Hip Osteoarthritis

Genetics, epidemiological risk factors and burden of the disease

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Chapter 1

Introduction

Osteoarthritis (OA) is the most common degenerative joint disease, characterized by progressive damage of the articular cartilage, osteophyte formation and alterations in the subchondral bone. OA is associated with an extremely high burden in terms of health and economics on individuals, communities, and health systems; This burden is largely attributable to the effects of disability, comorbid disease, and the expense of OA-treatment [1,2]. According with worldwide estimates 9.6% of men and 18.0% of women aged over 60 years have symptomatic osteoarthritis [3]. Hip and knee are joints commonly affected by OA; in these joints, structural damage is accompanied by clinical symptoms as joint pain, stiffness, loss of function and different degrees of disability.

General aspects of hip osteoarthritis

Activity, Disability, Life Perception and Mortality

OA has a major impact on physical and mental health of individuals. Hip and knee OA have been strongly associated with increased disability and difficulty in activities of daily life [4,5]. OA has also effects on individual's mental health; subjects with OA usually experience pessimism and distressing feelings as consequence of the pain and difficulty in daily activities [6]. In general, individuals with OA report lower levels of quality of life and a negative perception of life compared to individuals without OA [5-7]. This might -in part- be due to difficulties and frustration of not being able to manage the disease, especially the pain and disability. A total of 10% to 25% of the remaining quality-adjusted survival of persons aged 50 to 84 years are lost due to knee OA [8]. More than 60% of individuals with OA have one or more comorbidity [7], which contributes to an increased the burden of the disease and possibly leading to a shorter and less-productive life. In this respect, it has been suggested that subjects with hip or knee OA have a higher mortality risk compared with general population [8, 10, 11]. Together all this evidence make OA one of the most important diseases in elderly that represents a substantial loss in productivity and major costs for individuals and for our society.

Definition and Prevalence

Prevalence of hip OA varies according to the definition and method used to evaluate the affected joint. It also depends on the age and ethnic origin of the investigated population, being radiographic OA higher in older individuals of Caucasian-origin and symptomatic OA in African-American women [12,13]. There are many different definitions circulating in literature; they can be divided into two major categories: the first is based

only on radiological evidence of joint damage, while the second category is defined by both radiographical damage as well as clinical symptoms, such as pain and stiffness. For definitions based on radiographic damage, several scoring systems exist, which mainly focus on cartilage damage (seen at the X-ray as joint space narrowing: JSN) and presence of bone spurs on joint margins (osteophytes). The Kellgren and Lawrence score (KL) (Table 1a) and measuring of minimal Joint Space Width (mJSW) including Croft score (Table 1b), are two of the radiological definitions most frequently used to evaluate hip OA. Both methods show good reproducibility and strong association with joint pain, being better for measure of mJSW [14-17]. A definition of OA based on the presence of symptoms and radiographic alterations has been defined by the American College of Rheumatology [18].

The severity and progression of hip OA is also commonly assessed by the methods previously described: Kellgren and Lawrence and the degree of Joint Space Narrowing (JSN) on radiographs, while Kellgren and Lawrence score appears as a strong predictor of progression of hip OA defined only by the same method [19]. JSN has a higher sensitivity detecting structural progression, pain and predicting future joint replacement [20-22]. Changes in mJSW of at least 0.5 mm/year or 20% decrease compared to baseline joint space in a period of two years have been presented as relevant change for radiological progression of hip OA with clinical implications [22-24]. Several definitions of hip OA have been used in epidemiological, clinical and genetic studies. This variety is partially explained by the absence of successful pharmacological treatments and the lack of knowledge regarding its etiological factors, leading researchers to explore other alternative definitions. It is also due to the fact that a definition as KL implies several underlying sub-phenotypes (osteophytes/ sclerosis/ narrowing/ joint replacement). Some of them, may only be present in part of the population affected by OA, and maybe more frequent in one gender.

Table 1a. Kellgren and Lawrence grading system based on radiographic findings for OA.

Grade		Description
0	No osteoarthritis	
1	Doubtful	Possible narrowing of the joint space and possible osteophytes around femoral head; or osteophytes alone.
2	Mild	Definite narrowing of the joint space, definite osteophytes and slight sclerosis.
3	Moderate	Marked narrowing of the joint space, definite osteophytes, some sclerosis and cyst formation and deformity of femoral head and acetabulum.
4	Severe	Gross loss of joint space with sclerosis and cysts, marked deformity of femoral head and acetabulum and large osteophytes.

A hip classified as KL grade 2 or higher is defined as being OA.

Table 1b. Croft measurement of minimal Joint Space Width (mJSW) on radiographs.

Grade	Description
0	MJS>2.5mm
1	MJS>1.5 mm and ≤2.5mm
2	MJS≤1.5 mm

Minimal Joint Space Width (mJSW) is the shortest distance between the femoral head and the acetabulum.

Heritability and familial aggregation

OA is a multifactorial disease with a significant genetic component that has been demonstrated using twin-pairs and sibling risk studies. It has been estimated that between 40-60% of risk for OA might be explained by genetic factors. However, there are differences in the degree of heritability between different joints reported by these studies, being for example higher for hip OA than for knee OA. In 2000, two different genetic studies presented evidence of the high heritability and familial aggregation of hip OA. A twins-study reported that genetic factors accounted for approximately 60% of the variation in population liability to hip OA [25].

Additionally, hip OA has demonstrated a high familial aggregation which has been estimated using sib recurrence risk [26]. Sib recurrence risk (λs) is the risk ratio for a relative of an affected individual, compared with the population prevalence. In general, someone having a first degree relative affected has between 4 to 6 times more chances of having hip OA than someone without relatives affected [27]. A sib-pair study in ~600 index patients and sibpairs, found an age adjusted odds ratios in siblings between 4.9 and 6.4 for probable and definite hip OA, respectively [27]. Differences in risk estimates are not only due to differences in OA severity but also between those receiving a THR and those with just radiographic hip OA, suggesting that genetic influences are going beyond the changes seen on a radiograph perhaps influencing clinical symptoms [27].

Differences in degree of heritability between joints and phenotypic differences displayed in the same joint between affected individuals (explored along this thesis) point to the fact that specificity, severity degree and correct discrimination between the phenotypes that define the disease is needed to reduce heterogeneity when searching for the implicated genes.

Risk factors for hip osteoarthritis and correlated traits

Genetics

Different methods have been explored trying to identify the genes associated with OA that could explain its high heritability. Linkage and candidate gene studies have been used in the last decades trying to elucidate the genetics of OA with a "relative success". In the case of hip OA, some genes received especial attention which were previously found associated with chondrodysplasias and were related to collagen (*COL2A1*), inflammation (*IL1, IL4R, IL6*), estrogen (*ESR1*), bone (*LRP5, VDR*), apoptosis (ANP32A), in addition to genes found from linkage studies (*DIO2, FRZB*), as the most cited in literature [29-34]. However, in spite of the plausible functionality of those candidate genes, only modest associations with hip OA have been found, with difficulties for the association to be replicated in general populations [35-37]. It might be in part due to the few selected variants for candidate genes to be tested in a usually small sample size. However, the limited success from candidate genes studies is also due to the lack of understanding of the etiology of the disease. It does not allow establishing the relevant biological pathways implicated and correctly define the set of genes to be tested. OA is considered as a complex disease; complex in nature and of multi-factorial cause, arising from a combination of environmental and numerous genetic factors (most of which have not yet been identified). Complex diseases do not obey the single-gene dominant or single-gene recessive Mendelian pattern of inheritance [38, 39]. They are attributable in part to allelic variants present in more than 1–5% of the population (38). Therefore, OA might be considered an oligogenic disease with several common and uncommon variants from different genes that need to be discovered. Nevertheless, difficulties finding the genes that explain the high heritability of complex diseases is not specific for OA, it has been similar to other complex diseases [40].

Genome-wide association studies (GWAS) have been a successful approach to investigate the association between common genetic variation, traits and diseases [40]. In fact until now, many significant associations have been found for common and/or complex traits with common variants [41]. However, identification of genetic variants robustly associated with OA at a genome-wide significant level (GWS=Pvalue$\leq 5 \cdot 0 \times 10^{-8}$) has been more difficult than for other diseases. Trying to search for OA-associated genetic variants, GWAS on OA have used different phenotypes or a combination of them trying to increase the power of the studies and/or trying to find common susceptibility variants for OA at different joint sites (Hip OA by Kellgren and Lawrence (KL>=2),

Total Hip Replacement) (THR), Total Knee Replacement (TKR)). With the GWAS approach, *GDF5* and a locus on chromosome 7 were identified in association with hand and knee OA in Caucasian, and not with hip OA [42-44]. *GDF5* has been found associated with hip OA only in Asian populations as it was a variant in *DVWA* [45]. Genetic associations differ between Asiatic and European descendants and in spite of some concordance between knee and hip OA there is evidence of a genetic joint specificity. Table 2 shows the variants that have been reported across several GWAS performed until now, to be associated with hip OA at GWS level (Table 3). The locus on chromosome 13 localized in the *MCF2L* gene was associated with hip and/or knee OA, having stronger association with knee than with hip OA [46]. The last meta-analysis including patients with severe OA (from the arcOGEN consortium), identified 6 novel loci associated with hip and/or knee osteoarthritis at GWS level using different radiological definitions [47].

Table 2. Genetic loci associated with Hip OA identified using the Genome Wide Association approach.

SNP	Chr. location	Nearest gene	Phenotype used	Effect OR(95%CI)	P value	Ref.
rs11842874	13q34	MCFL2	Hip OA (KL or THR)	1.11 (1.03–1.19)	3.54×10^{-03}	42
rs10492367	12p27.9	PTHLH/ *KLHDC5*	Hip OA (KL or THR)	1·14 (1·09–1·20)	$1·48 \times 10^{-08}$	43
rs10948172	6p21.1	SUPT3H/ CDC5L	Hip OA in males	1·14 (1·09–1·20)	$7·92 \times 10^{-08}$	43
rs9350591	6q12	FILIP1/ SENP6	Hip or Knee OA	1·18 (1·12–1·25)	$2·42 \times 10^{-09}$	43
rs11177	3p21.1	GLN3	THR or TKR	1·12 (1·08–1·16)	$1·25 \times 10^{-10}$	43
rs4836732	9q33	ASTN2	THR–female	1·20 (1·13–1·27)	$6·11 \times 10^{-10}$	43
rs835487	12q24.1	CHST1L	THR	1·13 (1·09–1·18)	$1·64 \times 10^{-08}$	43
rs12982744	19p13.3	DOT1L	mJSW and Hip OA-in males	1·17 (1.11-1.23)	7.8×10^{-09}	This thesis
rs6094710	20q13.1	NCOA3	Hip OA	1.28 (1.18-1.39	5.6×10^{-08}	44

Results from these GWAS evidenced large heterogeneity, finding differences in the effect of the variants reported between genders and joints. Finally, a two-stage meta-analysis on more than 78,000 participants, recently discovered a new locus for hip OA near the *NCOA3* (nuclear receptor co-activator 3) gene [48].

Bone Related Factors

Hip Geometry. Biomechanical factors seem to have an important role in the development and progression of hip OA. The shape of the joint, including geometrical variance results in differences in biomechanical loading patterns in the joint. First evidences on the relation of OA and incongruence in hip morphology were presented in Wiberg's doctoral thesis about dysplasia in 1939 [49]. In hip with dysplasia, the majority part of the femoral head is not covered by the acetabulum (Figure 1a). Later on, dysplasia was presented as a potential factor for development of hip OA in younger and older individuals [50-52]. On the other hand, excessive coverage or retroversion of the acetabulum known as Coxa profunda deformity (the floor of the acetabulum touches the ilio-ischial line) will affect the normal spatial relationship between the femoral head and the acetabulum restricting flexion of the femoral neck [53]. Coxa profunda has been mentioned as one of the possible causes of impingement [53], other type of incongruence between femoral head and acetabulum. Impingement has also been related to hip OA in younger and older populations [54-57]. Different types of impingement at the hip joint can occur (Figure 1b & 1c). Additionally, wider femoral neck and differences in the external shape of the hip joint have been found to be associated with prevalent and incident cases of hip OA [58-59].
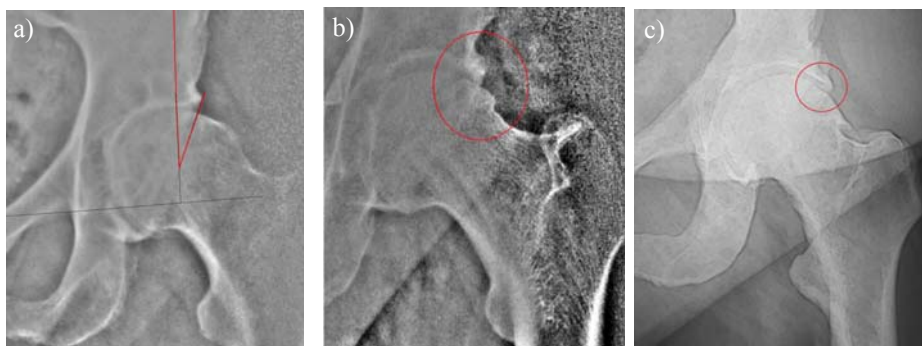
Figure 1 Dysplasia and different type of impingement of the hip joint. **a).** Radiograph shows a decreased (abnormal) Wiberg or Center Edge Angle <15 degrees. **b).** Radiograph shows a typical Cam impingement at the transition between femoral head and neck. **c).** Radiograph shows a mixed impingement with a Cam deformity at the transition between femoral head and neck and a pincer deformity at the acetabular roof.

Other geometry measures analyzed in relation with hip OA have been less studied and include pelvic, acetabular and hip size measures and angles: femoral head, neck length and/or hip axis length, pelvic width, offset (distance between the center of femoral head

and the most lateral point of the major trochanter) and neck shaft angle (represent the inclination of the femoral head respect to the vertical axis), between others.

Recently, Statistical Shape Modeling (SSM) has been used to measure the differences in hip morphology in relation with hip OA. SSM is a comprehensive method to quantify the external shape of an object in this case the hip joint, including acetabulum and pelvis in the radiographic images. SSM analysis has identified different types of variations of femoral shape associated to clinical or radiological hip OA [59, 60].

Bone Mineral Density (BMD) and Fractures. In the last years, more attention was given for the role of subchondral bone changes in the initiation of OA. Not only changes in bone shape as previously described but, also bone mineral density (BMD) and subchondral bone mechanical properties differences have been reported in the presence of radiographic signs of hip OA [61-63]. Not only locally increased density of subchondral bone, but also systemically higher BMD have been reported in the presence of radiographic signs of hip OA. There is accumulated evidence that a higher BMD is associated to an increased risk for radiographic OA in hip, knees, spine and hands [64-68]. BMD changes have been found near the affected joint (for example increased FN-BMD in hip OA patients), and also at other distant skeletal sites in patients with radiographic OA. It has been widely discussed whether higher BMD plays a role in OA development or progression. This relationship can be studied using genetics. It might be that common pathways play a role in BMD and OA. In a recent study it was shown that; the association between lumbar disc degeneration and BMD is attributable to variants in common genes [68]. The possible pleiotropic genes implicated in this relation need to be investigated. Although higher BMD is a protective factor for osteoporotic fractures. OA and osteoporosis can co-exist [66, 69,70] and some reports even suggested that subjects with OA may have increased fracture risk [71-73].

Growth and Developmental OA-associated factors

Hip Joint Development

Human hip development is an ordered and sequenced processes that starts with the formation of the limb bud at 4 weeks of life, followed by extensive cell multiplication and differentiation where primitive chondroblast condense at proximal, central and distal centers which will fusion to model the femur [74]. Although hip differentiation continues until approximately 20 weeks of development, the major anatomic structures of the hip are visible microscopically by the eighth week (Figure 2) [75]. During this initial bone

development, the mesenchyme receives patterning signals that determine the shape, size and number of mesenchymal condensations [76]. Cells in the condensations differentiate into chondrocytes. These chondrocytes deposit an extracellular matrix that is cartilage-specific, undergo unidirectional proliferation forming vertical columns, exit the cell cycle becoming hypertrophic and die. This sequence of events is responsible for the longitudinal bone growth and hip joint formation [77]. The pathways of chondrocyte and osteoblast differentiation are thus interconnected during endochondral bone formation and must be coordinated.

Key transcription factors and signalling molecules coordinate this complex process of chondrocyte and osteoblast differentiation. Molecules that provide such patterning information are polypeptides of the Wnt, Hedgehog, fibroblast growth factor (FGF), Parathyroid hormone related peptide (PTHrP) and the TGF-β superfamily (including BMPs subfamily also called Growth and differentiation factors (GDFs), epidermal growth factor (EGF) that is antagonistic to BMPs (78), as well as a series of transcription factors of the Hox, Pax, homeodomain- containing, Forkhead and basic helix–loop–helix (bHLH) families and SMAD factors [76, 79].

Consequently, any change or disturbance of this coordinate differentiation process of cartilage and bone might have important consequences on bone and cartilage including differences in bone size (height) defects in cartilage and bone structure, maintenance and/or healing that depending of its severity might be found at birth (chondrodysplasia) or later in life as higher risk for bone and cartilage diseases as OA.

Association of Height with OA

Epidemiological and genetic studies have associated height with the risk of OA; it has been found that individuals of short stature are at higher risk for hip and knee OA [44, 80]. Since bone and cartilage tissue share a common process of endochondral bone formation during growth and development, the hypothesis of common biological pathways regulating variation in both (OA and height) seems very plausible [81-83].

Common variants in the OA-associated locus *GDF5* contribute to variation in height and increase susceptibility to hip and knee OA in Asian and European populations [83-85]. *GDF5* is part of the TGF beta signalling pathway and is involved in the development of cartilage in the legs and other long bones. *SMAD3* is another example of a gene where genetic variants are involved in both OA and height. Early-onset joint abnormalities,

including OA have been reported in subjects with mutations in *SMAD3* gene [82, 86]. SMAD proteins function as a transcriptional modulator activated by transforming growth factor-beta. Common genetic variations in the TGF-beta signalling pathways have been reported as significant contributors to height differences [87].

However, there are more factors and possibly other unknown variants that might contribute to explain the association between height and OA. Despite advances in understanding the molecular processes that underlie joint development and growth the discovery of new variants and genes implicated in both traits: height and OA will give a better understanding of these mechanisms.

AIM AND OUTLINE

The main objectives of this thesis were: 1) To identify genetic and bone-related risk factor for hip OA using quantitative and qualitative OA-endophenotypes and 2) To determine the risk for osteoporotic fractures and mortality in subjects with OA. **Chapter 2**, consists of three studies focusing on the identification of common genetic variants associated with joint space width and hip OA. The first analysis on minimal joint space width identified variants in *DOT1L* associated with cartilage thickness and OA **(Chapter 2.1),** a final meta-analysis of minimal joint space width discovering new variants associated with both, cartilage thickness and hip OA **(Chapter 2.2)** and a bivariate analysis of joint space width and height **(Chapter 2.3). Chapter 3** contains two studies analysing the contribution of two bone related factors: hip geometry **(Chapter 3.1)** and bone mineral density **(Chapter 3.2)** in the prediction of hip OA. **Chapter 4** analyses the osteoporotic fracture risk in subjects with different types of hip OA **(Chapter 4.1)** and lumbar disc degeneration **(Chapter 4.2)**. **Chapter 5**, will describe the association of hip and knee OA with mortality and how different comorbidities have an important role in this relation. Finally, **Chapter 6** will provide a general discussion.

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Chapter 2

**Genetics of cartilage thickness
and hip osteoarthritis**

Chapter 2.1

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

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Chapter 2.1

ABSTRACT

Hip osteoarthritis (OA) 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 OA and specifically in hip OA have yielded only few loci, which is partly explained by heterogeneity in the 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 hip OA. 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 = 4.8×10^{-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 hip OA (P = 1×10^{-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 in mouse limbs supports a role for DOT1L in chondrogenic differentiation and adult articular cartilage. *DOT1L* is also expressed in osteoarthritic articular chondrocytes. Silencing of *Dot1*l inhibited chondrogenesis in vitro. *Dot1*l knockdown reduces proteoglycan and collagen content, and mineralization during chondrogenesis. In the ATDC5 chondrogenesis model system, DOT1L interacts with TCF and Wnt signalling. These data are a further step to better understand the role of Wnt-signaling during chondrogenesis and cartilage homeostasis. DOT1L may represent a therapeutic target for OA.

INTRODUCTION

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 and 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* (2-3) a locus on chromosome 7q22 near the orphan receptor *GPR22* (4-5) and a variant in *MCF2L* (6). The low number of identified loci can be explained by relatively low power caused by insufficient sample sizes and by phe-

notype 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). In this study we combined GWAS and functional studies to identify new genes involved in cartilage thickness and OA. We first performed a discovery GWAS on minimal JSW (mJSW) of the hip in 6,523 participants from the Rotterdam cohorts 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 (Table S1 for cohort specifics). Additionally, we analyzed association of the genetic variants with hip OA in 3,717 cases and 10,013 controls. Further, we carried out functional genetic studies using cell culture experiments in human and mouse tissues.

RESULTS

A GWAS on mJSW of the hip was performed in 6,523 participants from the Rotterdam cohorts I and II (RS-I and RS-II, see Table S1 for cohort specifics). We applied extensive quality control measures (see Table S2 for details on quality control and exclusion criteria) leaving a total of 2,455,290 SNPs for association analysis. Genomic control inflation factors for the P values of the RSI and RSII GWAS were low (lambda = 1.02 and 1.01 respectively), and the interquantile-quantile plot (Fig. S1) also indicated no substantial population stratification due to cryptic relatedness, population substructure or other biases.

After meta-analyzing 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 $P < 5 \times 10^{-8}$ (Fig. 1a). A total of 18 SNPs were GWS and clustered around 1 locus on chromosome 19p13.3. The top SNP rs12982744 ($P = 4.5 \times 10^{-10}$) 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 (Fig. 1b). We additionally found 8 loci with suggestive evidence for association ($5 \times 10^{-8} < P < 1 \times 10^{-5}$, Table S3). 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, see Table S1 for details).
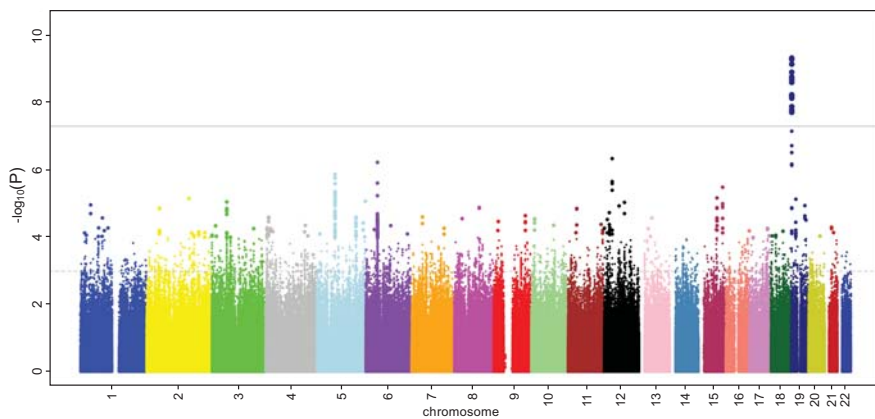
Figure 1a Association results by chromosome. The -log P -values for each of the 2.5 million tests performed as part of the genome-wide association of Minimal Joint Space (MJS) of the hip. The black solid horizontal line corresponds to P-value threshold of $5{\times}10^{-8}$ (genome-wide significance).
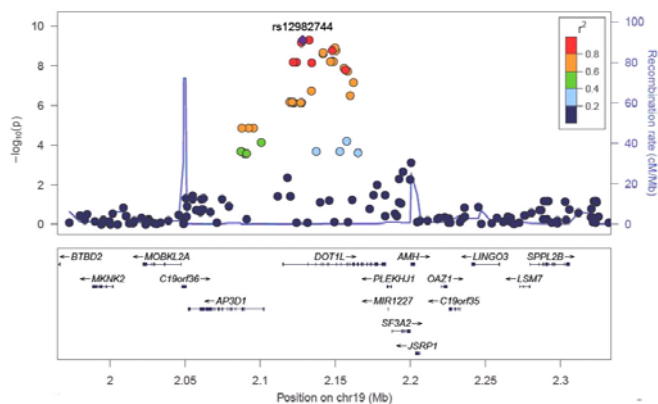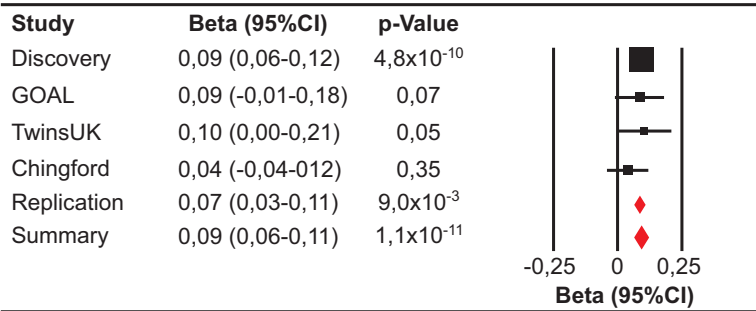

Figure 1b Regional association plot for the novel locus of joint space width (19 p31.3). SNPs are plotted by position in a 400kb window against association with mJSW (–log10 p). The purple diamond highlights the most significant SNP in discovery analysis. Blue peaks indicate recombination rates. The SNPs surrounding the most significant SNP are color coded to reflect their LD with this SNP (from pairwise r2 values from the HapMap CEU). Genes, exons and the direction of transcription from the UCSC genome browser are depicted underneath the plot.

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 = $9{\times}10^{-3}$, Figure 2).

Results from the Rotterdam Studies and the replication cohorts were combined in a joined meta-analysis. The combined analysis including discovery and replication studies showed strong evidence for association of the *DOT1L* locus with mJSW in the general population
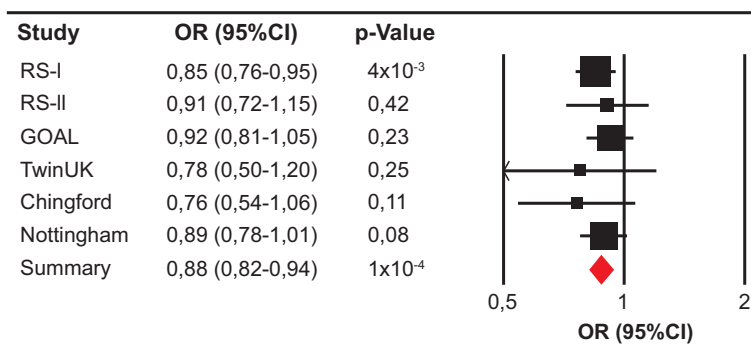
(beta: 0.09 mm/allele; P =1.1×10^{-11}, I^2=0%). These associations were corrected by age and gender. The minor G-allele of rs12982744 (MAF=0.39) is associated with an increased joint space width of 0.09 mm per copy of the G allele. This implicates that homozygote carriers of the rs12982744 G-allele 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 (Table S1). Risk for hip OA was calculated using logistic regression analysis and was adjusted for age and gender. As shown in Fig. 3, the minor allele of rs12982744 was significantly associated with a 12% reduced risk for hip OA (O.R: 0.88, CI: 0.82-0.94; P = 1.5×10^{-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 (O.R: 0.88, CI: 0.82-0.94; P = 1.1×10^{-4}). We observed that also in people without radiographic hip OA, the association with mJSW was present (Table S4, beta: 0.06 mm, SE: 0.011; P =7.3×10^{-9}). This 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.

Study	Beta (95%CI)	p-Value
Discovery	0,09 (0,06-0,12)	4,8x10^{-10}
GOAL	0,09 (-0,01-0,18)	0,07
TwinsUK	0,10 (0,00-0,21)	0,05
Chingford	0,04 (-0,04-012)	0,35
Replication	0,07 (0,03-0,11)	9,0x10^{-3}
Summary	0,09 (0,06-0,11)	1,1x10^{-11}

Figure 2 Forest Plots for rs12982744. Black squares represent effect estimate and 95%CI for each study, and the red diamond is a summary effect estimates. Minimal joint space width measurements units are in millimeters.

The G allele of the identified SNP (rs12982744) was previously found to be associated with increased height (11). 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.

| Study	OR (95%CI)	p-Value
RS-I	0,85 (0,76-0,95)	4×10^{-3}
RS-II	0,91 (0,72-1,15)	0,42
GOAL	0,92 (0,81-1,05)	0,23
TwinUK	0,78 (0,50-1,20)	0,25
Chingford	0,76 (0,54-1,06)	0,11
Nottingham	0,89 (0,78-1,01)	0,08
Summary	0,88 (0,82-0,94)	1×10^{-4}

OR (95%CI)

Figure 3 Risk for Hip OA. Values represent odds ratios (OR) and 95% confidence intervals (95%CI).

The associated polymorphisms are annotated in the *DOT1L* gene (Fig. 1b). DOT1L is an evolutionarily conserved histone methyltransferase, 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 (12-13). 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. 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 (14-15). As depicted in Fig. 4a and Fig. S2, 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 (Fig. 4b). Interestingly, type I collagen levels were 1.7-fold higher in *Dot1l-* cells at D21. Moreover, evaluation of matrix metalloproteinases (MMPs) mRNA level allowed to show a different pattern in *Dot1l-* cells compared to controls, as seen in Fig. S3. Indeed, Mmp9 expression was dramatically increased in *Dot1l-* cells (35-fold increase at D21), while Mmp13 was decreased by 1.7-fold at the same time. Mmp2 expression did not differ between *Dot1l-* and controls.

Figure 4 Functional analysis of Dot1l during chondrogenesis. Stable ATDC5 clones were established using either the control noninterfering pGIPZ or the pGIPZ-shmiRNA directed against mouse Dot1l. Three different antibiotic-resistant clones were selected. Knockdown efficiency was assessed by quantitative RT-PCR. Stably-transfected ATDC5 clones were cultured as micromasses as described previously (14, 15). Each condition was performed in triplicate. Total RNA from was isolated after 1, 7, 14, or 21 d. 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.
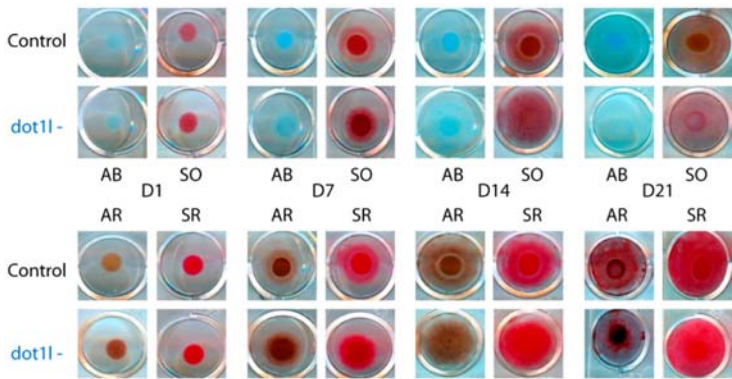

Figure 4a *Dot1l* knock-down reduces proteoglycan and collagen content, and mineralization during chondrogenesis. Stainings were performed on ATDC5 micromass cultures stably transfected with either control or Dot1l shmiRNA producing vector, over 21 days (D). (AB = Alcian blue, SO = safranin O, AR = alizarin red, SR = sirius red).
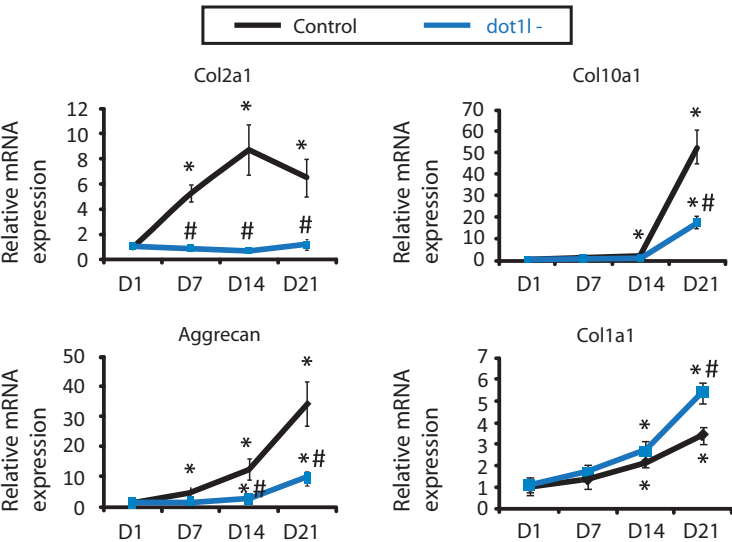

Figure 4b *Dot1l* knock-down reduces mRNA expression of markers of chondrogenesis. mRNA levels were normalized to S29 (reference gene) (n=3). Quantitative Real-Time PCR conditions and primers are available upon request.
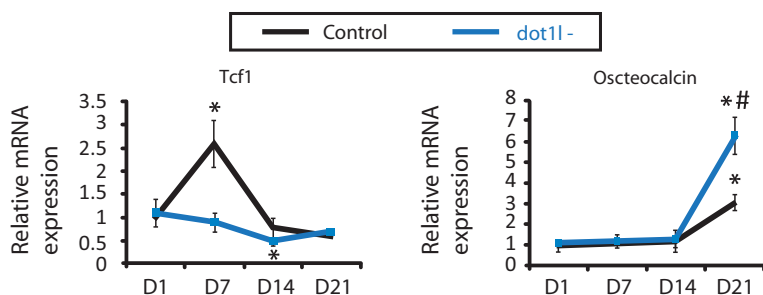
Figure 4c *Dot1l* knock-down affects Wnt signaling during chondrogenesis. mRNA levels of Wnt target genes Tcf1 and osteocalcin were normalized to S29 (reference gene) (n=3).
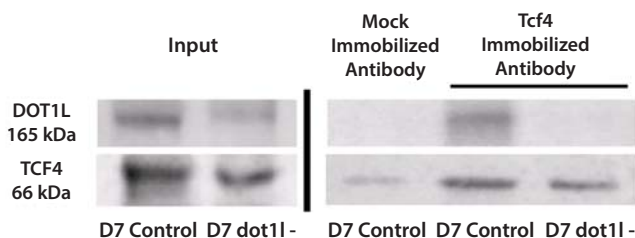
Figure 4d DOT1L interacts with the Wnt signaling pathway transcription factor TCF4. Coimmunoprecipitation of DOT1L and TCF4 using 100 µg of total proteins (input) from micromass cultures (at day 7 – D7) of either control or Dot1l knocked-down cells. Proteins were isolated from ATDC5 micromasses. CoIPs 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.
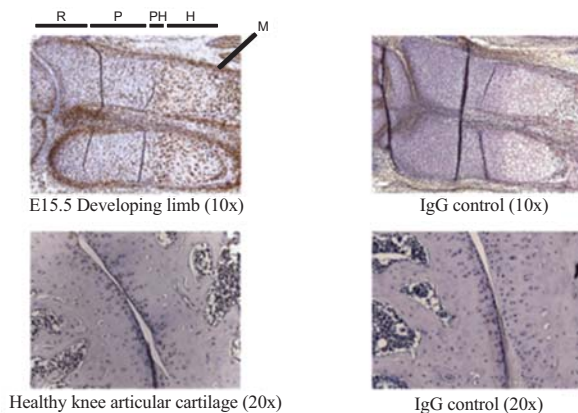
Figure 4e *DOT1L* is expressed during joint development and in mature articular cartilage of mice. Immunohistochemistry on paraffin embedded EDTA decalcified adult knee sections and non-decalcified embryonal sections, was performed with rabbit anti-Dot1L antibody (5µg/ml). After overnight incubation of the sections at 4°C, 1:100 peroxidase goat anti-rabbit IgG was applied and peroxidase activity was determined using DAB. In the developing limb (E15.5), expression was detected in resting (R), proliferating (P), prehypertrophic (PH) and hypertrophic (H) chondrocytes, as well as in the mesenchyme surrounding the bones (M). Immunohistochemistry also detected expression in articular cartilage chondrocytes in healthy mice knee (age 9 weeks). IgG as a negative control is also shown.

