ≤ 0.05 was considered statistically significant. Unless otherwise stated, SPSS version 15.0 software (SPSS INC., Chicago, USA) was used for all analyses.

RESULTS

Baseline characteristics

In Table 1 the characteristics of the 7 studies are given. In total, there were 2,364 hip OA cases and 6,773 controls, 1,983 knee OA cases and 4,706 controls and 1,431 hand OA cases and 3,883 controls available for the meta-analysis.

Association analyses

Previously, it has been described that there is high linkage disequilibrium (LD) across the *ESR2* region and that even between different haplotype blocks within the *ESR2* gene there is high LD (13). In this study we also observed high correlations ($r^2>0.7$) between rs1256031, rs1256044 and rs1256061 and therefore results are

Table 2. Risk of OA according to rs1256031 (tSNP2) genotypes in the Rotterdam Study-I

		Women Rotterdam Study-I			Men Rotterdam Study-I		
Phenotype	*Genotype*	*Nr cases/total[1] (%)*	*OR (95% CI)[2]*	*P*	*Nr cases/total[1] (%)*	*OR (95% CI)[2]*	*P*
	TT	43/534 (8.1)			34/408 (8.3)		
Hip OA	TC	80/861 (9.3)	1.36 (1.08-1.70)	0.009	58/621 (9.3)	1.01 (0.77-1.33)	0.93
	CC	46/363 (12.7)			23/269 (8.6)		
	TT	122/477 (25.6)			60/344 (17.4)		
Knee OA	TC	272/837 (32.5)	1.09 (0.93-1.28)	0.30	83/503 (16.5)	0.85 (0.67-1.07)	0.16
	CC	98/346 (28.3)			30/233 (12.9)		
	TT	172/510 (33.7)			83/418 (19.9)		
Hand OA	TC	300/836 (35.9)	1.13 (0.97-1.30)	0.11	134/644 (20.8)	0.95 (0.78-1.15)	0.58
	CC	131/359 (36.5)			54/291 (18.6)		

OA: osteoarthritis; P: P-value; [1]Number of cases/total number of subjects with radiographs scored for the presence of OA; [2]additive model adjusted for age and BMI

Table 3. Allele and genotype frequencies of rs1256031 (tSNP2) for hip OA cases and controls

Study	**Minor allele**	**MAF %**		**N TT**		**N TC**		**N CC**	
		Case	*Control*	*Case*	*Control*	*Case*	*Control*	*Case*	*Control*
Rotterdam Study-I	C	48.6	45.5	77	865	138	1344	69	563
Chingford Study	C	48.6	44.7	68	159	118	247	61	105
GARP	C	48.1	45.1	28	228	55	339	24	157
Greek cases	C	53.1	43.0	12	83	22	128	15	47
Oxford Study	C	45.6	45.7	321	224	517	342	227	161
SOF	C	43.0	44.8	114	416	189	675	63	274
Spanish cases	C	46.5	44.7	73	137	117	186	56	93

MAF: minor allele frequency; N: number of subjects

only presented for rs1256031 (Figure 1). Of all 7 tSNPs tested, rs1256031 showed a significant association with hip OA in women of RS-I. In Table 2, risk of OA by different genotypes of rs1256031 is given for men and women of RS-I. In RS-I, an allele dose effect was observed for the C allele of rs1256031 with a 36% increased risk of hip OA in women (adjusted for age and BMI: OR1.36, 95%CI 1.08-1.70, *P*=0.009). In addition, in women a trend was observed in the same direction for hand OA (OR 1.13, 95%CI 0.97-1.30, *P*=0.11). No significant associations between rs1256031 and risk of OA were observed for men of RS-I. Haplotype analysis did not add additional information (data not shown). There were no statistical significant associations in

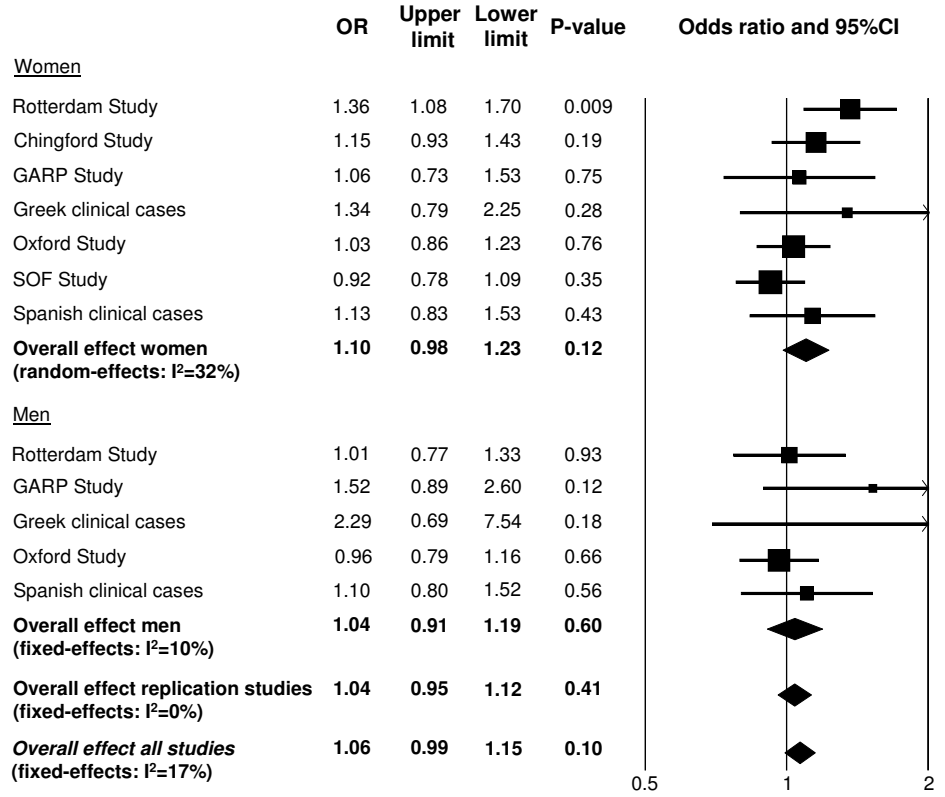


Figure 2. Forest plot for the association of tSNP2 (rs1256031) with hip OA adjusted for age and BMI. Crude odds ratios for the Oxford Study and Spanish cases (data on BMI and/or age was not available for the majority of subjects).

RS-I between common genetic variation in the *ESR2* gene and OA in a dominant or recessive model.

Replication studies were genotyped for rs1256031 since this SNP was associated with hip OA in women of the Rotterdam Study. The allele and genotype frequencies for hip OA cases and controls in each study are presented in Table 3. The association observed with hip OA in the Rotterdam Study was not supported by the replication studies. Results of the meta-analysis for hip OA are shown as a forest plot for both men and women separately and combined in Figure 2. The replication studies together showed an OR of 1.10 (95%CI 0.98-1.23, P-value 0.12) in women. The meta-analysis showed a crude OR of 1.06 (95%CI 0.99-1.15, P-value 0.09) for hip OA, OR 1.02 (95%CI 0.94-1.10, P-value 0.62) for knee OA and OR 1.03 (95%CI 0.94-1.15, P-value 0.44 (random-effects, I^2=43%)) for hand OA. Additional adjustment for age and BMI or stratification according to gender did not essentially change the results.

DISCUSSION

In this study, we showed by meta-analysis of 7 studies summarizing 2,364 hip OA cases and 6,773 controls, 1,983 knee OA cases and 4,706 controls and 1,431 hand OA cases and 3,883 controls, that common genetic variation in the *ESR2* gene is not likely to be associated with an increased risk of osteoarthritis. However, we have to note that we had 80% power to detect odds ratio's of 1.14 and therefore we cannot exclude that smaller effects may exist. As only novel data was included in this meta-analysis, the risk of publication bias is eliminated by this study.

The significant association of SNP rs1256031 and hip OA in the Rotterdam Study-I was only present in women, not in men. We have previously reported that this SNP was associated with an increased risk of vertebral and fragility fractures specifically in women (13). It was hypothesized that significant effects are not observed in men since elderly men have higher estradiol levels compared to postmenopausal women. These higher serum levels of estrogens in men may mask an impaired ESR2 signalling caused by the genetic variation and may also explain why we observed an association between SNP rs1256031 of the *ESR2* gene and hip OA only in women. However, the relationship between rs1256031 and hip OA was not supported by replication studies. Previously, Patsopoulos *et al.* showed that claims of sex-related differences in genetic association studies are most often spurious or insufficiently documented (14). Also in this study, where we initially did see a sex-specific association, replication studies could not corroborate this result. As the effect sizes in men and women are similar it is unlikely that interaction is present between the SNP and gender. This observation does not rule out a very subtle difference in the association between males and females and rs1256031 genotypes, but the current study is underpowered to robustly assess this.

The small case-control study by Fytili *et al.* (158 cases, 193 controls) reported that individuals carrying long alleles of the c.1092+3607 (CA)n repeat polymorphism of the *ESR2* gene have a 4.5-fold increased risk of knee OA. Since this repeat polymorphism was not studied in this meta-analysis and it is not known whether this repeat is in LD with rs1256031 we cannot conclude that we did or did not replicate the finding of the case-control study of Fytili and co-workers (9).

At this moment, genome-wide association studies (GWAS) are state-of-art studies to indentify novel genetic loci involved in complex diseases like OA. In the genome-wide association studies published to date, common genetic variation in the *ESR2* gene has not been found associated with OA (15-18). In addition, GWAS on bone-related traits like bone mineral density (BMD) did also not observe any genome-wide significant associations between common genetic variation in the *ESR2* gene and BMD (19-21).

In conclusion, it is not likely that there is an association between common genetic variation in the *ESR2* gene and hand, hip or knee OA although associations with very small effect sizes can not be excluded.

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Chapter 2.3

Serum C-reactive protein levels and genetic variation in the *CRP* gene are not associated with the prevalence, incidence or progression of osteoarthritis independent of body mass index

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ABSTRACT

Background: The objective was to study the relationship between serum C-Reactive Protein (CRP) levels, genetic variation in the *CRP* gene and prevalence-, incidence- and progression of radiographic osteoarthritis (ROA) in the Rotterdam Study-I (RS-I). Also, a systematic review of studies assessing the relationship between OA and CRP levels is given.

Methods: We examined 861 hand-, 718 knee-, and 349 hip OA cases and 2,806 controls of RS-I for the association between CRP levels and genetic variation in the *CRP* gene and ROA using one-way AN(C)OVA and logistic regression, respectively. PubMed was searched for articles published between January 1992-August 2009 assessing the relationship between CRP levels and OA.

Results: In RS-I prevalence of knee OA, not hip or hand OA, was associated with 14% higher serum CRP levels compared to controls (P=0.001), this association disappeared after adjustment for age and especially BMI (P=0.33). Genetic variation of the *CRP* gene was not consistently associated with prevalence, incidence or progression of OA within RS-I.
For the systematic review, we included 18 studies (including RS-I) on serum CRP levels and prevalence, incidence or progression of OA. We found consistent higher crude CRP levels in prevalent knee OA cases compared to controls. Among the studies with adjustment for BMI (n=3), no association was observed between serum CRP levels and prevalence of knee OA (meta-analysis P=0.61).

Conclusions: There is no evidence of association between serum CRP levels or genetic variation in the *CRP* gene with prevalence, incidence or progression of OA independent of BMI.

INTRODUCTION

Osteoarthritis (OA) is characterized by cartilage destruction, bone remodelling and synovitis. Although OA is not a classical inflammatory arthropathy like rheumatoid arthritis, there are indications that in OA local inflammation is present. It is known from *in vivo* and *in vitro* studies that chondrocytes can respond to chemokines and cytokines in the synovial fluid and joint tissues (1). In addition, OA patients have increased levels of catabolic enzymes and inflammatory mediators such as Interleukin-Iβ (IL-1β) and Tumor Necorsis Factor α (TNF- α) in the synovial fluid (1). For these reasons, it is believed that local inflammatory processes might play a role in the pathogenesis of OA.

C-reactive protein (CRP) is an acute phase protein, which is produced by hepatocytes and adipocytes and regulated by pro-inflammatory cytokines (2,3). CRP is an established marker for severe systemic infections, like sepsis (4). Previous studies showed that CRP levels are associated with age and with measures of obesity and cardiovascular disease (5-7).

Several studies have investigated serum CRP levels in relation to OA to determine if systemic inflammation plays a role in the pathogenesis of OA. In 1975, Acheson and Collart were the first to show that elevated serum CRP levels are associated with OA (8). In 1992 an immunoassay was developed to perform high sensitive CRP (hs-CRP) measurements, which is accurate in detecting low levels of CRP (9,10). The first study using hs-CRP measurements showed that OA patients had higher serum CRP levels compared to age-matched controls (11). Since then, several studies (n=17) have been carried out assessing the relationship betweenn prevalence, incidence and/or progression of OA and CRP levels, but with conflicting results (2, 12-27).

To examine the causal effect of a variable on disease, i.e., CRP levels on OA, the method of Mendelian randomization could be used (28). It is a method of testing for a causal relationship between CRP and OA. For example, if genetic variation in the *CRP* gene is associated with higher CRP levels, and the same variation is also related to an increased risk for OA, this suggests that CRP is involved in OA causally. So far, one study investigated the role of common genetic variation in the *CRP* gene in relation to OA (12). This study showed that genetic variation in the *CRP* gene (haplotype AAGGA, frequency 5%) was associated with higher hs-CRP levels and with an increased risk of severe hand OA (OR 2.3, 95%CI 1.2-4.3, *P*=0.009) in 42 severe hand OA cases and 70 controls.

We report data from a large population-based cohort study, the Rotterdam Study-I, (RS-I), assessing the relationship between serum CRP levels and radiographic OA (ROA). We also investigated whether common genetic variation in the *CRP* gene, known to be associated with CRP levels (29), is associated with prevalence, incidence

and/or progression of OA. In addition, we give an overview of all studies published on the relationship between serum hs-CRP levels and prevalence, incidence, and/or progression of OA. We performed a meta-analysis on the relationship between CRP levels and OA.

MATERIALS & METHODS

Association analyses of the Rotterdam Study-I

The Rotterdam Study-I (RS-I): Details of RS-I are given in Appendix 1. In total, there were 349 hip OA cases and 3,065 controls, 718 knee OA cases and 2,306 controls, 861 hand OA cases and 2,164 controls at baseline. For progression of hip OA there were 461 cases and 1,149 controls, for progression of knee OA 208 cases and 627 controls, for incidence of hip OA 220 cases and 2,519 controls and for incidence of knee OA 210 cases and 1,360 controls.

OA phenotypes in the RS-I: Radiographs were scored for the presence of a total joint replacement (TJR) and ROA of the hip, knee and hand according to the Kellgren and Lawrence (K/L) score. Knee and hip OA were defined as a K/L score ≥2 of one or both joints or a TJR. Hand OA was defined as presence of K/L scores ≥2 in 2 out of 3 hand joint groups (DIPs, PIPs, CMC1/STT) of each or both hands (31). Incidence of knee and/or hip OA was defined as a K/L<2 at baseline and TJR or K/L≥2 at follow-up. Progression of hip- and knee OA was determined in subjects which have a K/L score ≥1 at baseline. Progression of hip OA was defined as a TJR or joint space narrowing ≥1.0 mm during follow-up (32,33). Progression of knee OA was defined as a TJR or an increase in K/L score ≥1 during follow-up. Subjects with a TJR due to fracture were excluded from all analyses. Controls were free of OA at the joint site studied, but were allowed to have OA at other joint sites. For example, if knee OA was studied, controls were free of knee OA, but were allowed to have hip or hand OA. One subject could be considered a case for multiple phenotypes. For example, one subject can be a hip OA case and knee OA case.

Pain assessment: At baseline, hip, knee and hand symptoms in the previous month were assessed by interview. Hip, knee or hand pain was defined as pain in the right and/or left joint in the month preceding the interview.

CRP measurement: At baseline (1990-1993) and follow-up (1996-1999), blood was drawn by venous puncture, initially stored at –20ºC and thawed and assayed for hs-CRP using Rate Near Infrared Particle Immunoassay (Immage® Immunochemistry System, Beckman Coulter, USA). This method can accurately measure protein concentrations from 0.2 to 1440 mg/l, with a within-run precision <5.0%, a total precision <7.5% and a reliability coefficient of 0.995 (34).

Genotyping methods: Genomic DNA was extracted from peripheral venous blood samples according to standard procedures using salting-out and phenol extraction methods. Genotypes were determined using the Taqman allelic discrimination assay. The Assay-by-Design service (www.appliedbiosystems.com) was used to set up a Taqman allelic discrimination assay for three tagging SNPs. The primers and probes used are available on request and conditions of the assay were as previously described (35). The selection of the three haplotype tagging single nucleotide polymorphism (SNP) using the Seattle SNPs Program for Genomic Applications (http://pga.gs.washington.edu/) has been described elsewhere in detail (34). In short, the SNPs rs1130864(C>T), rs1205(C>T) and rs3093068(C>G) were selected to estimate haplotypes on the basis of their presence in existing literature and on their proximity to the *CRP* gene. We used genotype data of each of the 3 SNPs to infer frequency of the haplotype alleles present in the population using the program PHASE version 2.1 (36). Haplotypes with an estimated probability <95% were excluded from the analyses. In total, 4 haplotypes were constructed and were coded as the numbers 1 through 4 in order of descending frequency in the population: haplotype 1=CTC, 2=TCC, 3=CCC, 4=CCG.

Statistical analyses: Analyses were carried out only in subjects with a CRP level <10 mg/l, thereby excluding 311 subjects with possible acute inflammatory conditions. All analyses were performed using natural log-transformed hs-CRP levels since the distribution was not normal in the population. After transformation the distribution of CRP levels was normal in the population (data not shown). The CRP levels in the results section and the tables are shown as means. The associations between baseline characteristics of RS-I and OA phenotypes were assessed using one-way AN(C)OVA. Association analyses of CRP levels with hip, knee and hand OA (prevalence, incidence, progression), with pain within OA cases and with haplotypes were performed using one-way AN(C)OVA and, if applicable, linear regression. In addition, to detect possible threshold effects, sex-specific groups were created using tertiles and the median as cut-off for CRP levels. One-way AN(C)OVA was also used to assess the relationship between delta CRP levels (log-transformed) and incidence or progression of knee or hip OA (mean follow-up time 6.3 years). A logistic regression was performed to assess the relationship between CRP tertiles/median and OA. This logistic regression was subsequently adjusted for age and body mass index (BMI) and sex-specific age and BMI adjusted tertile/median groups were created to perform a logistic regression fully adjusted for gender, age and BMI. To study the relationship between *CRP* haplotypes and OA, a logistic regression model was applied. All analyses were performed crude and adjusted for gender, age and BMI. In addition, the association analyses for incidence/progression of OA were also adjusted for follow-up time. Also, adjustment for usage of analgesics and/or anti-inflammatory drugs and/or coronary heart disease (defined as myocardial infarction, coronary artery bypass

grafting or percutaneous transluminal coronary angioplasty) was performed, but only reported in the results section if this adjustment changed the results. To adjust for multiple testing we performed a Bonferroni correction after which the threshold for statistical significance was set at $P<8x10^{-4}$ (0.05/60 tests). For the individual SNPs, allele frequencies were estimated by allele counting and Hardy-Weinberg Equilibrium (HWE) was tested using Haploview 4.0. All analyses were performed using SPSS 15.0 for Windows.

Literature Study

Identification of studies: Relevant articles were identified by a systematic search using the database of PubMed with ["CRP" or "C-reactive protein"] and ["osteoarthritis" or "OA"] as keywords. Subsequently, the search was extended by screening reference lists of the included studies for review. The following inclusion criteria applied for this review: a) listed in PubMed, b) publication date between January 1992 and August 2009, c) publication in the English language, d) study in humans, e) the article represents original data, f) the disease of interest is OA, g) subjects with and without OA are compared in the study, h) the study reports on hs-CRP measurements in serum, i) the study investigates the relationship between OA and CRP levels presenting odds ratio's (ORs), difference in median or mean, j) the full-text article was available.

Methodological quality assessment: All studies were scored for the presence of the following criteria: 1) information on recruitment of cases, 2) information on recruitment of controls, 3) size of the study (n>100 cases or controls), 4) information for all subjects on age, gender and BMI, 5) description of CRP measurement, 6) OA definition according to American College of Rheumatology (ACR) criteria, a total joint replacement due to primary OA, the K/L score or Croft classification system, 7) clear description of statistical methods, 8) CRP levels were log-transformed if not following a normal distribution, 9) adjustments were made for age, gender and BMI in the analyses, 10) information on exclusion of subjects with a state of acute inflammation (i.e., subjects with CRP>20 mg/l), 11) results are presented as either mean, tertiles or median with P-values or ORs with 95% confidence limits. Positive scores were summed up to indicate an overall internal validity score with a maximum of 100% if all items were scored positive. A study was considered to be of high quality if the methodological score was ≥ 60% (37).

Meta-analysis: For meta-analyses purposes the program comprehensive meta-analysis by Biostat (www.meta-analysis.com) was used. If heterogeneity existed (I^2>25%) a random-effects model (DerSimonian and Laird) was used for the analysis, otherwise a fixed effects model (inverse variance method) was applied.

RESULTS

Results of the Rotterdam Study-I

Serum CRP levels and OA: In Table 1 the baseline characteristics of RS-I are shown. Overall, women more frequently had OA (3-18%) compared to men (with the exception of prevalent hip OA), they were 2-4 years older (with the exception of incident or progressive knee OA) and had 1-2 units higher BMI (with the exception of prevalent hip OA). Table 2 shows the results of the association of CRP levels and prevalence, incidence and progression of OA in RS-I. Subjects with prevalent knee OA had 14% higher CRP levels compared to control subjects (*P*=0.001). After additional adjustment for age and gender, the association remained significant with a P-value of 0.01. After adjustment for BMI the association disappeared and the P-value was 0.33.

Of all association analyses performed on CRP levels and incidence or progression of hip or knee OA and on prevalence of hip or hand OA, only incidence of hip OA was associated with 13% higher CRP levels (*P*=0.04 adjusted for age, gender, BMI and follow-up time). Yet, after adjustment for multiple testing this result did not remain significant (a P-value $<8x10^{-4}$ was considered statistically significant after Bonferroni correction). In addition, when OA prevalence, incidence and progression were studied across CRP tertiles and median to reveal a possible threshold effect, we did not observe any differences (data not shown).

Longitudinal change in serum CRP and OA: Change in serum CRP levels between baseline measurements and 5 years of follow-up was not associated with incidence or progression of knee or hip OA (Table 3). Additional adjustment for usage of anti-inflammatory drugs or pain killers did not change the results (data not shown).

Serum CRP levels and pain in OA cases: Subjects with radiographic OA with pain did not have higher or lower CRP levels compared to ROA subjects without pain. These results did not change after adjustment for age, gender, BMI, usage of pain killers and/or anti-inflammatory drugs. Subjects with hip OA had 4% lower CRP levels if they experienced pain compared to subjects without pain (*P*=0.84), for knee OA this percentage was 8% (*P*=0.25) and for hand OA 3% (*P*=0.69) (Figure 1).

Genetic variation in the CRP gene and OA: Genotype distributions of the three haplotype tagging SNPs were in HWE. Haplotype alleles were present with the following frequencies: haplotype 1 (CTC) in 32.8%; haplotype 2 (TCC) in 31.7%; haplotype 3 (CCC) in 29.5% and haplotype 4 (CCG) in 5.9%.

Genetic variation of the *CRP* gene was strongly and consistently associated with serum CRP levels (34), but no consistent associations were observed with prevalence, incidence or progression of OA (data not shown).

Table 1. Baseline characteristics of RS-I

OA phenotype		N	% women		Age		BMI	
			%	*P-value*	*Mean (sd)*	*P-value*	*Mean (sd)*	*P-value*
Prevalent knee OA	Controls	2306	56%	$5x10^{-18}$	67.7 (7.6)	$2x10^{-36}$	25.9 (3.4)	$2x10^{-39}$
	Cases	718	74%		71.9 (8.3)		27.9 (4.1)	
Incident knee OA	Controls	1360	54%	$1x10^{-5}$	65.8 (6.5)	0.44	26.0 (3.4)	$1x10^{-6}$
	Cases	210	70%		66.1 (6.7)		27.3 (3.7)	
Progression knee OA	Controls	627	61%	0.005	67.0 (7.0)	0.79	26.7 (3.6)	$3x10^{-5}$
	Cases	208	72%		66.9 (6.9)		28.0 (4.1)	
Prevalent hip OA	Controls	3065	58%	0.24	66.0 (7.0)	$5x10^{-22}$	26.2 (3.6)	0.23
	Cases	349	61%		69.9 (7.9)		26.5 (3.6)	
Incident hip OA	Controls	2519	56%	$2x10^{-4}$	65.4 (6.5)	$7x10^{-5}$	26.3 (3.5)	0.045
	Cases	220	69%		67.3 (6.6)		26.8 (3.7)	
Progression hip OA	Controls	1149	50%	$4x10^{-5}$	65.4 (6.6)	$2x10^{-5}$	26.1 (3.3)	0.02
	Cases	461	61%		67.0 (6.8)		26.6 (3.8)	
Prevalent hand OA	Controls	2164	52%	$1x10^{-17}$	64.9 (6.7)	$1x10^{-49}$	26.1 (3.5)	$6x10^{-9}$
	Cases	861	69%		69.0 (6.9)		26.9 (3.6)	

P-value one-way ANOVA; OA: osteoarthritis; n: number of subjects; sd: standard deviation; BMI: body mass index (kg/m^2)

Table 2. Association between CRP levels and knee, hip, and hand osteoarthritis (OA) in RS-I

	Disease status	N	CRP levels	Difference[1]	*P*[2]	CRP levels adjusted	Difference[1]	*P* adjusted[2]
Prevalent knee OA	Absent	2306	1.53	14%	0.001	1.59	-4%	0.33
	Present	718	1.74			1.53		
Incident knee OA	Absent	1360	1.43	1%	0.92	1.44	-3%	0.63
	Present	210	1.44			1.39		
Progression knee OA	Absent	627	1.45	3%	0.61	1.47	-3%	0.61
	Present	208	1.50			1.42		
Prevalent hip OA	Absent	3065	1.48	5%	0.30	1.49	0%	0.99
	Present	349	1.56			1.49		
Incident hip OA	Absent	2519	1.45	14%	0.04	1.45	13%	0.04
	Present	220	1.66			1.64		
Progression hip OA	Absent	1149	1.46	-2%	0.65	1.47	-4%	0.42
	Present	461	1.43			1.41		
Prevalent hand OA	Absent	2164	1.48	5%	0.12	1.52	-4%	0.92
	Present	861	1.56			1.46		

CRP levels presented as means; *P*: P-value; [1]difference = CRP level cases/CRP level controls; [2]P-value is calculated using AN(C)OVA and is subsequently adjusted for age, gender and BMI; adjustment for follow-up time for analysis involving incident or progression of OA

Table 3. Change in serum CRP levels and incidence and progression of knee and hip OA in RS-I during 5 years of follow-up

	Disease status	N	ΔCRP levels	*P*[1]	ΔCRP levels adjusted	*P* adjusted[1]
Incident knee OA	Absent	1135	+1.59	0.87	1.59	0.92
	Present	93	+1.56		1.57	
Progression knee OA	Absent	588	+1.53	0.92	1.53	0.92
	Present	147	+1.52		1.52	
Incident hip OA	Absent	2261	+1.53	0.80	1.53	0.73
	present	188	+1.56		1.57	
Progression hip OA	Absent	1100	+1.54	0.93	1.54	0.99
	Present	283	+1.53		1.54	

CRP levels presented as means; [1]P-value is calculated using AN(C)OVA and is subsequently adjusted for age, gender, BMI and follow-up time

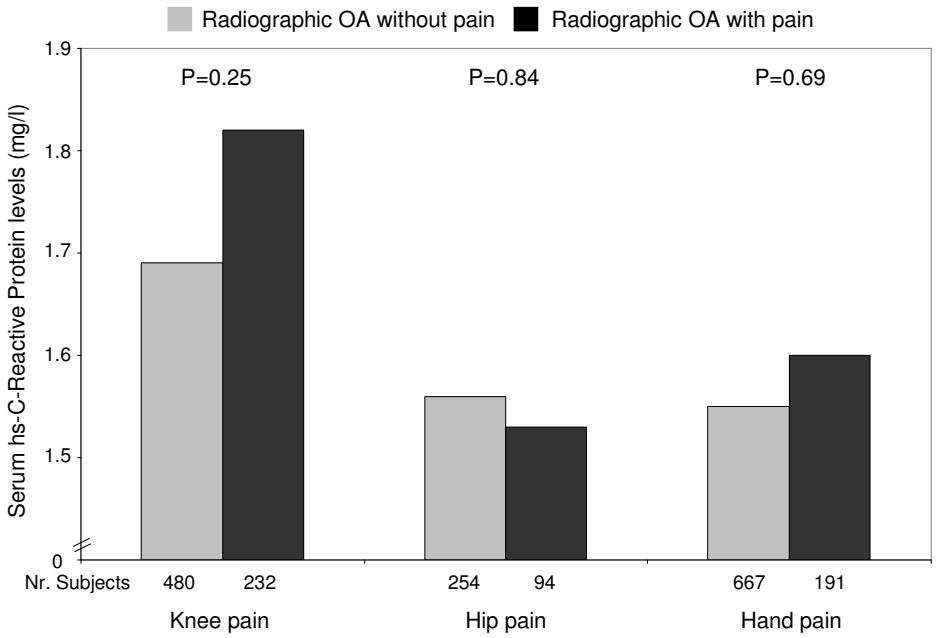

Figure 1. Association between hs-CRP levels and pain in radiographic OA subjects (adjusted for gender, age and BMI).

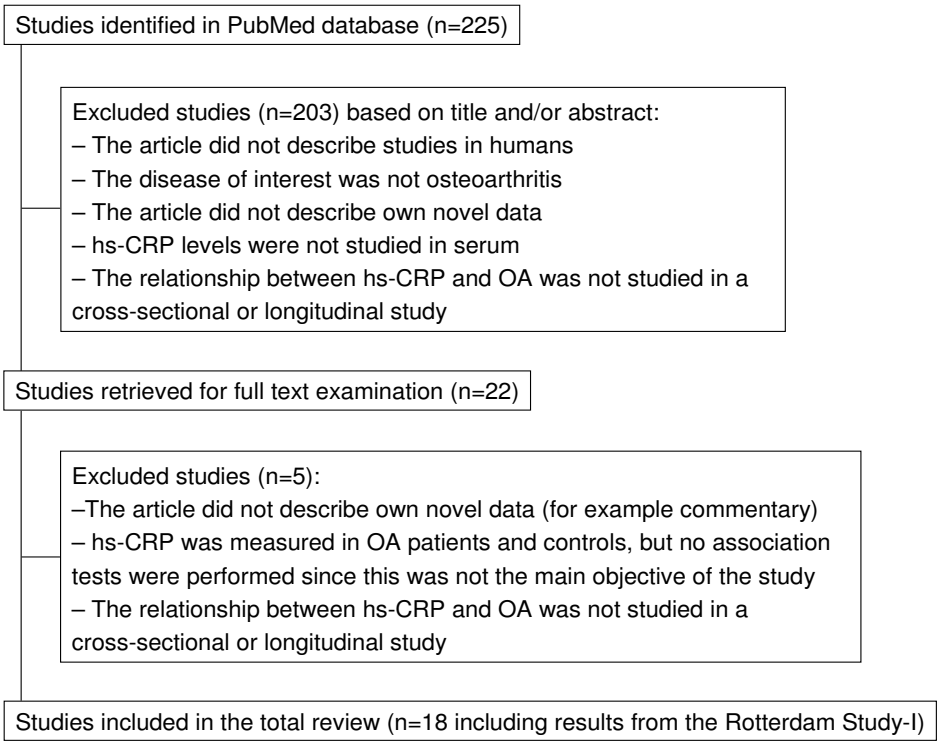

Figure 2. Flow chart of the selection process.

Results of the literature study

In total, there were 225 studies identified in the database, of which 203 studies were excluded on the basis of the title and/or abstract. Of the 22 studies retrieved for full examination, 5 studies were excluded because they did not fulfil the inclusion criteria after reading the complete manuscript. Finally, 18 studies (17 from the literature search + results from RS-I) were included in the review. In Figure 2, the flow-chart of the selection process is depicted.

Methodological quality assessment: In Table 4 the 11 aspects of quality of the studies as mentioned in the methods section are given. In summary, there were 2 studies without complete information on recruitment of both cases and controls (21,22). For example, the criteria were given for subjects to be considered a case, but the source population of recruitment and the method of recruitment were not given. Many studies (n=10) were small and included less than 100 cases or controls. All studies provided detailed information on the statistical methods used within their study. In total 5 out of 18 studies performed adjustments for age, gender and BMI (15,19,20,24, RS-I). A study was considered of high quality if the methodological

Table 4. Results of the methodological quality assessment of the articles included in this review

Reference	Recruitment clearly described		Study size[1]	Definition of OA[2]	Information on age, gender and BMI	Adjustment for gender, age and BMI	Description CRP measurement	Exclusion acute inflammation	Log transformation	Statistical methods clearly described	Results shown[3]	Methodological score
	Cases	Controls										
Bos et al.(11)	+	+	+	+	+	-	-	-	+	+	+[4]	73%
Chen et al.(12)	+	+	-	+	+	-	+	-	+	+	+	73%
Conrozier et al.(13)	+	-	-	+	-	-	+	+	+	+	+	64%
Conrozier et al.(14)	+	+	-	+	+	-	+	+	+	+	+	82%
Engström et al.(15)	+	+	+	+	+	+	+	-	+	+	+[5]	91%
Garnero et al.(16)	+	+	-	+	+	-	+	-	+	+	+	73%
Hulejová et al.(17)	+	-	-	+	-	-	+	+	-	+	+	55%
Kerkhof et al.	+	+	+	+	+	+	+	+	+	+	+	100%
Kraus et al.(18)	+	+	+	+	+	+	+	-	+	+	+	91%
Mazières et al.(19)	+	+	+	+	+	+	+	-	+	+	+	91%
Melikoglu et al.(20)	-	-	-	+	-	-	+	-	-	+	+	36%
Otterness et al.(21)	-	-	-	+	-	-	+	-	+	+	+	45%
Pearle et al.(2)	+	-	-	+	+	-	+	-	-	+	+	55%
Sharif et al.(22)	+	-	-	+	-	-	+	-	+	+	+	55%
Sharif et al.(23)	+	+	-	+	+	+	+	-	+	+	+	81%
Sowers et al.(24)	+	+	+	+	+	-	+	-	+	+	+	81%
Spector et al.(25)	+	+	+	+	+	-	+	+	+	+	+	91%
Stürmer et al.(26)	+	+	+	+	+	-	+	-	+	+	+	81%

A positive score implies that complete information is available; - implies that information is not completely available or the feature was not applied (i.e. for adjustments for age/gender/BMI this was either not applied or information was incomplete in the paper); [1]positive if > 100 cases or controls; [2]standard criteria are: OA defined as a K/L >=2, Croft grade >= 1, ACR criteria or total joint replacement; [3]results shown as mean (P-value) or tertiles/median (odds ratio); [4]only P-value is given; [5]CRP >=3 mg/l versus CRP < 1 mg/l

Table 5. Results of the included studies on CRP levels and prevalence of OA

Reference	Joint	Nr cases	Nr controls	Statistical analysis	Outcome (crude)		Outcome (BMI adjusted)	
					Difference in CRP (cases vs controls)[1]	*P-value*	*Difference in CRP (cases vs controls)*[1]	*P-value*
Bos et al.(12)	Knee	-	739	Linear mixed model	-	0.06	-	>0.05
	Hand	103	739		-	>0.05	-	>0.05
Chen et al.(13)	Hand	36	45	One-way ANOVA	8.26 vs 7.68	< 0.05	-	-
Conrozier et al.(14)	Hip	45	33	Mann Withney *u* test	2.93 vs 1.40	0.006	-	-
Garnero et al.(15)	Knee	67	67	Student's unpaired t-test	3.03 vs 2.50	0.12	-	0.76
Hulejová et al.(18)	Hip	55	30	Mann Withney *u* test	9.8 vs 1.2[2]	< 0.001	-	-
Kerkhof et al. (current study)	Knee	718	2,306	Linear regression/AN(C)OVA	1.74 vs 1.53	0.001	1.53 vs 1.59	0.33
	Hip	349	3,065		1.56 vs 1.48	0.30	1.49 vs 1.49	0.99
	Hand	861	2,164		1.56 vs 1.48	0.12	1.46 vs 1.52	0.92
Kraus et al.(19)	Knee or hip	386	276	One-way AN(C)OVA	5.88 vs 3.38	<0.05	-	0.07
Melikoglu et al.(21)	Knee	115	30	Independent sample t test	4.0 vs 4.0[2]	> 0.05	-	-
Otterness et al.(22)	Knee or hip	39	20	Wilcoxon Rank test	5.03 vs 1.64	0.0004	-	-
Pearle et al.(2)	Knee or hip	52	-	Mann Withney *u* test	3.40 vs 0.29[2]	0.007	-	-
Sharif et al.(23)	Knee	167	51	Student's unpaired t-test	3.25 vs 0.93	<0.0001	-	-
Sowers et al.(25)	Knee	122	903	One-way ANOVA	4.82 vs 2.09	<0.0001	-	-
Spector et al.(26)	Knee	105	740	Mann Withney *u* test + ANOVA	2.4 vs 0.7[3]	<0.001	-	-
Stürmer et al.(27)	Knee	368	567	Linear regression model	2.5 vs 1.7	<0.0001[4]	-	-
	Hip	402	567		2.5 vs 1.7		-	-

- implies that information is not completely available or the feature was not applied; [1]difference in CRP mean is shown (mg/l) unless indicated otherwise; Adj.: adjustment; BMI: body mass index; [2]CRP geometric mean is given (mg/l); [3]difference in median CRP is given; [4]hip or knee OA cases compared with controls

Figure 3. Forest plot for the association between serum hs-CRP levels and knee OA adjusted for BMI.

score was ≥ 60% and this applied to 13 out of 18 studies (72%). Five studies were of less quality with a methodological score ranging from 36-55% (2,18,21-23).

Association between CRP and prevalence of OA: The characteristics of the 14 studies assessing the relationship between serum CRP levels and prevalence of OA are shown in Supplementary Table 1 in Appendix 2 (2,12-14,17-19,21-23,25-27,RS-I). Combining all cases and controls there were in total 3,990 OA cases and 6,566 controls. In Table 5 the results are given for the relationship between CRP levels and prevalent OA of these 14 studies. Mean CRP levels in OA cases range from 1.6 to 9.8 mg/l and in controls from 0.9 to 7.7 mg/l. Statistically significant ($P<0.05$) results were observed in 10 out of 14 studies and they showed that subjects with OA (hip, knee or hand) have higher levels of CRP compared to controls (13,14,18,19,22,23,25-27,RS-I). For hip and hand only few studies were performed, which prevented us from drawing firm conclusions. For knee OA 11 studies were performed. Results are indicative of a positive relation between crude serum CRP levels and knee OA, since only three small studies do not show a significant association ($P>0.05$) whilst all other studies, including the two largest studies, did show a positive association between CRP levels and knee OA unadjusted for BMI (RS-I,19).

A meta-analysis could not be performed on the relationship between crude CRP levels and knee, hip or hand OA since 9 out of 14 studies report inexact P-values or geometric means are not provided. A meta-analysis assessing the relationship between CRP levels adjusted for BMI and hand- or hip OA could not be performed since there was only one study which had this information available (RS-I). We did perform a meta-analysis on knee OA and CRP levels adjusted for BMI, which included 3 studies with in total 1,171 cases and 2,649 controls (RS-I,17,19). The meta-analysis did not provide evidence of association of serum CRP levels and knee OA independent of BMI (random-effects P-value=0.61, I^2=54%) (Figure 3).

Association between CRP and incidence or progression of OA: The review included 7 studies which investigated the relation between CRP levels and incidence and/or progression of OA including results of RS-I (Supplementary Table 2 in Appendix 2) (15,16,20,24-26). The results of the studies on CRP levels and incidence or

Table 6. Results of the included studies on CRP levels incidence/progression of OA

Reference	OA outcome	Nr cases	Nr controls	Statistical analysis	Outcome (crude)		Outcome (BMI adjusted)	
					Difference in CRP (cases vs controls)[1]	*P-value*	*Difference in CRP (cases vs controls)*[1]	*P-value*
Conrozier et al.(15)	Progression hip	10	23	Mann Withney *u* test	5.61 vs 1.94[2]	0.01	-	-
Engström et al.(16)	Incident knee	89	5082	Cox regression model	1.62 vs 1.40	> 0.05	-	>0.05
	Incident hip	120	5044		1.64 vs 1.40	> 0.05	-	>0.05
Kerkhof et al. (current study)	Incident knee	210	1360	Logistic regression	1.44 vs 1.43	0.92	1.39 vs 1.44	0.63
	Incident hip	220	2519		1.66 vs 1.45	0.04	1.64 vs 1.45	0.04
	Progression knee	208	627		1.50 vs 1.45	0.61	1.42 vs 1.47	0.61
	Progression hip	461	1149		1.43 vs 1.46	0.65	1.41 vs 1.47	0.42
Mazières et al.(20)	Progression hip	200	133	Cox regression model	-	-	0.78 (0.57-1.08)[3]	0.13
Sharif et al.(24)	Progression knee	38	52	Logistic regression	-	-	1.90 (1.01-3.28)[4]	0.04
Sowers et al.(25)	Incident knee	83	942	One-way ANOVA	4.31 vs 2.04	< 0.0001	-	-
Spector et al.(26)	Progression knee	31	39	Mann Withney *u* test + ANOVA	2.6 vs 1.3[2]	0.006	-	-

A positive score implies that complete information is available; - implies that information is not completely available or the feature was not applied; [1]difference in mean (mg/l) or OR with 95%CI unless indicated otherwise; BMI = body mass index; Adj: adjustment; [2]CRP medians compared between cases and controls; [3]upper CRP tertile compared with the two lower CRP tertiles; [4]CRP levels as independent continuous variable in logistic regression with OA progression as outcome

progression of OA are given in Table 6. A meta-analysis could not be performed for these studies since results of the studies are shown in different ways, i.e., difference in mean or ORs using CRP levels as continuous variable or with a threshold of 3 mg/l. In addition, different OA outcomes were assessed; progression of hip or knee OA and incidence of hip or knee OA. Five out of 7 studies report statistically significant differences in crude CRP levels in relation to incidence or progression of OA (15,24-26, RS-I), of which only 2 studies adjusted for age and BMI (23, RS-I). The study of Sharif *et al*. on CRP levels and progression of knee OA showed higher CRP levels in subjects with progression of knee OA compared to controls (OR 1.90, 95%CI 1.01-3.28, *P*=0.04) (24). This was not supported by the 6 times larger dataset of RS-I which showed slightly lower levels of CRP in progression of knee OA cases compared to controls after adjustment for gender, age, BMI and follow-up time (*P*=0.61). In RS-I subjects with incidence of hip OA had 13% higher serum CRP levels compared to controls (*P*=0.04 after adjustment for age, gender, BMI and follow-up time), but this

was not supported by data of Engström *et al*. which showed an OR of 1.1 (95%CI 0.6-1.9) for subjects with a CRP level ≥ 3mg/l versus subjects with CRP <1 mg/l (16).

DISCUSSION

In the Rotterdam Study-I (RS-I), the largest study published to date on serum CRP levels and OA, we showed an association between knee OA and serum CRP levels similar as in previously published studies, but this association was fully driven by BMI (2,18,19,22,23,25-27). In addition, a meta-analysis including 3 studies on serum CRP levels adjusted for BMI and knee OA, did not show evidence of an association (*P*=0.61). Although we could not perform a meta-analysis on the crude CRP levels in relation to knee OA, a consistent positive association between CRP levels and knee OA was seen in 8 out of 11 studies. However, this association is mediated through BMI and highlights the complex interaction between BMI and/or body fat, CRP and OA as is also discussed in a paper by Engstrom *et al*. (16).

Within RS-I, an association between CRP levels and incidence of hip OA (*P*=0.04) was observed, which remained significant after adjustment for age, gender, BMI and follow-up time. However, this association did not remain significant after Bonferroni correction and was also not observed in the study of Engström *et al*. (16). We therefore believe that this result is false-positive due to multiple testing. In addition, also no association was observed between CRP levels and pain or between change in CRP levels and incidence or progression of knee or hip OA during 5 years of follow-up. This result provides further evidence that serum hs-CRP is not a marker for acute inflammation in OA and that acute systemic inflammation is not present in OA.

One of the limitations of this study is the fact that there were only 3 studies included in the meta-analysis on knee OA and serum CRP levels adjusted for age and BMI. Although these 3 studies did not provide evidence of an association between serum CRP levels and knee OA independent of BMI, a larger meta-analysis might strengthen this conclusion even more. The presence of pain is assessed in the Rotterdam Study by a questionnaire. More precise measurement for pain, such as the WOMAC pain score or the use of the VAS scale were not performed. The conclusions concerning the analysis of CRP levels and pain in ROA cases should therefore be taken with caution.

Genetic analysis in RS-I revealed that genetic variation in the *CRP* gene was not consistently associated with CRP levels. One can identify a causal relationship in observational studies using the method of Mendelian randomization (28). If common genetic variation in a gene is associated with your intermediate phenotype, which are CRP levels in this study, and genetic variation is also associated with OA in the same

direction as the association between the genetic variation and CRP levels, you can assume there is a causal relationship between CRP levels and OA. In this study we did not observe evidence of such a causal relationship between CRP levels and OA.

There was a large difference in mean CRP levels measured in the different studies. In control subjects the mean CRP levels ranged from 0.9 to 7.7 mg/l and in cases from 1.6 to 9.8 mg/l. This could be due to different techniques used to measure CRP or the fact that in some studies subjects with CRP levels above a certain level are excluded since this might be an indication of an acute inflammatory disease while other studies did not make such exclusions.

Although CRP is studied in relation to OA very often, we cannot provide evidence of a robust association independent of BMI. A recent paper showed that for most study designs and settings, it is more likely for a research claim to be false than true (38). It was also argued that studies are more likely to be false when the studies conducted are smaller, when effect sizes are smaller, and when there is great flexibility in definitions and analytical methods. For all studies performed on CRP and OA at least some of these characteristics apply and in our opinion, the message of this paper could also be applied to the studies conducted on serum CRP levels and OA.

In conclusion, results of RS-I, the largest cohort study up to now to study the relationship between serum CRP levels and genetic variation in the *CRP* gene in relation to prevalence, incidence and progression of OA, does not provide evidence of association between CRP levels and prevalence, incidence, or progression of OA independent of BMI. These results are corroborated by the systematic review in which we did not find evidence to support an independent effect of CRP levels on OA. In our opinion, future studies should focus more on local inflammation processes involved in OA rather than on systemic inflammation.

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Chapter 2.4

Large scale meta-analysis of interleukin-1 beta and interleukin-1 receptor antagonist polymorphisms on risk of radiographic hip and knee osteoarthritis and severity of knee osteoarthritis

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ABSTRACT

Background: The objective of this study was to clarify the role of common genetic variation in the Interleukin-1β (*IL1B*) and Interleukin-1R antagonist (*IL1RN*) genes on risk of knee and hip osteoarthritis (OA) and severity of knee OA by means of large-scale meta-analyses.

Methods: We searched PubMed for articles assessing the role of *IL1B* and *IL1RN* polymorphisms/haplotypes on the risk of hip and/or knee OA. Novel data was included from 8 unpublished studies. Meta-analyses were performed using fixed- and random-effects models with a total of 3,595 hip OA and 5,013 knee OA cases, and 6,559 and 9,132 controls, respectively. The role of *ILRN* haplotypes on radiographic severity of knee OA was tested in 1,918 cases with Kellgren-Lawrence (K/L) 1 or 2 compared to 199 cases with K/L 3 or 4.

Results: The meta-analysis of 6 published studies retrieved from the literature search and 8 unpublished studies showed no evidence of association between common genetic variation in the *IL1B* or *IL1RN* genes and risk of hip OA or knee OA ($P>0.05$ for rs16944, rs1143634, rs419598 and haplotype C-G-C (rs1143634, rs16944 and rs419598) previously implicated in risk of hip OA). The C-T-A haplotype formed by rs419598, rs315952 and rs9005, previously implicated in radiographic severity of knee OA, was associated with reduced severity of knee OA (OR=0.71 95%CI 0.56-0.91; $P=0.006$, $I^2=74\%$), and achieved borderline statistical significance in a random effects model (OR=0.61 95%CI 0.35-1.06 $P=0.08$).

Conclusions: Common genetic variation in the Interleukin-1 region is not associated with prevalence of hip or knee OA but our data suggest that *IL1RN* might have a role in severity of knee OA.

INTRODUCTION

Osteoarthritis (OA) is a multifactorial disease of the musculoskeletal system primarily involving the joints of the knee, hip, hand and spine. The prevalence of OA increases with age and is estimated to affect 40% of people over the age of 70 years (1). There is a large body of evidence that synovial inflammation is implicated in many of the signs and symptoms of OA, including joint swelling and effusion (2). This synovitis is cytokine-driven and there is convincing evidence that chondrocytes contribute to cytokine production leading to cartilage matrix degradation and in fact a number of variants in genes encoding for cytokines, in particular Interleukin-1 (*IL-1*), *IL-6* and *IL-10* involved in inflammation, have been reported to be associated with risk of OA as shown in a recent review (3-4). Chondrocytes are known to respond to IL-1 beta and alpha (IL1B, IL1A) by decreasing synthesis of matrix components and increasing synthesis of matrix metalloproteinases (3). The IL-1 receptor antagonist (*IL1RN* gene) could antagonise the effects of both IL-1 alpha and beta. In addition, it was recently shown that carriers of the *IL1RN* C-T-A haplotype had significantly lower synovial fluid levels of IL-10 and trends towards lower levels of IL-6 and IL1B (5). It is therefore expected that carriers of this haplotype are able to antagonise the effects of IL-1 and therefore reduce the risk of OA.

Several studies have investigated the role of polymorphisms in the *IL-1* gene on knee and hip OA, in particular *IL1B* C+3954T (rs1143634), *IL1B* A-511G (rs16944) and the *IL1RN* 86bp intron 2 variable number tandem repeat (VNTR) (tagged by rs419598), but results are conflicting (6-15). One haplotype, C-G-C (rs1143634, rs16944 and rs419598), was associated with an increased risk of hip OA in 2 studies (in total 144 cases and 1,501 controls) (10, 14). However, this could not be replicated by another study (370 cases, 544 controls) (6). Recently, a small meta-analysis (n=1,238 hip, knee and hand OA cases and 1,260 controls) has been published on the Interleukin-1 region and OA, but remained inconclusive (16). In that meta-analysis, some studies (n=4) with data available on allele frequencies of single nucleotide polymorphisms (SNPs) rs16944, rs419598 or rs1143634 (or SNPs/VNTR in linkage disequilibrium with these 3 SNPs) and knee and/or hip OA data were not included in the final analysis.

In 2009, Attur and colleagues explored the role of *IL1RN* variants on radiographic severity (5). It was shown in two studies (n total=130) that carriers of the C-T-A haplotype (rs419598/rs315952/rs9005) had a significantly decreased risk for severe knee OA (OR 0.14, 95%CI 0.05-0.37, $P<0.0001$ for the haplotype analysis). The CC/CT genotype at rs419598, was also reported in the same study to be significantly associated with radiographic severity (OR 0.22 95%CI 0.10-0.49).

Our scope was to clarify the role of rs1143634, rs16944 and rs419598 *IL1B* and *IL1RN* polymorphisms on risk of knee and hip OA. To do so, we have carried out a large meta-analysis of both published data (n=6) and unpublished new studies (n=8), comprising a total 3,595 hip OA cases and 6,559 controls, and 5,013 knee OA cases and 9,132 controls. Because one of these variants has also been implicated in severity of knee OA, a meta-analysis on severity of knee OA with rs419598 was also carried out in 8 new studies plus the original report. To detect association between severity of knee OA and the C-T-A haplotype, 3 studies and the original report were meta-analysed.

MATERIALS & METHODS

Study populations: unpublished studies with novel data

Descriptions of the Chingford Study, Estonian Cohort Study (ECS), the Genetics OsteoArthritis and Progression (GARP) Study, Hertfordshire Cohort Study (HCS), Nottingham Case-Control Study (NCCS), Rotterdam Study-I (RS-I) and III (RS-III) and the TwinsUK Study are given in Appendix 1.

In the Chingford Study, GARP Study, RS-I, RS-III, TwinsUK Study, hip OA was defined as definite joint space narrowing (JSN) and knee OA as at least one definite osteophyte and definite JSN or at least two definite osteophytes. Severe hip OA and knee OA were defined as a K/L score ≥ 3 or a total joint replacement (TJR). In the HCS knee OA was defined as K/L$\geq$2 at the tibiofemoral compartment. Cases in the NCCS are clinical patients which have been referred to the hospital with symptomatic, clinically severe hip or knee OA and the majority had undergone unilateral or bilateral THR or TKR within the previous 5 years.

In this meta-analysis, 5 studies are available with data on common genetic variation in the IL-1 region for hip OA and 8 studies for knee OA. The baseline characteristics and sample size of these studies are shown in Table 1a. In total 7 cohort studies originating from 3 countries were included. In addition, severity of knee OA was studied in 7 of these studies and in one additional study (CS, GARP, HCS, NCCS, RSI, RSIII, TwinsUK and GOAL).

Study populations: published studies

We searched PubMed for relevant articles assessing the relationship between genetic variation in the Interleukin-1 region and knee and hip OA. In Table 1b the baseline characteristics and sample size of 6 studies identified by our search are given. Since not all studies published allele and/or haplotype counts we contacted the authors if necessary to obtain haplotype and allele counts to perform a meta-analysis with a

Table 1a. Baseline characteristics of unpublished studies assessing the relationship between common genetic variation in the Il-1 region and risk of hip and knee OA

Study		Chingford Study	Estonia Cohort	GARP Study	Hertfordshire Cohort Study	Nottingham Case-Control Study	Rotterdam Study-I	Rotterdam Study-III	TwinsUK	Total
Study characteristics	Type of study	cohort	cohort	cohort	cohort	case-control	cohort	cohort	cohort	
	Origin	UK	Estonia	Netherlands	UK	UK	Netherlands	Netherlands	UK	
Controls	Definition	No ROA	No ROA	-	No ROA	No ROA & no symptoms	No ROA	No ROA	No ROA	
	Number[1]	547/671	430	-[2]	772	750	2115/2777	1514	708/722	**6836/ 4920**
	Age mean (range)	63.6 (54-76)[3]	46.5 (32-59)	-	64.9 (59-71)	66.4 (43-93)	66.1 (55-89)	55.8 (46-89)	52.0 (32-70)	
	BMI mean (range)	24.8 (17-42)[3]	27.7 (15-45)	-	26.6 (17-45)	26.6 (17-42)	25.7 (16-60)	27.5 (14-57)	24.3 (16-37)	
	% women	100%	70%	-	51%	56%	54%	56%	100%	
Hip OA cases	Definition	ROA	-	COA/ROA	-	THR	ROA	-	ROA	
	Number	95	-	81	-	1126	512	-	70	**1884**
	Age mean (range)	66.1 (55-76)	-	63 (62-65)	-	68.5 (40-90)	68.2 (55-93)	-	56.6 (41-79)	
	BMI mean (range)	24.9 (19-37)	-	26.5 (26-27)	-	27.7 (15-50)	26.5 (18-43)	-	25.3 (16-40)	
	% women	100%	-	75%	-	63%	59%	-	100%	
Knee OA cases	Definition	ROA	ROA	COA/ROA	ROA	ROA + 80% TKR	ROA	ROA	ROA	
	Number	264	65	115	143	1174	866	151	104	**2882**
	Age mean (range)	65.9 (55-76)	51.0 (36-60)	61.6 (60-63)	65.2 (59-71)	69.3 (40-96)	70.3 (55-94)	58.0 (47-81)	58.9 (41-79)	
	BMI mean (range)	27.3 (19-45)	30.6 (21-47)	28.0 (27-29)	29.4 (21-48)	29.9 (16-51)	27.7 (18-50)	29.9 (19-48)	27.5 (21-52)	
	% women	100%	66%	82%	42%	56%	73%	57%	100%	

GARP: Genetics osteoARthritis and Progression Study; ROA: radiographic osteoarthritis; COA: clinical osteoarthritis; THR: total hip replacement; TKR: total knee replacement; [1]knee OA controls and hip OA controls respectively if both phenotypes are present in one study; [2]Controls of the Rotterdam Study I are used as controls for the GARP Study; [3]Average for hip and knee controls

Table 1b. Baseline characteristics of published studies assessing the relationship between common genetic variation in the *Il-1* region and risk of hip and knee OA

Study		Bristol Study	Chinese Study	Czech Study	GOAL	Oxford Study	Turkish Study	Total
Study characteristics	Reference	Smith *et al.*(13,14)	Ni *et al.*(10)	Ruzickova *et al.*(11)	Limer *et al.*(7)	Loughlin *et al.*(5,8)	Sezgin *et al.*12)	
	Type of study	case-control	case-control	case-control	case-control	case-control	case-control	
	Origin	UK	China	Czech Republic	UK	UK	Turkey	
	Age mean (range)	62[1]	58	54.1[2]	66.5 (45-86)	73 (56-90)	61.3 (41-83)	
	BMI mean (range)	-	-	-	29.3 (17-58)	-	29.9 (21-44)	
	% women	52%	68%	68%[3]	49%	61%	75%	
Controls	Definition	Unrelated healthy blood donors	Healthy controls[4]	Healthy individuals	No ROA & no symptoms	Unaffected spouses	No OA according to ACR criteria	
	Number[5]	195	487	170	820	557	67	**2296/ 1639**
Hip OA cases	Definition	THR	-	-	Croft score ≥ 3	THR	-	
	Number	29	-	-	1299	383	-	**1711**
Knee OA cases	Definition	COA/ROA	COA/ROA	COA/ROA	COA/ROA	TKR	ACR criteria	
	Number	141	453	50	1247	133	107	**2131**

ROA: radiographic osteoarthritis; COA: clinical osteoarthritis; THR: total hip replacement; TKR: total knee replacement; [1]Mean age in cases; [2]Median age in cases; [3]percentage women in cases; [4]healthy controls from the Center at Physical Examination; [5]knee OA controls and hip OA controls respectively if both phenotypes are present in one study

minimum amount of bias. We were not able to retrieve allele counts for the controls of one Japanese Study (7) and for the London samples published by Smith *et al*. excluding participants from the Chingford study (15). Therefore, these samples were not included in the meta-analysis. In addition, in the study of Meulenbelt *et al*. a subset of RS-I was used (10). For this study we have now included the complete Rotterdam Study-I and therefore results of Meulenbelt *et al.* are not shown separately.