Because DOT1l was previously linked to beta-catenin signaling, we investigated whether mRNA expression of Wnt target genes was affected in *Dot1l-* cells. As seen in Fig. 4c, Tcf1 levels (positively regulated by Wnt/β-catenin signaling) were increased in control ATDC5s at D7 (2.5-fold), but no induction was detected in Dot1l- cells. Other Wnt target genes *Axin2* and *c-Myc* followed the same pattern (Fig. S4). Moreover, Osteocalcin level (negatively regulated by Wnt/β-catenin signaling) was increased by 2.8-fold at D21 in control cells, but the up-regulation was of 6.2-fold in *Dot1l-* ATDC5s (2.2-fold more than in control cells). Taken together, 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 Transcription Factor 4 (TCF4), a transcription factor interacting with β-catenin (Fig. 4d). These functional analyses seemed relevant in vivo, because DOT1L is very strongly present during chondrogenesis in mouse developing limbs and still found in articular cartilage as seen in Fig. 4e. Interestingly, *DOT1L* mRNA was clearly detected in adult human chondrocytes freshly extracted (without any passage) from articular cartilage obtained from patients with osteoarthritis (Fig. S5).

DISCUSSION

This study identified a genetic variant in the *DOT1L* gene robustly associated with joint space width and hip OA. 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.

DOT1 is an evolutionarily conserved histone methyltransferase, which was initially identified as a disruptor of telomeric silencing in Saccharomyces cerevisiae (16). The mammalian homolog, DOT1L, has been shown to be required for embryogenesis, hematopoiesis, and cardiac function (17-20). 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 (12-13). We are unique providing evidence that demonstrate 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. The differential effect of *Dot1l* silencing on different MMPs further highlights its complex role in cartilage biology.

Wnt signaling is critical in the formation of cartilage and bone and in the development of the synovial joint (21) Mutants in the Wnt have been shown to cause developmental abnormalities early in life (see for example WNT3 (22)). Variants with a less dramatic effect on function, such as the one discovered in this study, result in a mild phenotype with late onset. The same has been observed in the BMP-signaling pathway (another key developmental pathway). Mutations in the *GDF5* gene result in severe chondrodysplasia and skeletal malformations (23), while a milder variant that influences *GDF5* expression levels, results in a slightly elevated risk for knee OA later in life (2, 24).

The exact same variant that we found associated with cartilage thickness has previously been found associated with height both in young and old individuals (11, 25), which suggests a role in skeletal formation. Although the specific differentiation process in the growth plate and articular cartilage are different, common signaling pathways such as the Wnt cascades are involved (26). 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 in skeletal development.

OA 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) 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 OA affecting the knee and less significantly for hip OA (6). 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 HOA, where degeneration of articular cartilage is the most important feature, the approach to identify genetic 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, intrarater and interrater reliability has been significantly higher for joint space measurement than for KL (10, 27) and the findings that decline of JSW in OA proceeds in a linear manner (28) and that JSW is predictive of long- term progression of joint space narrowing (29) make measurement of JSW suitable for clinical trials and prioritize the identification of genes responsible for cartilage formation and homeostasis.

The effect we report of the *DOT1L* variant on cartilage thickness is modest, similar in magnitude to most of the identified variants involved in risk of complex diseases. Consequently, one might prematurely anticipate that the clinical relevance for this variant is by default small. However, the effect size of an identified variant does not necessarily reflect importance of the gene for a disease. Variants that strongly disrupt pivotal genes are unlikely to result in a late onset disease that affects 40% of the population over the age of 70. As DOT1L function is linked to Wnt signaling, genetic variation may not only contribute to cartilage thickness with reduced cartilage volume, a likely risk factor for OA development, but also to the deleterious processes that are activated when osteoarthritis is progressing. Accordingly, DOT1L might be a target for the design of new anti-osteoarthritis drugs that could be used in the prevention and treatment of OA. Additionally, carriers of *DOT1L* variant might have a different response to possible treatments targeting cartilage repair.

Another potential application of OA genetics is improved measurement of the disease process in combination with other variants of modest but consistent effect forming a "genetic risk score". A previous study has suggested that when several genetic markers are added up, the aggregated genetic risk is substantial and similar in magnitude to classical risk markers such as obesity or knee injury (30) and which may help to identify individuals at risk of OA years before disease onset.

Our results are not directly generalizable to other ethnicities, such as the Asian population. Asian populations have a high prevalence of large joint OA despite a much lower prevalence of obesity, suggesting etiological differences with regards to European-descent patients. Specifically, strong evidence of heterogeneity in the genetic contribution to OA between Asian and European populations has been widely reported (3, 31-34). In particular, no loci influencing hip OA have been consistently reported in both ethnic groups. Although the lack of inclusion of Asian patients may reduce the generalizability of our results, it has the advantage, (by concentrating on a clearly defined phenotype in

homogenous Caucasian samples of Dutch and UK origin) of reducing heterogeneity and thus achieving sufficient statistical power.

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 OA. 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 (35). This might represent an exciting opportunity for the development of disease modifying drugs for OA.

MATERIALS AND METHODS

GWAS Meta-analysis. Genotyping of the samples in the discovery cohorts (RS-I and RS-II) was carried out with the Illumina HumanHap 550v3 Genotyping BeadChip. The Beadstudio GenCall algorithm was used for genotype calling and quality control procedures, as described previously (36). The following quality control inclusion filters were applied: call rate >=97.5%, MAF >=1%, P for Hardy-Weinberg equilibrium <1×10^{-6} (See Table S2 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 (37), 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. 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 I2 metrics used to quantify between-study heterogeneity (www.sph.umich.edu/csg/abecasis/metal). The medical ethics committee of Erasmus University Medical School approved the study and written informed consent was obtained from each participant.

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 $<1\times10^{-6}$ (Table S2). 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) (38). 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. Results from the Rotterdam Studies 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 $=5\times10^{-8}$ after adjusting for all common variant tests in the human genome.

The replication studies were approved by the respective institutional review board and informed consent was obtained from all participants involved.

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 transferring (Sigma) and 3×10^{-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 2×10^{-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 3×10^{-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 colourless.

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 (MicrosonTM, 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 2 h 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 2 h 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, Mmp2, Mmp9 and Mmp13 (primers available upon request) in the ATDC5 micromasses. To assess Dot1l knock-down efficiency, primers were: forward 5'-CGAGGAAATCCCAGATCTCA-3', reverse 5'-ATGGCCCGGTTGTATTTGT-3'. The following PCR conditions were used: incubation for 10 min at 95°C followed by 40 amplification cycles of 15 sec 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 reaction. Results are expressed using the comparative threshold method (39) and were normalized to housekeeping gene S29 mRNA level (forward 5'-CCAGCAGCTCTACT-GGAGTCA-3', reverse 5'-GCCTATGTCCTTCGCGTACT-3'). Expression of DOT1L was also analyzed in articular chondrocytes (freshly isolated) from osteoarthritis patients undergoing knee prosthesis surgery.

Statistical analysis and 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.

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Chapter 2.1

SUPPORTING INFORMATION

Table S1. Characteristics of the study populations (N=13,085).

	Discovery Studies			Replication Studies	
	RS1	RS2	TwinsUK	GOAL	Chingford
Number	4773	1750	613	3084	745
Female (%)	56.6	54.4	93.2	48.5	100
Age (years)	67.7 (7.8)	64.8 (7.9)	53.4 (7.7)	66.6 (7.9)	62.0 (5.9)
Height (cm)	167.6 (9.3)	168.6 (9.2)	163.0 (6.8)	166.5 (9.1)	161.6 (5.9)
Weight (kg)	73.8 (11.7)	77.6 (13.3)	66.6 (12.1)	81.1 (15.9)	66.4 (11.2)
BMI (kg/m2)	26.3 (3.6)	27.2 (3.9)	25.1 (4.0)	29.3 (5.3)	25.4 (4.0)
mJSW (mm)	3.80 (0.84)	4.38 (0.90)	3.21 (0.74)	3.29 (1.13)	3.62 (0.69)
Definition of HOA	K/L score 2 of one or both joints or a TJR*		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
NrHOA cases/controls	771/443 6	166/179 5	48/565	1263/1821	88/657
Study design	Population-based		Twins pairs	Case-control	Population-based
Rationale, study design and methods references (SR)	(1-3)		(4-5)	(6)	(7)

Full names of studies: Rotterdam study I (RS1), Rotterdam Study II (RS2), TwinsUK, Genetics of Osteoarthritis and Lifestyle (GOAL) study, The Chingford Study, The Nottingham case-control study. Means are given with standard deviations between brackets. BMI: body mass index, mJSW: minimum hip joint space width, Nr HOA: number of hip osteoarthritis cases and controls NA: not available. *Subjects with a TJR due to fracture were excluded from all analyses. ** Exclusion criteria were: other major arthropathy (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. SR: Supplemental References are given at the end of the document.

Table S2. Quality control procedures and exclusion criteria for individuals of the GWA and replication studies.

Study	Genotyping Platform(s) / Chip(s)	Quality control inclusion filters/ Description			Sample QC / Other exclusions
		Call rate	MAF	P HWE	
RS-I	Illumina HumanHap 550K V.3 DUO	≥97.5%	≥1%	$<1 \times 10^{-6}$	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.
RS-II	Illumina / HumanHap 550 V.3 DUO	≥97.5%	≥1%	$<1 \times 10^{-6}$	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
TwinsUK	Illumina HumanHap 300 & 550 Illumina HumanCNV370 Duo	≥98%	≥1%	$<1 \times 10^{-6}$	1. autosomal heterozygosity <0.33 or >0.37 2. ethnic outliers (using STRUCTURE) 3. missing JSW or height measurements
Chingford	SNP genotyping system using fluorescence resonance energy transfer (FRET) quencher cassette oligos. KASPAR chemistry Competitive allele-specific (PCR).	N.A.	N.A.	N.A.	missing JSW or height measurements
GOAL					missing JSW or height measurements
Nottingham					missing hip OA status

Table S3. Markers with suggestive evidence for association from the GWAS on minimal joint space width mJSW (p: $10^{-5} > p > 5 \times 10^{-8}$).

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	4.30×10^{-7}
6p21.1	rs10948155	T/C	0.65	SUPT3H	-0.079	0.016	7.70×10^{-7}
19p13.1	rs11665774	A/G	0.49	SLC27A1	0.07	0.015	4.00×10^{-6}
5q13	rs11738020	T/C	0.43	PIK3R1	-0.068	0.015	5.50×10^{-6}
15q26	rs2380165	A/G	0.68	BLM	-0.076	0.017	5.60×10^{-6}
1p34.3	rs11206937	A/G	0.23	TRIT1/BMP8B	0.079	0.018	7.30×10^{-6}
15q23	rs12907468	A/T	0.36	TLE3	-0.075	0.017	7.80×10^{-6}
8q21.3	rs12544183	T/G	0.1	RUNX1T1	-0.126	0.028	8.50×10^{-6}

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

Table S4. Association results of rs12982744.

Type of Analysis		Discovery studies		Replication studies		Combined analysis	
mJSW							
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)	$4,5 \times 10^{-10}$	0,07 (0,02-0,12)	$9,0 \times 10^{-3}$	0,09 (0,06-0,11)	$1,1 \times 10^{-11}$
All	Age/gender/height	0,09 (0,06-0,12)	$4,5 \times 10^{-9}$	0,07 (0,02-0,12)	$8,0 \times 10^{-3}$	0,08 (0,06-0,11)	$1,6 \times 10^{-10}$
controls	Age/gender	0,07 (0,04-0,09)	$8,6 \times 10^{-7}$	0,06 (0,02-0,09)	$2,0 \times 10^{-3}$	0,06 (0,04-0,08)	$7,3 \times 10^{-9}$
controls	Age/gender/height	0,06 (0,04-0,09)	$3,1 \times 10^{-6}$	0,05 (0,02-0,09)	$3,0 \times 10^{-3}$	0,06 (0,04-0,08)	$4,2 \times 10^{-8}$
hipOA							
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)	$6,0 \times 10^{-3}$	0,89 (0,81-0,97)	$8,0 \times 10^{-3}$	0,88 (0,82-0,94)	$1,5 \times 10^{-4}$
All	Age/gender/height	0,86 (0,77-0,96)	$5,1 \times 10^{-3}$	0,89 (0,81-0,97)	$7,0 \times 10^{-3}$	0,88 (0,82-0,94)	$1,1 \times 10^{-4}$

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.
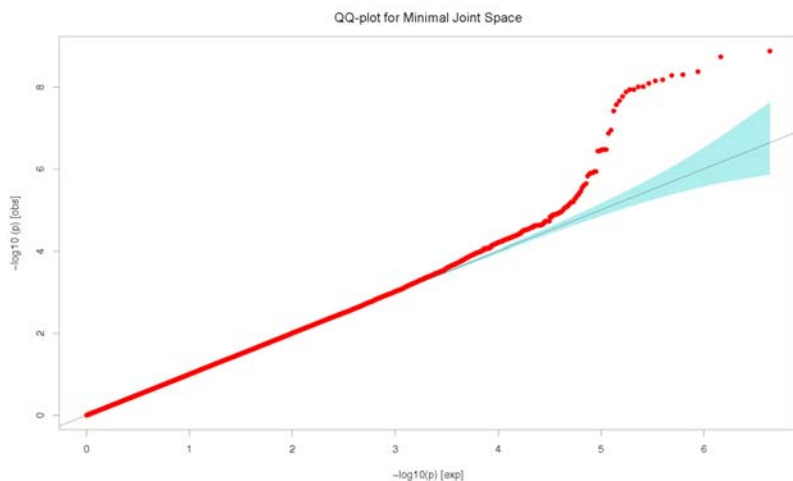
Figure S1 Quantile-Quantile plot (Q_Q plot) for minimal joint space width. The plot compares additive model statistics to those expected under the null distribution using fixed-effects for all analyzed HapMAP CEU imputed SNPs passing quality control criteria.
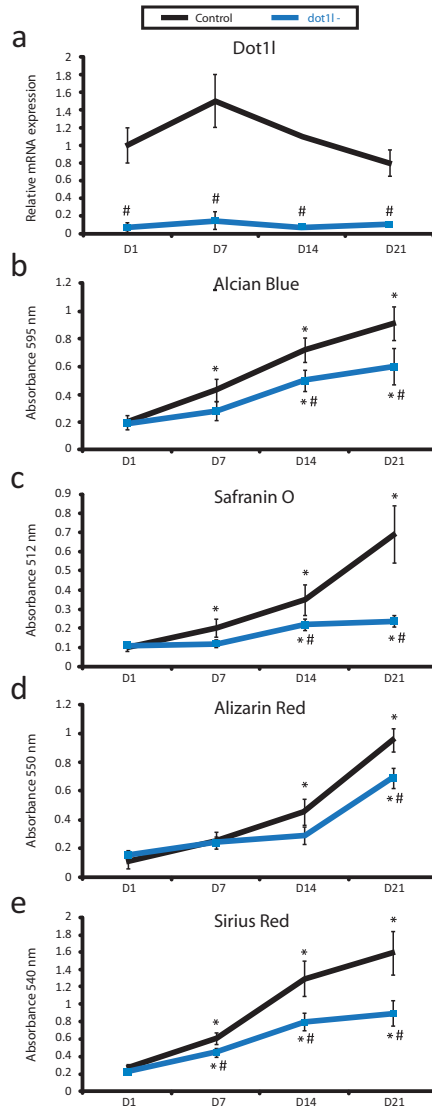
Figure S2 *Dot1l* knock-down efficiency during chondrogenesis and staining quantification. **A**, *Dot1l* knock-down is effective during chondrogenesis. mRNA levels were normalized to S29 (reference gene) (*n*=3). **B**, Proteoglycan content (Alcian blue staining) is decreased in *Dot1l*- cells. Stainings 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 #: $p<0.05$.

53
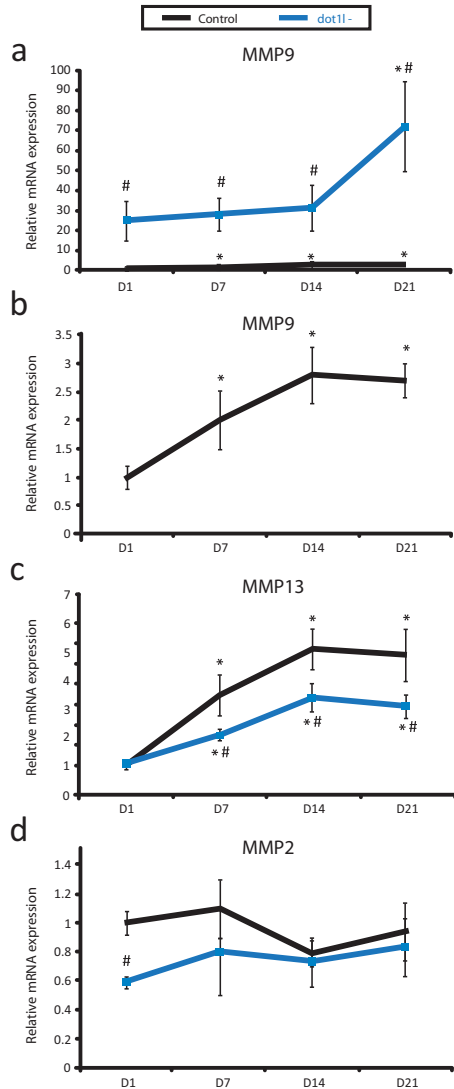
Figure S3 *Dot1l* knock-down modifies matrix metalloproteinases (MMPs) expression pattern during chondrogenesis. mRNA levels of *Mmp9* (panel A, B for control alone), *Mmp13* (panel C) and *Mmp2* (panel D) were normalized to *S29* (reference gene) (n=3). Statistically significant differences vs. day 1 (D1) (internal control condition) are indicated as *: $P<0.05$, and #$P<0.05$ for comparison to the GIPZ (control vector)-transfected cells.
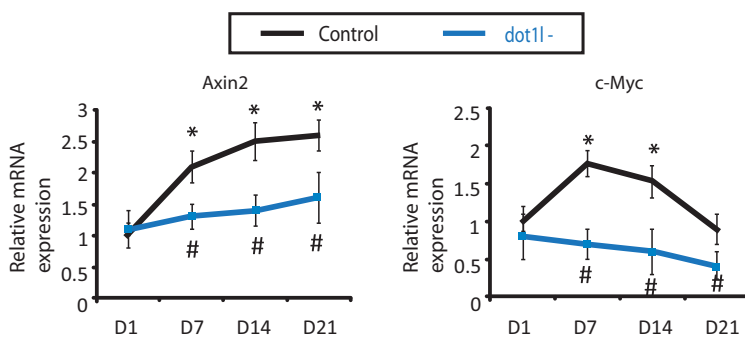
Figure S4 *Dot1l* knock-down affects Wnt signaling during chondrogenesis. mRNA levels of Wnt target genes c-Myc and Axin2 were normalized to S29 (reference gene) (*n*=3). Statistically significant differences vs. day 1 (D1) (internal control condition) are indicated as *P<0.05, and #P<0.05 for comparison to the GIPZ (control vector)-transfected cells.
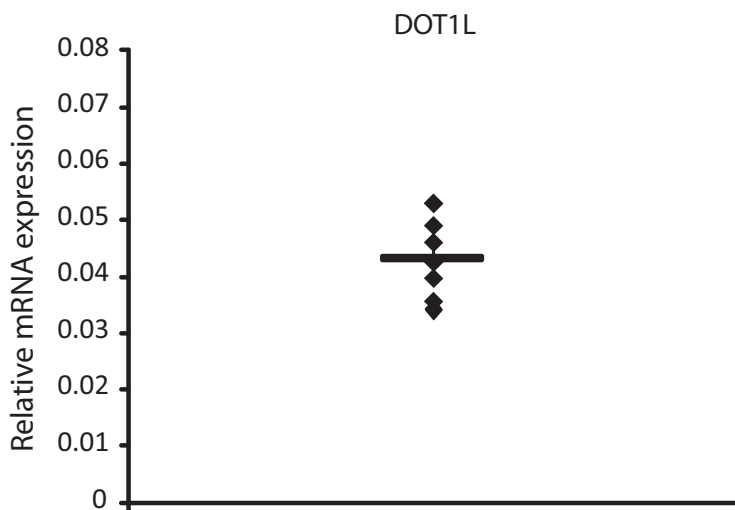
Figure S5 *DOT1L* is expressed in adult human chondrocytes freshly isolated from osteoarthritic articular cartilage. mRNA levels of *DOT1L* was normalized to S29 (reference gene) (n=7 different patients). Horizontal bar indicates mean relative expression.

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Chapter 2.2

Identification by GWAS meta-analysis of new signals and pathways that confer risk for hip osteoarthritis

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Chapter 2.2

ABSTRACT

Background: Hip Osteoarthritis (OA) is the one of the most disabling diseases with a prevalence of around 7% in elderly. Hip OA is principally characterized by a reduction in cartilage thickness which is represented by narrowing of the joint space width on a radiograph. Minimal Joint Space Width (mJSW) has demonstrated to be a successful proxy for measuring cartilage thickness and to identify genetic variants related with hip OA. Objective: We aim to identify genes involved in cartilage thickness and hip OA through the use of mJSW as endophenotype of interest. We conducted a genome-wide association study of mJSW in a discovery set of 13,013 participants from five different cohorts (Discovery: Rotterdam Study I and II, TwinsUK, SOF and MrOS) using standardized age-, gender and population stratification-adjusted residuals from linear regression. We replicated signals with a $P<1x10^{-5}$ in 6,168 individuals from 6 independent cohorts: GARP, JoCo, Chingford, GOAL, GOGO and CHECK. Results were combined in a joined meta-analysis using inverse variance weighting (METAL). Association with hip OA was tested using logistic regression in 36,341 participants in total of 11 cohorts. We identified 5 genome-wide significant (GWS) loci ($P<5\cdot0\times10^{-8}$) on chromosomes 2, 5, 6 and 19. Top SNPs were localized on the intronic region of *TGFA:* rs2862851, 200 Kb forward strand from *SUPT3H* (rs10948155), in the intronic region a transcript variant (protein coding) of *RUNX2* (rs12206662), an upstream gene variant (rs10471753) which closest genes is *PIK3R1* and finally a intronic variant in *DOT1L*. The identified GWS variants (excepting rs10471753 and rs10948155) showed association with hip OA at *P* value <=0.003.

Our findings provide insight into the genetics of cartilage development and hip OA that might be used as future therapeutic targets. We identified signals in genes with important roles in endochondral differentiation process, growth and development.

INTRODUCTION

In spite of the advances to understand osteoarthritis (OA), the absence of effective therapeutic targets demonstrates that a better comprehension of its causes and pathophysiological mechanisms are needed. Genetics studies have the possibility of revealing associated genes and pathways involved in the disease, increasing understanding of OA and offering future possibilities for treatment. The genetic component of hip OA have been estimated to be around 60%. However, only few genetic variants have been successfully identified [1, 2].

Recent findings in the search for genetic variants did not explain the high genetic component of OA. However, it has revealed that some of the genes associated with OA tend to be related to processes of synovial joint development and bone formation, including differentiation of cartilage and bone tissue (endochondral ossification) between others [3]. Some of the signals that have been associated with OA, have been previously associated with skeletal traits as height and cartilage thickness [1, 2]. Genes involved in joint formation and differentiation processes might have an important role in OA and might determine the future risk for joint disease.

Minimal Joint Space Width (mJSW) is a proxy for cartilage thickness measured on hip radiographs which has previously demonstrated to be a successful endophenotype to identify genes with a role in hip OA [4]. Therefore, we aim to identify genes implicated in cartilage thickness using minimal Joint Space Width (mJSW) as a proxy and to determine whether those genes have a role in hip OA.

MATERIALS AND METHODS

Statistical Analyses

Discovery GWAS Meta-analysis. We conducted genome-wide association studies of mJSW for each cohort of the discovery stage: Rotterdam Study I (RS-I), Rotterdam Study II (RS-II), TwinsUK, SOF and MrOS using standardized age-, gender and population stratification (four principal components) adjusted residuals from linear regression. The R version 2.10.1 (The R Foundation for Statistical Computing http://www.r-project.org/) and GRIMP were used for analyses [5]. Results from the Rotterdam Study and the other cohorts used in the discovery stage were combined in a joined meta-analysis using inverse variance weighting with METAL [6]. Genomic control correction

was applied to the standard errors and *P*-values before meta-analysis. SNPs with a *P* value $\leq 5 \times 10^{-6}$ were selected for replication.

Replication. Selected signals from the discovery stage were taken for replication in seven studies: the Genetics of Osteoarthritis and Lifestyle (GOAL) study, the Chingford study, CHECK (Cohort Hip & Cohort Knee), the Genetics of Generalized Osteoarthritis (GOGO) the Johnston County Osteoarthritis Project (JoCo) and additionally the Nottingham OA case-control study for association with Hip OA. Association of the SNPs with mJSW was additionally adjusted for height to test its independence. Secondary analyses included: association of the top SNPs with hip OA using logistic regression analysis (age and gender adjusted and by study centres when it was pertinent).

Discovery Cohorts: description, genotyping and QC

Rotterdam Study. The Rotterdam study is a population-based prospective cohort study ongoing since 1990 to study determinants of chronic disabling disease [10]. The Rotterdam Study I (RS-I) is the first cohort of 7,983 persons living in the Ommoord district of Rotterdam in the Netherlands. All subjects were aged 55 years and older and recruitment started in 1990. The Rotterdam Study II (RS-II) started in 1999 when 3,011 participants moved into the study since they became 55 years of age or moved into the study district. Genotyping of the samples in the discovery cohorts (RS-I and RS-II) was carried out with the Illumina HumanHap 550v3 Genotyping BeadChip. The Beadstudio GenCall algorithm was used for genotype calling and quality control procedures, as described previously [11]. The following quality control inclusion filters were applied: call rate $\geq$=97.5%, MAF>= 1%, *P* for Hardy-Weinberg equilibrium $<1 \times 10^{-6}$ (Table S1 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 [5], which uses genotype dosage value (0–2, continuous) as a predictor in a linear regression framework. We included only imputed SNPs that had a good imputation quality leaving a total of 2,451,799. 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 I2 metrics used to quantify between-study heterogeneity (www.sph.umich.edu/csg/abecasis/metal).

The medical ethics committee of Erasmus University Medical School approved the study and written informed consent was obtained from each participant.

TwinsUK. The TwinsUK 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 [9]. These unselected twins were recruited from the general population through national media campaigns in the United Kingdom. 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 >=1×10^{-6} (Table S1). 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) [10].

SOF. The Study of Osteoporotic Fractures (SOF) is a prospective multicenter study of risk factors for vertebral and non-vertebral fractures[11]. The cohort at the baseline visit is comprised of 9,704 community dwelling women 65 years old or older recruited from populations-based listings in four U.S. areas: Baltimore, Maryland; Minneapolis, Minnesota; Portland, Oregon; and the Monongahela Valley, Pennsylvania. Women enrolled in the study were 99% Caucasian with African American women initially excluded from the study due to their low incidence of hip fractures. The SOF study recruited only women. Among the 9,704 SOF participants enrolled at the baseline visit, 6795 participants provided blood samples and consented to genetic testing. Among these 6795 SOF participants, DNA samples from 4,117 participants had sufficient DNA quantity and were submitted to the Broad Institute for whole-genome genotyping.

All DNA samples eligible for whole-genome genotyping were genotyped using Sequenom iPLEX technology for a 24-SNP "fingerprint" panel. The Illumina HumanOmni1 Quad genotyping array, containing 1,016,423 probes, was used for whole-genome genotyping in MrOS and SOF samples. Quality control procedures for SNPs and samples are described in Table S2. In addition to the SNP filters described in Table S2, SNPs with GenTrain scores <0.6 or cluster separation scores <0.4 were excluded. Additional samples were excluded based on: (1) genotypic sex mismatch using X and Y chromosome probe intensities, (2) relatedness among genotyped samples using the kinship coefficient that estimates probability that alleles are identical-by-descent, and (3) gross chromosomal abnormalities detected using the LogR Ratio and B allele frequency. Among the 3,924 SOF samples that underwent whole-genome genotyping, 3,682

samples had acceptable call rates. Among these 3,682 SOF samples, 4 were removed due to relatedness and 53 were removed due to gross chromosomal abnormalities, leaving 3,625 SOF samples with whole genome genotyping data that passed QC.

MrOS. The Osteoporotic Fractures in Men Study (MrOS) is a multi-center prospective, longitudinal, observational study of risk factors for vertebral and all non-vertebral fractures in older men, and of the sequelae of fractures in men [12, 13]. The MrOS study population at the baseline visit consists of 5,994 community dwelling, ambulatory men aged 65 years or older from six communities in the United States (Birmingham, AL; Minneapolis, MN; Palo Alto, CA; Monongahela Valley near 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 MrOS 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, (6) ability to understand and sign an informed consent, and (7) 65 years or older. To qualify as an enrolled, the participant had to provide written informed consent, complete the self-administered questionnaire (SAQ), attend the clinic visit, and complete at least the anthropometric, DXA, and vertebral X-ray procedures. The MrOS cohort recruited only men.

Genomic DNA from participants in the Osteoporotic Fractures in Men (MrOS) Study was extracted from whole blood samples collected at the baseline visit using the Flexigene protocol (Qiagen, Valencia, CA, USA) at the University of Pittsburgh. Among the 5,994 MrOS participants enrolled at the baseline visit, 5,551 participants provided blood samples and consented to genetic testing. DNA samples from these 5,551 participants were submitted to the Broad Institute for whole-genome genotyping. Among the 5,506 MrOS samples that underwent whole-genome genotyping, 5,189 samples had acceptable call rates. Among these 5,189 MrOS samples, 1 was removed due to relatedness and 37 were removed due to gross chromosomal abnormalities, leaving 5,151 MrOS samples with whole genome genotyping data that passed QC.

Replication Cohorts: description, genotyping and QC

GOAL. The Genetics of Osteoarthritis and Lifestyle (GOAL) study and the Nottingham OA case-control study have been previously described [14]. Hip OA cases were recruited from hospital orthopaedic surgery lists in Nottingham. Cases 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.

Chingford. The Chingford study is a prospective population-based longitudinal cohort, which includes women derived from the age/sex register of a large general practice in North London [15]. For these studies' 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.

GARP. Genetics ARthrosis and Progression study (GARP). The GARP study is aimed at identifying determinants of osteoarthritis susceptibility and progression. The study is based on sibships of white Dutch ancestry with predominantly symptomatic osteoarthritis at multiple sites. Patients (probands) aged between 40 to 70 years with symptomatic osteoarthritis in the hands, knees, or hips—diagnosed by rheumatologists, orthopaedic surgeons, and general practitioners in Leiden, The Hague, Delft, Haarlem, and Amsterdam—were informed of the ongoing study by mail. Interested probands were subsequently sent a mailed questionnaire about demographic data, medical history, symptoms and signs of osteoarthritis, and family history of osteoarthritis. Subsequently probands with osteoarthritis at multiple sites and with a positive family history were requested to introduce a sibling "with joint complaints," who was also sent a questionnaire. After obtaining informed consent, all sibships underwent a physical examination and were assessed by a single medical doctor (NR) at the outpatient clinic. Patients with secondary osteoarthritis and familial syndromes with a Mendelian inheritance pattern were excluded. Osteoarthritis developing under the following conditions was considered secondary: major congenital or developmental diseases and bone dysplasias; major local factors such as severe scoliosis and hypermobility; certain metabolic diseases associated with joint disease such as haemochromatosis and Wilson's disease; inflammatory joint diseases such as rheumatoid arthritis; other bone diseases such as Paget's disease and osteochondritis; intra-articular fracture. Genotypes of the GARP study and controls were performed by a fluorescent 5' exonuclease assay from a predesigned SNP TaqMan Genotyping Assay (Applied Biosystems, Foster City, CA).

Genotyping quality was manually checked. The accuracy was determined from the 8-10% duplicate samples and was100% and genotyping success rate was > 85%.

JoCo. The Johnston County Osteoarthritis Project (JoCo) is an ongoing population-based prospective cohort study of the occurrence of knee and hip OA in African Americans and Caucasians in Johnston County, North Carolina [16, 17]. This project was designed as a long-term study of ethnic differences in OA occurrence and progression. The samples were collected from six townships among the 17 townships in Johnston County because they contained the largest proportion of African American residents. The participants were initially recruited at the baseline between 1990 and 1997 and were followed up between 1999 and 2004. Additional new individuals were enrolled in 2003–2004 to enrich the sample for African Americans and younger individuals who were deliberately targeted for inclusion. A total of 2,583 participants from the Johnston County Cohort were selected from the total study population for genotyping. Participants selected for inclusion in the present association study were Caucasians of European ancestry (68% of genotyped cohort) of both sexes (35% men).

GOGO. The Genetics of Generalized Osteoarthritis (GOGO) Study is a collaborative study involving seven academic sites, five in the United States (US) and two in the UK. Recruitment began in all sites in 2000 and was completed in 2002. Participants were recruited inform Rheumatology clinics, hospital databases of OA patients, pre-existing OA cohort, ad from community, by advertisements. A qualifying family consisted of a least two siblings with self-reported Caucasian ethnicity who fulfilled clinical GOGO and OA criteria (bony enlargements of >=3 joints distributed bilaterally, including bone enlargement of at least one DIP joint, and no more than three swollen metacarpophalangeal joints). In a family the first individual that met clinical GOGO and OA criteria was designated the proband. A total of 1145 families were recruited [18]. DNA was extracted from whole blood that was collected on the day of participants assessment. All GOGO participants with longitudinal data were genotypes at the DAvid H. Murdock Research Institue (Kannapolis, NC), using the illumina BeadChip that consisted of 500 K HapMap SNPs and about 60,000 custom SNPs. The microarrays were philantropic gifts to Duke University, teh coordination center for the GOGO study, based on the stipulation that discovery results be published and not patented.