Meta-analysis

For the meta-analysis we were able to include 14 studies on knee OA for one or more variants and 8 studies on hip OA with a total number of up to 3,595 hip OA cases and 6,559 controls and 5,013 knee OA cases and 9,132 controls. In addition, one study (n=130 knee ROA cases from 2 cohorts) published data on radiographic severity of knee OA and common genetic variation in the IL-1 region (5). We included this study in the meta-analysis of severity of knee OA. One study with already published data on the relationship between knee and hip OA and common genetic variation in the IL-1 region, provided also unpublished data on severity of knee and hip OA (GOAL Study) (8) and was therefore also included in the meta-analysis on severity of knee OA.

Laboratory methods

GARP Study: The genotypes of rs1143634, rs16944 and rs419598 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.). Control subjects of the Rotterdam Study-I were used as controls for the GARP study.

Rotterdam Study-I and III: Genotypes were subtracted from the genome-wide association (GWAS) dataset of RS-I and III). Genotyping of the samples with the Illumina HumanHap550v3 Genotyping BeadChip was carried out at the Genetic Laboratory of the Department of Internal Medicine of Erasmus Medical Center, Rotterdam, the Netherlands. The Beadstudio GenCall algorithm was used for genotype calling and quality control procedures were as described previously (17-18). Missing genotypes for RS-I and III were imputed as described previously (19). Subsequently, genotypes of rs1143634, rs16944, rs419598, rs315952 and rs9005 were subtracted using PLINK software V1.07 (20). All five polymorphisms were in HWE in controls in both studies (P>0.05, data not shown).

TwinsUK Study: Genotypes were subtracted from the GWAS dataset of the TwinsUK study (19) using the same methods as for RS-I and RS-III. All polymorphisms were in HWE in controls (P>0.05, data not shown).

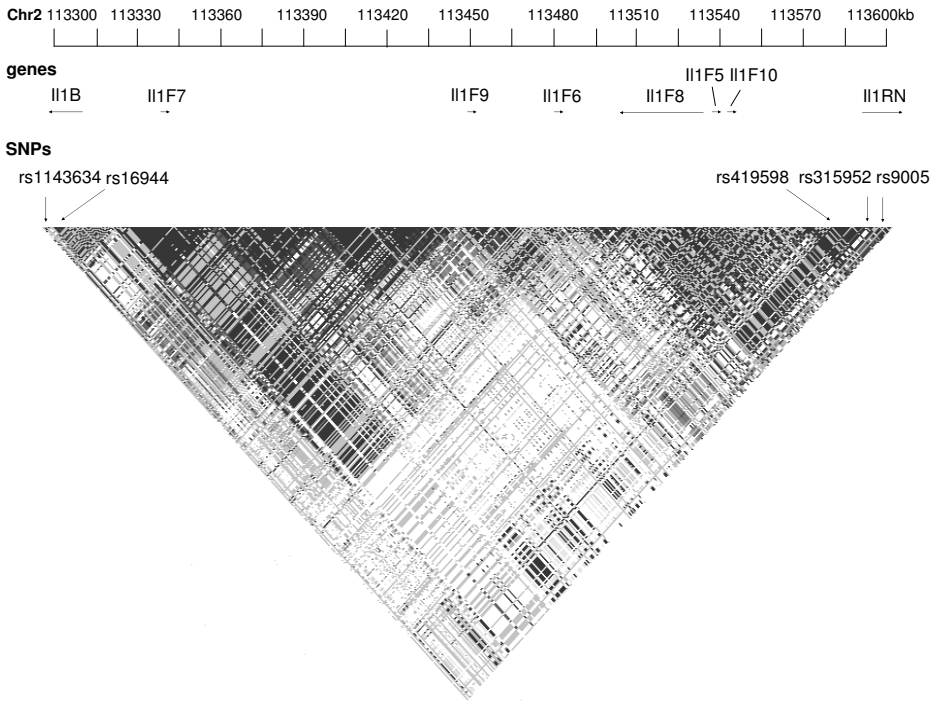


Figure 1. Linkage Disequilibrium (LD) plot of the Interleukin-1 region including the SNPs involved in the meta-analysis. The darker the region, the higher the LD.

Other studies: For the NCCS, HCS, CS and ECS study participants, genomic DNA was extracted from peripheral blood leukocytes of affected individuals and controls using standard protocols. Genotyping was carried out by Kbioscience Ltd, Hertfordshire UK. The *IL-1* SNPs were genotyped using the KASPar chemistry, which is a competitive allele-specific PCR SNP genotyping system using FRET quencher cassette oligos. Genotyping accuracy, as determined from the genotype concordance between 52 duplicate samples was 99.35% for all three SNPs. All three polymorphisms were in Hardy-Weinberg equilibrium in controls (P>0.05).

Haplotype estimation

In Figure 1 we show the linkage disequilibrium (LD) plot for *Il-1*. Linkage disequilibrium is low between the rs16944, rs1143634 and rs419598 (lowest D'=0.38 and r^2=0.02). For all new studies as well as for GOAL we estimated haplotypes on a population level using the program Haploview v 4.1 (21). In all studies, 7 common haplotypes were present for hip OA (rs1143634, rs16944 and rs419598). For the remainder of published studies the haplotype frequencies reported by authors were

Table 2. Genotype and haplotype frequencies for knee severity replication studies

Study	NCCS	HCS	CS	GOAL	GARP Study	RS-I	RS-III	TwinsUK
K/L score 1-2								
N	399	310	180	329	161	1283	490	145
rs419598 CC/CT	45.4%	44.8%	57.6%	50.5%	45.3%	46.1%	46.1%	53.1%
C-T-A haplotype	N/A	N/A	N/A	N/A	N/A	24.9%	24.6%	27.9%
K/L score 3-4								
N	981	45	164	796	58	103	40	56
rs419598 CC/CT	48.8%	66.7%	54.5%	50.1%	43.1%	45.6%	37.5%	48.2%
C-T-A haplotype	N/A	N/A	N/A	N/A	N/A	21.3%	19.9%	25.6%

Results for the C-T-A haplotype refer to: rs419598-rs315952-rs9005 C-T-A haplotype; K/L: Kellgren/Lawrence score; N: number of subjects; N/A: not applicable; NCCS: Nottingham Case-Control Study; HCS: Hertfordshire Cohort Study; CS: Chingford Study; RS: Rotterdam Study

used and the reader is referred to the original studies (see Supplementary Table 1a and 1b in Appendix 2).

Statistical analysis

Allele and genotype odds ratios were calculated by comparing the allele and genotype frequencies between cases and controls. Three SNPs, previously implicated in risk of hip and knee OA, rs16944, rs1143634 and rs419598, were tested for association with knee and hip OA. In addition, the haplotype C-G-C or 1-1-2 which was reported as significantly associated with hip OA in two previous studies (rs1143634, rs 16944 and rs419598), was tested for association with hip OA (10,14).

To be consistent with the previously published study by Attur *et al*. we classified patients as severe knee OA case if the K/L score of the knee was 3 or 4 and as mild to moderate knee OA with a K/L score of 1 or 2 (5). The studies with data for this type of analysis are CS, GARP, GOAL, HCS, NCCS, RS-I, RS-III and TwinsUK, totalling 3,297 individuals with K/L 1 or 2, and 2243 with K/L 3 or 4 (Table 2). These were combined to 130 individuals with K/L 1 or 2 from the original US study and individuals with K/L 3 or 4. In addition, for TwinsUK, RS-I and RS-III were able to estimate the C-T-A haplotype consisting of, respectively rs419598, rs315952 and rs9005. A meta-analysis was performed for both the C allele of rs419598 and for the C-T-A haplotype with severity of knee OA. We carried out both fixed effects and random effects meta-analyses as follows:

Meta-analyses: We synthesized the effect estimates in each study using fixed- and random effects models. In fixed effects calculations it is assumed that the true effect of risk allele is the same value in each study, whereas in random effects calculations the risk allele effects for the individual studies are assumed to vary around some overall average effect.

Table 3. Meta-analyses association results for common genetic variation in the *IL-1* region and risk of hip and knee OA

Genetic variation	Phenotype	N cases	N controls	Association results			Heterogeneity statistics				Statistical power
				OR	*95%CI*	*P-value*	I^2	*Q*	*df*	*P-value*	*OR 80%*[2]
rs1143634	Knee OA	4429	8549	1.03[1]	0.95-1.12	0.43	21%	12.7	10	0.24	1.088
rs16944		4761	8770	1.02	0.96-1.08	0.57	0%	6.4	10	0.78	1.078
rs419598		4900	9195	1.05[1]	0.97-1.14	0.24	32%	17.5	12	0.13	1.082
rs1143634	Hip OA	3634	7918	0.97	0.90-1.04	0.40	0%	3.7	6	0.72	1.096
rs16944		3605	7725	1.04[1]	0.95-1.14	0.35	36%	9.4	6	0.16	1.088
rs419598		3619	7897	1.00	0.93-1.08	0.97	0%	5.7	6	0.46	1.093
C-G-C haplotype		3654	8131	1.04[1]	0.93-1.17	0.52	44%	12.4	7	0.09	1.148

All association results are fixed-effects odds ratios unless indicated otherwise; OA: osteoarthritis; OR: odds ratio; df: degrees of freedom; [1]random-effects model; C-G-C haplotype:rs1143634-rs16944-rs419598; [2]80% power to detect ORs with alpha=0.05

We assessed the presence of heterogeneity using the Cochran's Q statistic (22). The heterogeneity was quantified by using the I^2 (23). In the absence of at least moderate inter-study heterogeneity within samples (I^2<25%) we conducted a Mantel-Haenszel meta-analysis of data from the samples to assess the overall evidence of association (24). For the random effects models we used the DerSimonian - Laird method which incorporates the heterogeneity between studies. The overall treatment effect is estimated by a weighted average of the individual effects with weights inversely proportional to the variance of the observed effects. The statistical significance of the DerSimonian –Laird odds ratio was estimated using the Z-statistic (the point estimate to its standard error). If evidence of heterogeneity existed, defined as either P <0.10 for the Q-statistic and/or I^2>25%, a random-effects model was applied for the meta-analysis.

Statistical power was computed using Quanto 1.2.4 (University of Southern California, USA, (http://hydra.usc.edu/gxe).

RESULTS

Statistical power

For the statistical power for each meta-analysis, given the frequency of the minor allele and the sample sizes available, it was estimated that we had 80% power to find associations with an OR=1.09-1.15 (depending on the allele frequency and on the

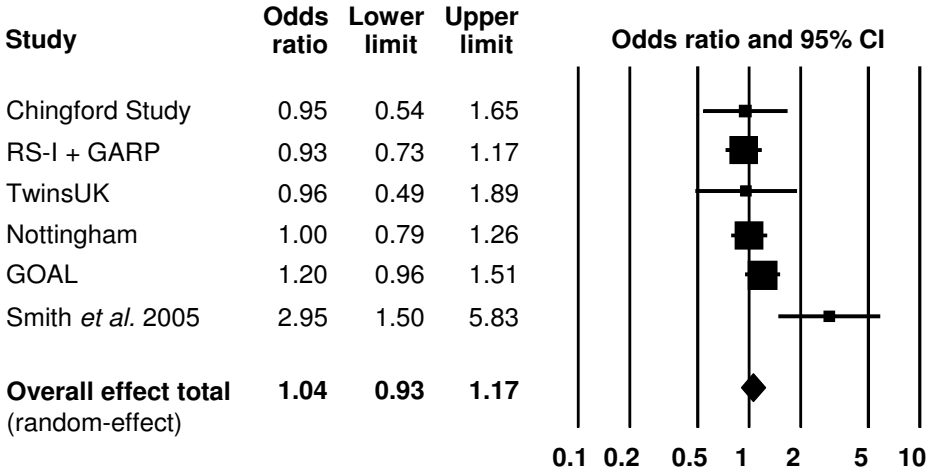


Figure 2. Study specific estimates and summary association (random-effects) between risk of hip OA and rs1143634-rs16944-rs419598 "C-G-C" haplotype on risk of hip OA.

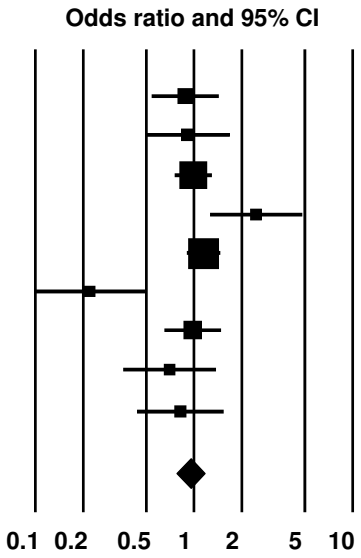
number of studies with data for each SNP) for hip OA and OR= 1.08-1.09 for knee OA with $P<0.05$ (Table 3).

Association between genetic variation in the IL-1 region and risk of hip and knee OA
The allele and haplotype frequencies for cases and controls in each study are presented in Supplementary Table 1a and 1b in Appendix 2, respectively for unpublished and published studies. The summary results of the hip and knee OA meta-analyses for rs1143634, rs16944 and rs419598 and haplotype C-G-C for hip OA are presented in Table 3. No significant associations were observed between rs16944, rs1143634 or rs419598 and hip or knee OA ($P>0.05$). No association was seen between the C-G-C haplotype and hip OA OR=1.04 (95%CI 0.93-1.17, $P=0.52$) (Figure 2).

Association between genetic variation in the IL-1 region and risk of severe knee OA
The genotype and haplotype frequencies for severe knee OA cases (K/L 3 and 4) and controls (K/L 1 or 2) in each study are presented in Table 2. No evidence of association between risk of severe knee and the *ILRN* SNP rs419598 region was observed (Figure 3a). Specifically, rs419598 had an OR of 0.92 (95%CI 0.71-1.20, $P=0.54$) for severe knee OA. Very strong heterogeneity ($I^2=70\%$, Q-statistic $P=0.002$) was observed for this analysis. Excluding the initial significant report and data from the Hertfordshire Cohort Study (which shows a significant association in the opposite direction) no between study heterogeneity remained ($I^2=0\%$) and the effect of this genotype became OR=1.00 (95%CI 0.87-1.15; $P=0.97$) indicating no role for this genotype in severity of knee OA. When a fixed-effects meta-analysis was performed

A

Study	Odds ratio	Lower limit	Upper limit
Chingford Study	0.88	0.55	1.43
GARP	0.91	0.50	1.67
GOAL	0.99	0.76	1.28
Hertfordshire	2.46	1.27	4.76
Nottingham	1.15	0.91	1.45
NYUHJD	0.22	0.10	0.49
RS-I	0.98	0.66	1.47
RS-III	0.70	0.36	1.36
TwinsUK	0.82	0.44	1.52
Overall effect total (random-effect)	**0.92**	**0.71**	**1.20**



B

Study	Odds ratio	Lower limit	Upper limit
NYUHJD	0,14	0,05	0,38
RS-I	0.82	0.58	1.15
RS-III	0.76	0.43	1.34
TwinsUK	0.89	0.54	1.46
Overall effect total (random-effect)	**0.61**	**0.35**	**1.06**

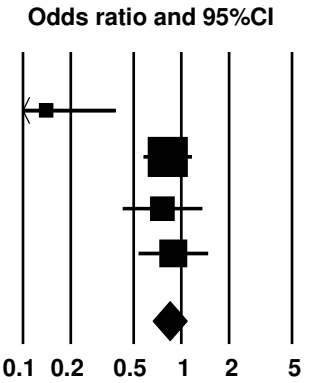


Figure 3. Study specific estimates and summary association (random-effects) between severity of knee OA defined as K/L 1 or 2 vs K/L 3 or 4 and (**A**) rs419598 CC/CT genotype, (**B**) haplotype rs419598, rs315952 and rs9005 "C-T-A".

for the haplotype reported to be associated with OA radiographic severity (Figure 3b) a trend was observed in the same direction in all three replication studies and combined with the initial report from Attur and co-workers (5) a summary effect of OR=0.71 (95%CI 0.56 -0.91, *P*=0.006) was observed. Nevertheless, the extremely strong effect reported by the first study introduces significant heterogeneity (I^2=74%, Q-statistic *P*=0.008) and a random-effects meta-analysis resulted in OR=0.61 (95%CI 0.35-1.06, *P*=0.08) (Figure 3b).

DISCUSSION

This meta-analysis on common genetic variation in the Interleukin-1 region and risk of hip and knee OA is the largest to date, including all available published studies (n=6) and unpublished novel data (n=8), and shows no evidence for a consistent association with knee or hip OA. In this study we had 80% power to detect OR=1.09-1.15 for hip OA and OR=1.08-1.09 for knee OA with α=0.05. Therefore it is not likely that we have observed false-negative associations with regards to risk. Nevertheless, we find that the *IL1RN* C-T-A haplotype may indeed have a role in severe knee OA which is consistent with the well known IL1's established role as a regulator of cartilage degradation (3, 5). Since there is a limited sample size of subjects of a non-Caucasian origin, we cannot exclude that there might be evidence of association between the SNPs studied and OA in subjects from a different ethnic origin.

So far, the literature has been inconclusive on the role of *IL-1* polymorphisms and/or haplotypes in risk of knee and hip OA, probably due to low sample sizes of individual studies. An attempt was made by Moxley and colleagues to perform a meta-analysis, but the results remained inconclusive (16). One of the reasons for this could be that they did not include all published studies on genetic variation in the *Il-1* region. More importantly the authors did not add unpublished novel data. This approach resulted in not only low power to detect statistically significant associations, but could potentially also lead to publication bias. There were not enough published studies examining the same genetic variant in relation to OA to study presence of publication bias.

In two previous publications the C-G-C haplotype was associated with an increased risk of hip OA, although this could not be replicated by another larger study (6). In this meta-analysis, which had 25 times more cases compared to the first two publications, we could not find evidence of an association between this haplotype and hip OA. Therefore we conclude that the previous two observations were false positive (10, 14). We also have to note that there is low LD between the three SNPs (2 SNPs in the *IL1B* gene and 1 SNP in the *IL1RN* gene) in all Caucasian populations studied and therefore an analysis of haplotypes in Caucasian populations is not appropriate, which is true for this study and all previous publications.

Recently, a small study (n=130 cases) showed that genetic variation in the *IL-1* region was associated with severity of knee OA (5). We find very strong heterogeneity in the association between the *ILRN* variant rs419598 and knee OA severity and overall there is no significant effect. Yet, when we tested the C-T-A haplotype associated with severe knee OA we found that in all studies it had a lower frequency among severe OA cases than non severe cases suggesting that it might be truly involved in this phenotype. We observed a borderline significant effect in the random-effects

model for the C-T-A haplotype and severe knee OA, but power was limited for this analysis and therefore a larger sample size or functional studies are needed to confirm the role of *ILRN* in severe knee OA.

In conclusion, common genetic variation in the Interleukin-1 region is not associated with prevalence of hip or knee OA but our data suggest that *IL1RN* might have a role in severity of knee OA.

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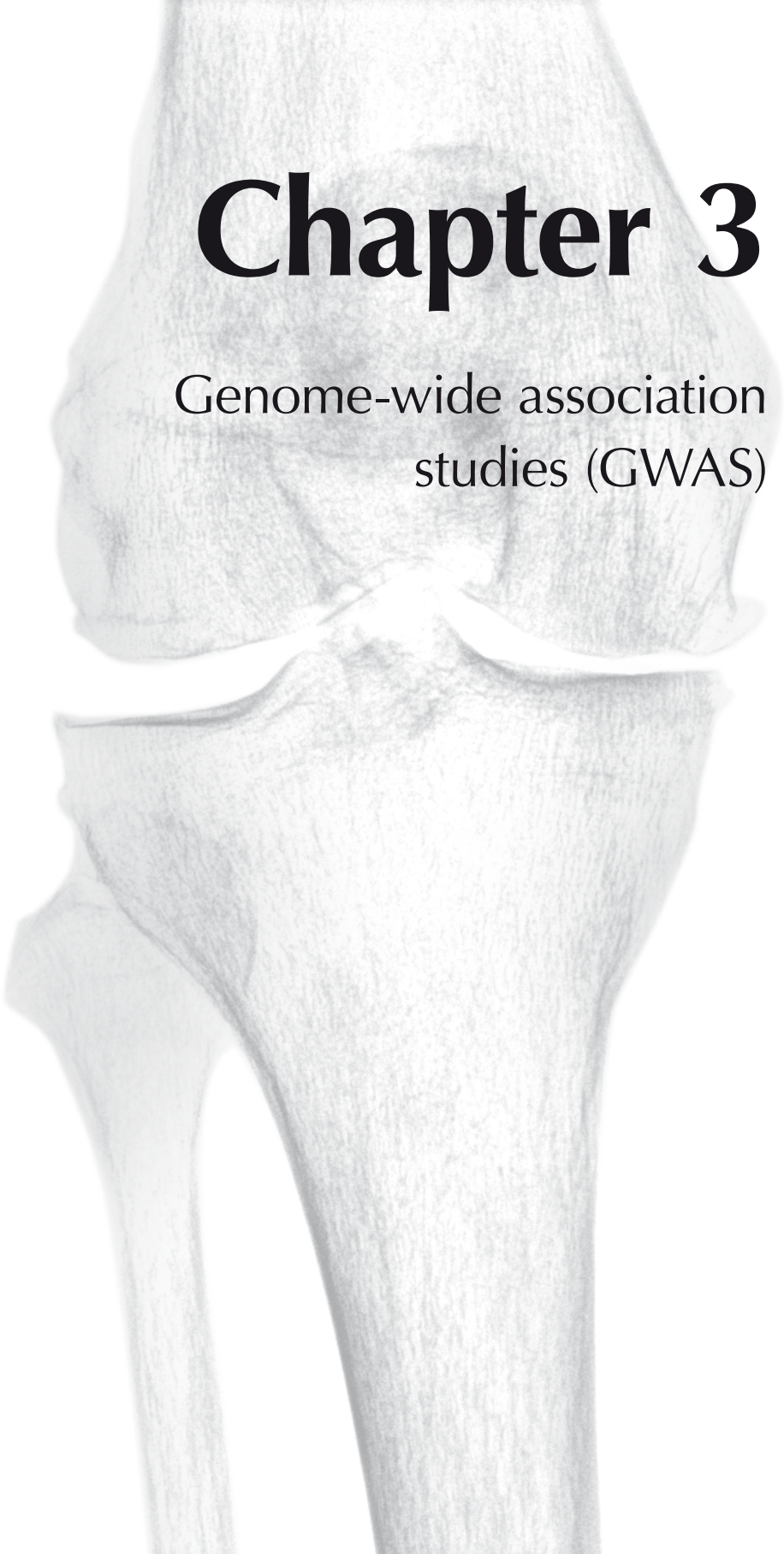

Chapter 3

Genome-wide association studies (GWAS)

Chapter 3.1

The TREAT-OA consortium: Recommendations for standardization and phenotype definitions in genetic studies of osteoarthritis

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ABSTRACT

Background: The objective of this study was to address the need for standardization of osteoarthritis (OA) phenotypes by examining the effect of heterogeneity among symptomatic (SOA) and radiographic osteoarthritis (ROA) phenotypes.

Methods: Descriptions of OA phenotypes of the 28 studies involved in the TREAT-OA consortium were collected. We investigated whether different OA definitions result in different association results by creating various hip OA definitions in one large population based cohort (the Rotterdam Study-I) and testing those for association with gender, age and BMI using one-way ANOVA. For radiographic OA, we standardized the hip, knee and hand ROA definitions and calculated prevalence's of ROA before and after standardization in 9 cohort studies. This procedure could only be performed in cohort studies and standardization of SOA definitions was not feasible at this moment.

Results: In this consortium, all studies with symptomatic OA phenotypes (knee, hip and hand) used a different definition and/or assessment of OA status. For knee, hip and hand radiographic OA 5, 4 and 7 different definitions were used, respectively. Different hip OA definitions do lead to different association results. For example, we showed in the Rotterdam Study-I that hip OA defined as "at least definite JSN and one definite osteophyte" was not associated with gender (P=0.22), but defined as "at least one definite osteophyte" was significantly associated with gender (P=3x10^{-9}). Therefore, a standardization process was undertaken for radiographic OA definitions. Before standardization a wide range of ROA prevalence's was observed in the 9 cohorts studied. After standardization the range in prevalence of knee and hip ROA was small.

Conclusions: Phenotype definitions influence the prevalence of OA and association with clinical variables. ROA phenotypes within the TREAT-OA consortium were standardized to reduce heterogeneity and improve power in future genetics studies.

INTRODUCTION

The Translational Research in Europe Applied Technologies for OsteoArthritis (TREAT-OA) consortium was established in January 2008 to address the generalisability and utility of genetic and biochemical risk factors (www.treatoa.eu). The two main goals of TREAT-OA are 1) to develop efficient diagnostics for risk and progression of osteoarthritis (OA) and 2) to identify new targets for therapeutic interventions. This will be done by identification of genes and biochemical markers consistently associated with risk and progression of OA, but also by defining the roles of these genes in molecular pathways involved in disease aetiology, for example by the development of *in vivo* transgenic animal OA model systems.

A major goal of the consortium is to identify new genes consistently associated with risk and progression of OA. To reach this goal, large-scale genome-wide association studies (GWASs) and meta-analyses are being performed. To date, research within the TREAT-OA consortium has resulted in the identification of a novel genetic locus on chromosome 7q22 that is associated with knee and hand OA (1), which was confirmed by a yet unpublished GWAS meta-analysis on knee OA. In addition, the ataxin 2 binding protein 1 gene (2) and the prostaglandin-endoperoxide synthase 2 gene (3) have been found associated with, respectively hand and knee OA.

One of the difficulties in these genetic analyses, and also in general in epidemiological research of OA is heterogeneity of the definition of the phenotype under study. Heterogeneity of the definition of the phenotype among different studies reduces power to find consistent associations in any disease (4). Two working groups of HuGEnet and NCI-NHGRI have published recommendations for replication studies in genetic epidemiology studies (5-7). One of their recommendations was to try to investigate the same or a very similar phenotype in replication studies. Specifically for OA, the American College of Rheumatology (ACR) criteria were developed to define clinical OA within a secondary care setting (8) and the OARSI-OMERACT initiative proposed definitions for radiological progression of hip and knee OA (9). The problem of heterogeneity in genetic association studies of OA has been highlighted (10) and therefore standardized radiographic OA (ROA) phenotypes were used in our recent GWAS and subsequent meta-analysis (1). However, symptomatic (SOA) and ROA phenotypes were both used within the same meta-analysis. For ROA, several grading systems exists, but the most widely and consistently used system is the Kellgren and Lawrence (K/L) grading system (11). Among major cohort studies, K/L scores are interpreted differently, especially for the knee and hip, despite the fact that they all refer to the original description (12-14).

In the current study, we have examined the effect of heterogeneity among symptomatic (SOA) and radiographic osteoarthritis (ROA) phenotypes on associa-

tion analyses, to address the need for standardization of osteoarthritis phenotypes to enhance power for future association studies. We further provide recommendations for standardization of OA phenotypes.

MATERIALS & METHODS

Study Populations

We collected data for 28 studies currently involved in the TREAT-OA consortium on the following 9 items: 1) reference article, 2) study design, 3) ethnic origin, 4) country of origin, 5) joint site(s) studied 6) radiographic or symptomatic OA definition, 7) availability of age and/or BMI data, 8) percentage of women in the study and 9) availability of follow-up data. Table 1 describes the characteristics of all studies evaluated. A short description of all studies involved in the TREAT-OA consortium is given in the Supplementary data in Appendix 1.

OA definitions

OA phenotypes can be categorized into symptomatic OA and radiographic OA, and this information was collected from all studies. Subsequently, we asked for the exact OA definition used in that particular study. For example, if a study used a K/L score and used the cut-off value defined by a summary grade of 2 or more to define OA cases, the exact description of a K/L of 2 was requested (e.g. definite osteophytes with possible JSN versus definite osteophyte(s) only) or a reference article was asked were the exact interpretation of the K/L score was given.

Data analysis of OA phenotypes within the Rotterdam Study-I (RS-I)

Within RS-I radiographic features are scored separately for hip OA (such as osteophytes, sclerosis and joint space narrowing at the lateral, superior and axial site of the hip joint) (50). In addition, total hip replacement and the presence of pain during the last month are recorded. To discover if differences in case definitions result in different association results, we created all hip OA case definitions used by studies of the consortium within RS-I. Association analyses were performed to study the relationship between different OA definitions of the hip and age, gender and body mass index (BMI). One-way ANOVA was used to assess the relationship between hip OA and the clinical variables. The analyses were carried out using SPSS version 15.0.

Standardization of phenotypes

Consensus on which ROA phenotype to use within the TREAT-OA consortium was based on the ROA definition as originally described by Kellgren and Lawrence and

Table 1. Overview of all studies involved in the TREAT-OA consortium

Study	Reference article	Study design	Ethnic origin	Country of origin	Joint site	ROA/ SOA	Age/ BMI	% women	Follow-up data
GWAS data									
arcOGEN consortium		Case-control	Caucasian	United Kingdom	Knee, hip	ROA/ SOA	-	60%	Not available
Chingford Study	Hart *et al.* (15)	Cohort	Caucasian	United Kingdom	Knee, hip	SOA	+	100%	Available
Nottingham Case-Control Study	Valdes *et al.* (16)	Case-control	Caucasian	United Kingdom	Knee, hip	SOA	+	53%	Not available
Oxford Study	Chapman *et al.* (17)	Case-control	Caucasian	United Kingdom	Knee, hip	SOA	-	55%	Not available
Sheffield Study	Gordon *et al.* (18)	Case-control	Caucasian	United Kingdom	hip	SOA	+[1]	53%	Not available
TwinsUK	Spector *et al.* (19)	Cohort	Caucasian	United Kingdom	Knee, hip	ROA	+	100%	Available
VIDEO	Not available yet	RCT	Caucasian	United Kingdom	Knee	SOA	+	60%	Available in 2011
Other									
deCODE	Ingvarsson *et al.* (20) and Stefansson *et al.* (21)	Case-control	Caucasian	Iceland	Knee, hip, hand	SOA	+[1]	58%	Not available
Framingham Osteoarthritis Study	Hunter *et al.* (22)	Cohort	Caucasian	United States	Knee, hand	ROA	+	56%	Available
GARP	Riyazi *et al.* (23)	Cohort	Caucasian	Netherlands	Knee, hip, hand	SOA/ ROA	+	65%	Available
Health 2000	Kaila-Kangas *et al.* (24)	Cohort	Caucasian	Finland	Hip, knee	SOA	+	55%	Available
Rotterdam Study-I	Hofman *et al.* (25)	Cohort	Caucasian	Netherlands	Knee, hip, hand	ROA	+	59%	Available
Rotterdam Study-II	Hofman *et al.* (25)	Cohort	Caucasian	Netherlands	Knee, hip, hand	ROA	+	56%	Available
Rotterdam Study-III	Hofman *et al.* (25)	Cohort	Caucasian	Netherlands	Knee, hip, hand	ROA	+	57%	Available in future
TwinsUK	Spector *et al.* (19)	Cohort	Caucasian	United Kingdom	Knee, hip, hand	ROA	+	100%	Available

GWAS: genome-wide association study; ROA: radiographic osteoarthritis; SOA: symptomatic osteoarthritis; BMI: body mass index; RCT: randomized clinical trial; Age/BMI +: age and BMI data are available; GARP: Genetics osteoARthritis and Progression;[1]age is available for all subjects, BMI only for part of the subjects

Table 1. Continued

Study	Reference article	Study design	Ethnic origin	Country of origin	Joint site	ROA/ SOA	Age/ BMI	% women	Follow-up data
De novo genotyping									
Chingford Study	Hart *et al.* (15)	Cohort	Caucasian	United Kingdom	Knee, hip, hand	ROA	+	100%	Available
Chinese Case-Control Study	Miyamoto *et al.* (26)	Case-control	Asian	China	Knee	SOA	+[2]	75%	Not available
Dentist &Teacher Study	Solovieva *et al.* (27)	High risk Cohort	Caucasian	Finland	Hand	ROA	+	100%	Only symptoms
Estonian Studies	Tamm *et al.* (28)	Cohort	Caucasian	Estonia	Knee	ROA	+	65%	Available
Finnish OA cases	Näkki *et al.* (29)	Case-control	Caucasian	Finland	Hand, knee	SOA/ ROA	+	76%	Not available
Greek clinical cases	Fytili *et al.* (30)	Case-control	Caucasian	Greece	Knee	SOA	+	78%	Not available
Hertfordshire Cohort Study	Abdin-Mohamed *et al.* (31)	Cohort	Caucasian	United Kingdom	Knee, hand	ROA	+	50%	Available[3]
Japanese Case-Control Study	Miyamoto *et al.* (26)	Case-control	Asian	Japan	Knee, hip	SOA	+[2]	80%	Not available
Japanese Cohort Study	Miyamoto *et al.* (26)	Cohort	Asian	Japan	Knee	ROA	+	75%	Available[4]
KANON	Frobell *et al.* (32)	High risk Cohort	Caucasian	Sweden	Knee	ROA	+	26%	Available in 2011
LUMEN	Englund *et al.* (33)	High risk Cohort	Caucasian	Sweden	Knee	ROA	+	21%	Available
Malmo Diet Cancer study	Lohmander *et al.* (34)	Cohort	Caucasian	Sweden	Knee, hip	SOA	+	65%	Available
Osteoporotic ractures in men study	Orwoll *et al.* (35)	Cohort	Caucasian	United States	Hip	ROA	+	0%	Available
Nottingham Case-Control	Valdes *et al.* (16)	Case-control	Caucasian	United Kingdom	Knee, hip	SOA	+	53%	Not available
Spanish clinical cases	Rodriguez-Lopez *et al.* (36)	Case-control	Caucasian	Spain	Knee, hip, hand	SOA	+	65%	Not available
Study of Osteoporotic Fractures	Nevitt *et al.* (37)	Cohort	Caucasian	United States	Hip	ROA	+	100%	Available
The ROAD Study	Muraki *et al.* (38)	Cohort	Asian	Japan	Knee	ROA	+	65%	Available in 2010

GWAS: genome-wide association study; ROA: radiographic osteoarthritis; SOA: symptomatic osteoarthritis; BMI: body mass index; Age/BMI +: age and BMI data are available for all subjects; [2]only for the cases data on age and BMI is available; [3]available for clinical data, not available for x-ray data; [4]for part of the subjects follow-up data is available

the feasibility of its use within each of the studies (11). Total joint replacements (TJR) due to primary OA visible on radiographs are considered as OA. TJR due to fractures and other diseases were excluded as much as possible. After a consensus was reached between consortium members, the cohort studies either shared their data with our research group (Rotterdam Study) who standardized the definitions (data of TwinsUK, Chingford Study) or performed the standardization process themselves (other replication studies) if they were able and willing to standardize their ROA definition. The prevalence of OA was calculated by dividing the number of prevalent ROA cases over controls. Before standardization, controls were defined as the absence of OA, according to the definition used by each study, at the joint site studied.

After standardization, controls were defined as the absence of OA, according to the standardized definition as described in the results section, at the joint site studied.

RESULTS

Study populations

Since the start of the TREAT-OA consortium in 2008, the number of teams collaborating with the consortium has grown to include 28 teams participating as of April 2010 (a description of each study is given in Appendix 1). The studies originate from Europe, the United States of America and Asia. In 24 of the 28 studies (86%), the majority of subjects included are women (63% on average). With respect to genetic data, there are in total 11 studies with GWAS data, 2 studies in which part of the subjects have GWAS data and 15 studies without GWAS data.

OA definitions

In total, there were 11 studies using a symptomatic definition of OA and 15 studies with a radiographic definition. Two studies could not be classified as completely symptomatic or radiographic (SOA/ROA).

Radiographic OA (ROA): For knee OA, there are 14 studies using radiographic definitions of knee OA shown in Table 2a with a detailed description of the knee ROA definition. A total of 12 studies used the K/L score, of which 11 studies used a cut-off value of 2 to define knee ROA and 1 study used a more stringent cut-off of 3. Two studies, which are both high risk cohorts, used a definition of OA not according to a standard classification system. As is shown in Table 2a, four different interpretations are given for the K/L score of the knee considering a cut-off value of 2 although all studies used the original K/L atlas. In Table 2b-c, results are given for hand and hip ROA, respectively, in a similar way as for knee ROA.

Table 2a. Description of the radiographic knee OA definition according to 14 studies of the TREAT-OA consortium

Study	Classification System	Cut-off value for OA	Exact OA definition
Chingford Study	K/L score	2	One definite osteophyte
Estonian Studies	K/L score	2	Definite osteophytes
Finnish cases	K/L score	3	Definite osteophytes + definite JSN and/or joint deformation
Framingham Osteoarthritis Study	K/L score	2	Definite osteophytes and possible JSN
GARP	K/L score	2	Definite osteophytes and possible JSN
Hertfordshire Cohort Study	K/L score	2	Definite osteophytes
Japanese Cohort Study	K/L score	2	One definite osteophyte
KANON	-	-	JSN grade ≥ 2 or sum of 2 marginal osteophyte grades from the same compartment ≥ 2 or grade 1 JSN + grade 1 osteophytes in the same compartment
LUMEN	-	-	JSN grade ≥ 2 or sum of 2 marginal osteophyte grades from the same compartment ≥ 2 or grade 1 JSN + grade 1 osteophytes in the same compartment
Rotterdam Study-I	K/L score	2	Definite osteophytes and possible JSN
Rotterdam Study-II	K/L score	2	Definite osteophytes and possible JSN
Rotterdam Study-III	K/L score	2	Definite osteophytes and possible JSN
The ROAD Study	K/L score	2	One definite osteophyte
TwinsUK	K/L score	2	One definite osteophyte

K/L: kellgren and Lawrence; JSN: joint space narrowing; - no standard classification system is used to define OA; GARP: Genetics osteoARthritis and Progression

Table 2b. Description of the radiographic hand OA definition according to 9 studies of the TREAT-OA consortium

Study	Classification System	Cut-off value for OA	Exact OA definition
Chingford Study	K/L score	2	≥3 joints (DIP/PIP/CMC1) affected[1]
Dentist &Teacher Study	Modified K/L score	2	≥2 joints (DIP/PIP/MCP) affected[2]
Finnish OA cases and families	K/L score	2-3	K/L ≥ 3 for index cases and K/L ≥2 for their siblings (DIP bilateral)
Framingham Osteoarthritis Study	K/L score	2	K/L ≥ 2 (one definite osteophyte): joint specific definitions (i.e., DIP OA, PIP OA etcetera)
GARP	K/L score	2	≥3 joints (DIP/PIP/CMC1) affected[3]

Table 2b. Continued

Study	Classification System	Cut-off value for OA	Exact OA definition
Hertfordshire Cohort Study	-	-	Presence of Heberden's or Bouchard's nodes
LUMEN	-	-	Presence of OA (JSN grade ≥2 or osteophyte grade ≥2 or JSN grade 1 plus osteophyte grade 1) in at least 1 DIP or PIP joint in each hand symmetrically or at least 2 DIP/PIP joints in the same hand in a pattern consistent with primary OA (in the same row or ray) or the CMC1 joint bilaterally.
Rotterdam Study-I	K/L score	2	2 out of 3 hand joint groups (DIP/PIP/CMC1 or TS) affected[1]
TwinsUK	K/L score	2	≥3 joints (DIP/PIP/CMC1) affected[1]

OA: osteoarthritis; K/L: Kellgren and Lawrence; - no standard classification system is used to define OA; DIP: distal interphalangeal joint; PIP: proximal interphalangeal joint; CMC1: first carpometacarpal joint; TS: trapezioscaphoid joint; MCP: metacarpophalangeal joint; GARP: Genetics osteoARthritis and Progression;[1]affected means K/L ≥ 2 (=definite osteophyte) in each or both hands; [2]affected means modified K/L ≥ 2 (=a single radiographic sign indicative of OA, slight to moderate lowering of the joint space, sometimes subluxation, minimal osteophytes, degeneration cysts or slight marginal sclerosis, each of the latter signs without a clear narrowing of joint space but little if any additional pathology) irrespective of right or left hand; [3]affected means K/L ≥ 2 (=definite osteophyte) irrespective of left or right hand

Table 2c. Description of the radiographic hip OA definition according to 7 studies of the TREAT-OA consortium

Study	Classification System	Cut-off value for OA	Exact OA definition
Chingford Study	K/L score	2	Definite osteophyte
GARP Study	K/L score	2	Definite JSN + definite osteophyte
MrOS	Modified Croft grade	2	Presence of either definite JSN or definite osteophytes plus at least 1 of 5 other features: osteophytes, JSN, sclerosis, cysts or femoral head deformity
RS-I	K/L score	2	Definite JSN + definite osteophyte
RS-II	K/L score	2	Definite JSN + definite osteophyte
SOF	Modified Croft grade	2	Presence of either definite JSN or definite osteophytes plus at least 1 of 5 other features: osteophytes, JSN, sclerosis, cysts or femoral head deformity
TwinsUK	Croft grade	1	Definite osteophytes

OA: osteoarthritis; K/L: Kellgren and Lawrence; JSN: joint space narrowing; GARP: Genetics osteoARthritis and Progression; MrOS: Osteoporotic Fractures in Men Study; RS-I: Rotterdam Study-I; RS-II: Rotterdam study-II; SOF: Study of Osteoporotic Fractures

For hand ROA, most studies (7 out of 9) used the K/L score to define hand OA, with the exception of two studies (31, 33). The interpretation of this K/L score is the same for all these studies, but there are 4 different hand ROA definitions based on the number of joints included. For example, 2 studies define OA in one hand joint as at least one definite osteophyte, but hand OA is defined as "≥3 joints (DIP/PIP/CMC1) affected" in one study and "2 out of 3 hand joint groups (DIP/PIP/CMC1 or STT) affected" in another study. Hip ROA was defined by the (modified) Croft grade in 3 studies and by the K/L score in 4 studies. Also for hip ROA there is no consensus on the interpretation of the K/L score as 2 different interpretations are present among the studies. This includes both "definite JSN and a definite osteophyte" OR "one definite osteophyte". The Croft grade cut off of 1 as a criterion for hip ROA, is defined as definite osteophytes and does not include JSN.

Symptomatic OA (SOA): In Table 3a, results are given for hand (n=2) SOA. These definitions differed for each study. In summary, hand SOA was defined by either ACR criteria or by patient records. For knee OA, there are 10 studies using clinical definitions of knee OA, which are shown in Table 3b. In total, 4 of these 10 studies defined knee OA as ROA + symptoms, but the inclusion of patients was done in 4 different ways. For example, one study used a K/L score ≥ 2 (defined as one definite osteophyte) + medial joint space > 1 mm + pain to include patients, whilst another study used a K/L score ≥ 3 + symptomatic OA and treated on a regular basis. The other 6 studies included patients on the basis of total joint replacements due to primary OA or a combination of a TJR or ROA and clinical symptoms of OA.

Hip SOA was defined as a THR by 3 studies although the assessment was different for all 3 studies (i.e., based on hospital records versus based on the description of a rheumatologist) (Table 3c). In addition, 2 studies defined SOA of the hip as symptoms of OA + ROA, but the definition of ROA is unclear and inclusion based on symptoms differs. Furthermore, there were 3 additional studies defining hip SOA again in another way (i.e., incident THR or either clinical records of SOA or a THR).

Data analysis of OA phenotypes within the Rotterdam Study-I (RS-I)

In Table 4, association results are given for the relationship between age, gender and BMI and different hip OA definitions. When hip OA was defined radiographically as "one definite osteophyte" subjects with hip OA were more frequently men compared to controls (mean difference of 10%, $P=3x10^{-9}$), whilst subjects with a THR were more frequently women compared to controls (mean difference of 21%, $P=0.001$). When radiographic OA definitions were compared, we observed that hip ROA defined as "one definite osteophyte" were more frequently men compared to controls ($P=3x10^{-9}$), whilst hip OA defined as "definite JSN and one definite osteophyte" was not associated with gender ($P=0.22$). When analyzing SOA, we did not observe clear

Table 3a. Description of the symptomatic hand OA definitions according to 2 studies of the TREAT-OA consortium

Study	OA definition based on:	Exact OA definition
deCODE	Patients records at hospitals and health centres	Included on the basis of clinical examination by an experienced examiner, supported by a radiograph for >60% of the cases
Spanish clinical cases	ACR criteria	Patients were complaining of hand OA and followed in the Rheumatology Unit. The ACR criteria were used for inclusion in the study

OA: osteoarthritis; ACR: American College of Rheumatology

Table 3b. Description of the symptomatic knee OA definitions according to 10 studies of the TREAT-OA consortium

Study	OA definition based on:	Exact OA definition
Chinese Case-Control Study	K/L grade + symptoms	K/L ≥ 2 (=one definite osteophyte) + pain with rest and/or night pain of over 5-month duration. Exclusion of inflammatory, posttraumatic, post septic arthritis, dysplasias
deCODE	Hospital records of TJR	TKR. A clinician reviewed the patients records to verify the diagnosis
Greek clinical cases	TJR due to OA reported by specialist	TKR + K/L ≥ 2 (=definite osteophytes + possible JSN)
Health 2000	Clinical records of OA or TKR	History, records and a standardized clinical diagnosis of previously diagnosed knee OA or knee arthroplasty due to OA based on convincing findings OR at least moderately restricted mobility OR slightly restricted mobility and either of the following: documented history of previously diagnosed knee OA but not convincingly presented grounds for the diagnosis or typical symptoms of knee OA
Japanese Case-Control Study	K/L grade + symptoms	Symptomatic OA and treated on a regular basis + K/L ≥ 3
MDC Study	Incident knee arthroplasty/ osteotomy from national Swedish hospital discharge register	First knee arthroplasty or high tibial osteotomy + diagnosis of OA according to the International Classification of Disease (ICD) 9 and 10
Nottingham Case-Control	Clinically severe knee OA based on hospital orthopaedic surgery lists	Referred to the hospital with symptomatic, clinically severe knee OA and the majority had undergone unilateral or bilateral TKR within the previous 5 years. Pre-operative knee radiographs were examined to confirm the diagnosis. Exclusion based on another major arthropathy, Paget's disease
Oxford Study	Severe symptomatic knee OA + K/L grade	Signs and symptoms of OA sufficiently severe to require TKR + K/L ≥ 2 (exact definition unknown). Exclusion based on dysplasia
Spanish clinical cases	TJR	TKR, a rheumatologists considered patients to suffer from severe primary OA. Exclusion based on inflammatory, infectious, traumatic or congenital joint pathology and lesions due to crystal deposition or osteonecrosis
VIDEO	K/L grade + pain	K/L ≥ 2 (=one definite osteophyte) + medial joint space width > 1mm + knee pain

OA: osteoarthritis; TJR: total joint replacement; TKR: total knee replacement; JSN: joint space narrowing; MDC: Malmö Diet and Cancer

Table 3c. Description of the symptomatic hip OA definitions according to 8 studies of the TREAT-OA consortium

Study	OA definition based on:	Exact OA definition
deCODE	Hospital records of TJR	THR. A clinician reviewed the patients records to verify the diagnosis
Health 2000	Clinical records of OA or THR	History, records and a standardized clinical diagnosis of previously diagnosed hip OA or hip arthroplasty due to OA based on convincing findings OR at least moderate restrictions in extension or in inner rotation or in outer rotation OR slight restrictions in extension, inner rotation, outer rotation or at least moderately restricted abduction-adduction and either of the following: documented history of previously diagnosed hip OA but no grounds for the diagnosis is given or typical symptoms of hip OA
Japanese Case-Control Study	Symptoms + radiographs	Subjects are symptomatic and were treated in participating institutions on a regular basis + radiographic signs of hip OA (exact definition unknown)
MDC Study	Incident hip arthroplasty from national Swedish hospital discharge register	First hip arthroplasty in combination with a contemporaneous diagnosis of hip osteoarthritis according to the International Classification of Disease (ICD) 9 and 10
Nottingham Case-Control	Clinically severe hip OA based on hospital orthopaedic surgery lists	Referred to the hospital with symptomatic, clinically severe hip OA and the majority had undergone unilateral or bilateral THR within the previous 5 years. Pre-operative hip radiographs were examined to confirm the diagnosis. Exclusion based on another major arthropathy, Paget's disease, overt child hip disease, THR due to trauma or terminal illness
Oxford Study	Severe symptomatic hip OA + K/L grade	Signs and symptoms of OA sufficiently severe to require THR + K/L ≥ 2 (exact definition unknown). Exclusion based on dysplasia
Sheffield Study	THR	Subjects had undergone THR for clinical, idiopathic OA that was confirmed radiographically prior to joint replacement (exact radiographic definition uknown)
Spanish clinical cases	TJR	THR, a rheumatologists considered patients to suffer from severe primary OA. Exclusion based on inflammatory, infectious, traumatic or congenital joint pathology and lesions due to crystal deposition or osteonecrosis

OA: osteoarthritis; TJR: total joint replacement; THR: total hip replacement; MDC: Malmö Diet and Cancer

differences in association results for the different definitions of SOA, but the number of cases for SOA is much lower than for ROA, therefore results should be taken with caution.

Standardization of phenotypes

Consensus was reached for the knee and hip OA definition based on the ROA definition as originally described by Kellgren and Lawrence and at the feasibility within each of the studies. It was agreed that the knee ROA definition used within the

Table 4. Association results of different hip OA case definitions (prevalence) and gender, age and BMI in RS-I

OA phenotype	Number		Gender (%women)			Age (mean)			BMI (mean)		
	cases	*controls*	*cases*	*controls*	*P*	*cases*	*controls*	*P*	*cases*	*controls*	*P*
Radiographic OA											
Definite JSN and one definite osteophyte (original K/L ≥ 2)	242	3037	54%	58%	0.22	68.1	65.7	$3x10^{-8}$	26.3	26.3	0.99
One definite osteophyte	1906	1373	54%	64%	$3x10^{-9}$	66.1	65.5	0.009	26.2	26.4	0.07
Symptomatic OA											
Total hip replacement	64	3215	78%	57%	0.001	71.2	65.7	$8x10^{-11}$	26.9	26.3	0.18
ROA (original K/L ≥ 2) + pain	58	3221	79%	57%	0.001	69.9	65.8	$3x10^{-6}$	26.7	26.3	0.38
ROA (original K/L ≥ 3) + pain	23	3256	70%	58%	0.26	70.0	65.8	0.003	26.4	26.3	0.88

P: P-value; OA: osteoarthritis; K/L: Kellgren and Lawrence score; JSN: joint space narrowing; BMI: body mass index; RS-I: Rotterdam Study-I

TREAT-OA consortium is the original K/L score (11) defined as "definite osteophytes and possible joint space narrowing" at the tibio-femoral (TF) joint. If studies did not score possible JSN as a separate feature, the definition used was: "at least 2 definite osteophytes OR one definite osteophyte plus definite JSN". Hip ROA, which was the most poorly specified in the original scores, was defined as "at least definite joint space narrowing". For hand ROA, consensus was not reached within the consortium, due to the fact that different studies graded different joints for hand OA, thus limiting the possibility to generate a single definition. As an alternative, thumb OA was put forward as an interesting phenotype to study, because of the high correlation with pain and disability (51). Consensus was reached on a definition for thumb OA which is "at least one definite osteophyte (= original K/L grade ≥ 2) in either the left or right first carpometacarpal (CMC1) joint".

In Table 5, the number of cases and controls for each study are given after standardization of phenotypes (both SOA and ROA). In total, there are 13,119 knee OA cases and 61,538 controls, 9,521 hip OA cases and 59,345 controls and 4,913 hand OA cases and 41,863 controls with DNA and phenotype data within the TREAT-OA consortium.

To evaluate the effect of standardization of the ROA phenotypes, we calculated the prevalence of knee and hip ROA in 8 Caucasian and 1 Japanese cohort study before and after standardization of the ROA definition. In Table 6, the mean age and BMI are shown for the 9 cohorts. The Framingham Osteoarthritis Study, The Hertfordshire Cohort Study, The Osteoporotic Fractures in Men Study, The Rotterdam study-I, the

Table 5. Number of cases (including incident cases) and controls in each study involved in the TREAT-OA consortium according to standardized phenotypes

Study	Knee OA		Hip OA		Thumb OA	
Radiographic OA	*cases*	*controls*	*cases*	*controls*	*cases*	*controls*
Chingford Study	80	560	34	702	356	620
Dentist & Teacher Study	-	-	-	-	36	507
Estonian Studies	70	441	-	-	-	-
Framingham Osteoarthritis Study	419	1,674	-	-	913	2,783
Hertfordshire Cohort Study	156	831	-	-	78	179
Japanese Cohort Study	226[1]	486	-	-	-	-
KANON	NA[1]	NA	-	-	-	-
LUMEN	152[1]	317	-	-	55	197
Osteoporotic fractures in men study	-	-	389	3,660	-	-
Rotterdam Study-I	1,017[2]	2,452	581[2]	3,183	868[3]	2,516
Rotterdam Study-II	NA[2]	NA	NA[2]	NA	NA[2]	NA
Rotterdam Study-III	136	922	NA[2]	NA	-	-
Study of Osteoporotic Fractures	-	-	364	3,668	-	-
The ROAD Study	541	2,426	-	-	-	-
TwinsUK	149	1,436	105	1,253	393	1,565
Subtotal radiographic OA	2946	11,545	1,473	12,466	2,699	8,364
Symptomatic/Radiographic OA						
Finnish OA cases	113	210	-	-	-[4]	-[4]
GARP	161	720	106	720	151	720
Subtotal symptomatic/ radiographic OA	274	930	106	720	151	720

NA: not applicable; [1]number of cases and controls unstandardized;[2]complete dataset available summer 2010; [3]scoring of radiographs in progress, complete dataset available in 2011; [4]available in the near future

ROAD Study and the Study of Osteoporotic Fractures are on average 14 years older than The Chingford Study, the Rotterdam Study-III and TwinsUK.