CHECK. It is a multi-centre cohort formed by the Dutch Arthritis Association (DAA) with over 1,000 participants with pain of hip and/or knee expected to develop knee and or hip OA: CHECK (Cohort Hip & Cohort Knee). The objective of CHECK is to study the course of complaints, the mechanisms that cause joint damage, and to identify

markers for diagnosis and prognosis, as well as to identify prognostic factors that predict and explain the course of OA. On entry, all participants had pain of knee or hip, were aged 45-65 years. They had not yet consulted their physician for these symptoms, or the first consultation was within 6 months before entry. Any other pathological condition that could explain the existing complaints was excluded(e.g. other rheumatic disease, previous hip or knee joint replacement, congenital dysplasia, osteochondritis dissecans, intra-articular fractures, septic arthritis, Perthes' Disease, ligament or meniscus damage, plica syndrome, Bakers cyste). The study was approved by the medical ethics committees of all participating centres, and all participants gave their written informed consent before entering the study. Blood samples were collected at baseline and de-novo genotyping of the selected SNPs for replication was carried out with Sequenom iPLEX and Taqman Allelic Discrimination genotyping Genomic DNA was extracted from samples of peripheral venous blood according to standard procedures. 4 ng genomic DNA was dispensed into 384-wells plates using a Caliper Sciclone ALH3000 pipetting robot (Caliper LS, Mountain View, CA, USA). Genotyping was done using Sequenom iPLEX genotyping and Taqman Allelic Discrimination. For this, sequences containing the SNP site and at least 100 bp of flanking sequence on either side of the SNP were used. Briefly, 2 ng genomic DNA was amplified in a 5 ul reaction containing $1 \times$ Taq PCR buffer (Sequenom), 2 mM MgCl2, 500 uM each dNTP, 100 nM each PCR primer, 0.5 U Taq (Sequenom). More detail have been previously described [19].

The replication studies were approved by the respective institutional review board and informed consent was obtained from all participants involved.

OA definition:

Radiographic hip OA was defined in the RS-I, RS-II, Twins-UK, Chingford, and JoCo studies using Kellgren and Lawrence (K/L) scores and JSN. HOA cases were defined as a K/L score >=2 and JSN >=2 on either side of the hip or THR due to OA. Hip OA controls were defined as no THR for OA and K/L score <=1 and JSN <=1. In GOGO, hip OA was defined as KL grade >=2 and excluded individuals with joint replacement.

In MrOS and SOF cohorts, Radiographic hip OA case-control was defined by a modified Croft grade [20]. Radiographic hip OA cases in the GOAL and Nottingham OA studies were defined by having THR, and controls were radiographically free of hip OA, as previously described [14]. In GARP, hip OA was defined as pain or stiffness in the groin and hip region on most days of the preceding month in addition to femoral or acetabular osteophytes or axial joint space narrowing on radiographs or prosthesis due to OA. In GOGO, hip OA was defined as KL grade >=2, or minimal joint space width <=2.5 mm,

or the combination of joint space narrowing grade >=2 and any osteophyte of grade >=1, or history of joint replacement for OA.

Biological relevance and Pathways. We also investigated whether significant relevant connections existed between the associated loci, using two different computation approaches. Molecular function, pathways and biological processes were analysed using (Protein ANalysis THrough Evolutionary Relationships) PANTHER [21, 22]. These analyses included: the function of the protein by itself or with directly interacting proteins at a biochemical level, the function of the protein in the context of a larger network of proteins that interact to accomplish a process at the level of the cell or organism and a pathway that also explicitly specifies the relationships between the interacting molecules. Additionally we used Gene Relationships Across Implicated Loci (GRAIL). GRAIL is a text-mining algorithm to search for connectivity between genes near the associated SNPs, based on existing literature on PubMed text data source (till August 2012) was run on the selected variants ($P<1.0\times10^{-5}$) lifted over the human genome hg18 coordinates with gene size correction turned on [23].

RESULTS

Association of minimal JSW (mJSW) of the hip and genetic variants was performed in a discovery set including 13,013 individuals (see Table S2 for cohort specifics). We applied extensive quality control measures (see Table S1 for details on quality control and exclusion criteria) leaving a total of 2,385,183 SNPs for association analysis. Genomic control inflation factors for the P values of the RS, TwinsUK, MrOS, SOF GWAS were low (lambda = 1.02, 1.01, 1.02 and 0.99 respectively), and the interquantile-quantile plot (Fig. S1) also indicated no substantial population stratification due to cryptic relatedness, population substructure or other biases. The estimated median lambda combining the discovery cohorts was 1.04. In the discovery stage, signals from five different loci achieved GWS threshold (Fig.1).

Top SNPs of these 5 regions and 13 top SNPs from loci with a *P* value $<1\times10^{-5}$ were selected for replication in another 6,168 individual from six different cohorts. Table 1 shows the association results of each SNP in the meta-analysis of mJSW. We declared results genome wide significant at $P<=5\times10^{-8}$ after adjusting for all variants tested. A total of 6 SNPs replicated at nominal significance in the correct direction, when discovery and replication were combined.
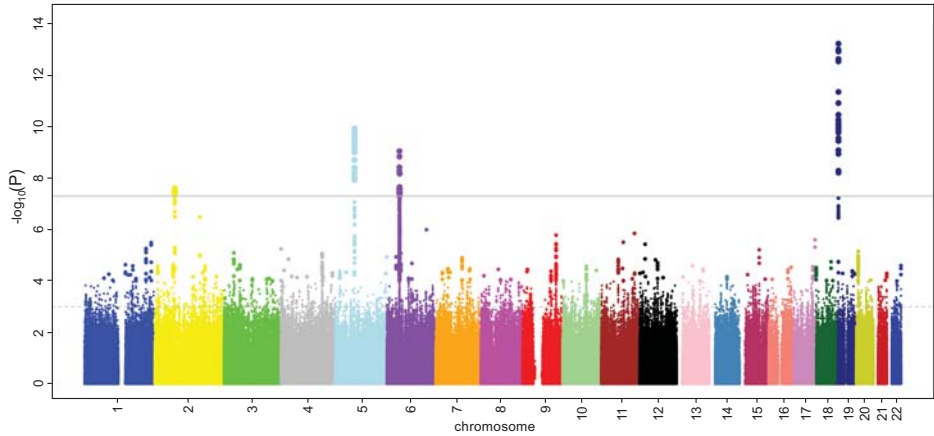
Figure 1 Manhattan Plot for Association of Cartilage Thickness in the Discovery page. Association results by chromosome. The log P values for each of the 2.5 million tests performed as part of the genome-wide association of minimal joint space (MJS) of the hip in the discovery stage. The gray solid horizontal line corresponds to P value threshold of $5×10^{-8}$ (GWS). Dotted gray line corresponds to a P value of $1×10^{-5.}$

After replication, five of 18 loci had genome-wide significance ($P<=5·0×10^{-8}$) and 4 were suggestive for association being close to this threshold (Table 1, SNPs with $P<=5×10^{-6}$).

Effect size estimates for all loci were lower in the replication than in the discovery analysis (Table 1). The most associated signal for mJSW was rs1180992 localized in the intronic region of *DOT1L*. This signals was also associated with hip OA in our meta-analysis with a *P* value of $9.7×10^{-5}$ (all-subjects). This variant is very close to rs12982744 (D=1, r^2=1), previously found associated with mJSW hip OA [1, 4]. The next signal was located on chromosome two, rs2862851 localized in the intronic region of *TGFA,* GWS for mJSW (P=$1.6×10^{-10}$) and with strong association with hip OA (OR=1.06, 95% confidence interval (CI)= 1.03-1.09, *P*= $6.9×10^{-5}$).

A variant at 554 Kb of *SUPT3H*, rs10948155, was associated to mJSW in the discovery and replication meta-analysis with a final *P* value of $4.1×10^{-10}$. This variant is in high LD with rs10948172 (D'=0.95 and r^2=0.90), previously associated with hip OA in males at borderline GWS level [2]. However, the variant in our study, rs10948155 (t), was only modestly associated with hip OA: (OR:0.96 (0.93-0.99), Table 2, *P*=$2.3×10^{-2}$).

Table 1. Results meta-analyses of minimal Joint Space Width (mJSW), discovery, replication and final results.

SNP	Chr	Position	A1	A2	Freq1	Discovery				Replication					Combined results				GENE
						Beta	SE	P	N.	Beta	SE	P	N.	I2	Beta	SE	P	N.	
rs10495106	1	217017809	t	c	0.937	0.119	0.026	6.16E-06	13013	0.055	0.037	1.3E-01	5349	51.4	0.097	0.021	1.2E-05	18362	TGFB2
rs6429001	1	235495143	a	g	0.832	0.078	0.017	3.14E-06	13013	-0.006	0.039	8.8E-01	2220	72.6	0.065	0.015	2.4E-05	15233	RIR2
rs6437120	2	158493396	t	c	0.503	-0.064	0.013	4.00E-07	13013	0.006	0.019	7.5E-01	5352	89.7	-0.043	0.011	5.3E-06	18365	ACVR1/UPP2
rs2862851	2	70566310	t	c	0.485	-0.070	0.013	2.86E-08	13013	-0.062	0.018	**4.8E-04**	5353	0	-0.068	0.010	**1.6E-10**	18366	TGFA
rs2061109	3	35380584	c	g	0.503	0.057	0.013	8.80E-06	13013	0.003	0.030	9.2E-01	2221	64.9	0.048	0.012	1.5E-04	15234	ARPP-21
rs2236995	4	1674340	t	g	0.487	0.057	0.013	6.43E-06	13013	0.038	0.018	**3.2E-02**	5353	51.2	0.047	0.010	**1.5E-07**	18366	SLBP
rs13148031	4	145679788	a	g	0.596	0.057	0.013	9.75E-06	13013	0.016	0.018	3.6E-01	5353	67.8	0.044	0.010	3.7E-05	18366	HHIP/GYPA
rs10471753	5	217017809	c	g	0.609	0.082	0.013	1.52E-10	13013	0.019	0.019	3.3E-01	4764	87.3	0.063	0.011	**4.9E-09**	17777	PIK3R1
rs7739938	6	139823110	a	g	0.026	-0.314	0.065	1.13E-06	12401	-0.030	0.026	2.5E-01	4763	93.7	-0.076	0.024	1.3E-04	17164	CITED2
rs10948155	6	44795935	t	c	0.667	0.081	0.013	1.22E-09	13013	0.045	0.019	**1.5E-02**	5353	66.9	0.068	0.011	**4.1E-10**	18366	SUPT3H
rs12206662	6	45484199	a	g	0.068	-0.187	0.032	7.93E-09	12401	-0.093	0.035	**7.3E-03**	4455	71.4	-0.146	0.024	**6.0E-10**	16856	RUNX2
rs4837613	9	118349346	c	g	0.487	-0.062	0.013	1.96E-06	12401	0.002	0.019	9.1E-01	4449	88.4	-0.041	0.011	3.69E-05	16850	ASTN2
rs6592847	11	78889654	a	g	0.281	0.070	0.015	3.65E-06	12401	-0.014	0.056	8.1E-01	1011	53.0	0.065	0.015	9.5E-06	13412	NARS2
rs496547	11	118081673	a	t	0.343	-0.064	0.013	1.54E-06	13013	-0.046	0.022	**3.8E-02**	3866	0	-0.059	0.011	**4.7E-07**	16879	TREH
rs11045356	12	20683368	a	g	0.168	0.078	0.017	4.43E-06	13013	-0.052	0.036	1.5E-01	2212	81.4	0.064	0.016	1.0E-04	15225	PPE3A
rs2703529	17	74709369	a	g	0.071	-0.114	0.024	2.88E-06	13013	0.006	0.034	8.6E-01	5353	84.0	-0.079	0.020	4.3E-05	18366	HRN3P3
rs1880992	19	2127403	a	g	0.398	0.096	0.013	9.12E-14	13013	0.091	0.029	**1.5E-03**	2221	0	0.095	0.012	**1.1E-14**	15234	DOT1L
rs717433	20	7786135	t	c	0.758	0.067	0.015	7.97E-06	13013	0.058	0.033	8.3E-02	2221	0	0.066	0.014	3.9E-06	15234	HAO1

Discovery cohorts: RS-I, RS-II, TwinsUK, SOF, MrOS.
Replication cohorts: CHECK, Chingford, GARP, GOAL, GoGo, JoCo.
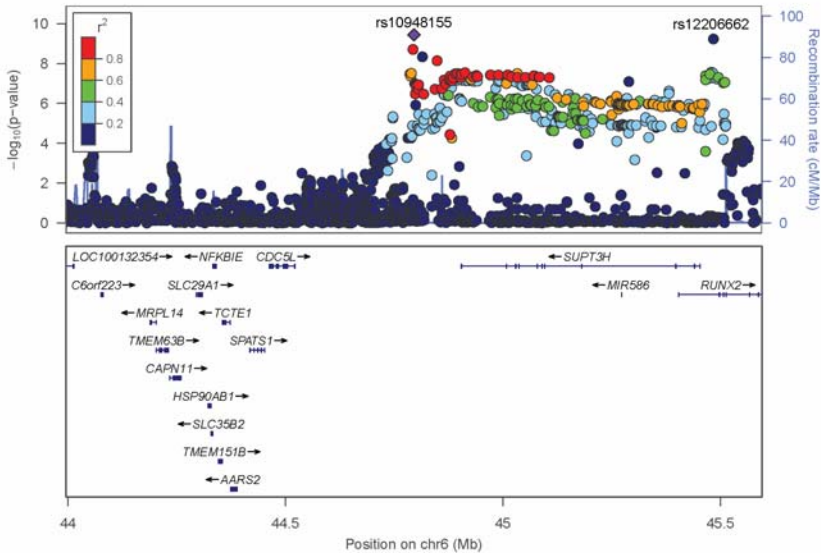
Figure 2 Regional association plot for the locus of JSW (6p 21). SNPs are plotted by position in a 800-kb window against association with mJSW (-log10 P). The purple diamond highlights the most significant SNP in discovery analysis close to *SUPT3H*. SNP rs12206662 on *RUNX2* gene showing low correlation with rs10948155. Blue peaks indicate recombination rates. The SNPs surrounding the most significant SNP are color coded to reflect their LD with this SNP (from pairwise r2 values from the HapMap CEU). Genes, exons and the direction of transcription from the University of California at Santa Cruz genome browser are depicted underneath the plot.

A variant in *RUNX2*: rs12206662, having a minor allele frequency of less than 10% (a-allele=9%) had the strongest effect on mJSW and hip OA (table 1, Beta=-0.146, P=6.0×10^{-10} for mJSW and table 2, OR=1.15 (1.07-1.24), P= 1.3×10^{-4} for association with hip OA. This SNP on *RUNX2* was independent of the SNP rs10948155 close to *SUPT3H*, being localized at ~700Kb and showing no correlation with the mentioned variant (Figure 2). Finally, between the GWS signals is rs10471753, a intergenic variant closer to *PIK3R1* (~450 Kb) than to *SLC30A5* (~750Kb). This variant was only associated with mJSW (Table 1, P=4.9×10^{-9}) and not with hip OA (Table 2, P=7.6×10^{-1}). Other suggestive signals for association with mJSW at a P<=5·0×10^{-6} that were replicated but did not achieve the GWS threshold were: rs717433 (approximately 60kbp from *HAO1*), rs496547 a downstream gene variant located 3' of *TREH* and correlated with rs494459 SNP previously associated with height at GWS level (r2=0.290, D'=1) and an intron variant on *SLBP* (rs2236995) in high LD (r^2=0.65, D'=1) with rs2247341 previously associated with height. Another gene close to this variant that might be a good candidate for its role in bone development and maintenance is *FGFR3*. From these suggestive signals, only the variant in *SLBP* showed significant association with hip OA (Table 2, OR=0.96 (0.93-0.98) P= 2.5×10^{-3}).

Table 2. Association of top hits for mJSW with hip osteoarthritis in meta-analysis of 10,065 cases and 26,276 controls.

SNP	Chr	position	A1	Freq1	Beta	SE	P	NEAREST GENE
rs10495106	1	217017809	t	0.935	-0.055	0.031	8.0E-02	TGFB2
rs6429001	1	235495143	a	0.828	-0.013	0.021	5.2E-01	RIR2
rs2862851	2	70566310	t	0.467	0.058	0.015	**6.9E-05**	TGFA
rs6437120	2	158493396	t	0.507	-0.005	0.015	7.2E-01	ACVR1/UPP2
rs2061109	3	35380584	c	0.513	-0.011	0.016	4.8E-01	ARPP-21
rs2236995	4	1674340	t	0.484	-0.038	0.015	**2.5E-03**	SLBP/FGFR3
rs13148031	4	145679788	a	0.592	0.018	0.016	2.6E-01	HHIP/GYPA
rs10471753	5	67854708	c	0.614	0.005	0.016	7.6E-01	PIK3R1
rs10948155	6	44795935	t	0.668	-0.037	0.016	**2.3E-02**	SUPT3H
rs12206662	6	45484199	a	0.078	0.140	0.037	**1.3E-04**	RUNX2
rs7739938	6	139823110	a	0.078	0.069	0.054	1.9E-01	CITED2
rs4837613	9	118349346	c	0.490	-0.028	0.015	7.1E-02	ASTN2
rs496547	11	118081673	a	0.359	0.009	0.016	5.8E-01	TREH/DOX6
rs6592847	11	78889654	a	0.248	-0.006	0.019	7.5E-01	NARS2
rs11045356	12	20683368	a	0.181	-0.053	0.022	**1.5E-02**	PPE3A
rs2703529	17	74709369	a	0.076	0.018	0.029	5.4E-01	HRN3P3
rs11880992	19	2127403	a	0.386	-0.064	0.016	**9.7E-05**	DOT1L
rs717433	20	7786135	t	0.777	0.005	0.019	7.8E-01	HAO1

Cohorts included: Treat-OA meta-analysis (arcOGEN part 1, deCODE, EGCUT, Framingham, GARP, RSI, RSII, RSIII, TwinsUK), SOF, MrOS, CHECK, GOAL, Nottingham, JoCo, GoGo, Chingford

Additional adjustment for height did not have effect on the association with mJSW of the described signals showing an independent effect (possibly pleiotropic) on both traits. All other additional signals selected in the discovery stage lacked genome-wide significance and did not replicate, including a intron variant in *ASTN2* (rs4837613), in high LD (D'=0.91, r^2=0.73) with a variant previously reported as GWS for association with total hip replacement in females (rs4836732) [2].

All other additional signals selected in the discovery stage lacked genome-wide significance and did not replicate, including a intron variant in *ASTN2* (rs4837613), in

high LD (D'=0.91, r^2=0.73) with a variant previously reported as GWS for association with total hip replacement in females (rs4836732) [2].

Biological process in common for some of these nine described signals associated with hip OA and/or cartilage thickness were identified using PANTHER and are described in Supplementary table 4. They include: cell connection, cell cycle, cellular process, developmental process and metabolism. The pathways found for these signals according with the analysis using PANTHER are the EGF receptor signalling pathway (*TGFA*), gonadotropin realising hormone receptor pathway (*TGFB2*), TGF-beta signalling pathway (*TGFB2*) and several pathways for *PIK3R1*, including Insulin/IGF pathway protein kinase B signalling, PI3 kinase pathway, angiogenesis and Endothelin signalling pathway between others.

We used GRAIL to identify significant connections between the SNPs associated to cartilage thickness selected at the discovery stage ($P<1\times10^{-5}$). We found the next 13 keywords describing functional connections of our locis: 'transforming', 'growth', 'hedgehog', 'factor', 'beta', 'phosphodiesterase', 'cardiac', 'alpha', 'mice', 'differentiation', 'bone', 'development', 'osteoblast'.

Group of genes and significance of the connections between each other were calculated by GRAIL and are presented in suplementary table 5. The candidate genes with most significant connections with other genes according with GRAIL and significant for its association with hip OA are: *TGFA* and *RUNX2* which showed high similarity with a GRAIL *P*-value 5.5×10^{-4} and 9×10^{-3}, respectively. Connections between genes are represented in the Supplementary figure 2.

DISCUSSION

This study identified nine novel loci that are associated with cartilage thickness, five surpassed genome-wide significance and four of them were suggestive for association with cartilage thickness. From these loci, three were associated with hip OA after correction for multiple testing and two more were significant at a nominal level. Seven of the nine genes that here we reported associated with mJSW have been reported in literature as having a role in early development and growth (except *HA01* and *PIK3R1*). In addition, variants in six of them also previously associated with height at GWS level 24, 25].

The most significant variants for association with cartilage thickness and hip OA were localized on different regions: chromosome 2p13, 6p21 and the previous reported locus of chromosome 19p31.1. Rs2862851 on chromosome 2 lies in the intronic region of the Transforming Growth Factor Alpha (*TGFA*). This gene encodes a growth factor that is a ligand for the epidermal growth factor receptor, which activates a signalling pathway for cell proliferation, differentiation and development. TGFA is present in limb-forming mesoderm and in apical ectodermal ridge (AER)-forming ectoderm and may be important for initial limb formation [25]. *Tgfa* knockout mice have a delay in bone development specifically the conversion of hypertrophic cartilage to true bone [26]. Furthermore, TGF inhibit articular chondrocyte anabolic capacity and induces endothelin receptor A expression in osteoarthritis [27, 28]. Interestingly, the *tgfa*-knockout mice expressed less RUNX2 in their cartilage growth plates than controls did [26], further corroborating our findings.

On chromosome 6, we found rs12206662, a transcript protein coding variant of Runt-related transcription factor 2 (*RUNX2*). *RUNX2* negatively regulates osteoblast differentiation and skeletal morphogenesis and acts as a scaffold for nucleic acids and regulatory factors involved in skeletal gene expression [29]. RUNX2 is considered a master transcription factor controlling chondrocyte hypertrophic differentiation and its expression has been demonstrated in human articular cartilage [30]. Rs12206662 showed the strongest effect on cartilage thickness and hip OA between the analysed loci. There is some evidence that modifications in this gene cause chondrocyte hypertrophy; a hallmark for OA-cartilage. We here provided evidence that this gene plays an important role in the development of hip OA in general population thought variations in cartilage thickness.

Rs10948155, associated with cartilage thickness is localized between *SUPT3H* and *CDC5L* and it was highly correlated with other SNPs that have been previously associated with OA and height. Reported association with OA and height for SNPs in this area might involve regulation of *RUNX2.* Moreover, no functional data has been presented to support this argument. Here we found two distant and independent signals from each area, with a different frequency and effects on both traits, cartilage thickness and hip OA.

The reported SNP on *DOT1L* is in high linkage with a previously reported SNP (rs12982744) associated with both traits cartilage thickness and OA, being the same signal [1]. *DOT1L* was identified using the same approach; through a GWAS association

study on mJSW as a proxy for cartilage thickness and later on rs12982744 demonstrated to be also associated with hip OA in males at a GWS level [4].

We found a novel loci on chromosome 5, rs10471753, which closest gene is *PIK3R1*, Phosphatidylinositol 3-kinase phosphorylates the inositol ring of phosphatidylinositol at the 3-prime position. Phosphatidylinositide 3-kinases (PI 3-kinases or PI3Ks) are a family of enzymes involved in cellular functions such as cell growth, proliferation, differentiation, motility, survival and intracellular trafficking, involved in cancer, body fat, leptin and insulin metabolism [31-33]. Recently, it has been found that *PIK3R1* mutations are a major cause of SHORT syndrome. SHORT syndrome is a rare multi-systemic disease, characterized by short stature, eye anomalies, characteristic facial features, lipodystrophy, hernias, hyperextensibility and delay dentition. This gene has been proposed to participate in pathways involve in control of skeletal myogenesis by HDAC & calcium/calmodulin-dependent kinase (CaMK), G, Growth Hormone, EGF IGF-1 Signaling Pathways. Erk and PI-3 Kinase are Necessary for Collagen Binding in Corneal Epithelia.

Other signals associated with cartilage thickness are rs10495106 and r1717433, which annotated closest gene are *TGFB2* and *HAO1*, respectively. *TGFB2* encodes a member of the transforming growth factor beta (TGFB) family of cytokines, which are multifunctional peptides that regulate proliferation, differentiation, adhesion, migration, and other functions in many cell types by transducing their signal through combinations of transmembrane type I and type II receptors (TGFBR1 and TGFBR2) and their downstream effectors, the SMAD proteins. Disruption of the TGFB/SMAD pathway has been implicated in osteoarthritis and cancer [34, 35]. Other SNP in the *TGFB2* has been previously associated with height [24]. Hydroxyacid Oxidase/Glycolate Oxidase 1 (*HAO1*) is one of three related genes that have 2-hydroxyacid oxidase activity yet differ in encoded protein amino acid sequence, tissue expression and substrate preference. HAO1 have a role in metabolism of amino acids and derivatives and has been associated with colorectal cancer [36]. It is unknown whether *HAO1* is associated with muscle-skeletal traits.

The closest gene to rs496547 is trehalase (*TREH*), brush-border membrane glycoprotein that encodes an enzyme that hydrolyses trehalose, a disaccharide formed from two glucose molecules found mainly in fungi, plants, and insects. Deficiency of TREH produces isolated trehalose intolerance causing gastrointestinal symptoms after ingestion of edible mushrooms [37, 38]. Another variants in this gene have been associated with the risk of height and glioma [24, 39].

histone mRNAs [40]. According with our results and its association with height at GWS level, it seems that this gene also have an active role in muscle-skeletal traits.

Between the set of words from GRAIL that best describe the signals associated with cartilage thickness are words reflecting the role of these genes in bone differentiation process that start early, during embryonic development, and conclude in adulthood after establishment of definitive bone and cartilage characteristics. Key transcription factors and signalling molecules that coordinated cartilage and bone development also regulate adult physiology. RUNX2, Wnt, EGF, IGF, TGFB superfamily between others are known to have a very important role in the processes of endochondral bone formation and development of the hip joint, including chondrocyte proliferation and differentiation (Figure 3). Some of the genes associated with cartilage thickness have been also identified in the analysis of height, showing a possible pleiotropic effect for these traits.
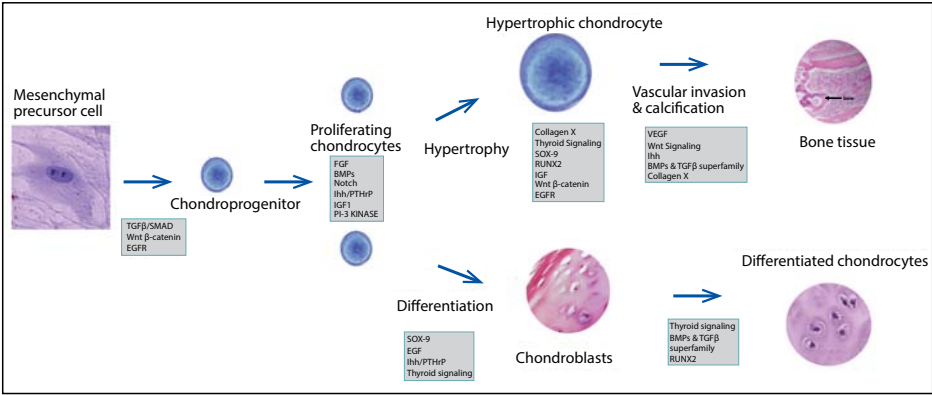
Figure 3   Schematic diagram providing an overview of the influence of signaling pathways and transcription factors during the processes of condensation, chondrogenesis, chondrocyte proliferation, differentiation, hypertrophy and calcification during endochondral bone and cartilage formation. Some of the genes associated with cartilage thickness: DOT1L (Wnt signaling), PIK3R1 (IGF & PI-3 Kinase), RUNX2, TGFA (EGFR) and possibly TGFB2 (not replicated) are part of these signaling pathways. Signaling and growth factors are shown in light gray boxes.

Deregulation of genes and mechanisms during endochondral ossification might have a role in hip OA initiation and progression. Chondrocyte apoptosis has been identified as involved in OA progression [41]. In addition, it seems that induction of a transcriptional activator Runx2 in chondrocytes under mechanical stress contributes to the pathogenesis of OA through chondrocyte hypertrophy, between other possible mechanisms [41]. Understanding of the regulatory processes that finally shape the hip joint, including formation of articular cartilage and healing will be relevant to identify the best therapeutics targets for hip OA. Expression and functional studies with the genes here described are warranted.

Rs2236995 is an intronic variant of Stem-Loop Binding Protein (*SLBP*). This gene encodes a protein that binds to the stem-loop structure in replication-dependent histone mRNAs. Histone mRNAs do not contain introns or polyadenylation signals, and are processed by endonucleolytic cleavage. The stem-loop structure is essential for efficient processing but this structure also controls the transport, translation and stability of

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Supplementary Table 1. Baseline characteristics of the studies included in the analyses of minimal Joint Space Width (mJSW).

Cohort Studies	n	Female (%)	Characteristics				Nr.Cases/ controls	HOA definition
			Age (year)	Height (cm)	BMI (kg/m2)	mJSW (mm)		
RS-I	4773	59	67.7(7.8)	167.6 (9.3)	26.3 (3.6)	3.80(0.8)	771/4436	K/L 2
RS-II	1750	54	64.8 (7.9)	168.6 (9.2)	27.2 (3.9)	4.38 (0.9)	166/1795	K/L 2
SOF	2612	100	70.9 (5)	159.5 (5.8)	26.9 (4.7)	2.96(0.5)	243/2185	Croft grade
MrOS	3266	0	77.2 (5.3)	173.8 (6.7)	27.4 (4)	3.30 (0.7)	411/2512	Croft grade
Twins-UK	612	100	53.4 (7.7)	163 (6.8)	25.1 (4)	3.21 (0.7)	48/565	JSN and KL2
GOAL	3131	48	66.6 (7.9)	166.5 (9.1)	29.3 (5.3)	3.29 (1.1)	1263/1821	JSN2 & KL2
CHECK	815	79	55.9 (5.2)	169.5 (8.3)	26.4 (4.6)	3.5 (0.9)	291/547	K/L 2
GOGO	838	80	69.2 (8.6)	162.0 (8.4)	28.5 (6.1)	3.2 (0.8)	359/898	KL >=2 mJSW <=2.5 mm JSN >=2 & osteophyte grade >=1
Chingford	589	100	62.0 (5.9)	161.6 (5.9)	25.4 (4)	3.62 (0.7)	88/657	KL >=2THR
JoCo	422	60						KL >=2THR
GARP	312	82	60 (6)	168.0 (6.8)	26 (5.3)		93/219	Pain+(OsteophytesJSNaxialTHR)
Nottingham	2120	62	67.7 (9.2)	166.7 (5.3)	27.2 (4.6)	NA	1381/739	KL >=2THR

Abbreviations; KL=Kellgren and Lawrence score. JSN=Joint Space Narrowing. THR: Total Hip Replacement. Values are expressed as means with standard deviations (sd) or percentages (%). Nottingham only provided information on OA.

Chapter 2.2

Supplementary table 2. Quality control and inclusion criteria.

Study	Genotyping platform(s)	Genotype calling algorithm	Genotyping center	SNP inclusion criteria				Other criteria
				Call rate (%)	MAF (%)	HWE P-value	# SNPs meeting inclusion criteria	
Discovery								
RS-I	Illumina HumanHap 550K V.3 Duo	BeadStudio	Genetic Lab, Department Internal Medicine, Erasmus MC, NL	≥97%	≥1%	$<10^{-6}$	512,349	1) sex mismatch with typed X-linked markers, 2) excess autosomal heterozygosity > 0.336~ FDR > 0.1%, 3) duplicates and/or first or second degree relatives using IBS probabilities > 97% from PLINK, 4) ethnic outliers using IBS distances > 3SD from PLINK
RS-II	Illumina HumanHap 550K V.3 Duo	BeadStudio	Genetic Lab, Department Internal Medicine, Erasmus MC, NL	≥97%	≥1%	$<10^{-6}$	466,389	1) genotypic sex mismatch, 2) relatedness by estimated IBD, 3) gross chromosomal abnormalities by BAF in > 5 chromosomes, 4) non-European ethnic outliers from PCA
MrOS	Illumina HumanOmni1 Quad	BeadStudio	Broad Institute	≥97%	≥1%	$<10^{-4}$	740,713	1) genotypic sex mismatch, 2) relatedness by estimated IBD, 3) gross chromosomal abnormalities by BAF in > 5 chromosomes, 4) non-European ethnic outliers from PCA
SOF	Illumina HumanOmni1 Quad	BeadStudio	Broad Institute	≥97%	≥1%	$<10^{-4}$	740,713	1) Autosomal heterozygosity 2 SD from mean, 2) non-European ancestry from PCA with HapMap3 populations, 3) sample mismatch based on IBD probabilities
TwinsUK	HumanHap610Q, 1M-Duo, and 1.2M-Duo	Illuminus (1)	Wellcome Trust Sanger Institute, Hinxton, UK	≥98%	≥1%	$<10^{-6}$	303,940	
Replication in silico								
GARP	Taqman Genotyping Assay						NA	

Study	Array	Calling software	Genotyping location	Call rate	MAF	HWE		QC exclusion criteria
GOGO	Illumina BeadChip 550K	BeadStudio	David H. Murdock Research Inst.	>99%	>5%	NA	NA	1) Autosomal heterozygosity 2 SD from mean, 2) non-European ancestry from PCA with HapMap3 populations, 3)sample mismatch based on IBD probabilities
Chingford	Illumina HumanHap610Q	Illuminus (1)	Wellcome Trust Sanger Institute, Hinxton, UK	NA	≥1%	<10-6	NA	
JoCo	Illumina Infinium 1M Duo	BeadStudio	Expression Analysis Inc. (Durham, NC)	NA	≥0.5%	<10-4	NA	1) sex/gender mismatch 2) First-degree relative 3) disagreement between reported and genotypic race
Replication de novo								
GOAL	KASPar chemistry, which is a competitive allele-specific PCR SNP genotyping system using FRET quencher cassette oligos.	NA	Kbiosciences, Hoddesdon, UK	NA	NA	NA	NA	NA
CHECK	Sequenom iPLEX and Taqman allelic Discrimination KASPar chemistry, which is a competitive allele-specific PCR SNP genotyping system using FRET quencher cassette oligos.	NA	Genetic Laboratory Department, Internal Medicine, Erasmus	NA	>1%	<10-6	18	NA
Nottingham	KASPar chemistry, which is a competitive allele-specific PCR SNP genotyping system using FRET quencher cassette oligos.	NA	Kbiosciences, Hoddesdon, UK	NA	NA	NA	NA	NA

1. Teo YY et al (2007) A genotype calling algorithm for the Illumina BeadArray platform. 23: 2741–2746

Supplementary table 3. Information on genotyped SNPs.

| SNPs | RS-I & RS-II | | MrOS | | SOF | | Twins-UK | | GARP | | GOGO | Chingford | JoCo | | GOAL | CHECK | Nottingham |
	Imp	Imp Q	Imp	Imp Q	Imp	Imp Q	Imp	Imp Q	Imp	Imp Q	Imp	Imp	Imp	Imp Q	Imp	Imp	Imp
rs10471753	0	0.998	1	0.998	1	0.998	1	0.995	1	0.995	proxy	NA	1	1	0	0	NA
rs10495106	0	0.985	1	0.99	0	0.999	1	0.931	1	0.937	proxy	proxy	0	1	0	0	NA
rs10948155	1	0.995	1	0.981	1	0.981	0	0.997	1	0.998	0	0	0	0.995	0	0	NA
rs11045356	1	0.998	1	0.934	1	0.934	0	0.815	1	0.982	0	0	0	0.998	NA	NA	NA
rs11880992	0	0.989	1	0.992	1	0.992	1	0.977	1	0.987	proxy	proxy	1	0.979	NA	NA	NA
rs12206662	0	0.490	1	0.718	1	0.718	NA	NA	1	0.516	NA	proxy	1	0.506	0	0	0
rs13148031	0	0.998	1	0.998	1	0.998	1	0.988	1	0.992	proxy	proxy	1	0.995	0	0	NA
rs2061109	0	0.967	1	0.996	1	0.996	1	0.97	1	0.958	proxy	proxy	1	0.958	NA	NA	NA
rs2236995	1	0.998	1	0.987	1	0.987	0	0.997	0	1.000	0	0	0	1	NA	0	0
rs2703529	1	0.995	0	NA	1	0.996	0	0.995	1	0.998	0	0	0	0.995	0	0	NA
rs2862851	0	0.991	1	0.998	1	0.998	1	0.995	1	0.994	proxy	proxy	1	0.994	0	0	0
rs4837613	0	0.957	1	0.957	1	0.957	NA	NA	NA	NA	proxy	NA	1	0.965	0	0	NA
rs496547	0	0.998	1	0.987	1	0.987	1	0.982	1	0.995	NA	NA	1	0.999	0	0	NA
rs6429001	1	0.994	0	NA	1	0.992	0	0.975	0	1.000	0	0	0	0.999	NA	0	NA
rs6437120	0	0.985	1	0.978	1	0.979	1	0.791	1	0.981	proxy	proxy	1	0.992	0	NA	NA
rs6592847	0	0.868	1	0.886	1	0.886	NA	NA	NA	NA	NA	proxy	0	0.989	NA	NA	NA
rs717433	0	0.911	1	0.974	1	0.974	1	0.971	1	0.900	proxy	proxy	1	0.958	NA	NA	NA
rs7739938	0	0.346	1	0.446	1	0.446	NA	NA	1	0.406	proxy	NA	1	0.374	0	0	NA

Abbreviations: Imputation (Imp):(yes=1 / no=0 or proxy)

Supplementary Table 4. Results of analysis in PANTHER.

Gene	Molecular Function	Biological Process
RUNX2	Transcription factor activity.	Signal transduction;regulation of transcription from RNA polymerase II promoter;signal transduction;ectoderm development;mesoderm development; sex determination;Embryonic development;skeletal system development;nervous system development;hemopoiesis
TGFA	Cytokine activity;growth factor activity	Cell cycle;cytokine-mediated signaling pathway;cell-cell signaling;cell cycle;cytokine-mediated signaling pathway;cell-cell signaling
SLBP	RNA binding	Protein metabolic process
TGFB2	Growth factor activity	Female gamete generation;cell surface receptor linked signal transduction;cell surface receptor linked signal transduction;ectoderm development; mesoderm development;skeletal system development;heart development;muscle organ development
PIK3R1	Protein binding;kinase regulator activity	intracellular signaling cascade;intracellular signaling cascade
TREH	Hydrolase activity, hydrolyzing N-glycosyl compounds	Disaccharide metabolic process
HAO1	Oxidoreductase activity;isomerase activity;ligase activity	Carbohydrate metabolic process
DOT1L	Methyltransferase activity	Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process

Supplementary Table 5.   GRAIL data for selected individual SNPs of interest (P<1x10^{-5}).

GENE	GRAIL-P val ue	SELECTED SIMILAR GENES (Rank in parentheses)
TGFA	0.00056	TGFB2(2), CITED2(53), PIK3R1(358), TACC3(737), RUNX2(993), AMH(1571)
TGFB2	0.0010	TGFA(5), CITED2(45), RUNX2(472), PIK3R1(818), TACC3(856), AMH(1546), AP3D1(1585), HHIP(1957)
CITED2	0.00415	TGFB2(24), TGFA(90), RUNX2(834), TACC3(980), AP3D1(1059), PIK3R1(1101)
DOT1L/AMH	0.00677	PLEKHJ1(2), SF3A2(11), TGFB2(88), TGFA(89), CITED2(288), TACC3(506), RUNX2(1048), AP3D1(1248), DDX6(1495), PIK3R1(1771), HHIP(1780)
PAPPA	0.00764	TGFB2(123), TGFA(135), PIK3R1(370), CITED2(447), RUNX2(1140), AP3D1(1478), AMH(1616)
RUNX2	0.00905	CITED2(81), TGFB2(112), TGFA(256), PIK3R1(901), AP3D1(1069), TACC3(1616), HHIP(1803)
HHIP	0.02133	CITED2(203), TGFB2(256), TGFA(296), RUNX2(692), TACC3(1088), PIK3R1(1364), AP3D1(1949)
TACC3	0.04850	CITED2(160), TGFA(406), TGFB2(517), DDX6(825), TXLNB(1396), SF3A2(1489), AP3D1(1727)
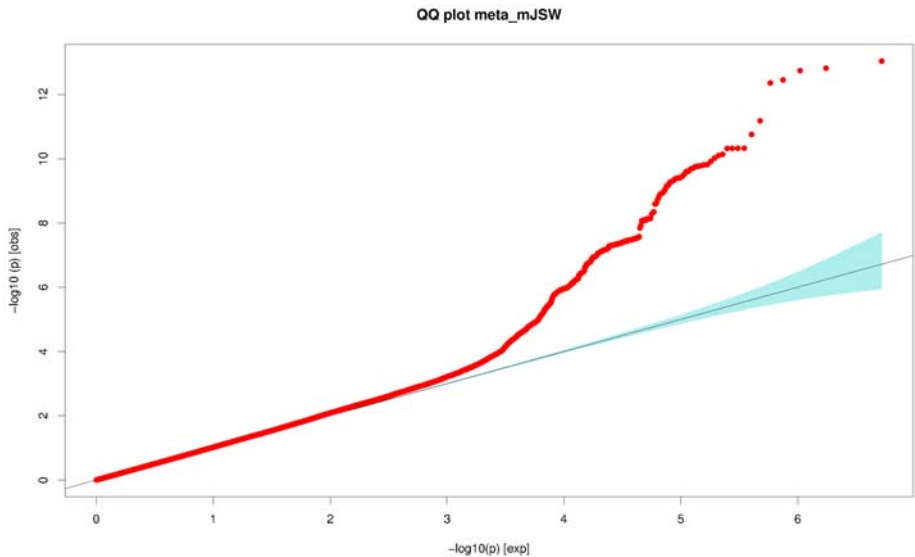
Supplementary Figure 1   Interquantile-quantile plot for the analysis of mJSW in the discovery cohorts.
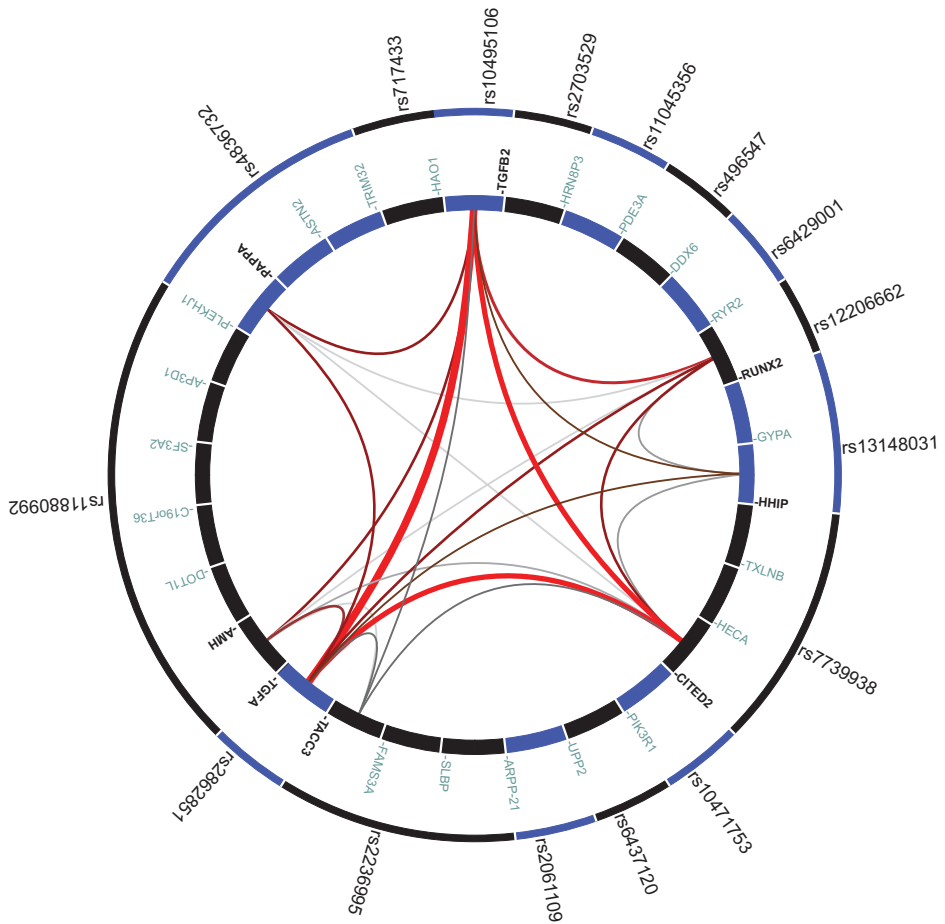
Supplementary Figure 2   Graphical representation of the connections between the selected SNPs associated with cartilage thickness ($P<1\times10^{-5}$) and corresponding genes using GRAIL. GRAIL analysis identified non-random connectivity ($P<0.05$) between associated genes (Black color text). Thicker and redder lines imply stronger literature-based connectivity.

Chapter 2.3

Bivariate analysis of correlated traits: joint space width and height

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et al

Chapter 2.3

ABSTRACT

Hip osteoarthritis (OA) is characterized by cartilage degradation leading to narrowing of the joint space width (JSW). Hip OA has been associated with height and part of the genetic variants associated to hip OA are localized in genes that also explain variation in height. Additionally, both traits JSW (a proxy for cartilage thickness) and height have a common origin during development; chondrocyte differentiation during endochondral bone formation, which determines cartilage thickness as well as the length of the bones. We aim to identify common variants associated to cartilage thickness of the hip and height. We used a bivariate analysis of the traits. The meta-analysis of hip-JSW and height datasets was performed using an inverse variance-weighted method. This method combines single trait genome-wide association results of correlated traits using Z-score test statistic and Fisher's methods based meta-analysis. For JSW, we used meta-analysed results from two cohorts: RS-I and RS-II in 6482 participants, while for height, we used the meta-analysed result from the GIANT consortium. We considered as Genome-wide significant a combined Fisher P value $<= 5 \times 10^{-8}$ when the variants were associated in both traits with a *P* value $<= 5 \times 10^{-4}$ for JSW and a *P* value $<= 5 \times 10^{-5}$ for height. Association with OA was tested using logistic regression. We identified variants in or near eight different genes: *DOT1L, DYM, FBXW11, TEAD1, IGF2BP3, SUPTH3, ASTN2 and IER3* associated with cartilage thickness. The variants in *DOT1L, SUPTH3* were associated with OA in males and *ASTN2* in females (P=2.1×10^{-4}, P=2.4×10^{-5} and P=3.5×10^{-3}, respectively). Variants in these genes were previously reported to be associated with height. These genes might provide a framework for joint formation during development, embryogenesis and in general have an important role in endochondral ossification process, including formation of cartilage thickness and hip joint.

INTRODUCTION

Osteoarthritis (OA) is a degenerative joint disease characterized by progressive damage of the articulate cartilage that on radiographs can be identified as narrowing of the joint space, new bone formation and alterations in subchondral bone. Heritability studies have shown that more than half of the variation in susceptibility to osteoarthritis in the population is explained by genetic factors, but these factors have been difficult to identify [1, 2].

Epidemiological studies have associated height with the risk for hip osteoarthritis [3]. Well known signalling pathways involved in growth and differentiation of the skeleton are the TGF-beta and Wnt-signalling pathways between others. Variants in genes having a role in these pathways have been identified in association with OA and height and deregulation of these signalling pathways has been shown to be involved in OA and height [4-10]. Common variants in the growth differentiation factor 5 (*GDF5*) gene contribute to variation in height and increase susceptibility to hip and knee osteoarthritis in Asian and European populations [6, 11, 12]. *GDF5* is part of the TGF beta signalling pathway and is involved in the development of cartilage in the legs and other long bones. *SMAD3* is another example of a gene where genetic variants are involved in both OA and height [5, 14]. Early-onset joint abnormalities, including osteoarthritis have been reported in a big percentage of subjects with mutations in *SMAD3* gene, while more common variation was also reported as significant contributor of height differences. Recently a DNA variant in the *DOT1L* gene was found to be associated with variations in Joint Space Width (JSW) and hip OA. This same variant was previously found to be associated with height [7, 14, 15]. JSW is a good surrogate for cartilage thickness in hips and it is used to radiographically define hip OA [16, 17]. Narrowing of the JSW is considered to be a strong predictor of progression of hip OA [18].

Since there is a common biological process underlying cartilage thickness (represented by JSW) and height (endochondral bone formation), we hypothesize that these two traits might be under genetic influence of more and still unknown shared genes. A significant excess of shared signals between osteoarthritis and height has been reported [19]. However, the responsible signals have not been identified.

Using a bivariate analysis, it is possible to identify associated genetic loci that have an effect on correlated phenotypes [20-22]. This method takes benefit of the mutual correlation between traits, in this case JSW and height, to increase the power to identify signals that are relevant for both traits. Using this method, we aim to identify those

variants playing a role in both cartilage thickness and height. Identifying the genes involved in both traits will help to elucidate unknown aspects of joint formation that are relevant to understand the pathogenesis of important diseases of the hip joint including hip OA.

METHODS

Study design, population and data collection procedure

We used data of the Rotterdam study, a large prospective population-based cohort study among men and women ≥55 years of age. In summary, the objective of the study is to investigate the determinants, incidence and progression of chronic disabling diseases in the elderly [23]. A total number of 6,482 participants from Rotterdam study I (RS-I) and II (RS-II) with measures of JSW and genetic data were included. The medical ethics committee of Erasmus Medical Center approved the study and written informed consent was obtained from each participant.

Clinical evaluation and physical examination

At baseline, medical information and physical examination of participants of the RS-I and RS-II including measurements of height and weight were obtained.

Radiographic assessment

Weight-bearing antero-posterior pelvic radiographs were taken with both of the patient's feet positioned in 10° internal rotation and the x-ray beam centered on the umbilicus. At baseline and follow up, the JSW of the hip joints was measured using a 0.5-mm graduated magnifying glass laid directly over the radiograph. JSW is defined as the distance between the superior part of femoral head and the acetabulum at three different positions: lateral, superior and medial (Figure 1). Hip osteoarthritis was defined as a grade 2 or more using Kellgren and Lawrence score (at least one hip with definite narrowing and at least possible femoral osteophyte(s).

Data analysis procedure

Correlation between variables, height and JSW adjusted for age and gender was done using Pearson correlation coefficient. The variable of JSW (lateral, superior or medial) with the highest correlation with height was used for the bivariate analysis for both traits.
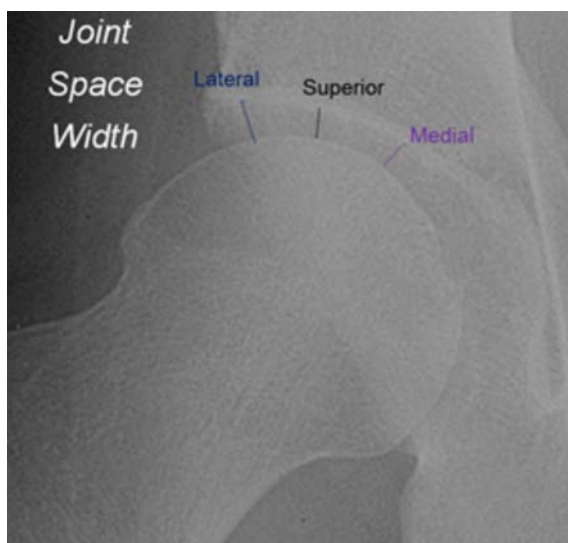
Figure 1   Radiograph from Rotterdam study I (RS-I) showing the Joint Space Width (JSW) measured at different locations of hip joint. JSW measured at lateral, superior and medial locations.

Meta-analysis of single traits:

JSW meta-analysis

Residuals from linear regression analysis of JSW in 6,482 participants from RS-I and RS-II corrected for age, gender and population stratification (four principal components) were used for genetic association analysis in each cohort. Creation and quality control of the GWAS data for the Rotterdam study has been described earlier. In short, we used MACH for imputation with reference to HapMap release 22 CEU [24]. 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, which uses genotype dosage value (0-2, continuous) as a predictor in a linear-regression framework [25]. The following quality control inclusion filters were applied: call rate >97.5%, MAF >1%, *P* for Hardy-Weinberg equilibrium $<1 \times 10^{-6}$ (see Table 1 for details on quality control and exclusion criteria). The total number of genotypes SNPs that passed these filters were 512,349 for RS-I and 466,389 for RS-II. Genomic control correction was done to the standard error and P-values before meta-analysis. We included only imputed SNPs that had a good imputation quality leaving a total of 2,355,290. The summary statistics of RS-I and RS-II were meta-analysed using METAL applying inverse-variance methodology assuming fixed effects.

Height meta-analysis

Meta-analyzed results of Genome Wide Association Study (GWAS) of height (GIANT consortium) in 183,727 individuals from different populations were used for the bivariate analysis.Results from meta-analysis of height are freely available in the website of GIANT consortium: (http://www.broadinstitute.org/collaboration/giant/index.php/ GIANT_consortium_data_files). Other methodological details have been described previously [7, 14].

Bivariate analysis

The method used combines single trait genome-wide association results of correlated traits using Z-score test statistic and Fisher's methods based meta-analysis [26]. In this case we used meta-analyzed results of JSW from two cohorts: RS-I and RS-II and meta-analyzed results from GIANT as previously described [14]. To detect associations of the same and opposite directions, two tests were performed. First, the directional meta-analysis; with the original results of each trait, followed by a second where the direction of association was flipped in one dataset and an opposite-allelic effect analysis was performed. The observed chi-square value was divided by the lambda value to derive the corresponding chi-square meta-adjusted value and therefore the P-adjusted values. The final step consists of combining the two independent P-adjusted distributions using the Fisher's methods for combining independent P-values [26]. The resulting chi-square value distribution with four degrees of freedom (two times the number of traits) provide the overall association evidence for each genetic variant taking underlying correlation and directionality of the effects into accounts. In both traits the genomic control corrected results were used for final Fisher meta-analysis. Further details of the bivariate analysis have been previously described [27]. Variants associated in both traits with a *P* value $<=5x10^{-4}$ for JSW and with a *P* value $<=5x10^{-5}$ for height, were considered. Conditional analyses were used to determine whether two associated variants had an independent effect on the trait (JSW). It was done adding the associated variants into the linear model for joint space width. Two variants are considered as independent when in the same model have an significant effect on JSW. Analyses were performed in R package and Plink [28]. We considered as Genome-wide significant a combined Fisher *P*-value $<= 5x10^{-8}$.

Association with hip OA

Association of the top SNPs found in the bivariate analysis with hip OA was verified using the TREAT-OA meta-analysis that includes 5,244 hip OA cases and 17,836 controls. 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 2 definite osteophytes and possible joint space narrowing or a (partial or total) knee 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 knee 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. Details of the studies cohorts included are presented in Supplementary table 1.

Association of each of the top SNPs (P<=5x10^{-8}) found using the bivariate analysis of height and JSW was tested using logistic regression age and gender adjusted or gender stratified. We considered a significant association with OA when the P value was equal or less than 0.0062 (P<=6.2x10^{-3}).

RESULTS

Table 1 presents the baseline characteristics of the study populations, quality control procedures and exclusion criteria of individuals for GWAS of Superior JSW in RS-I and RS-II. JSW is thicker at the superior site of the hip joint, more than at medial or lateral sides. Table 2 shows the correlation coefficients between measures of JSW at different locations of the hip joint and height. We found a significant correlation between JSW at different locations of the hip and height (Table 2). Superior JSW was the most correlated phenotype of the hip joint with height (Table 2, r^2: 0.18, P<0.001). Therefore we used superior JSW as selected trait for the bivariate analysis with height, considering that for this type of method higher correlation between studied variables represents an advantage to increase power to localize common variants for both traits [31]. The GWAS on superior JSW was performed in 6,482 participants from RS-I and RS-II (Figure 2).

Meta-analysed results from this GWAS was used for bivariate analysis with the meta-analysed results obtained in the height-study as it was previously explained. After quality control (QC) measures a total of 2.359.324 SNPs remained for the association analysis. Figure 2 shows the results of the association of all remaining SNPs after QC with Superior JSW. Since strong association of some known variants with height might inflate the final P value in the bivariate analysis, we selected SNPs with at least $P<=5x10^{-5}$ for height and $P<=5x10^{-4}$ for superior JSW and final Fisher P value $<=5x10^{-8}$. We only detected significant associations for both traits in the directional meta-analysis (effects with the same direction for both traits).

A total of eight different independent signals were identified. The strongest signal related to both: JSW and height was rs2864419 (Table 3, $P=1.2x10^{-20}$ for combined Fisher P value), is localized on the intronic region of *DOT1L* gene previously reported associated to both traits: height and JSW at genome wide significant level, being the same signal ($r^2=0.87$ with rs12982794) [32, 33]. Followed by rs2156250 on Chr 18q21 in the intronic region of the *Dymeclin (DYM)* gene. This SNP represent the same signal as rs9967417, previously reported for association with height and according with our results, it is associated with superior JSW with a P value of $4.8x10^{-5}$ (Table 3).

Additionally, rs1368380 was associated with both traits at a GWS level (Table 3, P value=$3.6x10^{-11}$). Rs1368380 is a variant in a regulatory region of *FBXW11* A previously reported SNP for height is at around 100 Kbp from rs1368380 (rs12153391, $r^2=0.03$) [14].

Rs9888179 is localized on Chr. 11, between two genes: *PARVA* and *TEAD1*, *TEAD1* being the closest gene reported as responsible for the associated with height (rs7926971, $r^2=0.58$) and more biological plausible for its relation with growth and size [14].

Rs10950947, an intronic variant in the *IGF2BP3* gene was associated with JSW and height, with a combined P-fisher=$1.2x10^{-9}$. A linked variant (rs12534093, $r^2=0.55$), was previously associated with height.

Table 1. Characteristics of the study populations, quality control procedures and exclusion criteria for individuals of the GWA of superior JSW.

Variables	Description of the Studies	
	RS1	RS2
Number	4770	1712
Female (%)	56.6	54.4
Age (years)	67.7 (7.8)	64.5 (7.8)
Height (cm)	167.6 (9.2)	168.8 (9.2)
Weight (kg)	73.8 (11.7)	77.6 (13.3)
BMI (kg/m2)	26.3 (3.6)	27.2 (3.9)
Superior JSW (mm)	4.80 (0.82)	5.26 (0.82)
Lateral JSW (mm)	4.69 (0.93)	5.02 (1.0)
Medial JSW (mm)	4.45 (0.83)	4.51 (0.86)
Nr HOA cases/controls	771/4436	166/1795
Genotyping Platform(s) / Chip(s)	Illumina HumanHap 550K V.3 DUO	
Call rate* selection	$\geq$97.5%	
Sample QC / Other exclusions	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.	
Genotyping facility	Genetic Laboratory Dept Internal Medicine Erasmus MC, The Netherlands	

Means are given with standard deviations between brackets. BMI: body mass index, JSW: Joint Space Width of the hip, Nr HOA: number of hip osteoarthritis (OA) cases and controls NA: not available

Table 2. Correlation between measures of JSW at different joint locations and Height.

Measures of JSW	Lateral JSW	mJSW	Superior JSW	Height*
Lateral JSW	1	0.48	0.51	0.092
Medial JSW	0.48	1	0.64	0.095
Superior JSW	0.51	0.64	1	0.184

* Pearson coefficient (r^2) for correlation between the measures of JSW at different location of the hip joint and height after correction for age and gender. Correlations between joint space width (JSW) at any location of the hip joint and height were significant ($P<0.001$).
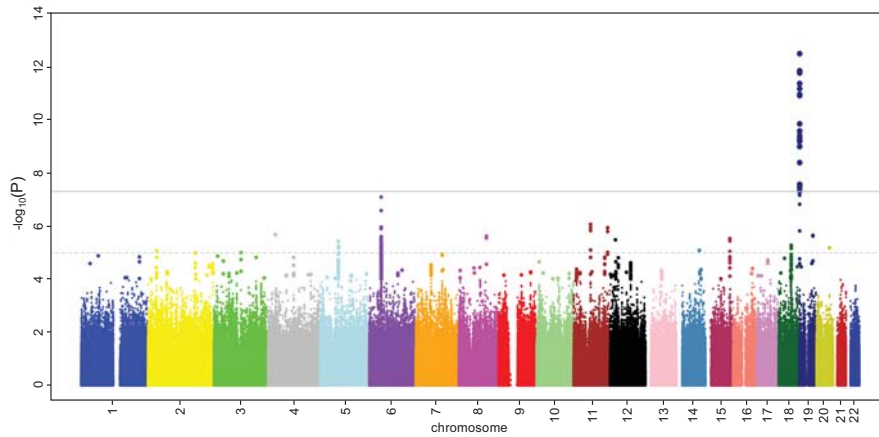
Figure 2 Manhattan plot: -log p-values for each of the ~2.4 million tests performed for the genome wide association analysis on Superior JSW of the hip. The gray dashed line correspond to a p-value of $5x10^{-8}$. The gray solid horizontal line corresponds to the p-value threshold of $5x10^{-8}$.

On chromosome 6, rs10948194, localized in the intronic region of *SUPTH3* and associated at GWS level in the bivariate analysis of both traits, height and superior JSW (Table 3, *P*-Fisher=$6.9x10^{-9}$). The SNP is correlated with rs10948172 (D'=1, r^2=0.31), previously shown associated with hip OA in males at a borderline GWS level (*P* value= $6x10^{-7}$) [34]. Another SNP rs9472414 moderated linked to rs10948194 was reported in association with height in the GIANT meta-analysis (r^2=031).

Rs2208562 localized in the intronic region of the astrotactin 2 gene (*ASTN2*). The SNP reported as associated with height at GWS level (rs751543) is an intronic variant in *PAPPA* with a very low correlation with rs2208562 (r^2=0.006). On the other hand, rs4836732 is another intronic variant of ASTN2 and was previously reported as associated with total hip replacement due to OA only in females   (D'=0.88, r^2=0.53).

Finally, rs12527415 (Table 3, *P*-fisher: $2.9x10^{-8}$), is a intergenic variant localized around 86 kbp of *IER3* and 90 kbp from the *FLOT1* gene. Immediate Early Response 3 (*IER3*) has a role in cellular stress response, inflammation and tumorigenesis [35]. This genomic locus has not been associated at GWS level with height before.

The association of these signals with JSW did remain after correction for height demonstrating an independent effect and indicating a possible pleiotropic effect of these genes on both traits.

Table 3. Top SNPs associated at a Genome Wide Significant (GWS) level with Joint Space Width (JSW) and Height.

SNP	Chr	Position	A1	Freq-1	Beta	JSW SE	JSW P	Height P	Combined P-Fisher	Gene
rs1368380	5	171218237	t	0.43	0.0695	0.017	6.2E-05	6.8E-08	3.6E-11	FBXW11
rs10948194	6	45415421	t	0.55	-0.083	0.018	4.8E-06	4.5E-05	6.9E-09	SUPTH3
rs1257415	6	30862519	t	0.29	-0.068	0.019	3.4E-04	1.9E-05	2.9E-08	IER3/FLOT1
rs10950947	7	23470279	a	0.62	0.063	0.018	4.7E-04	3.5E-07	1.2E-09	IGF2BP3
rs2208562	9	118384349	t	0.61	-0.063	0.018	4.6E-04	1.1E-05	2.1E-08	ASTN2
rs9888179	11	12739177	t	0.39	0.074	0.018	4.1E-05	2.0E-07	5.7E-11	PARVA/TEAD1
rs2156250	18	45004909	a	0.56	-0.071	0.017	4.9E-05	1.6E-15	2.5E-17	DYM
rs2864419	19	2134175	t	0.46	0.126	0.017	3.3E-13	2.9E-09	1.2E-20	DOT1L

Association with OA was significant for rs2864419 (*DOT1L*) and rs2208562 (*ASTN2*) at a P<0.0062 (Table 4, P=5.1×10^{-4} and 3.5×10^{-3} respectively, for association in all subjects). Previously, a variant in high LD with rs2864419 was reported as GWS only in males with OA [36].

A signal in *ASTN2* was previously reported associated at GWS level with OA in females (arcOGEN stage 2 meta-analysis) [34]. Therefore, we analysed the association with hip OA in each gender. We found the variant rs10948194 significantly associated with hip OA in males and not in females (Table 4, P=2.4×10^{-5} and P=0.80 respectively). The betas from association of this SNP with hip OA are significantly different between females and males (P<0.001). Similarly, there is a significant difference in the betas between genders for the variants in *ASTN2* and *DOT1L*, having *ASTN2* variant stronger effect in females and *DOT1L* in males (P<0.005 and P<0.001, for differences in betas respectively). The other signals (close or on *FBXW11, IER3, IGF2BP3* and *TEAD1*) were not significantly associated with hip OA. Although rs9888179 (*PARVA/TEAD1*) reached a nominal significance (Table 4, P=0.05).

Five genes from the list here presented as candidate genes for the association between the variants associated with cartilage thickness and height are in at least one pathway. *SUPT3H* and *DOT1L* are present in the Transcriptional misregulation in cancer in human. Additionally, *DOT1L* is present in the Wnt signalling pathway. Dymeclin has been found expressed in cartilage during early development and it is involved in two secretion pathways: GOLM1 and PPIB, known in endochondral bone formation being interacting partners. Two of the candidate genes (*FBX11* and *TEAD1*) are present in the Hippo signalling pathway. The Hippo signalling pathway coordinates cell proliferation, apoptosis, and differentiation, and has emerged as a major regulator of organ development and regeneration. Transcriptional regulators in the Hippo pathway, Tead4 and Yap1, are required for general vertebrate epimorphic regeneration as well as for organ size control in appendage regeneration in Xenopus [37]. Given the roles of the Hippo pathway in directing cell fate and tissue regeneration has been proposed that regulatory elements in this pathway will be essential for regenerative medicine [38].

Chapter 2.3

Table 4. Association of top SNPs from bivariate analysis (JSW-Height) and hip osteoarthritis in the TREAT-OA meta-analysis.

SNP	Chr	Gene	Association all subjects (n=22709)			Association in females (n=12812)			Association in males (n=9897)		
			Beta	SE	P	Beta	SE	P	Beta	SE	P
rs1368380	5	*FBXW11*	0.014	0.025	0.57	0.054	0.033	0.10	-0.038	0.036	0.30
rs10948194	6	*SUPTH3*	0.06	0.025	0.014	0.008	0.032	0.80	0.15	0.036	2.4E-05
rs1257415	6	*IER3/FLOT1*	-0.003	0.028	0.92	0.027	0.037	0.46	0.019	0.041	0.64
rs10950947	7	*IGF2BP3*	0.0003	0.025	0.99	0.0006	0.034	0.98	-0.012	0.037	0.75
rs2208562	9	*ASTN2*	-0.073	0.025	0.0035	-0.099	0.033	0.003	-0.056	0.037	0.133
rs9888179	11	*PARVA/TEAD1*	0.049	0.025	0.050	0.053	0.033	0.11	0.045	0.037	0.23
rs2156250	18	*DYM*	-0.0034	0.025	0.89	-0.0057	0.033	0.86	-0.0084	0.036	0.82
rs2864419	19	*DOT1L*	-0.086	0.025	5.1E-04	-0.0364	0.033	0.27	-0.1346	0.036	2.1E-04

DISCUSSION

In this bivariate analysis using GWAS of two correlated traits, we identified new signals on chromosomes 5, 6, 7, 9, 11, 18 and 19 that are associated with joint space from the superior side of the hip joint, representing cartilage thickness and height. The SNPs reported here might have pleiotropic effect on both traits: height and cartilage thickness since the effect of the variants was independent of the association with height. We confirmed our previous hypothesis that these two traits are under genetic influence of common genes, some of them being identified using this approach. Additionally, we found that three of these variants were associated with hip OA, showing gender specificity.

It has been hypothesized that genes underlying certain skeletal disorders might have pleiotropic effects. Pleiotropy results when a gene has different phenotypic effects depending on circumstances of expression of the gene during development and aging [39, 40]. The discovery of genes with independent effect on both traits (as it was the case of *DOT1L)* supports this hypothesis. There have been other previous examples of variation in genes that have effect on height, cartilage thickness and osteoarthritis: *GDF5 and IHH [4, 6, 14].* Those genes share in common participation in important processes of chondrocytes differentiation during endochondral bone formation which might be the case for some of the other genes reported in this bivariate analysis of height and joint space width. There are other effects in common, part of endochondral ossification processes as hypertrophy of chondrocytes, cartilage volume and hip geometry (joint size, orientation and shape) that might influence the correlation between these traits (height, cartilage thickness and hip osteoarthritis). Chondrocytes hypertrophy, joint size (longitudinal and cross-sectional) and hip geometry are possibly influenced by common genes as it has been previously demonstrated for *GDF5* [41]. Chondrocytes hypertrophy is an essential step required for longitudinal bone growth and for maintaining the cartilage synovial joint surface [42]. Abnormal chondrocyte hypertrophy in articular cartilage has been associated with osteoarthritis [43]. Additionally, adult height correlates with joint dimensions and substantial genetic correlations have been found between femoral geometric traits, height and initiation and progression of hip osteoarthritis [44, 45].