The result of the standardization of knee and hip OA phenotypes is shown in Figure 1. Results for the thumb OA phenotype are not shown since all studies use the same definition. The standardized hip OA definition is "at least definite JSN or a THR visible on the radiograph due to primary OA". In the SOF and MrOS Study a minor adjustment was made and hip ROA was defined as: "at least medial JSN (grade≥3) or lateral JSN (grade≥2) or a THR visible on the radiograph due to primary OA". The

Table 5. Continued

Study	Knee OA		Hip OA		Thumb OA	
Symptomatic OA	*cases*	*controls*	*cases*	*controls*	*cases*	*controls*
arcOGEN consortium	4,287[5]	4,287	4,107[5]	4,107	-	-
Chinese Case-Control Study	1,200[5]	1,500	200	1,500	-	-
deCODE	1,033	32,482	1,571	32,482	1,822	32,482
Greek clinical cases	228	344	67	344	-	-
Health 2000	237	6,048	132	6,151	-	-
Japanese Case-Control Study	900	3,400	-	-	-	-
Malmo Diet Cancer Study	471	471	551	551	-	-
Nottingham Case-Control	1,355[5]	237	1,011[5]	730	-	-
Spanish clinical cases	188	294	303	294	241[6]	294
Subtotal symptomatic OA	9,899	49,063	7,942	46,159	2,063	32,776
Total	**13,119**	**61,538**	**9,521**	**59,345**	**4,913**	**41,863**

OA: osteoarthritis; [5]recruitment in progress; [6]hand OA according to ACR criteria, thumb OA definition not possible

standardized knee ROA definition is "at least definite osteophytes and possible JSN or a TKR visible on the radiograph due to primary OA".

Before standardization the prevalence of knee OA ranged between 10-55%, of hip OA between 2-33%. After standardization the prevalence of knee OA ranged from 8-25% and hip OA between 4-10%. When comparing cohorts with the same age range, the prevalence of knee ROA was 8-12% in the younger cohorts and 16-25% in the cohorts with subjects of an older age. To show that the differences in age are indeed the cause of the lower prevalence of knee OA in 3 cohort studies, we studied the prevalence of knee ROA in one relatively young and one old cohort with a wide age range, respectively TwinsUK and RS-I. The prevalence of knee ROA ranged from 10-15% in subjects aged 65 years and younger. In subjects aged 65 years and older, the prevalence ranged from 29-34% for the 2 studies.

DISCUSSION

A wide range of OA definitions were used in the 28 studies participating in the TREAT-OA consortium. Since heterogeneity in phenotype definitions will reduce power to find consistent associations, radiographic OA phenotypes were standardized within the consortium.

There are some research fields in which specific attention is given to phenotype definitions. This mainly concerns studies in the field of neuroscience (i.e., bipolar

Table 6. Baseline characteristics of 6 cohort studies with ROA phenotypes involved in the standardization process

Study	Mean age (range)	Mean body mass index (range)
Chingford Study	54 (44-67)	26 (17-47)
Framingham Osteoarthritis Study	64 (29-93)	26 (14-54)
Hertfordshire Cohort Study	65 (59-71)	27 (17-48)
Osteoporotic Fracture in Men Study	77 (69-97)	27 (18-50)
ROAD Study	70 (23-94)	23 (13-37)
Rotterdam Study-I	68 (55-94)	26 (15-59)
Rotterdam Study-III	57 (45-89)	28 (14-57)
Study of Osteoporotic Fractures	71 (65-91)	27 (16-59)
TwinsUK	54 (37-76)	25 (15-51)

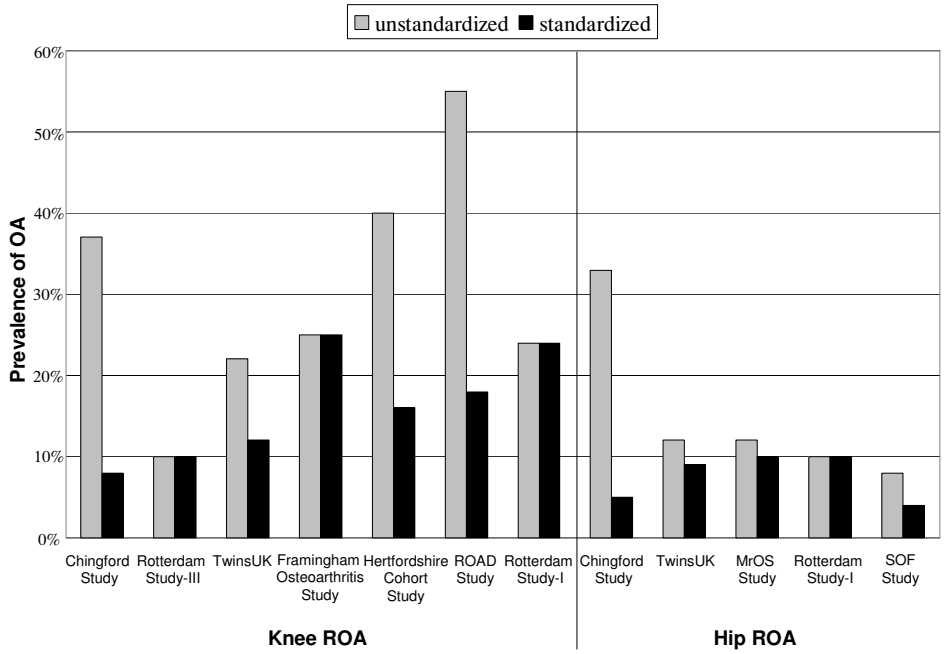

Figure 1. Prevalence of knee and hip OA before and after standardization of the radiographic OA (ROA) phenotypes.

disorder or schizophrenia) (52) and obesity (53). In contrast, published research involving osteoarthritis, osteoporosis and heart disease does not usually discuss phenotype definitions. Our results showed that OA definitions should be standardized since association results differ when varying ROA and SOA definitions are used within the same study. In addition, it was recently shown that the ability to detect hip

OA genetic associations is influenced by proper phenotyping (54). We showed by standardizing of ROA phenotypes, that similar ROA prevalence's could be obtained.

For hip ROA, a distinction can be made between atrophic OA (presence of JSN without osteophytes), hypertrophic OA (presence of osteophytes without JSN) or a composite score (both JSN and osteophytes) (55). It is known that these different forms of hip ROA have different risk factors (56-57). In addition, atrophic OA shows to be a more progressive form of OA than hypertrofic OA (58). Since some studies interpret a K/L score ≥ 2 as one definite osteophyte, whereas other studies interpret this as definite JSN and one definite osteophyte, a difference in association results would be expected. Although the standardized definition agreed upon by the consortium is based on JSN (hip ROA = at least definite JSN, with or without osteophytes), a majority of the subjects (78 and 80% in the Rotterdam Study-I and III, respectively) have both JSN and osteophytes. This definition can therefore also be seen as a composite score. Although less often used than the composite score of hip ROA, hypertrophic hip and atrophic hip ROA definitions should also be standardized. We suggest using "presence of at least one definite osteophyte at the femoral head without definite JSN" as preferred definition for hypertrofic OA and "definite JSN without the presence of any osteophytes at all locations" as atrophic OA which was also used in a previous study by Javaid *et al*. (55).

It was difficult to reach consensus on the hand ROA definition, since different studies scored different joints. To overcome this problem, a subtype for clinically relevant OA was suggested within the consortium: thumb OA, associated with pain and disability (51, 59), will be used within the consortium. The definition of ROA of the thumb is "at least one definite osteophyte in either right or left CMC1 joint". We recommend for future studies on ROA to always specify the exact OA definition. A statement such as "we defined OA as a K/L ≥ 2" should be avoided or the interpretation of this K/L score should be given.

Since all studies involved in the consortium defined SOA differently, or at least assessed the OA status differently, it is likely that heterogeneity is a problem in studies on SOA. Standardization of SOA would in principle be possible if studies had pain, clinical assessment data for study subjects, as well as radiographic grade for the index joints, age, BMI, for both cases and controls. The design of some studies is such however that there is no radiographic characterization for cases and controls, which is necessary if SOA would be defined based on both symptoms and radiographs, and only a diagnosis of TJR for an indication of OA is present. These are extant studies and to collect homogenous SOA studies would require a huge investment of resources as well as time. However, there remains a lack of consensus and guidelines about how SOA should be assessed. For example, the American College of Rheumatology (ACR) defines signs of OA as stiffness <30 minutes, crepitus, bony tenderness, bony enlarge-

ment, no palpable warmth and pain in or around the joint. The presence of these traits in subjects over the age of 50 (preferably accompanied by radiographic evidence of OA) is commonly used in the design of randomized clinical trials (RCTs) (60). But these criteria were developed in a clinic setting so the sensitivity and specificity of a diagnosis based on these criteria in a community or primary care settings, are as yet unknown.

Most of the SOA cases included in the TREAT-OA consortium are total joint replacement cases with a primary indication of OA. Although it is possible to define TJR as the main clinical outcome representative of severe symptomatic large joint OA in itself, as has been proposed for RCTs (61), this might not be the best option. Recent studies on this topic have revealed considerable heterogeneity in the radiographic severity, functional disability and pain suffered by TJR candidates (62). In addition, the pain and disability components among subjects undergoing TJR are significantly correlated with risk factors that also impact on ROA such as BMI, age, sex, whilst being poorly correlated with radiographic severity (62-63). Further, not all patients with severe symptomatic OA can or are willing to get a TJR either because of lack of access to healthcare, or they may be afraid of surgery, or have co-morbidities that make them ineligible etcetera (64). TJR patients are usually recruited in secondary care settings and might in some instances represent a non-random subset of severe symptomatic OA. In summary, additional research is needed to reach consensus for in- and exclusion criteria and definitions of clinical/symptomatic OA studies. We suggest that more thought should be given to the establishment of clear guidelines for future research using symptomatic OA cohorts, as this would have implications not just for genetic studies, but also for the assessment of biomarkers, imaging and interventional studies.

Genome-wide association studies (GWAS) and meta-analyses have been (1, 65) and will continue to be performed within the TREAT-OA consortium in order to identify genes consistently associated with risk and progression of OA. Presently, there are few genes discovered for OA by means of GWAS, and this may be explained by heterogeneity of phenotypes and the limited sample size used in the discovery GWAS samples up to now. For example, in a previous GWAS, ROA and SOA definitions were used within one meta-analysis (1). It has been shown before that ROA shows only modest correlation with clinical features of OA (66-67). In addition, we showed in this study that the association between SOA and age, gender and BMI is different compared to ROA. Although the sample size would decrease using stratification methods, the statistical power might increase if there is a reduction in the heterogeneity in the phenotype definition. Therefore, we recommend that for future GWASs additional work is needed to standardize or stratify on ROA and SOA.

Fortunately, in the TREAT-OA consortium studies on ROA have access to the source material and individual features of ROA are scored separately. This enables us to easily establish standardized phenotypes across cohorts.

Additionally, other phenotypes or possible predictors such as hypertrophic vs. atrophic forms of OA, joint shape, MRI based features, severe ROA (K/L≥3 versus K/L=0) or generalized OA may expand our definitions of the OA phenotypes and may increase the number of consistent associations in genetic studies. However, consensus among OA epidemiologist on OA phenotypes should be reached within the OA field, prior to the performance of these association studies.

In conclusion, standardization of radiographic OA phenotypes was carried out in the TREAT-OA consortium to reduce heterogeneity as much as possible. Standardization of symptomatic OA phenotypes, although desirable, was not possible due to the case-control study design of the studies. In the future, more precise OA phenotypes and stratification according to symptomatic and radiographic OA phenotypes are highly recommended.

RECOMMENDATIONS

1	Future studies on OA should always specify the exact OA definition. A statement such as "we defined OA as a K/L ≥ 2" should be avoided or the interpretation of this K/L score should be given.
2	The use of standardized ROA definitions is recommended in association studies with knee ROA defined as "at least 2 moderate definite osteophytes and possible JSN at the tibio-femoral joint", hip ROA as "at least definite JSN" and thumb ROA as "at least one moderate definite osteophyte at the CMC1 joint".
3	Atrophic hip ROA is suggested to be defined as "definite JSN without the presence of any osteophytes at all locations" and hypertrophic hip ROA as "presence of at least one moderate definite osteophyte at the femoral head without definite JSN".
4	Consensus is needed on in- and exclusion criteria and phenotype definitions of SOA studies. More thought should be given to the establishment of clear guidelines for future research using clinical OA cohorts
5	For future GWASs additional work must be done to stratify on age/BMI and especially ROA and SOA.
6	Expansion of OA phenotypes is not discouraged. Other phenotypes such as joint shape, MRI based features, severe ROA (K/L ≥ 3 versus K/L = 0) or generalized SOA/ROA may expand our definitions of the OA phenotypes, but consensus among OA epidemiologist on these new OA phenotypes should be reached, prior to the performance of these association studies.

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Chapter 3.2

A genome-wide association study identifies an osteoarthritis susceptibility locus on chr7q22

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ABSTRACT

Background: Osteoarthritis (OA) is the most common, incurable, age-related disease of the synovial joints. The aim of this study was to identify novel genes involved in OA by means of a genome-wide association study (GWAS).

Methods: We tested 500,510 Single Nucelotide Polymorphisms (SNPs) in 1,341 OA cases and 3,496 Dutch Caucasian controls. SNPs associated with at least two OA phenotypes were analysed in 14,938 OA cases and approximately 39,000 controls. The meta-analyses were performed using the program Comprehensive Meta-analysis, a P-value $<1x10^{-7}$ was considered genome-wide significant.

Results: The C-allele of rs3815148 on chromosome 7q22 (MAF 23%, intron 12 of the *COG5* gene) was associated with a 1.14-fold increased risk (95%CI 1.09-1.19) for knee and/or hand OA ($P=8x10^{-8}$), and also with a 30% increased risk for knee OA progression (95%CI: 1.03-1.64, $P=0.03$). This SNP is in almost complete linkage disequilibrium with rs3757713 (68kb upstream of *GPR22*) which is associated with *GPR22* expression levels in lymphoblast cell lines ($P=4x10^{-12}$). Immunohistochemistry experiments showed absence of Gpr22 in normal mouse articular cartilage or synovium. However, Gpr22 positive chondrocytes were found in the upper layers of the articular cartilage of mouse knee joints that were challenged by in vivo papain treatment or in the presence of interleukin-1 driven inflammation. Gpr22 positive chondrocyte-like cells were also found in osteophytes in instability-induced OA. Since the *GPR22* gene encodes a G-protein-coupled receptor, this is potentially an interesting drug target.

Conclusions: Our findings reveal a novel common variant on chromosome 7q22 to influence susceptibility for prevalence and progression of OA.

INTRODUCTION

Osteoarthritis (OA), the most common degenerative, age-related disease of the synovial joints, is commonly characterized by cartilage degradation, formation of osteophytes and subchondral sclerosis. Unfortunately, there are no curative and hardly any symptomatic treatment options available for this disease. OA is a complex disease in which both environmental and genetic factors play an important role. Primary OA has an estimated heritability of 40% for the knee, 60% for the hip and 65% for the hand (1). Identifying the genes underlying the genetic background could give new insights into the pathophysiology of OA and could potentially lead to new drug targets.

To date, genetics of OA mainly focuses on genome-wide linkage and candidate gene studies. Results of these studies have been controversial due to lack of power and replication. Currently, only two genes have been found consistently associated with OA in Caucasians: Growth Differentiation Factor 5 (*GDF5*) (2) and Iodothyronine-Deiodinase enzyme type 2 (*DIO2*) (3). Recently, a novel approach has become available to search for important risk genes involved in complex traits: a hypothesis-free genome-wide association study (GWAS). The first pooled-DNA GWAS for OA discovered the C-allele of the rs4140564 Single Nucleotide Polymorphism (SNP) (minor allele frequency (MAF) 8%), 75kb upstream of the Prostaglandin-Endoperoxide Synthase 2 (*PTGS2*) gene, to be associated with prevalent knee-OA in Caucasian women (n=387 cases and 255 controls in pooled GWAS and 1,177 cases and 2,372 controls in the replication studies) (4). In addition, Miyamoto *et al*. (5) identified the SNP rs7639618 in the Double Von Willebrand factor A (*DVWA*) gene to influence susceptibility in knee OA (using 94 cases and 658 controls in the discovery study), but this could not be replicated in Caucasian populations with in total 2,119 knee OA cases, 2,325 hip OA cases and 3,313 controls (6,7).

We here report a GWAS in the population-based prospective Rotterdam Study on hip, knee, and hand OA with replication in 11 additional studies with in total 14,938 cases and approximately 39,000 controls (depending on the OA phenotype studied). Since OA can occur at different joint sites, we tried to identify SNPs that showed a consistent pattern of association across multiple joint sites. This method will likely identify more general susceptibility genes for OA rather than site-specific genes. In addition to the relation with prevalence of OA, we also examined the relationship between the genome-wide significant SNP(s) and progression of knee OA.

MATERIALS AND METHODS

Study design

In Figure 1, we present a flow chart of the study design. We used a two-stage design to test the association of 500,510 SNPs with hip, knee, and hand OA and urinary urinary C-terminal cross-linked telopeptide of type II collagen (CTX-II) levels, a marker for cartilage degradation. Within the first stage, the SNPs were tested for association with the four OA phenotypes in women of the Rotterdam Study to identify signals that were consistent across multiple OA phenotypes. Data were available on genotypes and phenotypes of 248 hip OA cases and 1,411 controls, 515 knee-OA cases and 1,047 controls and 578 hand-OA cases and 1,038 controls. If one of the following criteria was fulfilled, the SNP was followed up into the replication stage: 1) hip, AND knee, AND hand OA AND CTX-II levels with a P-value <0.05, or 2) one of the radiographic OA phenotypes with a P-value $<10^{-4}$ AND one other radiographic OA phenotype with a P-value <0.05. The direction of the association had to be the same for the different phenotypes, i.e., if a SNP was associated with an increased risk for knee OA, there also had to be an association with an increased risk of hand OA and/or hip OA. In total, 12 loci were selected on the basis of these criteria and were tested

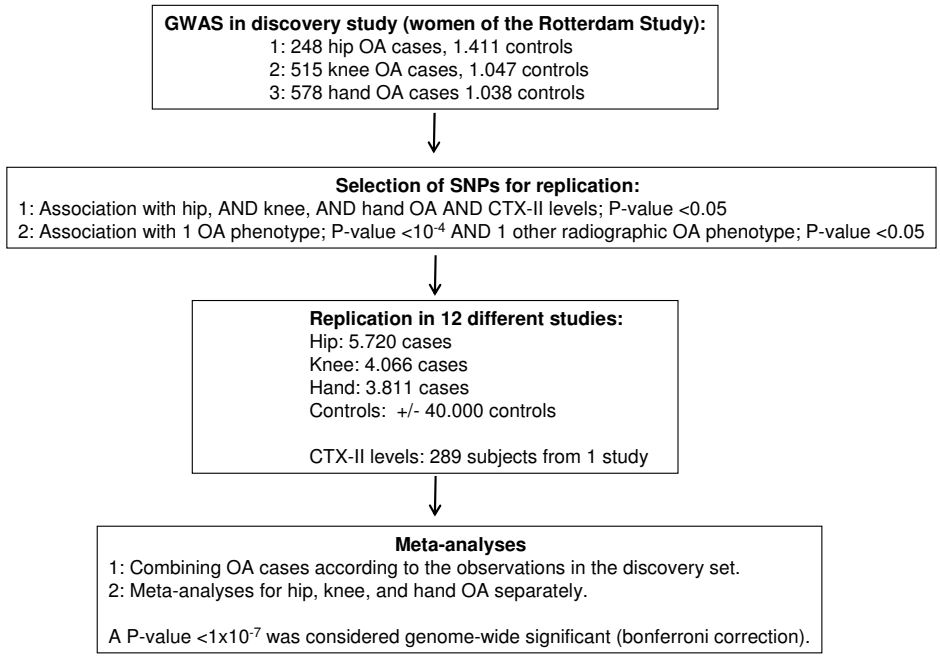

Figure 1. Flow chart of the study design.

for association with OA in 11 additional studies. In total, there were 5,968 hip OA cases and 40,420 controls, 4,581 knee OA cases and 38,730 controls, and 4,389 hand OA cases and 35,694 controls available for association analysis.

Phenotype definitions

For this study, radiographic definitions of OA were standardized as follows: knee OA was defined as a Kellgren/Lawrence (K/L) score >=2 (definite osteophytes and possible joint space narrowing), hand OA as 2 out of 3 hand joint groups (distal interphalangeal joints/proximal interphalangeal joints/carpometacarpal joint) affected with a K/L score >=2 (definite osteophyte) and hip OA as definite joint space narrowing. Controls were defined as having a K/L<2 for the knee and hip, respectively. Subjects with hand data were a control if they did not fulfill the criteria for hand OA. Subjects affected with hand OA, but not with knee OA, were included as controls for the knee OA analyses. Clinical studies all used total joint replacement of the hip or knee as outcome for hip and knee OA.

The ACR criteria were used to define hand OA for the Spanish cases (8), deCODE used a definition of either squaring or dislocation of the thumb or at least two nodes at the DIP joints. In the Rotterdam Study and the Chingford Study progression of knee OA was defined as a K/L grade >=1 at baseline and an increase in at least one K/L grade during follow-up.

Study populations

Table 1 provides an overview of all populations participating in this study. An extensive description of the studies participating in this GWAS is given in Appendix 1.

Biochemical measurements

Urinary C-terminal cross-linked telopeptide of type II collagen (CTX-II), a marker of degradation of mineralized cartilage was measured as described before (9,10). The concentration of CTX-II (ng/liter) was standardized to the total urine creatinine (mmol/liter).

Laboratory methods and quality control

GWAS Rotterdam Study (discovery study)*:* genotyping of the samples with the Illumina HumanHap550v3 Genotyping BeadChip was carried out at the Genetic Laboratory of the Department of Internal Medicine of Erasmus Medical Center, Rotterdam, the Netherlands. The Beadstudio GenCall algorithm was used for genotype calling and quality control procedures were as described previously (11). For this study, the following quality control filters were applied: SNP call rate >= 95%, minor allele frequency >= 5%, P-value HWE >=$1x10^{-6}$. After quality control 500,510 SNPs

Table 1. Baseline characteristics of studies included in the GWAS meta-analysis of OA-phenotypes

	Discovery	**Replication studies**										
	Rotterdam Study[1]	*deCODE*	*TwinsUK*	*Framingham Study*	*Chingford Study*	*GARP*	*Spanish cases*	*Greek TJR cases*	*Oxford Study*	*Nottingham case-control*	*SOF*	*MrOS*
Hip OA cases/controls	413/2474	1630/31654	33/425	NA	97/685	109/720	307/294	110/344	1402/871	929/236	541/1602	397/1540
Knee OA cases/controls	698/1893	1068/31654	53/476	419/1674	269/568	154/720	262/294	368/344	555/871	735/236	NA	NA
Hand OA cases/controls	832/2055	2725/31654	64/425	NA	286/546	241/720	241/294	NA	NA	NA	NA	NA
N CTX-II	1100	NA	NA	NA	289	NA	NA	NA	NA	NA	NA	NA
Case/control or cohort	cohort	case-control	cohort	cohort	cohort	case-control	case-control	case-control	case-control	case-control	cohort	cohort
OA definition	radiographic	clinical	radiographic	radiographic	radiographic	radiographic/ clinical	clinical	clinical	clinical	clinical	radiographic	radiographic
Mean age (range)	67(55-94)	69(19-99)	54(37-76)	64(29-93)	54 (44-67)	60(30-79)	66(32-94)	61(20-90)	67(55-89)	68(40-92)	70(65-90)	73(65-91)
Mean BMI (range)	26(16-56)	26(14-60)	25(15-51)	26(14-54)	26 (17-47)	27(19-47)	31(18-53)	26(17-34)	NA	28(15-52)	27(15-58)	28(18-51)
% women	59%	58%	100%	56%	100%	63%	66%	72%	55%	62%	100%	0%
GWA platform	Illumina HumanHap 550v3	Infinium HumanHap 300	Infinium HumanHap 300	Affymetrix GeneChip® Human Mapping	NA	NA	NA	NA	NA	NA	NA	NA

[1]number of cases and controls include both men and women of the Rotterdam Study; For the discovery set, only women are analysed, men are analysed as replication study; GWA: genome-wide association; OA: osteoarthritis; NA: not applicable/available

remained for association analyses. The intensity cluster plots were visually inspected for the top-hits of the Rotterdam Study and no abnormalities were discovered. Genomic inflation factors were calculated for all analyses and there was no evidence of population stratification with lambdas of 1.01 for hip and hand OA, 1.00 for knee OA and 1.02 for CTX-II levels.

Replication cohorts: For detailed information of laboratory methods and quality control we refer to the supplementary materials. In summary, all samples of deCODE were assayed with the Infinium HumanHap300 or humanCNV370 SNP chips (Illumina). Samples of TwinsUK were genotyped with the Infinium HumanHap 300 assay (Illumina, San Diego, USA) at the Duke University Genotyping Center (NC USA), Helsinki University (Finland) and the Wellcome Trust Sanger Institute. The Framingham Osteoarthritis Study genotyped all individuals using the Affymetrix GeneChip® Human Mapping 500K array set and the 50K supplemental array set focused on coding SNPs and SNPs tagging protein-coding genes (Santa Clara, California) as part of the SHARE initiative. All other studies were genotyped using the MassArray iPLEX Gold from Sequenom for the replication of the top-hits of the Rotterdam Study except for the Study of Osteoporotic Fractures (SOF) and MrOS (Osteoporotic Fractures in Men) study which were genotyped using Taqman.

Statistical analysis

Association analyses: All associations with OA phenotypes for all non-familial studies were tested using an allelic chi-square test (1df) assuming an additive effect for each SNP tested. Analyses for the Rotterdam Study, Chingford Study, Oxford cohorts, the Nottingham case-control study, the Spanish TJR and hand OA cases and the Greek TJR cases, were carried out using PLINK V1.02 software (12). CTX-II levels were log-transformed and converted to z-scores adjusted for age and subsequently analysed using an allelic chi-square test (1df). The SOF and MrOS Study performed logistic regression analyses using the program SAS version 9. deCODE used a likelihood procedure described in a previous publication for the association analyses (13) to calculate two-sided P-values and odds ratio for each individual allele, assuming a multiplicative model for risk, i.e., that the risk of the two alleles a person carries multiply. Some of the individuals in the Icelandic case-control groups were related to each other, causing the $\chi 2$ test statistic to have a mean >1 and median $>0.675^2$. In addition, the inflation factor was estimated by using genomic controls by computing the median of the 300754 $\chi 2$ statistics and dividing it by 0.675^2 as previously described (14). For all analyses of deCODE adjustments for the inflation factor were made.

For the TwinsUK study, robust standard errors were calculated and logistic regression was performed using the program Stata version 10 (Stata Corporation, USA) for hip, knee and hand OA. The analysis used in the Framingham Osteoarthritis Study is

a logistic regression using GEE (generalized estimating equations) to control for the dependency among family members. Analyses were performed using R software.

In the GARP study, standard errors were estimated from the variance between the sibling pairs (robust standard errors) to adjust for the family relationship among sibling pairs (2). Robust standard error analyses using Stata SE8 software (Stata Corporation, USA).

In the Rotterdam Study and the Chingford Study, the rs3815148 SNP was also tested for association with progression of knee OA using a logistic regression model adjusted for gender and follow-up time in SPSS version 15.0.

Meta-analysis: A SNP-specific meta-analysis was performed for the combined- and individual phenotypes using the program Comprehensive Meta-analysis by Biostat (www.meta-analysis.com). For the combined phenotype meta-analyses, the analyses were performed on, for example, hand and/or knee OA cases versus controls that had neither hand nor knee OA, if in the discovery study a SNP was associated with knee and hand OA using the criteria specified in the methods section earlier. With this method of analyses, no overlap between cases or controls existed in the analyses. All OA cases from the replication studies were included in the meta-analyses when applicable. For example, if a study had hip, knee and hand OA cases and in the discovery study a SNP was associated with only hip and knee OA, then the hip and knee OA cases were used as cases in the meta-analyses. If the heterogeneity metric I^2 exceeded 25% a random-effects model (DerSimonian and Laird) was also used for the analysis, otherwise only a fixed effects model (inverse variance method) was applied. The analyses were performed on the total population of all studies and were subsequently stratified for gender to reveal, if any, gender-specific associations. For family-based studies, association results of men and women combined are shown because of relatedness among men and women in the study. A P-value $<1x10^{-7}$ was considered genome-wide significant (=bonferroni correction: 0.05/500,510).

Association analyses with previously published SNPs: Within the Rotterdam Study, allelic chi-square tests (1df) assuming an additive model were performed for the SNPs discovered in previous studies which are mentioned in the introduction section. The rs225014 and rs12885300 SNPs of the *DIO2* gene, rs4140564 75kb upstream of the *PTGS2* gene, rs7639618 of the *DVWA* gene and rs6088813 of the *GDF5* gene were tested for association with hip, knee and hand OA. A P-value <0.05 was considered significant.

mRNA expression analyses and immunohistochemistry experiments

Synovial biopsies from patients undergoing arthroscopy of the knee for meniscal and cartilage injury at the Division of Orthopedics, University Hospitals Leuven, were

used for in vitro culture in Dulbecco's Modified Eagle Medium/10% serum, using plastic adherence as described by Lories *et al*. (15).

Histological and flow cytometric analysis revealed that the synovia did not show signs of chronic inflammation. Articular chondrocytes and meniscal cells were isolated from knee cartilage and meniscus obtained from patients with osteoarthritis undergoing knee replacement surgery as described previously (16). All procedures were approved by the Ethical committee for clinical research at KU Leuven and patient informed consent was obtained. Synovial fibroblast-like cells at passage 4 and freshly isolated chondrocytes (P0) were used for quantitative PCR using Assays on Demand provided by Applied Biosystems. Gene expression levels were determined by 2-delta Ct relative to the housekeeping gene beta-actin. Immunohistochemistry was performed on mouse knee tissue after induction of papaïn, methylated bovine serum albumin induced arthritis or medial menicus instability induced arthritis as described before (17,18). Rabbit anti-Gpr22 antibody was obtained from Sigma-Aldrich and used at 5 µg/ml overnight. Secondary antibody was peroxidise-conjugated goat anti-rabbit (Jackson Immunoresearch Laboratories).

RESULTS

Association results

In the first stage, 500,510 SNPs were tested for association with osteoarthritis in women of the Rotterdam Study. Because power to pick up genome-wide associations with individual phenotypes was limited, we applied a strategy which uses the advantage of the multiple phenotypes available in our discovery study (see methods) to search for common variants showing consistent associations with OA at multiple joint sites. In total, 18 SNPs in 12 loci were identified using this approach (Table 2).

In the second stage, these 12 loci were tested for association with OA in 11 additional studies. The 12 loci were tested for association with the OA-phenotype that formed the basis for selection in the discovery phase. In total, there were 5,968 hip OA cases and 40,420 controls, 4,581 knee OA cases and 38,730 controls, and 4,389 hand OA cases and 35,694 controls available for association analysis. In Table 3 the results of the meta-analysis are shown for these 12 SNPs for the combined phenotypes and in Supplementary Table 1 in Appendix 2 for the individual phenotypes. From these 18 SNPs, we observed 3 loci to be significantly associated with OA. The C allele of the SNP rs3815148 (A>C, MAF 23%) annotated in intron 12 of the component of oligomeric golgi complex 5 (*COG5*) gene, was associated with knee and/or hand OA in the discovery study (Table 3) and also in the meta-analysis with an OR of 1.14, 95% confidence interval (CI) 1.09-1.19, a P-value of $8x10^{-8}$ and

Table 2. Identified top-hits of the GWAS in the Rotterdam Study

							Radiographic OA						uCTX-II	
rs-number	**locus**	**chr**	**bp-position**	**MAF**	**annotation**		**P-knee**		**P-hip**		**P-hand**			
Novel loci					*Nearest Gene*	*Location*	*P*	*OR*	*P*	*OR*	*P*	*OR*	*P*	*beta*
rs10465850	1	1	65471791	0.46	JAK1	-347kb	0.009	1.22	0.02	1.26	0.01	1.20	0.02	1.07
rs12402320	2	1	152968030	0.30	KCNN3	intronic	0.03	1.19	0.04	1.23	0.01	1.22	0.01	1.07
rs4656364	3	1	160545479	0.10	NOS1AP	intronic	0.04	1.29	$9x10^{-6}$	1.88	0.96	1.01	0.49	1.02
rs3963342	4	4	43995005	0.33	KCTD8	intronic	0.04	1.18	0.02	1.26	0.03	1.18	0.04	1.05
rs10248619	5	7	50718584	0.21	GRB10	intronic	0.002	1.33	0.03	1.28	0.04	1.21	0.01	1.10
rs7791286	5	7	50824286	0.17	GRB10	intronic	0.02	1.25	0.02	1.33	0.01	1.28	0.03	1.07
rs3815148	6	7	106725656	0.23	COG5	intronic	$7x10^{-5}$	1.42	0.81	1.03	0.05	1.18	0.96	1.00
rs1548524	6	7	106731799	0.24	COG5	intronic	$9x10^{-5}$	1.41	0.72	1.05	0.04	1.19	0.96	1.00
rs997311	6	7	106740269	0.24	COG5	intronic	$8x10^{-5}$	1.41	0.74	1.04	0.03	1.20	0.96	1.00
rs959396	7	9	37001289	0.38	PAX5	intronic	$3x10^{-6}$	0.69	0.04	0.81	0.93	0.99	0.91	0.95
rs12352822	8	9	119587103	0.15	TLR4	-69kb	$2x10^{-5}$	0.60	0.001	0.57	0.30	0.89	0.19	0.95
rs873598	9	10	120413174	0.45	GPR10	-68kb	0.002	0.79	0.004	0.75	0.007	0.82	0.02	0.95
rs880844	10	12	107486497	0.24	ISCU	intronic	0.02	1.23	$3x10^{-5}$	1.56	0.16	1.13	0.18	1.05
rs741542	10	12	107488184	0.24	ISCU	3'down-stream	0.02	1.23	$3x10^{-5}$	1.56	0.16	1.13	0.18	1.05
rs11651351	11	17	71478457	0.07	ACOX1	intronic	0.02	0.68	0.02	0.57	0.03	0.70	0.01	0.89
Known Loci														
rs4911494	12	20	33435328	0.40	GDF5/UQCC	coding/ intron	0.03	0.84	0.91	1.01	$2x10^{-5}$	0.72	0.40	0.98
rs6088813	12	20	33438595	0.40	GDF5/UQCC	intronic	0.02	0.84	0.91	1.01	$1x10^{-5}$	0.72	0.40	0.98
rs6087705	12	20	33464664	0.40	GDF5/UQCC	5'upstream	0.03	0.84	0.91	1.01	$1x10^{-5}$	0.72	0.40	0.98

Chr: chromosome; Bp: base pair; MAF: minor allele frequency; OR: odds ratio; P-values are from allele-based association analysis

Table 3. Meta-analysis results: association of the 12 top hits with OA phenotypes

SNP	Chr	Minor allele	MAF	Annotation		Association with OA		uCTX-II (beta)	
				nearest gene	*location*	*OR (95%CI)*	*P*	*beta*	*P*
rs10465850	1	T	43%	JAK1	-347kb	1.02 (0.98-1.05)[1]	0.42	-0.99[a]	0.82
rs12402320	1	T	27%	KCNN3	intron	1.05 (0.99-1.12)[1]	0.12	1.07	0.005
rs4656364	1	C	7%	NOS1AP	intron	1.00 (0.89-1.13)[2,4]	0.95	1.00	0.97
rs3963342	4	C	25%	KCTD8	intron	1.04 (0.97-1.12)[1,4]	0.29	1.05	0.07
rs10248619	7	T	19%	GRB10	intron	1.09 (1.04-1.14)[1]	$5x10^{-4}$	1.05[1]	0.26
rs3815148	7	C	23%	COG5	intron	1.14 (1.09-1.19)[3]	$8x10^{-8}$	0.98[1]	0.37
rs959396	9	G	38%	PAX5	intron	0.94 (0.86-1.04)[2,4]	0.26	0.95	0.01
rs12352822	9	G	10%	TLR4	-69kb	0.99 (0.86-1.15)[2,4]	0.93	1.01[1]	0.89
rs873598	10	T	49%	GPR10	-68kb	0.98 (0.91-1.06)[1,4]	0.65	0.95	0.03
rs741542	12	T	24%	ISCU	3'downstream	1.03 (0.91-1.16)[2,4]	0.65	1.02	0.43
rs11651351	17	T	7%	ACOX1	intron	0.89 (0.74-1.08)[1,4]	0.24	0.91[1]	0.33
rs6088813	20	C	35%	GDF5	intron	0.91 (0.84-0.98)[3,4]	0.01	0.99	0.65

Chr: chromosome; MAF: minor allele frequency (HapMap); OA: osteoarthritis; OR: odds ratio; CI: confidence interval; [1]association with hand and/or hip and/or knee OA; [2]hip and/or knee OA; [3]hand and/or knee OA; [4]random effects model

consistent results across the datasets for knee and/or hand OA (P-value Q-statistic for heterogeneity=0.39, I^2=5%).

The SNPs rs10248619 (T>C, MAF 19%, situated in the *GRB10* gene) and rs6088813 (A>C, MAF 35%, situated in the promoter region of the *GDF5* gene) were both associated with OA in the meta-analysis, but did not reach genome-wide significance (*P*=0.0004 and *P*=0.01, respectively). There were no gender-specific associations observed.

Given its significant associations with 2 OA phenotypes in the discovery study and the meta-analysis (knee and/or hand OA), we further focussed on SNP rs3815148.

In Figure 2 the forest plot for the association of the C allele of rs3815148 with knee and/or hand OA is given. The estimated effect sizes were similar for knee OA alone (OR 1.16) and hand OA alone (OR 1.14). Interestingly, this SNP was also associated with progression of knee OA in the Rotterdam Study (OR 1.31, 95%CI: 1.01-1.69) and a non-significant trend was observed in the Chingford Study (OR 1.26, 95%CI: 0.73-2.17) (Supplementary Figure 1 in Appendix 2). The meta-analysis for progression of knee OA combining the results of the Rotterdam Study and the Chingford Study showed an OR of 1.30 per C-allele (95%CI: 1.03-1.64, *P*=0.03). We performed a sensitivity analysis in which the Rotterdam Study as discovery study was excluded from the meta-analysis of the SNP rs3815148 and observed that the association remained highly significant (OR 1.12, 95%CI: 1.06-1.18, $P=3x10^{-5}$).

Females	Nr cases	Nr controls	OR and 95% CI	P-value
Rotterdam Study (discovery study)	961	1.343	1.32 (1.15-1.51)	$7x10^{-5}$
deCODE	2.625	17.886	1.14 (1.06-1.22)	$4x10^{-4}$
GARP	462	818	1.06 (0.80-1.40)	0.68
Framingham Osteoarthritis Study	246	926	0.85 (0.67-1.07)	0.17
Chingford Study	432	407	1.03 (0.81-1.31)	0.80
Oxford cohorts	320	376	1.32 (1.03-1.68)	0.03
Greek TJR cases	305	196	1.16 (0.80-1.68)	0.43
Spanish cases	422	271	1.12 (0.75-1.68)	0.59
TwinsUK	169	381	1.01 (0.74-1.38)	0.95
Nottingham case-control study	432	108	1.08 (0.77-1.51)	0.67
Overall effect females (random-effect: I^2 = 34%)	**6.374**	**22.712**	**1.12 (1.03-1.22)**	**$6x10^{-3}$**
Males				
Rotterdam Study	393	1.268	1.09 (0.90-1.31)	0.39
deCODE	835	14.596	1.20 (1.05-1.36)	$5x10^{-3}$
GARP	110	622	1.00 (0.58-1.71)	1.00
Framingham Osteoarthritis Study	173	748	1.13 (0.86-1.49)	0.38
Oxford cohorts	218	448	1.04 (0.79-1.37)	0.77
Greek TJR cases	63	148	0.66 (0.35-1.26)	0.21
Spanish cases	77	274	1.28 (0.79-2.10)	0.32
Nottingham case-control study	310	101	1.10 (0.76-1.58)	0.60
Overall effect males (fixed-effect: I^2 = 0%)	**2.179**	**18.205**	**1.13 (1.04-1.23)**	**$6x10^{-3}$**
Overall effect replication studies (fixed-effect: I^2 = 0%)	**7.423**	**39.193**	**1.12 (1.06-1.18)**	**$3x10^{-5}$**
Overall effect all studies (fixed-effects I^2 = 5%)	**8.384**	**40.536**	**1.14 (1.09-1.19)**	**$8x10^{-8}$**

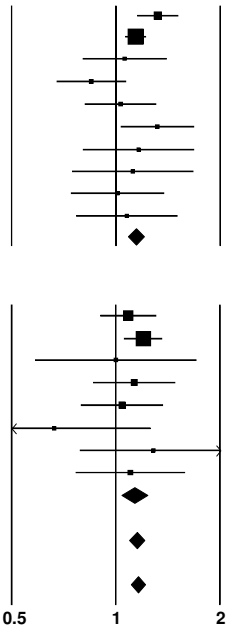

Figure 2. Forest plot for the association of the C allele of rs3815148 with hand and/or knee osteoarthritis (allelic model).

Functional analysis for the rs3815148 SNP

Although this SNP is annotated in the *COG5* gene, there is a very large linkage disequilibrium (LD) block of over 500 kb in which this SNP resides with >400 SNPs which are highly correlated (Supplementary Figure 2 in Appendix 2 and in color in Appendix 3) (19). The functional variant is likely to be one of the variants in LD with rs3815148 and could be located in any of the five genes annotated within this region, component of oligomeric golgi complex 5 (*COG5*), HMG-box transcription factor 1 (*HBP1*), dihydrouridine synthase 4-like (*DUS4L*), protein kinase, cAMP-dependent, regulatory, type II, beta (*PRKAR2B*) or G protein-coupled receptor 22 (*GPR22*). Therefore, we performed *in silico* and *in vitro* functional analysis to try to identify the gene underlying this association.

There are no human mutations reported in the *COG5*, *HBP1*, *DUS4L*, *PRKAR2B* and *GPR22* genes resulting in clinical phenotypes. Expression databases show that *DUS4L*, *COG5* and *HBP1* are ubiquitously expressed, with *PRAKR2B* especially in the immune system and brain and *GPR22* mainly in heart and brain (http://www.genecards.org/). Furthermore, *DUS4L*, *COG5* and *HBP1* are expressed in cartilage according to the integrated cartilage gene database (http://bioinfo.hku.hk/iCartiGD/main), whilst *GPR22* and *PRKAR2B* are not present in this database. For the *GPR22* gene and the *PRKAR2B* gene, animal models are described in the literature, but skel-

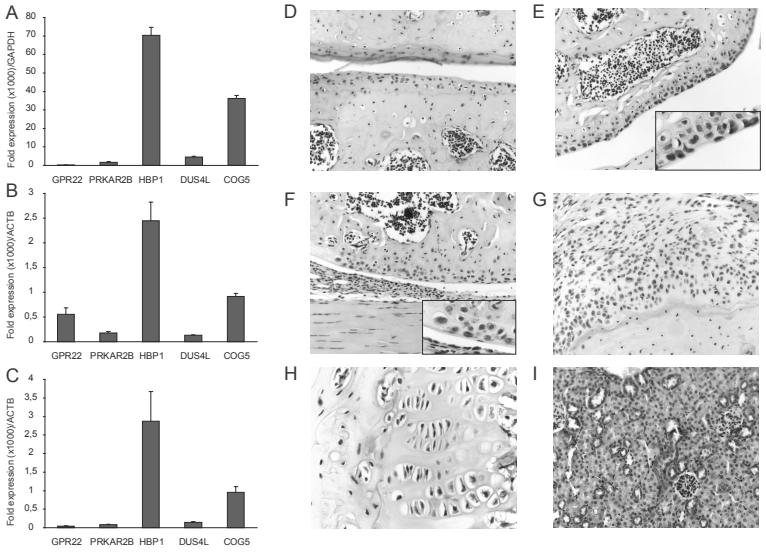

Figure 3. (**A**) Quantitative PCR analysis of gene expression levels (*Gpr22*) in expanded synovial fibroblasts (n=3 sets), (**B**) freshly isolated chondrocytes (n=5 sets) and (**C**) meniscal cells (n=3 sets). Data are shown as mean +/- SEM. Fold expression is multiplied by 1000. (**D**) Immunohistochemistry shows the absence of Gpr22 protein in normal articular cartilage. (**E** + insert) Gpr22 positive chondrocytes (brown cytoplasm) are found in articular chondrocytes of mice with papain-induced osteoarthritis, (**F** + insert) in mBSA induced arthritis, (**G**) and in developing osteophytes in instability induced osteoarthritis. No Gpr22 protein signal is found in the synovium. (**H**) Growth plate chondrocytes were negative for Gpr22. (**I**) Kidney sections were used as a positive control. (Magnification 200x in D, E, F and I, 100x in G and 400x in H). A color figure is presented in Appendix 3.

etal and joint tissues were not studied in depth. It is known that knock-out mice of the *PRKAR2B* gene have diminished white adipose tissue (20) and show decreased physical activity (21), while *GPR22* gene knock-out mice develop heart failure in response to induced stress (22). Also, we tested whether rs3815148 or linked SNPs affected expression of any of the 5 genes in lymphoblast cell lines (n=400) (23) and/or in the cortex of the brain (n=193) (24) using publically available databases. In the expression database of the brain, only the *DUS4L* and *COG5* genes were represented and no clear significant associations were observed between SNPs in the region surrounding the rs3815148 SNP and expression levels of those 2 genes. In the lymphoblast cell lines, there was a significant association between rs3757713 (D'=1.0, r^2=0.95 with rs3815148) and expression levels of *GPR22* ($p=4x10^{-12}$, Supplementary Figure 3 in Appendix 2). We also studied mRNA expression levels of the 5 genes of interest in cells derived from several tissues of the joint. Expression analyses by quantitative PCR in cultured synovial fibroblasts and primary chondrocyte cultures showed detectable

transcripts of all 5 genes with *COG5* and *HBP1* being most abundantly expressed, while *DUS4L*, *PRKAR2B* and *GPR22* are very low in all cell types (Figure 3A-C, a color picture is depicted in Appendix 3).

As *GPR22* showed the only remarkable association with altered expression in the lymphoblast cell lines, we studied the presence of Gpr22 protein in knee joints from normal and osteoarthritic mice. Immunohistochemistry did not show Gpr22 positive cells in normal mouse articular cartilage or synovium. However, Gpr22 positive chondrocytes were found in the upper layers of the articular cartilage of mouse knee joints that were challenged by in vivo papain treatment or in the presence of interleukin-1 driven inflammation. Gpr22 positive chondrocyte-like cells were also found in osteophytes in instability induced osteoarthritis (Figure 3D-I, a color picture is depicted in Appendix 3).

Analysis of SNPs shown to be associated with OA from the literature

The SNPs rs225014 and rs12885300 situated in the *DIO2* gene, rs4140564 of the *PTGS2* gene and rs7639618 of the *DVWA* gene were tested for association with hip, knee and hand OA in the Rotterdam Study. Results for these SNPs and for the *GDF5* gene (rs6088813) which was also selected for replication in this GWAS, are shown in Supplementary Table 2 in Appendix 2. The T allele of SNP rs12885300 (*DIO2* gene) had a trend towards a protective effect for hip OA (OR 0.84, 95%CI 0.68-1.03). The T-allele of SNP rs7639618 (*DVWA* gene) was associated with an increased risk of hand OA (OR 1.18, 95%CI 1.02-1.38). The C allele of rs6088813 (*GDF5*) was associated with a decreased risk of hand OA (OR 0.82, 95%CI 0.73-0.92) and a non-significant trend in the same direction for knee OA (OR 0.79, 95%CI 0.89-1.01). No other statistically significant associations were observed.

DISCUSSION

In this paper we present a GWAS of OA to find DNA sequence variation associated with OA at multiple joint sites and with progression of knee OA.

The C allele of the SNP rs3815148 showed an increased risk of 14% for knee and hand OA and was also associated with progression of knee OA (increased risk of 30% per allele). This DNA variant is located in intron 12 of the *COG5* gene, while there is high linkage disequilibrium in the surrounding region (Supplementary Figure 2 in Appendix 2). *In silico* and *in vitro* functional experiments showed that the T allele of the rs3757713 SNP (D'=1, r^2=0.95 with rs3815148) was associated with expression levels of *GPR22* in lymphoblasts (P-value $4x10^{-12}$) and immunohistochemistry experiments showed presence of the Gpr22 protein in cartilage and osteophytes in

OA-induced mouse models, whilst this protein was absent in normal cartilage. In our opinion, this indicates that the *GPR22* gene might be the causal gene in the association found. *Gpr22*$^{-/-}$ mice were only tested for heart-specific outcomes (20), it would be interesting to study their skeletal phenotype as well. To our knowledge, ligands that may bind to GPR22 have not been identified yet, which makes this receptor a so called "orphan receptor". Since the *GPR22* gene encodes a G-protein-coupled receptor, this is potentially an interesting drug target. Although we present results to suggest *GPR22* as the gene underlying the genetic association, the understanding of the precise mechanism remains unknown and it is possible that one or more of the other genes in the same LD block are important for OA. Further research into the other genes in this locus is necessary to assess whether *GPR22* is the true underlying gene.

In this study, we focused on a spectrum of OA phenotypes for several reasons. Because multiple joint-sites can be affected by OA, we searched for loci that showed association with more than one OA phenotype. This method is likely to identify more general susceptibility genes for OA rather than site-specific aspects. In addition, in our discovery study we had limited power to identify joint-specific alleles, such as the *GPR22* SNP, to be modestly associated with OA. We realize that our broad phenotype approach can introduce heterogeneity and that we could have increased the chances of missing true OA susceptibility alleles that are site-specific. In studies where the OA phenotype was defined as clinical OA, many controls did not have radiographic information and therefore may have asymptomatic OA. In theory, there is a possibility that this might influence results and some associations could have been missed within studies that have not phenotyped their control subjects radiographically. This, however, would only lead to an underestimation of our results and therefore only false-negatives would be a concern. Therefore, we cannot fully exclude other SNPs, except the rs3815148 SNP, as being associated with OA. Unfortunately, only subjects of 1 replication study, the Chingford Study, were genotyped for all SNPs and had CTX-II levels available for analysis and therefore power was too low to obtain genome-wide significant results for CTX-II for any of the SNPs

The consistent and robust pattern of association we observe for the *GPR22* locus together with the expression data provides sufficient evidence to implicate this locus in OA etiology. However, the expression patterns observed did not clarify the exact underlying mechanism and therefore more experiments are warranted. While we observed several other potentially interesting loci, these did not reach genome-wide significance and so larger GWAS sample sizes and meta-analysis will be useful to identify further OA susceptibility loci, including those that might act at a joint-site specific level.

To date, genome-wide linkage and association studies have shown that there are DNA variants in 4 genes found to be consistently associated with OA (2-5,25). These are the rs225014 and rs12885300 SNPs of the *DIO2* gene, rs4140564 of the *PTGS2* gene, rs7639618 of the *DVWA* gene and rs143383 (rs6088813 proxy SNP with D'=1.0 and r^2=0.93) of the *GDF5* gene. In Supplementary Table 2, the association results of those 5 SNPs are shown for the Rotterdam Study based on the GWAS dataset. The 2 SNPs found to be associated with hip OA in females in the *DIO2* gene were discovered through a linkage study (3). Those 2 SNPs were not associated with hip OA in the Rotterdam Study although the rs12885300 SNP showed a trend towards a decreased risk in female carriers of the T allele which is the same direction as described in the paper of Meulenbelt *et al.* (3). The SNP in the *PTGS2* gene was not associated with knee OA in females of the Rotterdam Study. In the original papers by Valdes *et al.* (4) and Meulenbelt *et al.* (3) an association with a more severe OA phenotype (total joint replacement or a K/L grade >=3) was described, which could be an explanation for the lack of replication in the Rotterdam Study using the less severe, radiographic knee OA phenotype (K/L >=2). In addition, the *DVWA* SNP was not associated with knee OA in our population. An increased risk for hand OA was observed for the T allele of rs7639618 SNP (*DVWA*), however this effect is in the opposite direction compared to the association observed in the GWAS for knee OA by Miyamoto *et al.* (5). This SNP was also not associated with knee OA in other Caucasian studies (6,7), which could indicate that this SNP has different effects in different ethnic populations. The *GDF5* gene, identified with our search strategy showed an association with knee and/or hand OA with an OR of 0.91 and 95%CI: 0.84-0.98 in the meta-analysis. This was previously also shown by Miyamoto *et al.* (25), Chapman *et al.* (2), Vaes *et al.* (26) and in a large meta-analysis (n=5085 cases and 8135 controls) performed within the TREAT-OA consortium (27) for knee OA. Although the *GDF5* locus does not reach genome-wide significance, it is considered a true and consistent association in the OA field.

In conclusion, we have identified a novel locus involved in susceptibility for prevalence and progression of OA, SNP rs3815148 close to the *GPR22* gene.

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Chapter 3.3

Genome wide association and functional studies identify the *DOT1L* gene to be involved in cartilage thickness and hip osteoarthritis

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ABSTRACT

Background: Hip osteoarthritis (HOA) is one of the most disabling and common joint disorders with a large genetic component which is, however, still ill defined. To date, genome-wide association studies (GWAS) in osteoarthritis (OA) and specifically in HOA have yielded only few loci, which is partly explained by the heterogeneity in OA definition. Therefore, we here focused on radiographically measured joint space width (JSW), a proxy for cartilage thickness and an important underlying intermediate trait for HOA.

Methods/Results: In a GWAS of 6,523 individuals on JSW of the hip, we identified the G allele of rs12982744 on chromosome 19p13.3 to be associated with a 5% larger joint space width (P=4.8x10^{-10}). The association was replicated in 4,442 individuals from 3 UK-cohorts with an overall meta-analysis P-value of 1.1×10^{-11}. The SNP was also strongly associated with a 12% reduced risk for HOA (P=1x10^{-4}). The SNP is located in the *DOT1L* gene, which is an evolutionarily conserved histone methyltransferase, recently identified as a potentially dedicated enzyme for Wnt target gene activation in leukemia. Immunohistochemical staining of Dot1l protein during mouse limb bud development supports a role for DOT1L in chondrogenic differentiation and adult articular cartilage. Silencing of *Dot1l* inhibited chondrogenesis.
Dot1l knock-down reduces proteoglycan and collagen content, and mineralization during chondrogenesis. In the *in vitro* ATDC5 chondrogenesis model system, DOT1L interacts with TCF and Wnt signalling.

Conclusions: These data are a further step in better understanding the role of Wnt-signaling during chondrogenesis and cartilage homeostasis. DOT1L may represent a therapeutic target for osteoarthritis.

INTRODUCTION

Osteoarthritis (OA), the most common, age-related disease of the synovial joints, results in a substantial reduced quality of life due to pain and disability. Current clinical management of OA focuses on pain control. In severe cases, joint prosthesis surgery may be the unique solution. There are currently no targeted therapies that maintain homeostasis of the joint or stimulate cartilage repair. OA is characterized by progressive destruction of articular cartilage, subchondral bone sclerosis and osteophyte formation.

OA has a large genetic component, which varies between the joint studied (1). Several GWAS on OA have been published, but up to now few signals have been identified with reproducible association (2-6). In Caucasians, only 3 loci reach the genome wide significance threshold. These include a variant influencing expression of *GDF5* (3, 6) a locus on chromosome 7q22 near the orphan receptor *GPR22* (4-5) and a variant in *MCF2L* (2). The low number of identified loci can be explained by relatively low power caused by insufficient sample sizes and by phenotype heterogeneity, which is a well-known problem in epidemiology of OA (7). The diagnosis of OA is based on a combination of parameters including both clinical features (pain and stiffness) as well as a structural damage score (the most widely used is the Kellgren & Lawrence score), which includes formation of new bone spurs (osteophyte formation) and reduction of the joint space width (JSW) indicating cartilage degradation. JSW is considered to be the surrogate for cartilage thickness in the joint and change in minimal JSW is the primary structural endpoint used in clinical trials and epidemiological studies of knee and hip OA (8-10). Heritability analyses showed that mJSW at the hip and change in mJSW have a genetic component estimated between 40-42% (11).

In this study we combined GWAS and functional studies to identify new genes involved in cartilage thickness and osteoarthritis. We first performed a discovery GWAS on minimal JSW (mJSW) of the hip in 6,523 participants from the Rotterdam Study-I and II (RS-I and RS-II) and replication included population from three independent UK studies (n=4,442) in which mJSW was measured (Supplementary Table 1 in Appendix 2 for cohort specifics and a description of studies is given in Appendix 1). Additionally, we analyzed association of the genetic variants with hip osteoarthritis in 3,717 cases and 10,013 controls. Further, we carried out functional genetic studies using cell culture experiments and animal models.