We identified variants in/near *DOT1L, DYM, FBXW11, TEAD1, IGF2BP3, SUPTH3, ASTN2 and IER3* associated to cartilage thickness. Variants in these genes were previously reported as associated with height excepting *IER3.* For the majority of these genes (except for *DOT1L*) their expression in articular cartilage and joint tissues have

not been evaluated. It has been demonstrated that variants in *DOT1L, SUPTH3* and *ASTN2* are associated with hip OA [33, 34, 36]. However, the variants here reported are only in moderate LD with those that have been previously reported, excepting the *DOT1L* variant. *ASTN2* encodes a protein that is expressed in the brain and may function in neuronal migration. It has been associated with cognition and bipolar disorder, migraine and schizophrenia and recently with total hip replacement in females [34, 46-48]. According with our results, the association of *ASTN2* with OA seems to be mediated through its possible role in cartilage thickness (development/maintenance). The expression and function of *ASTN2* in joint tissue (cartilage and/or bone) needs to be demonstrated. The variants in *DOT1L, SUPT3H* and *ASTN2* showed to be gender specific. It might be considered that part of the complexity of OA rely on these genetic gender differences which need to be elucidated. In addition, *DOT1L* and *SUPT3H* interact in the same signaling pathway involved in transcriptional misregulation in cancer.

Our bivariate analysis used JSW at the superior site of the hip joint, which seems to be thicker than JSW at other joint sites in both studied populations (both older than 65 years). Therefore, we might conclude that this site of the hip joint is probably less affected by cartilage degeneration during OA. Subsequently, not all the identified signals necessarily showed association with hip OA. The reported signals might have a role during joint development, embryogenesis and in general endochondral ossification process, including formation of cartilage thickness and not necessarily in OA. One example of a gene involved in skeletal development and here reported associated with cartilage thickness but not with hip OA is *DYM*. *DYM* encodes a protein which is necessary for normal brain function and skeletal development having a crucial role on chondrocyte differentiation. Mutations in this gene are associated with two types of recessive osteochondrodysplasia syndromes which involve skeletal defects, short stature and radiological changes in different joints [49, 50]. Additionally, Dym mutant mice exhibit disorganized chondrocytes surrounded by thicker ossified regions containing numerous small islands of chondrocytes [51]. Degeneration of articular cartilage in OA is also associated with changes in chondrocytes some of them similar to those observed in Dym mutant mice during endochondral bone formation[52]. It might be the case that sever forms of mutation in this gene are required to produce a OA-like phenotype.

Rs1368380 is localized on *FBXW11*. *FBXW11* encodes a member of the F-box protein family which is characterized by approximately 40 aminoacid motif, the F-box. The F-box proteins constitute one of the four subunits of ubiquitin protein ligase complex called SCFs (Skp1-Cul1-F-box protein). This gene has a role in protein translation, cell

grow and survival [53]. *FBXW11* is implicated in the Hedgehog signalling pathway and its expression is negatively regulated by Wnt/beta-catenin pathway. Additionally, we found here that this gene together *TEAD1* are part of the Hippo signalling pathway, which has been studied as a potential target for limb and tissue regeneration. It might be also a possibility in the field of regeneration of cartilage tissue

Another signal was localized on the Insulin Growth Factor-2 Binding Protein 3 (*IGF2BP3*), that is a Glioblastoma-specific marker that activates Phosphatidylinositol 3-Kinase/Mitogen-activated Protein Kinase (PI3K/MAPK) Pathways by Modulating IGF-2. *IGF2BP3* interacts with Insulin-like growth factor 2 which has a major role as growth promoting hormone during gestation [54].

Altogether these elements show the relevance of identification of the variants associated with both traits: height and cartilage thickness and candidates genes for this association that participate in relevant pathways that might play a role in OA.

In conclusion, we found variants in different genes associated with cartilage thickness on the superior part of the hip joint and height, possibly underlying common processes of endochondral bone formation. Mild forms of variation in these genes might produce small to moderate phenotypic changes in cartilage and surrounding joint structures that might predispose to OA. Replication of these findings in other populations and studying effect of expression and mutation in these genes on articular cartilage of the hip joint will be pursued.

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Supplementary table 1. Studies included in TREAT-OA GWAS meta-analysis.

Study	N_cases (n=5244)	N_controls (n=17836)	Genotyping platform	Imputation method
arcOGEN	1728	4896	Illumina Human610 (cases) + Illumina 1.2M Duo (controls)	Impute
deCODE	2318	2318	Infinium HumanHap 300 + humanCNV370	Impute
EGCUT	64	2531	Illumina HumanCNV370 or HumanOmniExpress	Impute
GARP	106	1671	Illumina Infinium HD Human660W-Quad	Impute
RSI	760	3233	Illumina HumanHap550v3	MACH
RSII	159	1472	Illumina HumanHap550v3	MACH
RSIII	41	1487	Illumina HumanHap550v3	MACH
TwinsUK	68	228	Infinium HumanHap300	Impute

Chapter 3

Bone related factors predictive of hip OA:
hip geometry and bone mineral density

Chapter 3.1

The contribution of hip geometry to the prediction of hip osteoarthritis

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ABSTRACT

To determine how well measures of hip geometry can predict radiological incident hip osteoarthritis (OA) compared to well known clinical risk factors. The study population is part of the Rotterdam Study, a prospective population-based cohort. Baseline pelvic radiographs were used to measure hip geometry by two methods: Statistical Shape Modeling (SSM) and predefined geometry measures (PGP). Incident hip OA (Kellgren and Lawrence (KL) $\geq=2$) was assessed in 688 participants after 6.5 years without radiographic OA at baseline. The ability to predict hip OA was quantified using the area under the Receiver Operating Characteristics curve (AUC). Comparison of the two methods showed that both contain information that is not captured by the other method. At 6.5 years-follow up 132 hips had incident HOA. Five PGPs (Wiberg angle, Neck Width, Pelvic Width, Hip Axis Length and Triangular Index) and 2 SSM (modes 5 and 9) were significant predictors of HOA (p=0.007). Hip geometry added 7% to the prediction obtained by clinical risk factors (AUC= 0.67 (geometry), 0.66 (gender, age, BMI) and combining both: AUC=0.73, respectively). Mode 12 (associated with position of the femoral head in acetabulum) and Wiberg angle were predictors of OA in participants without radiological signs at baseline (KL=0). Contribution of variables to predict hip OA at a longer follow decreased however, was still significant for hip geometry (p=0.01). Hip geometry has a moderate ability to predict OA in participants with and without initial signs of OA, similar to and largely independent of the predictive value of clinical risk factors.

Keywords: Hip Geometry, hip osteoarthritis, prediction.

INTRODUCTION

Variations from what it is considered a "normal hip morphology" have been associated with hip osteoarthritis (OA). The reason might be that certain morphologies of the hip joint result in mechanical loads that increase stress at the articular surface (1-3). Certain extreme morphologies, such as congenital hip dysplasia can cause hip at a relatively young age (4, 5). For individuals with a less unfavourable morphology of the hips, OA might only develop when other risk factors are present as well and consequently hip OA occurs at older age.

Different approaches to quantify hip geometry exist. Generally, many predefined geometry parameters (PGP) applicable to radiographs have been described in literature that measure distinct traits of the hip joint (acetabulum, pelvis and proximal femur) in terms of distances, areas or angles. Within the group of PGPs, the Wiberg angle also known as Center Edge Angle (measuring acetabular dysplasia and position of the hip in relation to the acetabulum), femoral neck width and the triangular index (measuring asphericity of the femoral head) have been associated to the development and progression of hip OA (6-9). Other parameters have also been associated to hip OA in small studies without conclusive evidence: neck shaft angle, hip axis length, spherical sector, offset and pelvic width (9-13). In general all these PGPs have been studied in small separate efforts and their contribution to the prediction of incident hip OA is not well known.

Alternatively, the geometry of the hip joint might be quantified in a more general sense using Statistical Shape Models (SSM). SSM offer a relatively new and conceptually different approach that captures the entire shape. Each of the SSM measures, which are called modes, describes a distinct pattern of variation present in a population (14). SSM analysis has identified some distinct aspects of femoral shape that have been associated to clinical or radiological hip OA (15-17). However, it is unknown whether these modes capture the same aspects of hip geometry as the predefined geometry measures. In addition, it is unknown if hip geometry can be used to identify subjects that will develop hip OA in the future.

The aim of this study was to compare these two approaches to quantify hip geometry (PGP and SSM) with respect to predicting OA, and more specifically to determine the contribution of hip geometry to the prediction of incident radiographic hip OA.

METHODS

Study population

We used data of the Rotterdam study, a large prospective population-based cohort study among men and women 55 years of age. The study design and rationale are described elsewhere in detail (18). In summary, the objective of the study is to investigate the determinants, incidence and progression of chronic disabling diseases in the elderly. The medical ethics committee of Erasmus Medical Center approved the study and written informed consent was obtained from each participant. The baseline measurements were conducted between 1990 and 1993. In total, 7,983 participants were examined. The present study used a randomly selected sample of 750 subjects from Rotterdam study I (RS-1) among participants with a Kellgren and Lawrence score (KL) at baseline ≤1 in both hips. Participants with hip fracture and participants with low quality radiographs or with artifacts on both hips were not included. Only subjects with completed follow up were included. This resulted in a total of 1,283 hips from 688 participants.

Clinical evaluation and physical examination

At baseline, medical information and physical examination, including measurements of height and weight were obtained.

Radiographic assessment

Weight-bearing antero-posterior pelvic radiographs were taken with both of the patient's feet positioned in 10° internal rotation and the x-ray beam centered on the umbilicus. Both at baseline and at 2 follow-up visits (mean time to follow-up: 6.5 and 11 years) hip joints were scored using the KL-grading system, by two independent observers who were trained by an experienced physician in OA and advised by a radiologist (19). The presence of OA features (osteophytes and joint space narrowing) was evaluated using as reference an atlas of individual radiographic features in OA. The final KL-score was a composite score according to the presence of both features: narrowing of the joint space (superior, medial, axial) and superior osteophytes (femoral and/or acetabular) scored from 0-3 according to the atlas (20). Incident hip OA, determined at each follow-up visit separately, was defined as a KL of 2 or more (Definite narrowing of the joint space and at least possible osteophytes; equivalent to grade 1 in the atlas for each feature) or a total hip replacement (THR). KL was scored for both hip joints. Kappa statistic for KL-score was 0.68 (inter-rater reliability).

Statistical Shape Modeling (SSM)

A set of 67 points were placed by one observer (MC) and used to delineate the contours of proximal femur, pelvis and acetabulum to create the statistical shape model (Figure 1). Using the freely available ASM toolkit (Cootes et al. Manchester, UK), we constructed an SSM of the 1,283 hips (Figure 1a). The independent modes of variation in hip shape were extracted by Principal Component Analysis. The first 24 modes that were used in this study, explained 90% of the variance in hip shape (Supplementary figure 1).

Predefined Geometry Parameters (PGP)

Using the contour points of the SSM, we automatically calculated 12 geometry parameters (PGP) that describe different aspects of the femoral head, acetabulum, femoral neck and pelvis: triangular index, head radius, neck shaft angle, head-neck ratio, spherical sector, Wiberg angle, neck width, neck length, hip axis length, Isquiopubic index, pelvic width and offset (Figure 1b and 1c). Explanation about methods for calculating PGPs was included in the supplementary data (supplementary table 1).
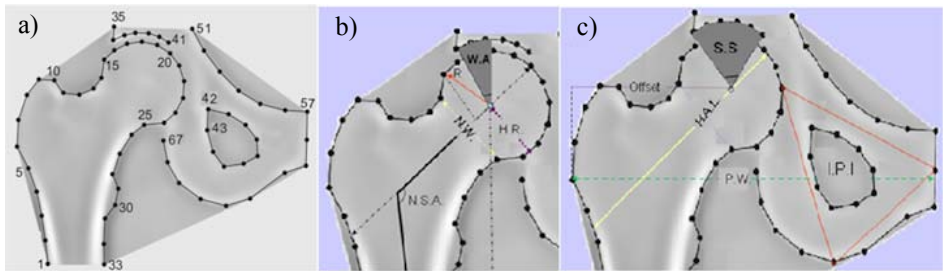
Figure 1   a) set of 67 points were used to delineate the contours of proximal femur, pelvis and acetabulum to create the Statistical Shape Model (SSM). **b & c)** Schematic representation of the predefined geometry parameters used in this study. **b)** Neck Width (NW), Head Radius (HR), Wiberg angle (W) "in dark gray", Neck Shaft Angle (NSA), Triangular Index (TI), dotted line (red line shows resulting Radius (R)). **c)** shows in dark gray: Spherical Sector (SS), Pelvic Width (PW), Isquiopubic Index (IPI) "orange triangle", Hip Axis Length (HAL) and Offset.

Intra and Inter-Observer agreement for shape modes and geometry parameters

A subset of 46 hips was used to measure within and between observer agreement in shape modes and predefined geometry measurements. Two observers (JW and MCC) placed the 67 points for each hip. Intraclass Correlation coefficients were used to analyze intra and inter-observers agreement.

Supplementary figure 2a and 2b show the intra- and inter-observers agreement values for each of the modes and predefined geometry parameters, respectively. For the shape modes, we found a mean ICC value of 0.78 for intra- and 0.80 for inter-observer agreement. Some modes had an ICC below 0.7: mode 7, 9, 12 and 13 (Supplementary figure 2a). Mean ICC for the predefined geometry parameters were 0.92 for intra- and 0.86 for Inter-observer agreement. Almost all PGPs had an ICC above 0.7 (Supplementary figure 2b). Triangular-index had a low inter-observer ICC but a high intra-observer ICC (Supplementary figure 2b. 0.54 and 0.90 respectively). For this parameter it is necessary to fix specific points around the femoral head, which apparently was done slightly different by the two independent readers.

Correlation among PGPs and among modes

We studied correlations between all predefined geometry measures using Pearson correlation test statistic (Supplementary Table 2, r-squared). We observed a high number of significant correlations between the PGPs, most significant were the correlations (r-squaredx100) between hip-axis length and head radius (72%), head radius and neck width (69%), triangular index and neck width (67%), neck width and pelvic width (55%), neck width and hip- axis length (54%), spherical sector and Wiberg (52%), offset and hip-axis length (45%). We defined moderate correlation as a $R^2>70\%$ and high correlation as a $R^2>80\%$ between two parameters.

Theoretically, all modes should be independent of each other. However, we found some significant correlations between some of the higher (explaining less variance in shape) modes, which could be due to mild non-linear correlation between variation in points.

Variation in geometry explained by modes

We examined how much of the variation in PGPs is captured by the modes. Using linear regression we found that all 24 modes together explain between 37% and 95% of the variation in each predefined geometry parameter (Supplementary Figure 3a). These percentages are lower for parameters that represented angles or ratios like spherical sector and head/neck ratio (Supplementary Figure 3a, Neck-shaft angle: 64%, Wiberg angle: 60% and triangular index: 57% and head/neck ratio: 37% respectively).

Variation in modes explained by PGP

Similarly, we examined how much of the modes were explained by the PGPs (Supplementary figure 3b). A model for each mode was constructed, including the PGPs that were significant for that respective mode. Only significant PGPs contributed to explain the variation in modes. In general the PGPs explained only a small part of the total variation in hip shape as represented by SSM. The selected PGPs explained a high proportion of variation only for the two first modes (Supplementary figure 3a, R²: 0.5 and 0.53 respectively). PGPs explained between 30% and 50% of the variation for modes between 4 and 8 and generally less than 30% after mode 9 (Supplementary Figure 3b). For each mode a different set of geometry parameters was significant.

Statistical Analysis

We calculated Pearson's correlation coefficients within the set of modes and within the set of geometry parameters. R^2 from linear regressions was used to estimate the proportion of variance in each mode of the SSM explained by PGPs and the percentage of variation in each PGP explained by the 24 modes of the SSM.

The associations between the SSM modes and the PGPs with incident hip OA (defined as KL>=2 or having had a THR at follow up) were determined using Generalized Estimating Equations (GEE) which takes the correlation between left and right sides into account. The analyses were further adjusted for gender, age, height and BMI. When two PGPs were correlated (R²?0.7) the most significant was included in the final model. To correct for multiple testing we set for significance a *P*-value of 0.05 divided by the number of parameters tested (Bonferroni adjustment). Testing the SSM modes, we considered *P*-values lower than 0.0021 (=0.05/24) to be significant. Similarly, *P*-values lower than 0.0042 were considered significant for the analysis of the 12 PGPs. All *P*-values lower than 0.05 were reported.

To assess the contribution of PGPs and SSM modes to the prediction of incident hip OA, multivariable GEE models were constructed using the significant PGPs and SSM modes. These models were compared using DeLong's method (21) on the area under the ROC curves (AUC). SPSS v.15 (SPSS Inc., Chicago, IL, U.S.A) and MedCalc v12.2.1.0 (MedCalc Software bvba, Belgium) were used for the statistical analyses.

RESULTS

Baseline characteristics

Baseline characteristics of the study population are described in table 1. The mean age of the population was 65.6 years. At first follow up, 119 subjects (132 affected hips) had incident hip OA. At second follow up only 56 new incident cases were registered (comprising 65 new incident hips with OA). Participants with incident OA (KL>=2) at follow up were at baseline older, taller and more often females (Table 1). Results for intra- and inter-observer agreements for the assessment of the geometry parameters of both SSM and PGP methods, correlation between variables of each method and percentage of variation explained of the measures of one method by the measures of the other method are presented as supplementary material.

Table 1. Baseline characteristics of the study subjects.

Baseline Characteristics	No-OA (n=569)	OA (n=119)	P value
Age (years)	65 (0.27)	68(0.58)	< 0.0001
Female*	315 (55.4)	84 (70.6)	0.002
Height (cms)	168.6(0.55)	170.3(0.20)	0.03
Weight (Kg)	74.7(0.4)	75.9(0.89)	0.24
BMI (kg/m^2)	26.4(0.29)	26.7(0.10)	0.37
KL 0*	280(49.2%)	13 (10.9%)	< 0.0001
KL 1*	289 (50.8%)	106 (89.1%)	

No-OA: subjects without hip osteoarthritis (OA) after 6.5 years follow up. OA= cases with radiological osteoarthritis (KL2) at first follow up. N=688 individuals, 1,283 hips. Presented values are means with SD between brackets for continuous variables and numbers with percentages between brackets for categorical variables.

Statistical Shape Modes

Mode 5 and mode 9 were significantly associated with incident hip OA after Bonferroni adjustment for multiple testing ($P_{threshold}$0.0021, table 2). We used the graphic tool of the ASM toolkit to visually interpret the shape variations that each mode represented. Mode 5 appeared to represent internal and external rotation of the femur and

corresponding variation in the pelvis, such that the femur is placed slightly in and out of the acetabulum (Figure 2). Higher risk of incident OA appears to be associated with less covering of the femoral head by the acetabulum. (Odds Ratio per standard deviation in mode 5 (OR): 1.54, and 95% Confidence Interval (CI): 1.30-1.85). Mode 5 was the variable more significantly associated with narrowing of the joint space at follow up (p=0.001), positively associated to the Wiberg angle and negatively to the triangular-index (p< 0.01, not in table). Visually, mode 9 mainly represents variation in length of the femoral neck, due to variation in the superior neck (Figure 2). Mode 9 was negatively associated to the Wiberg angle and positively to the triangular-index. A higher risk for incident hip OA corresponded to mode 9 values that represent a shorter neck (OR: 1.40, CI: 1.14-1.72).

Mode 12 was the only geometry parameter associated to incident OA in participants that had KL=0 at baseline (Table 4, OR:1.69, CI: 1.24-2.30). It appears to represent variation in acetabular version with corresponding rotation of the femur (Figure 2). Mode 12 was positively associated to the spherical sector, triangular-index and pelvic width (more covering of the femoral head and wider pelvis) ($P<=0.01$) and to a higher risk for OA in subjects without initial osteoarthritic changes (OR: 1.69, CI: 1.24-2.30).
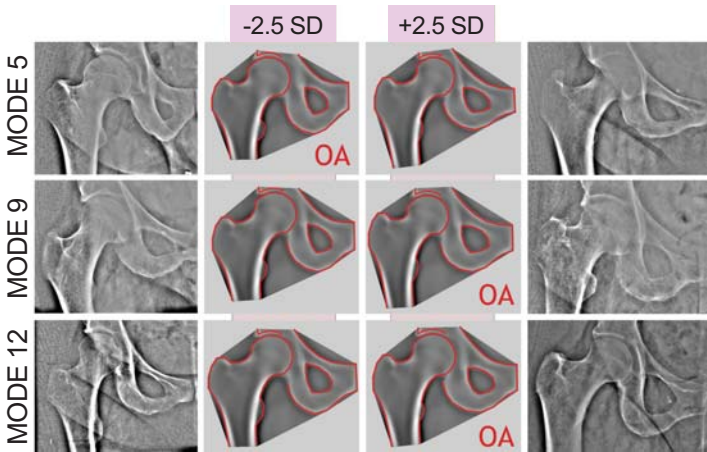
Figure 2   A visual representation of the extremes of the range of variation of SSM modes 5, 9 and 12 (-2.5 and +2.5 times the population standard deviation). The left and right columns contain true radiographs of subjects with extreme scores on the specific modes.

Predefined geometry parameters

After Bonferroni adjustment, higher values for neck width, pelvic width, hip axis length and triangular index and lower values for the Wiberg angle corresponded to a higher risk

for incident hip OA at first follow up (Table 2, *P*<0.0042). Adjustment for pelvic rotation (FOI) and hip size (scaling factor) did not influence the association with incident hip OA for these parameters. Gender was negatively correlated to scaling factor (r^2=-0.56) and influenced the association of PGPs, especially those "bone size related parameters" and OA.

Table 2. Association between hip geometry (shape modes and predefined geometry parameters) and incident hip OA at first follow up.

Parameters	6.5 years incidence hip OA n. cases =119 (132 hips)	
	*OR (CI)	P value
SSM		
Mode 5	0.65 (0.54-0.77)	< 0.0001
Mode 6	0.80 (0.66-0.97)	0.026
Mode 7	1.23 (1.02-1.50)	0.034
Mode 8	1.31 (1.08-1.58)	0.005
Mode 9	1.40 (1.14-1.72)	0.001
Mode 10	1.35 (1.11-1.64)	0.003
Mode 12	1.22 (1.02-1.45)	0.026
PGP		
Wiberg	0.76 (0.63-0.92)	0.004
Spherical Sector	1.26 (1.06-1.50)	0.011
Head Radius	1.41 (1.09-1.82)	0.01
Neck Width	1.60 (1.24-2.05)	2.45×10^{-4}
Hip Axis Length	1.49 (1.18-1.90)	0.001
Pelvic Width	1.43 (1.16-1.75)	0.001
Triangular Index	1.93 (1.54-2.43)	< 0.0001

*Odds ratios (OR) and Confidence Interval (CI) are presented as for SD change in each parameter. All Statistical Shape modes (SSM) and Predefined Geometry Parameters (PGP) with p0.05 are presented. Each parameter was analysed in relation with incidence of hip osteoarthritis (OA) at first follow up after adjustment for gender, age and BMI.

Prediction models of incident hip OA

Hip geometry alone (PGPs+modes) demonstrated moderate discriminative value for incident hip OA at first follow up (Table 3, AUC: 0.67). This value was similar to the

predictive value based on demographic parameters that are known to associate with hip OA (age, gender, height, BMI; AUC: 0.66). Addition of PGP and modes increased the predictive value of demographic risk factors by 5% and 6% respectively (Table 3, p=0.014 for PGPs and p=0.002 for modes). Inclusion of the combination of PGPs and modes did not further increase the predictive power. The inclusion of all modes and PGPs that are associated to incident hip OA at p<0.05 (from table 2), increased the area under the ROC only 2% (N.S.)

Table 3. Area under ROC curves for models to predict hip OA at 6.5 and 11 years follow up.

Models	First follow-up 6.5 years		Second Follow-up 11 years	
	AUC-ROC (95%CI)	P value	AUC-ROC (95%CI)	P value
Baseline Characteristics	0.66 (0.64-0.69)	Ref.	0.56 (0.53-0.59)	Ref.
1) PGP + modes	0.67 (0.64-0.69)	0.88*	0.66 (0. 63-0.69)	0.06*
2) Base+modes	0.72 (0.67-0.76)	0.002*	0.59 (0.56-0.62)	0.27*
3) Base+PGP	0.71 (0.68-0.73)	0.014*	0.67 (0.64-0.70)	0.016*
4) Base+PGP+ modes	0.73 (0.71-0.76)	0.007*	0.68 (0.65-0.71)	0.011*
5) Base+KL	0.83 (0.81-0.85)	<0.0001*	0.57 (0.54-0.60)	0.72*
6) Base+KL+PGP+modes	0.82 (0.80-0.84)	0.42**	0.68 (0.65-0.71)	0.014**

AUC-ROC= Areas under the receiving operator characteristics curve and 95% confidence interval (95%CI). *P values are given for AUC comparison between baseline characteristics (gender, age, BMI and height) and the following models: 1) Only geometry parameters; Predefined Geometry Parameters (PGP): Wiberg, neck width, pelvic width, hip axis length triangular index and modes: 5 and 9, 2) Baseline characteristics and modes (5 and 9), 3) Baseline characteristics and the Predefined Geometry Parameters (PGP), 4) Baseline characteristics and geometry: Predefined Geometry Parameters (PGP) and modes, 5) Baseline characteristics and Kellgren and Lawrence score (KL) ** P value for comparison of the full model with model 5 (Baseline characteristics and Kellgren and Lawrence score (KL).

Additionally, we analyzed the contribution of the selected SSM modes and PGPs to the prediction of the incident cases at second follow up (n=56) compared also to the demographic risk factors. In general, predictive values decreased at second follow up for all variables. Prediction given by baseline characteristics was 10% lower. The contribution of the selected PGPs (Wiberg angle, neck and pelvic width, hip axis length and triangular index) compared to baseline characteristics was around 11% (Table 3, P=0.016 for ROC' curves comparison with baseline characteristics). The two selected SSM modes did not add to the prediction at second follow-up.

Table 4. Incident hip OA at first follow up and modes based on selection of KL=0 or KL=1 at baseline.

	KL=0 O.R. (CI)	Pvalue	O.R.(CI)	KL=1 Pvalue
SSM				
mode5	0.43 (0.26-0.70)	6.6×10^{-4}	0.71 (0.58-0.87)	$8.0 * 10^{-4}$
mode6	1.04 (0.65-1.67)	0.86	0.80 (0.64-1.00)	0.05
mode7	1.52 (0.88-2.61)	0.13	1.19 (0.93-1.51)	0.17
mode8	0.94 (0.59-1.50)	0.80	1.41 (1.12-1.78)	0.003
mode9	1.41 (0.83-2.34)	0.20	1.30 (1.04-1.64)	0.023
mode10	1.10 (0.74-1.63)	0.64	1.36 (1.09-1.69)	0.007
mode12	1.69 (1.24-2.30)	9.4×10^{-4}	1.01 (0.80-1.28)	0.94
PGP				
Wiberg	0.44 (0.26-0.73	0.001	0.74 (0.59-0.93)	0.009
Spherical Sector	0.93 (0.66-1.32)	0.70	1.33 (1.07-1.64)	0.010
Head Radius	1.54 (0.93-2.56)	0.09	1.37 (1.02-1.84)	0.039
Neck Width	1.31 (0.76-2.27)	0.33	1.57 (1.06-2.33)	0.025
Hip Axis Length	1.70(1.11-2.62)	0.015	1.47(1.14-1.90)	0.003
Pelvic Width	1.42 (0.91-2.21)	0.12	1.26 (0.99-1.60)	0.06
Triangular Index	1.26 (0.60-2.62)	0.54	1.69 (1.32-2.17)	< 0.0001

Association of SSM= Statistical Shape Modes, PGP= Predefined Geometry Parameters and incident hip OA at first follow up was evaluated according with the Kellgren and Lawrence score (KL) at baseline (KL=0 or KL=1). Values presented are odds ratios (O.R) and 95% confidence intervals (CI) per SD change in the respective parameter.

Since many of the KL=1 cases probably have early OA and thus do not represent true incident OA, we stratified the group according to KL at baseline. For the KL=0 cases, mode 5 and 9 still showed association with incident hip OA, albeit not significant anymore for mode 9 (Table 4). In these subjects, mode 12 also gave a significant association to incident hip OA.   Mode 12 contributed around 9% to the prediction of OA given by baseline characteristics in individuals with KL=0 at baseline (Not in table, ROC: 0.61, CI: 0.54-0.67). Of the PGPs, only Wiberg angle reached significance when hips with KL=0 were selected (Table 4), though the effects of the other PGPs did not disappear.

Predictions based on hip geometry for OA were of very low specificity for incidence OA using the first follow up the sensitivity was 0.998 while specificity was very low, around 0.05. Thus, the probability that a positive prediction was a true positive is around 0.104 while the probability that it was a false positive is 0.896 for any particular positive result (+OA). On the other hand, the probability that a negative prediction was a true negative was around 0.995 while the probability that it was a false negative is very low: 0.0047.

DISCUSSION

In this study we investigated the extent to which hip geometry contributes to the prediction of radiological incident hip OA. In line with previous studies, we showed that distinct aspects of hip shape are clearly associated to incident hip OA. Additionally, we demonstrated that hip geometry can improve the prediction given by clinical risk factors in subjects with or without initial signs of OA (KL=0/1). Ability of hip shape to predict incident hip OA was similar and slightly better than the common demographic risk factors age, gender, height and BMI. Hip geometry contributes between 8 and 12% to the prediction given by clinical risk factors for participants with or without initial radiographic changes respectively. Two different approaches were used to quantify hip geometry: predefined measures and a Statistical Shape Model (SSM). In the first approach, we selected specific measures from literature, which have been shown to be related to mechanical load on the hip or to OA. The second hypothesis-free approach used a SSM to find measures of hip shape that represent distinct patterns of correlated aspects of hip geometry within the total variation of hip shape in our cohort. In theory, the SSM represents the most complete information on hip shape. Indeed, the predefined geometry measures could only partly explain the variation in the SSM modes. Vice versa, the modes explained well the measures of size, but could not fully describe those measures that represented angles and ratios. Both methods appear to contain information that is not captured well by the other method; since they describe different aspects of hip geometry we consider that depending of the study' subject they might complement each other.

Since the OA process alters the shape of the bones in the affected joint, the question arises whether the geometry aspects associated with OA represent a cause or consequence of OA. The strength of the association for many measures was similar in subjects with KL=0 at baseline when compared to the overall association, suggesting that these measures might represent shape variants that pre-date radiological OA. Exceptions were Neck Width, Triangular Index, Spherical Sector and a few modes that showed a lower association with OA and thus might reflect changes in shape related to bone remodelling during early OA. Interestingly, Wiberg angle and mode 12 were predictors of OA in subjects without initial signs of OA (KL=0), and also contributed to the prediction of incident OA at second follow up. These measures might thus represent shape aspects that are a causing factor for OA. However, these statements remain only as tentative explanations, since we had low power in the group with KL=0 to derive conclusions on the differences between the groups (KL= 0/1).On the other hand, for many of the association with hip geometry parameters the relation was stronger or only

present for those with KL=1. This indicates that at least part of the observed associations might actually represent active bone remodelling that it is known to occur at early stages of OA and that might be considered as an important components of the pathogenic process that leads to OA (22). Alternatively, bone adaptation in OA can be mechano-regulated with structural changes that might occur independent of cartilage degeneration (23).

According with our results, subjects with OA had higher values of bone size related parameters and gender adjustment increased the strength of the associations between geometry and OA. It was not the case for adjustment of the scaling factor. Association between bone size, geometry and OA might share common etiological factors; It has been recently discussed that genes implicated in bone formation and growth have also a role in OA (pleiotropic effects), what could be part of the explanation. Prediction of OA decreased for all variables using a longer follow up. The demographic risk factors for OA, gender, age, height and BMI lost their predictive value for the 11 years follow-up. The predictive power of geometry also decreased to a value between 6 and 16%. The decrease in predictive value of geometry at second follow up was not exclusive for shapes but, it was more pronounced for them. It might be explained by the sensitivity of shapes to detect early OA-changes, including bone remodelling. On the other hand, larger variations in geometry might cause OA earlier in life as is the case for subjects with severe dysplasia and impingement where OA develops several years earlier than for subjects without these large geometrical differences (24, 25).

Many shape aspects that were found to be relevant for hip OA, appear to be related to the congruency between femur and acetabulum determined by asphericity of the femoral head, more specifically the shape of the superior head-neck junction, and the shape of the acetabular socket (acetabular dysplasia). Asphericity of the femoral head results in impingement of the head against the labrum, eventually resulting in damage which might trigger the development of OA. Typical is the anterolateral prominence or cam deformity which is thought to be formed during adolescence as a result of physical activity (26, 27). Also less severe forms of asphericity like a flattening of the head-neck junction (pistol grip deformity) have been associated to OA (9). These shape aspects are generally measured by the alpha angle or the triangular index, while other measures like the width of the neck or the head-neck ratio are also influenced. This study further supports these findings, with significant associations of the triangular index, neck width.Our results corroborate earlier publications on mild dysplasia as a risk factor for hip OA (7), in the current study indicated by the effects of the Wiberg angle and mode 5. Besides, the association of high values of the spherical sector with increased OA-risk supports the

idea that deep placement of the femoral head predisposes for OA as in cases of protrusion acetabuli where there is a progressive migration of the femoral head into the pelvic cavity (28, 29).

The conclusions of this study extend only to cases of radiographic OA since we did not consider information on clinical OA symptoms. Other limitation derives from the interpretation of geometry from 2D x-ray images that might be simultaneously influenced by true changes in geometry and by positional variation in the bones. Thus, we cannot be certain whether the found association with OA is due to true geometry variants or due to differences in bone position, especially since variation in position of the bones could reflect hip pain and OA-related limitations in internal rotation of the hip. Visual inspection of the modes of the SSM, however subjective, does give some indication of whether positional variation plays a role (30). In the same manner, interpretation of what modes represent is subjective of nature. Thus the diagnostic value of SSM is rather limited. Further, although reproducibility of the predefined geometry measures was good, we did not validate these measures with the same geometry parameters measured by hand using the original protocols. Finally, in spite of the significant predictive value of geometry for hip OA in general, predictions based on hip geometry were of very low specificity. Clinical utility of hip geometry need to be tested in groups at higher risk, for example in groups at higher genetic risk for OA.