MATERIALS & METHODS

GWAS meta-analysis

GWAS analysis of the Rotterdam Studies: Genotyping of the samples in the discovery cohorts (RS-I and RS-II) was carried out with the Illumina HumanHap550v3 Genotyping BeadChip. The Beadstudio GenCall algorithm was used for genotype calling and quality control procedures, as described previously (12). The following quality control inclusion filters were applied: call rate ≥97.5%, MAF ≥1%, *P* for Hardy-Weinberg equilibrium $<1x10^{-6}$ (Supplementary Table 2 in Appendix 2 for details on quality control and exclusion criteria). The total number of genotyped SNPs that passed these filters was 512,349 for RS-I and 466,389 for RS-II. Imputation was done with reference to HapMap release 22 CEU using the maximum likelihood method implemented in MACH (http://www.sph.umich.edu/csg/abecasis/MACH/index.html). Analysis of imputed genotype data accounted for uncertainty in each genotype prediction by using the dosage information from MACH. For this analysis, MACH2QTL, was used via GRIMP (13), which uses genotype dosage value (0–2, continuous) as a predictor in a linear regression framework. Genomic control correction was applied to the standard errors and P-values before meta-analysis. We included only imputed SNPs that had a good imputation quality leaving a total of 2,455,290 SNPs. The summary statistics of RS-I and RS-II were meta-analysed using METAL applying inverse-variance methodology assuming fixed effects with Cochran's Q and I^2 metrics used to quantify between-study heterogeneity (www.sph.umich.edu/csg/abecasis/metal).

Replication analysis: All samples from the TwinsUK cohort for this study were genotyped with the HumanHap610Q (Illumina). The following quality control filters were applied: call rate ≥98%, MAF ≥1%, *P* for Hardy-Weinberg equilibrium $\geq 1x10^{-6}$ (Supplementary Table 2 in Appendix 2). The total number of genotyped SNPs that passed these filters was 598,207 SNPs. Imputation was done with reference to HapMap release 22 CEU using the IMPUTE software package (v2) (14). For the GOAL, Nottingham and Chingford study participants, genomic DNA was extracted from peripheral blood leukocytes of affected individuals and controls using standard protocols. Genotyping was carried out by Kbioscience Ltd. SNPs were genotyped using the KASPar chemistry, which is a competitive allele-specific polymerase chain reaction (PCR) SNP genotyping system using fluorescence resonance energy transfer (FRET) quencher cassette oligos. Association between rs12982744 and mJSW in the replication cohorts was analysed by linear regression including age and gender as covariates. In addition, separate analyses were carried out including age, gender and height as covariates. The R version 2.10.1 (The R Foundation for Statistical Computing http://www.r-project.org/) was used for analysis.

Overall meta-analysis: Results from RS-I and RS-II and the replication cohorts were combined in a joined meta-analysis using inverse variance weighting with METAL as described before. We declared results genome wide significant at $\alpha=5x10^{-8}$ after adjusting for all common variant tests in the human genome.

Cell culture experiments

ATDC5 cells were cultured in growth medium (1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium) (Gibco) containing 1% antibiotic-antimycotic (Gibco), 5% fetal bovine serum (Gibco), 10 µg/ml human transferrin (Sigma) and $3x10^{-8}$ M sodium selenite (Sigma). Cells were maintained in a humidified atmosphere of 5% CO_2 and 95% O_2 at 37°C. Stable ATDC5 clones were established using either the control non-interfering pGIPZ (Thermo Fisher) or the pGIPZ-shmiRNA directed against mouse *Dot1l* construct (Thermo Fisher). Arrest-In transfection reagent (Thermo Fisher) was used for transfection. After 24 hours, selection with 1 µg/ml puromycin (Invitrogen) was initiated and continued for 10 days. In the end, three different antibiotic resistant clonal colonies were isolated and grown independently. Knock-down efficiency was assessed by qRT-PCR. Stably-transfected ATDC5 clones were cultured as micromasses: trypsinized cells were resuspended in medium at a concentration of $2x10^7$ cells/ml. Three drops of 10 µl of this cell suspension were placed in a well of a standard 24-well culture plate. The cells were allowed to adhere for 3 h at 37°C, and then 0.5 ml medium was added to each well. For induction of chondrogenesis, the cells were cultured in growth medium containing 1% antibiotic-antimycotic, 5% fetal bovine serum, supplemented with an ITS premix containing 10 µg/ml insulin, 5 µg/ml human transferrin and $3x10^{-8}$ M sodium selenite for 2 weeks (Gibco). 5 µg/ml human transferrin (Sigma) was additionally added to reach a final concentration of 10 µg/ml. Alpha-MEM medium (Gibco) containing 5% fetal bovine serum (Gibco), and the same mix of insulin, human transferrin and sodium selenite was added supplemented with 7 mM beta-glycerolphosphate (Sigma) from day 14 until day 21. The medium was replaced daily. Each condition was performed in triplicate. Total RNA from ATDC5 cell micromasses was isolated after 1, 7, 14 or 21 days in culture using the Nucleospin RNA II kit (Macherey-Nagel). Some ATDC5 micromasses were fixed in 95% ice-cold methanol for 30 minutes at 4°C. After washing with water, they were stained for 1h in either Alcian Blue (0.1% Alcian Blue 8GX, (Sigma) in 0.1 M HCl pH 0.2), Safranin O (Klinipath), Alizarin Red (1% Alizarin Red S (Sigma) in water pH 4.2) or Sirius Red (0.1% Direct Red 80 (Sigma) in a saturated aqueous solution of picric acid). To remove unbound staining, cells were washed with water until the washing solution remained colorless.

Co-Immunoprecipitation (CoIP) analyses: Proteins were isolated from ATDC5 micromasses using the IP Lysis/Wash buffer (Thermo Fisher) supplemented with 5%

Protease Cocktail Inhibitor (Sigma) and 1 mM phenylmethanesulfonyl (Sigma). After two homogenization cycles (7 sec) with an ultrasonic cell disruptor (Microson™, Misonix), total cell lysates were centrifuged 10 min at 13,000*g*, and supernatant containing proteins was collected. CoIP were performed using the ProFound™ Co-Immunoprecipitation Kit (Thermo Scientific). Columns were conditioned following manufacturer's recommendations, to activate a gel slurry retained in a spin-column system, ensuring the proper binding of antibodies. Antibody binding to the column was performed using 100 µg of either a mock antibody (donkey anti-goat IgG) as a control or an anti-TCF4 antibody (Millipore) in the gel slurry, followed by an overnight incubation at 4°C under constant mixing. The day after, the columns were washed, and 100 µg of the lysate's proteins were incubated for 2h at room temperature. After four washings, retained proteins were eluted using 50 µl of Elution Buffer (Thermo Fisher) pH 3, and stored at -80°C.

Western Blot analyses: 20 µl of the elution fraction, supplemented with Laemmli Buffer (Sigma) was heated for 5 minutes at 95°C, chilled at room temperature and separated on a 4-12% Bis-Tris gel (Invitrogen). Proteins were then transferred onto a polyvinylidene fluoride membrane (Millipore). After 2h in blocking buffer (TBS-0.1% Tween (TBST) supplemented with 5% non-fat dry milk), membranes were washed three times with TBST and incubated overnight at 4°C with primary antibodies. The antibody against Dot1l (Abcam) was used at a 1/1000 dilution, while antibody against Tcf4 (Millipore) was used at a 1/500 dilution. After three washings with TBST, each blot was incubated for 1 h at room temperature with either anti-rabbit IgG (for Dot1l) or anti-mouse IgG (for Tcf4) conjugated with HRP (both from Jackson ImmunoResearch Laboratories, West Grove, USA) at 1/10,000 dilution in blocking buffer. After four washings in TBST, protein bands were detected by chemiluminescence with the SuperSignal West Femto Maximum Sensitivity Substrate system (Thermo Scientific) according to manufacturer's recommendations. Images were acquired with the LAS-3000 mini CCD camera (Fujifilm).

cDNA synthesis and Quantitative Real-Time PCR: Complementary DNA (cDNA) was synthesized of 500 ng RNA isolated from ATDC5 micromasses using the RevertAid H minus First Strand cDNA synthesis kit (Fermentas). The Maxima®SYBRgreen qPCR master mix system (Fermentas) was used to analyze differential mRNA expression of *Col2a1*, *Col10a1, Col1a1*, *Aggrecan*, *Tcf1* and *Osteocalcin* (primers available upon request) in the ATDC5 micromasses. To assess *Dot1l* knock-down efficiency, primers were: forward 5′-CGAGGAAATCCCAGATCTCA-3′, reverse 5′-ATGGCCCG-GTTGTATTTGT-3′. The following PCR conditions were used: incubation for 10 min at 95°C followed by 40 amplification cycles of 15s of denaturation at 95°C followed by 45 sec of annealing-elongation at 60°C. Melting curve analysis and 1% agarose gel migration of amplicons were performed to determine the specificity the PCR reac-

tion. Results are expressed using the comparative threshold method (15) and were normalized to housekeeping gene *S29* mRNA level (forward 5′-CCAGCAGCTCTACT-GGAGTCA-3′, reverse 5′-GCCTATGTCCTTCGCGTACT-3′).

Statistical analysis- cell culture experiments: Data presented are representative of the three independent clonal colonies. Results are expressed as the mean ± SD of three independent replicates. Comparisons were made by ANOVA, followed by Fisher's *t post-hoc* test, using the Statview™ 5.0 software (SAS Institute Inc). A value of $P<0.05$ was considered significant.

Immunohistochemistry on mouse tissues: Immunohistochemistry on paraffin embedded EDTA decalcified adult knee sections and non-decalcified embryonal sections, was performed with rabbit anti-Dot1l antibody (Ab64077, Abcam, Camebridge, UK) (5µg/ml). After overnight incubation of the sections at 4°C, 1:100 peroxidase goat anti-rabbit IgG (Jackson Immunoresearch, Suffolk, UK) was applied for 30 minutes and peroxidase activity was determined using DAB. Rabbit IgG (Santa Cruz Biotechnologies, Santa Cruz, CA) was used as negative controls.

RESULTS

GWAS meta-analysis of minimal JSW

A GWAS on minimal JSW (mJSW) of the hip was performed in 6,523 participants from the Rotterdam cohorts I and II (RS-I and RS-II, see Supplementary Table 1 in Appendix 2 for cohort specifics). We applied extensive quality control measures (Supplementary Table 2 in Appendix 2) leaving a total of 2,455,290 SNPs for association analysis. Genomic control inflation factors for the P-values of the RS-I and

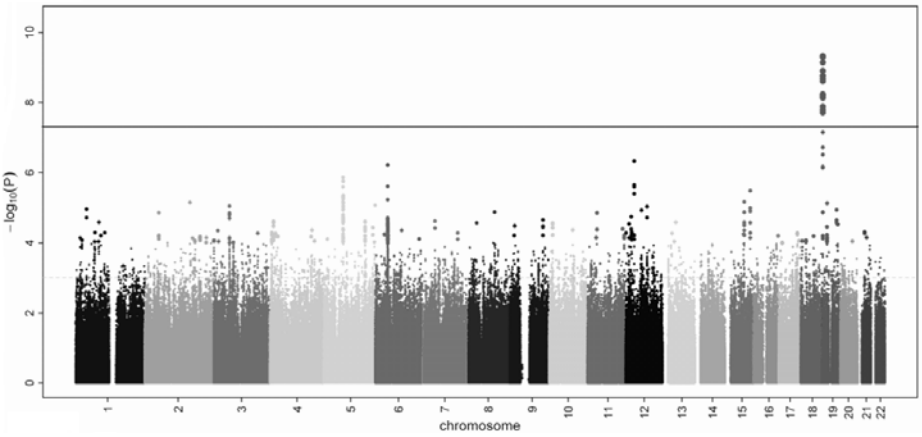

Figure 1. Manhattan plot for P-values of the GWAS meta-analysis of the Rotterdam Studies.

RS-II GWAS were low (λ=1.02 and 1.01, respectively), and the interquantile-quantile plot also indicated no substantial population stratification due to cryptic relatedness, population substructure or other biases (Supplementary Figure 1 in Appendix 2). After meta-analysing the association results of RS-I and RS-II, we identified a significant association on chromosome 19 that satisfied our genome-wide significance (GWS) threshold of $P<5x10^{-8}$ (Figure 1).

A total of 18 SNPs were GWS and clustered around 1 locus on chromosome 19p13.3. The top SNP rs12982744 ($P=4.5x10^{-10}$) is localized in the first intron of the gene DOT1-like, histone H3 methyltransferase (*DOT1L*). This SNP is in high LD with the other 17 GWS SNPs representing the same signal (Figure 2, a color picture is depicted in Appendix 3). We additionally found 8 loci with suggestive evidence for association ($5x10^{-8}<P<1x10^{-5}$) (Supplementary Table 3 in Appendix 2).

To validate the association with *DOT1L,* we performed a replication study using three independent UK studies: TwinsUK, Chingford, and GOAL (n=4,442 in total). Association between rs12982744 and mJSW in the replication cohorts was analysed by linear regression including age and gender as covariates. The association of rs12982744 with mJSW was replicated (beta: 0.07 mm/allele; $P=9x10^{-3}$, Figure 3). Results from the Rotterdam Studies and the replication cohorts were combined in a joined meta-analysis (Figure 3). The combined analysis including discovery and replication studies showed strong evidence for association of the *DOT1L* locus with minimal joint space width in the general population (beta: 0.09 mm/allele; $P=1.1x10^{-11}$, $I^2=0\%$). These associations were corrected by age and gender. The minor G allele of rs12982744 (MAF=0.39) is associated with an increased joint space width of 0.09 mm per copy of the G allele. This implicates that homozygote carriers of the rs12982744 G-allele have approximately 5% thicker cartilage than the reference group.

We further investigated whether rs12982744 was influencing the risk for hip OA. This was examined in all the five studies described previously and one additional large case-control study (Nottingham); the total sample size was 3,717 cases and 10,013 controls for this analysis (Supplementary Table 1 in Appendix 2).

Risk for hip OA was calculated using logistic regression analysis and was adjusted for age and gender. The results were combined in a meta-analysis in an additive model. As shown in Supplementary Figure 2 in Appendix 2, the minor allele of rs12982744 was significantly associated with a 12% reduced risk for hip OA (OR: 0.88, 95%CI: 0.82-0.94; $P=1.5x10^{-4}$, $I^2=0\%$; analysis adjusted for age and gender), with consistent effects in all cohorts studied. Additional adjustment for height did not affect the association (OR: 0.88, 95%CI: 0.82-0.94; $P=1.1x10^{-4}$). We observed that also in people without radiographic hip OA, the association with mJSW was present (Supplementary Table 4 in Appendix 2, beta: 0.06 mm, SE: 0.011; $P=7.3x10^{-9}$). This

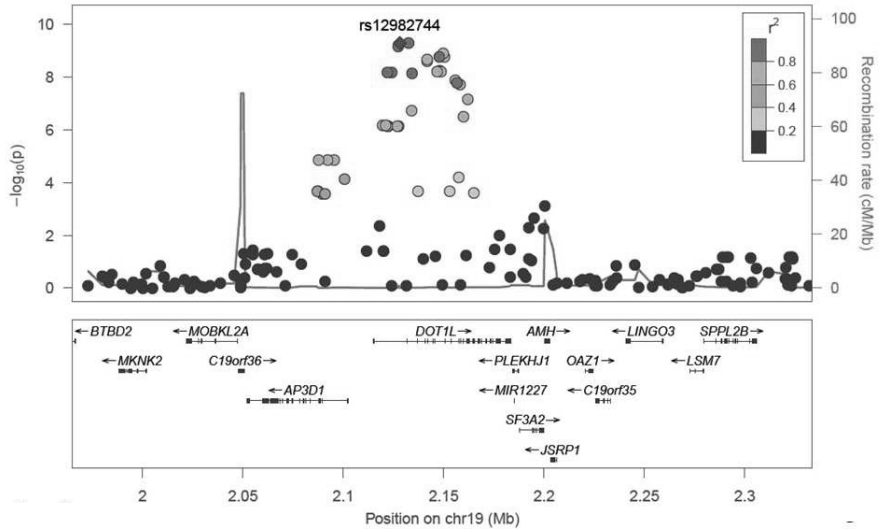

Figure 2. Regional association plot for rs12982744. A color figure is presented in Appendix 3.

Study	Beta	Lower limit	Upper limit	P-value
Discovery (RS-I + RS-II)	0.09	0.06	0.12	4.8×10^{-10}
Chingford	0.04	-0.04	0.12	0.35
GOAL	0.09	-0.01	0.18	0.07
TwinsUK	0.10	0.00	0.21	0.05
Overall effect replication (fixed-effect)	0.07	0.03	0.11	9.0×10^{-3}
Overall effect total (fixed-effect)	**0.09**	**0.06**	**0.11**	$\mathbf{1.1 \times 10^{-11}}$

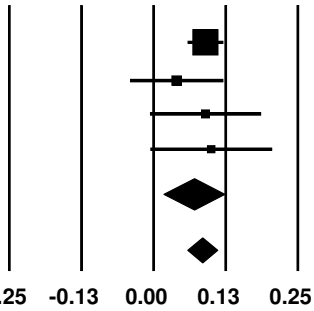

Figure 3. Forest plot for the association between minimal joint space width (JSW) and rs12982744.

suggests that the association with cartilage thickness is present already before onset of OA, and possibly implicates involvement of this DNA-variant on the articular cartilage during development and growth.

The G allele of the identified SNP (rs12982744) was previously found to be associated with increased height (16-17). This is in line with the thicker cartilage that was found in the current study. We therefore tested whether our findings with mJSW were affected by differences in stature, by including height as a covariate in the analysis. This did not substantially change the results. It suggests that this locus has independent pleiotropic effects on height as well as mJSW of the hip.

The associated polymorphisms are annotated in the *DOT1L* gene (Figure 2). DOT1L is an evolutionarily conserved histone methyltransferase, identified as an essential

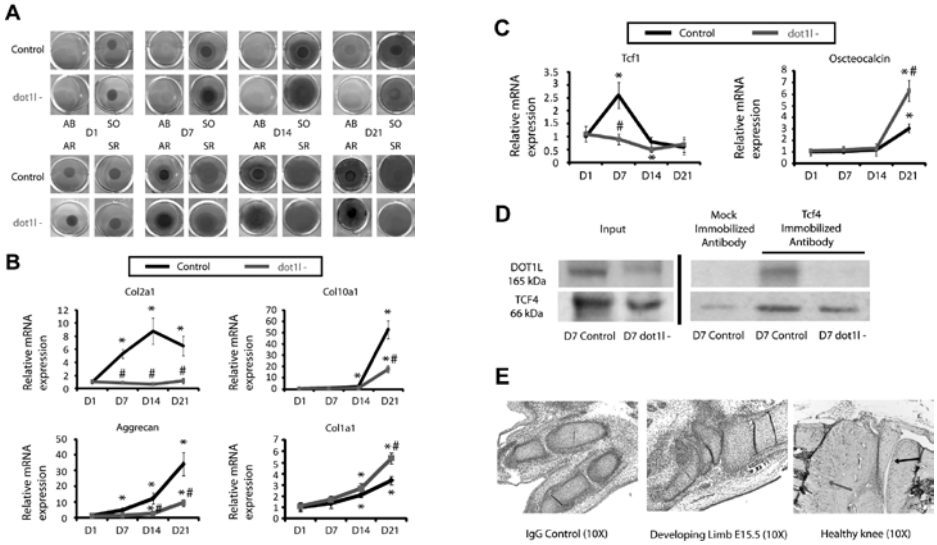

Figure 4. Functional analysis of *Dot1l* during chondrogenesis. Stable ATDC5 clones were established using either the control non-interfering pGIPZ or the pGIPZ-shmiRNA directed against mouse *Dot1l*. Three different antibiotic resistant clones were selected. Knock-down efficiency was assessed by qRT-PCR. Stably-transfected ATDC5 clones were cultured as micromasses as described before (20,21). Each condition was performed in triplicate. Total RNA from was isolated after 1, 7, 14 or 21 days. Data presented are representative of the three independent clonal colonies. Results are expressed as the mean ± SD of three independent replicates. Comparisons were made by ANOVA, followed by Fisher's *t post-hoc* test. Statistically significant differences *vs.* day 1 are indicated as *: $P<0.05$, and *vs.* control-transfected cells as #: $P<0.05$. (**A**) *Dot1l* knock-down reduces proteoglycan and collagen content, and mineralization during chondrogenesis. Stainings were performed on ATDC5 micromass cultures stably transfected with either control or *Dot1l* shmiRNA producing vector, over 21 days (D). (AB = Alcian blue, SO = safranin O, AR = alizarin red, SR = sirius red). (**B**) *Dot1l* knock-down reduces mRNA expression of chondrogenesis markers of chondrogenesis. mRNA levels were normalized to S29 (reference gene) (n=3). Quantitative Real-Time PCR conditions and primers are available upon request. (**C**) *Dot1l* knock-down affects Wnt signaling during chondrogenesis. mRNA levels of Wnt target genes *Tcf1* and osteocalcin were normalized to *S29* (reference gene) (n=3). (**D**) DOT1L interacts Wnt signaling pathway transcription factor TCF4. Co-immunoprecipitation of DOT1L and TCF4 using 100 µg of total proteins (input) from micromass cultures (at day 7 – D7) of either control or *Dot1l* knocked-down cells. Proteins were isolated from ATDC5 micromasses. CoIPs were performed and 20 µl of elution fraction was probed after protein binding on either mock (donkey anti-goat IgG) or TCF4 column-immobilized antibody. (**E**) DOT1L is expressed during joint development and in mature articular cartilage of mice. Immunohistochemistry on paraffin embedded EDTA decalcified adult knee sections and non-decalcified embryonal sections, was performed with rabbit anti-Dot1L antibody (5µg/ml). After overnight incubation of the sections at 4°C, 1:100 peroxidase goat anti-rabbit IgG was applied and peroxidase activity was determined using DAB. Immunohistochemistry detected very strong expression in chondrocytes in the developing mouse. A color figure is presented in Appendix 3.

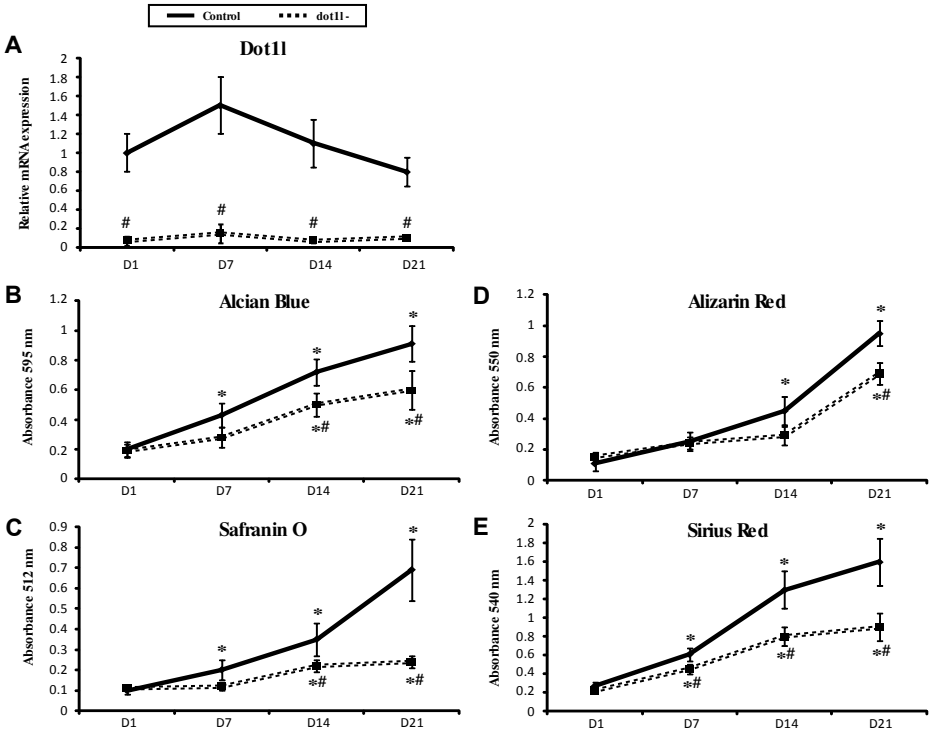

Figure 5. DOT1L knock-down efficiency during chondrogenesis and staining quantification.
(**A**) DOT1L knock-down is effective during chondrogenesis. mRNA levels were normalized to *S29* (reference gene) (n=3). (**B**) Proteoglycan content (Alcian blue staining) is decreased in DOT1L- cells. Stainings were performed on ATDC5 micromass cultures, stably transfected with either control or DOT1L shmiRNA producing vector, over 21 days (**D**). Absorbance was evaluated at 595 nm. (n=3). (**C**) Proteoglycan content (Safranin O staining) is strongly reduced in DOT1L- cells. Stainings were performed on ATDC5 micromass cultures, stably transfected with either control or DOT1L shmiRNA producing vector, over 21 D. Absorbance was evaluated at 512 nm. (n=3). (D) Mineralization is decreased in DOT1L- cells. Alizarin red stainings were performed on ATDC5 micromass cultures, stably transfected with either control or DOT1L shmiRNA producing vector, over 21 D. Absorbance was evaluated at 550 nm. (n=3). (**E**) Collagen content is decreased in Dot1l- cells. Sirius red stainings were performed on ATDC5 micromass cultures, stably transfected with either control or DOT1L shmiRNA producing vector, over 21 D. Absorbance was evaluated at 540 nm. (n=3). Statistically significant differences *vs.* D1 (internal control condition) are indicated as *: $P<0.05$, and *vs.* control-transfected cells as #: $P<0.05$.

and dedicated enzyme for Wnt target gene activation in the intestine and needed for the expression of genes that require high levels of Wnt signaling in Drosophila (18-19). We hypothesized that *DOT1L* is the culprit gene underlying the association with mJSW and height by influencing chondrogenic differentiation, which is important in growth and joint formation.

Cell Culture Experiments

We examined the function of Dot1l during chondrogenesis in ATDC5 cells which exhibit a multistep process of chondrogenic differentiation analogous to that observed during endochondral bone formation (20-21). As depicted in Figure 4a (in color in Appendix 3) and Figure 5, ATDC5 cells stably-transfected with plasmid overexpressing shmiRNA directed against *Dot1l* (*Dot1l*-) synthetized less sulphated proteoglycans than control cells, demonstrated by the weaker Alcian blue and safranin O staining, respectively decreased by 1.35- and 2.5-fold. Moreover, mineralization in the micromasses was less, efficient, as shown by the 1.4-fold decrease in Alizarin Red staining, which was restricted to the core of the micromasses in *Dot1l*- cells. Collagen content, revealed by Sirius red staining, was also 1.8 fold reduced in these cells. These data indicate that chondrogenesis is severely affected by *Dot1l* knock-down. These observations were supported by mRNA analyses. Indeed, type II collagen expression was not increased in cells with *Dot1l* knock down, while type X collagen and aggrecan induction was 3.3-fold and 4-fold reduced compared to normal ATDC5 cells (Figure 4b). Interestingly, type I collagen levels were 1.7-fold higher in *Dot1l*- cells at D21.

As *DOT1L* was previously linked to β-catenin signalling (18-19) we investigated whether mRNA expression of Wnt target genes was affected in *Dot1l*- cells. As seen in Figure 4c, Tcf1 levels (positively regulated by Wnt/β-catenin signaling) were increased in control ATDC5s at D7 (2.5-fold), while no induction was detected in *Dot1l*- cells. Other Wnt target genes *Axin2* and *c*-Myc followed the same pattern (Figure 6). Moreover, Osteocalcin level (negatively regulated by Wnt/ β-catenin signaling) was increased by 2.8-fold at D21 in control cells, while the up-regulation was of 6.2-fold in *Dot1l*- ATDC5s (2.2-fold more than in control cells). Altogether,

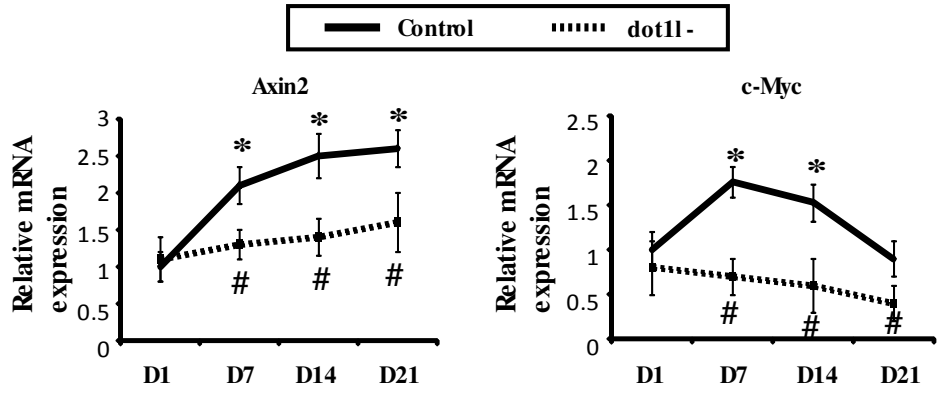

Figure 6. *Dot1l* knock-down affects Wnt signaling during chondrogenesis. mRNA levels of Wnt target genes c-Myc and Axin2 were normalized to *S29* (reference gene) (n=3). Statistically significant differences vs. D1 (internal control condition) are indicated as *: $P<0.05$, and #: $P<0.05$ for comparison to the GIPZ (control vector) transfected cells.

these elements suggest a role for DOT1L in the Wnt/β-catenin signaling cascade in developing chondrocytes.

Co-immunoprecipitation experiments strengthened these observations, as DOT1L was found to directly interact with TCF4, a transcription factor interacting with β-catenin (Figure 4d).

These functional analyses seemed relevant in vivo, as DOT1L is very strongly present during chondrogenesis in mouse developing limbs and still found in adult cartilage (articular cartilage, black arrow and growth plate, red arrow) as seen in Figure 4e.

DISCUSSION

This study identified a genetic variant in the *DOT1L* gene robustly associated with joint space width and hip osteoarthritis. We used an *in vitro* chondrogenesis model and ex vivo expression studies in mice to functionally characterize the role of *Dot1l* in chondrogenesis. We found that DOT1L is involved in chondrogenic differentiation presumably through its role in canonical Wnt-signaling.

The exact same variant that we found associated with cartilage thickness has previously been found associated with height both in young and old individuals (16-17), which suggests a role in skeletal formation. Although the specific differentiation process in the growth plate and articular cartilage are different, common signalling pathways such as the Wnt cascades are involved (22). Interestingly the association between the *DOT1L* genetic variant and cartilage thickness was present also in people without OA. This indicates that the association with cartilage thickness is present already before onset of OA, and possibly implicates involvement of this DNA-variant on normal formation of the articular cartilage during development, in agreement with a role for this variant on skeletal development.

DOT1L is an evolutionarily conserved histone methyltransferase, which was initially identified as a disruptor of telomeric silencing in *Saccharomyces cerevisiae* (23). The mammalian homolog, DOT1L, has been shown to be required for embryogenesis, hematopoiesis, and cardiac function (24-27). DOT1L was recently identified as an essential and dedicated enzyme for Wnt target gene activation in the intestine and needed for the expression of genes that require high levels of Wnt signaling in Drosophila (18,19). We here provide the first evidence demonstrating a role for DOT1L in chondrogenesis. Knock down of *Dot1l* resulted in a reduced chondrogenic differentiation in the ADTC5-cells. We additionally observed a pronounced reduction in expression of Wnt-targeted genes. Together with the proven physical interaction

of DOT1L and TCF4 proteins, this suggests that Dot1l influenced chondrogenic differentiation by regulating transcription of Wnt target genes.

Osteoarthritis is a complex disease with a large genetic component. Twins studies have shown that the influence of genetic factors for hip OA is about 60% (1). Nevertheless, it has been difficult to find genes involved in OA and especially in hip OA. From the few genetic signals found, only one has shown a modest association with hip OA (6). *GDF5* polymorphisms (3), and a locus on chromosome 7q22 near the *GPR22* gene (4,5) have been consistent associated with knee OA only across different European populations. Recently, a locus on chromosome 13, localized in the *MCF2L* gene that regulates a nerve growth factor (NGF) points to pronounced association with osteoarthritis affecting the knee and less significantly for hip OA (2). These few signals have been found using the traditional composite definitions of OA, which have features of structural damage to the joint (Kellgren and Lawrence score of 2 or more including joint replacement) as well as clinical parameters such as pain. This may lead to considerable heterogeneity and consequently low power. In the case of hip OA, where degeneration of articular cartilage is the most important feature, the approach to identify genetics variants of OA studying only one of the components of the physiopathology (cartilage thickness) can result in less heterogeneity in the phenotype definition and therefore in more power to pick up true signals. Both intra-rater and inter-rater reliability has been significantly higher for joint space measurement than for K/L (10, 28) and the findings that decline of JSW in OA proceeds in a linear manner (29) and that JSW is predictive of long-term progression of joint space narrowing (30) make measurement of JSW suitable for clinical trials and prioritize the identification of genes responsible for cartilage formation and homeostasis.

Considering the known important function of Wnt signaling pathway in cartilage and bone formation and the role of DOT1L in chondrogenesis here presented, DOT1L may represent a therapeutic target for modulation, and thus therapeutic intervention in osteoarthritis. It is apparent that DOT1L and its associated methylation activity are regulated in an extremely complex way. As such, the regulation of DOT1L activity and the functional consequences of manipulation of DOT1L need to be further elucidated before efficient treatments can be developed. Future studies are therefore warranted to determine how to target DOT1L in a selective and tissue specific manner. There are already initiatives for targeting DOT1L in other pathologies, having in mind that DOT1L has a key role in other normal cellular processes (31). This might represent an exciting opportunity for the development of disease modifying drugs for osteoarthritis.

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Chapter 3.4

Meta-analysis of genome-wide association studies confirms a susceptibility locus for knee osteoarthritis on chromosome 7q22

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ABSTRACT

Background: Osteoarthritis (OA) is the most prevalent form of arthritis and accounts for substantial morbidity and disability, particularly in older people. It is characterised by changes in joint structure, including degeneration of the articular cartilage, and its aetiology is multifactorial with a strong postulated genetic component.

Methods: A meta-analysis was performed of four genome-wide association (GWA) studies of 2,371 cases of knee OA and 35,909 controls in Caucasian populations. Replication of the top hits was attempted with data from 10 additional replication datasets.

Results: With a cumulative sample size of 6,709 cases and 44,439 controls, one genome-wide significant locus was identified on chromosome 7q22 for knee OA (rs4730250, $P=9.2\times10^{-9}$), thereby confirming its role as a susceptibility locus for OA.

Conclusions: The associated signal is located within a large (500 kb) linkage disequilibrium block that contains six genes: *PRKAR2B* (protein kinase, cAMP-dependent, regulatory, type II, β), *HBP1* (HMG-box transcription factor 1), *COG5* (component of oligomeric golgi complex 5*), GPR22* (G protein-coupled receptor 22), *DUS4L* (dihydrouridine synthase 4-like) and *BCAP29* (B cell receptor-associated protein 29). Gene expression analyses of the (six) genes in primary cells derived from different joint tissues confirmed expression of all the genes in the joint environment.

INTRODUCTION

Osteoarthritis (OA) is the most prevalent form of chronic joint disease and accounts for substantial morbidity and disability, particularly among older people. It is characterised by loss of joint homeostasis. The articular cartilage cannot maintain its integrity and is progressively damaged; the subchondral bone envelope is thickened changing loads in the bone-cartilage biomechanical unit, the synovium shows signs of inflammation and bony spurs (osteophytes) appear at the edges of the bone. Its aetiology is multifactorial with a significant genetic component as shown by twin and family studies (1-2).

Many genetic variants have been considered as potential risk factors for OA, but most of the reported associations are inconclusive or not replicated. A recent large-scale meta-analysis found evidence that the *GDF5* locus on chromosome 20 was associated with the increased risk of knee OA in Caucasians (3-6). Other genome-wide data have reported an association with the *DVWA* gene in Asians but not Caucasians (7) and a *PTGS2* variant that replicated but did not reach genome-wide significance (GWS) (8). Recently, a genome-wide association (GWA) study identified a locus on chromosome 7q22 which has an association with combined knee OA and/or hand OA phenotype (9).

In this study we have synthesized available data from four GWA studies under the auspices of the Translational Research in Europe Applied Technologies for Osteoarthritis (TREAT-OA) consortium (www.treatoa.eu). A total of 2,371 cases of knee OA and 35,909 controls were available for this first stage of the analysis. The most significant signals were further investigated in additional samples of European descent and single nucleotide polymorphisms (SNPs) that reached GWS were further evaluated in Asian samples.

MATERIALS & METHODS

Study design

A detailed description of all samples used in this study is provided in Appendix 1. A three-stage design was used for the identification of any potential associations between sequence variants and knee OA in populations of European ancestry. We first synthesized the available data from four GWA studies (deCODE, Rotterdam Study, Framingham, Twins UK) using inverse variance fixed effects models. The variants that reached the $2x10^{-5}$ level of significance were selected for further replication. These SNPs were followed up in eight additional European cohorts (arcOGEN, Greek, Spanish, Finnish, Nottingham, Chingford study, GARP, Estonian and Swedish). The

SNPs that replicated in the follow-up samples were genotyped in two additional European samples (deCODE (Icelandic) and Swedish). One cohort provided computer-generated replication from an ongoing GWA study (arcOGEN, 12 SNPs were directly genotyped and 6 were imputed) while de novo replication was performed in the other cohorts. Furthermore, the top hits were followed up in Asian populations (Chinese and Japanese samples). The effect sizes from the meta-analysis of the GWA studies and the effect sizes from the replication effort were all combined to provide an overall estimate. We also synthesised the effect estimates of the European and Asian samples to provide a global summary effect estimate.

Phenotype definitions

Study subjects with a radiographic Kellgren and Lawrence (K/L) grade ≥2 (10) or total knee replacement were included as cases in the analysis. When clinical criteria were considered (Greek, Spanish and GARP study groups), the ACR rheumatology classification criteria were used (11). Subjects who had no known affected joints among those assessed acted as controls. For example, in a cohort that assesses knee, hip and hand OA, controls were participants with no affected hip or hand joints for the knee OA analysis. Population-based controls were used for the arcOGEN study.

Genotyping and imputation

Samples from the GWA studies were genotyped using the Infinium HumanHap300 (Illumina) for deCODE and Twins UK samples, HumanHap550v3 Genotyping BeadCHip (Illumina) for the Rotterdam Study and the Affymetrix GeneChip Human Mapping 500K for the Framingham cohort. The number of SNPs genotyped ranged from 314,075 to 500,510. Imputations were performed to increase the coverage. All the top SNPs studied had acceptable imputation quality. The genotyped and imputed SNPs that successfully passed the quality control criteria (n=2,335,627) were considered for the analyses. Detailed information on genotyping platform, quality control and imputation methods for each cohort are shown in Table 1 and in Appendix 2.

The replication samples for the Greek, Spanish, Finnish, Chingford and GARP studies were genotyped using the MassArray iPlex Gold from Sequenom. Replication genotyping was carried out by a genotyping contractor (Kbiosciences Ltd, Hertfordshire, UK) using a competitive allele-specific PCR SNP genotyping system for the Nottingham and the Estonian cohort. The additional 622 Icelandic cases and the samples from the Swedish cohort were genotyped by deCODE genetics using the Centaurus (Nanogen) platform (12). Detailed information on genotyping is provided in the published online supplement.

Table 1. Technical details of genotyping, quality control criteria and SNP imputation methods

Study	deCODE	Rotterdam	Framingham	TwinsUK
Array type	Infinium HapMap 300	Illumina HapMap550v3	Affymetrix GeneChip® Human Mapping 500	Infinium HapMap 300
Genotyping calling algorithm	Illuminus	Beadstudio		Illuminus
Exclusion of SNPs used for imputation	Call rate<98%	Call rate<98%	Call rate<97%	Call rate<98%
Imputation method	IMPUTE	MACH	MACH	IMPUTE
Imputation backbone (NCBI build)	HapMap CEU release 22 build 36	HapMap CEU release 22 build 36	HapMap CEU release 22 build 35	HapMap CEU release 22 build 36
Data handling and statistical tests	R, SNPTEST	MACH2QTL	R	PLINK and R

Statistical analysis

A*ssociation analysis:* Each team performed an association test per gender for knee OA under a per-allele model. The λ inflation factor was calculated per gender-specific effect size using the genomic control method (13) and the standard errors were corrected by the square root of the λ inflation factor was calculated per gender-specific effect size using the genomic control method13 and the standard errors were corrected by the square root of the λ inflation factor ($SE_{corrected} = SE_{observed} \times \sqrt{\lambda}$) . Robust standard errors were estimated to adjust for the family relationships (Framingham and GARP studies). Robust standard errors were estimated to adjust for the family relationships (Framingham and GARP studies).

Meta-analysis: The effect size for each SNP (OR per copy of minor allele as per HapMap) was calculated using inverse variance fixed effects models (14), synthesizing all the sex-specific effect sizes and the corrected standard errors. Analyses combining men and women were also performed. In family studies the results from men and women combined were used to account for relatedness between women and men within families. Meta-analyses of the GWA studies were performed using the METAL software (www.sph.umich.edu/csq/abecasis/metal). Between-study heterogeneity was tested using the Cochran Q statistic, which is considered significant at $P<0.1$. The extent of inconsistency across studies was quantified using the I^2 metric which ranges from 0 to 100% (15). Heterogeneity is considered low, moderate, high and very high for 0–24%, 25–49%, 50–74% and >75%, respectively (16). We also computed the 95% CI for the I^2 (17). The calculation was repeated with random effects models for all SNPs that were further evaluated in replication datasets. Meta-analyses of the 18 top hits were performed using Stata Version 10.1.

Assessment of credibility: In order to assess the credibility of the top hit, we calculated the Bayes factor under a spike and smear prior to using as an alternative

an average genetic effect corresponding to an OR of 1.2 and a conservative agnostic prior of 0.0001% (18).

Functional analysis

Two methodological approaches were used to investigate the functional role of genes identified by GWA studies: (1) by assessing their expression in primary human joint cells (synovial fibroblasts, chondrocytes and meniscal cells) and its change in response

Table 2. Characteristics of all studies

Team	Cases/ Controls[1]	Platform	Age mean (range)	BMI mean (range)	F[2] (%)	OA definition	Control definition
GWA studies							
deCODE	1033/32482	Infinium HapMap300	69(19-99)	26(14-60)	58%	TKR	Health care records
Framingham	419/1674	Affymetrix GeneChip®	64(29-93)	26(14-54)	56%	Radiographic	Radiographic
Rotterdam	868/1464	Illumina HapMap550v3	67(55-94)	26(16-56)	59%	Radiographic	Radiographic
TwinsUK	51/289	Infinium HapMap300	54(37-76)	25(15-51)	100%	Radiographic	Radiographic
Replication cohorts - 1							
arcOGEN	1643/4894	Illumina610Q	NA	NA	71%	Radiographic/ clinical	General population
Chingford	64/236	NP	63 (54-77)	26 (17-43)	100%	Radiographic	Radiographic
Finnish	112/210	NP	67 (51-74)	29 (20-42)	75%	TKR	Population-based
Greek	368/606	NP	61(20-90)	26(17-34)	72%	Clinical	Clinical
GARP	161/758	NP	60(30-79)	27(19-47)	63%	Radiographic/ clinical	Radiographic/ clinical
Spanish	262/294	NP	66(32-94)	31(18-53)		TKR/clinical	Clinical
Nottingham	647/237	NP	66 (40-97)	27 (15-51)	53%	TKR	Radiographic and clinical
Estonian	69/456	NP	47 (32-60)	28(15-47)	69%	Radiographic	Radiographic
Replication cohorts - 2							
deCODE	622/32482	Illumina and Centaurus (Nanogen)	77 (40-99)	29 (19-49)	63%	TKR	Population-based
Swedish	390/839	NP	62 (46-73)	29 (18-51)	63%	TKR+concomitant clinical & radiographic OA	General population without TKR

[1]nr of knee OA cases and controls; [2]females; NP: not pertinent; TKR: total knee replacement

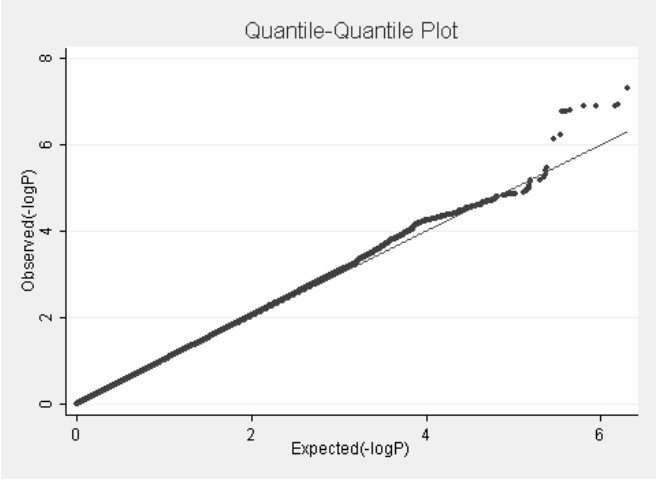

Figure 1. Quantile-Quantile (QQ) plot comparing the meta-analysis association results of the four studies with those expected by chance.

to the proinflammatory cytokines tumour necrosis factor α and interleukin 1β as well as comparing their gene expression profiles during chondrocyte dedifferentiation (3D pellet cultures vs monolayer culture); and (2) by assessing their expression dynamics by whole mount in situ hybridisation using zebrafish (*Danio rerio*) embryos aged 6 h (shield), 10 h (bud), 13 h (5–9 somites) and 1, 2, 3 and 4 days to explore their role during embryogenesis.

RESULTS

Meta-analysis of GWA studies and replication of top findings

The descriptive characteristics of the GWA studies used for the meta-analyses are from Iceland (deCODE), the Netherlands (Rotterdam Study), USA (Framingham) and the UK (Twins UK). The characteristics of these studies are shown in Table 2 and Appendix 1. The four GWA datasets included a total of 2,371 cases and 35,909 controls. A quantile-quantile (QQ) plot comparing the meta-analysis association results of the four studies with those expected by chance showed an excess of SNP associations indicating a likely true association signal (Figure 1). Data analysis showed the strongest association on chromosome 7q22 with a P-value of 5.06×10^{-8} for rs4730250 localized in dihydrouridine synthase 4-like gene (*DUS4L)* (Figure 2). Other associated signals in the 7q22 gene cluster were in high linkage disequilibrium (LD) ($r^2>0.8$) with the top signal (Figure 2).

We selected for follow-up in replication samples all SNPs with a P-value $<2 \times 10^{-5}$ in the meta-analysis association results. A total of 18 SNPs from 10 chromosomal

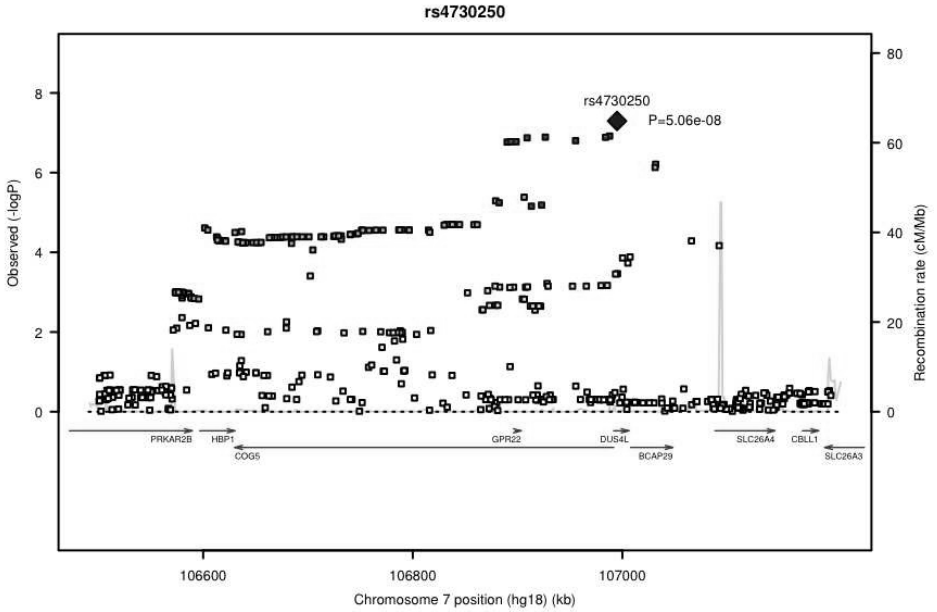

Figure 2. Regional association plot for the top signal rs4730250. A color figure is presented in Appendix 3.

loci satisfied this criterion (see Supplementary Table 1 in Appendix 2). However, as some of those SNPs were fully equivalent in the HapMap-CEU dataset, a total of 11 non-identical SNPs were tested for replication in 3,326 cases and 7,691 controls from eight European studies (see Table 2). Two SNPs (rs4730250 and rs10953541), both located at 7q22, replicated nominally ($P<0.05$) in the combined analysis of the follow-up samples with P-values of 6.3×10^{-4} and 8.3×10^{-3}, respectively. The two SNPs rs4730250 and rs10953541 were then further genotyped in two additional replication sets.

Both SNPs reached GWS in a meta-analysis of all European sample sets (GWA datasets and replication cohorts, Table 3). A total of 6,709 cases of knee OA cases and 44,439 controls were analyzed. SNP rs4730250 was genome-wide significant with a per-allele summary OR of 1.17 (95% CI 1.11 to 1.24) and a P-value of 9.2×10^{-9}. The minor allele frequency was 0.17 in the combined dataset. Low heterogeneity was observed (I^2=15%, 95%CI 0-48%) which was not statistically significant (P=0.26 for Cochran Q statistic, Figure 3).

No gender-specific effects were seen. The summary estimates did not differ significantly in men and women (P=0.74, test of homogeneity, Figure 3). Analysis of both sexes together in all cohorts did not alter the results (OR 1.17, 95%CI 1.07 to 1.27, $P=4.1 \times 10^{-8}$). The summary effect sizes of all loci under study are shown in

Table 3. Summary odds ratios and 95% confidence intervals of SNPs in the analysis including all European descent data

SNP	Minor allele	Chr	Gene	MAF	OR (95% CI) fixed-effect	P-value	I^2 (95% CI)	Q
rs4730250	G	7	DUS4L	0.17	1.17 (1.11-1.24)	$9.2x10^{-9}$	15(0-49)	0.26
rs10953541	T	7	BCAP29	0.24	1.17 (1.10-1.23)	$3.9.x10^{-8}$	19 (0-54)	0.23
rs3749132	A	2	ARHGAP25	0.07	1.17 (1.05-1.30)	$4.1x10^{-3}$	47 (0-74)	0.04
rs886827	C	7	GLI3	0.27	1.07 (0.99-1.16)	$9.0x10^{-2}$	65 (43-80)	0.001
rs1886695	G	20	CPNE1	0.16	0.89 (0.84-0.95)	$1.8x10^{-4}$	42 (2-66)	0.02
rs10071956	T	5	Intergenic	0.38	1.12 (1.06-1.19)	$5.1x10^{-5}$	15 (0-53)	0.29
rs6816070	G	4	LDB2	0.42	0.91 (0.86-0.95)	$1.3x10^{-4}$	0 (0-54)	0.46
rs661924	T	10	NEBL	0.39	1.11 (1.05-1.17)	$1.8x10^{-4}$	30 (0-67)	0.18
rs436354	G	5	ZDHC11	0.17	1.19 (1.01-1.30)	$1.8x10^{-2}$	41(2-63)	0.06
rs1994104	T	12	intergenic	0.13	0.88 (0.80-0.96)	$3.1x10^{-3}$	46 (2-70)	0.02
rs9857056	G	3	intergenic	0.12	1.11 (1.02-1.20)	$1.7x10^{-2}$	72 (43-87)	0.001

Chr: chromosome; MAF: minor allele frequency; OR: odds ratio

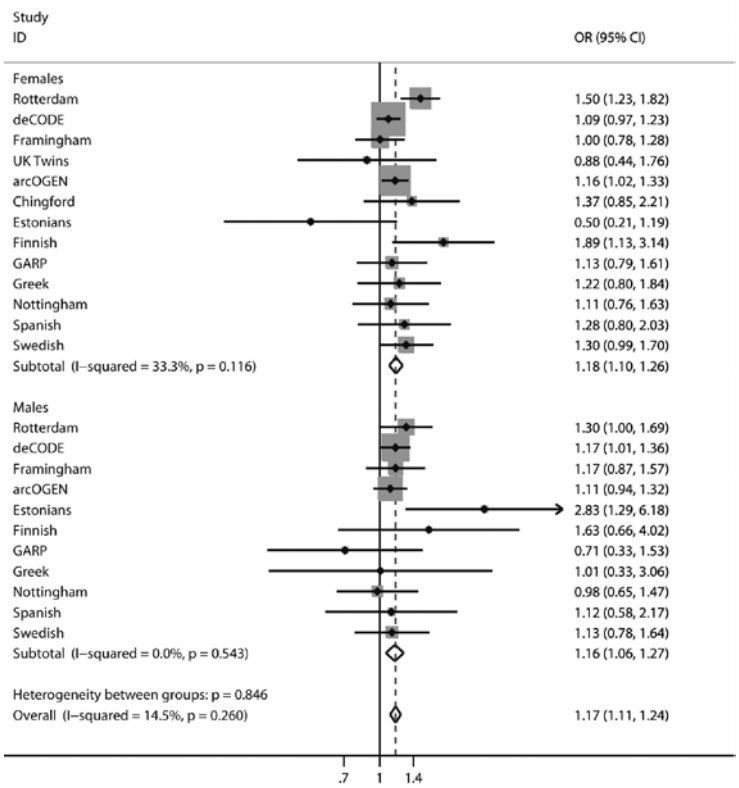

Figure 3. Forest plot of study-specific estimates (black boxes) and summary OR estimates and 95% CIs (diamonds) for the association between SNP rs4730250 knee OA.

Table 3 and the results from the random effects analysis for the top hits are shown in Supplementary Table 2 in Appendix 2.

The two significant SNPs at 7q22, rs4730250 and rs10953541, are highly correlated (D'=1, r^2=0.63 in HapMap-CEU) and are likely to represent the same underlying association signal as shown by conditional association analysis (see Supplementary Table 3 in Appendix 2). Age and body mass index are considered to be significant risk factors for the development of knee OA (19-25). We performed an analysis where the top hit was adjusted for these risk factors in deCODE samples and the Rotterdam Study. The association of the top hit remained largely unchanged in analyses adjusted for body mass index and age (data not shown).

In order to assess the credibility of the associations of the two SNPs, we calculated the Bayes factor (18) under a spike and smear prior using an average genetic effect corresponding to an OR of 1.2 and a conservative agnostic prior (assuming no prior knowledge of the association) of 0.0001%. The posterior credibility of these associations was 98% and remained similarly high even with a small alternative effect size of 1.1.

We also tested if the observed signal at the 7q22 region was replicated in East Asian samples (Japanese and Chinese cohorts). The total numbers of cases of knee OA and controls assessed were 1183 and 1245, respectively. rs12535761 was used as a proxy for rs4730250. The two SNPs are in strong LD (r^2=1, D'=1 in HapMap Asian samples). The finding was not replicated in the Asian samples with a summary effect size of 1.03 (95%CI 0.85-1.25). A meta-analysis including both European and Asian samples with 7,892 cases and 45,684 controls yielded a global summary effect of 1.15 (95%CI 1.10-1.22) with a P-value of 5.7×10^{-8} for rs4730250 with low heterogeneity (I^2=19%).

Expression patterns of genes in 7q22 cluster

The associated signal at 7q22 is located within a large (500 kb) LD block which contains six genes: *PRKAR2B* (protein kinase, cAMP-dependent, regulatory, type II, β), *HPB1* (HMG-box transcription factor 1), *COG5* (component of oligomeric golgi complex 5), *GPR22* (G protein-coupled receptor 22), *DUS4L* (dihydrouridine synthase 4-like) and *BCAP29* (B cell receptor-associated protein 29). We performed additional experiments to get more information about the genes in the cluster and their potential role in joint biology and pathology. Analysis of mRNA expression data in a chondrocyte pellet indicates that *BCAP29*, *COG5*, *DUS4L* and *HBP1* expression levels were higher than in monolayer cultures, suggesting that they are expressed in an environment that more accurately recapitulates articular cartilage (see Supplementary Figure 1 in Appendix 2). In contrast, no difference was seen for *GPR22* and *PRKAR2B* mRNA expression. In a zebra fish model, the expression of all genes

was detectable from the shield stage onwards (see detailed results in Supplementary Figure 2 in Appendix 2 and in color in Appendix 3).