The advantage of the use of predefined geometry measures is that it seems simple and intuitive. However, some measures correlate hampering statistical analysis and interpretation of findings. This drawback is absent when SSM is used, since all modes are theoretically independent, although we found some mild correlations in the higher modes. When we combined the two methods in a predictive model for OA, we observed that the PGPs did not contribute additionally to the prediction of incident hip OA made by the shape modes, indicating that the majority of relevant geometry information for OA is contained in the modes of the SSM.

In conclusion, this study confirms that hip geometry is strongly associated with OA and it is able to predict OA similar to known risk factors. Some variations in hip geometry are associated with early osteoarthritic changes but others might precede radiologic OA contributing to the prediction of incident OA in subjects without radiological evidence of OA.

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AUTHOR CONTRIBUTIONS

All authors contributed to the research project's design and conception; data analysis and interpretation; and in the writing, revising and final approval of the manuscript. Castaño Betancourt MC, Waarsing JH and van Meurs JBJ take responsibility for the integrity of the work presented to the Journal.

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Conflict of interest. None of the authors have conflicts of interest related to the manuscript.

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SUPPLEMENTARY METHODS

In order to calculate the PGPs, we derived a few reference points and lines from the manually placed contour points. All measures were defined by a combination of manually placed landmark points, reference points and lines derived from the landmark points and the contours. A complete description of the geometry parameters and how they were calculated are presented in Supplementary table 1. Additionally, a reference line was formed between the points (point 42) in the corner of the foramen on the radiographs of both left and right side. It was used as horizontal reference line representing the inclination of the pelvis. To control for possible pelvis rotation/inclination and size differences that could affect some measurements we used "the Foramen obturator index (FOI)" from Tonnis [22] and a scaling factor; The scaling factor was then defined as the ratio of this summed distance and the mean of these summed distances of all images. It was used as a covariate to adjust the predefined geometry parameters in the comparison of predefined geometry parameters and modes. All geometry parameters were calculated using Matlab.

Supplementary table 1. Description of predefined Geometry Parameters.

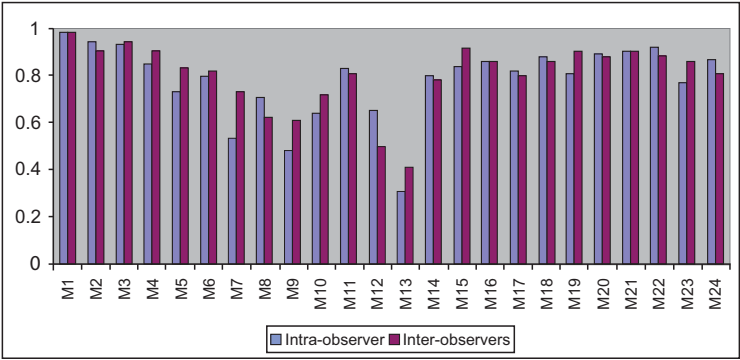| Predefined Geometry Parameter	Description
1. Hip Axis length (HAL)	Distance from pelvic rim to the lateral side of the femur along the neck axis. The neck axis was defined by the line through the center of the head (see fig. 1b, HR) and the center of the neck. The center of the neck was defined as the average of the points on the contour of the neck. HAL is the length of this line from where the line intersects with the contour of the pelvic rim to where the line intersects with the lateral side of the femur.
2. Neck Shaft Angle (NSA)	Angle of inclination of the femoral neck. It was calculated as the angle between the neck axis (see HAL) and the shaft axis. This latter was found by drawing a line through the center of the shaft (average of the 4 most distal points of the femur) and a point place at the major trochanter (Fig. 1a, point 11).
3. Triangular Index (TI)	Index for assessing asphericity of the femoral head. The triangular index was calculated by drawing a line perpendicular to the neck-shaft axis (see HAL) starting at a point on the neck-shaft axis, placed a distance equal to half the radius of the head laterally from the center of the head (see HAL). The line ends at a point where the line intersects with the superior contour of the head or neck. The triangular index was defined as the difference between the length of this line and the radius of the head.
4. Spherical Sector (SS)	Angle formed by two lines drawn that connect the Center of the femoral head (see HAL) to the lateral and medial edges of the acetabular sourcil (Figure 1a, points 36 and 41). (Bombelli R).
5. Angle of Wiberg	Or Center Edge (CE) angle. Angle formed by the vertical reference line (perpendicular to the horizontal reference line) through the center of the femoral head (see HAL) and the line connecting the center of the femoral head to the most exterior aspect of the acetabular margin (point 36).
6. Head Neck ratio (H/N)	Ratio between head radius (see HR) and minimal neck width (see NW).
7. Pelvic Width (PW)	The length of the line, perpendicular to the vertical reference line, between the most lateral side of the femur and the most medial side of the pelvis. The length is found by projecting the most lateral and most medial points onto a line that runs perpendicular to the vertical reference line.
8. Femoral Offset	Length of the line, perpendicular to the vertical reference line that runs from the center of the head to the most lateral side of the femur (similar to PW).
9. Isquio-pubic Index (IPI)	Relation between the Pubic length and ischium length, defined by the triangle formed by points 41 (A), 58 (B) and 62 (C) in figure 1a. IPI= length of pubis(AB)/length of ischium(AC)*100
10. Head radius (HR)	The center of the head and the radius of the head were found by fitting a circle to the medial points of the femoral head (points 17 to 23 in figure1a).
11. Neck Width (NW)	Minimal neck width was found by the minimal distance between a curve fitted to the points of the superior neck and a curve fitted to the points of the inferior neck.
12. Neck Length (NL)	Distance between the center of the femoral head (see Head radius) and the intersection of the neck axis (see HAL) and the shaft axis (see NSA).

Supplementary table 2. Correlation (r^2) between Predefined Geometry parameters.

PGP	Wiberg	SS	HR	NW	H/N	NSA	HAL	PW	Offs	IPI	TI	Scaling
Wiberg	1	0.52	-0.18	-0.11	-0.04	-0.22	-0.29	-0.06	0.19	-0.11	0.002	-0.10
SS	0.52	1	-0.13	-0.06	-0.05	-0.01	-0.15	-0.03	0.02	-0.18	0.03	-0.08
HR	-0.18	-0.13	1	0.69	0.14	0.13	0.72	0.62	0.32	-0.04	0.56	0.57
NW	-0.11	-0.06	0.69	1	-0.48	-0.97	0.54	0.56	0.36	-0.13	0.67	0.48
H/N	-0.04	-0.05	0.14	-0.48	1	0.28	0.11	-0.08	-0.15	0.10	-0.2	-0.07
NSA	-0.22	-0.01	0.13	-0.9	0.28	1	0.27	-0.23	-0.45	0.03	-0.24	-0.12
HAL	-0.29	-0.15	0.72	0.54	0.11	0.27	1	0.66	0.45	-0.03	0.52	0.61
PW	-0.06	-0.03	0.62	0.56	-0.08	-0.23	0.66	1	0.72	-0.01	0.62	0.67
Offset	0.19	0.02	0.32	0.36	-0.15	-0.45	0.45	0.72	1	-0.12	0.52	0.55
IPI	-0.11	-0.18	-0.04	-0.13	0.10	0.03	-0.03	-0.01	-0.12	1	-0.50	-0.09
TI	0.002	0.03	0.56	0.67	-0.2	-0.24	0.52	0.62	0.52	-0.50	1	0.51
Scaling	-0.10	-0.08	0.57	0.48	-0.07	-0.12	0.61	0.67	0.55	-0.09	0.51	1
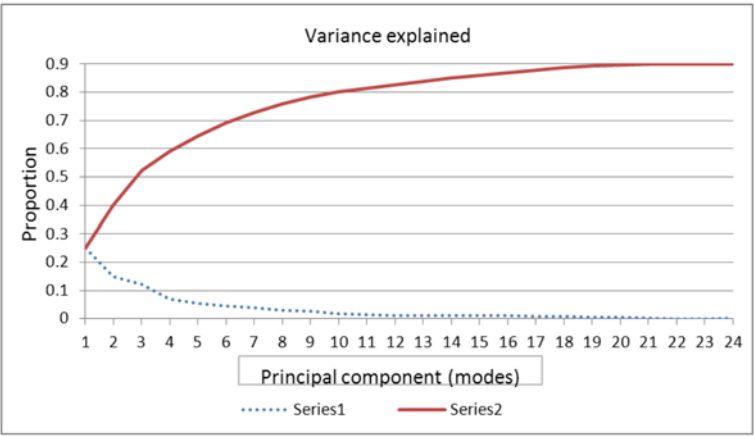
Supplementary figure 1   Individual and cumulative variance explained by each of the 24 SSM modes.

Supplementary figure 2a. Intraclass correlation coefficient for SSM modes.
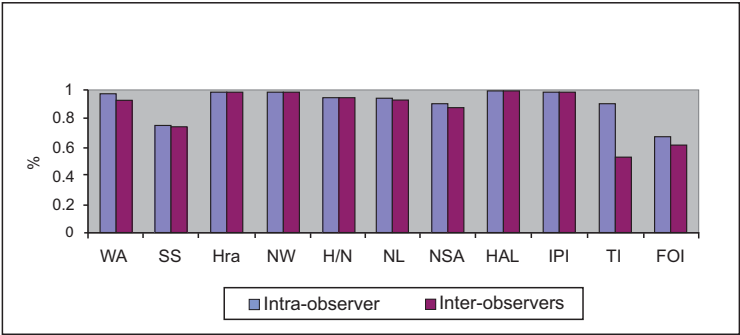
Supplementary figure 2b   Intraclass correlation coefficient for predefined geometry parameters.
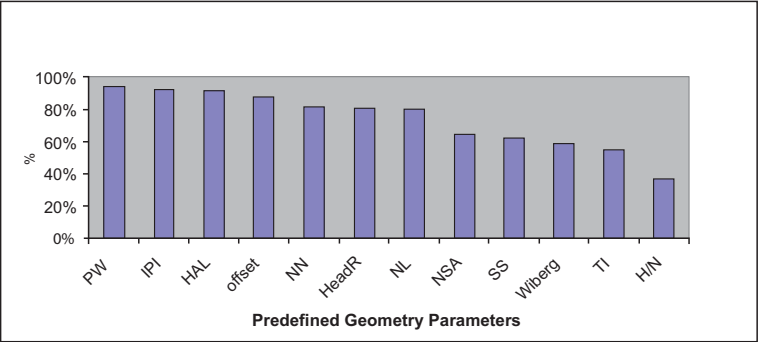
Supplementary figure 3a Variance in predefined geometry parameters explained by SSM modes.
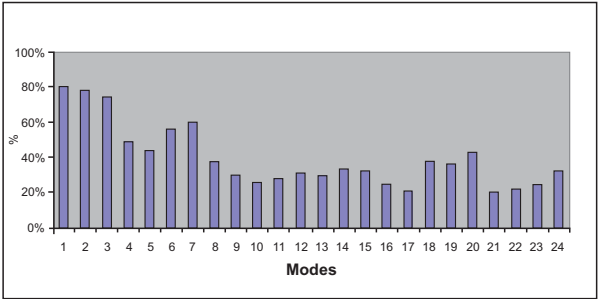
Supplementary figure 3b Variation in SSM modes explained by predefined geometry parameters.

Chapter 3.2

Dual energy X-ray absorptiometry analysis contributes to the prediction of hip osteoarthritis progression

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ABSTRACT

To determine if structural bone parameters obtained from dual energy X-ray absorptiometry (DXA) contribute to the prediction of progression of hip osteoarthritis (OA) and to test if the difference between the most affected (OA) hip and the contralateral hip adds to this prediction. The study group involves a prospective cohort of 189 patients that met the ARC classification criteria for hip OA. Progression was defined as 20% joint space narrowing or total hip replacement within a two years follow up. Software was developed to calculate geometrical aspects and BMD in different regions of interest of the proximal femur. Logistic regression was used to test if Kellgren and Lawrence (K-L) scores and DXA parameters can predict "progression" of OA. Models were compared using -2log likelihood tests, R^2 Nagelkerke and areas under the Receiver Operator Characteristic curves, assessed using 10-fold cross validation.The model that included the DXA variables was significantly better in predicting hip OA progression than the model with K-L score of the affected side alone ($P<0.01$). The addition of the differences in DXA parameters between the most affected and contralateral hip in the superior part of the femoral head, trochanteric and intertrochanteric area further improved the prediction of progression ($P<0.05$). K-L score of the affected side was still the most significant single variable in the models. DXA parameters can significantly contribute to the prediction of progression in patients with hip OA. The analysis of the DXA differences between the hips of the patient represents a small but significant contribution to this prediction. These analyses show the importance of bone density changes in the etiology of OA.

Key Words: hip osteoarthritis, progression, densitometry, femoral head.

INTRODUCTION

Osteoarthritis (OA) is a degenerative joint disease characterized by progressive damage of the articulate cartilage, occasional inflammation of the synovium, osteophytosis and alterations in the subchondral bone. It is often hypothesized that subchondral bone changes play an important role in either initiation or progression of OA [1-2]. Changes in bone shape, bone mineral density (BMD) and subchondral bone mechanical properties were reported in the presence of radiographic signs of hip OA [3-8]. A number of studies were performed that correlate radiographic osteoarthritis and/or clinical symptoms with bone measurements based on DXA that are typically performed in relation to osteoporosis. These measures concern BMD in the hip or spine at specific regions of interest such as e.g. the femoral neck. This data is rather confusing and conflicting in many aspects. An increased local and remote BMD has been reported in patients with radiographic hip OA [9], suggesting an inverse relationship between OA and osteoporosis. This was confirmed by Goker et al. [10] in patients that underwent total hip replacement, where the subjects with high progression of Joint Space Narrowing (JSN) at their contralateral hip had elevated BMD in both hip and spine.

Antoniades et al. only found this inverse relationship between local BMD and osteophytosis and not with JSN [11]. Other studies report an inverse relationship only in the affected hip and even a decreased BMD at remote sites and the contralateral hip [12-13]. This was further substantiated by Sandini et al, finding higher Bone Mineral Content (BMC) and larger area in the DXA data from patients with hip OA [14]. Changed muscle conditions and weight bearing may alter the load conditions in OA and local bone density changes may be the result of adaptation to an altered load distribution through the bone structure. Altogether, there seems to be conflicting data concerning the relationship between bone related parameters in OA. The variables that have been analyzed using DXA are often defined only in regions of interest that are relevant for osteoporosis, for which DXA has been specifically designed. These regions are e.g. the femoral neck and vertebral body. Beck and co-workers have designed methods to analyze a number of other parameters that are related to biomechanical aspects of the narrowest region of the proximal femur, an area of high interest in osteoporosis [15]. However, for OA other regions might be of more interest, such as the subchondral bone BMC or BMD.

The rate of progression of hip OA varies largely between patients. Some patients with radiographic signs of initial hip OA do not show disease progression for years. In other cases the disease progresses relatively fast, e.g. needing total hip replacement after less

than two years after onset of the first symptoms. The determinants of this progression are largely unknown [16]. It is also unclear what the role is of BMD, BMC or morphological bone variations on progression of hip OA. Better understanding of the involvement of alterations in the bone might allow early identification of cases and maybe even provide opportunities for early intervention. Therefore, this study aims to determine if structural bone geometry and density parameters as determined by hip DXA scans in the proximal femur, contribute to the prediction of OA progression. Furthermore, we tested if the difference in these DXA-based variables between the most affected and contralateral hip adds to this prediction. Since left-right differences are independent of biological variation in bone size or density we hypothesize that these are better predictors of disease progression.

MATERIALS AND METHODS

Study Population

This study includes primary care patients with osteoarthritis of the hip derived from the "Glucosamine sulphate in hip osteoarthritis" (GOAL) trial of the Erasmus Medical Center, with data collected at baseline and every three months up to two years follow-up. Details of the study have been described earlier [17]. In summary, patients were eligible for inclusion in the GOAL cohort when they met one of the American College of Rheumatology (ACR) criteria for hip OA [18]. Patients that had already undergone hip replacement surgery or those on the waiting list for joint replacement were not included in the study. In addition, eligible patients with a Kellgren & Lawrence (K-L) score of 4, people with renal and/or hepatic disease, diabetes mellitus or with disabling co-morbidity were excluded. Sex, age, height, weight, duration of complaints and body mass index (BMI) were registered or measured in all OA patients. For this study only participants with bilateral radiographs and dual energy X-ray absorptiometry (DXA scans) of adequate quality measured at baseline and after two years follow up were included in the analyses. The Ethical Committee of Erasmus MC approved the study protocol, and patients provided written informed consent.

Radiographic Assessments

A strict protocol was used to enable correct measurements of joint space narrowing at baseline and two years follow up. Pelvic radiographs were taken in weight bearing position with the patient's hips at 15° internal rotation. From the digitized x-rays the

minimal joint space width (JSW) was assessed at the medial, axial, superior and lateral points of the joint or any other site where the JSW was minimal. The intraclass correlation coefficient of the minimal joint space width measurement was 0.98. All the radiographs were scored at baseline according to the Kellgren-Lawrence score from grades from 0 "no OA" to 4 "severe OA" [19].

DXA Scan Analysis

DXA-scans (DPX-Lunar GE) from both hips were made at baseline ensuring 15° internal rotation of the hips, similar to the protocol used for the radiographs. A software tool was developed that enables evaluating bone geometry and density parameters from DXA scans in specified (non-conventional) regions of interest in the hip. Regions of interest (ROI) of which we calculated BMD, BMC and area size included the femoral head (divided in quarter and arcs), femoral neck, acetabulum, trochanteric and inter-trochanteric areas. Figure 1 present a detailed definition of all the DXA parameters. The analysis was performed using Matlab (version 7.1.0, MathWorks Inc, Natick, Massachusetts). The software calculated the parameters in a semi-automatic way. The major and minor trochanters were indicated manually, as was the size and position of the femoral head according to the location of the bony margins of the acetabulum or acetabular rim, which were used as points of reference; all other parameters were measured automatically. The neck axis was positioned in the middle of the femoral neck, bisecting the centre of the neck. The femoral axis was determined as a line parallel to the femoral shaft passing through the middle point localized between the most external margins of the femur. Geometry parameters and regions of interest (ROI) for BMD, BMC or area measurements included the femoral head, femoral neck, acetabulum, trochanteric and inter-trochanteric areas. Figure 1 and figure 2 show a detailed definition of all the DXA parameters.

Progression of hip osteoarthritis

We defined progressive cases as those patients that presented joint space narrowing (JSN); a decreased joint space width (JSW) compared to baseline of twenty percent (20%) or more was considered positive for progression of hip OA according to previously described criteria [20]. It takes in account for the big variability in the joint space that exists between individuals. We included in the progression group also those patients that received a total hip replacement (THR) during the 2-year follow up.
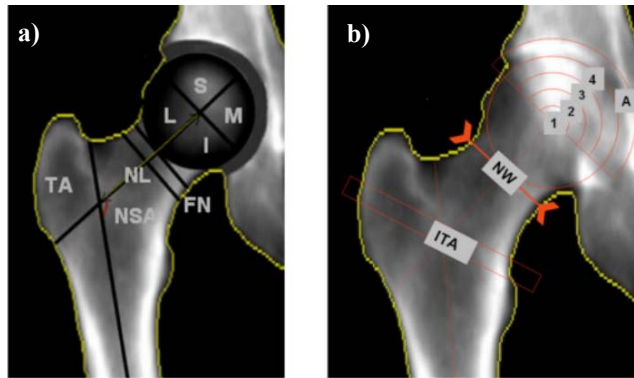
Figure 1 DXA image that shows the parameters that are determined in the software of the DXA analysis. **a)** Trochanteric area (TA), Neck shaft angle (NSA), femoral neck length (NL): line from the center of the femoral head to the intersection point of the femoral shaft and femoral neck (FN). The femoral head was divided in four quarters: Superior (S), Medial (M), Inferior (I), and lateral (L). **b)** Arcs dividing the upper part of the femoral head in four sub regions ranging from the center of the subchondral region and acetabular area (A), neck width (NW) measured on the narrowest neck region and intertrochanteric area (ITA). For all areas the BMD, BMC and area size were determined.
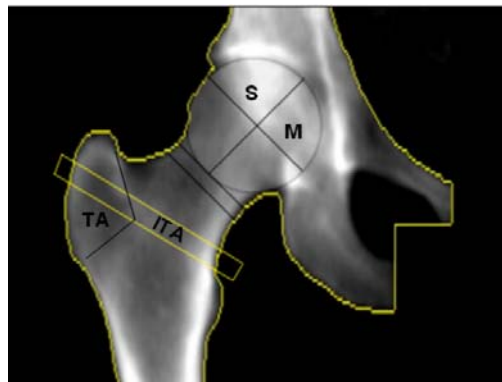
Figure 2 DXA image that shows the parameters of the DXA scan that are part of model 5, which provides the overall best prediction of OA progression. Superior area size (S), superior and medial (M) BMD and BMC from the femoral head, Intertrochanteric and trochanteric area size (ITA and TA) respectively.

Statistical Models

We evaluated a number of statistical regression models with different combinations of the following variables: baseline Kellgren and Lawrence (K-L); baseline DXA-based parameters (both geometry and BMD or BMC related parameters); the K-L score difference between the most affected and contralateral hip at baseline (ΔK-L) and the difference between the most affected vs. contralateral DXA-based parameters at baseline (ΔDXA). All models were adjusted for age, weight, height, and sex. To reduce the

number of DXA variables to a significant subset we used a backward stepwise method using the Likelihood ratio test. Progression of OA was predicted using five different models: the first model (1) was used to investigate the contribution of the K-L score of the most affected side to the prediction of progression (K-L); model 2 was used to investigate the contribution of the DXA based parameters of the most affected side to progression (DXA); model 3 revealed how the combination of DXA parameters and the K-L score of the most affected side contribute to the prediction of progression (DXA + K-L); model 4 was used to test if adding the K-L difference within hips to the K-L score of the affected side only improved the prediction of progression of model 1 (K-L + ΔK-L); and model 5 was used to test if the difference of the most affected (OA) and contralateral hip between the DXA parameters added to the prediction based on K-L score of the affected side (K-L+ΔDXA).

The likelihood-ratio test was used to determine if the differences between the models were significant [21]. Using the software package "R" we calculated "An Information Criterion (AIC)" values of the various models. R is a programming language and open source software environment for statistical computing and graphics widely used for data analysis. AIC is an index of the amount of information that is lost when the model is used to describe the data [22]. The preferred model is the one with the AIC value closest to zero. In all regression models areas under the Receiver Operator Characteristic curves (ROC) were determined and used to compare the discriminatory capacity of the models. The Areas under the Curve (AUC) represent the prediction probability that a randomly selected pair of diseased and non-diseased subjects will be correctly classified. A perfect predictive model has the value AUC=1.0. Conversely, a non-informative test has AUC=0.5. True positive and true negative rate were separately analysed to identify the percentage of OA cases and non-cases correctly predicted by the models. In addition, Nagelkerke R² was used to measure the proportion of variability in a data set that is accounted for by the statistical models. Nagelkerke's R^2 is a modification of the Cox and Snell coefficient to assure that it can vary from 0 to 1. Ten-fold cross validation was used to reduce the error due to over-fitting for the statistical estimates (AIC and AUC). All statistical analysis were performed using SPSS, version 14 (SPSS inc., Chicago USA) and R version 2.7.2 (Free software foundation, Inc, Boston USA).

RESULTS

Participant characteristics and progressors characteristics

Out of the 222 patients that were enrolled in the trial, 189 patients had DXA scans of sufficient quality to be included in the current study. Using our definition for progression 43 out of 189 patients (22.8%) were considered to have developed radiographic progression of hip osteoarthritis after 2 years of follow- up (Table 1). Of the 43 patients that progressed, 17 (39.5%) received a total hip replacement and 26 had a JSN of 20% or more. We did not find significant differences in age, sex, weight and height between the progression and non-progression groups (Table 1). The majority of the progressors were found among patients with a K-L score of 2 and 3. There were no progressors in the group with a baseline K-L score of zero (Table 1). JSW decreased with increasing K-L score, with slightly (but not significantly) lower baseline values for the progressor group (Table 2). The biggest differences in BMD or BMC between progressors and non-progressors were found in the regions close to the joint space (superior and medial part of the head and the outer arcs 3 and 4, Table 3 and Figure 1). As expected, these values were higher (Z-score 0.39 to 0.48) for the progressors. The area of the entire femoral head (all four quarters) and the femoral neck width also were significantly higher in the progressor group (Table 3).

Table 1. Baseline population characteristics of studied population.

Characteristic	Total n = 189	Progressor n = 43	Non-progressor n =146
Age (years) mean +/-SD	63.5+/-9.0	64.2+/-8.7	63.2+/-9
- Age 41-60, n (%)	72 (38)	16 (37)	56 (38)
- Age 60-70, n (%)	117 (62)	27 (63)	90 (62)
Female, n (%)	131 (69)	26 (60)	105 (72)
Height, mean +/- SD	1.69+/-.08	1.69+/-.08	1.69+/-.08
Weight, mean +/- SD	78.8+/-12.5	80+/-11.5	78.5+/-12.8
BMI (kg/m²), mean +/- SD	27.7+/-4.0	27.9+/-3.3	27.7+/-4.2
K-L score 0	12	0	12
K-L score 1	95	6	89
K-L score 2	57	21	36
K-L score 3	25	16	9

Table 2. JSW at baseline and follow up in progressor and non-progressor groups according to KL score at baseline. Values represent JSW in mm (mean and SD) at baseline and 2 years follow up.

	Progressors		Non-progressor	
KL score	JSW bas	JSW fu	JSW bas	JSW fu
0	N.A	N.A	3.0 (0.7)	3.0 (0.6)
1	2.67 (0.9)	2.31 (1.2)	2.8 (0.5)	2.8 (0.6)
2	1.62 (0.83)	1.15 (0.64)	1.89 (0.75)	1.93 (0.73)
3	0.75 (0.7)	0.57 (0.7)	0.8 (0.9)	0.8 (1.1)

Model results

The Kellgren and Lawrence score (K-L) proved to be a significant predictor for progression. After cross-validation the area under the Receiver Operator Curve (AUC) for Model 1 was 0.76 (Table 4). The true positive rate (TPR) of this model is 37.2%.

In the next model we analyzed the DXA scan parameters of the affected side. The backward stepwise regression left only three variables in the model: the BMC of the medial part of the femoral head, the BMC of the inferior part of the femoral head and the BMC of the femoral neck (Model 2). After cross-validation the model's performance was inferior to K-L in model 1 (AUC=0.69, table 4). Similarly the true positive rate (TPR) of this model was lower (9.3%).

In Model 3 we combined the predictors from model 1 (K-L score) with the predictors from Model 2 (the three BMC DXA variables), which resulted in a model with reasonable good predictive performance after cross-validation (AUC = 0.83). The difference in AUC score of this model with the previous two models proved to be significant ($P<0.05$). The TPR of 34.92 was slightly less than model 1, table 4.

In Model 4 we added the K-L score difference (ΔK-L) between the hips of each patient as a predictor to model 1 (K-L score of the affected side only). Adding ΔK-L resulted in a significant increase in AUC ($P<0.05$) compared to model 1. Both the AUC (0.82) and the TPR (34.9%) were similar to the values for model 3, table 4.

Table 3. DXA variables for progressors and non-progressors. Values represent the distance between the mean value of each variable for progressors and non progressors and the population mean in units of the standard deviations. Z is negative when the group's mean is below the population mean. P value was adjusted by gender, age, height and weight.

Variables	Z-score non-progressors	Z-score progressors	Adjusted p-value
BMC			
Femoral Neck (FN)	-0.07	0.16	0.17
Intertrochanteric Area (ITA)	0.02	0.07	0.9
Trochanteric area (TA)	-0.01	0.02	0.6
Superior quart femoral head (S)	-0.13	0.44	0.009
medial quart femoral head (M)	-0.10	0.39	0.019
inferior quart femoral head (I)	-0.07	0.24	0.08
lateral quart femoral head (L)	-0.08	0.27	0.06
acetabular arc (A)	-0.10	0.36	0.01
arc4	-0.12	0.45	0.003
arc3	-0.13	0.48	0.001
arc2	-0.11	0.37	0.02
arc1	-0.07	0.24	0.19
Areas / size			
Femoral Neck (FN)	-0.08	0.21	0.6
Intertrochanteric area (ITA)	0.02	-0.02	0.16
Trochanteric area (TA)	0	0	0.4
Superior quart femoral head (S)	-0.15	0.47	0.002
medial quart femoral head (M)	-0.12	0.50	0.002
inferior quart femoral head (I)	-0.15	0.47	0.003
lateral quart femoral head (L)	-0.15	0.49	0.003
Acetabular arc (A)	-0.08	0.20	0.04
arc4	-0.01	0.04	0.005
arc3	-0.15	0.06	0.001
arc2	-0.10	0.10	0.007
Arc1	-0.07	0.32	0.2
Geometry			
Neck width (NW)	-0.14	0.38	0.04
Neck length (NL)	0.00	-0.04	0.41
Neck shaft angle (NSA)	-0.02	0.08	0.7

In the last model (Model 5) we combined K-L of the affected side (Model 1) with the difference in DXA values between the most affected and contralateral hip. The backward regression resulted in a different set of DXA parameters than those identified by Model 2: The area size of the superior part of the femoral head, the area of the major trochanter, the intertrochanteric area and both the BMD and BMC of the superior part and medial

part of the femoral head were selected (Fig 2). This model is significantly different to the model that only includes K-L score of the affected side (Model 1) and to the model that uses the K-L score difference and the value of the K-L score of the affected side (Model 4) based on comparing AUC differences after cross-validation ($P<0.05$). The AUC of Model 5 (0.84) was not different from the AUC of Model 3 (K-L + DXA most affected side; AUC: 0.83), but the model is much better in the prediction of progressive cases (With a TPR of 51.2%). Additionally, this model has the lowest -2Log Likelihood ratio and AIC value (Table 4).

Table 4. Models using clinical, radiological and DXA variables. Abbreviations: K-L: Kellgren and Lawrence score of the affected side. The difference in values between affected hip and contralateral side is expressed in percentage (%). Positive values represent an increase in the affected hip. No applicable (NA) in the cases that the variable only reflect the affected side. Level of significance codes: '***' p value<0.001, '**' p value<0.01, '*' p value<0.05. All models were corrected for patient characteristics. True positive rate (TPR) and true negative (TNR) columns correspond to the percentage correctly predicted by the models. *Area under the curve value obtained after 10-fold cross validation process.

Variables	% Diff. & P	-2 Log	R²	AIC	AUC	TNR	TPR
KL score affected side:	30.7 ***	159.5	0.31	163.5	0.76	93.2	37.2
DXA affected side:		184.2	0.15	192.2	0.69	97.3	9.3
- BMC medial part femoral head	13.9 ***						
- BMC inferior part femoral head	7.2 ***						
- BMC femoral neck	5*						
DXA affected side + KL:		148.6	0.38	158.6	0.83	93.9	34.9
- BMC medial part femoral head	13.9 *						
- BMC inferior part femoral head	7.2 *						
- BMC femoral neck	5**						
- KL affected side	NA***						
KL affected side + Delta KL		154.0	0.35	160	0.82	93.9	34.9
- KL score affected side	NA***						
- Delta KL	32*						
DXA ROI'S difference:		135.6	0.45	153.6	0.84	91.7	51.2
- Difference superior area fem head	16.5*						
- Difference trochanteric area size	2*						
- Difference BMD sup. part fem. head	5.7**						
- Difference BMC sup. part fem. head	9**						
- Difference BMD med. part fem. head	4.6**						
- Difference BMC med. part fem. head	4**						
- Difference Intertrochanteric area size	-4.5*						
- KL score affected side	NA***						

DISCUSSION

In this study we analyzed how well selected DXA parameters of the hip that were specifically chosen to be relevant for osteoarthritis, together with the accepted Kellgren and Lawrence score contribute to the prediction of OA progression.

We found that both the K-L score and the selected DXA parameters alone were not good predictors for OA progression, with K-L performing marginally better than the DXA parameters alone. Interestingly, when both models were combined the resulting model exhibited a small but significant increase in performance as shown by the increase in AUC. Apparently, the DXA parameters that were investigated in this study refer to measures of OA that are relatively independent of the Kellgren & Lawrence score.

Many of the DXA parameters themselves however, were not independent but highly correlated among each other. The number of DXA variables used in the regression models was reduced using the backward stepwise method in the likelihood ratio test. Therefore the resulting regression models are dependent on the backward stepping procedure and other models that include other parameters (representing similar aspects) might work just as well. What is important here is not so much the meaning of the specific parameters used in the regression models, but the potential of DXA parameters for the prediction of OA progression, which justifies a more in depth study.

We further investigated if the prediction based on DXA parameters would improve when the difference between most affected and contralateral side was used rather than the affected side itself. We assumed that looking at the DXA difference between the most affected and contralateral side would correct at least partly for the biological variation in bone sizes and bone density. Thus, this measure could highlight how the disease process has affected the bone and therefore be a better predictor for disease progression. Even though the AUC for the model that included this ΔDXA (model 5) was only slightly higher than the AUC of model 3 (DXA parameters of the most affected side and K-L score of the most affected side), the percentage of correctly classified progression cases (TPR) is much higher than in model 3. Additionally, this model (model 5), showed a better statistical performance, lowest –2Log, AIC and higher R^2 (Table 4: -2Log: 135.6, AIC: 153.6 and R^2: 0.45) than any other model.The definition of progression in this study included patients with both JSN (more than 20%) and patients that received a total hip replacement (THR) within the follow-up period of 2 years [20]. The latter is maybe a possible limitation of this study, because we cannot determine if the THR patients truly exhibited joint space narrowing. We tested the effect of excluding the THR patients to

the models in a sensitivity analysis. In all models the exclusion of THR cases affects the percentage of correct predictions and AUC. However, the general trends were similar and the model that included the difference between the most affected- and the contralateral side (model 5) still remained the best predictive model.

Other limitations of this study are related to the relatively short follow-up and the inaccuracies inherent to the DXA measurements. The limitations of the DXA method itself have been exposed previously by other authors [23]. Radiological progression of OA is better defined when patients have longer follow up. In addition the study population is rather heterogeneous with patients that varied in (subjective) pain scores and ranged from mild OA (K-L 0 and 1) to advanced stages (K-L 2 and 3). It seems likely that the more degenerated joints at baseline progress differently than a joint in the early phase of the disease. In terms of our definition of progression it is clear that advanced OA joints with an already small JSW do not have to progress much to reach a 20% narrowing. The majority of the progressors are in the K-L scores 2 and 3 and since a K-L score of 4 was an exclusion criterion we have no patients with extreme low JSW (Table 2).