DISCUSSION

This study provides further evidence for a knee OA signal localizing to the 7q22 cluster region and associated with knee OA. The statistical credibility and confidence of this evidence is very high, based on the calculations of the Bayes factor. The same locus has been identified and proposed as an OA susceptibility locus from the Rotterdam Study for the prevalence and progression of OA (9). Our study and the earlier Rotterdam Study do include overlapping populations. However, our study was specifically targeting the knee OA phenotype. An additional three European cohorts and two Asian populations were used for further replication. Our study uses the largest sample size in the genetics of knee OA research to date with almost 8,000 cases of knee OA analyzed.

The most significant hits identified by our study are located within a large (500 kb) LD block that contains six genes: *PRKAR2B*, *HPB1*, *COG5*, *GPR22*, *DUS4L* and *BCAP29*. The top hit rs4730250 is annotated in intron 3 of the *DUS4L* gene. Any of the genes at the 7q22 region may confer risk for knee OA as the LD pattern across the region is high.

The gene expression data support the epidemiological findings but do not exclude any of the six candidate genes. Specifically, the zebrafish experiments show that both *COG5* and *DUS4L* are expressed in developing cartilage, supporting the notion that either of these genes could have a biological function during chondrogenesis. The studies in the dedifferentiation model of human chondrocytes (3D vs 2D culture) show that *BCAP29*, *COG5*, *DUS4L* and *HBP1* all have different expression patterns in 3D culture (chondro-like cells) from 2D culture (dedifferentiated cells), suggesting that these four genes may play a role in cartilage metabolism.

A major issue in the field of OA is the definition of the disease phenotypes (4, 26). Different criteria may introduce bias and dilute the effect. The cases in our study were defined either clinically by the presence of a knee replacement or radiographically using the K/L system. The K/L system is, however, far from perfect and can be affected by differences in the position of the knee in which the X-rays were obtained, observer biases, interpretation of grading criteria and random error (27-28). Similarly, there are no standard criteria for replacing knee joints. This may introduce heterogeneity and move the observed effects towards the unity and so underestimate the true strength of an association. In our study we synthesized data with a standardized definition of the phenotype; however, small individual locus effects with ORs in the range of

1.1–1.2 as for other chronic diseases may well be plausible for knee OA, explaining the paucity of other significant hits despite the reasonable large-scale effort. These findings highlight that even larger collaborative studies and improved standardization of the phenotypes are needed to better understand and identify further genetic variants of OA. Moreover, even though we were able to accumulate a large sample size, the power of the study to detect very small effect sizes in the range of 1.05–1.15 is inadequate. For example, identification of a GWS signal with an effect size of 1.15 and minor allele frequency of 20% with 80% power would require almost 7,000 additional cases of knee OA.

Our results confirm that the 7q22 chromosomal region confers risk for knee OA which, along with our functional work, implicates six possible genes. Further in-depth genetic analysis of the locus including deep sequencing of the region and functional work including in vitro assays and animal models will be required to deepen our understanding of the underlying molecular pathways associated with the disease.

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Chapter 3.5

A genetic variant in the *NCOA3* gene is associated with hip osteoarthritis

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Manuscript in preparation

ABSTRACT

Background: The aim of this study was to discover novel genes involved in hip OA by means of a large genome-wide association study (GWAS) meta-analysis to gain more insights into the pathogenesis of the disease.

Methods: Hip OA was defined as "definite joint space narrowing" or "a total hip replacement". In the discovery stage, association results from GWASs of 8 studies, in total 5,244 cases and 17,836 controls, were meta-analyzed applying an inverse variance method using METAL software. The top signals with $P<1x10^{-6}$ were pursued in the replication stage where data of 5,010 hip OA cases and 17,151 controls was available originating from 8 studies. Results of the first and second stage were combined to get an overall estimation of effect size and P-value. A P-value $<5x10^{-8}$ was considered statistically significant.

Results: Meta-analysis results of the discovery stage showed 5 independent signals with $P<1x10^{-6}$, which were followed up in the replication stage. After replication, one genome-wide significant signal was identified. The A-allele of rs6490710 (minor allele frequency 4%) was significantly associated with a 26% increased risk of hip OA in an additive model with an OR of 1.26 ($P=4.6x10^{-8}$). This SNP is annotated in the nuclear receptor co-activator 3 (*NCOA3*) gene in a region with copy number variation and is in complete LD with a missense SNP (rs6094752).

Conclusions: Genetic variation annotated in the *NCOA3* gene is associated with hip osteoarthritis. Functional studies are needed to examine the role of this gene in the pathophysiology of OA.

INTRODUCTION

Osteoarthritis (OA) is a complex disease in which both environmental and genetic factors play an important role. The estimated heritability for OA ranges from 40-65% depending on the joint site (1). This means that a substantial proportion of variation in risk for OA can be attributed to genetic variation, i.e., polymorphisms in genes involved in the aetiology of OA. Knowledge about the genetic factors that contribute to the disease is important, because this can give us new insights in the pathogenesis and may identify new targets for prevention and treatment.

Over the last few years, a limited number of genome wide association studies (GWAS) studies have been published in Asians and Caucasians, but up to now few signals show reproducible association throughout different ethnicities. In Caucasians, 3 loci have reached the so-called genome-wide significance level (P=5x10^{-8}), at which level associations are now considered real (1 out of 20 false positives) (2). These loci include variants near the *GDF5* gene (originally originating from a candidate study) (3-4), a locus on chr7q22 near the orphan receptor *GPR22* (5-6), and a variant near the *MCF2L* gene (7).

The fact that so far not many loci were discovered could well be due to low power for detecting small effect sizes resulting from small sample sizes and/or phenotype heterogeneity. This is supported by results from the first stage of the arcOGEN consortium where no genome-wide significant hits were observed in a discovery study of 3,177 cases and 4,894 controls (8). On the other hand, differences in phenotype definitions can results in heterogeneity and thereby reduce the power of a study. We recently showed that many studies have different interpretations of K/L scores and clinical and radiographic outcomes are analyzed within one meta-analysis (9).

In this study a large-scale GWAS meta-analyses for hip OA is performed within the Translational Research in Europe Applied Technologies for OsteoArthritis (TREAT-OA) consortium. With in total 5,244 hip OA cases and 17,836 controls in the discovery phase, we identified a novel gene involved in hip OA at a genome-wide significance level.

MATERIALS & METHODS

Study design

A two-stage design was used for the identification of potential associations. The variants that surpassed the P<1x10^{-6} threshold in the discovery effort were selected for further follow-up. In silico and de novo replication was sought for these signals in 8 additional studies. In the discovery stage 5,244 cases and 17,836 controls were

included, in the replication stage 5,010 cases and 17,151 controls, with in total 10,254 cases and 34,987 controls in the final meta-analysis.

Study populations and phenotype definition

Discovery stage: A detailed description of all studies is given in Appendix 1. In summary, the arcOGEN consortium, the EGCUT study and deCODE are studies including symptomatic OA cases. The Rotterdam Study and TwinsUK defined OA on the basis of X-rays where at least definite joint space narrowing or a (partial or total) hip replacement due to OA were considered as OA. OA cases in the GARP study have symptomatic OA at multiple joint sites and for this study hip OA was defined in the same way as for the Rotterdam Study and TwinsUK Study. For this study, the arcOGEN consortium used controls from population-based, unrelated UK controls which came from 5 distinct sources: the 1958 Birth Cohort and the UK Blood Donor Service from the Wellcome Trust Case Control Consortium 2 study, the 1958 Birth Cohort from the Type 1 Diabetes Genetics Consortium (T1DGC) study, the Avon Longitudinal Study of Parents and Children (ALSPAC) study and the People of the British Isles (PoBI) study.

Replication stage: A description of the replication studies (Greek TJR cases, MrOs Study, Spanish TJR cases, SOF Study, MDC Study) is given in Appendix 1. In addition, the Paprika Study is a long term follow-up study of patients that have undergone a total hip replacement (10). Random controls (n=2377) were subjects participating in the Leiden Longevity Study (11). Written informed consent was obtained from each participant.

Genotyping and Imputation: To allow for meta-analysis across different marker sets, imputation of polymorphic HapMap European CEU SNPs was performed using MACH (12) or IMPUTE (13). An overview of all studies and the genotyping platforms and imputation method used is given in Table 1. Three cohorts (arcOGEN, MrOS and SOF) provided 'in silico' replication whereas 'de novo' replication was performed in other cohorts (Swedish, EGCUT, Paprika study, Greek, Spanish). Two research centers (Ioannina, Greece and Erasmus MC Rotterdam, the Netherlands) performed both the Quality Control (QC) and meta-analyses. A QC protocol was set up including validation of the results file format, reports for range of values and elimination of potential biases (i.e., extremely large beta's or SEs). Files were cross-validated between the two research centers after QC and after meta-analyses to check for inconsistencies. SNPs with a MAF <1%, imputation quality <0.30 (MACH) or <0.40 (IMPUTE) and beta's >4 or <-4 were excluded for further analysis.

Statistical Analysis: Each team provided the beta coefficients from an additive logistic model adjusting for sex as a primary analysis. To identify potential sex-specific hits each team performed an analysis in males and females only. The effects sizes of the discovery effort were corrected for relatedness using the lambda inflation factor.

Table 1. Studies included in TREAT-OA GWAS meta-analysis

Study	N cases	N controls	Lambda	N SNPs	Genotyping platform	Imputation method
Discovery stage						
arcOGEN stage 1	1728	4896	1.058	2.454.242	Illumina Human610 (cases) + Illumina 1.2M Duo (controls)	Impute
deCODE	2318	2318	1.182	2.399.690	Infinium HumanHap 300 + humanCNV370	Impute
EGCUT	64	2531	0.994	2.242.156	Illumina HumanCNV370 or HumanOmniExpress	Impute
GARP	106	1671	1.294	2.406.007	Illumina Infinium HD Human660W-Quad	Impute
RS-I	760	3233	1.009	2.450.385	Illumina HumanHap550v3	MACH
RS-II	159	1472	0.993	2.442.419	Illumina HumanHap550-Duo	MACH
RS-III	41	1487	0.962	2.397.764	Illumina Human660W-Quad	MACH
TwinsUK	68	228	0.993	2.358151	Infinium HumanHap300	Impute
Total discovery	**5,244**	**17,836**	**1.028**	**2.567.279**	-	-
Replication stage						
arcOGEN stage 2	1763	6157	-	-	Illumina Human610 (cases) + Illumina 1.2M Duo (controls)	Impute
EGCUT	141	1341			Illumina HumanCNV370 or HumanOmniExpress	Impute
Greek TJR cases	93	361	-	-	Single base extension using SNaPshot Multiplex Kit (Applied Biosystems, Foster City, USA))	-
MrOS[1]	446	2837	-	-	Illumina Omni 1 array (1.1 million probes)	-
Paprika Study	600	2377	-	-	Sequenom (MassARRAY iPLEX Gold)	-
Spanish TJR cases	697	783	-	-	Single base extension using SNaPshot Multiplex Kit (Applied Biosystems, Foster City, USA))	-
SOF[1]	761	2376	-	-	Illumina Omni 1 array (1.1 million probes)	-
Swedish cases	509	919	-	-	Centaurus (Nanogen)(16)	-
Total replication	**5,010**	**17,151**	-	-	-	-
Total	**10,254**	**34,987**	-	-	-	-

[1]proxy SNPs were used if the SNP was not on the array as imputation data was not available yet. rs6094752 as proxy for rs6094710 (r^2=1), rs10943623 for rs1577792 (r^2=0.8), rs10445324 for rs17610181 (r^2=1)

For the meta-analysis, the effect size for each SNP was calculated using inverse variance fixed effects models for the pooled and the sex-specific analysis. Between-study heterogeneity was tested using the Cochran Q statistics and it was quantified using the I^2 metric. Meta-analyses were performed using the METAL software (www.sph.umich.

edu/csq/abecasis/metal). Between-study heterogeneity was tested using the Cochran Q statistic, which is considered significant at $P<0.1$. The extent of inconsistency across studies was quantified using the I^2 metric which ranges from 0 to 100% (14). Heterogeneity is considered low, moderate, high and very high for 0–24%, 25–49%, 50–74% and >75%, respectively (15). A P-value $<5x10^{-8}$ was considered statistically significant.

RESULTS

The 8 GWAS datasets in the discovery stage included 5,244 hip OA cases and 17,836 controls. After QC, 2,567,279 SNPs were meta-analyzed. The results of the meta-analysis had a genomic inflation factor of λ=1,028. In Figure 1, the Manhattan plot is shown indicating P-values for each SNP in the GWAS meta-analysis. The SNPs on chromosome 6 and 20 remain just under the genome-wide significance limit of $5x10^{-8}$. The QQ plot showed an excess of signals in the lower P-value range, indicating a likely true signal (data not shown).

In Table 2 association results for the top signals for the discovery stage and discovery and replication stages combined is shown. In the discovery stage, five independent loci reached the threshold of $P<1x10^{-6}$. The most significant SNP was rs6094710 with an OR of 1.39 and $P=5.6x10^{-8}$. A sixth signal was selected for the replication stage: this SNP was just below the significance threshold of $1x10^{-6}$, but was previously shown to be associated with height (17). As the *GDF5* gene is an

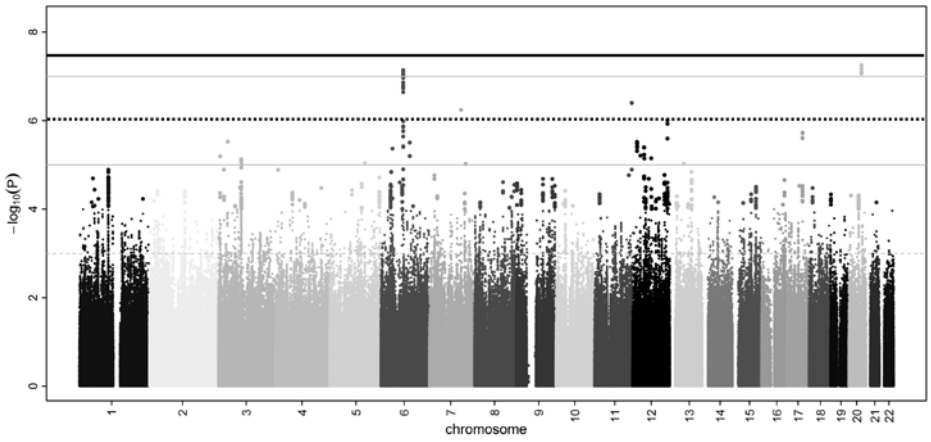

Figure 1. Manhattan plot for P-values for the GWAS meta-analysis. Bold line $P=5x10^{-8}$, dashed line $P=1x10^{-6}$.

Table 2. Association results for the GWAS meta-analysis of 8 GWA studies and the replication stage on hip OA

SNP	chr	gene	location	allele1	allele2	MAF	discovery stage		discovery + replication	
							OR	*P*	*OR*	*P*
rs6094710	20	NCOA3	5'UTR	A	G	4%	1.39	$5.6x10^{-8}$	1.26	$4.6x10^{-8}$
rs1577792	6	HMGN3	3'UTR	A	G	39%	1.14	$7.3x10^{-8}$	1.09	$7.7x10^{-7}$
rs640070	11	OPCML	intron	C	T	3%	1.69	$4.0x10^{-7}$	NA	NA
rs5009270	7	FLJ39575	3'UTR	A	G	30%	1.15	$5.7x10^{-7}$	1.10	$1.5x10^{-6}$
rs10773046	12	DNAH10	Intron	G	A	45%	0.88	$9.7x10^{-7}$	0.91	$1.2x10^{-7}$
rs17610181	17	NACA2	Exon[1]	A	G	14%	1.14	$1.9x10^{-6}$	1.14	$1.5x10^{-6}$

Chr: chromosome; gene: nearest gene; location: location in gene; allele 1: coded/effect allele; allele 2: noncoded allele; AF: minor allele frequency allele 1; OR: odds ratio; P: P-value; I^2: measure of heterogeneity; [1]SNP results in stop codon

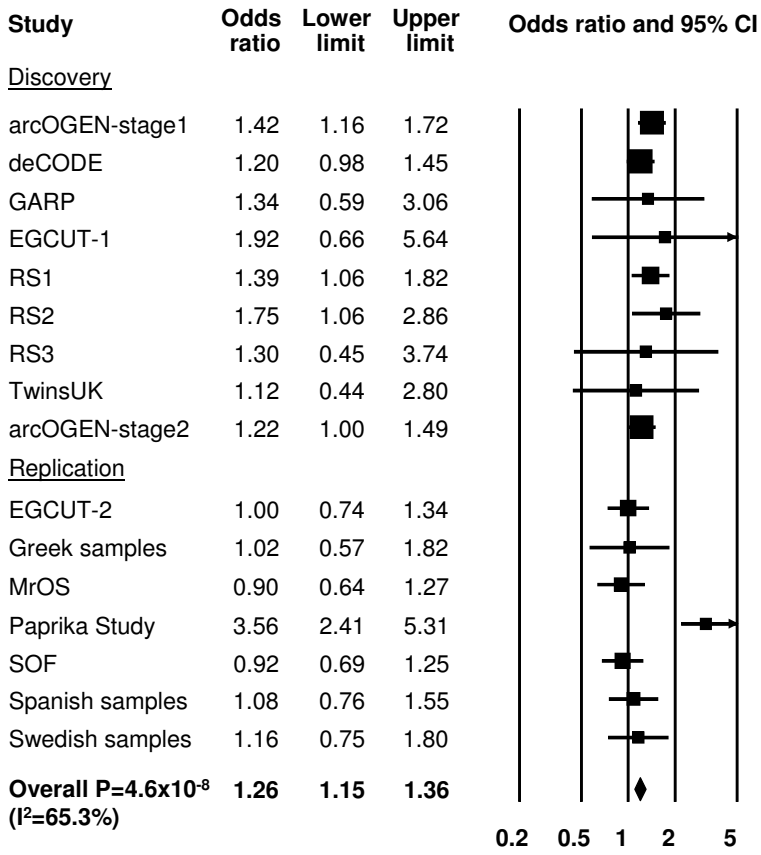

Figure 2. Forest plot for the association between rs6094710 and hip OA.

example in which genetic variation in the gene was also associated with both height and OA (4, 18), we believe that this signal was therefore also worth pursuing.

Two research centers checked imputation errors for the top signals. Imputed genotypes were compared to actual genotypes (de novo genotyping was performed) in a random sample of the Rotterdam Study and TwinsUK. Only rs640070 does not seem to be imputed accurately (data not shown). This SNP was therefore excluded from further analyses. The SNP rs6094710 reached the genome-wide significance level of $5x10^{-8}$. Large heterogeneity was present for this analysis (I^2= 65.3% *P*=0.00015), which originated from the results of the Paprika Study which showed a very large effect size compared to the other studies. A random effects model resulted in OR 1.27, 95%CI 1.09-1.49, *P*=0.002 for the association between rs6094710 and hip OA. A forest plot for the association between this SNP and hip OA is shown in Figure 2.

To visualize the genetic architecture (linkage disequilibrium, recombination hotspots) surrounding this locus a regional association plot was created for the GWAS discovery meta-analysis results of stage 1 (Figure 3 and in color in Appendix 3). rs6094710 is annotated in the 5'UTR of the nuclear receptor coactivator 3 (*NCOA3*) gene and that there are no other genes in the same LD block which are likely to underlie the association observed. Stratification according to sex did not reveal any additional loci reaching the level of genome-wide statistical significance. rs6094710, is in complete LD with rs6094752 which is a missense SNP (MAF 3%) leading to an amino acid change at position 218 in the protein (Arg>Cys). Bioinformatic analysis, using Polyphen: http://genetics.bwh.harvard.edu/pph/ and FASTSNP http://fastsnp.ibms.sinica.edu.tw/pages/input_SNPListAnalysis.jsp, of this change predicted this change to be probably damaging.

DISCUSSION

In this GWAS meta-analyses a novel locus for hip OA was discovered, the rs6094710 SNP which is annotated in the 5'UTR of the *NCOA3* gene. Carriers of the A allele have an increased risk (per copy of the allele) of 26% for hip OA. As there are no other SNPs correlated with rs6094710 which are located in another gene and there are no other genes close by the newly identified locus, the *NCOA3* gene is a likely candidate gene underlying the association with hip OA. The top SNP, rs6094710, is in complete LD with rs6094752 which is a missense SNP leading to an amino acid change at position 218 in the protein (Arg>Cys). Bioinformatic analysis of this change predicted this change to be probably damaging.

The *NCOA3* gene is a nuclear receptor co-activator that interacts with nuclear receptors, for example the estrogen receptor, and certain other transcription factors,

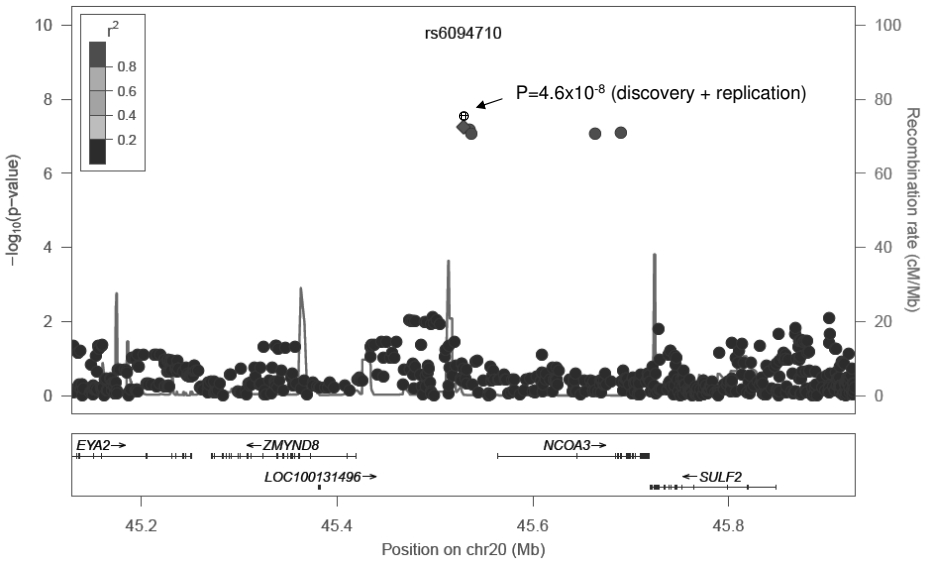

Figure 3. Regional association plot for rs6094710. A color figure is presented in Appendix 3.

it recruits histone acetyltransferases and methyltransferases for chromatin remodeling and facilitates target gene transcription (19). Previously, *Ncoa3* knockout mice were generated through homologous recombination in embryonic stem cells (20). These mice showed growth retardation and reduced adult body size, but the molecular mechanism responsible for this growth retardation remains largely unknown. In addition, female mice exhibited abnormal development and function of their reproductive system and estrogen levels were significantly lower in the knockout mice compared to the wild type (20). This gene is also known as a breast cancer susceptibility gene (21). A relationship between breast cancer and OA has never been established and it is therefore unlikely that breast cancer would be a confounding factor. It could be that subjects who are carrier of the A allele of rs6094710 die at an earlier age due to breast cancer, but this would lead to an underestimation of the effect of this SNP on OA. One hypothesis could be that the *NCOA3* gene is involved in the oestrogen signalling pathway and through this pathway is affecting OA risk. However, it is shown that there is only limited evidence for a protective effect of oestrogen therapy for hip OA and no clear associations between female hormonal aspects and hip OA were found in reviews (22-23). The exact functional consequences of SNP rs6094752 are unknown, but as the frequency of the SNP is low (~4%) and an amino acid change is probably underlying the genetic association observed, it is possible that a functional variant is discovered in this study. Functional experiments are needed to unravel the biological mechanism of this amino acid change in relation to OA.

Large heterogeneity was observed in the meta-analysis for rs6094710. However, this heterogeneity was introduced by a single study, the Paprika Study, which had a very large effect size (OR3.56) in the same direction as the discovery effect. A random-effects model needs to be applied in case of significant heterogeneity, but this results in a lower power to observe genome-wide significant results, which was also the case in this study. Although the P-value using a random-effects model was not genome-wide significant anymore, we do believe that the finding of the association between rs6094710 and hip OA is real. In the forest plot it is shown that consistent results across multiple studies are found and the study which introduced heterogeneity showed an effect size in the same direction as the overall effect size.

There was only 1 locus discovered in this large multi-cohort effort, which could have several reasons. First, power was still limited even with 5,244 cases in the discovery stage to find associated SNPs with small effect sizes. Second, only SNPs with a frequency >1%, with expected small effect sizes, included in the HapMap (www.hapmap.org) database are examined in relation to hip OA. Updates for imputation are appearing frequently and imputation based on the latest version of the 1000G project might increase our change of finding more loci involved in OA (24). It is believed by most scientists that multiple common variants are involved in common diseases, such as OA. However, GWAS has shown to be successful for some complex traits, whilst for others it was less successful. It could therefore well be that for some common disease the less frequent genetic variants, i.e., those with MAFs 0.1-5%, are playing a larger role in the pathogenesis of the disease. A GWAS meta-analysis for hip OA using imputation based on the 1000G project might identify some of these loci. A third reason for the limited amount of new genetic loci found is heterogeneity due to phenotype definitions (9). Radiographic and symptomatic OA phenotypes were used by different studies in this meta-analysis. In the future, more specific phenotypes, for example only symptomatic OA or only subjects with severe OA on an X-ray, will be meta-analysed to discover new loci involved in OA.

In conclusion, a novel locus involved in hip OA, located in the *NCOA3* gene, was discovered through a large-scale meta-analysis of genome-wide association studies. The exact underlying mechanism leading to a higher risk of OA remains to be elucidated by functional experiments.

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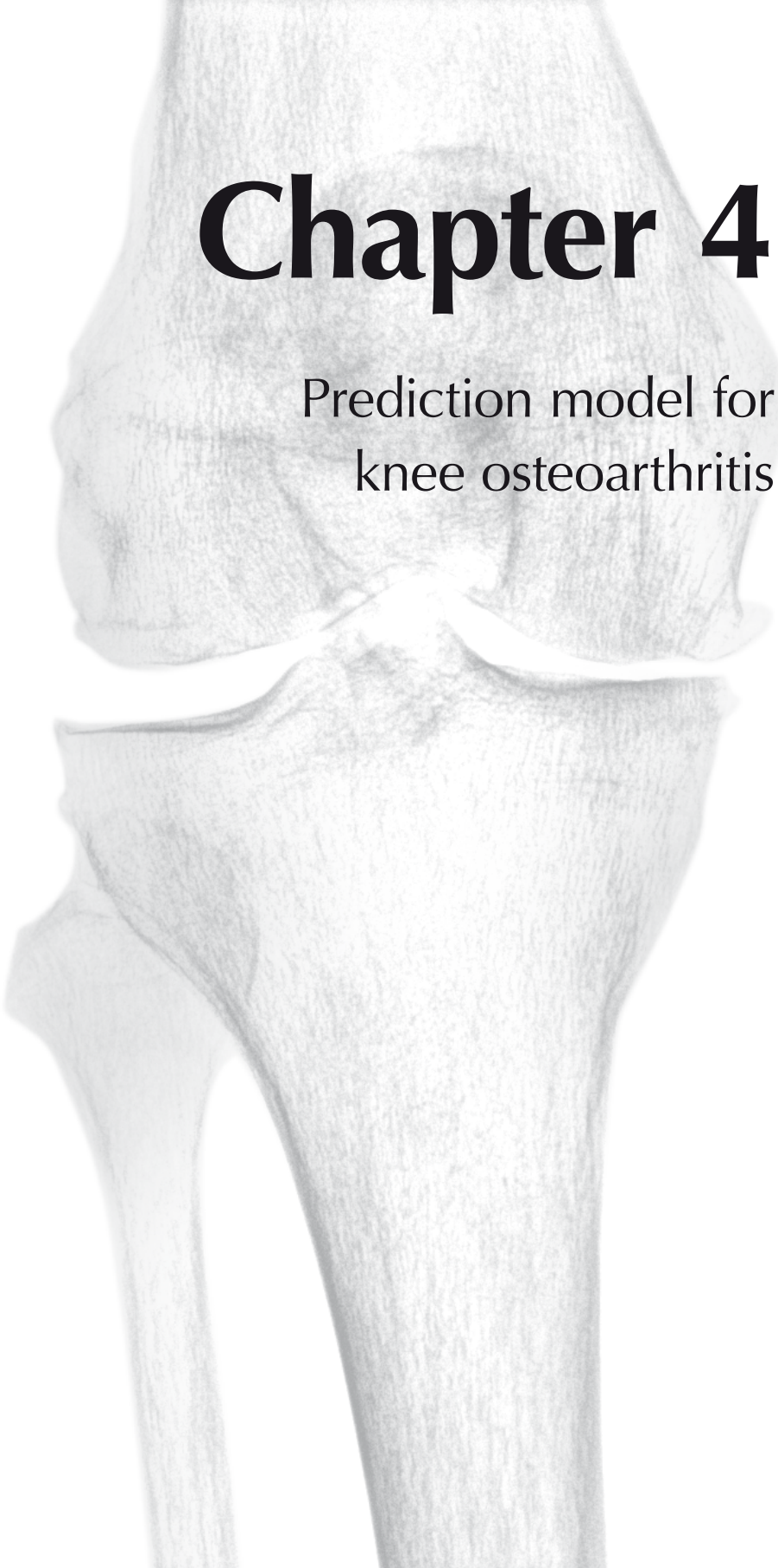

Chapter 4

Prediction model for knee osteoarthritis

Chapter 4.1

A prediction model for knee OA including clinical, genetic and biochemical risk factors

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ABSTRACT

Background: Identification of subjects at high risk for knee osteoarthritis (OA) is warranted for biochemical and pharmaceutical research and for prevention/monitoring in the general practice. Although some risk factors for knee OA are established, it is unknown how they perform in prediction models. The objective of this study was to create a risk prediction model for incident knee OA in an elderly population and assess the discriminative value of this model in an independent study.

Methods: 474 incident knee OA cases and 2,154 controls from the Rotterdam Study-I are included in this study. Univariate and multivariate analyses are performed for clinical variables (age, gender, BMI, pain, disability, general health, smoking and educational level), X-ray variables (baseline KL score of the knee, hand OA, hip OA) and biochemical markers (uCTX-II). The full (all risk factors included) multivariate model will be tested on discrimination (ROC curve, AUC) and calibration (Hosmer-Lemeshow) in the Rotterdam Study-II.

Results: The multivariate analysis showed the strongest association(s) between knee OA and gender (OR 1.69), BMI (OR 1.28), hand OA (OR 1.45), knee pain (OR 1.62) and baseline KL score (OR 6.97). In the Rotterdam Study-I there was moderate predictive value for incident knee OA based on the genetic score alone in subjects aged <65 years of age (AUC 0.65), whilst it was only 0.55 in subjects aged ≥65 years. The AUC for gender, age and BMI in prediction for knee OA was 0.66 in RS-I. Addition of the questionnaire variables or questionnaire variables + genetic score did not change the AUC (AUCs of 0.67 for both models). Addition of uCTX-II levels also did not improve the AUC. However, when adding the knee baseline KL score to the model the AUC increased to 0.79. Applying an external validation, similar results were observed in the Rotterdam Study-II with AUCs of 0.59, 0.61, 0.61 and 0.86 for, respectively, the same models.

Conclusions: We showed that "Questionnaire" variables, genetic markers, OA at other joint sites and CTXII levels do not add much predictive value to age, gender and BMI, at least not in an elderly population. A baseline K/L score of 1 however, is a good predictor of future knee OA. Furthermore, the genetic risk score alone has moderate predictive value in subjects aged <65 years of age, but is low in subjects aged ≥65 years.

INTRODUCTION

Osteoarthritis (OA) is a complex disease in which both environmental and genetic factors play an important role. The burden of OA is increasing due to a higher life expectancy and population structure, whilst there are no curative treatment options for the disease.

Identification of subjects at a high risk of OA is necessary for two main reasons. First, if subjects in a general population, who are at a high risk of developing OA can be identified, preventive strategies (and monitoring of that person) could be applied, if available, by for example the family doctor. Nowadays it is common practice for family doctors to warn their patients for the hazards of smoking. But what if we know that i.e., by decreasing weight with 10% in overweight people the risk of OA can be reduced? Should advice on prevention of OA then also be implemented in daily practice of general practioners? Second, in clinical and biochemical research it is most cost-effective to include subjects in a study who are at a high risk of developing OA on a short term. This would make it feasible to efficiently test new treatment strategies in OA patients.

Recently, the first risk prediction model for incident knee OA was presented in a small study of 99 cases and 179 controls with 12 years of follow-up (1). They evaluated, in a high risk cohort study (individuals were recruited from questionnaire studies for knee pain), 3 different models for incident radiographic and symptomatic knee OA and progression of knee OA using conventional risk factors such as age, gender, BMI, family history of OA, occupational risk and joint injury. External validation of these models in the Osteoarthritis Initiative (OAI) and the Genetics of Osteoarthritis and Lifestyle (GOAL) study showed poor to good discrimination with area-under-the-curves (AUCs) ranging from 0.52 for progression of knee OA in OAI, to 0.60 for radiographic and symptomatic knee OA in OAI and to 0.79 for symptomatic knee OA in the GOAL study. There are two main limitations to this study. First, as also the authors acknowledge, the internal cohort was small. Second, only basic clinical/questionnaire based variables are included in the model, ignoring other risk factors such as genetic and biochemical risk factors.

The objective of this study is to create and compare risk prediction models for knee OA, including clinical, genetic and biochemical risk factors, in a large population-based cohort study, the Rotterdam Study (RS). Validation of the model is assessed in an independent population-based study with similar population characteristics.

MATERIALS & METHODS

Study design

This study was conducted using a two stage design. In stage 1, the risk prediction model was created in the Rotterdam Study I (RS-I). Validation of this model was assessed in stage 2; calibration and discrimination were established in an independent cohort study with similar population characteristics, the Rotterdam Study II (RS-II).

Study populations

The Rotterdam Study is a large prospective population-based cohort study of men and women aged 55 years and older. The study design and rationale are described elsewhere in detail (2). In summary, the objective of the study is to investigate the determinants, incidence and progression of chronic disabling diseases in the elderly. The Rotterdam Study-I (RS-I) is the first sub-cohort of 7,983 persons, aged 55 years and over living in Rotterdam in the Netherlands. Since the start of the study there have been several follow-up visits to the research centre. All participants were examined at baseline in detail(2). In summary, a home interview was conducted (~2 hours) and subjects had an extensive set of examinations at the research centre (~5 hours). Subjects were selected if they were free of OA at baseline, had baseline and follow-up X-ray data and had data available for all risk factors assessed in this study at baseline (n=474 incident knee OA cases and 2154 controls). RS-I was extended in 1999 with 3,011 participants using the same inclusion criteria (the Rotterdam Study II (RS-II)). The medical ethics committee of Erasmus University Medical School approved the study and written informed consent was obtained from each participant.

Risk factor assessment in RS-I and RS-II

Age, gender and BMI: Age and gender were assessed during the home interview. Height and weight were measured at baseline examination with the subject in a standing position with indoor clothing without shoes and the BMI was calculated (kg/m^2).

Questionnaire based risk factors: A detailed questionnaire on joint complaints, and duration of the complaints was performed on all participants by trained interviewers (3). Knee pain was defined as pain during the last month during most of the days. The Stanford Health Assessment Questionnaire (HAQ) was used to assess disability. The HAQ formed part of a one hour home interview carried out by one of nine intensively trained interview assistants. It measures disability in eight categories Special attention was paid to standardisation of the scoring system of the HAQ. Participants were asked whether they scored their general health, better worse or the same compared to subjects from the same age. The reference category (0) in this study was better or the

same, participants who rated their general health as worse were scored as 1. Subjects who never smoked were scored 0, former or current smokers as 1. Educational level was assessed as highest educational level attained with 1 = primary education, 2 =primary education + a higher non completed education, 3= lower vocational education, 4=lower secondary education, 5=intermediate vocational education, 6=general secondary education, 7=higher vocational education, 8=university.

Genetic risk score: the genetic risk score was calculated as Σrisk allele (non-weighted) or Σ risk allele * $OR_{meta\text{-}analysis}$ (weighted). The SNPs selected for this genetic risk score are all SNPs with a P-value $<1x10^{-7}$ in Caucasian populations. This included rs143383 (*GDF5*) (4), rs11842874 (*MCF2L*) (5), rs10953541 (chr7q22) (6-7), rs4730250 (chr7q22) (6-7) and 5 additional SNPs from the latest yet unpublished arcOGEN GWAS study (8).

Biochemical marker: Urinary C-terminal cross-linked telopeptide of type II collagen (uCTX-II) is measured as described before (9). The concentration of uCTX-II (ng/liter) was standardized to the total urine creatinine (mmol/liter).

X-ray risk factors: variables included in the prediction model were the baseline Kellgren and Lawrence (K/L) score of 0 or 1, hand OA defined as 2 out of 3 joint groups (DIP, PIP and CMC1 or STT) affected by at least one definite osteophyte (K/L≥2) (10) and hip OA defined as at least definite joint space narrowing (11).

Outcome assessment: knee osteoarthritis

Weight-baring knee radiographs were scored by 6 trained readers for the presence of radiographic OA of the knee according to the K/L score (12-13). Approximately 10% of all X-rays were scored by all readers and interobserver reliability expressed as correlation statistics were 0.71 for RS-I and 0.68 for RS-II. Knee ROA was defined as a Kellgren-Lawrence (K/L) score ≥2 (= at least 2 definite osteophytes and possible joint space narrowing) of one or both joints. Incidence of knee ROA is defined as a K/L score <2 at baseline and a K/L≥2 at follow-up.

Statistical analysis

Creation of the risk prediction model: Imputation of missing data was performed for continuous variables with less than 10% missing data based on the correlation with all other risk factors. Only those subjects were selected which had data available for all variables. Standardized scores ((x-x)/sd) were made for continuous variables, such that the OR in the multivariate models is expressed as % of in- or decreased risk per standard deviation (sd). First, univariate analyses (ANOVA) are performed in RS-I to assess the relationship between risk factors and incident knee ROA. Subsequently multivariate logistic regression models are created with first including gender, age, BMI and questionnaire based variables (MV1), followed by the addition of the genetic

risk score (MV2) and finally addition of the x-ray risk factors (MV3). In a subset of RS-I data (135 incident knee OA cases and 794 controls) on uCTX-II levels are available. Therefore, risk prediction models were also created in this subset with again gender, age, BMI and questionnaire variables in MV1; gender, age, BMI and uCTX-II (MV2); gender, age, BMI, questionnaire variables, x-ray variables, genetic risk score and uCTX-II (MV3). All analyses were performed using SPSS version 17 and all analyses were adjusted for follow-up time.

Validation of the risk prediction model: Calibration indicates how close the risks predicted by the model are to the actual observed risks and the Hosmer-Lemeshow χ^2 statistics for goodness-of-fit were used to compare observed and predicted risks. Small χ^2 values and large P-values indicate good calibration. Calibration is assessed in the independent cohort study (RS-II). Discrimination examines the probability that an individual with the disease will be assigned a higher risk than an individual without disease. It says therefore something about the ability to correctly classify cases and controls. The area under the receiver operating characteristic (ROC) curve (AUC) was used to assess discrimination in RS-I (internal validation) and RS-II (external validation). An AUC of 0.50 means that this test has no discriminative abilities, flipping a coin would be as much predictive. An AUC of 1.00 means perfect discrimination where the test has a 100% sensitivity and 100% specificity. Internal validation was assessed in RS-I and external validation in RS-II.

RESULTS

Population characteristics

In total, 474 incident knee OA cases and 2,154 controls had data available for all risk factors in RS-I. Of these participants, 54% was female, they had an average of 65.1 (±6.4) years, an average BMI of 26.1 (±3.4) kg/m^2 and the mean follow-up time was 9.4 (±2.2) years. Subjects of RS-II were similar in population characteristics with 54% women, average of 63.2 (±6.6) years, average BMI of 26.9 (±3.8) kg/m^2, but with a shorter follow-up period of on average 4.1 (±0.6) years.

Risk prediction models

The genetic risk score was normally distributed in RS-I (Figure 1) and RS-II (data not shown). As the odds ratio's for all SNPs are within a small range (1.11-1.21) the weighted risk score shows the same results as the non-weighted (data not shown) and therefore only results of the non-weighted risk score are shown.

The results of the univariate analysis for the relationship between risk factors and incident knee OA in RS-I and the multivariate analyses are shown in Table 1. As

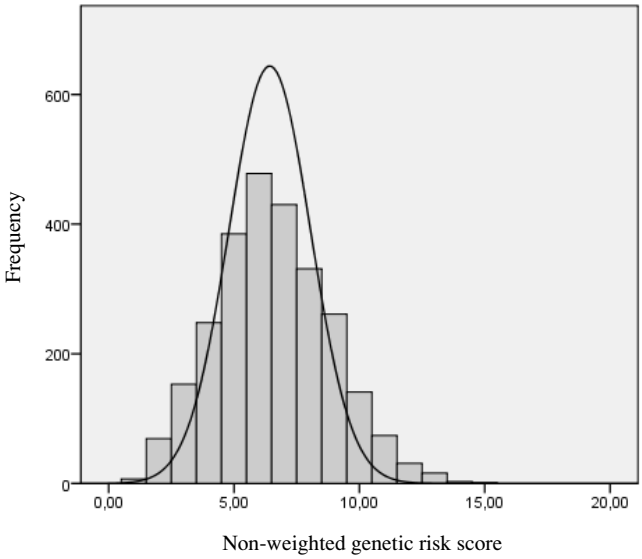

Figure 1. Histogram for the non-weighted genetic risk score in RS-I.

shown in the full multivariate model (MV3), the strongest associations are observed for the baseline KL score of 0 or 1 (OR 6.97), gender (OR 1.69), BMI (OR per standard deviation 1.28), knee pain (OR 1.62) and hand OA (1.45).

Validation of the risk prediction models: The Hosmer-Lemeshow χ^2 statistics for goodness-of-fit showed a good calibration for all risk prediction models in RS-II (Table 2). The area-under-the-curve is as to be expected higher in RS-I where the risk prediction model was created compared to the independent external validation cohort RS-II. A model including only age, gender and BMI resulted in an AUC of 0.66 in RS-I. The addition of questionnaire based variables did not add much predictive value (increase in AUC of 0.01). Subsequently adding the genetic risk score to the model also did not improve the model, whilst addition of X-ray variables increased the AUC up to 0.79 in RS-I. This increase in AUC is explained by the baseline KL score, not by the risk factors hand and/or hip OA (data not shown). In RS-II similar trends in AUCs were observed as in RS-I, with the AUCs being smaller for each model compared to the AUCs in RS-I. In Figure 2 the ROC curves are shown for RS-I for the 4 multivariate models as specified in Table 2.

In a subset of RS-I (135 cases and 794 controls), data was available on a biochemical marker, uCTXII levels. Risk prediction models were created in the same way as mentioned before in RS-I and were tested for discrimination in RS-I and RS-II. In Figure 3 the ROC curves are shown for 4 models as specified in Table 3. Comparing the genetic risk score with uCTXII levels, similar results were obtained, namely no significant increase in AUC compared to MV2.

Table 1. Univariate and multivariate association models to assess the relationship between risk factors and incident knee OA in RS-I

	Univariate			Multivariate 1			Multivariate 2			Multivariate 3		
Risk factor	*OR*	*95%CI*	*P*	*OR*	*95%CI*	*P*	*OR*	*95%CI*	*P*	*OR*	*95%CI*	*P*
Gender (%F)	2.13	1.72-2.64	$4.3x10^{-12}$	1.94	1.51-2.48	$2.0x10^{-7}$	1.88	1.45-2.44	$1.6x10^{-6}$	1.69	1.28-2.22	$2.1x10^{-4}$
Age (yrs)	0.99	0.89-1.10	0.84	0.99	0.88-1.10	0.81	0.99	0.88-1.10	0.79	0.89	0.79-1.00	0.049
BMI (kg/m²)	1.41	1.28-1.55	$5.0x10^{-12}$	1.36	1.23-1.50	$9.0x10^{-10}$	1.36	1.23-1.50	$9.8x10^{-10}$	1.28	1.15-1.42	$7.8x10^{-6}$
Knee pain[1]	1.81	1.42-2.32	$2.4x10^{-6}$	1.65	1.27-2.14	$1.8x10^{-4}$	1.64	1.26-2.13	$2.1x10^{-4}$	1.62	1.22-2.15	0.001
Disability index[1]	1.08	0.98-1.19	0.11	0.97	0.87-1.08	0.62	0.98	0.88-1.09	0.64	0.95	0.84-1.07	0.41
General health[1]	0.95	0.63-1.42	0.79	0.86	0.56-1.34	0.52	0.86	0.55-1.34	0.51	0.99	0.2-1.59	0.98
Smoking[1]	0.66	0.53-0.81	$7.8x10^{-5}$	0.92	0.73-1.17	0.51	0.92	0.73-1.17	0.50	0.98	0.76-1.26	0.85
Educational level[1]	0.95	0.90-1.00	0.07	1.02	0.96-1.08	0.56	1.02	0.96-1.08	0.56	1.02	0.95-1.09	0.60
Hand OA[2]	1.82	1.47-2.25	$4.7x10^{-8}$	-	-	-	-	-	-	1.45	1.14-1.85	0.003
Hip OA[2]	1.08	0.75-1.53	0.69	-	-	-	-	-	-	1.02	0.68-1.52	0.94
Baseline knee KL score (0/1)[2]	7.29	5.77-9.21	$3.1x10^{-62}$	-	-	-	-	-	-	6.97	5.48-8.86	$1.9x10^{-56}$
Genetic score	1.17	1.06-1.29	0.002	-	-	-	1.04	0.94-1.16	0.44	1.12	0.99-1.25	0.07

P: P-value; OR: odds ratio; CI: confidence interval; For continuous variables the OR per increase in standard deviation is shown (age, BMI, disability index, genetic score); [1] "questionnaire" variables; [2] X-ray variables

Table 2. Validation of the risk prediction models: calibration and discrimination

Model	Discrimination: AUC (95%CI)		Calibration: Hosmer-Lemeshow P-value	Variance explained
	Internal (RS-I)	*External (RS-II)*		
1	0.66 (0.64-0.69)	0.60 (0.52-0.67)	0.19	6%
2	0.67 (0.64-0.70)	0.62 (0.55-0.69)	0.82	8%
3	0.67 (0.64-0.70)	0.62 (0.55-0.69)	0.88	8%
4	0.79 (0.77-0.82)	0.86 (0.82-0.90)	0.63	34%

AUC: area under the curve; CI: confidence interval; RS-I: Rotterdam Study I; RS-II: Rotterdam Study II; Model 1: gender, age, BMI; Model 2: gender, age, BMI and questionnaire variables; Model 3: gender, age, BMI, questionnaire variables and genetic risk score; Model 4: gender, age, BMI, questionnaire variables, genetic risk score and x-ray variables

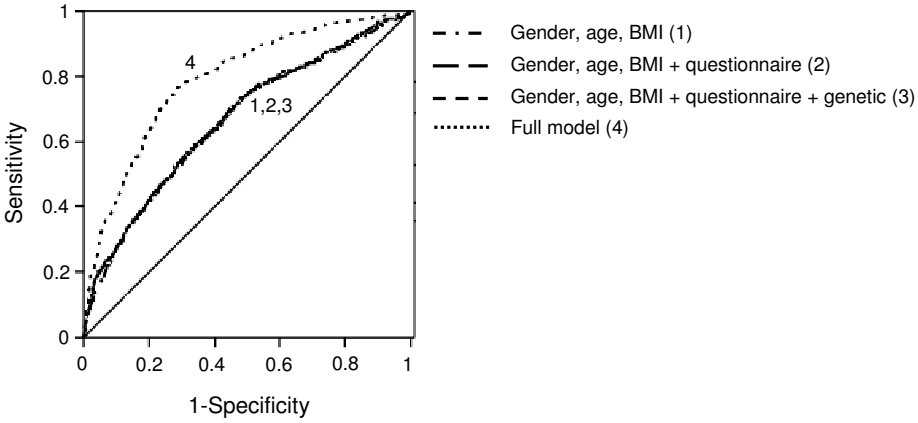

Figure 2. ROC curves for the four prediction models in RS-I.

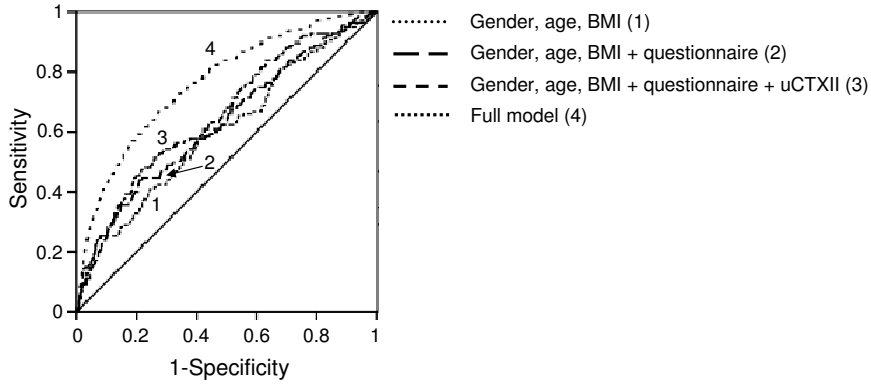

Figure 3. ROC curves for four prediction models including uCTX-II levels in RS-I (model based on a subset of the data of RS-I).

Table 3. Discrimination, expressed as AUC, for risk prediction models including uCTX-II in a subset of RS-I

	RS-I	RS-II
	AUC (95%CI)	*AUC (95%CI)*
Age, gender and BMI (1)	0.60 (0.55-0.66)	0.60 (0.52-0.67)
(1) + "questionnaire" variables (2)	0.62 (0.57-0.67)	0.63 (0.55-0.70)
(1) + (2) + uCTXII (3)	0.63 (0.58-0.68)	0.64 (0.57-0.71)
(1) + (2) + (3) + X-ray variables + genetic risk score	0.77 (0.72-0.81)	0.85 (0.81-0.89)

Table 4. Discriminative ability for incident knee OA based on separate groups of risk factors in RS-I and RS-II

	RS-I	RS-II
	AUC (95%CI)	*AUC (95%CI)*
Age, gender and BMI	0.66 (0.64-0.69)	0.60 (0.52-0.67)
Questionnaire based variables	0.62 (0.57-0.67)	0.57 (0.49-0.64)
Genetic risk score	0.61 (0.58-0.64)	0.51 (0.44-0.59)
uCTX-II levels	0.62 (0.59-0.65)	0.60 (0.52-0.67)
X-ray variables	0.76 (0.74-0.79)	0.84 (0.81-0.88)

All models were adjusted for follow-up time; AUC: area-under-the-curve; CI: confidence interval; RS: Rotterdam Study

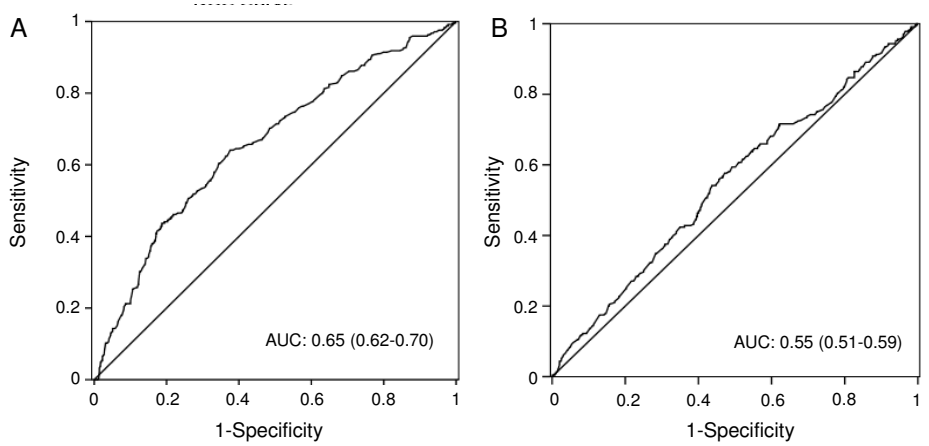

Figure 4. ROC curves for the genetic risk score alone in RS-I stratified according to age 65 years. (**A**) ROC curve for incident knee OA according to the genetic risk score only in subjects aged <65 years (RS-I). (**B**) ROC curve for incident knee OA according to the genetic risk score only in subjects aged ≥65 years (RS-I).

It would be of interest to know what the discriminative abilities are for the genetic risk score only as this risk factor is stable and already present from birth and prediction might therefore already be possible at young age. To estimate the predictive value of the genetic risk score alone, but also for questionnaire based variables, X-ray

variables and CTX2 alone, we created risk prediction models in RS-I and RS-II for these groups separately. The AUCs with 95%CI are depicted in Table 4.

As we hypothesized that the heritability of OA is higher in younger subjects, we also expect that discriminative abilities of genetic risk prediction models will increase when studying younger subjects. Therefore, the study population was stratified according to age <65 years of age (245 cases and 1,176 controls in RS-I) or ≥ 65 years of age (229 cases and 978 controls in RS-I). The AUC for the genetic risk score only was 0.65 (95%CI 0.62-0.70) in subjects aged < 65 years (Figure 4a), whilst it was only 0.55 (95%CI 0.51-0.59) in subjects aged ≥65 years (Figure 4b). These analyses were adjusted for follow-up time. Subjects aged <65 years of age were similar to subjects aged ≥65 years in terms of gender (*P*=0.70) and BMI (P=0.88), but had on average 1.0 year longer follow-up time (P=4.4x10^{-33}) and less frequent a baseline K/L score of 1 (35% versus 42%, P=1.9x10^{-4}) (data not shown).

DISCUSSION

We developed different types of risk prediction models to have an estimate of the discriminative abilities of models including very basic risk factors such as age, gender and BMI and the additive value of less conventional risk factors like a genetic risk score and uCTXII levels. The discriminative ability between subjects who develop OA versus subjects who remain free of OA was moderate when including only gender, age and BMI. Addition of either clinical/questionnaire based variables, a genetic risk score or a well-established biochemical marker, uCTXII level (9), did not add much predictive value to the model. When adding the baseline KL score for the knee to the model the AUC increased dramatically up to 0.86 in the independent validation study, RS-II.

Although this model is clearly not recommended for use in daily practice as the model is too complicated and labour intensive, there are some important lessons to be learned. In the Netherlands, it is not common practice for all radiologists to report minor OA-like features such as enclosed in a K/L score of 1. However, we showed that a K/L score of 1 is by far the best predictor of future knee OA even better than age, gender and BMI alone. Therefore this finding is highly relevant for the general practioner who requested this X-ray and we would recommend all radiologists to report such minor findings.

The genetic risk score is not a very good predictor of future knee OA in an elderly population as we showed in this study. This is not very surprising or disappointing. There are many other diseases like for example Diabetes Mellitus type II where the same results are observed (for review see (14)). There are several reasons why

discrimination between future OA cases and controls based on a genetic risk score is still difficult. First, there are only a few variants known which alter the risk of knee OA. In the genetic risk score 9 SNPs were included, which all have relatively small effects and therefore high predictive value is not to be expected. In addition, only genetic factors which arise from GWAS are included. We have to keep in mind that only 0.1% of the human genome is studied in these GWAS meta-analyses and therefore much more genetic factors will be involved in the aetiology of OA and might have an additional predictive value. In addition, genetic prediction can be context specific; it can be dependent on age or BMI. For example, the genetic risk prediction might be better in subjects which are not overweight. Moreover, the current study was conducted in an elderly population, aged 55 years and over with an average age of 65.1 years. In a large twin study for hip fracture (n=1055 hip fractures) it is shown that the heritability decreases with age from 68% at the age of 69 to 3% above the age of 79 years (15). Predictive value of genetic markers may therefore be larger when assessing the discriminative value at younger age. By stratifying the study population according to age at baseline, we showed that the AUC for the genetic risk score for subjects aged <65 years was much higher compared to the AUC for subjects aged ≥65 years. Perhaps it will be possible in the future to predict the risk of OA at birth using genetic markers. Whether this is desirable remains a question debate. However, in that case the prediction model should be created within a birth cohort and subjects should be followed for 60-70 years until they develop OA. There are such birth cohorts available, for example the Generation R Study (16), or Finnish birth cohorts. Another, possibly more feasible way to examine genetic risk score prediction during life, is to examine predictive value of genetic markers in a study with a long follow-up time, although one has to be aware of selection bias. The Rotterdam Study is such a study and in the near future OA data from 15-years of follow-up will become available.