Different hypotheses exist about the role of BMD changes during the OA process. We had defined different regions of interest of which some were close to the joint with a putative effect on osteoarthritis development. Not only femoral head regions were found to be relevant, but also the more distant regions such as the femoral neck and trochanteric regions. The difference in intertrochanteric area size (between affected and contralateral hip) had a negative correlation with progression and might be the consequence of muscular dysfunction of the hip abductor group that has been found in patients with hip OA [24, 25].

We also identified an increase in size at the femoral head and trochanter and increased BMD and BMC of the superior and medial part of the most affected femoral head compared to the contra lateral side in the group of patients where the disease progressed (Figure 2). The BMD and BMC increase in the head regions is in concordance with published literature and we suppose that the differences are acquired as part of the osteoarthritis process and subsequent bone adaptation. However we cannot exclude the possibility that some of these left-right differences existed previous to the onset of the disease.

CONCLUSIONS

We have shown that DXA scans of the hip contain information that can be used to predict OA progression. Patients that presented OA progression had a higher BMC in the medial and inferior region of the femoral head compared to those that did not progress. Also, the bone mass in these regions was higher in the most affected hip compared to the contralateral side. These differences between the most affected hip and the contralateral hip appear promising to predict progression of the disease. Further study of DXA scans with improved resolution could lead to the development of useful clinical tools to diagnose OA and predict the chances of fast progression.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

MC participated in the design of the study, analysis of DXA images, performed the statistical analysis and drafts the manuscript. JL conceived the study and elaboration of the program used to analyse the DXA images. RF participated in the statistical analysis and helped to draft the manuscript. RR selected the cohort and collected patient's information, SB participated in the study design and helped to draft the manuscript. HW conceived the study, and participated in its design and coordination and helped to draft the manuscript. JW participated in the design of the study and design of the program used to analyse the DXA images, helped to perform the statistical analysis and draft the manuscript. All authors read and approved the final manuscript.

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Chapter 4

**Osteoporotic fracture risk
in osteoarthritis**

Chapter 4.1

Bone parameters across different types of hip osteoarthritis and its relation to osteoporotic fracture risk

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ABSTRACT

Atrophic type of hip osteoarthritis (OA) is characterised by cartilage degradation without formation of osteophytes. Individuals with atrophic OA have been less studied and it is unknown whether they differ from osteophytic types regarding bone tissue. We here examined BMD, hip structural properties and fracture risk in individuals with atrophic OA type compared to individuals with osteophytic types (normotrophic/hypertrophic) and to individuals without OA. This study is part of the Rotterdam Study, a large prospective population based cohort study. We examined 5006 participants who were assessed for osteoarthritis, BMD, and geometry measures at baseline and incident non-vertebral osteoporotic (OP) fractures (mean follow-up time of 9.6 years). We estimated differences in bone characteristics between the OA-groups and controls. Cox's proportional hazards regression was used to calculate OP fracture risk.

Results. Participants with atrophic OA had systemically lower BMD compared to normotrophic type and controls respectively (6.5 and 9% in total body and 4 and 5% in skull-BMD respectively). Participants with osteophytic OA had approximately 4 and 5% higher total body- and skull-BMD, wider femoral neck and greater bone strength (12 and 5% higher section modulus) compared to controls or atrophic OA. However, the risk of OP fractures was almost 50% higher in the atrophic group compared to controls (HR: 1.48, p: 0.008). It was not explained by differences in BMD, falling, disability or corticosteroid use. Individuals with atrophic hip OA have an increased risk for OP fractures not fully explained by systemically lower BMD compared to controls.

Keywords: Osteoarthritis, atrophic, hypertrophic, hip, joint space, osteophytes, bone mineral density, bone geometry, osteoporotic fractures.

INTRODUCTION

Hip osteoarthritis (OA) is a common joint disorder and a major cause of pain and disability in the elderly population. OA is characterised by cartilage degradation, new bone formation and changes in the subchondral bone. Although OA was once considered a primary disorder of articular cartilage, it is now generally appreciated that bone structure play a role in OA pathology.

Individuals with OA are known to present higher bone mineral density (BMD) and hence, be protected against osteoporosis (1-4). However, both conditions can co-exist (5-7) and some reports even suggest that subjects with OA may have increased fracture risk (8-11).

Hip bone geometry parameters have been shown to influence the risk of fracture (12-18). Differences in bone geometry have also been observed in subjects with hip OA as compared with controls, presenting with wider femoral necks and alterations of mass distribution which are associated with both incident and prevalent OA (19). Yet, only few studies have examined the relation of hip OA with fracture risk and none of them considering the different subtypes of the disease (2, 4, 8, 10-11, 20-22).

Radiographic hip OA is frequently defined by the presence of both cartilage degeneration (radiographically defined as joint space narrowing (JSN)) and formation of new bone spurs at the joint margins (osteophytosis; OPH). However, both radiographic features are not always present, which makes it possible to sub-classify OA in different types (23-25). The most frequently studied form of hip OA (classical or "normotrophic") presents with both JSN and OPH. When only OPH are present is called "hypertrophic" and when only JSN is observed, it is called "atrophic" (23). The atrophic form of hip OA is far less studied. However, it is suggested that subjects with atrophic OA have a higher risk of presenting hip joint destruction (26-27) and faster disease progression (28). In addition, patients with atrophic OA have been shown to present with micro-architectural disorganization, lower bone volume and thinner trabeculae than controls, as assessed by bone histomorphometry of iliac crests (27). Therefore, atrophic OA might be the result of reduced bone forming capacity.

The aim of our study was to examine bone density and structural properties across individuals with different types of OA: atrophic and osteophytic (normotrophic/hypertrophic) and their relation with fracture risk as compared to controls.

METHODS

The Rotterdam Study

The Rotterdam Study (RS) is a large prospective population-based cohort study of men and women aged 55 years and older. The design and rationale of the study has been described in detail elsewhere (29). In summary, the objective of the study is to investigate the determinants, incidence and progression of chronic disabling diseases in the elderly. The medical ethics committee of Erasmus University Medical School approved the study and written informed consent was obtained from each participant. RS-I refers to the baseline examination of the original cohort that included 7,983 participants. For this study we used data from 5,006 (62.7%) participants for whom both hip OA and BMD data were available.

Clinical assessment

At baseline, trained interviewers performed an extensive home interview on demographic characteristics, medical history, risk factors for chronic diseases and use of medication that was verified by a physician. We also used information about smoking (current and former vs. never), use of analgesics (in the last month: yes/no). Falling was assessed using structured personal interviews by trained medical research nurses. A faller was defined as an individual with a history of one, two or more falls without precipitating trauma (e.g., car accident or sport injury) in the 12 months preceding the baseline interview. A lower limb disability index was obtained by calculating the mean score of answers to questions concerning rising, walking, bending, and getting in and out of a car. The index is represented by a continuous score ranging from 0 to 3, where 0 indicates no impairment and 3 indicates severe impairment. Detailed definitions for lower limb disability are described elsewhere (30). Height and weight were measured at baseline examination with the subject in a standing position with indoor clothing without shoes.

Corticosteroids use: Persons who received a corticosteroid prescription for oral, rectal, parenteral, or inhaled use within 1 month before the index date were defined as current users; all others were considered non-current users.

CRP measurement: At baseline (1990–3) and follow-up (1996–9), blood was drawn by venous puncture, initially stored at –20°C and thawed and assayed for hs-CRP using Rate Near Infrared Particle Immunoassay (Immage Immunochemistry System; Beckman Coulter, Brea, California, 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.

Radiographic assessment and hip osteoarthritis definition

Weight-bearing antero-posterior radiographs of the hip were obtained at 70 kV, a focus of 1.8, and a focus-to-film distance of 120 cm, using High Resolution G 35 _ 43–cm film (Fuji Photo Film Company, Kanagawa, Japan). Radiographs of the pelvis were obtained with both of the patient's feet positioned in 10° internal rotation and the x-ray beam centred on the umbilicus. Hip radiographs were scored for the presence of OA features (osteophytes and joint space) by two independent observers who were trained by an experienced physician in OA and advised by a radiologist. The trained observers used as reference an atlas of individual radiographic features in osteoarthritis (31). After each set of approximately 250 radiographs the scores of the two trained observers were evaluated. Whenever the score of the two readers differed more than one grade (osteophytes) or more than 30% for JSW (Intraclass Correlation Coefficient (ICC) a consensus reading was carried out. Readers were blinded to all data of the participants. Additionally, there was no indication of sex or age on the X-rays. Joint space width (JSW) of the hip was measured using a 0.5-mm graduated magnifying glass laid directly over the radiograph. Superior and inferior compartments of the femoral head were reviewed in both hips as lateral, superior and axial (32). We determined JSN at axial, lateral and superior sites for all hips. The axial site was most frequently affected by JSN, this was true in normotrophic as well as atrophic hip OA. The Intraclass correlation coefficient (ICC) for the minimal joint space was 0.73 (0.69-0.77) and the Kappa statistic for femoral osteophytes was 0.76. These estimates were an average that included ICC taken from the first till the last reading sessions.

Table 1. Definition of OA according to bone response.

	Description	Type of hip OA
1	Participants without JSN or OPH (33)	No OA
2	Participants with OPH and JSN	Normotrophic
3	Participants with only OPH	Hypertrophic
4	Participants with only JSN	Atrophic

Ref= People without Joint Space Narrowing (JSN) or Osteophytes (OPH) were taken as reference.

We defined joint space narrowing (JSN) as a JSW equal or lower than 2.5 mm in at least one compartment. We defined osteophytosis (present/absent) if at least 1 definite femoral osteophyte was present. These two features, JSN and osteophytosis were used as dichotomous outcomes and used to classify participants as presenting with one of four different types of hip OA including: No OA, Normotrophic OA, Hypertrophic OA and Atrophic OA (Table 1).

BMD and bone geometry measurements

DXA BMD (g/cm²) of the right proximal femur and lumbar spine were measured at baseline using a Lunar DPX-L densitometer (Lunar Radiation Corp., Madison, WI, USA) and analyzed with DPX-IQ v.4.7d software as described previously (35-36). Total body scans were performed at the third follow-up visit (mean follow-up 6.5 years) using a Prodigy^{TM} fan-beam densitometer (GE Lunar Corporation Madison, WI) and analyzed with the enCORE^{TM} software. The software employs an algorithm that divides body measurements into areas corresponding to total body, head, trunk, arms and legs. All analyses were verified by a trained technician who performed adjustments when necessary. Hip structural analysis (HSA) software was used to measure hip bone geometry from the DXA scan at the narrow neck region across the narrowest point of the femoral neck (NN). BMD and narrow neck width (outer diameter) were measured directly from mineral mass distribution, while section modulus (an index of bending strength) and buckling ratio (index of cortical bone instability) were calculated using algorithms previously described (35).

Assessment of incident fracture

All events, including fractures and death were reported by general practitioners (GPs) in the research area (covering 80% of the cohort) by means of a computerized system. Information from GPs outside the research area was obtained by regular checking of patient records by research physicians. All reported events were verified by two trained research physicians, who independently reviewed and coded the information. Subsequently, all coded events were reviewed by a medical expert for final classification. Subjects were followed from their baseline visit until January 1, 2007 or until a first fracture or death occurred resulting in a mean fracture follow-up duration of 9.7 years (SD=5.1 years). All fractures that were considered not osteoporotic (fractures caused by cancer and all hand, foot, skull, and face fractures) were excluded.

Statistical Analysis

We compared the baseline characteristics of the study population and the DXA- derived BMD and geometry parameters from the narrow neck region (NN) between the OA types and controls using analysis of variance (ANOVA). Categorical variables were tested using the chi squared statistic. All the BMD measurements and the DXA-derived geometry analyses were adjusted for gender and age. We also presented BMD from femoral neck (FN), total body and skull-BMD. Additionally, Z-scores values were included to compare differences between different types of OA and controls. DXA-derived geometry analyses were adjusted by NN-BMD to test its independence. Cox's proportional hazards regression was used to study association between the different types of hip osteoarthritis and fracture risk. Hazard ratios with 95% confidence interval were reported. We introduced in the Cox regression model for OP fractures type of hip osteoarthritis as categorical variable. The analysis was adjusted by gender, age, height, weight (additionally by FN-BMD to evaluate the effect of BMD and corticosteroid use). All analyses were run using SPSS V. 15.0.

RESULTS

Table 2 shows the baseline characteristics of individuals with the different types of OA and those without OA. Participants with OA were on average older and more frequently females except for the hypertrophic OA group, where more men were present. There were no significant differences in height, weight and BMI between the groups. Participants in the hypertrophic or normotrophic groups had more disability in lower limb compared to participants without OA.

Table 3 presents the results of the adjusted baseline bone and geometry parameters measured by DXA at the femoral narrow neck region (NN). BMD from NN, FN, and LS BMD was higher in the osteophytic groups compared to controls (Table 3). The atrophic group had similar BMD to controls and significantly lower compared to osteophytic groups (*P* values<0.05, not in table). In spite that total BMD and skull BMD were measured at a later time point having less participants (2018) to include in the analysis, head and total BMD followed the same trend, higher in osteophytic groups (N.S) and significantly lower BMD-values in atrophic group compared to controls (Not in table, *P*=0.007 for head BMD and 0.002 for Total body BMD). Figure 1 shows the Z-scores of the BMD values between the different OA groups compared to controls.

Table 2. Baseline characteristics.

Variables	OA Cases (n=1289)				ANOVA P-value
	No OA* (n=3717)	Normotrophic (n=178)	Hypertrophic (n=925)	Atrophic (n=186)	
Gender female (%)	58	64.6	48.6**	63.4	0.84
Age (years)	67.2±5.6	71.3±6.0**	69.2±6.4**	68.7±5.3**	3.7×10^{-20}
Height (cm)	166.5±9.5	167.2±9.7	166.4±9.0	166.9±10.7	0.80
Weight (kg)	72.9±11.9	72.3±12.3	73.2±12.6	72.8±11.3	0.73
BMI (Kg/cm²)	26.3±3.7	26.0±3.5	26.4±3.9	26.2±3.2	0.69
LLD	0.29±0.75	0.49±0.43**	0.39±0.65**	0.32±0.26	4.4×10^{-4}
Total CRP (mg/l)	3.06±5.4	2.24±2.7	3.20±6.5	3.42±5.2	0.61
Falling > 1 per year (%)	14.5	21.9	14.3	18.8	0.35

*No-OA: No definite osteophytes or narrowing. Values for each joint at baseline: mean ± SD for continuous variables and frequency (% of women) for categorical variables. P value from x² test or ANOVA. **P values<0.01. Height and BMI were gender and age adjusted, Weight was gender, age and height adjusted and LDD was gender, age and weight adjusted. CRP and falling were adjusted for gender, age and BMI. BMI is body mass index and LLD is lower limb disability.

Table 3. Hip bone parameters measured at the NN-region & FN/LS BMD.

DXA parameter	OA Cases (n=120wq9)				ANOVA P-value
	No-OA (n=3507)	Normotrophic (n= 167)	Hypertrophic (n=874)	Atrophic (n= 168)	
LS-BMD	1.09±0.18	1.16±0.21**	1.11±0.18*	1.07±0.19	1.1×10^{-6}
FN-BMD	0.87±0.13	0.93±0.15**	0.88±0.13*	0.86±0.13	5.2×10^{-8}
Total-BMD	1.12± 0.12	1.15± 0.13	1.13± 0.13	1.08± 0.11*	0.002
Skull-BMD	1.94± 0.28	2.01± 0.26	1.96± 0.28	1.85± 0.31*	0.008
NN-BMD	0.70±0.13	0.75±0.15**	0.72±0.13*	0.69±0.11	1.4×10^{-4}
Neck Width (cm)	3.21±0.32	3.34±0.38**	3.29±0.33**	3.19±0.28	1.1×10^{-19}
NN-cortical buckling	13.73±3.1	14.48±3.3	14.16±3.0	13.56±2.7	1.9×10^{-15}
NN-section modulus	1.15±0.33	1.29±0.44**	1.21±0.37**	1.14±0.33	8.6×10^{-28}

Values are means and standard deviations (sd) adjusted for age and gender. LS: Lumbar spine and FN: femoral neck BMD. ^Total body BMD and Skull BMD were measured in 2018 individuals and hip structural parameters in 4007 individuals. P value from ANOVA are means and P values<0.05, **P values<0.01.
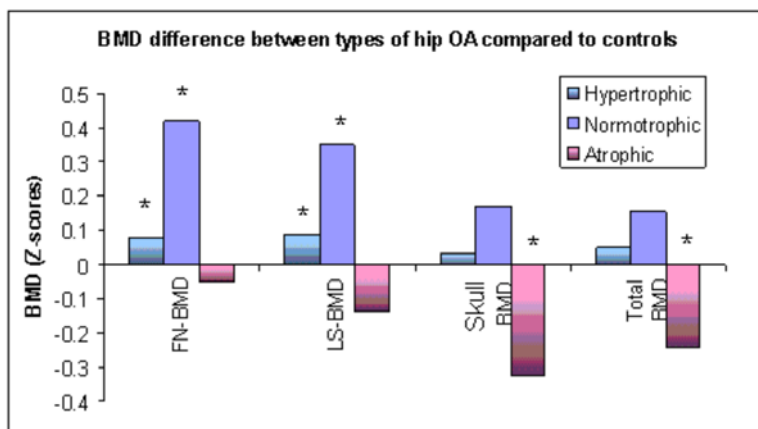
Figure 1 BMD differences between different types of hip OA and controls. Values represent bone mineral density (BMD) Z-scores from Femoral Neck (FN), Lumbar Spine (LS), skull and total body. * Represent P values <0.05. Comparison was made between types of OA (hypertrophic, normotrophic, atrophic) and controls. The analysis was adjusted for gender and age.

As compared to subjects without OA, participants with normotrophic or hypertrophic OA had 4.1 and 2.5% wider femoral necks and 12.3 and 5% greater bone strength (higher section modulus) respectively. No significant differences were found for these parameters in subjects with atrophic OA compared to controls.

In total, 1071 participants with OA-scored radiographs sustained a fracture during the period of investigation. Participants with the atrophic hip OA type had almost 50% increased risk for fractures as compared to controls with or without BMD adjustment (Figure 2, HR: 1.48, CI: 1.11-1.98, p: 0.008 and HR: 1.44, CI: 1.08-1.92, p: 0.012 respectively). Normotrophic and hypertrophic group did not differ significantly in fracture risk from that of controls (Figure 2, after BMD adjustment: normotrophic HR: 1.18, CI: 0.84-1.66, p: 0.35; hypertrophic HR: 0.89, CI: 0.74-1.06, p: 0.20 and without BMD adjustment: Normotrophic HR: 0.98, CI: 0.70-1.36, Hypertrophic HR: 0.88, CI: 0.74-1.05). Additional adjustment for lower limb disability did not change the risk estimate as it was expected because disability in the atrophic group was not significantly different from controls (Table 2, *P*>0.05). Similarly, the adjustment for falling did not change the risk estimates. Falling at baseline was not different between OA types and controls (table 2, *P*>0.05 for each OA group compared to controls). Finally, adjustment for use of corticosteroids at baseline changed the risk estimates to a slightly higher fracture risk for participants with atrophic type (HR: 1.69, CI: 1.19-2.41, *P*: 0.004). The risk for osteoporotic fractures remained no significant for participants with other OA types.
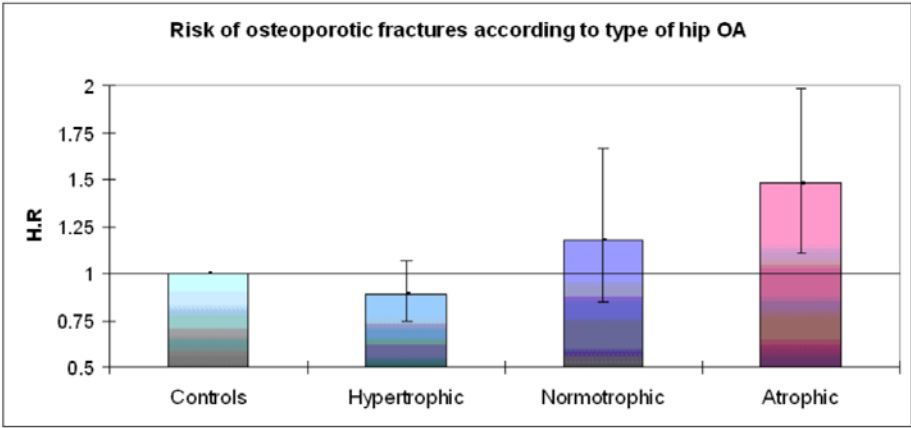
Figure 2   Osteoporotic fracture risk by OA type. Values presented are Hazard ratios (H.R.) and their 95% CI from Cox regression model. P values are for comparison of risks with controls after adjustment for age, height, weight and FN-BMD.

DISCUSSION

In this large prospective population-based study we found differences in bone structural geometry across individuals with different types of OA. Individuals with osteophytic OA (both normotrophic and hypertrophic) had higher BMD, wider femoral necks and greater bone strength than individuals with atrophic OA and individuals without OA. While individuals with atrophic OA had lower head and total BMD than individuals without OA, no differences were observed in parameters of hip geometry. A 50% increased of osteoporotic fracture risk was observed in individuals with atrophic OA as compared to both subjects with osteophytic OA and without OA.

Previous studies have found increased BMD and differences in DXA-derived geometry values in hips with OA as compared to controls (19, 37-40). However, the majority of those studies used the classical definition of OA where cartilage degradation (joint space narrowing) and osteophytes need to be present. Such definition excludes individuals with atrophic forms (only joint space narrowing), despite some reports indicating that these individuals present with clear signs of cartilage degradation (24) and rapid disease progression (41) in the absence of osteophytes. In line with our findings, Javaid *et al.* found in subjects from the Study of Osteoporotic Fractures (SOF), that individuals with an osteophytic type of hip OA had wider femoral necks and displacement of the center of mass (19). In general, Individuals with atrophic type of hip OA have been discarded from previous works analyzing the relation of OA and osteoporosis.

Even though we found no differences in hip structural parameter in subjects with the atrophic form of OA we did find a consistent systemically lower BMD than subjects without OA and with osteophytic forms of OA. The fact that the BMD of subjects with atrophic OA is also lower than that of subjects without OA suggests this phenomenon is not explained alone by the absence of osteophytes (i.e.artifactual elevation of BMD in participants with osteophytic forms). This seems to be the result of a systemic process considering that total body BMD is also decreased; and the fact that this decrease is also evident on skull BMD, a site less prone to be influenced by environmental factors, mechanical strain and weight bearing (42). Overall, these measurements are less affected by artefactual elevations arising from osteoarthritic changes.

We evaluated if the observed differences in skeletal properties observed across the different types of OA were translated in differences in fracture risk. Even though participants with osteophytic types of OA had higher bone mineral density, wider femoral neck and greater bone strength than controls we observed no significant differences in fracture risk. Inversely, subjects with atrophic hip OA who did not differ in DXA-derived geometry but who had lower BMD, had a ~50% increased risk for osteoporotic fractures, which was still present after BMD adjustment. Further, this increased risk is not likely to be explained by lower BMD consequence of immobility or differences in falling risk since correction for lower limb disability and falling did not essentially modify the risk estimate. Finally, adjustment for corticosteroid use made more pronounced the osteoporotic fracture risk difference between the atrophic group and controls. It is known that prior and current exposure to corticosteroids confers an increased risk of fracture (43).

There are possible explanations to the increased osteoporotic risk fracture in participants with the atrophic type of osteoarthritis. Subjects affected with the atrophic OA type might have lower bone quality, characteristics that are not captured by DXA or DXA-derived geometry measures. Indeed, earlier studies of hip replacement patients showed that patients with OA had lower bone volume and thinner trabeculae than controls (27). The most affected were patients with hip joint destruction that was three times more common in atrophic cases than in the other OA types. The study of bone properties using other methods different than DXA scans could elucidate bone differences between OA types that might have influence on fracture risk.

Examination of biomarkers of bone metabolism might also clarify important differences between OA types. In our study we did not have data available on markers of cartilage

and bone resorption and formation which could help in the classification of the OA subtypes.

We consider that our study has some limitations. Even though we have a large prospective population with prospective fracture assessment less susceptible to biases, dividing in OA types limits sample size and hence the power of the study. A possible selection-bias due to different response-rate and inclusion of participants according with availability of hip radiographs and DXA scans has been previously discussed (44). There was a high response-rate in the Rotterdam study (>=80%) and therefore selection-bias for this reason will be limited. Yet people who refused to participate were generally older (especially above the age of 80) and more often seriously ill. Additionally, the fact that subjects had to be mobile enough to visit the research centre for radiographs and DXA examinations caused a possible health selection bias in our study population. Taking into consideration these aspects, it can be expected that our risk estimates are biased towards lower levels.

Other limitation is given by DXA scan's inherent properties and missing information on markers of bone metabolism. In spite of the information on bone structure that is gained using HSA, the major limitation of HSA is imposed by the two-dimensional nature of DXA (36). The ideal situation could be presented by adding CT-measurements to assess 3-d structural configurations. Finally, there is a limitation inherent to reproducibility of manual measurement of JSW among different observers. Interobserver reproducibility of ICC using manual measurement techniques ranges from 0.71 to 0.78 for JSW measurements (45). Our ICC-measures fall within these boundaries. The variability in measuring JSW was principally due to difficulties in determination of exact points on femoral head and acetabulum and directions to measure joint space in the different sites of the hip joint (lateral, superior, and medial). However, this ICC estimate was an average that includes interobserver values for the first reading session were observes had less expertise. JSW is considered a reliable method for measuring progression of osteoarthritis in hip joints with higher reliability compared to scoring of osteophytes (25, 46).

In conclusion, we identify BMD and DXA-derived differences principally between the atrophic and osteophytic sub-types of hip OA. It confirms that different types of OA exist with specific bone structural properties and relation with fracture risk. Classifying individuals in sub-types of hip OA can help identifying distinct aetiologies and pathogenic courses of the disease, which may be translated in appropriate therapeutic interventions.

Acknowledgments, Competing interests, Funding

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Chapter 4.2

Association of lumbar disc degeneration with osteoporotic fractures: the Rotterdam study and meta-analysis from systematic review

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ABSTRACT

To investigate the relation between Lumbar Disc Degeneration (LDD) and all type of osteoporotic (OP) fractures including vertebral. This study is part of the Rotterdam study, a large prospective population-based cohort study among men and women aged 55 years and over. In 2,819 participants spine radiographs were scored for LDD (osteophytes and Disc Space Narrowing (DSN)) from L1 till S1, using the Lane atlas. Osteoporotic (OP) fracture data were collected and verified by specialists during 12.8 years. We considered two types of vertebral fractures (VFx): Clinical VFx (symptomatic fractures recorded by medical practitioners) and Radiographic VFx (using the McCloskey-Kanis method). Meta-analysis of published studies reporting an association of LDD features and VFx was performed. Differences in Bone Mineral density (BMD) between participants with and without LDD features were analyzed using ANOVA. Risk of OP-fractures was analyzed using Cox regression. In a total of 2,385 participants, during 12.8 years follow up, 558 suffered an OP-fracture. Subjects with LDD had an increased OP fracture risk compared to subjects without LDD (HR: 1.29, CI: 1.04-1.60). LDD-cases have between 0.3-0.72 standard deviations more BMD than non-cases in all analyzed regions including total body and skull BMD (P<0.001). Only males with LDD had increased risk for OP-fractures compared to males without LDD (adjusted-HR: 1.80, 95%CI: 1.20-2.70, P=0.005). The risk was also higher for VFx in males (HR: 1.64, CI:1.03-2.60, P: 0.04). The association LDD-OP-fractures in females was lower and not significant (adjusted- HR:1.08, 95%CI: 0.82-1.41). Meta-analyses showed that the risk of VFx in subjects with LDD has been studied only in women and there is not enough evidence to confidently analyse the relationship between LDD-features (DSN or/and OPH) and VFx due to low power and heterogeneity in phenotype definition in the collected studies. Conclusions: Subjects with LDD, especially males have a higher osteoporotic fracture risk, in spite of systemically higher BMD.
Author keywords: Lumbar Disc degeneration, osteoporotic fractures, Bone mineral density, disc space narrowing, vertebral fractures.

INTRODUCTION

Lumbar Disc Degeneration (LDD) and osteoporosis are two age-related skeletal diseases which are very prevalent in elderly and known to be related to pain, increased morbidity and disability in this population [1, 2]. In Europe, the mean prevalence of vertebral fractures (VFx) in women between 60 and 64 years is 17% and this increases up to 35% when they are aged 75 years or more[1]. Both, osteoporotic (OP) fractures and LDD occur also in men however, it has been more studied in women [3, 4]. The relationship between LDD and bone health is unclear. As it has been previously shown, the presence of LDD is associated with higher spine Bone Mineral Density (BMD) [5-7]. In addition, LDD has been found associated with higher BMD of the femoral neck, which suggests a systemic increased BMD in subjects affected by LDD [5, 6, 8]. In this respect LDD behaves very similar to knee or hip osteoarthritis (OA), where also an increased systemic BMD has been found [9, 10]. In theory, the higher BMD found in subjects with LDD should corresponds to lower fracture risk compared to subjects without LDD. However, the few studies examining the relationship between LDD and vertebral fractures (in women) found conflicting results [7, 11-14]. In part it might be explained by the different radiological definitions used for both, LDD (based exclusively on presence of osteophytes (OPH) or Disc Space Narrowing (DSN)) and vertebral fractures (scored by different methods). Additionally, there are no studies examining the relationship between LDD and all types of OP fractures which would indicate whether the increased BMD found in LDD cases correspond to a decreased fracture risk.

Therefore, we investigated the relation between LDD and all type of osteoporotic fractures including vertebral, in a large prospective cohort that includes men and women. In addition, we performed a systematic review or previously published studies.

MATERIALS AND METHODS

The Rotterdam Study

This study is part of the Rotterdam study (RS), a large prospective population-based cohort study among men and women 55 years of age and older. The study design and rationale are described elsewhere in detail [15]. The objective of the study is to investigate the determinants, incidence and progression of chronic disabling diseases in the elderly. The baseline measurements were conducted between 1990 and 1993. In total, 7983 participants were examined. The current study was performed in 2,385 study participants for whom data on incident vertebral fractures, BMD and LDD was available.

The medical ethics committee of Erasmus University Medical School approved the study and written informed consent was obtained from each participant. Data Collection for potential risk factors.

Home interviews on medical history was performed by trained interviewers. Smoking habit was categorized binary as current or former versus never. The lower limb disability index used was composed of the mean score from six different questions regarding activities of daily living, using a modified version from the Stanford Health Assessment Questionnaire [16]. At baseline measurement, medical information and physical examination including height and weight were obtained. Body mass index (BMI) was calculated by dividing weight by height squared (kg/m²).

Radiographic Assessment of LDD

Each vertebral level from L1 to S1 was reviewed for the presence and severity of osteophytes (OPH) and vertebral narrowing (Disc Space Narrowing (DSN)), using the Lane atlas [3, 4]. In this atlas the categories are as follows: grade 0 = none; grade 1 = mild; grade 2 = moderate; and grade 3 = severe. DSN was defined as present when there was a grade 1 narrowing at two or more vertebral levels. Because of the small proportion of subjects without osteophytes, we used a higher cut-off value for this feature; OPH was positive when there were osteophytes of at least grade 2 at two or more vertebral levels. When DSN and OPH were both positive and present at 2 or more levels, the participant was assigned as "LDD case". The definition suggested for LDD was previously found as the best related to clinical symptoms including lumbar pain [17]. A severity score for each participant was calculated adding the individual scores of DSN and OPH (1-3) of all intervertebral levels.

BMD measurements

DXA BMD (g/cm²) of the right proximal femur and lumbar spine were measured at baseline using a Lunar DPX-L densitometer (Lunar Radiation Corp., Madison, WI, USA). Total body scans were performed at the third follow-up visit (mean follow-up 6.5 years) using a ProdigyTM fan-beam densitometer (GE Lunar Corporation Madison, WI) and analyzed with the encoreTM software. The software employs an algorithm that divides body measurements into areas corresponding to total body, head, trunk, arms and legs. Other methodological details have been described previously [18].

Assessment of osteoporotic fracture

Follow-up started either on January 1 1991 or at the time of inclusion into the study. For this analysis, follow-up ended either at January 1, 2007 or, when earlier, at the participant's death or loss to follow-up. For 80% of the study population, medical events were reported through computerized general practitioner diagnosis registers. For the remaining 20%, research physicians collected data from the general practitioners' medical records of the study participants. All collected fractures were verified by reviewing discharge reports and letters from medical specialists. Fracture events were coded independently by two research physicians according to the International Classification of Diseases, 10th revision (ICD-10). Finally, an expert in osteoporosis reviewed all coded events for final classification. Fractures coded as incident vertebral fractures were considered clinical fractures if they were identified on radiographs when subjects with symptoms (principally pain) visited the medical practitioner. All fractures that were considered not osteoporotic (fractures caused by cancer and all hand, foot, skull, and face fractures) were excluded. The period of follow-up was calculated as the time from enrolment in the study to the first fracture, death, or the end of the planned follow-up period, whichever occurred first. The participants were followed for the occurrence of fracture for approximately 12.8 years (±3.1 SD yr.).

Assessment of prevalent and incident radiographic vertebral fracture

Radiographic vertebral fracture: both at baseline, between 1990 and 1993, and at the second follow-up visit, between 1997 and 1999, a trained research technician obtained lateral radiographs of the thoracolumbar spine of subjects who were able to come to the research center. The follow-up radiographs were available for 2,819 individuals, who survived an average of 6.3 years after their baseline center visit and who were still able to come to our research center. All follow-up radiographs were evaluated morphometrically in Sheffield by the McCloskey-Kanis method, as described previously [19]. If a vertebral fracture was detected, the baseline radiograph was evaluated as well. If the fracture was already present at baseline, it was considered a prevalent fracture. All vertebral fractures were confirmed by visual interpretation by an expert in the field to rule out artifacts and other etiologies, such as pathological fractures [20]. Participants with missing data on one or more risk factors were excluded (n=434).

Literature Study

Relevant articles were identified by a systematic search using the database of PubMed with the words ["spine osteoarthritis" or "spine OA" or "disc degeneration"] and [21] as keywords in the title or abstract. The following inclusion criteria applied for this review: a) listed in PubMed, b) publication in the English language, c) study in humans, d) the article represents original data, e) subjects with and without disc degeneration features are compared in the study in relation to vertebral and/or osteoporotic fractures, f) the full-text article was available. Methodological quality assessment is found in the supplementary material.