Conventional risk factors based on questionnaires and uCTXII levels did also not add much predictive value to the basic model of age, gender and BMI. As age, gender and BMI are already rather strong predictors of OA at older age in the population studied, additive predictive value can only be achieved when risk factors with relatively large effect sizes are added to the model. All conventional risk factors for OA studied in this model and uCTXII levels have moderate effect sizes and it is therefore not surprising that these factors add little predictive value to the age, gender and BMI model. We showed that all risk factor groups by itself (so age/gender/BMI or genetic risk score or questionnaire based variables etcetera) all have limited and rather similar predictive value.

We have to note that there are a few potentially important risk factors for knee OA for which data was not available in our study that were not included in this study.

These are heavy work, injury and physical activity. However as noted above, reviews showed that occupational risk and physical activity have moderate effect sizes (OR <2) (17-18) and therefore not much additive predictive value is to be expected from these risk factors on top of the baseline variables as shown also for other risk variables in the current model (like for example knee pain or hand OA), although this should be formally tested. Injuries are a major risk factor (19), although recall bias is an issue to overcome in most epidemiological studies, and in the future the predictive value of this risk factor should therefore be investigated in a risk prediction model for knee OA.

The risk models shown in this study are not directly applicable in daily practice. This should be seen as one of the first steps towards a risk prediction model which can be applied in a clinical setting. If one would create a prediction model to be used by for example general practioners, it is important to select the study population to which you want to apply the model very carefully and to make the model as pragmatic as possible. In conclusion, age/gender/BMI or "questionnaire based" risk factors or a genetic risk score or uCTXII levels alone are not very good predictors of incident knee OA. Also, these risk factors combined had a relatively low predictive value for knee OA. In contrast, the baseline KL score of the knee is the best predictors for future knee OA, at least in an elderly population.

ACKNOWLEDGMENTS

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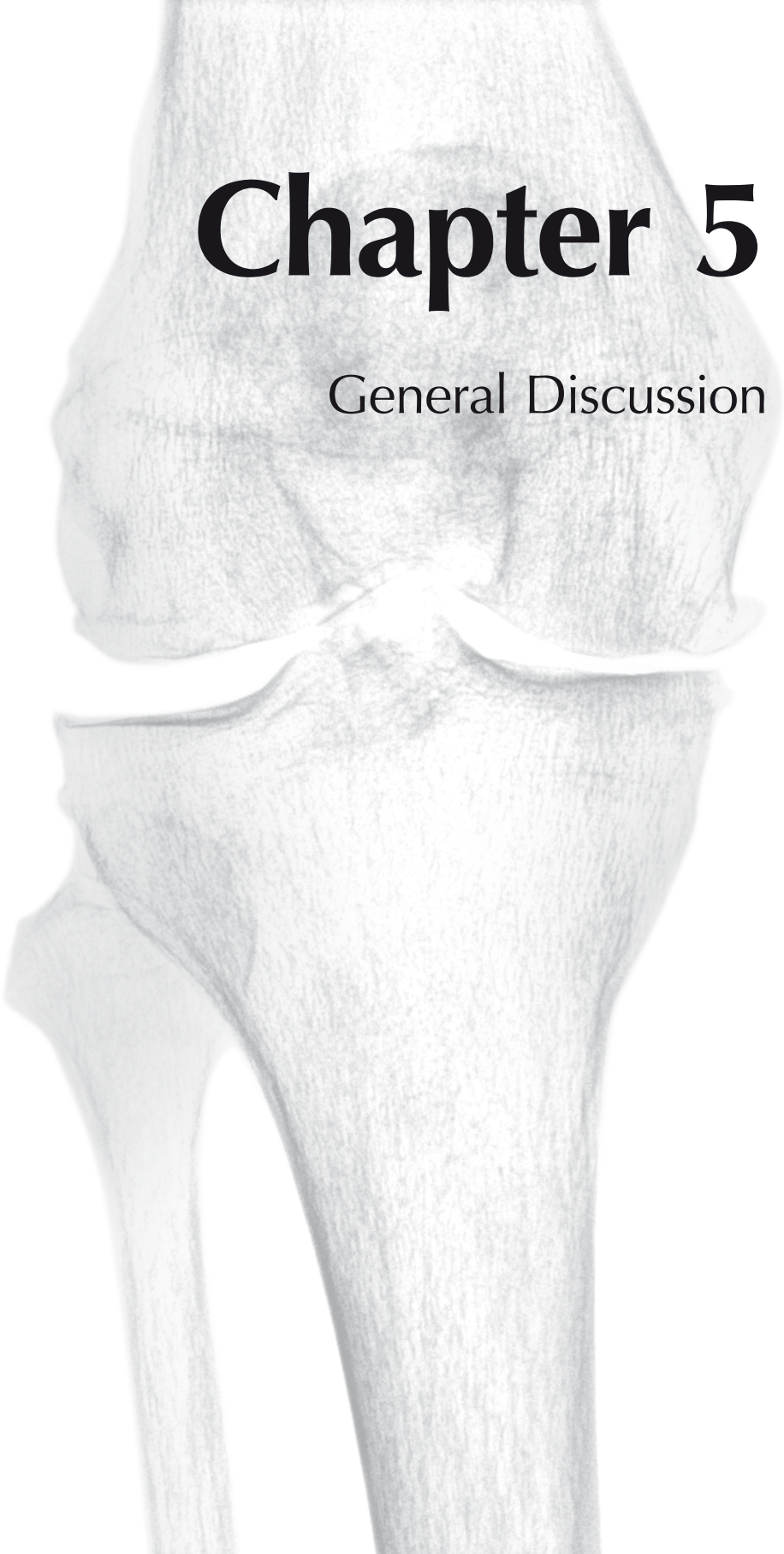

Chapter 5

General Discussion

The overall objective of this thesis was to identify genes involved in osteoarthritis (OA) by means of large scale genetic association studies using both the candidate gene approach and hypothesis-free genome-wide association studies. In this chapter all findings of this thesis are brought together, the modest number of loci discovered for OA so far are discussed and the challenges of phenotype definitions are placed in a broader context. Furthermore, (dis)advantages of the use of a large population-based cohort study are discussed and suggestions for future research, including next-generation sequencing, are given.

GENES INVOLVED IN OA

In this thesis genetic variation in many genes has been studied and some have been found to indeed be involved in the pathophysiology of OA whilst others are not. In this paragraph a short discussion on the most important genes studied in this thesis is given.

FRZB

In 2004 the first publication appeared on the relationship between a genetic variant (rs7775) in the *FRZB* gene and hip OA in women (1). We showed in *Chapter 2.1* by meta-analysis of published studies and adding results of the Rotterdam Study and the Chingford Study, that genetic variants in three genes of the Wnt signalling pathway, *FRZB*, *LRP5* and *LRP6*, are not associated with hip, hand or knee OA. These findings were further corroborated in an even larger meta-analysis where no association between hip or knee OA was found with this variant (2). Although 2 SNPs studied in the *FRZB* gene are not associated with OA, this does not necessarily mean that this gene is not involved in the pathogenesis of OA. There are multiple possible explanations why no consistent association was observed between the *FRZB* SNP rs7775 and OA. The first possibility is that there truly is no association between rs7775 and OA. Second, only 2 SNPs in the *FRZB* gene were studied in relation to OA, not all SNPs in the gene or surrounding the gene were studied on a large scale. It could be that there are functional variant(s) in the *FRZB* gene which are not studied which contribute to OA susceptibility. Third, different study populations, different OA phenotype definitions and different study designs are used to assess the relationship between *FRZB* variants and OA which could introduce heterogeneity and therefore power is too low to detect significant associations. Recent genome-wide association studies which use standardized OA definitions as much as possible, also do not find SNPs annotated in or nearby the *FRZB* gene to be associated with OA (3-6). Does this mean then that Wnt signalling is not important in OA or that the *FRZB* gene is not important?

The answer is rather straightforward: no. The fact that there is no genetic association found that affects the risk for OA does not necessarily imply that this gene is not important in the pathophysiology of OA. There are several lines of evidence that show the importance of Wnt-signaling and *FRZB* in the formation of the skeleton. *Frzb*$^{-/-}$ mice do not show apparent skeletal abnormalities or an increased incidence of OA upon aging compared to wild type mice. However, in arthritis induced mouse models, cartilage damage was significantly enhanced as compared to the wild type mice (7). In addition, increased MMP3 levels were observed in the cartilage of *Frzb*$^{-/-}$ mice (7). As osteoarthritis is a whole joint disease not only affecting the cartilage, changes in bone properties of the *Frzb*$^{-/-}$ mice are of interest. Cortical bone mineral density, content and thickness were significantly increased compared to wild type mice resulting in higher bending forces needed to obtain the same degree of deformation in the *Frzb*$^{-/-}$ mice (7). In conclusion, rs7775 in the *FRZB* gene is not associated with OA, but there seems to be a role for FRZB and especially for the Wnt signalling pathway in OA. For a review on Wnt signalling and OA see (8).

Chr7q22

In this thesis we have shown that the chr7q22 locus is involved in knee OA (*Chapters 3.2 and 3.4*). As was shown by the linkage disequilibrium (LD) plot, there are 6 genes annotated in this region and any of these genes or a combination of multiple genes could be involved in the pathogenesis of OA. A literature search did not point out a clear candidate gene. eQTL analysis showed, in a lymphoblast cell line, that there was a significant association between rs3757713 (D'=1.0, r^2=0.95 with rs3815148) and mRNA expression levels of *GPR22*. In addition, immunohistochemistry experiments showed presence of the Gpr22 protein in cartilage and osteophytes in OA-induced mouse models, whilst this protein was absent in normal cartilage (Chapter 3.2). Although this result is suggestive that the *GPR22* gene, which is an orphan G-coupled protein receptor, is the gene underlying the genetic association, the exact role in for example chondrogenesis is unclear.

Recently, additional functional work has been performed by the Laboratory for Skeletal Development and Joint Disorders at the Katholieke Universiteit of Leuven in Belgium, to unravel the biological mechanism behind this genetic association. They showed that stable over-expression of *GPR22* in the ATDC5 chondrogenic cell line alters the mRNA level of genes involved in the early proliferative phase (type II collagen is strongly reduced, aggrecan is reduced). In addition, it enhances the expression of genes involved in the late hypertrophy-mineralization phase (type X collagen expression increased). This indicates that alterations in chondrogenesis have taken place in such a way that the cells tend to differentiate only towards hypertrophy which is a characteristic of diseased cartilage such as in OA (9). During chondrogenesis,

quantification of Alcian Blue, Safranin O, Sirius red and Alizarin red staining were used to evaluate proteoglycans, collagens and mineralization content, respectively. *GPR22* overexpression reduced the overall collagenic content and to a minor extent the proteoglycan synthesis, while increasing the mineralization of the extracellular matrix. This increase in mineralization occurs when there are hypertrophic cells in the culture, which is in line with the findings of the alterations in mRNA levels shown in this paragraph. *ERK1/2* activation, a subfamily of the MAP kinases, is the most predominant pathway involved in hypertrophy and enhanced in OA like conditions (for review see (10)). The research group in Leuven showed that *ERK 1/2* activation is enhanced when *GPR22* is over-expressed.

Altogether, *GPR22* is the most likely candidate gene underlying the genetic association between genetic variation in the chr7q22 region and knee OA observed in this thesis. As GPR22 is a G-coupled protein receptor, this could be a new drug target. As the ligand for GPR22 is unknown, efforts need to be made to identify the ligand and explore the possibilities for drug development.

DOT1L

We showed in *Chapter 3.3* that genetic variation in the *DOT1L* gene is associated with cartilage thickness and hip OA. The G allele of the rs12982744 SNP was previously associated with increased height (11), which is in line with the thicker cartilage observed in our study. In addition, by functional experiments, we found that DOT1L is involved in chondrogenic differentiation presumably through its role in canonical Wnt-signaling. The Wnt-signalling pathway is known for its role in embryogenesis, bone and cartilage differentiation (for reviews see (8, 12)). Taken into account these results, we hypothesize that sensitivity for OA is partly determined during embryogenesis. One could see OA as a developmental disease in such a way that the cartilage thickness and bone characteristics at birth are important predictors of future OA. This hypothesis is not completely new; in 2008 it was suggested that OA susceptibility genes may play a role both in early developmental processes, for example in skeletal morphogenesis leading to malformations of joints and later in life the propensity of articular chondrocytes to become hypertrophic, which will lead to failure of cartilage homeostasis (13). This might explain differences in pathogenesis for example for hip and knee OA. The exact underlying developmental differences, including genetic factors determining part of these differences, need to be discovered to get new insights into the pathogenesis of OA.

NCOA3

In *Chapter 3.5* we present data from the most recent GWAS meta-analysis of the TREAT-OA consortium, and showed association between genetic variants in the

NCOA3 gene with hip OA. The mechanism of action of this gene on cartilage, bone and perhaps also muscle are unknown and functional experiments are warranted to elucidate the biological actions of this gene. This gene is a nuclear co-activator receptor gene and is therefore a candidate target for therapeutic options. It was found that NCOA3 interacts with a variety of nuclear hormone receptors, including RAR (14), thyroid hormone receptor (15), vitamin D receptor (16) and the oestrogen receptor (17). NCOA3 seems also to be involved in steroid dependent cancers, such as breast cancer (18-20). For example, expression of NCOA3 and its co-expression with members of the EGFR family in breast cancer is associated with a poor outcome in response to Tamoxifen therapy (21). Further work is needed to determine the value of this co-activator receptor as possible therapeutic cancer target. Regarding the role of NCOA3 in bone, cartilage and muscle pathology and homeostasis little is known. Are there specific targets in this pathway, what are the exact mechanisms? If we can answer these questions, perhaps a therapeutic target for OA might be found as well.

MISSING HERITABILITY

By 2010, genome-wide association studies have discovered >1,000 SNP associations for more than 150 polygenic traits (22). For osteoarthritis, only the *GDF5* gene (23), chr7q22 locus (4-5), *MCF2L* gene (3), *DIO2* gene (24), *NCOA3* gene (*Chapter 3.5*) and 5 additional loci from a yet unpublished GWAS study from the arcOGEN consortium, are found to be associated to OA in Caucasians at genome-wide significance level. In Asian populations, the *DVWA* and *ASPN* genes were also found to be involved in OA (25-26). Compared to some other diseases, the number of discovered loci is low. In addition, only a small part of the total variance is explained by these variants, while the total heritability for OA is substantial (40-60%). In complex genetics, the term missing heritability is often used to refer to the fact that only a very small percentage of the variation in a disease is explained by the variants discovered, but the remaining is still missing. This is also true in OA where the genetic variants contribute 3% to the explained variance (in the Rotterdam Study-I).

Power

One of the most obvious reasons to explain the low number of loci found is power. In the latest TREAT-OA GWAS meta-analysis (*Chapter 3.5*) ~5,200 cases were included in the discovery stage. More loci are expected to be discovered if the sample size in the discovery stage of the GWAS meta-analysis would increase. However, increasing power by adding more cases is not always sufficient to find new loci. In Figure 1 an

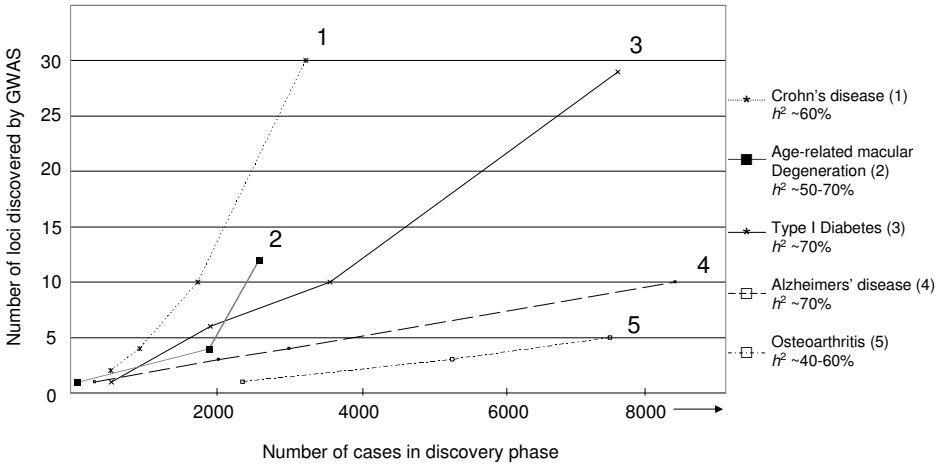

Figure 1. Number of loci identified through GWAS by number of cases in the discovery stage for different complex diseases. h^2: heritability estimate.

overview is given of the number of SNPs identified through GWAS by number of cases in the discovery stage for 5 complex diseases.

One can clearly see that there are large differences between diseases. Diabetes type I, Crohns' disease and age-related macular degeneration have been very successful in finding loci, whilst Alzheimer's disease and osteoarthritis have been less successful. This could be due to heterogeneity in the disease definition as shown in *Chapter 3.1*. Osteoarthritis is defined in many different ways, both symptomatically and radiographically, and increasing heterogeneity leads to a dramatic decrease in power (27). For example, Type I Diabetes is more straightforward to classify and therefore misclassification issues are of less importance.

Another explanation could be age. Studies with subjects with type I Diabetes and Crohns' disease are usually younger compared to subjects in studies on OA and Alzheimer's disease. It was shown for hip fracture that the heritability drops dramatically with increasing age (28). The same is likely to be true for other complex age-related diseases such as OA and Alzheimer's disease. By studying younger subjects this pitfall in genetic research could be overcome. Although there is no clear relationship between the heritability of a disease and the number of loci found (see Figure 1), the disease with the fewest loci discovered so far (OA) is also the disease with the lowest heritability.

Rare variants

Although it is clear that a part of the heritability of common diseases is explained by common variants, as exemplified by the large number of common variants found associated, it is becoming clear that common variants do not fully explain the heri-

tability of these diseases. The common disease common variant hypothesis, which states that multiple genetic variants with small effects underlie common diseases, can also be partly incorrect. It is now believed that part of the heritability is likely to be explained by low frequent genetic variants. The first reports are beginning to appear that this might be true (29). To prove this hypothesis, associations of such relatively rare (MAF 0.5-5%) SNPs or structural genetic variants with common diseases should be discovered. There are a few examples known where rare structural variants that affect genes are involved in common diseases such as mental retardation, epilepsy and schizophrenia (29). Such rare de novo (copy number) mutations can be responsible for a part of the missing heritability of OA as is also seen in for example schizophrenia (30). To prove that this hypothesis also holds true for OA at least 2 criteria need to be met. First, the sample size should be large enough to have sufficient power to find associations between rare genetic variations and a common disease like OA. Second, coverage of rare DNA variation in genetic studies should be high. As exome sequencing becomes affordable and the number of studies with sequencing data is increasing, it is expected that at least the second criterion will be met in the near future. Also, rare de novo (copy number) mutations can be responsible for a part of the missing heritability of OA as is also seen in for example schizophrenia (30).

The missing heritability is probably to be found in the ~99.9% of the genome which has not been studied yet. The fact that only ~0.1% of the human genome is studied in the GWA studies performed in the past 5 years is also a likely explanation for missing heritability. Recently, the 1000 genomes project provided a map of human genome variation from population scale sequencing. The aim of this project was "to discover, genotype and provide accurate haplotype information on all forms of human DNA polymorphisms in multiple human populations" (31). Compared to the International HapMap Project (32) this project aims, amongst others, to discover novel variants in the lower allele frequency range. This project will make it possible to study low frequency and rare variants in the near future which might partly fill in the gap of unexplained heritability.

Gene-gene and gene-environment interaction

Undetected epistasis (gene-gene interactions) remains one of the explanations for the missing heritability. Recent research on genetic interactions indicated that coding and regulatory variants of the same gene often modify the functional impact of each other (33). Challenges remain to identify these functional variants, but in the future this might become important in characterizing the genetic sources of phenotypic variation in OA.

Epigenetics

Last but not least, epigenetics have not been studied on a large scale for OA. Epigenetics is the study of heritable changes in gene expression or cellular phenotype caused by mechanisms other than changes in the underlying DNA sequence. The two most studied mechanisms are those of DNA methylation, including genomic imprinting, and histone deacetylation. Studying DNA methylation in a epigenome-wide association study could give new insights into the pathogenesis of OA. However, specific considerations need to be taken into account regarding the study design as DNA methylation patterns are specific to tissues and developmental stages and they change over time (for more information on epigenome-wide association studies see review (34)).

OA PHENOTYPES

In *Chapter 3.1* we showed that many different definitions of OA are being used in (genetic) epidemiological studies and this could lead to a reduction in power to find true associations. We therefore recommend standardizing phenotype definitions as much as possible in future (genetic) epidemiological studies. Although this is desirable, we realise this is also a huge challenge. Case-control, but also cohort studies, have collected their subjects in the past on certain in- and exclusion criteria and for most studies it is simply not possible to study multiple OA phenotypes like for example radiographic OA or symptomatic OA separately. The challenge is therefore to collect as many studies as possible with similar OA phenotypes and combine the results of those studies. Consensus between researchers, willingness to collaborate and perseverance are needed to make these meta-analyses to a success. When more distinct phenotypes, for example osteophytosis or joint space narrowing, of OA are studied on a larger scale then they have been performed in the past, new insights could be achieved. In addition, symptomatic versus asymptomatic OA can be studied, but also MRI features like cartilage volume and bone marrow lesions, OA at multiple joint sites at young age, OA free at old age and total joint replacements could be of great interest in relation to OA. OA is a disorder of the joints, but also factors like pain perception and muscle strength play a role in the burden people with OA experience. Especially for genetic studies, more (ethiological) homogenous phenotypes such as joint space narrowing, cartilage volume or osteophytosis could be of interest. By studying more distinct phenotypes of OA as mentioned above, perhaps novel loci can be found which are related to a small piece of the puzzle that is called OA. Large collaborative efforts, like the TREAT-OA and arcOGEN consortium,

should be expanded with as much studies as possible to make GWAS studies on sub-phenotypes a success.

OA PREDICTION

We showed in *Chapter 4* that discrimination between people that will develop knee OA in the near future or not, in an elderly population, is hardly possible with the current knowledge. With increasing knowledge about risk factors for OA, risk prediction might become available in the future and can be applied in different areas. If there are medicine available which could cure OA it might be desirable to identify subjects at a high risk of OA to monitor them and treat them as early as possible when the first symptoms arise. Genetic risk prediction is already close to clinical application in the field of pharmaco-genetics. The best known examples arise from cancer research where it was shown by multiple studies that subjects with a certain genotype respond better to treatment compared to subjects with another genotype (for examples see (35-38)). For example, the *SHMT1* 1420 T-allele was associated with a longer progression-free survival and overall survival in patients with metastatic colorectal cancer treated with first-line FOLFIRI+Bevacizumab (37). When medicines become available for OA, this will be an area where genetics can contribute significantly. In addition, there do not seem to be much adverse effects of genetic counselling. In a review it was shown that genetic counselling (in familial cancer) does not lead to adverse outcomes (39). Adverse psychological effects are not to be expected from genetic profiling based on recent studies of hereditary breast or colorectal cancer syndromes or type 2 diabetes (40-41). Also, in clinical trials ideally subjects are included which are at a high risk to develop the disease. In this way follow-up time could be shorter and less subjects need to be included which reduces costs. If we could identify those high risk subjects, this would be of great benefit for developing new treatment strategies.

There are also several limitations and disadvantages to risk prediction. One of the most relevant questions to ask when discussing risk prediction is: do people want to know if they are at a high risk of OA later in life? And does this change when people age? To my knowledge, there are no studies which examined the willingness of subjects to assess their OA risk. It is difficult to generalize results from diabetes or cardiovascular disease studies based on the assumption that these diseases have a much greater impact on life expectancy than OA. Research to answer this question is therefore needed. If we assume that people want to know their OA risk, but there are no preventive strategies and no treatment options would it then be desirable to know? Screening a (sub) population for a large number of susceptibility alleles is only

socially and economically justifiable if there are effective interventions or preventive strategies available (42-43). It will be economically unwise for health care systems to screen a whole population and subsequently take preventive actions for all subjects at a high risk for common diseases. Triaging genetic screening based on family history is an option to lower the number of people tested (44), but often at least part of the family history is unknown. Moreover, insurance companies might reject people based on their risk of disease. As there are currently limited treatment options and preventive strategies for OA with only small effects, risk prediction for OA is therefore not desirable at this moment.

In summary, (genetic) risk prediction for OA in clinical practice is not desirable at this moment as there are no good (preventive) treatment options for the disease. However, identification of high-risk subjects will be beneficial in research settings, but is not accurate yet. Whether risk prediction in the future will be available is dependent on numerous factors amongst which health-care costs.

(DIS) ADVANTAGES OF THE ROTTERDAM STUDY

There are a number of advantages of a large-scale population based cohort study, such as the Rotterdam Study, when studying genetics of OA. The size of the study is the most important factor. Worldwide there is no other population-based cohort study on OA of a magnitude like the Rotterdam Study. The study is large both quantitatively (number of subjects) as well as qualitatively (deep phenotyping) for numerous traits. Besides the detailed information on OA status at different joint sites, there is also data available on many co-morbidities, general characteristics, body composition and many other determinants of common age-related diseases. This makes it possible to study OA in a broader context, adjust for many confounders and study interactions between risk factors. Another main advantage of the Rotterdam Study is its longitudinal design. There is a long period of follow-up time, which gives the opportunity to not only study OA cross-sectional, but also answer research questions on incidence and progression of OA. A prediction model as shown in *Chapter 4* would not have been possible without a longitudinal design.

The main disadvantage of the use of data from the Rotterdam Study for OA research is the fact that the study was not designed specifically for OA. In epidemiology the slogan "do more less well" is often heard. For OA this means that there are no detailed questions on pain and disability due to OA available in all participants nor is there detailed data available at baseline on muscle strength or sports activities. However, within the Rotterdam Study efforts are made to expand the phenotypes for OA and more detailed information on especially clinically relevant factors will be

available in the near future. In addition, due to the long follow-up time of the study some measurements taken at baseline might not be the most appropriate ones to date. For example, MRI is becoming more and more important in OA research although the exact usefulness of this imaging technique is still a matter of debate. MRIs of the knee are, at this moment, only available in RSIII-1, thereby reducing the possibility to study joint structures and soft-tissue surrounding the joint in detail.

In summary, the main advantages of a large population-based cohort study like the Rotterdam Study definitely outweigh the disadvantages, especially for genetic epidemiological research questions.

IMPLICATIONS FOR CLINICAL PRACTICE

There are some direct implications of the findings shown in this thesis for daily clinical practice. We showed in *Chapter 4* that the baseline K/L score of the knee is a strong predictor of future knee OA and recommend that radiologists should report such findings to general practioners.

The main goal of genome-wide association studies is not to identify subjects at a high risk of developing a disease, although clinicians regularly believe this is the objective of GWAS. Identification of novel genes involved in OA and thereby discovering new pathways and create new insights into the pathogenesis of the disease is the main goal. The finding of the chr7q22 locus and knee OA and DOT1L is such an example. The genes underlying this genetic association have at first sight no obvious relation to OA. It is likely that new OA biology is found and future functional experiments could be promising in discovering new drugs for OA. Therefore, indirectly implications for clinical practice can be huge for genetic association studies.

FUTURE RESEARCH

The results presented in this thesis are among the first major breakthroughs in genetic OA research. We showed three new loci to be involved in OA which are all future candidates for drug development. As there are still only 10 independent loci found in relation to OA, much work needs to be done to unravel the genetic background of OA. As there is no cure for OA and even symptomatic treatment options are scarce, OA is an interesting and challenging disease to study. There is definitely hope for the future if we keep in mind the progress over the past 5 years. Before 2006, irreproducible candidate gene studies were performed which prevented us to discover genes for OA. This is mainly due to lack of knowledge about the pathophysiology of OA, but

also power issues are important. Advances in high-throughput genotyping methods enabled researchers to perform large GWA studies. Results of a few of these studies are presented in this thesis. The genetic research field is now at the edge of shifting towards a new era, next generation sequencing. In this paragraph recommendations for future research are given.

Study population

All large-scale genetic association studies included subjects at older age with the majority of patients aged >65 years. It is thought that genetic effects are larger in younger subjects, For example, it was shown on a study of hip fracture that the heritability dramatically decreases with age (28). In addition, we showed in *Chapter 4* that risk prediction based on a genetic risk score is weak in subjects below the age of 65 (AUC of 0.55), whilst it is moderate for subjects aged 65 years and over (AUC of 0.65). It is therefore highly recommended to perform genetic association studies in younger subjects. Especially longitudinal cohort studies including subjects aged 30-40 years can contribute significantly in terms of genetic studies and in creating risk prediction models, but sample sizes need to be large and multi-cohort studies are necessary. To our knowledge, there are no population-based longitudinal cohort studies in younger subjects studying osteoarthritis, with the exception of high-risk cohorts (45), and the initiation of such studies is encouraged. In addition, selecting many cases with a strong family history will possibly increase the probability of finding new loci for OA.

Expansion of OA phenotypes

In future OA research expansion of OA phenotypes is recommended. Distinct OA phenotypes should be analyzed for their association with genetic variants. For example, severe radiographic OA defined as a K/L score of at least 3, MRI features or OA at multiple joint sites. It might be that if we select subjects with a more severe phenotype, K/L score of 3 or 4, and compare those subjects with a K/L score of 0, that the power to find significant associations increases. At this moment, there is no proof yet that this will result in additional loci, new studies are therefore needed to find out of such an approach will be beneficial. In addition, a whole new research area in the OA field could be set up with a focus on developmental variations/abnormalities. One could think of for example hip shape in childhood in relation to OA in late adulthood. Although such studies are expensive, have ethical issues and the lost to follow-up will be high, it would be very interesting to study joint shape, bone, cartilage and muscle characteristics from birth and during childhood, in relation to OA at older age.

Technological developments

At this moment and in the coming years there are/will be new technological developments in genetic research. Whole exome sequencing (WES), where the protein-coding regions of the DNA are sequenced, has become affordable for large studies during the last 2 years. This method has proven to be successful in the identification of genes for Mendelian disorders (for examples see (46-48)). WES allows studying low frequency and rare variants in relation to OA. It can also contribute to the detection of structural variants and re-sequencing of a targeted gene (49) or region for fine mapping (i.e., chr7q22 region) could be done. The first study which identified a new gene for a common disease using next generation sequencing methods was on type 1Diabetes (50). By re-sequencing the previously reported associated LD block, they were able to identify significantly associated low frequency variants (MAF <3%) in the IFIH1 gene.

Although there are many opportunities of these new techniques to discover genetic determinants for complex diseases, several challenges need to be overcome. WES has the potential to identify rare variants, but some exons are not targeted by the current capture kits (~3% of RefSeq coding exons have <5x coverage in the latest kits (51)). Separating true variation from mapping and sequencing errors is a big challenge in next-generation sequencing. In addition, the amount of data derived from WES is enormous (in the range of terabytes for one single run) and a good informatics infrastructure is essential.

To increase the chance of detecting robust associations with the phenotype of interest one should include as much samples as possible. Two other approaches would be to include cases which are either affected severely or have a strong familial background to increase the chance of finding low frequency variants for OA. Also powerful analytical approaches have been suggested where aggregated analysis of multiple rare variants across one locus is performed (52-54). Altogether, new techniques are evolving rapidly and the past taught us that there are probably upcoming solutions for technical and analytical issues. This gives us the unique opportunity to study the genome/exome in more detail and hopefully this will provide us new insights into OA biology.

Last, but not least, finding new loci in OA is not the final destination. We need to know which genes are underlying these loci, what the functional consequences are, which new pathways are discovered and finally how we can translate this to drug development. (Financial) efforts are needed to answer these questions. One area of research would be RNA-seq, which refers to the use of high-throughput sequencing technologies to sequence cDNA in order to get information about one's RNA. RNA-seq would provide information on how different alleles of a gene are expressed, could detect post-transcriptional modifications such as splice-effects or identify gene fusions. In the Rotterdam Study RNA-seq data will be available in the near future

which allows us to study the genetic loci found in GWAS and WES studies in more detail.

In summary recommendations for future research would be to increase the sample size, study sub-phenotypes, include younger subjects and expanding analysis to the exome and in the future whole genome, epigenetics and interactions, which might unravel the missing heritability for OA.

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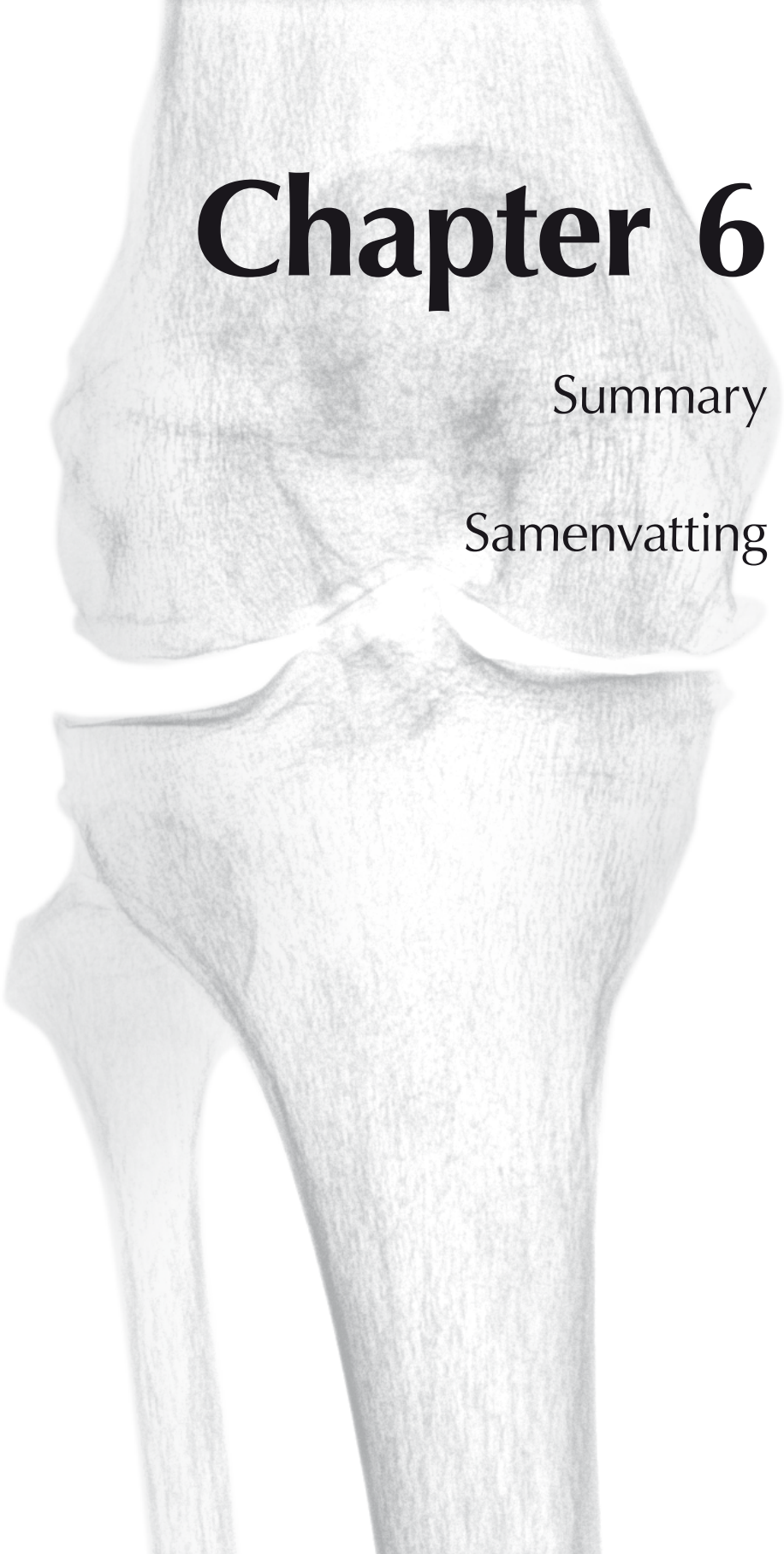


Chapter 6

Summary

Samenvatting

SUMMARY

Osteoarthritis (OA) is a chronic disabling disease, characterized by pain and disability, affecting mainly the elderly population. Only scarce treatment options are available without any cure for the disease.

The risk factors for OA remain largely undiscovered, but it is known that besides the environment also genetic factors play an important role in the etiology of the disease. The overall objective of this thesis was to identify genes involved in osteoarthritis. First by means of candidate gene association studies followed by hypothesis-free genome-wide association studies (GWAS), both individual and large-scale meta-analyses.

In **Chapter 2** the association of single nucleotide polymorphisms (SNPs) in candidate genes and hip, knee and hand OA are presented. In **Chapter 2.1** polymorphisms in three genes of the Wnt signaling pathway, *FRZB*, *LRP5* and *LRP6*, are studied in relation to hand, knee and hip OA in the Rotterdam Study and the Chingford Study. Subsequently a meta-analysis of all published studies, including the new data from the Rotterdam and Chingford Study, was performed for the *FRZB* rs7775 (Arg324Gly) SNP for hip and knee OA. No associations were observed between any of the polymorphisms studied in the *LRP5*, *LRP6* and *FRZB* genes and OA at the hip, knee or hand.

In **Chapter 2.2** results of a candidate gene association study on genetic variation in the *ESR2* gene and risk of OA is shown. We showed by meta-analysis of 7 studies summarizing 2,364 hip OA cases and 6,773 controls, 1,983 knee OA cases and 4,706 controls and 1,431 hand OA cases and 3,883 controls, that common genetic variation in the *ESR2* gene is not likely to be associated with an increased risk of osteoarthritis. However, we have to note that we had 80% power to detect odds ratio's of 1.14 and therefore we cannot exclude that smaller effects may exist.

The relationship between serum high sensitive C-reactive protein (hs-CRP) levels and OA and between genetic variation in the *CRP* gene and OA is described in **Chapter 2.3**. First we showed that serum hs-CRP levels are associated with knee OA in the Rotterdam Study-I. However, after adjustment for body mass index (BMI) this association disappeared. We conducted a literature review to obtain information on all published studies which assessed the relationship between hs-CRP levels and OA and we showed by meta-analysis that there is no relationship between hs-CRP levels and progression, incidence or prevalence of OA of the knee, hip and/or hand, at least not independent of BMI. In addition, we showed that genetic variation in the *CRP* gene was not associated with OA at those three joint sites.

To clarify the role of common genetic variation in the Interleukin-1β (*IL1B)* and Interleukin-1R antagonist (*IL1RN)* genes on risk of knee and hip osteoarthritis (OA)

and severity of knee OA, large-scale meta-analyses were performed in **Chapter 2.4**. PubMed was searched for articles assessing the role of *IL1B* and *IL1RN* polymorphisms/haplotypes on the risk of hip and/or knee OA (n=6) and novel data was included from 8 unpublished studies. We showed that there is no evidence of association between common genetic variation in the *IL1B* or *IL1RN* genes and risk of hip OA or knee OA (P>0.05 for rs16944, rs1143634, rs419598 and haplotype C-G-C (rs1143634, rs16944 and rs419598) previously implicated in risk of hip OA). The C-T-A haplotype formed by rs419598, rs315952 and rs9005, previously implicated in radiographic severity of knee OA, was associated with reduced severity of knee OA (OR=0.71 95%CI 0.56-0.91; P=0.006, I^2=74%). Summarized, common genetic variation in the Interleukin-1 region is not associated with prevalence of hip or knee OA but our data suggest that IL1RN might have a role in severity of knee OA.

These studies were all candidate gene association studies for which *a priori* knowledge on the disease is essential and as genotyping was still relatively expensive at the time these studies were conducted, only a few SNPs in the genes were studied. However, as perhaps also genes from unidentified pathways or genes involved in other tissues than cartilage or bone can be important for OA, we also attempted to identify novel genes involved in OA by means of hypothesis-free GWAS (**Chapter 3**).

First, in **Chapter 3.1** challenges in (genetic) epidemiological research in the field of OA are given with the focus on phenotype definitions. In this chapter, the need for standardization of OA phenotypes is addressed by examining the effect of heterogeneity among symptomatic (SOA) and radiographic osteoarthritis (ROA) phenotypes within the TREAT-OA consortium (a consortium established in January 2008 to address the generalisability and utility of genetic and biochemical risk factors (www.treatoa.eu). We showed that all studies (n=13) with SOA used a different definition and/or assessment of OA status for SOA of the knee, hip and hand. For knee, hip and hand ROA, 5, 4 and 7 different definitions were used, respectively. Hip OA defined as "definite JSN and one definite osteophyte" was not associated with gender (P=0.22), but defined as "total hip replacement" was significantly associated with gender (P=0.001). This indicates that differences in phenotype definitions can introduce heterogeneity when combining data of different studies. A standardization process was carried out and the prevalence of hip and knee ROA was similar in 9 cohort studies after standardization. Standardization of SOA phenotypes was not possible due to the case-control study design of the studies.

In **Chapter 3.2** results of the first GWAS study of the Rotterdam Study are shown. In this study we tested 500,510 Single Nucelotide Polymorphisms (SNPs) in 1,341 OA cases and 3,496 Dutch Caucasian controls. SNPs associated with at least two OA-phenotypes were analysed in 14,938 OA cases and approximately 39,000 controls. The C-allele of rs3815148 on chromosome 7q22 (MAF 23%, intron 12 of the

COG5 gene) was associated with a 1.14-fold increased risk (95%CI: 1.09-1.19) for knee- and/or hand-OA (P=8x10^{-8}), and also with a 30% increased risk for knee-OA progression (95%CI: 1.03-1.64, P=0.03). This SNP is in almost complete linkage disequilibrium with rs3757713 (68kb upstream of *GPR22*) which is associated with *GPR22* expression levels in lymphoblast cell lines (P=4x10^{-12}). Immunohistochemistry experiments showed absence of *GPR22* in normal mouse articular cartilage or synovium. However, *Gpr22* positive chondrocytes were found in the upper layers of the articular cartilage of mouse knee joints that were challenged by *in vivo* papain treatment or in the presence of interleukin-1 driven inflammation. *Gpr22* positive chondrocyte-like cells were also found in osteophytes in instability-induced OA. Since the *GPR22* gene encodes a G-protein-coupled receptor, this is potentially an interesting drug target. In summary, these findings reveal a novel common variant on chromosome 7q22 to influence susceptibility for prevalence and progression of OA. A GWAS meta-analysis of the TREAT-OA consortium later confirmed this locus to be involved in knee OA at a genome-wide significance level of P<5x10^{-8} (**Chapter 3.4**). No additional loci for knee OA were observed in this relatively small GWAS meta-analysis of 2,371 knee OA cases and 35,909 controls in the discovery phase.

Chapter 3.3 describes a GWAS of joint space width of the hip. In this GWAS of 6,523 individuals from a population based study in the Netherlands on joint space width of the hip, we identified the G-allele of rs12982744 on chromosome 19p13.3 to be associated with a 5% larger joint space width (P=4.8x10^{-10}). The association was replicated in 4442 individuals from 3 UK-cohorts with an overall meta-analysis P-value of 1.1x10^{-11}. The SNP was also strongly associated with a 12% reduced risk for hip OA (P=1x10^{-4}). rs12982744 was previously found associated with height, where the G-allele was associated with increased height. The SNP is located in the *DOT1L* gene, which has been recently linked to Wnt-signalling. Immunohistochemical staining of the *Dot1l* gene during mouse limb bud development supports a role for DOT1L in chondrogenetic differentiation and adult articular cartilage. In the ATDC5 chondrogenesis model system, silencing of *Dot1l* inhibited chondrogenesis and negatively affected Wnt signalling. We further demonstrated that Dot1l interacts with TCF and Wnt signalling in developing chondrocytes.

In **Chapter 3.5** the latest preliminary results on the second GWAS meta-analysis of the TREAT-OA consortium are described. In the discovery stage, association results from GWASs of 8 studies, in total 5,244 cases and 17,836 controls, were meta-analyzed. Meta-analysis results of the discovery stage showed 5 independent signals with P<1x10^{-6} and were followed up in the replication stage (5,010 hip OA cases and 17,151 controls). Replication in 8 studies resulted in one genome-wide significant signal. The A allele of rs6490710 (minor allele frequency 4%) was significantly associated with a 26% increased risk of hip OA in an additive model with an OR of

1.26 and $P=4.6x10^{-8}$. This SNP is annotated in the nuclear receptor co-activator 3 (*NCOA3*) gene in a region with copy number variation and is in complete LD with a missense SNP (rs6094752). Functional studies are needed to examine the role of this gene in the pathophysiology of OA.

Finally, in **Chapter 4**, results of these genetic studies are brought together in a prediction model for knee OA. Identification of subjects at high risk for knee osteoarthritis (OA) is warranted for biochemical and pharmaceutical research and for prevention/monitoring in the general practice. Although some risk factors for knee OA are established, it is unknown how they perform in prediction models. The objective of this study was therefore to create and compare risk prediction models for incident knee OA in an elderly population and assess the discriminative value of this model in an independent study. 474 incident knee OA cases and 2,154 controls from the Rotterdam Study-I are included in this study. Discrimination and calibration of the models was tested in RS-II. The multivariate analysis in RS-I showed the strongest association(s) between knee OA and gender (OR 1.69), BMI (OR 1.28), hand OA (OR 1.45), knee pain (OR 1.62) and baseline KL score of 1 (OR 6.97). The genetic risk score alone has a low predictive value with an AUC of 0.61 in RS-I and 0.51 in RS-II. However, when stratifying on age, moderate predictive value is observed in subjects aged <65 years of age (AUC=0.65 in RS-I), but remains low in subjects aged ≥65 years (AUC=0.55 in RS-I). In RS-I the AUC for gender, age and BMI in prediction for knee OA was 0.66. Addition of the questionnaire variables or questionnaire variables + genetic score did not change the AUC (AUCs of 0.67 for both models). Addition of CTXII levels also did not improve the AUC. However, when adding the knee baseline KL score to the model the AUC increased to 0.79. Applying an external validation, similar results were observed in the Rotterdam Study-II with AUCs of 0.60, 0.62, 0.62 and 0.86 for, respectively, the same models. In summary, we showed that the baseline KL score for the knee is the best predictor of future knee OA in the elderly. "Questionnaire" variables, genetic markers, OA at other joint sites and CTXII levels do not add much predictive value to age, gender and BMI, at least not in an elderly population.

SAMENVATTING

Artrose is een chronische, invaliderende ziekte, gekenmerkt door pijn, stijfheid en disfunctioneren, die voornamelijk bij oudere mensen voorkomt. Op dit moment zijn er slechts enkele behandelingsopties, maar genezing van de ziekte is onmogelijk. Veel risicofactoren voor artrose zijn nog onbekend, maar we weten in ieder geval dat zowel omgevingsfactoren (bijvoorbeeld overgewicht of trauma) en genetische factoren een rol spelen bij het ontstaan van de ziekte. Het doel van dit proefschrift is om nieuwe genen te ontdekken die betrokken zijn bij artrose. Allereerst door middel van kandidaat gen studies, gevolgd door zogeheten genoom-wijde associatie studies.

In **Hoofdstuk 2** worden de kandidaat gen studies beschreven. In deze studies gaat men uit van de al beschikbare kennis over de biologische achtergrond van artrose. Genen worden geselecteerd waarvan men verwacht dat deze betrokken zullen zijn bij het ontstaan van artrose. Vervolgens wordt genetische variatie in deze genen, over het algemeen de zogenaamde "single nucleotide polymorphisms" ofwel SNPs onderzocht in relatie tot artrose. Men vergelijkt hierbij de frequentie van deze SNPs in gezonde individuen (controles) met mensen met artrose (cases). In **Hoofdstuk 2.1** zijn SNPs in 3 genen van de Wnt signaling cascade, *FRZB*, *LRP5* en *LRP6*, onderzocht in relatie met hand, heup en knie artrose in de Rotterdam Studie en de Chingford Studie. Wij lieten zien dat geen van de SNPs die bestudeerd zijn betrokken zijn bij artrose en een meta-analyse (analyse waarbij de resultaten van meerdere studies gecombineerd worden) laat ook geen relatie zien tussen de *FRZB* rs7775 SNP en artrose.

In **Hoofdstuk 2.2** worden resultaten getoond van een kandidaat gen studie betreffende het oestrogeen receptor beta (*ESR2*) gen. In een meta-analyse van 7 studies met in totaal 2364 heup artrose cases en 6773 controles, 1983 knie artrose cases en 4706 controles en 1431 hand artrose cases en 3883 controles wordt geen associatie gevonden tussen SNPs in het *ESR2* gen en artrose.

De relatie tussen CRP waardes gemeten in bloed (CRP waardes zijn een marker van ontsteking) en artrose en tussen genetische variatie in het *CRP* gen en artrose is beschreven in **Hoofdstuk 2.3**. Wij lieten in de Rotterdam Studie zien dat mensen met knie artrose hogere CRP waardes hebben dan mensen zonder knie artrose, maar dat dit veroorzaakt wordt doordat die mensen ook een hogere BMI hebben. Mensen met een hogere BMI hebben namelijk vaker knie artrose, maar ook hogere CRP waardes. Vervolgens werd er een literatuur studie uitgevoerd om alle artikelen te achterhalen waarbij de relatie tussen CRP waardes en artrose is onderzocht. In een meta-analyse kon er geen verband worden gelegd tussen hogere CRP waardes en artrose, in ieder geval niet onafhankelijk van BMI. Tevens zagen we ook geen relatie tussen genetische variatie in het *CRP* gen en artrose van de knie, heup of hand.

Grootschalige meta-analyses zijn uitgevoerd om de rol van genetische variatie in het Interleukine 1β (*IL1B*) en Interleukine 1R antagonist (*IL1RN*) gen te bepalen in relatie tot knie en heup artrose en in relatie tot de ernstigheid van knie artrose. Dit is beschreven in **Hoofdstuk 2.4**. Er was data beschikbaar van 6 reeds gepubliceerde studies en nieuwe data van 8 andere studies. Wij lieten zien dat er geen relatie is tussen genetische variatie in de *IL1B* en *IL1RN* genen en risico op heup of knie artrose (P>0.05 voor rs16944, rs1143634, rs419598 en haplotype C-G-C (combinatie van rs1143634, rs16944 en rs419598). Wel lijkt er een verband te bestaan tussen het C-T-A haplotype gevormd door rs419598, rs315952 en rs9005 en de ernstigheid van knie artrose. Mensen die drager zijn van dit haplotype hebben 41% minder kans op ernstige artrose vergeleken met mensen die geen drager zijn van dit haplotype (OR 0.71 95%CI 0.56-0.91; P=0.006).

Voor kandidaat gen studies zoals beschreven in hoofdstuk 2 is kennis over de ziekte nodig. Daarnaast was ten tijde dat deze studies uitgevoerd werden het laboratoriumwerk om genetische variaties te bepalen nog relatief duur en werden er dus ook maar enkele SNPs per gen onderzocht. Echter, aangezien het waarschijnlijk is dat er ook genen in nog onbekende signaling cascades zijn die betrokken zijn bij artrose, hebben we ook geprobeerd om nieuwe genen te identificeren door middel van hypothese vrije genoom-wijde associatie studies (GWAS) (**Hoofdstuk 3**). Meer dan 500.000 SNPs over het gehele genoom worden dan gegenotypeerd middels een chip en voor elk van deze 500.000 SNPs kan vervolgens de frequentie van het minst voorkomende allel vergeleken worden in mensen met versus mensen zonder artrose.

Allereerst wordt in **hoofdstuk 3.1** het probleem van gebruik van verschillende phenotype definities in artrose onderzoek aangekaart. De noodzaak voor standaardiseren wordt vermeld en het effect van de zogenaamde heterogeniteit tussen studies wordt onderzocht voor symptomatisch en radiografische artrose. Dit alles wordt gedaan binnen het TREAT-OA consortium, een consortium dat in 2008 is opgericht om de genetische en biochemische risicofactoren van artrose beter in kaart te brengen. In dit hoofdstuk laten wij zien dat alle studies (n=13) die symptomatisch artrose onderzoeken een verschillende definitie van heup, knie en hand artrose hanteren. Ook zagen we dat verschillende definities invloed hadden op de relaties van artrose met een aantal basale factoren zoals geslacht, leeftijd en BMI. Phenotype definities werden vervolgens zo goed als mogelijk gestandaardiseerd en de prevalentie van artrose voor en na standaardisatie werden vergeleken. Het blijkt dat de prevalentie van artrose van verschillende studies veel dichter bij elkaar komt te liggen na standaardisatie.

In **Hoofdstuk 3.2** worden de resultaten getoond van de eerste GWAS van de Rotterdam Studie. In deze studie werden 500,510 SNPs getest in 1341 artrose cases en 3496 controles. SNPs die met minimaal 2 artrose uitkomstmaten geassocieerd waren (bijvoorbeeld met knie en hand artrose) werden vervolgens geanalyseerd in

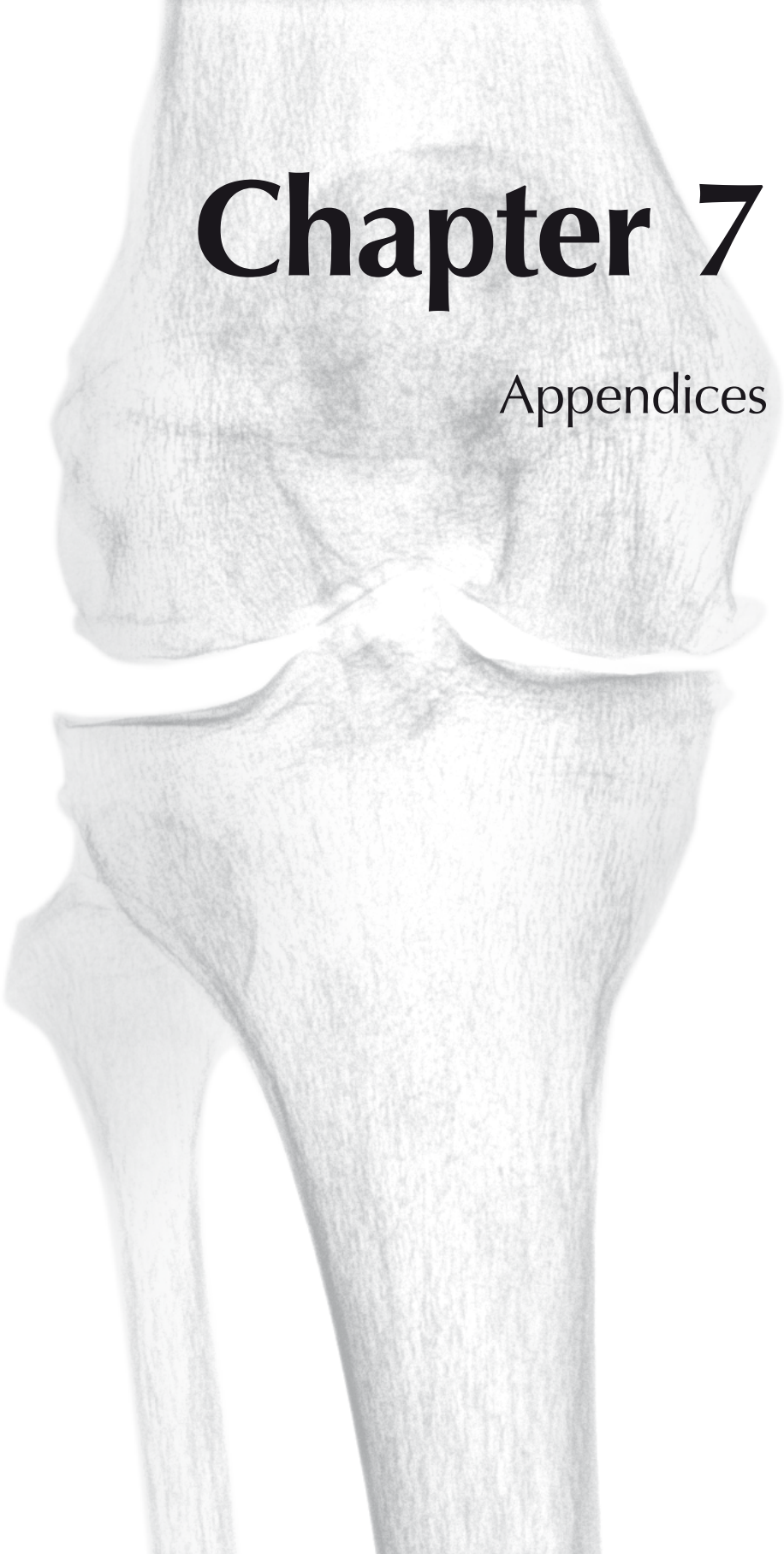
14.938 cases en ongeveer 39.000 controles. Het C allel van SNP rs3815148 op chromosoom 7q22 (MAF 23%, intron 12 van het COG5 gen) was geassocieerd met een 14% verhoogd risico op knie en/of hand artrose (OR1.14, 95%CI 1.09-1.19, P=8x10^{-8}). Tevens was dit allel geassocieerd met een 30% verhoogd risico op progressie van knie artrose (P=0.03). Deze SNP is gecorreleerd met SNP rs3757713 welke geassocieerd is met *GPR22* mRNA expressie levels in een lymfoblast cellijn. Functionele experimenten lieten zien dat het Gpr22 eiwit afwezig is in kraakbeen of gewrichtsvocht van gezonde muizen, maar aanwezig is in kraakbeencellen van muizen die artrose/artritis hadden. Tevens werd dit eiwit ook aangetroffen in osteofyten en kraakbeencellen van muizen die artrose hadden gekregen dmv een instabiele knie na een operatie. Aangezien GPR22 een G-coupled protein receptor is, is dit mogelijk een interessant eiwit voor nieuwe medicatie. Een latere GWAS meta-analyse van het TREAT-OA consortium bevestigde dat dit locus betrokken is bij knie artrose, waarbij de P-waarde genoom-wijd significant werd met P<5x10^{-8} (**Hoofdstuk 3.4**).

Hoofdstuk 3.3 beschrijft een GWA studie met de grootte van de gewrichtsspleet van de heup als uitkomstmaat. In het algemeen neemt de grootte van de gewrichtsspleet af naarmate de artrose vordert. In deze GWAS van 6523 mensen van de Rotterdam Studie, hebben wij ontdekt dat het G allel van rs12982744 op chromossom 19p13.3 geassocieerd is met een 5% grotere gewrichtsspleet (P=4.8x10^{-10}). Dit is vervolgens bevestigd in 4,442 mensen van 3 studies uit Groot-Brittannië met een uiteindelijke P-waarde van 1.1x10^{-11} in de meta-analyse. Deze SNP was overigens ook geassocieerd met een 12% verlaagd risico op heup artrose. rs12982744 is eerder gevonden in relatie tot lengte, waar het G allel geassocieerd was met een langere lengte. De SNP ligt in het *DOT1L* gen, welke recentelijk gelinked is aan Wnt-signalling. Functionele experimenten ondersteunen dat DOT1L waarschijnlijk een rol speelt in de differentiatie van kraakbeencellen en in volwassen kraakbeencellen en dat het een negatieve regulator is van Wnt signalling.

In **Hoofdstuk 3.5** worden de nieuwste resultaten van de tweede GWAS meta-analyse van het TREAT-OA consortium beschreven. In totaal waren er 8 studies met 5244 heup cases en 17.836 controles geincludeerd voor de meta-analyse. Er waren 5 signalen met P<1x10^{-6} en deze werden vervolgens onderzocht in de replicatie fase met 5010 heup artrose cases en 17.151 controles. Replicatie in 8 studies leidde tot 1 genoom-wijd significant resultaat. Het A allel van rs6490710 was geassocieerd met een 26% verhoogd risico op heup artrose (OR1.26, P=4.6x10^{-8}). Deze SNP ligt vlak voor het nuclear receptor co-activator 3 (*NCOA3*) gen en is in complete LD met een missense SNP (rs6094752). Functionele studies zijn nodig om de exacte rol van dit gen in de ontstaanswijze van artrose te achterhalen.

Tot slot worden in **Hoofdstuk 4** de resultaten van al deze studies samengebracht in een predictie (voorspelling) model. Het identificeren van mensen die knie artrose

krijgen is nodig voor zowel biochemisch en farmaceutisch onderzoek als voor preventie in de huisartsenpraktijk. Hoewel er enkele risicofactoren bekend zijn voor knie artrose, is het onbekend hoe goed deze ook daadwerkelijk de ziekte voorspellen. Het doel van deze studie was dan ook om meerdere predictie modellen voor knie artrose te maken en deze te vergelijken. De modellen werden gemaakt o.b.v. 474 incidente knie artrose cases en 2154 controles van de Rotterdam Studie I. Het onderscheidend vermogen, een maat om mensen met de ziekte van mensen zonder de ziekte te onderscheiden waarbij 0.50 gelijk staat aan kop of munt en 1.00 een perfecte onderscheiding is, werd vervolgens getest in de Rotterdam Studie II. De multivariate analyse in RS-I liet de sterkste associaties zien tussen knie artrose en geslacht (OR 1.69), BMI (OR 1.28), hand artrose (OR 1.45), knie pijn (OR 1.62) en de baseline K/L score van 1 (OR 6.97). De genetische risico score alleen heeft een lage voorspellende waarde (AUC=0.61 in RS-I en 0.51 in RS-II). Echter, we zien een redelijk voorspellend vermogen voor mensen <65 jaar (AUC=0.65 in RS-I), maar een laag voorspellend vermogen in mensen ≥65 jaar (AUC=0.55 in RS-I). In RS-II de area-under-the-curve (AUC) was 0.60 voor het model met enkel geslacht, leeftijd en BMI, 0.62 wanneer daar de vragenlijst en makkelijk verkrijgbare risicofactoren aan worden toegevoegd, 0.62 met toevoeging van de genetische risico score en 0.86 voor het complete model.



Chapter 7

Appendices

APPENDIX 1: DESCRIPTION OF STUDY POPULATIONS

arcOGEN consortium (GWAS data available)

The stage 1 samples, which were part of the discovery stage of this study, comprised 3,086 cases from existing DNA collections from 5 United Kingdom locations within the arcOGEN consortium (London, Nottingham, Oxford, Sheffield, and Southampton). Briefly, all were unrelated and of European origin, and all had primary OA of the hip or knee of radiographic KL grade ≥2, or clinical evidence of disease to a level requiring total joint replacement (TJR). The stage 2 cases, which were part of the replication effort of this study, were collected prospectively as part of the arcOGEN study at 9 locations across the UK (Edinburgh, London, Newcastle-Upon-Tyne, Nottingham, Oxford, Sheffield, Southampton, Wansbeck, and Worcester). The ascertainment criterion for the majority of cases (n=4,212) was primary OA that was severe enough for the individual to require joint replacement of the hip or of the knee; an additional small number (n=112) of cases were collected as part of a randomized, placebo controlled trial of vitamin D replacement (VIDEO Study) where the ascertainment criterion was radiographic disease of the knee, with a KL grade of ≥2. The prospective collections were approved by the National Research Ethics Service in the United Kingdom, and all subjects provided written, informed consent prior to inclusion.

Chingford Study: Is a prospective population-based longitudinal cohort. The Chingford study includes 1,003 women derived from the age/sex register of a large general practice (n >11,000) in North London, who are representative of the general UK population in terms of weight, height and smoking characteristics (1). The study design and rationale are described elsewhere in detail (2-4). All anthropometrical measurements were taken in a standardized manner, measured in standing position. Knee and hip pain were defined as having pain on most days of the month in at least one episode, in one or both joints during the last year. 47% of the cases of the Chingford Study are involved in the arcOGEN consortium.

Nottingham Case-Control Study (NCCS): All individuals were affected by knee or hip OA and were recruited in Nottingham both from families with a history of OA and from clinic populations (5). Hip and knee OA cases were recruited from hospital orthopaedic surgery lists. All had been referred to the hospital with symptomatic, clinically severe hip or knee OA and the majority had undergone unilateral or bilateral THR or TKR within the previous 5 years. Pre-operative knee or pelvis radiographs were examined to confirm the diagnosis. Subjects were excluded if they had another major arthropathy, Paget's disease, overt child hip disease, THR due to trauma or terminal illness. Controls were age-matched individuals from the same catchment area free from radiographic OA and over the age of 55. All research participants gave written informed consent to take part. Approval for recruitment of index knee and

hip OA cases and siblings of index hip OA cases was obtained from the research ethics committees of Nottingham City Hospital and North Nottinghamshire. 28% of the knee OA cases and 27% of the hip OA cases are included in the arcOGEN consortium.

Oxford Study: Subjects were ascertained using the criteria of signs and symptoms of OA sufficiently severe to require joint replacement surgery in the United Kingdom (6). The radiographic stage of the disease was a K/L grade >=2 in all cases. In addition, no cases suggestive of a skeletal dysplasia or developmental dysplasia were included. The controls comprised individuals with no signs or symptoms of arthritis or joint disease (pain, swelling, tenderness or restriction of movement). Only for a subset of the samples data on age was available. Ethical approval for the Oxford collection was obtained from the Oxfordshire Clinical Research Ethics Committee, MREC 02/2/108, with each participant providing informed consent for their sample to be used in OA genetics studies.

Sheffield Study: The Sheffield Study is a case-control study that was conducted to identify loci associated with prosthesis-related complications of total hip replacement. All subjects had undergone cemented total hip replacement for clinical, idiopathic, osteoarthritis that was confirmed radiographically prior to joint replacement. All subjects had K/L disease grade ≥ 2, and were free from any history of childhood hip disorders, inflammatory arthropathy or infection, and were not taking drugs known to affect bone metabolism. The characteristics of the subjects are described elsewhere (7). The study was approved by the North Sheffield research ethics committee, and all subjects provided written, informed consent prior to participation.

TwinsUK Study: The study participants were white monozygotic and dizygotic twin pairs from the TwinsUK adult twin registry, a group used to study the heritability and genetics of age-related diseases (8). Ethics approval was obtained from the Guy's and St. Thomas' Hospital Ethics Committee. Written informed consent was obtained from every participant. Samples included in this study were a subset over the age of 40 who had pelvis and anteroposterior weight-bearing knee X-rays. Only one individual from each twin pair was included. 23% of the cases of the TwinsUK Study are involved in the arcOGEN consortium.

VIDEO: This study is a placebo-controlled randomized controlled trial of 800 unit's cholecalciferol in men and women with knee OA. In total, 477 cases are included in this trial which are classified as knee OA patients according to the Kellgren and Lawrence grading system (9) (OA defined as one definite osteophyte) and patients had to suffer from knee pain and have a medial joint space width > 1mm. The study was approved by the local research ethics committee, and all subjects provided written, informed consent prior to participation.

Other

Chinese Case Control Study: The Han Chinese knee OA cases and controls were recruited from the Center for Diagnosis and Treatment of Joint Disease and the Center of Physcial Examination at Drum Tower Hospital. All subjects included in the study were Han Chinese living in and around Nanjing. All the patients had pain with rest and/or night pain of over 5-month duration. Other etiologies causing knee diseases such as inflammatory arthritis, posttraumatic or postseptic arthritis, skeletal dysplasia or developmental dysplasia were excluded (10). The controls had never any signs or symptoms of arthritis or joint diseases (pain, swelling, tenderness or restriction of movement). The Study was approved by the ethical committee of the Medical School of Nanjing University and informed consent was obtained from all patients and controls.

deCODE (GWAS data available): Hand OA cases were obtained from patient's records at hospitals and health care centers in Iceland (11). 2754 hand OA cases were included on the basis of clinical examination by an experienced examiner, supported by radiographs in over 60% of the cases, including all doubtful cases. Assessment was based on radiologists' descriptions and in doubtful cases from the radiographs. THR and TKR cases were recruited through a computer-aided search of hospital records. A clinician reviewed the patient's records to verify the diagnosis (12). Population controls were used, excluding all individuals with known signs of OA in any joint. The study was approved by the Data Protection Authority of Iceland and the National Bioethics Committee of Iceland. Informed consent was obtained from all participants.

Dentist and Teachers (D&T) Study: Hand OA among Finnish dentists and teachers: Subjects were identified through the registers of the Finnish Dental Association and the Finnish Teachers' Trade Union (comprising both the occupationally active and non-active) (13). In 2002, a questionnaire was sent to 436 female dentists (67% participation rate) and 436 female teachers (57% participation rate) randomly selected from the registers, using the place of residence (Helsinki or its neighboring cities) and age (45 to 63 years) as inclusion criteria. The mean number of years in occupation was 26 (SD 7, range 11–40) for the dentists and 24 (SD 7, range 1–37) for the teachers. 94% of the dentists and 98% of the teachers were occupationally active at the time the study was conducted. Participation in the study was voluntary and based on informed consent; altogether 543 women participated. The Hospital District of Helsinki and Uusimaa Ethics Committee for Research in Occupational Health and Safety approved the study proposal. Both hands of the participants were radiographed. Kodak x-ray films were exposed with Siemens x-ray equipment (48 kV, 10 mAs, focus film distance 115 cm). The radiographs were evaluated by an experienced radiologist who was blind to the occupation, age, and the participants' health

data. The workload on the hands during the dentists' work history was estimated in detail (14).

EGCUT: The Estonian cohort is from the population-based biobank of the Estonian Genome Project of University of Tartu (EGCUT) (www.biobank.ee). The whole project is conducted according to the Estonian Gene Research Act and all participants have signed the broad informed consent (15). The current cohort size is over 51,515, from 18 years of age and up, which reflects closely the age distribution in the adult Estonian population. Subjects are recruited by the general practitioners (GP) and physicians in the hospitals were randomly selected from individuals visiting GP offices or hospitals. Each participant filled out a Computer Assisted Personal interview during 1-2 hours at a doctor's office, including personal data (place of birth, place(s) of living, nationality etc.), genealogical data (family history, three generations), educational and occupational history and lifestyle data (physical activity, dietary habits, smoking, alcohol consumption, women's health, quality of life). Anthropometric and physiological measurements were also taken. Osteoarthritis was diagnosed by a specialist as a clinical finding and was usually confirmed by a radiograph (KL score>2). The OA cases for the current study had an ICD10 M16 and/or M17 diagnosis.

Estonian Cohort Study (ECS): The primary survey was conducted in two small South-Estonian towns, Elva and Võru where a postal questionnaire on knee problems was sent to all 1800 subjects aged 35-55 years (three family doctors' lists) (16). A total of 965 responses were obtained. Of all contacted subjects 407 participated in an in-depth clinical examination (KOOS questionnaire, functional knee tests, X-ray and ultrasonography of both knees) and gave blood samples for DNA and for other biomarkers. Moreover, 88 subjects (aged 35-55 years) were included which were undergoing arthroscopy at the Clinic of Traumatology & Orthopaedics of the Tartu University Hospital in Estonia. In all of them radiographs of the tibiofemoral (TF) joint and axial radiographs of the patellofemoral (PF) joint were taken. Two independent radiologists read the radiographs according to the grading system (grades 0–III) of Nagaosa *et al*. (17). The JSN and osteophytes grades were then used it to derive K/L score. Individuals with a K/L grade≥2 were considered cases, otherwise were classified as controls. Both studies were approved by the Ethics Committee of the University of Tartu and informed consent was obtained from all subjects.

Finnish OA cases: Knee OA cases were 113 patients visiting ORTON Orthopaedic Hospital, Helsinki, between 1994-2001 having primary bilateral knee OA severe enough to fulfill the criteria for knee arthroplasty: pain, walking disability and radiologically at least stage 3/4 osteoarthritic changes according to Kellgren and Lawrence (K/L) classification (18). They had not had a major knee trauma in the aetiology of OA, their pain or other OA symptoms began at a mean age of 52 y (SD 12 y), and mean age at first arthroplasty was 67 y (SD 8). The hand OA material (18) was based on the

set of severe DIP OA families. Eighty-five index cases with a primary criterion of ≥ 3rd degree K/L radiographic OA in DIP joints bilaterally and siblings of index cases with ≥ 2nd degree OA in DIP joints were included as affected individuals. In total the material includes 134 affected hand OA cases and 34 unaffected family members. Subjects with rheumatoid arthritis (RA) were excluded from both OA materials. The 210 control subjects were selected from the Finnish Twin Cohort study on opposite sex twins (19). The inclusion criteria were that they were born in 1938-1941, responded to a questionnaire in 1996-1997 and gave DNA samples for analyses. One twin from each twin pair was included in the control group if neither twin had physician diagnosed OA or RA, and neither twin reported that their mother, father, co-twin or any other sibling had OA or RA. Men and women were selected using the ratio of 1:3, similarly to our case series. The study was approved by the ethics committee of the Helsinki metropolitan hospital region and all individuals gave their informed consent.

Framingham Osteoarthritis Study (FOS) (GWAS data available): This study is a longitudinal population-based cohort study established in 1948 in Framingham, Massachusetts to examine risk factors for heart disease (20). In addition to the original cohort, a study of the offspring and their spouses of this cohort was initiated in 1971. The Framingham OA study, which includes participants of both cohorts, was developed to study the inheritance of OA (21). The Boston University Medical Center IRB approved the Osteoarthritis Protocol. Written informed consent was obtained from all subjects for both the osteoarthritis examination and for DNA acquisition and use. During the Framingham Offspring Cohort examination 5 visit (conducted during 1992–1994), a radiograph of both knees in full extension with weight-bearing was obtained, using a standardized protocol that included outlines of the feet in order to keep the rotation of the knee the same at follow-up evaluations. Knee radiographs were obtained at 0° and at 6° caudal, and the better of the 2 views (based on the optimal superimposition of the anterior and posterior margins of the medial tibial plateau) was selected for comparison with findings at the follow-up examination (22). A single bone-and-joint radiologist read PA hand films according to the reading protocols of the Framingham OA Study (23).

Genetics OsteoArthritis and Progression (GARP) Study (GWAS data available): A study from Leiden, the Netherlands, consisted of 192 sibling pairs concordant for clinical and radiographically (K/L score) confirmed OA at two or more joint sites among hand, spine (cervical or lumbar), knee or hip (24), random controls (N=720) were partners of the offspring of the Leiden longevity study (25). Written informed consent was obtained from each subject involved in the GARP study as approved by the ethical committees of the Leiden University Medical Center.

Greek TJR cases: The individuals included in the study were of Greek origin living in the district of Thessalia in central Greece (26). All of them had undergone a TKR/THR, meaning that all of them suffered from severe knee or hip OA, which is defined by a K/L grade >=2. None of the patients had evidence of arthritis due to another disease. All the controls had a K/L score of 0 and had undergone treatment for injuries or fractures. Patients with rheumatoid arthritis and other autoimmune diseases as well achondrodysplasias, infection-induced OA, and posttraumatic OA were not included in the study. This study was approved by the ethics committee of the Larissa University Hospital and all individuals gave their informed consent.

Health 2000 (GWAS data available): This study is a nationally representative population-based study of 8,028 persons aged 30 years or over. Of these, 78.4% participated in a health examination including standard clinical examination of the joints by a physician. In total, 2856 men and 3436 women were included. Knee- and hip OA were defined according to clinical records. Knee OA is defined as a documented history of previously diagnosed knee OA or knee arthroplasty due to OA based on convincing findings OR at least moderately restricted mobility OR slightly restricted mobility and either of the following: documented history of previously diagnosed knee OA but not convincingly presented grounds for the diagnosis or typical symptoms of knee OA (27). Hip OA is defined as a documented history of previously diagnosed hip OA or hip arthroplasty due to OA based on convincing findings OR at least moderate restrictions in extension or outer rotation OR slight restrictions in extension, inner rotation, outer rotation or moderately restricted abduction-adduction and either of the following: documented history of previously diagnosed hip OA but no grounds for the diagnosis is given or typical symptoms of hip OA (27-28).

Hertfordshire Cohort Study (HCS): The HCS is a population-based cohort study of men and women born and still resident in Hertfordshire designed to investigate the relationship between growth in infancy and the development of adult disease (29). In the late 1990s, 3000 men and women were recruited to this study which included a home interview and a subgroup (498 men and 468 women) underwent knee X-rays. Ethical approval was obtained from East and North Hertfordshire ethical committees and all participants gave written informed consent (5). Weight bearing anteroposterior and lateral semi-flexed radiographs of both knees were taken at the same hospital using the same radiographic equipment; a standard tube to film distance of 100 cm was used (29).

Japanese Case-Control Study: Subjects are individuals living in or around Tokyo, located in mainland Japan, and visited the participating clinical institutions. All individuals with OA were symptomatic and were treated in participating institutions on a regular basis. For each individual with knee OA, standard three-direction radiographs

were taken and for each individual with hip OA, anteroposterior radiographs were taken (10).

Japanese Cohort Study: This is a population-based cohort study (n=317) from habitants of Miyagawa village in the mainland of Japan. For each individual, standard three-direction knee radiographs were taken. All individuals recruited for this study were Japanese and received clinical and radiographic examinations by orthopaedic specialists. Rheumatoid arthritis (RA) and polyarthritis associated with auto-immune diseases were excluded, as were post-traumatic OA and infection-induced OA. Individuals who had clinical and radiographic findings suggestive of skeletal dysplasias and a definitely positive Mendelian family history of OA were also excluded from the study (10).

KANON: Subjects are from a cohort of patients with anterior cruciate ligament (ACL) injury which are part of a randomized controlled trial (RCT) (Controlled-Trials.com number, ISRCTN 84752559). Subjects were recruited and screened, at two different centers (Helsingborg hospital and University Hospital Lund), aged 18–35 years, having a high to moderate physical activity level and a not more than 4 weeks old ACL rupture. Eligible patients were randomized to surgical reconstruction or non-surgical treatment after having agreed to participate in the RCT and signed informed consent. All patients were assigned to an identical rehabilitation protocol. MRI of the knees was performed within a mean of 19 (standard deviation [SD] 6.5) days after injury using a 1.5 T imager (Gyroscan, Intera, Philips, Eindhoven, The Netherlands) with a circular polarized surface coil and at regular intervals thereafter. The MRI scans consisted of sagittal three-dimensional (3D) Water excitation fast low angle shot (FLASH) with repetition time (TR)/echo time (TE)/flip angle of 20 ms/7.9 ms/25°, sagittal T2) weighted 3D gradient echo (GRE) with TR/TE/flip angle of 20 ms/15 ms/50°. Both series were acquired with 15 cm field of view (FOV), 1.5 mm slice thickness, and 0.29 x 0.29 mm pixel size (30). Standardized standing postero-anterior X-ray films using the MTP-view (31) were obtained at baseline and at regular intervals thereafter. The Ethics Committee of the Lund University Faculty of Medicine approved the study, and informed consent was obtained from all participating subjects.

LUMEN: Patients who underwent isolated meniscectomy at Lund University Hospital in 1973, 1978, or 1983–1985 were retrospectively identified through the surgical coding system or by manual search of surgical records. A total of 456 patients fulfilled the criteria and were invited to undergo radiographic and clinical assessment in 1994, 1995, or 2000. In total, 70% of the subjects participated in the study (n=317). The control group comprised 68 individuals who have not undergone meniscectomy and who had no clinical meniscal or cruciate ligament injury. Controls were identified using national population records (32). In patients and controls, standing anteroposterior images of both knees in 15 degrees of flexion were. Axial views of

the patellofemoral joint were obtained with a vertical beam with the subject standing with the knee in 50° of flexion. A Siemens Basic Radiological System (Siemens, Erlangen, Germany) with a film-focus distance of 1.4m at 70 kV and 10 mA was used for patients who were followed up in 1994 and 1995, and for the control subjects. For patients who were assessed in 2000, we used a Phasix 60generator (CGR, Liege, Belgium) at 70 kV, 16 mA, film-focus distance 1.5m. The Ethics Committee of the Lund University Faculty of Medicine approved the study, and informed consent was obtained from all participating subjects (33).

Malmö Diet and Cancer Study (MDC): All men and women living in the city of Malmö in Sweden, who were born between 1923 and 1945 (men) or between 1923 and 1950 (women), were invited to participate in the MDC Study (34). The subjects were invited by letters and advertisements in newspapers. The cohort consisted of 28 449 subjects (11 246 men and 17 203 women) from the eligible population of approximately 74 000 individuals. The research ethical committee at Lund University approved the MDC Study (LU 51–90). Each participant signed a written informed consent. All participants were followed until the first osteoarthritis surgery, emigration from Sweden, death or 31 December 2005, whichever came first. Information on knee and hip arthroplasty for osteoarthritis and mortality were based on record linkage with the national Swedish hospital discharge register and the Swedish causes of death register. Knee osteoarthritis was defined as a first knee arthroplasty or high tibial osteotomy in combination with a contemporaneous diagnosis of osteoarthritis according to the International Classification of Disease (ICD) 9 and ICD-10, respectively. Hip osteoarthritis was defined as a first hip arthroplasty in combination with a contemporaneous diagnosis of hip osteoarthritis according to ICD-9 and ICD-10, respectively (34).

Osteoporotic Fractures in Men Study (MrOS): The MrOS Study is a multi-center prospective, longitudinal, observational study of risk factors for vertebral and all non-vertebral fractures in older men, and of the squealed of fractures in men (35-36). The study population consists of community dwelling, ambulatory men aged 65 years or older and were recruited from different clinical centers in the US: Birmingham, AL; Minneapolis, MN; Palo Alto, CA; Pittsburgh, PA; Portland, OR; and San Diego, CA. Inclusion criteria were designed to provide a study cohort that is representative of the broad population of older men. The inclusion criteria were: (1) ability to walk without the assistance of another, (2) absence of bilateral hip replacements, (3) ability to provide self-reported data, (4) residence near a clinical site for the duration of the study, (5) absence of a medical condition that (in the judgment of the investigator) would result in imminent death, and (6) ability to understand and sign an informed consent. To qualify as an enrollee, the participant had to provide written informed consent.

Research on Osteoarthritis/Osteoporosis Against Disability (ROAD) Study: The ROAD Study, started in 2005, involves the collection of clinical information from 4 cohorts with participants located in urban, mountainous and coastal areas. Currently, a baseline database including 3 cohorts with in total 3,040 participants is completed. The objectives of the study are to clarify the prevalence and estimate the number of people with musculoskeletal diseases represented by knee OA, lumbar spondylosis and osteoporosis (37). Plain radiographs with standing on both legs and the knee extended were taken with a horizontal X-ray beam unless otherwise described, using a Fuji 5000 Plus Reader on a 36 x 46 cm Fuji ST-VI Computed Radiography (CR) imaging plate (Fuji Medical Systems, Tokyo, Japan) with a 20 x 30 mm rectangular metal plate beside it as a magnification index. Rotation of the foot was adjusted to keep the second metatarsal bone parallel to the X-ray beam (38). The study was conducted with approval of the Institutional Review Boards (IRBs) of the University of Tokyo and the Tokyo Metropolitan Institute of Gerontology, and all participants provided written informed consent.

Rotterdam Study (RS) (GWAS data available): Is a large prospective population-based cohort study of men and women aged 55 years and older. The study design and rationale are described elsewhere in detail (39). In summary, the objective of the study is to investigate the determinants, incidence and progression of chronic disabling diseases in the elderly. The Rotterdam Study-I (RS-I) is the first cohort of 7,983 persons, aged 55 years and over living in Ommoord, Rotterdam in the Netherlands. This cohort was extended in 1999 with 3,011 participants using the same inclusion criteria (the Rotterdam Study-II (RS-II). A further extension, the Rotterdam Study-III (RS-III) was initiated in 2006. Since the start of the study there have been several follow-up visits to the research centre. The medical ethics committee of Erasmus University Medical School approved the study and written informed consent was obtained from each participant. All participants were examined at baseline in detail. In summary, a home interview was conducted (~2 hours) and subjects had an extensive set of approximately 1,500 examinations at the research centre (~5 hours). Amongst others, blood and urine is collected and x-rays of the knees, hips, hands and spine are taken. All these examinations were repeated every 3-4 years. Height and weight were measured at baseline examination with the subject in a standing position with indoor clothing without shoes. The presence of knee/hip/hand pain ("did you have joint complaints of your right/left knee/hip/hand during the last month") was asked during the home interview at baseline (40-41). Definitions for coronary heart disease, diabetes and lower limb disability are described elsewhere (42-44).

Spanish TJR and hand-OA cases: Patients were selected from consecutive patients, aged 55-75 years of age at time of the surgery, undergoing THR/TKR and patients complaining of hand OA that were followed in the Rheumatology Unit (45). All patients

were included if a rheumatologist considered them to suffer from severe primary OA. Exclusion criteria were inflammatory, infectious, traumatic or congenital joint pathology and lesions due to crystal deposition or osteonecrosis. Patients with hand OA were required to fulfill the ACR criteria. Controls were recruited among subjects older than 55 years of age undergoing preoperative work-up for elective surgeries other than joint surgery and who did not show clinical manifestations of OA. This study was approved by the Ethical Committee for Clinical Research of Galicia and all cases and controls gave their written informed consent to participate.

Study of Osteoporotic Fractures (SOF) (GWAS data available): Is a multicenter cohort study initiated in 1986 to determine risk factors for osteoporotic fractures in elderly women (46). Participants were all aged > 65 years at baseline and were recruited from population-based listings at 4 clinical centers in the US: Baltimore, MD; Minneapolis, MN; Monongahela Valley, PA (near Pittsburgh); and Portland, OR. Exclusion criteria for the parent study, the SOF, included bilateral hip replacement and an inability to walk unassisted. The SOF study was approved by the institutional review boards at each of the institutions involved. All subjects provided written informed consent at enrollment and at each clinical examination.

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APPENDIX 2: SUPPLEMENTARY DATA

Chapter 2.1 Radiographic osteoarthritis at three joint sites and *FRZB, LRP5* and *LRP6* polymorphisms in two population-based cohort studies

Supplementary Table 1. Baseline hip, knee, and hand OA risk by *FRZB* Arg200Trp genotypes in both study cohorts

	Allele frequency		Rotterdam Study			Allele frequency		Chingford Study		
Females	*Controls*	*Cases*	*Arg/Arg*	*Arg/Trp Trp/ Trp*	*OR (95% CI)*[1]	*Controls*	*Cases*	*Arg/Arg*	*Arg/Trp Trp/ Trp*	*OR (95% CI)*[1]
THR	0.13	0.12	48/1323 (3.6)[2]	15/412 (3.6)	0.97 (0.53-1.76)	NA	NA	NA	NA	NA
K/L hip	0.12	0.14	123/1323 (9.3)	49/412 (11.9)	1.30 (0.91-1.85)	0.12	0.11	195/586 (33.3)	55/176 (31.3)	0.92 (0.64-1.33)
K/L knee	0.12	0.13	365/1259 (29.0)	117/377 (31.0)	1.05 (0.80-1.36)	0.12	0.12	233/624 (37.3)	73/187 (39.0)	1.14 (0.80-1.63)
Hand OA	0.12	0.13	441/1281 (34.4)	153/404 (37.9)	1.15 (0.90-1.46)	0.12	0.10	84/512 (16.4)	20/154 (13.0)	0.76 (0.43-1.33)
Males										
THR	0.12	0.09	14/992 (1.4)	3/297 (1.0)	0.74 (0.21-2.60)	NA	NA	NA	NA	NA
K/L hip	0.12	0.14	84/992 (8.5)	31/297 (10.4)	1.28 (0.83-1.98)	NA	NA	NA	NA	NA
K/L knee	0.13	0.11	137/815 (16.8)	34/265 (12.8)	0.76 (0.50-1.15)	NA	NA	NA	NA	NA
Hand OA	0.12	0.12	205/1032 (19.9)	64/308 (20.8)	1.05 (0.76-1.46)	NA	NA	NA	NA	NA

[1]values are adjusted for age and BMI; [2]values are no. of subjects with OA outcome/total number (%); NA: not applicable; THR: total hip replacement; K/L: Kellgren/Lawrence

Supplementary Table 2. Baseline hip, knee, and hand OA risk by *LRP5* Ala1330Val genotypes in both study cohorts

	Allele frequency		Rotterdam Study			Allele frequency		Chingford Study		
Females	*Controls*	*Cases*	*Ala/Ala*	*Ala/Val Val/ Val*	*OR (95% CI)*[1]	*Controls*	*Cases*	*Ala/Ala*	*Ala/Val Val/ Val*	*OR (95% CI)*[1]
THR	0.21	0.13	48/1295 (3.7)[2]	14/480 (2.9)	0.77 (0.42-1.43)	NA	NA	NA	NA	NA
K/L hip	0.15	0.13	132/1295 (10.2)	38/480 (7.9)	0.76 (0.52-1.11)	0.15	0.14	181/529 (34.2)	56/191 (29.3)	0.78 (0.54-1.12)
K/L knee	0.15	0.13	370/1218 (30.4)	127/463 (27.4)	0.88 (0.68-1.13)	0.15	0.15	204/547 (37.3)	68/194 (35.1)	0.94 (0.66-1.36)
Hand OA	0.14	0.13	453/1267 (35.8)	148/451 (32.8)	0.88 (0.69-1.11)	0.14	0.15	68/450 (15.1)	26/157 (16.6)	1.02 (0.60-1.75)
Males										

Supplementary Table 2. Continued

	Allele frequency		Rotterdam Study			Allele frequency		Chingford Study		
THR	0.14	0.21	10/974 (1.0)	7/354 (2.0)	2.03 (0.76-5.44)	NA	NA	NA	NA	NA
K/L hip	0.14	0.15	85/974 (8.7)	33/354 (9.3)	1.07 (0.70-1.64)	NA	NA	NA	NA	NA
K/L knee	0.16	0.13	133/806 (16.5)	45/313 (14.4)	0.85 (0.58-1.24)	NA	NA	NA	NA	NA
Hand OA	0.14	0.14	203/1018 (19.9)	75/363 (20.7)	1.04 (0.77-1.42)	NA	NA	NA	NA	NA

[1]values are adjusted for age and BMI; [2] values are no. of subjects with OA outcome/total number (%); NA: not applicable; THR: total hip replacement; K/L: Kellgren/Lawrence

Supplementary Table 3. Baseline hip, knee, and hand OA risk by *LRP6* Ile1062Val genotypes in both study cohorts

	Allele frequency		Rotterdam Study			Allele frequency		Chingford Study		
Females	*Controls*	*Cases*	*Ile/Ile*	*Ile/Val Val/Val*	*OR (95% CI)*[1]	*Controls*	*Cases*	*Ile/Ile*	*Ile/Val Val/Val*	*OR (95% CI)*[1]
THR	0.19	0.23	37/1189 (3.1)[2]	25/601 (4.2)	1.35 (0.80-2.29)	NA	NA	NA	NA	NA
K/L hip	0.19	0.21	108/1189 (9.1)	63/601 (10.5)	1.17 (0.84-1.63)	0.18	0.20	153/501 (30.5)	89/240 (37.1)	1.37 (0.99-1.91)
K/L knee	0.20	0.20	318/1093 (29.1)	181/598 (30.3)	1.11 (0.88-1.40)	0.19	0.19	195/513 (38.0)	94/250 (37.6)	1.05 (0.75-1.46)
Hand OA	0.19	0.19	408/1152 (35.4)	200/579 (34.5)	0.97 (0.78-1.20)	0.19	0.17	69/427 (16.2)	30/200 (15.0)	1.04 (0.63-1.71)
Males										
THR	0.20	0.13	14/850 (1.6)	5/489 (1.0)	0.54 (0.19-1.52)	NA	NA	NA	NA	NA
K/L hip	0.20	0.21	73/850 (8.6)	47/489 (9.6)	1.09 (0.74-1.60)	NA	NA	NA	NA	NA
K/L knee	0.20	0.22	110/723 (15.2)	71/406 (17.5)	1.17 (0.84-1.64)	NA	NA	NA	NA	NA
Hand OA	0.19	0.23	160/876 (18.3)	117/516 (22.7)	1.22 (0.92-1.61)	NA	NA	NA	NA	NA

[1]values are adjusted for age and BMI; [2]values are no. of subjects with OA outcome/total number (%); NA: not applicable; THR: total hip replacement; K/L: Kellgren/Lawrence

Chapter 2.3 Serum C-reactive protein levels and genetic variation in the *CRP* gene are not associated with the prevalence, incidence or progression of osteoarthritis independent of body mass index

Supplementary Table 1. Characteristics of the included studies on CRP levels prevalence of OA

Reference	Study design	Mean age	Joint site	OA assessment	Definition OA cases
Bos et al.(11)	case-control	60 (median)	Knee, hand	Radiographic Symptomatic	GARP Study: Caucasian sibling pairs affected with symptomatic OA multiple sites. Knee/hand OA is defined as a K/L >=2.
Chen et al.(12)	cohort	54	Hand	Radiographic Symptomatic	CARRIAGE family study: hand OA defined according to three definitions: GOGO criteria, ACR criteria or any single joint involvement.
Conrozier et al.(13)	case-control	65[1]	Hip	Radiographic Symptomatic	Cases were selected from the Department of Rheumatology (France) who were referred for symptomatic hip OA and they fulfilled the ACR criteria.
Garnero et al.(16)	case-control	63	Knee	Radiographic Symptomatic	Cases were outpatients with knee OA who were attending the Department of Rheumatology of the Centre Hospitalier Lyon Sud who fulfilled the ACR criteria for knee OA.
Hulejová et al.(17)	case-control	64	Hip	THR	Cases were patients with hip OA (with indication for THR) who regularly attended the outpatient clinic of Institute of Rheumatology in Prague, Czech Republic.
Kerkhof *et al.*	cohort	67	Knee, hip, hand	Radiographic	The Rotterdam-Study-I is a population-based cohort study of men and women aged ≥ 55 years. Knee and hip OA were defined as a K/L score ≥2 of one or both joints or a TJR. Hand OA was defined as presence of a K/L score ≥2 in 2 out of 3 hand joint groups (DIPs, PIPs, CMC1/TS) of each or both hands
Kraus et al.(18)	cohort	61	Knee	Radiographic	Johnston County OA Project: population-based study in rural North Carolina, USA. Cases were defined as a K/L >=2 in either knee.
Melikoglu et al.(20)	case-control	60	Knee	Radiographic Symptomatic	Irish cases were diagnosed from clinical symptoms, examinations and radiographic findins. They fulfilled the ACR criteria for knee OA.
Otterness et al.(21)	case-control	55	Knee or hip	Radiographic Symptomatic	Cases had a diagnosis of idiopathic OA of the knee or the hip as defined by a radiological grade 1-3 by ACR criteria.
Pearle et al.(2)	case-control	63[1]	Knee or hip	Symptomatic	Patients with primary OA undergoing total hip or knee arthroplasty were identified at a single musculoskeletal specialty hospital and all met ACR criteria for idiopathic OA.
Sharif et al.(22)	case-control	58	Knee	Radiographic Symptomatic	Knee OA cases were defined according to clinical symptoms and radiographic findings.
Sowers et al.(24)	cohort	43	Knee	Radiographic	Population-based cohort of 1025 women who are enrolees in a study of musculoskeletal conditions at the mid-life. Knee OA was defined as K/L >=2.
Spector et al.(25)	cohort	54	Knee	Radiographic	Chingford Study: population-based cohort study of women derived from the age-sex register of a large general practice in North London. Cases were defined as K/L >=2.
Stürmer et al.(26)	case-control	63[1]	Knee or Hip	Radiographic Symptomatic	Patients under the age of 76 admitted to hospital for hip or knee joint replacement due to advanced radiographic OA were eligible for recruitment in four tertiary centres in southwest Germany. A K/L >=2 + symptoms was defined as knee OA.

THR: total hip replacement; K/L: Kellgren/Lawrence; [1]only mean age for cases given

Supplementary Table 2. Characteristics of the included studies on CRP levels and incidence/progression of OA

Reference	Study design	Mean age	Joint	OA assessment	Definition OA cases
Conrozier et al.(14)	case-control	64	Hip	Radiographic Symptomatic	Patients were selected from a cohort of 104 symptomatic hip OA patients. Rapidly **progressive** OA was defined as: (1) severe hip pain (2) symptom onset within the last two years (3) annual rate of joint space loss > 1 mm (4) ESR > 20 mm/h (5) absence of detectable inflammatory or crystal-induced joint disease. Slowly progressive hip OA was defined as a JSW loss < 0.2 mm/year.
Engström et al.(15)	cohort	58	Knee and hip	TKR/THR	Random part of the Malmö Diet and Cancer Study which is a population-based cohort. All participants were followed until the first OA surgery. **Incidence** of knee and hip OA was reported.
Kerkhof et al. (current study)	cohort	67	Knee and hip	Radiographic	The Rotterdam-Study-I is a population-based cohort study of men and women aged 55 years and older. Incidence and progression of hip and knee OA is reported. Incidence of knee and/or hip OA was defined as a K/L <2 at baseline and TJR or K/L ≥2 at follow-up. Progression of hip and knee OA was determined in subjects which have a K/L score ≥1 at baseline. Progression of hip OA was defined as a TJR or joint space narrowing ≥1.0 mm during follow-up. Progression of knee OA was defined as a TJR or an increase in K/L score ≥1 during follow-up.
Mazières et al.(19)	cohort	62	Hip	Radiographic Symptomatic	Outpatients who fulfilled the ACR criteria for hip OA were recruited by rheumatologists if they were aged between 50-75 years, experienced pain during daily activities and if the JSW was >1 mm at the narrowest point on the radiographs. **Progression** of hip OA was defined as a JSW decrease >0.5 mm or requirement for THR.
Sharif et al.(23)	cohort	65	Knee	Radiographic Symptomatic	Cases were taken from a cohort of consecutive outpatients with peripheral joint symptoms attributable to radiological evidence of OA. **Progression** of knee OA was defined as reduction in joint space width >=2 mm or TKR.
Sowers et al.(24)	cohort	43	Knee	Radiographic	Population-based cohort of 1025 women who are enrolled in a study of musculoskeletal conditions at the mid-life. **Incidence** of knee OA was reported.
Spector et al.(25)	cohort	54	Knee	Radiographic	Chingford Study: population-based cohort study of women derived from the age-sex register of a large general practice in North London. **Progression** of knee OA: at baseline a K/L >=2 and at follow-up an increase of at least 1 K/L grade

TKR: total knee replacement; THR: total hip replacement; K/L: Kellgren/Lawrence; ESR: erythrocyte sedimentation rate; JSW: joint space width

Chapter 2.4 Large-scale meta-analysis of interleukin-1 beta and interleukin-1 Receptor antagonist polymorphisms on risk of radiographic hip and knee osteoarthritis and severity of knee osteoarthritis

Supplementary Table 1a. Allele and haplotype frequencies for cases and controls for all studies with novel data (unpublished studies)

Study		Chingford Study		Estonia CS	GARP	Hertfordshire CS	Nottingham CCS	Rotterdam Study I		Rotterdam Study III		TwinsUK	
	SNP/haplotype	knee	hip					knee	hip	knee	hip	knee	hip
Controls	rs1143634 (C>T)	24.8	24.8	23.4	-[1]	22.9	22.8	24.5	24.0	25.1	25.6	23.8	23.7
	rs16944 (A>G)	31.8	31.9	35.8	-[1]	32.1	32.3	33.9	33.8	32.2	31.3	34.6	34.8
	rs419598 (T>C)	31.1	29.7	30.5	-[1]	27.9	27.3	26.1	26.0	25.4	25.0	27.7	27.6
	C-G-C	-	8.6	-	-[1]	-	8.8	-	8.4	-	8.1	-	7.3
Hip OA cases	rs1143634 (C>T)	25.8		-	24.7	-	22.0	25.0		22.6		25.7	
	rs16944 (A>G)	35.1		-	26.0	-	33.1	33.6		39.2		31.6	
	rs419598 (T>C)	26.1		-	24.4	-	27.6	24.9		28.1		30.0	
	C-G-C	8.2		-	10.3	-	8.8	7.4		7.5		7.0	
Knee OA cases	rs1143634 (C>T)	24.9	25.8	21.3	26.2	23.5		24.5		27.6		23.1	
	rs16944 (A>G)	33.7	33.8	32.3	33.2	33.2		34.0		29.1		32.5	
	rs419598 (T>C)	31.3	33.6	27.3	31.8	27.5		26.2		25.2		28.4	

Minor allele frequencies and haplotype frequencies are shown for each study in cases and controls; CS: cohort study; GARP: Genetics osteoARthritis and Progression Study; CCS: Case-Control Study; C-G-C haplotype: rs1143634-rs16944-rs419598; [1]controls of RS-I are used

Supplementary Table 1b. Allele and haplotype frequencies for cases and controls for all published studies

Study		Bristol Study	Chinese Study	Czech Study	GOAL	Oxford Study	Turkish Study
	SNP/haplotype						
Controls	rs1143634 (C>T)	20.0	-	-	24.5	24.1	22.4
	rs16944 (A>G)	35.1	48.6	-	32.3	31.8	-
	rs419598 (T>C)	34.4	7.1	15.0	25.9	28.4	14.2
	C-G-C	9.7	-		7.6	10.2	-
Hip OA cases	rs1143634 (C>T)	-	-	-	23.3	22.0	-
	rs16944 (A>G)	-	-	-	32.8	31.8	-
	rs419598 (T>C)	-	-	-	27.4	26.1	-
	C-G-C	24.1	-	-	9.0	9.7	-
Knee OA cases	rs1143634 (C>T)	26.8	-	-	22.1	28.0	21.5
	rs16944 (A>G)	30.1	50.9	-	33.6	33.1	-
	rs419598 (T>C)	29.1	7.1	27.0	28.7	24.3	13.1

Minor allele frequencies and haplotype frequencies are shown for each study in cases and controls; C-G-C haplotype: rs1143634-rs16944-rs419598

Chapter 3.2 A genome-wide association study identifies an osteoarthritis susceptibility locus on chr7q22

Supplementary Table 1. Meta-analysis results: association of the 12 top-hits with individual OA-phenotypes

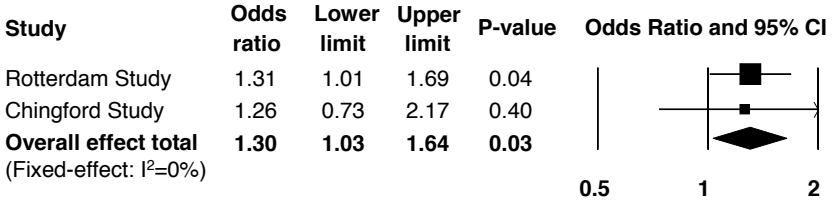
SNP	Chr	Minor allele	MAF	Knee OA		Hip OA		Hand OA		CTX-II (beta)	
				OR (95%CI)	*P*	*OR(95%CI)*	*P*	*OR(95%CI)*	*P*	*beta*	*P*
rs10465850	1	T	43%	1.03 (0.97-1.09)	0.32	0.99 (0.94-1.05)	0.77	1.05 (0.99-1.10)	0.10	-0.99[1]	0.82
rs12402320	1	T	27%	1.03 (0.95-1.12)	0.48	1.07 (0.98-1.16)	0.15	1.17 (1.06-1.29)	0.001	1.07	0.005
rs4656364	1	C	7%	0.97 (0.86-1.09)[1]	0.59	1.10 (0.91-1.32)[1]	0.33	1.01 (0.92-1.11)	0.84	1.00	0.97
rs3963342	4	C	25%	1.03 (0.97-1.09)	0.32	1.02 (0.94-1.11)[1]	0.67	1.04 (0.96-1.13)[1]	0.38	1.05	0.07
rs10248619	7	T	19%	1.03 (0.91-1.17)[1]	0.65	1.01 (0.90-1.14)[1]	0.82	1.04 (0.93-1.16)[1]	0.49	1.05[1]	0.26
rs10248619 (recessive)	7	T	19%	1.29 (1.04-1.61)	0.02	1.22 (1.01-1.49)	0.04	1.12 (0.92-1.36)	0.25	1.10	0.17
rs3815148	7	C	23%	1.16 (1.09-1.24)	$2x10^{-6}$	1.04 (0.98-1.10)	0.23	1.14 (1.06-1.23)	$3x10^{-4}$	0.98[1]	0.37
rs959396	9	G	38%	0.96 (0.84-1.09)[1]	0.51	0.96 (0.88-1.04)[1]	0.31	1.03 (0.97-1.08)	0.40	0.95	0.01
rs12352822	9	G	10%	0.99 (0.86-1.14)[1]	0.89	1.02 (0.87-1.19)[1]	0.81	1.02 (0.95-1.10)	0.59	1.01[1]	0.89
rs873598	10	T	49%	0.97 (0.89-1.04)[1]	0.35	0.96 (0.88-1.06)[1]	0.41	0.96 (0.88-1.05)[1]	0.33	0.95	0.03
rs741542	12	T	24%	1.02 (0.93-1.11)[1]	0.75	1.08 (0.95-1.22)[1]	0.24	1.04 (0.98-1.10)	0.21	1.02	0.43
rs11651351	17	T	7%	0.98 (0.79-1.22)[1]	0.86	0.91 (0.74-1.12)[1]	0.37	0.96 (0.77-1.19)[1]	0.70	0.91[1]	0.33
rs6088813	20	C	35%	0.90 (0.85-0.96)	$4x10^{-4}$	0.99 (0.93-1.05)	0.69	0.92 (0.82-1.05)[1]	0.12	0.99	0.65

Chr: chromosome; MAF: minor allele frequency (HapMap); OA: osteoarthritis; OR: odds ratio; CI: confidence interval; [1]random-effects model

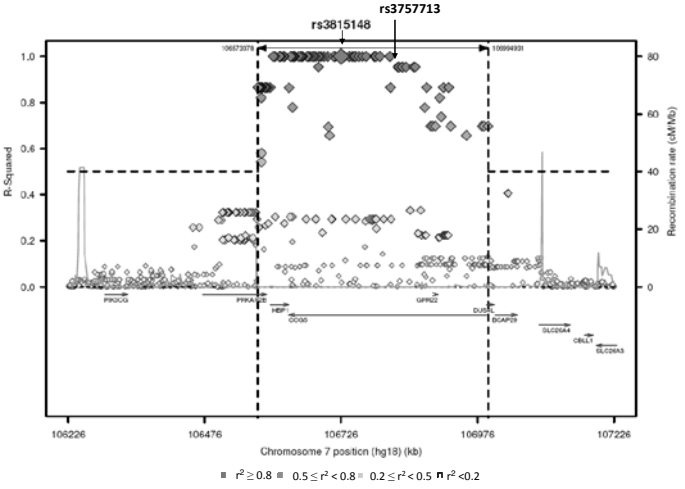
Supplementary Table 2. Association results of SNPs in the *DIO2*, *PTGS2*, *DVWA* and *GDF5* genes identified in previous genome-wide (linkage) studies

rs-number	Gene	Minor allele	Paper of first report	OA-phenotype in first report	Association tested in males/ females	Hip OA	Knee OA	Hand OA
						OR (95%CI) P-value	*OR (95%CI) P-value*	*OR (95%CI) P-value*
rs225014	DIO2	C	Meulenbelt *et al.* (22)	hip	females	1.1 (0.9-1.4) 0.28	1.0 (0.8-1.1) 0.53	1.1 (0.9-1.2) 0.44
rs12885300	DIO2	T	Meulenbelt *et al.* (22)	hip	females	0.8 (0.7-1.0) 0.09	1.1 (0.9-1.3) 0.34	0.9 (0.8-1.0) 0.13
rs4140564	PTGS2	C	Valdes *et al.* (23)	knee	females	1.0 (0.6-1.5) 0.81	1.2 (0.9-1.6) 0.31	0.9 (0.6-1.2) 0.45
rs7639618	DVWA	T	Miyamoto *et al.* (24)	knee, hip	females/males	0.9 (0.7-1.1) 0.25	1.1 (0.9-1.2) 0.53	1.2 (1.0-1.4) 0.03
rs6088813	GDF5	C	Miyamoto *et al.* (25)	knee, hip	females/males	1.0 (0.9-1.2) 0.77	0.9 (0.8-1.0) 0.08	0.8 (0.7-0.9) $7x10^{-4}$

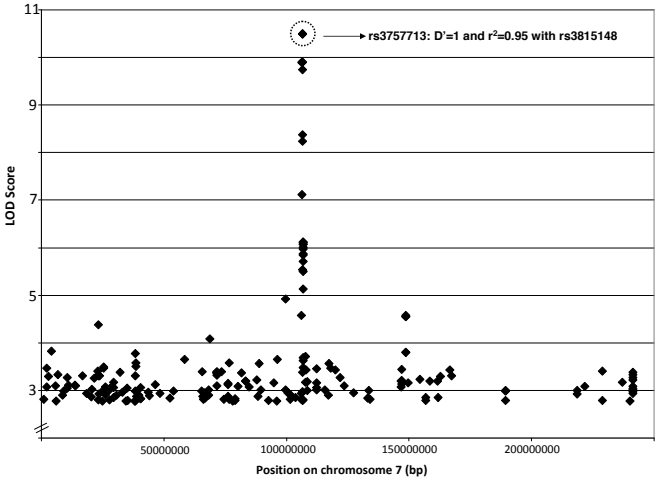
OA: osteoarthritis; OR: odds ratio; CI: confidence interval



Supplementary Figure 1 Chapter 3.2. Forest plot for the association of the rs3815148 SNP (GPR22 locus) with knee OA progression (allelic model).



Supplementary Figure 2 Chapter 3.2. Regional LD plot for the chromosome 7 locus (rs3815148 SNP, *GPR22*). r^2 measures are based on the HapMap CEU samples. Each diamond represents one SNP. The brightness of each diamond is proportional to the r^2 values for that SNP, the lower the r^2, the lighter the color of the diamond. A color figure is presented in Appendix 3.



Supplementary Figure 3 Chapter 3.2. Expression Quantitative Trait Locus (eQTL) for the GPR22 gene in EBV-transformed lymphoblast cell lines in a family study. The x-axis represents the chromosome location, the y-axis the LOD score for linkage between the genotyped SNPs and expression levels of the *GPR22* gene. Each diamond represents one SNP.

Chapter 3.3 Genome wide association and functional studies identify the *DOT1L* gene to be involved in cartilage thickness and hip osteoarthritis

Supplementary Table 1. Characteristics of the study populations (N=13,085)

	Discovery Studies		Replication Studies			
	RS-I	*RS-II*	*TwinsUK*	*GOAL*	*Chingford*	*Nottingham*
Number	4773	1750	613	3084	745	2120
Female (%)	56.6	54.4	93.2	48.5	100	61.7
Age (years)	67.7 (7.8)	64.8 (7.9)	53.4 (7.7)	66.6 (7.9)	62.0 (5.9)	67.7 (9.2)
Height (cm)	167.6 (9.3)	168.6 (9.2)	163.0 (6.8)	166.5 (9.1)	161.6 (5.9)	166.7 (15.3)
Weight (kg)	73.8 (11.7)	77.6 (13.3)	66.6 (12.1)	81.1 (15.9)	66.4 (11.2)	76.7 (15.3)
BMI (kg/m2)	26.3 (3.6)	27.2 (3.9)	25.1 (4.0)	29.3 (5.3)	25.4 (4.0)	27.2 (4.6)
mJSW (mm)	3.80 (0.84)	4.38 (0.90)	3.21 (0.74)	3.29 (1.13)	3.62 (0.69)	NA
Definition of hip OA	K/L score ≥2 of one or both joints or a TJR[1]		Joint space narrowing and a K/L score ≥2 of one or both joints	Definite joint space narrowing and K/L score ≥2 of one or both joints	K/L score ≥2 of one or both joints	K/L score ≥2 of one or both joints or TJR[2]
Nr cases/controls	771/4436	166/1795	48/565	1263/1821	88/657	1381/739
Study design	Population-based		Twin pairs	Case-control	Population-based	Case-control

RS-I: Rotterdam study I; RS-II: Rotterdam Study II; GOAL: Genetics of Osteoarthritis and Lifestyle study; Means are given with standard deviations between brackets. BMI: body mass index; mJSW: minimum hip joint space width; NA: not available; [1]Subjects with a TJR due to fracture were excluded from all analyses; [2]Exclusion criteria were: other major rthropathy (e.g., rheumatoid arthritis, ankylosing spondylitis); Paget's disease affecting the pelvis or femur; overt childhood hip disease (e.g., Legg-Calvé-Perthes disease, slipped femoral epiphysis, severe acetabular dysplasia); THR due to hip trauma or avascular necrosis of the femoral head; or terminal illness. The minimal JSW (mJSW) was defined as the shortest distance between the femoral head margin and the acetabulum. The inter-rater reliability by Intraclass Correlation Coefficient for the mJSW was 0.85 (0.80-0.89). The hip with the lowest JSW was taken as the mJSW of that individual

Supplementary Table 2. Quality control procedures and exclusion criteria for individuals of the GWA and replication studies

Study	Genotyping Platform(s) / Chip(s)	Quality control inclusion filters			Sample QC / other exclusions	SNPs that met QC criteria	Analyzed samples	Genotyping facility
		Call rate	MAF	P HWE				
RS-I	Illumina HumanHap 550K V.3 DUO	≥97.5%	≥1%	$<1x10^{-6}$	1) gender mismatch with typed X-linked markers; 2) excess autosomal heterozygosity > 0.336~FDR>0.1%; 3) duplicates and/or 1st or 2nd degree relatives using IBS probabilities >97% from PLINK; 4) ethnic outliers using IBS distances > 3SD from PLINK; 5) missing JSW measurements.	512,349	4,773	Genetic Laboratory Dept Internal Medicine Erasmus MC, The Netherlands
RS-II	Illumina / HumanHap 550 V.3 DUO	≥97.5%	≥1%	$<1x10^{-6}$	1) gender mismatch with typed X-linked markers; 2) excess autosomal heterozygosity > 0.336~FDR>0.1%; 3) duplicates and/or 1st or 2nd degree relatives using IBS probabilities >97% from PLINK; 4) ethnic outliers using IBS distances > 3SD from PLINK; 5) missing JSW measurements	466,389	1,750	Genetic Laboratory Dept Internal Medicine Erasmus MC, The Netherlands
TwinsUK	Illumina HumanHap 300/550 & Illumina HumanCNV370 Duo	≥98%	≥1%	$<1x10^{-6}$	1. autosomal heterozygosity <0.33 or >0.37 2. ethnic outliers (using STRUCTURE) 3. missing JSW or height measurements	598,207	612	Wellcome Trust Sanger Institute Hinxton, U.K.
Chingford	SNP genotyping using fluorescence resonance energy transfer (FRET) quencher cassette oligos. KASPAR chemistry competitive allele-specific PCR.	NA	NA	NA	missing JSW or height measurements	NA	747	Kbiosciences Ltd, Hoddesdon, UK.
GOAL					missing JSW or height measurements	NA	3,084	
Nottingham					missing hip OA status	NA	2,127	

RS-I: Rotterdam Study I; RS-II: Rotterdam Study II; NA: not applicable; QC: quality control; HWE: Hardy-Weinberg Equilibrium; MAF: minor allele frequency

Supplementary Table 3. Markers with suggestive evidence for association from the GWAS on minimal joint space width ($1x10^{-5}>P>5x10^{-8}$)

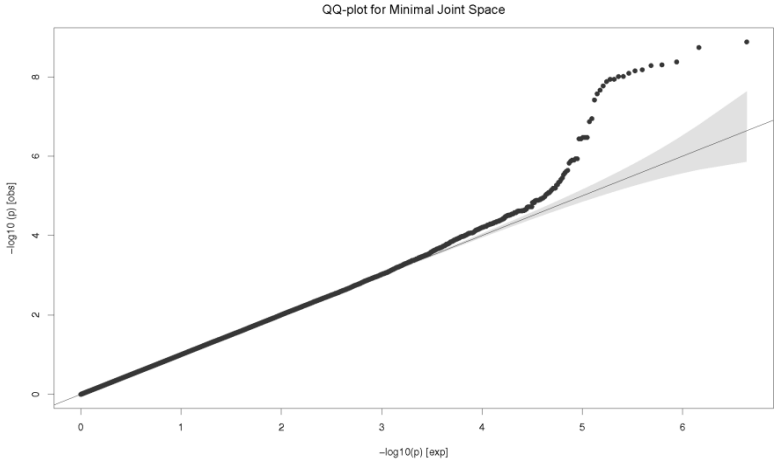
Locus	SNP	A1/A2	MAF	Closest Gene	Effect estimate		P-value
					Beta	SE	
12p11.2	rs4931462	T/G	0.33	OVOS2	-0.084	0.017	$4.3 x10^{-7}$
6p21.1	rs10948155	T/C	0.65	SUPT3H	-0.079	0.016	$7.7 x10^{-7}$
19p13.1	rs11665774	A/G	0.49	SLC27A1	0.07	0.015	$4.0 x10^{-6}$
5q13	rs11738020	T/C	0.43	PIK3R1	-0.068	0.015	$5.5 x10^{-6}$
15q26	rs2380165	A/G	0.68	BLM	-0.076	0.017	$5.6 x10^{-6}$
1p34.3	rs11206937	A/G	0.23	TRIT1/BMP8B	0.079	0.018	$7.3 x10^{-6}$
15q23	rs12907468	A/T	0.36	TLE3	-0.075	0.017	$7.8 x10^{-6}$
8q21.3	rs12544183	T/G	0.1	RUNX1T1	-0.126	0.028	$8.5 x10^{-6}$

A1: modeled allele; A2: the reference allele; MAF: modeled allele frequency; SE: standard error; Beta: delta mm per allele; SE: standard error

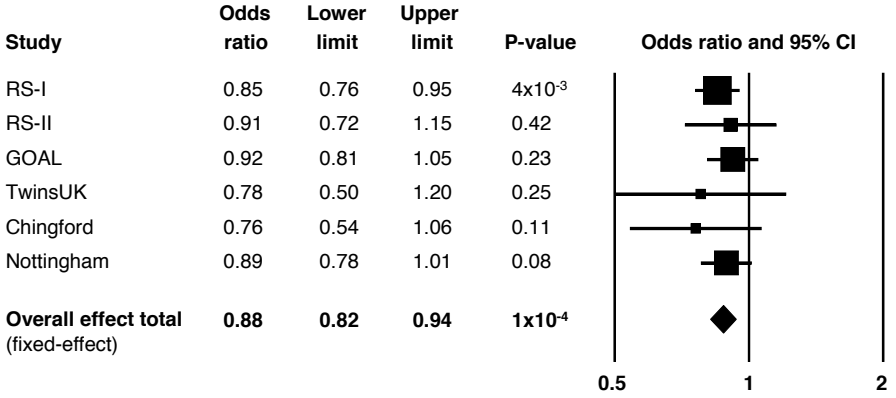
Supplementary Table 4. Association results of rs12982744

Type of Analysis		Discovery studies		Replication studies		Combined analysis	
Minimal JSW							
Subjects tested	*Adjustment*	*Beta(95% CI)*	*P-value*	*Beta(95% CI)*	*P-value*	*Beta(95% CI)*	*P-value*
All	Age/gender	0.09 (0.06-0.12)	$4.5 x10^{-10}$	0.07 (0.02-0.12)	$9.0 x10^{-3}$	0.09 (0.06-0.11)	$1.1 x10^{-11}$
All	Age/gender/ height	0.09 (0.06-0.12)	$4.5 x10^{-9}$	0.07 (0.02-0.12)	$8.0 x10^{-3}$	0.08 (0.06-0.11)	$1.6 x10^{-10}$
controls	Age/gender	0.07 (0.04-0.09)	$8.6 x10^{-7}$	0.06 (0.02-0.09)	$2.0 x10^{-3}$	0.06 (0.04-0.08)	$7.3 x10^{-9}$
controls	Age/gender/ height	0.06 (0.04-0.09)	$3.1 x10^{-6}$	0.05 (0.02-0.09)	$3.0 x10^{-3}$	0.06 (0.04-0.08)	$4.2 x10^{-8}$
Hip OA							
Subjects tested	*Adjustment*	*OR(95% CI)*	*P-value*	*OR(95% CI)*	*P-value*	*OR(95% CI)*	*P-value*
All	Age/gender	0.87 (0.79-0.96)	$6.0 x10^{-3}$	0.89 (0.81-0.97)	$8.0 x10^{-3}$	0.88 (0.82-0.94)	$1.5 x10^{-4}$
All	Age/gender/ height	0.86 (0.77-0.96)	$5.1 x10^{-3}$	0.89 (0.81-0.97)	$7.0 x10^{-3}$	0.88 (0.82-0.94)	$1.1 x10^{-4}$

mJSW: minimal joint space width. Beta: change in mm per allele; CI: confidence limit; OR: Odds Ratio; For all analysis the minor G-allele of rs12982744 was the modeled allele; Controls were all people not having hip OA



Supplementary Figure 1 Chapter 3.3. Quantile-Quantile plot (QQ plot) for minimal joint space width. The plot compares additive model statistics to those expected under the null distribution using fixed-effects for all analyzed HapMap CEU imputed SNPs passing quality control criteria.



Supplementary Figure 2 Chapter 3.3. Forest plot for the association of the rs12982744 SNP (*DOT1L* locus) with risk for hip OA. Values represent odds ratios (OR) and 95% confidence intervals (95%CI).

Chapter 3.4 Meta-analysis of genome-wide association studies confirms a susceptibility locus for knee osteoarthritis on chromosome 7q22

Supplementary Table 1. SNPs selected for replication

SNP rs number	Chr	Locus	P-value discovery
rs4730250	7	DUS4L	5.06×10^{-8}
rs6957186	7	COG5	1.21×10^{-7}
rs12535761	7	COG5	1.29×10^{-7}
rs763386	7	COG5	1.30×10^{-7}
rs10953541	7	BCAP29	6.16×10^{-7}
rs7785962	7	BCAP29	7.42×10^{-7}

Supplementary Table 1. Continued

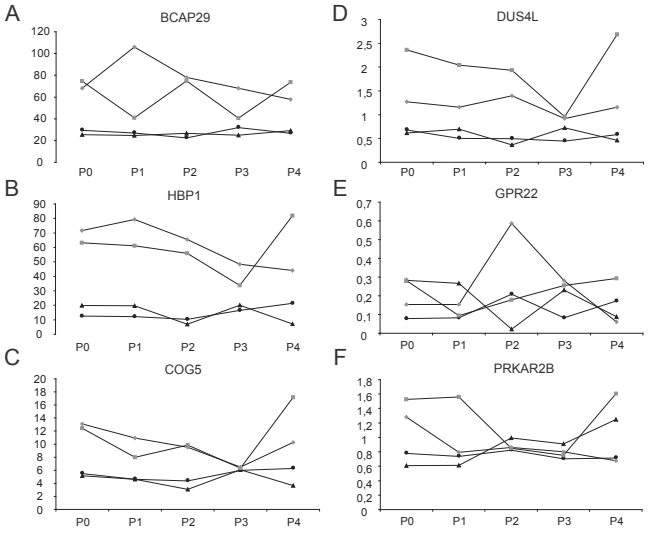
rs3749132	2	ARHGAP25	$3.58x10^{-6}$
rs886827	7	GLI3	$6.84x10^{-6}$
rs1886695	20	CPNE1	$9.25x10^{-6}$
rs3746410	20	CPNE1	$1.06x10^{-5}$
rs10071956	5	Intergenic	$1.16x10^{-5}$
rs6816070	4	LDB2	$1.39x10^{-5}$
rs661924	10	NEBL	$1.46x10^{-5}$
rs436354	5	ZDHC11	$1.51x10^{-5}$
rs1994104	12	Intergenic	$1.51x10^{-5}$
rs3787166	20	CPNE1	$1.72x10^{-5}$
rs9857056	3	Intergenic	$1.72x10^{-5}$
rs1823429	3	Intergenic	$1.85x10^{-5}$

Supplementary Table 2. Summary odds ratio for the top hits using random effects (RE) models

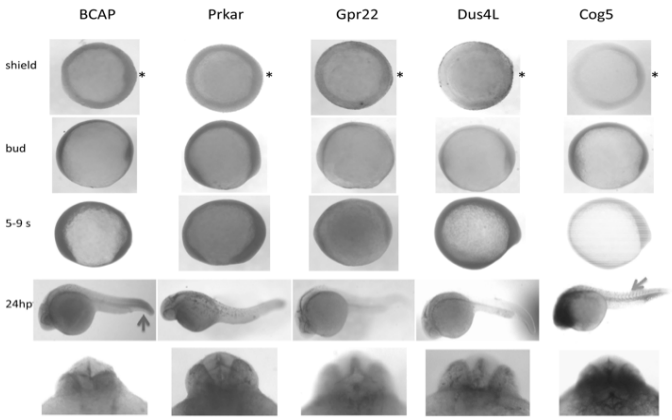
SNP	Minor allele	Chr	Position	Gene	MAF	OR (95% CI) random-effect	P-value
rs4730250	G	7	106994931	DUS4L	0.17	1.18 (1.11-1.26	$5.5x10^{-7}$
rs10953541	T	7	107031781	BCAP29	0.24	1.17 (1.09-1.23)	$7.9x10^{-7}$
rs3749132	A	2	68907001	ARHGAP25	0.07	1.18 (1.00-1.38)	0.041
rs886827	C	7	42285581	GLI3	0.27	1.07 (0.92-1.25)	0.37
rs1886695	G	20	33643949	CPNE1	0.16	0.88 (0.81-0.96)	$5.0x10^{-3}$
rs10071956	T	5	173093290	Intergenic	0.38	1.12 (1.05-1.20)	$3.4x10^{-4}$
rs6816070	G	4	16089455	LDB2	0.42	0.91 (0.86-0.95)	$1.3x10^{-4}$
rs661924	T	10	21353562	NEBL	0.39	1.12 (1.04-1.20)	$1.8x10^{-4}$
rs436354	G	5	783271	ZDHC11	0.17	1.21 (0.99-1.31)	0.06
rs1994104	T	12	83040643	intergenic	0.13	0.90 (0.77-1.05)	0.18
rs9857056	G	3	181698548	intergenic	0.12	1.16 (0.97-1.39)	0.10

Supplementary Table 3. Conditional analysis of the two top hits (effect of rs10953541 given that rs4730250 exists in the model)

Group	Effect (beta)	P-value
deCODE	0.153	0.088
Rotterdam	0.10	0.414



Supplementary Figure 1 Chapter 3.4. Gene expression levels of *BCAP29* (**A**), *HBP1* (**B**), *COG5* (**C**), *DUS4L* (**D**), *GPR22* (**E**) and *PRKAR2B* (**F**) were determined by real-time quantitative PCR in cell cultures from 2 donors. PCR analyses were performed in duplicates. Data are relative expression compared to housekeeping gene beta-actin multiplied by 100. Pellet cultures are shown in red, mono-layer cultures in black. The diamond and square represent individual patients. Pellet cultures were started with either fresh cells or at each cell culture passage (P). A color picture is presented in Appendix 3.



Supplementary Figure 2 Chapter 3.4. Whole mount in situ hybridization of zebrafish embryos at different stages of development. The stages are indicated on the left side of the figure and the gene names are on the top. At the shield stage, all embryos are vegetal view, shield to the right (asterisk). At the bud, 5-9 somite and 24hpf stages, the embryos are positioned anterior to the right, dorsal to the top. Below the panels containing the 24hpf embryos are corresponding frontal view of the head with dorsal to the top. The red arrow indicates tail specific expression and the blue arrow indicates the intersomitic expression. A color figure is presented in Appendix 3.

GWAS genotyping

The Rotterdam Study: Genotyping of the samples with the Illumina HumanHap550v3 Genotyping BeadChip was carried out at the Genetic Laboratory of the Department of Internal Medicine of Erasmus Medical Center, Rotterdam, the Netherlands. The Beadstudio GenCall algorithm was used for genotype calling and quality control procedures were as described previously (1). The following quality control filters were applied: SNP call rate >= 95%, minor allele frequency >= 5%, P-value HWE >=$1x10^{-6}$. After quality control 500,510 SNPs remained for association analyses. The intensity cluster plots were visually inspected for the top-hits of the Rotterdam Study and no abnormalities were discovered. Genomic inflation factors were calculated for all analyses and there was no evidence of population stratification with lambdas of 1.01 for hip- and hand-OA, 1.00 for knee-OA.

*TwinsUK:*Samples were genotyped with the Infinium HumanHap 300 assay (Illumina, San Diego, USA) at the Duke University Genotyping Center (NC USA), Helsinki University (Finland) and the Wellcome Trust Sanger Institute. The Illuminus calling algorithm was used for genotype calling. After strict quality control criteria were applied as described in (1) there were 314075 SNPs available for analysis. Imputation was performed using the IMPUTE software (v0.2.0)(2). At imputed loci, all genotypes with posterior probabilities < 0.9 were discarded and the imputed loci were filtered out using usual QC filters.

Framingham: Samples were genotyped using the Affymetrix GeneChip® Human Mapping 500K array set and the 50K supplemental array set focused on coding SNPs and SNPs tagging protein-coding genes (Santa Clara, California) as part of the SHARE initiative. Sample exclusion criteria included call rate < 97% and a per subject heterozygosity > ± 5 standard deviations from the mean. In addition, 2 participants were excluded for excessive Mendelian errors. Imputation was performed using MACH (version 1.0.15) to impute all autosomal SNPs using the publicly available phased haplotypes from HapMap (release 22, build 25, CEU population) as a reference population. A sample of 200 known unrelated participants with high call rates and low Mendelian errors were used to determine parameter estimates that were subsequently applied in a model for all subjects. Dosage estimates outputted by MACH were used in analysis.

deCODE: All samples were assayed with the Infinium HumanHap 300 or humanCNV370 SNP chips (Illumina), containing 317,503 tagging SNPs derived from phase I of the International HapMap project. All of the SNPs tested in this report passed quality filtering (a call rate >97%, a minor allele frequency >1%, not a significant distortion from HWE (P-value >10^{-7} on any of the three chip types used (humanHap300, humanHap300-duo and humanCNV370). Any samples with a yield <98% were excluded from the analysis. Imputation was done using the IMPUTE

software(2). The 622 additional cases in the replication analysis were genotyped using the Centaurus (Nanogen) platform

In silico replication

arcOGEN study: arcOGEN case samples were genotyped using Illumina Human 610-Quad BeadChips. Controls were typed using the Illumina 1.2M platform as part of the WTCCC2 project (www.wtccc.org.uk). Samples were excluded if their call rate was <97%, and if they showed gender discrepancies (estimated from genotypic data against external information). Individuals were also excluded on the basis of excess genome-wide heterozygosity or homozygosity. We identified samples that were accidentally duplicated or closely-related by calculating genome-wide IBD (given IBS information) for pairs of individuals. Multidimensional scaling (MDS) was performed in conjunction with data from the three HapMap phase II populations in order to identify and exclude individuals of non-European descent. SNPs were excluded from further analysis based on the following criteria: Call rate <95% if minor allele frequency (MAF)≥5% or call rate <99% if MAF<5%, HWE exact p values <0.0001 in cases or controls, and MAF <1%. Association analyses were carried out under the additive model. Imputation was carried out using IMPUTE (Marchini et al 2007) and imputed genotypes were analysed taking under account the full genotype probability distribution.

Replication de novo genotyping

Replication genotyping in the samples from Nottingham and Estonia was carried out by Kbioscience Ltd, Hertfordshire UK. SNPs were genotyped using the KASPar chemistry, which is a competitive allele-specific PCR SNP genotyping system using FRET quencher cassette oligos (http://www.kbioscience.co.uk/genotyping/genotyping-chemistry.htm). Genotyping accuracy, as determined from the genotype concordance between duplicate samples was 99.8%. All polymorphisms were in Hardy-Weinberg equilibrium in controls (all $p > 0.05$). Replication genotyping in the samples from Finland, Spain, Leiden, Greece, Chingford (not part of arcoGEN) was carried out at the Leiden University Medical Centre using the MassArray iPLEX Gold from Sequenom. All samples from Sweden were genotyped by deCODE genetics using the Centaurus (Nanogen) platform. In Asian populations (ROAD study) The top SNPs were genotyped by the Taqman assay. Further, 16 SNPs including the top SNPs were genotyped by the DigiTag2 assay, a multiplex single nucleotide polymorphism typing method(3). For the RIKEN Case-Control and Chinese case-control studies genomic DNA was extracted from peripheral blood leukocytes of study subjects using standard protocols. SNPs were genotypied using the multiplex PCR–based Invader assay28 (Third Wave Technologies) or TaqMan SNP genotyping assays (Applied Biosystems), or by direct

sequencing of PCR products using ABI 3700 DNA analyzers (Applied Biosystems), according to the manufacturers' protocols.

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1. Richards JB, Rivadeneira F, Inouye M, Pastinen TM, Soranzo N, Wilson SG, et al. Bone mineral density, osteoporosis, and osteoporotic fractures: a genome-wide association study. *Lancet* 2008;371(9623):1505-12.
2. Marchini J, Howie B, Myers S, McVean G, Donnelly P. A new multipoint method for genome-wide association studies by imputation of genotypes. *Nat Genet* 2007;39(7):906-13.
3. Nishida N, Tanabe T, Takasu M, Suyama A, Tokunaga K. Further development of multiplex single nucleotide polymorphism typing method, the DigiTag2 assay. *Anal Biochem* 2007;364(1):78-85.

APPENDIX 3: COLOR FIGURES

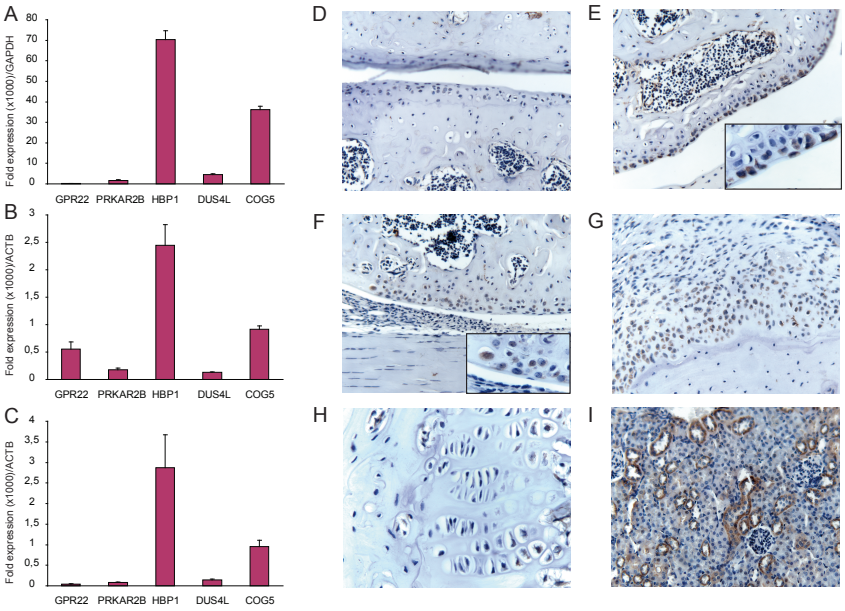
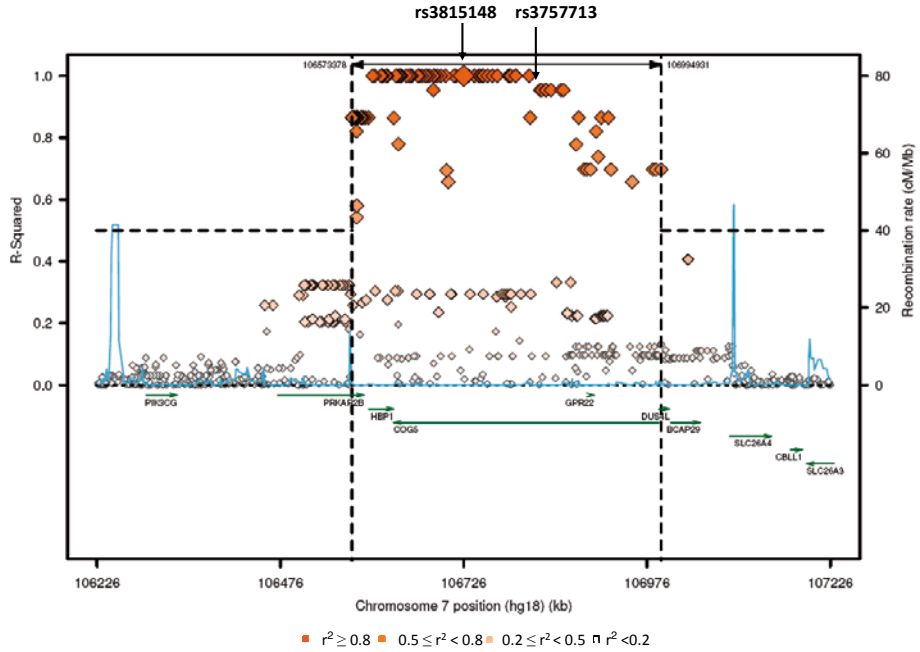


Figure 3 Chapter 3.2. (**A**) Quantitative PCR analysis of gene expression levels (*Gpr22*) in expanded synovial fibroblasts (n=3 sets), (**B**) freshly isolated chondrocytes (n=5 sets) and (**C**) meniscal cells (n=3 sets). Data are shown as mean +/- SEM. Fold expression is multiplied by 1000. (**D**) Immunohistochemistry shows the absence of Gpr22 protein in normal articular cartilage. (**E** + insert) Gpr22 positive chondrocytes (brown cytoplasm) are found in articular chondrocytes of mice with papain-induced osteoarthritis, (**F** + insert) in mBSA induced arthritis, (**G**) and in developing osteophytes in instability induced osteoarthritis. No Gpr22 protein signal is found in the synovium. (**H**) Growth plate chondrocytes were negative for Gpr22. (**I**) Kidney sections were used as a positive control. (Magnification 200x in D, E, F and I, 100x in G and 400x in H).



Supplementary Figure 2 Chapter 3.2. Regional LD plot for the chromosome 7 locus (rs3815148 SNP, *GPR22*). r^2 measures are based on the HapMap CEU samples. Each diamond represents one SNP. The brightness of each diamond is proportional to the r^2 values for that SNP, the lower the r^2, the lighter the color of the diamond.

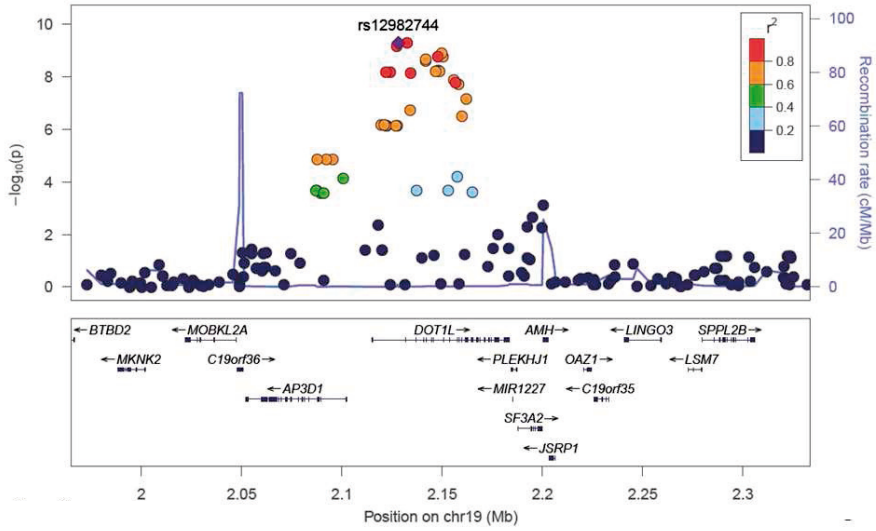


Figure 2 Chapter 3.3. Regional association plot for rs12982744.

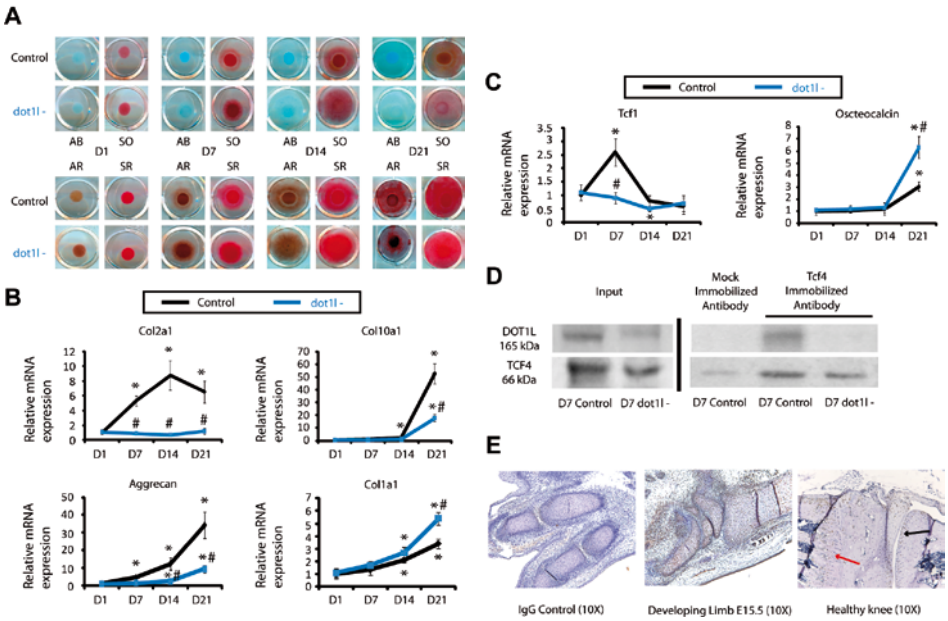


Figure 4 Chapter 3.3. Functional analysis of *Dot1l* during chondrogenesis. Stable ATDC5 clones were established using either the control non-interfering pGIPZ or the pGIPZ-shmiRNA directed against mouse *Dot1l*. Three different antibiotic resistant clones were selected. Knock-down efficiency was assessed by qRT-PCR. Stably-transfected ATDC5 clones were cultured as micromasses as described before (20,21). Each condition was performed in triplicate. Total RNA from was isolated after 1, 7, 14 or 21 days. Data presented are representative of the three independent clonal colonies. Results are expressed as the mean ± SD of three independent replicates. Comparisons were made by ANOVA, followed by Fisher's *t post-hoc* test. Statistically significant differences *vs.* day 1 are indicated as *: $P<0.05$, and *vs.* control-transfected cells as #: $P<0.05$. (**A**) *Dot1l* knock-down reduces proteoglycan and collagen content, and mineralization during chondrogenesis. Stainings were performed on ATDC5 micromass cultures stably transfected with either control or *Dot1l* shmiRNA producing vector, over 21 days (D). (AB = Alcian blue, SO = safranin O, AR = alizarin red, SR = sirius red). (**B**) *Dot1l* knock-down reduces mRNA expression of chondrogenesis markers of chondrogenesis. mRNA levels were normalized to S29 (reference gene) (n=3). Quantitative Real-Time PCR conditions and primers are available upon request. (**C**) *Dot1l* knock-down affects Wnt signaling during chondrogenesis. mRNA levels of Wnt target genes *Tcf1* and osteocalcin were normalized to *S29* (reference gene) (n=3). (**D**) DOT1L interacts Wnt signaling pathway transcription factor TCF4. Co-immunoprecipitation of DOT1L and TCF4 using 100 µg of total proteins (input) from micromass cultures (at day 7 – D7) of either control or *Dot1l* knocked-down cells. Proteins were isolated from ATDC5 micromasses. CoIPs were performed and 20 µl of elution fraction was probed after protein binding on either mock (donkey anti-goat IgG) or TCF4 column-immobilized antibody. (**E**) DOT1L is expressed during joint development and in mature articular cartilage of mice. Immunohistochemistry on paraffin embedded EDTA decalcified adult knee sections and non-decalcified embryonal sections, was performed with rabbit anti-Dot1L antibody (5µg/ml). After overnight incubation of the sections at 4°C, 1:100 peroxidase goat anti-rabbit IgG was applied and peroxidase activity was determined using DAB. Immunohistochemistry detected very strong expression in chondrocytes in the developing mouse.

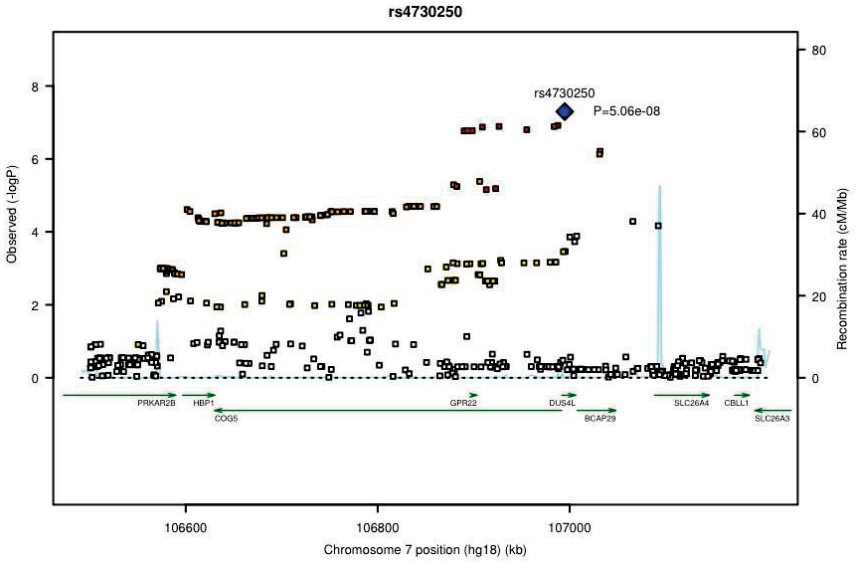
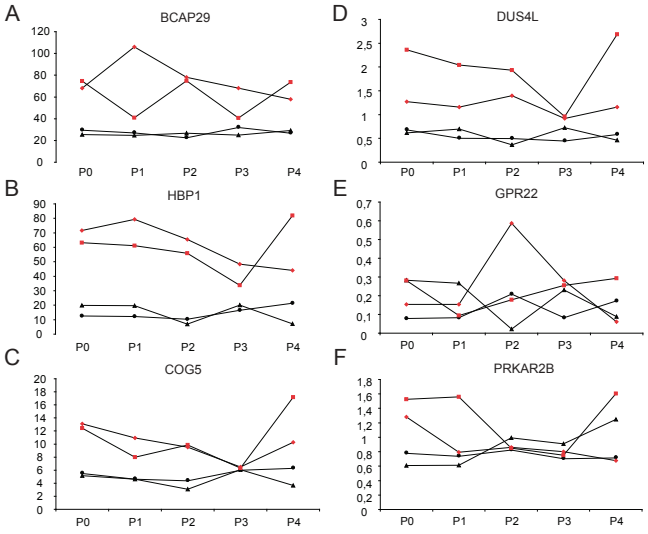


Figure 2 Chapter 3.4. Regional association plot for the top signal rs4730250.



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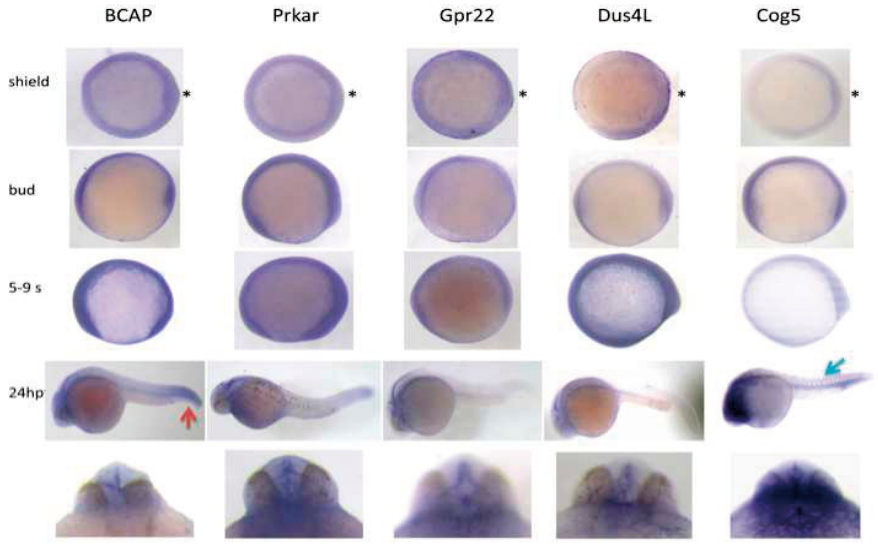

Supplementary Figure 2 Chapter 3.4. Whole mount in situ hybridization of zebrafish embryos at different stages of development. The stages are indicated on the left side of the figure and the gene names are on the top. At the shield stage, all embryos are vegetal view, shield to the right (asterisk). At the bud, 5-9 somite and 24hpf stages, the embryos are positioned anterior to the right, dorsal to the top. Below the panels containing the 24hpf embryos are corresponding frontal view of the head with dorsal to the top. The red arrow indicates tail specific expression and the blue arrow indicates the intersomitic expression.

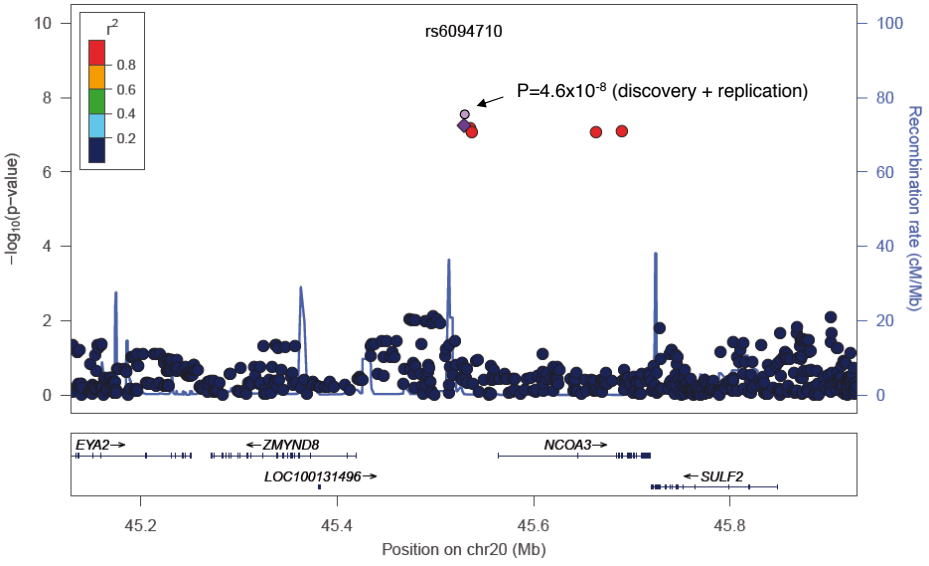

Figure 3 Chapter 3.5. Regional association plot for rs6094710.

DANKWOORD

Wat heb ik de afgelopen jaren met ontzettend veel plezier aan dit boekje gewerkt! Ik wil heel graag iedereen bedanken die heeft bijgedragen aan dit plezier en iedereen die heeft meegeholpen aan dit proefschrift. In het bijzonder…

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Pap en mam, "als je maar gelukkig bent" zeiden jullie altijd als ik vroeg of het goed was als ik iets wilde gaan doen. Nou, dat is gelukt! Dankzij jullie onvoorwaardelijke liefde en steun ligt uiteindelijk dit boekje er en ben ik zo trots als een pauw.

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Prof. Spector, dear Tim, I am very happy that you are in my committee. Many thanks for reading this thesis, but especially for all the opportunities I have been given in the TREAT-OA consortium. I have enjoyed working with you on many of the papers published in this thesis. With the TREAT-OA consortium, a new boost in genetics of OA has been given and I hope this will continue for a long time. Good luck with your new book!

Beste Prof. Bierma-Zeinstra, Sita, hartelijk dank voor alle inzichten die jij gegeven hebt in de meer klinische en epidemiologische vraagstukken. Jouw kennis over artrose is enorm en ik heb zeer veel van al onze bijeenkomsten en discussies geleerd. Vier jaar geleden begon ik mijn promotie onderzoek en toen moesten er nog héél veel röntgenfoto's gescoord worden. Mede dankzij jou heb ik snel en goed geleerd hoe dit moest en heb ik met de hulp van een legertje studenten alle röntgenfoto's bij kunnen lezen die nog niet beoordeeld waren.

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levert nieuwe ideeën en nieuwe inzichten op. Bedankt voor de samenwerking en alle gezellige meetings en congressen. Het etentje bij zonsondergang op Mallorca zit nog goed in mijn geheugen.

Beste Dr. Lories, Rik, je bent altijd enthousiast om nieuwe genen functioneel te onderzoeken en bereid om snel en kwalitatief hoogwaardige experimenten toe te voegen aan onze bevindingen. Gaan we er nog achter komen welk gen(en) het nu is op chromosoom 7? Ik zeg GPR22…

Dr. Oei, beste Edwin, jij hebt mij wegwijs gemaakt in de wereld van röntgenfoto's. Wat is normaal, op wat voor toevalsbevindingen moet actie ondernomen worden, is dit nu reuma? Allemaal vragen waar ik met jouw hulp antwoord op heb gekregen. Ik vind het dan ook erg fijn dat je in mijn promotiecommissie zitting wilt nemen.

Beste Prof. van Duijn, hartelijk dank voor uw bereidheid om zitting te nemen in mijn promotiecommissie en te opponeren bij de verdediging van mijn proefschrift.

Promoveren zou niet lukken zonder paranimfen en ik ben supertrots dat jullie mijn paranimfen willen zijn.

Rowena, Lieve Ro, jij had de durf om een grote overstap te maken van analist naar een sales functie, waarvoor je van mij een groot respect verdient. Sinds je niet meer bij het lab werkt, hebben we onze kaasfondue avondjes ingevoerd en die houden we er hopelijk nog vele jaren in. Het is zo fijn om vrienden te hebben waar je alles tegen kan zeggen, die altijd voor je klaar staan en waar je ontzettend mee kan lachen. Dank je wel dat jij zo een lieve vriendin voor mij bent en mijn paranimf wilt zijn!

Als we nou maar nooit meer een slang tegen komen dan wil ik verder nog wel meer leuke uitjes met jou beleven Marjolein. Ik heb ontzettend veel plezier met je gehad de afgelopen jaren en we hebben honderduit gekletst over onderzoek, maar ook over zoveel andere dingen. Toppie om jou als collega te hebben! Heel veel succes met het afronden van je boekje.

Ik zit inmiddels bijna 8 jaar bij het Genetisch Lab en het lab is flink gegroeid. Ik weet dus zeker dat ik mensen vergeet als ik nu iedereen bij naam ga noemen. Daarom, alle analisten (Pascal, Mila en Michael in het bijzonder), onderzoekers/promovendi (Martha dank je wel voor alle discussies over OA), (bio)informatici en al het andere ondersteunend personeel dank jullie wel voor alle hulp bij mijn onderzoek, de gezellige congressen, lunches, labuitjes en noem maar op!

Ik zou twee mensen tekort doen als ik ze niet persoonlijk zou noemen, want zij hebben veel bijgedragen aan dit boekje. Als een van de senior onderzoekers weet je altijd de juiste kritische vragen te stellen om zo het onderzoek nog beter te krijgen. Ook jouw statistische kennis heeft mij meerdere malen verder geholpen in de projecten. Dank je wel voor al je hulp Fernando. Lieve Lisette, van jou heb ik veel geleerd

over de grondbeginselen van de genetica en samen hebben we ontdekt hoe je een GWAS analyse moet doen. Wat is het goed dat wij niet meer op één kamer zitten, dat zou veel te veel geouwehoer worden zeker nu we allebei zo'n klein guppie hebben. Ik zal alle leuke uitstapjes niet snel vergeten! Hm dit is niet echt een samenhangend geheel, maar ach dat kenmerkt onze gesprekken denk ik.

Dirk, Tip, Annette, Martijn, Jacqueline, Vivienne en Julian, dank jullie wel voor jullie hulp bij het lezen van al die röntgenfoto's. Ik hoop dat jullie er geen trauma aan hebben over gehouden, want het waren er nogal wat. Ik zal alle consensusbesprekingen niet snel vergeten, want die waren vooral gezellig. Ik zal geen namen noemen, maar uitspraken als "ik zit graag op het invalidentoilet, want dan heb je zo'n lekker vrij gevoel" maakten dat de tijd erg snel ging tijdens de meetings. Dirk, een speciale dank voor jou, want jij hebt naast het lezen van de foto's veel extra werk verzet en dit altijd met enthousiasme gedaan. Heel veel succes allemaal met jullie co-schappen!

Alle studenten die hard gewerkt hebben aan het digitaliseren van al die röntgenfoto's hartelijk dank! Het lezen van de handenfoto's ging een stuk sneller sinds we niet meer elke keer alle mappen hoefden te pakken.

I would like to say thank you to all the TREAT-OA collaborators and all other investigators which I have been in contact with for the several papers published in this thesis. I cannot mention you all by name as there are simply too many and I will definitely forget someone, but I have appreciated the close collaborations very much. I would like to highlight one person, Ana Valdes. Dear Ana, thank you for all the projects we did together, for the joy we had during meetings, dinners and drinks and thank you for the opportunities you have given me. I wish you all the best in your career.

Mijn dank gaat ook uit naar alle co-auteurs, voor jullie bijdrage en waardevolle commentaren en suggesties op de manuscripten.

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Professor Pols, beste Huib, ondanks dat je nu in de raad van bestuur zit, houd je het wetenschappelijk onderzoek binnen onze groep goed in de gaten. Je komt op de ERGO-besprekingen altijd weer met nieuwe inzichten en de klinische relevantie zal nooit vergeten worden. Hartelijk dank!

Professor Hofman, beste Bert, dankzij jou ben ik ooit de Master Clinical Epidemiology gaan volgen en bij deze onderzoeksgroep terecht gekomen. Ik ben blij dat ik deze kans heb gekregen en dat ik zoveel kennis op heb kunnen doen.

Beste Cecile, hartelijk dank dat je mijn mentor wilde zijn voor mijn Master Public Health. Ik heb veel van je geleerd over predictiemodellen.

Alle deelnemers en alle mensen rondom het ERGO onderzoek, bedankt! Zonder jullie was geen enkele publicatie uit mijn proefschrift mogelijk geweest. Jan Heeringa, als coördinator ben je onmisbaar voor ERGO. In het bijzonder wil ik graag de mensen bedanken die mij geholpen hebben bij de data verzameling en alle computer/automatisering problematiek, Frank, Jolande, Nano, Rene, Edward en Roel. Een warm hart wordt de medewerkers op het ERGO centrum toegedragen. Door jullie toewijding en vriendelijkheid komen de deelnemers graag naar het onderzoekscentrum en heb ik met veel plezier mijn uitschrijfdiensten gedaan. Hannie, zonder jou had ik geen rontgenfoto's gehad, dank je wel!

Zonder ontspanning is hard werken haast onmogelijk. De borrels en feestjes van de afdeling Endocrinologie hebben hier zeker aan bijgedragen. Alle collega's op de 5e etage, hartelijk dank voor alle fijne jaren die ik op deze afdeling heb gehad!

Ook mijn lieve vrienden, hebben voor ontspanning en een luisterend oor gezorgd, jullie mag ik zeker niet vergeten. Misschien waren jullie direct gezien niet van invloed op de totstandkoming van dit boekje, maar indirect des te meer.

Er zijn nog twee belangrijke mensen die ik graag als laatste zou willen bedanken.

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Lieve Joyce, jij bent iemand die meestal liever op de achtergrond blijft, maar in dit geval kan dat niet. Ik kan namelijk niet anders dan met zeer lovende woorden over je spreken. Waar vind je een co-promotor die slim, creatief en (positief) kritisch is en altijd tijd weet vrij te maken om haar promovendi te kunnen begeleiden? Ik had de mazzel om zo'n co-promotor tegen te komen en ben je zeer dankbaar voor alles wat je voor me hebt gedaan. Jij hebt mij het vertrouwen gegeven om zelf projecten te kunnen initiëren en begeleiden, maar was er ook altijd als het tegen zat en ik een klankbord of een aanmoediging nodig had. Ik heb mezelf de afgelopen jaren enorm kunnen ontwikkelen dankzij jouw geloof in mij!

Laten we proosten
Op het leven
Laat het leven je omarmen
Sla je armen om de liefste
Want de liefste, dat ben jij
(Guus Meeuwis – Proosten)

LIST OF PUBLICATIONS

This thesis

Betancourt MC, Cailotto F, **Kerkhof JM**, Cornelis FMF, Doherty SA, Hart DJ, Hofman A, Luyten FP, Maciewicz RA, Mangino M, Metrustry S, Muir K, Peters MJ, Rivadeneira F, Wheeler M, Zhang W, Arden NK, Spector TD, Uitterlinden AG, Doherty M, Lories RJ, Valdes AM, van Meurs JBJ. Genome wide association and functional studies identify the *DOT1L* gene to be involved in cartilage thickness and hip osteoarthritis. *Submitted*.

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MC, Pols HA, Rivadeneira F, Shi D, Slagboom E, Spector TD, Stefansson K, Sudo A, Tamm A, Tamm AE, Tsezou A, Uchida A, Uitterlinden AG, Wilkinson JM, Yoshimura N, Valdes AM, van Meurs JB. Recommendations for standardization and phenotype definitions in genetic studies of osteoarthritis: the TREAT-OA consortium. *Osteoarthritis Cartilage* 2011;19(3):254-64.

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K, Blackburn H, Blanco FJ, Bumpstead S, Cupples LA, Day-Williams AG, Dixon K, Doherty SA, Esko T, Evangelou E, Felson D, Gomez-Reino JJ, Gonzalez A, Gordon A, Gwilliam R, Halldorsson BV, Hauksson VB, Hofman A, Hunt SE, Ioannidis JP, Ingvarsson T, Jonsdottir I, Jonsson H, Keen R, **Kerkhof HJ**, Kloppenburg MG, Koller N, Lakenberg N, Lane NE, Lee AT, Metspalu A, Meulenbelt I, Nevitt MC, O'Neill F, Parimi N, Potter SC, Rego-Perez I, Riancho JA, Sherburn K, Slagboom PE, Stefansson K, Styrkarsdottir U, Sumillera M, Swift D, Thorsteinsdottir U, Tsezou A, Uitterlinden AG, van Meurs JB, Watkins B, Wheeler M, Mitchell S, Zhu Y, Zmuda JM; arcOGEN Consortium, Zeggini E, Loughlin J. Insights into the genetic architecture of osteoarthritis from stage 1 of the arcOGEN study. *Ann Rheum Dis* 2011;70(5):864-7.

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PhD PORTFOLIO

Name:	Hanneke J.M. Kerkhof
Erasmus MC Department:	Internal Medicine – Genetic Laboratory
Research school:	NIHES/MolMed
PhD period:	January 1st, 2008 – May 11th, 2012
Promotor:	Prof. dr. André A.G. Uitterlinden
Supervisor:	Dr. Joyce B.J. van Meurs

1. PhD training	**Year**	**Workload**
Master Public Health	2009-2012	68.5 ECTS
(Inter) national Conferences		
- 13th World congress of the Osteoarthritis Research Society International - Rome, Italy	2008	Sept 18-21
- 36th European Symposium on Calcified Tissues -23-27 Vienna, Austria	2009	May
- 14th World congress of the Osteoarthritis Research 10-13 Society International - Montreal, Canada	2009	Sept
- Nederlandse Vereniging voor Calcium en Bot--12-13 stofwisseling - Zeist	2009	Nov
- European Human Genetics Conference - Gothenburg,	2010	June - 15th
World congress of the Osteoarthritis Research Society International - Brussels, Belgium	2010	Sept 23-26
- Nederlandse vereniging voor Calcium en Bot- stofwisseling - Zeist	2011	Nov 10-11
- 17th World congress of the Osteoarthritis Research	2012	April 26-29
Society International - Barcelona, Spain		

Seminars and Workshops	**Year**	**Workload**
- TREAT-OA meeting: kick off	2008	2 days
- 12th Molecular Medicine Day - Rotterdam	2008	1 day
- KNAW conference - The Role of DNA Polymorphisms in Complex Traits and Diseases - Amsterdam	2008	Mar 18-21
- TREAT-OA Symposium: Breaking boundaries in OA - London, UK	2009	Feb 1
- TREAT-OA meeting - London, UK	2009	April 20-21
- TREAT-OA meeting - Leiden	2009	Nov 9-10
- NCHA meeting - Leiden	2010	Jan 25
- Symposium: phenotype definitions in OA - Mallorca, Spain	2010	Oct 6-7
- TREAT-OA meeting - Mallorca, Spain	2010	Oct 7-8
- TREAT-OA meeting - Leuven, Belgium	2011	Oct 5-6

- NCHA meeting - Rotterdam	2011	Dec 14
- NCHA meeting - Amersfoort	2012	1 day
- 16th Molecular Medicine Day - Rotterdam	2012	1 day

Presentations

- "A Genome-Wide Association Study identifies a locus on chromosome 7q22 to influence susceptibility for osteoarthritis" - *Wetenschapsdagen*	2009	Poster
- "A Genome-Wide Association Study identifies a locus on chromosome 7q22 to influence susceptibility for osteoarthritis" *ECTS*	2009	Oral
- "A Genome-Wide Association Study identifies a locus on chromosome 7q22 to influence susceptibility for osteoarthritis" *OARSI*	2009	Poster
- "A Genome-Wide Association Study identifies a locus on chromosome 7q22 to influence susceptibility for osteoarthritis" *NVCB*	2009	Oral
- "A Genome-Wide Association Study identifies a locus on chromosome 7q22 to influence susceptibility for osteoarthritis" *artrose bijeenkomst* EMC	2009	Oral
- "Serum CRP and genetic variation in the *CRP* gene are not associated with osteoarthritis independent of BMI" *NVCB*	2009	Oral
- "Large scale meta-analysis of interleukin-1 beta and interleukin-1 receptor antagonist polymorphisms on risk of radiographic hip and knee osteoarthritis and severity of knee osteoarthritis" *OARSI*	2010	Poster
- "Recommendations for standardization and phenotype definitions in genetic studies of osteoarthritis: the TREAT-OA consortium" *OARSI*	2010	Poster
- "Suggestive evidence for a rare duplication (+/-260kb) on chr6q25-28 to be involved in knee OA" *Wetenschapsdagen*	2011	Poster
- "Prediction model for knee osteoarthritis" *NVCB*	2011	Oral
- "Prediction model for knee osteoarthritis" *Wetenschapsdagen*	2012	Poster
- "Prediction model for knee osteoarthritis" *MolMed day*	2012	Poster
- "Prediction model for knee osteoarthritis" OARSI	2012	Poster

Other

- Coding of fractures in the Rotterdam Study	2008-2009	~40 hours
- Scoring of X-rays on OA features in the Rotterdam Study	2008-2011	~600 hours
- Exit interview with participants of the Rotterdam Study	2009-2011	~80 hours
- Referee activities for various international scientific Journals (e.g., Arthritis Rheum, Osteoarthritis Cartilage)	2008-2012	

2. Teaching activities

Lecturing and supervising practicals	**Year**	**Workload**
- Vaardigheidsonderwijs second year medical students: Hypercortisolisme	2010	1 day
- Erasmus Summer Program - Genomics in Molecular Medicine	2010	1 day
- Molecular Medicine Postgraduate School - SNP's and Human Diseases	2009	Nov 2-6
- Molecular Medicine Postgraduate School - SNP's and Human Disease	2010	Nov 15-19

Teaching activities continued	**Year**	**Workload**
- Teaching in identification of OA on x-rays to medical students and PhDs	2009-2012	
Supervising students		
- Dirk Sluiter - Master thesis - Erasmus Medical School	2011	9 months
- Supervision of 8 medical students in scoring of x-rays Erasmus MC Rotterdam	2008-2012	6h/week

ABOUT THE AUTHOR

Hanneke (Johanna Maria) Kerkhof was born on October 17th 1983, in Nieuwegein. In 2001, she passed her secondary school at the "Cals College" in Nieuwegein. After studying one year of Health Sciences in Maastricht she was accepted, through a selection procedure, at the "Erasmus University Rotterdam" Medical School. In 2009 she graduated cum laude. During medical school Hanneke had many jobs such as nurse in a home for the elderly and assistant nurse in a team of students at the Department of Oncology and Geriatrics of the Erasmus Medical Center in Rotterdam, where she became head of the student's team later on. In August 2006 she achieved her registration as Master of Science in Clinical Epidemiology (cum laude). During her PhD she will attain a degree as Master Public Health (2009-expected graduation in spring 2012). Both research masters were conducted at the Netherlands Institute of Health Sciences (NIHES). In January 2008 Hanneke started the work described in this thesis at the Department of Internal Medicine at the Erasmus Medical Center in Rotterdam. She received young investigator awards in 2007 (OARSI) and 2009 (ECTS) for her work on the estrogen receptor beta gene and the discovery of the chr7q22 locus. Hanneke lives together with Nico Wiss and has a son, Tim.

WHAT A PAIN!

Osteoarthritis (OA) is the most common musculoskeletal disorder among the elderly and leads to pain, stiffness and disability. The prevalence of OA is rising with the obesity pandemic and the ageing population. Currently, there is no medication available for this disease other than standard pain medication. Therefore, the need for new insights into the pathogenesis of the disease and discovery of new drug targets is high. In this thesis, the identification of novel genes involved in OA is described and the need for standardization of phenotype definitions in OA research is addressed. In addition, a prediction model including clinical, genetic and biochemical risk factors for knee OA is provided.

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