Statistical Analysis

We compared the baseline characteristics of the study population and the FN- and LS-BMD between the LDD cases and controls using analysis of variance (ANOVA). Categorical variables were analyzed using chi squared test. Cox's proportional hazards regression was used to assess association between LDD and OP fractures (or only clinical vertebral fractures). The analyses were adjusted by gender (or stratified by gender), age, BMI, lower limb disability and FN-BMD as continuous variables. Departure from additive effect of the risk factors was tested using interaction terms in the model. All these analyses were made using SPSS V. 15.0. Meta-analyzed results and forest plots included in the literature study were obtained using the Comprehensive Meta-analysis Software Version 2, Biostat, Englewood NJ (2005). Power calculations were done with PS version 2.1.31.

RESULTS

Study population

Characteristics of the cohort comprising 2,385 participants with data for the two major outcomes: LDD and vertebral fracture are shown in table 1. At baseline, 362 participants had LDD (moderate OPH and mild-DSN) in two or more intervertebral levels. Subjects with LDD were older and heavier than controls. Also, LDD subjects had 0.72 and 0.32 S.D. higher LS- and FN-BMD at baseline compared to controls (Figure 1, $P<0.001$ for both, FN and LS-BMD differences). Additionally, figure 1 shows that total body- and skull-BMD (measured at a later time point) were also significantly increased in subjects with lumbar disc degeneration. All BMD analyses were adjusted for age, gender, BMI, lower limb disability.

Table 1. Baseline Characteristic for subjects according to LDD features in two or more levels.

Variables	Controls * n=2023(82)	LDD** n=362(18)	P value
Female	1161 (57)	207 (57)	0.9
Age (years)	63.7 (5.7)	65.9(6.2)	<0.001
BMI (kg/m^2)	26.2 (3.7)	26.9(3.8)	0.04
FN-BMD (g/cm^2)	0.88 (0.13)	0.91 (0.14)	<0.001
Total-BMD (g/cm^2)	1.10 (0.12)	1.12 (0.13)	0.001
Head-BMD(g/cm^2)	1.93 (0.28)	2.01 (0.29)	<0.001
Smoking (Current/former)	1367 (68)	233 (64)	0.18
Lower limb disability	0.16 (0.38)	0.21 (0.33)	<0.001
Falling (yes)	241 (12)	52 (14.5)	0.29
Prevalent Radiological VFx*	88(6.6)	22(7.4)	0.83

Abbreviations: LDD: Lumbar Disc Degeneration, **defined as mild Disc Space Narrowing (DSN) and moderate/severe Osteophytes (OPH) in two vertebral levels. *Controls: Participants with less than 2 levels affected by DSN and OPH per level. Values presented are mean and Standard Deviations (SD) for each continuous variable and numbers and percentage (%) for categorical variables. BMI (Body Mass Index), smoking, LDD (Lower Limb Disability, falling and prevalent radiographic-vertebral fractures (VFx) comparisons were adjusted for age and gender. All Bone Mineral Density (BMD) analyses were adjusted for age, gender, BMI and LDD.
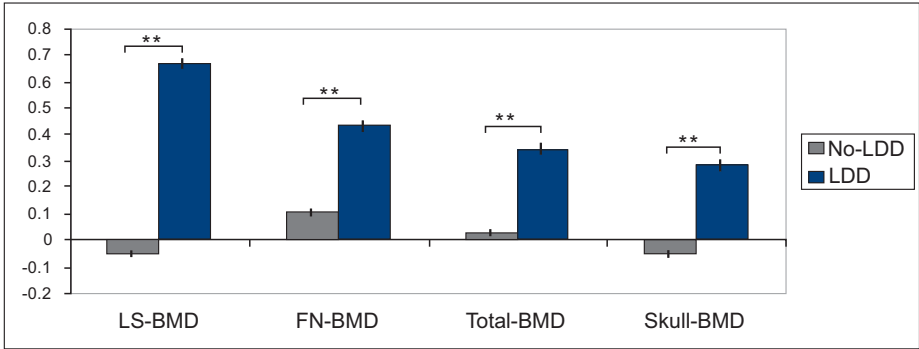
Figure 1 Bone Mineral Density (BMD; Z-scores) differences between subjects with Lumbar Disc Degeneration (LDD) and without LDD in four different regions: Lumbar Spine (LS), Femoral Neck (FN), Total Body and Skull. P values were adjusted by age, gender, height and BMI. **P value0.001. Total Body- and Skull-BMD were measured in a subset of 1,649 participants at a second follow up.

The number of prevalent radiographic-vertebral fractures was not different in the LDD group compared to the group without LDD after adjustment for age and gender (Table 1,

P=0.83). The mean LDD severity score was higher in males than in females (mean=6.6 (SD=4.3) for males and 5.9 (SD=4.4) for females (*P*<0.001, adjusted for age and BMI)). However, there was no statistically significant association between LDD-severity score and all type of OP-fractures (*P*=0.13).

Osteoporotic fracture risk

During 12.8 (SD=3, 12) years of follow-up, 558 participants suffered an osteoporotic (OP) fracture. Subjects with LDD had an increased risk of OP fractures compared to subjects without LDD (HR: 1.29, CI: 1.04-1.60). The risk slightly decreased after adjustment for age, gender, BMI, lower limb disability and FN-BMD (HR: 1.24 (0.99-1.55). We found a significant interaction between gender and LDD on fracture risk suggesting differences in OP fracture risk between genders (P for interaction term: 0.03). Therefore, we stratified the analysis according to gender and observed that only males with LDD had an increased OP fracture risk. (Table 2, Adjusted HR: 1.80 (1.20-2.70). *P*: 0.005 for males and HR: 1.08, CI: 0.82-1.41, *P*=0.59 for females).

Table 2. Risk of vertebral and Osteoporotic fracture in participant with Lumbar Disc Degeneration (LDD).

n.LDD/n	Clinical Vertebral Fractures (HR & 95% CI)				Osteoporotic Fractures (HR & 95%CI)			
	Unadjusted	P	Adjusted risk	P	Unadjusted	P	Adjusted risk	P
All (362/2385)	1.53 (0.98-2.40)	0.06	1.64 (1.03-2.60)	0.04	1.29 (1.04-1.60)	0.02	1.24 (0.99-1.55)	0.06
Males	1.41 (0.68-2.90)	0.36	2.34 (1.09-5.04)	0.03	1.88 (1.27-2.79)	0.002	1.80 (1.20-2.70)	0.005
Females	1.22 (0.71-2.10)	0.47	1.39 (0.78-2.50)	0.26	1.11 (0.85-1.43)	0.45	1.08 (0.82-1.41)	0.59

Risk for osteoporotic and clinical vertebral fractures in participants with Lumbar Disc Degeneration (LDD) defined as categorical variable according to presence of at least mild Disc Space Narrowing (DSN1) and moderate/severe osteophytosis (OPH) per intervertebral level in at least two intervertebral levels. Lumbar disc degeneration was evaluated only at lumbar spine (L1-L5). Risk of vertebral and osteoporotic fracture are Hazard ratio (HR) and 95% confidence interval (CI) unadjusted or adjusted for baseline Characteristics (gender, age, BMI, lower limb disability, femoral Neck Bone mineral density (FN-BMD). Number of clinical vertebral fractures=116, Number Osteoporotic fractures= 558.

Clinical Vertebral Fracture:

After the follow-up time, 21% of participants having fractures (n=116) had a clinically defined vertebral fracture. Participants with LDD had an increased hazard of having a clinical vertebral fracture during the follow-up (Table 2, adjusted-HR: 1.64, CI: 1.03-2.60, *P*: 0.04. As it was for overall OP-fractures, the hazard for a clinical vertebral fracture was higher only for males with LDD (HR: 2.34, CI: 1.09-5.04 in males and 1.39, CI: 0.78-2.50 for females).

Radiographic Vertebral Fracture:

During 6.3 years of follow-up, 106 participants had an incident radiographic-vertebral fracture. After adjustment for age, gender, BMI, FN-BMD and prevalent radiographic vertebral fracture, subjects with LDD had 2.14 increased odds of having a radiographic vertebral fracture. However, this was not statistically significant and the broad confidence interval revealed low power in the analysis (CI: 0.82-5.58, *P*=0.12). Hence, we reviewed the existent literature on the relationship between LDD and (vertebral) fractures.
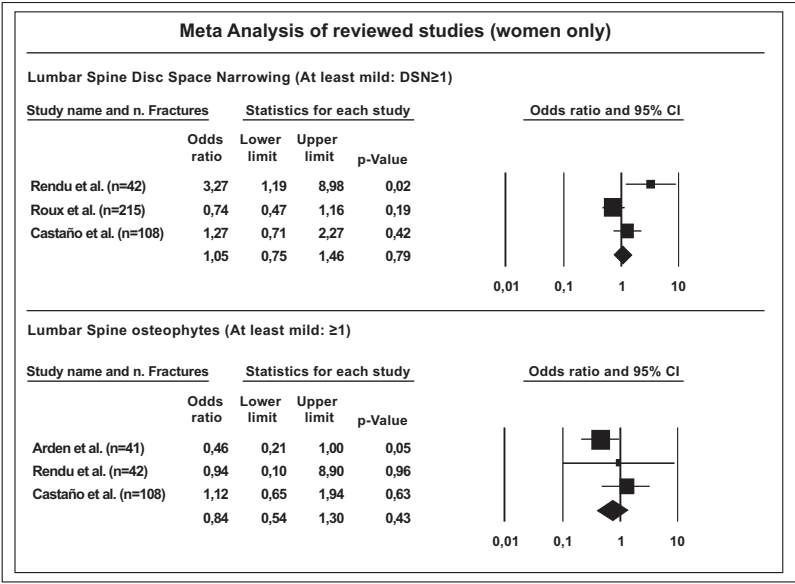
Figure 2 Meta-analysis of lumbar disc degeneration features and their relation with vertebral fractures in women. Values are Odd Ratios (OR) and 95% confidence interval. Abbreviations: Mild disc space narrowing (DSN>=1), minimal or mild osteophytes (OPH>=1). Studies were included when they had similar adjustments.

Results of the literature study:

A total of five studies (four from the literature search + current results from our study) analysing the relation LDD and radiographic vertebral fractures were included in this review. There were no studies analysing the relation of LDD with other types of fractures according with the selection criteria previously explained. Further details of the selection procedure of studies can be found in the Supplementary material. From these five selected studies, two were done in the same population therefore, only the most recent "longitudinal prospective" (Sornay et al.) was included in the meta-analysis (Figure 2). These studies fulfilled the inclusion criteria requirements and methodological quality assessment, including adjustment for age, gender, BMI and BMD in the analysis. Only one study (Roux et al.) did not perform BMD adjustment in the analysis of vertebral fracture risk.

All selected studies were done in post-menopausal women, one of them in women with osteoporosis [11]. In all studies, radiographic-LDD features were evaluated from the first till fifth lumbar segment (L1-L5). LDD was defined as presence of osteophytes (OPH), or disc space narrowing (DSN) in at least one intervertebral level. A detailed description of the studies and definition of LDD and vertebral fracture assessment is presented in the supplementary table 1.

Table 3 shows the results of the studies reviewed. Prevalence of at least minimal/mild osteophytes in the studied populations differs between 56 to 90% (Table 3). In the study of Roux et al. there was also a protective effect of OPH for vertebral fractures, however in that study, the association was not adjusted for BMD; what seems to modify the relationship osteophytes-Vertebral fractures (Table 2). Post-hoc power calculation demonstrated that to have 80% power to detect OR>=1.2 having an incidence of radiographic vertebral fractures of around 5%, a sample size of around 4200 participants would be needed. Consequently, confidence intervals are wide and associations of separate features of combined LDD definition did not reveal conclusive evidence (Figure 2, OR: 0.70, CI: 0.39-1.25, P: 0.23 and OR: 1.05, CI: 0.75-1.46, P: 0.79 for presence of at least mild osteophytes and disc space narrowing, respectively).

Table 3. Results of the Reviewed Studies for Association of LDD and Risk of Vertebral Fractures.

Reference	Definition VFx	n. VFx	OR (Confidence Interval) Unadjusted risk	OR (Confidence Interval) (BMD adjusted)	P adj.
Arden et al.[13]	Quantitative McCloskey	41	Not shown	LS-OPH: 0.46 (0.21-0.99) Thoracic-OPH: 3.57 (1.55-8.24)	<0.05
Sornay Rendu [14]	Semi-quantitative Genant	48	Lumbar spine OPH 1: 0.8 (0.4–1.6) DSN 1: 3.4 (1.5–7.8) OA Grade 2: 1.6 (0.9–2.3)	Lumbar spine 1.0 (0.5–2.1) 3.5 (1.5–8.3) 1.7 (0.9–3.2)	Not shown
Sornay Rendu [12]	Semi-quantitative Genant	42	LS-DSN: Not shown LS and Thoracic spine DSN1: 6.88 (1.64–28.9) OPH1: 1.39 (0.18–10.7) OA grade 2: 1.57 (0.81–3.01)	LS-DSN: 3.27 (1.19-8.98) LS and Thoracic spine DSN>=1: 6.59 (1.36–31.94) OPH>=1: 0.94 (0.10–8.97) OA grade 2: 0.92 (0.42–1.99)	Not shown
**Roux C et al [11]	Semi-quantitative Genant	215	DSN1: 0.83 (0.55–1.26) OPH1: 0.45 (0.21–0.99) LS-DSN2: 0.86 (0.56–1.33)	DSN 1: 0.67 (0.43–1.04) OPH 1: 0.38 (0.17–0.86) LS-DSN2: 0.74 (0.47–1.16)	0.071 0.02 0.191
This study	Quantitative McCloskey	108	DSN1: 1.26 (0.72–2.20) OPH1: 1.10 (0.43–2.84) OA grade 2: 1.00 (0.57–1.74) LDD: 1.14 (0.61-2.15)	DSN1: 1.27 (0.71–2.26) OPH1: 1.27 (0.48–3.34) OA grade 2: 1.05 (0.59–1.87) LDD: 1.14 (0.57–2.29)	0.42 0.63 0.86 0.70

DISCUSSION

This study shows that individuals affected with lumbar disc degeneration (LDD), in spite of having a systemically higher BMD, are not protected of osteoporotic fractures. Contrarily, male subjects with LDD had an increased risk for osteoporotic fractures, including clinical vertebral fractures. Results of the meta-analysis of the literature showed that LDD has been studied only in women for its association with radiographic vertebral fractures. Additionally, this is the first study analysing the association of LDD with all type of fractures in males and females.

The results of the meta-analysis were not conclusive regarding the relation of separate LDD features (osteophytes and disc space narrowing) and risk of radiographic vertebral fractures in females. We found a higher systemic BMD in participants with LDD. Participants with LDD had a higher bone mineral density not only in lumbar spine (where BMD measurements are known to be influenced by the presence of osteophytes), but also in femoral neck, total body and skull.

This is in line with previous findings of higher BMD in LDD patients not only in lumbar spine but also in other body regions [6, 7]. Measurement of skull-BMD has been shown to be less subjective to change during aging and influence of environmental and mechanical factors (strain and weight bearing) [22]. Higher skull-BMD in subjects with LDD suggests that a systemically higher BMD might be present before LDD. In spite of higher BMD in participants with LDD, we found a higher osteoporotic fracture risk. It is possible that the increased BMD in subjects with LDD is not enough to compensate for other detrimental effects of disc degeneration on trunk stability and flexibility that might result in an increased fracture risk. Loading on the spine is determined by a person's height, weight, muscle forces, and activity, but can also be affected by intervertebral disk degeneration [23-25]. Loss of disc height and its properties produce high tensile strains in the endplate and they have been shown as causal factors for "failure of the vertebra" [26, 27].

Additionally, disc degeneration can affect other structures (vertebra itself, muscles and ligaments) producing modification in the distribution of compressive and tensional forces through the column that in normal conditions are evenly distributed. Ligaments of the anterior region have changes as a consequence of LDD, causing its remodelling and thickening [28, 29]. Consequently ligaments loses elasticity and the trunk's flexibility decreases; this becomes evident during aging where the range of spine movement is severely affected. Individuals with LDD have more stiffness in trunk and lower legs that

could increase the reaction time during falling and other demanding occupational activities which are major situations where fractures occur in elderly [30, 31]. We found a higher osteoporotic fracture risk in males. Severity of LDD was higher in males, principally because of higher severity of disc space narrowing. Additionally, there is some evidence for an association between disc space narrowing and lower back pain especially in men proportionally increasing with a higher number of affected intervertebral disc spaces [17]. However, severity itself did not explain the increased OP fracture risk in males. Neither was the risk explained by other factors such as lower limb disability, which was found to be higher in males with LDD and other common risk factors including age and falling risk. In older males (>=65years), clinical vertebral fractures are caused by no known trauma or by low-energy trauma. It is known that most fractures occur in men with normal BMD and clinical vertebral fractures are particularly common in the oldest men [21]. Clinical vertebral fractures have been also related to important comorbidities, negative effects on quality of life and increased mortality [27-29].

The systematic review showed some important aspects. Previous studies analysing the association between LDD and fractures have been done only in females, the majority had small sample size and they defined LDD using only separate features. There were also important methodological differences between these studies in type (cross-sectional versus longitudinal) and fracture assessment. In spite of trying to meta-analyze the results of studies included in this review, using homogeneous definitions, power was still insufficient to draw significant conclusion on the relation of separate LDD features and radiological vertebral fractures. The review made evident that there is need for consensus in the definition of radiological LDD. In our opinion, more stringent radiological definitions are needed; some studies only consider osteophytes to define LDD. Osteophytes are a common feature in older populations and its prevalence depends of how stringent the definition is, reaching 90% for presence of minimal/ mild osteophytes. Presence of disc space narrowing and osteophytes (at least moderate) in the same intervertebral level should be considered when LDD is defined because it has been shown to be more clinically relevant; this combination radiological definition was found to best correlate with clinical symptoms: lumbar pain and stiffness [17, 26].

There are strengths and limitations in this study. This study is unique in examining the relation of LDD, osteoporotic and vertebral fractures in a large prospective cohort that includes males and females. In addition, the composed definition of several radiographic features used in this study is an advantage because it is more stringent and clinically relevant. We also examined separate LDD features in order to compare results with

earlier published studies. However, we concluded that even after the meta-analysis the number of radiographic vertebral fractures was insufficient to get conclusive evidence in the relation of radiographic vertebral fractures with separate LDD features. Finally, the conclusions of this study are limited to radiographic findings of LDD.

CONCLUSIONS

To conclude, we consider that subjects with LDD in spite of having higher systemic BMD are at higher risk of osteoporotic fractures, especially males for whom LDD seem more severe.The exact mechanisms to explain this association merits further investigation, considering that both, LDD and clinical vertebral fractures are common, associated with comorbidities and decreasing quality of life.

Acknowledgement

Authors' roles: Study design: MCB, JvM, FR. Study conduct: MCB. Data collection: EdS, SBZ, FR, LO, AH. Data analysis: LO and MCB. Data interpretation: MCB, LO, JvM and FR. Drafting manuscript: MCB and JvM. Revising manuscript content: AGU, JvM, SBZ, AH, EdS and FR. Approving final version of manuscript: all authors. MCB and JvM take responsibility for the integrity of the data analysis.

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Chapter 4.2

SUPPLEMENTARY MATERIAL

Radiographic Assessment of LDD

Lumbar lateral radiographs were obtained at 70 kV, a focus of 1.8, and a focus-to-film distance of 120 cm, using High Resolution G35_43–cm film (Fuji Photo Film Company, Kanagawa, Japan). Radiographs were scored by a single observer trained by a radiologist for the presence of the individual radiographic features of disc degeneration. The observer was blinded to clinical characteristics of the participants. Inter-observer reproducibility was assessed by a second independent observer who evaluated a random selection of 140 (5%) radiographs. The ICC was 0.83 for osteophytes and 0.77 for vertebral narrowing, indicating good reproducibility. More details have been described elsewhere [17].

Methodological quality assessment review

The presence of the following quality criteria was verified in all studies: 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 (or weight and height) 5) clear definition of disc degeneration and fracture, 6) clear description of statistical methods, 7) adjustments were made for age, gender, BMI and BMD in the analyses of fracture risk, 8) results are presented as OR with 95% confidence limits. Procedure to Select studies for Review: In total, there were 296 studies identified in PubMed. Only 36 had the selected words in the title or abstract. 28 studies were excluded on the basis of the abstract. Of the 8 studies retrieved for full examination, 3 studies were excluded because they did not fulfil the inclusion criteria after reading the complete manuscript.

Supplementary Table 1. Details of the Studies Included in this Review.

AUTHOR & REF.	TYPE OF STUDY	N	POPULATION	DEFINITION LDD	JOINTS ASSESSED FOR OA	N. LDD (LS) CASES/ CONTROLS (FEMALES)
Arden et al.	Population based Retrospective	939	Females	At least minimal osteophytes (DNS no evaluated)	Lumbar and thoracic spine	OPH: 248/444
Sornay Rendu	Cross-sectional	559	Females	0= No DSN, No OPH, 1= Mild osteophytes/DSN, 2= Moderate/severe DSN/OPH	Lumbar L1–L5 Thoracic 2 levels	OPH 1: 419/140 DSN 1:358/201 OA (Grade 2): 345/214
Sornay Rendu et al	Longitudinal prospective	634	Females	0= No DSN, No OPH 1= Mild osteophytes/DSN 2= Moderate/severe DSN/OPH	Lumbar L1–L5 Thoracic: 2 levels	OPH 1: 481/153 DSN 1:374/260 OA (Grade 2) =355/279
**Roux et al	Cross-sectional	410	Osteoporotic Females	*At least 1 DSN * 1 level of grade 2 (LS only) * At least 1 OPH	T4 to L4	DSN 1 : 265/145 LS (DSN2): 367/410 OPH1 : 370/40
This study	Population based Prospective	2354	Males (1008) Females (1346)	0= No DSN, No OPH, 1= Mild osteophytes/DSN, 2= Moderate/severe DSN/OPH LDD= DSN 1& OPH2 (2-IVL)	Lumbar L1-L5	DSN1: 875/471 OPH1:1219/127 DSN1/OPH1:860/508 +DSN 1& OPH2 : 207/1161

Abbreviations: OPH= osteophytes, DSN= Disc Space Narrowing, L1-L5: Lumbar level from first till fifth Intervertebral Level (IVL).

Chapter 5

Osteoarthritis and mortality

Chapter 5.1

Association of hip and knee osteoarthritis with mortality

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Ling Oei

Edwin Oei

et al

ABSTRACT

Osteoarthritis (OA) is the most prevalent joint disease affecting elderly individuals worldwide. However, limited information exists regarding its effect on mortality. To determine the effect of OA on mortality and its underlying causes. We assessed whether duration of OA influences mortality risk. We studied this in a prospective population based cohort of general population living in the city of Rotterdam (The Netherlands), consisting of 4,848 individuals 55 years and over with scored radiographs and data of covariates. Participants were evaluated for radiographic and clinical hip and knee OA at baseline and first follow-up visit. The OA-duration was estimated considering radiographic OA-evidence at baseline and follow up. Relationships of all-cause and cause-specific mortality and survival-time differences in years were assessed from first follow-up visit (between 1990-1993) through 2011. Radiological and clinically defined OA (hip or knee), was associated with higher overall mortality (Radiological-OA, Hazard Ratio [HR]: 1.36, 95%CI: 1.23-1.51). Rates were higher only for individuals with disease of long duration (OA>4years-HR: 1.44, 95%CI: 1.29-1.60). OA was associated with an increased hazard risk for all cause-specific mortality rates. Diabetes, dementia, overweight, difficulty in activities and walking disability were more common in individuals with OA, and partially explained the increased mortality (fully-adjusted HR: 1.23, 95%CI: 1.10-1.37). Subjects with hip- and knee-OA died on average three years earlier compared to subjects without OA ($P<0.001$). Hip and knee OA of long duration (>4 years) is associated with higher mortality of all causes, which is partly explained by comorbidities, disability and functional limitations.

INTRODUCTION

Osteoarthritis (OA) is the most common joint disease among the elderly and a leading cause of chronic pain and disability in the elderly population. It is known that osteoarthritis coincides with other diseases as obesity, cardiovascular disease (CVD) and diabetes (1-4). OA has a negative impact on general health, which translates into important differences in quality of life compared to non-affected individuals (4-7). It has been suggested that subjects with hip or knee OA have a higher mortality compared to the general population (4, 8, 9). Yet, this information comes from few studies that have several methodological problems (reviewed in 8), including small sample size as a major limitation. Two recent reports had large sample size, but were not cohort based (4, 9). The first estimated quality-adjusted life-years lost by combining the U.S. census and obesity data from national data sources with estimated prevalence of symptomatic knee osteoarthritis and then simulated the outcome using the Osteoarthritis Policy Model (4). The second manuscript selected individuals with joint pain from a general population and compared their mortality risk with estimates taken from national databases (9). This makes it difficult, if not impossible, to study the underlying cause of the association and in addition might limit the generalizability of the observations. The relationship between OA and mortality merits further investigation since OA is one of the most prevalent and disabling diseases in the elderly. Possible explanations for the deleterious effect of osteoarthritis on survival include: reduced levels of physical activity among persons with osteoarthritis due to disability, adverse effect of medications used to treat symptomatic OA, particularly non-steroidal anti-inflammatory drugs and presence of comorbidities, including obesity (4, 8, 9). Therefore, we aimed to determine the effect of OA on mortality and whether it is affected by the duration of the disease. We further explored the role of comorbidities, risk factors, functional limitations and/or disability.

METHODS

Study Population: The Rotterdam Study

The Rotterdam Study is a large ongoing prospective population-based cohort study of individuals 55 years and over in the city of Rotterdam. This study includes participants from the Rotterdam Study I cohort (RS-I) which was initiated in 1990 and consists of 7,983 individuals and the Rotterdam Study II cohort (RS-II) which was initiated in 2000 and consists of 3,011 individuals. The design and rationale of the studies have been described in detail elsewhere (10). In summary, the objective of the Rotterdam Study is to investigate the determinants, incidence and progression of chronic disabling diseases

in the elderly. The medical ethics committee of Erasmus University Medical Center approved the study and written informed consent was obtained from each participant.

Radiographic assessment and OA definition

Hip and knee radiographs were scored using the Kellgren and Lawrence (KL)-grading system at baseline (RS-I-1 and RS-II-1) and first follow up visits (RS-I-3 and RS-II-2) in each cohort (11). Clinical osteoarthritis (hip/knee) was defined as radiographic KL>=2 and complaints in the same joint during the month previous to interview. Further details of the radiographs' scoring can be found in the Appendix. A total of 4848 participants (3,376 participants from RS-I and 1,472 participants from RS-II) had scored knee and hip radiographs for osteoarthritis, both at baseline and follow-up (mean time between first and second radiographs was 6.29 and 4.15 years for RS-I and RS-II, respectively). From them, 115 participants had received a TJR, these were analysed separately for association with mortality. Consequently, analyses for the association between osteoarthritis and mortality were performed in 4,733 participants with the most updated OA status (RS-I-3 and RS-II-2).

We hypothesized that the effect of OA on mortality might be "cumulative", being stronger for those participants that had lived longer with osteoarthritis and its associated comorbidities and functional limitations. To test this hypothesis, we divided the radiographic osteoarthritis cases into new incident cases (no osteoarthritis at baseline) and cases with a longer duration (OA from baseline).

Assessment of cause-specific mortality

Participants from the Rotterdam Study are continuously monitored for major disease outcomes and mortality through computerized linkage of the study database to general practitioners' medical files. The cohorts' cause-specific mortality was coded according to the International Classification of Diseases, tenth Revision (ICD-10). This information was available until the first of January, 2011. All different causes of mortality were recorded according to ICD-10 codes and grouped into seven categories: cardio- and cerebrovascular diseases, cancer, chronic lung diseases (non-infected pulmonary diseases), dementia, external causes (composed mainly of severe fractures that lead to death –most of them hip fractures, also fatal accidents and suicides), infectious diseases and other causes (heterogeneous group composed of a minority of non-tumoral gastrointestinal, renal, haematologic and cerebral diseases, senility and

cachexia; and a majority of unspecified, unattended and sudden death).
http://www.who.int/classifications/icd/en/

Clinical assessment

At baseline and after six and four years follow up for RS-I and RS-II respectively, trained interviewers performed an extensive home interview on demographic characteristics, medical history, risk factors for chronic diseases and medication use; Questionnaires included aspects of joint pain, activities of daily living, walking disability and ability in Activities of Household and Leisure (from now called "functional limitations"), smoking, use of analgesics and socio-economic status (SES)/ educational level. Height and weight were measured to calculate Body Mass Index (BMI). Incidence of major outcomes and diseases as CVD (including stroke), cancer, dementia and type-2 diabetes were collected through medical registers. Further methodological details are explained in the Appendix

Statistical Analyses

Cox proportional hazards models were used to assess the relation between hip and/or knee osteoarthritis (clinical/radiographic) and mortality after exclusion of the participants with total joint replacement at follow-up (n=4733). We imputed the missing covariates data at follow up for 527 participants by using multiple imputation for each cohort with overall mortality, log transformed survival time, sex, age, smoking, BMI, comorbidities (cardiovascular disease, dementia, diabetes) use of analgesics, knee and/or hip OA, walking disability and functional limitations as variables in the imputation model, to create 50 imputed datasets (12, 13). We evaluated the quality of the imputation using Monte Carlo error (14).

The proportional hazard assumption of the Cox models was assessed using the Schoenfeld residuals-based test. HRs reported in this paper did not violate the proportionality assumption. Log likelihood statistics was used to evaluate the goodness of fit of the models.

We examined the association between OA and mortality-related risk factors (BMI, walking and functional disability, smoking, analgesic use and SES) including comorbidities, (CVD, diabetes mellitus, cancer and dementia) using logistic regression analysis after adjustment for age, cohort study and gender.

Multivariate Cox models for total mortality were adjusted by gender, age and cohort study. The full regression model included all comorbidities and related risk factors associated with radiographic/clinical OA and mortality. Additionally, we examined each of the seven specific causes of mortality in participants with osteoarthritis using Cox models for each of them after adjustment for age, gender and cohort study and after adjustment for comorbidities and risk factors included in the previous models (Model 2). Meta-analyzed results of the association between osteoarthritis and mortality for both cohorts are presented. All Analyses were performed using SPSS (version 17) and/or Stata (version 12).

Survival Analyses

Differences in Hazards between osteoarthritis and no-osteoarthritis groups were converted to survival difference in years. Mean survival time was estimated as the area under the survival curve using Kaplan and Meier. The Log-rank test was used to test for equality of survival time between osteoarthritis groups (hip/knee/hip and knee OA) compared to participants without osteoarthritis at any joint site.

RESULTS

Clinical Characteristics and Mortality-related Factors

There were some differences between the two analysed cohorts (Appendix Figure 1, baseline characteristics). Compared to RS-II, participants from RS-I were 2% more females, on average 5 years older, had 5% lower BMI, 11% lower SES and 4% more smokers. They also had more CVD, cancer, dementia, radiological osteoarthritis, joint complaints and walking disability than participants from RS-II; which in part might be due to their older age (Appendix Figure 1). In spite of these differences between cohorts, we found consistent results of all variables in their relation with mortality (appendix Figure 1). In RS-I, during a mean follow-up period of 9.6 years, 1,473 subjects died. In RS-II, 137 participants died during a mean follow-up of 5.7 years. Older age, male gender, CVD, diabetes mellitus, dementia, walking disability, functional limitations, current or former smoking, radiological hip and knee OA, lower SES were significant factors associated with higher mortality in both cohorts. A total of 115 participants underwent TJR before starting the follow up period for mortality. Those participants with TJR were 20% more females and 3 years older compared to participants without TJR (both: *P*<0.001, not in table). A separate analysis of mortality in participants with total joint replacement at baseline due to osteoarthritis was performed considering that

elderly patients with osteoarthritis who have undergone TJR might be healthier than participants with OA without replacement (15). They indeed had a decreased mortality compared to participants without replacement without achieving statistical significance (HR: 0.80 (0.53-1.04, *P*=0.08, full adjusted model).

Mortality Risk in Participants with Osteoarthritis

We had a total of 4,733 individuals assessed for OA-status at baseline and first follow-up. OA (radiographic/clinically defined) was significantly associated with overall mortality (Table 1. HR for radiological OA: 1.36, 95%CI: 1.23-1.51 and HR for clinical OA: 1.36, 95%CI: 1.19-1.55, respectively). The association with mortality was stronger in females compared to males (Table 1). We examined whether the effect of OA on mortality is dependent on disease duration and therefore stratified the analysis into cases that had osteoarthritis from baseline versus new incident cases. A total of 1,334 participants (28.2%) had osteoarthritis from baseline and 387 (8.2%) were new incident osteoarthritis-cases (Figure 1). The increased mortality risk was confined to those individuals that already had OA from baseline (Figure 1. HR: 1.44 [1.29-1.60], *P*<0.001), while the new osteoarthritis cases did not have an increased mortality risk (Figure 1. HR: 1.05 [0.86-1.28]). This analysis was adjusted for age, gender and cohort study.
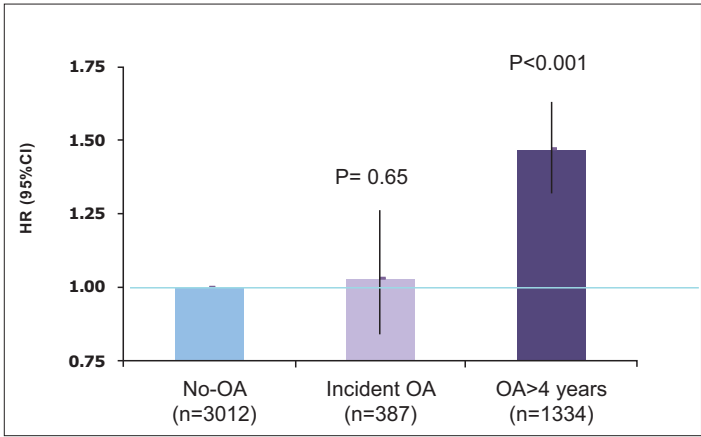
Figure 1 Association of Osteoarthritis with Mortality according with the disease duration. Values presented are Hazard Ratios and 95% confidence intervals (CI).

Table 1. Association of radiological and clinical osteoarthritis with total mortality.

Variables	Mortality all			Females			Males		
	N events/N	Hazard Risk 95%CI	P Value	n.events/N	Hazard Risk 95%CI	P Value	n.events/N	HazardRisk 95%CI	P Value
Radiological OA[a]									
Model 1	1610/4733	1.36 (1.23-1.51)	<0.001	846/2721	1.48 (1.28-1.71)	<.001	764/2012	1.23 (1.06-1.44)	0.01
Model 2		1.23 (1.10-1.37)	<0.001		1.32 (1.14-1.54)	<.001		1.15 (0.98-1.34)	0.081
Clinical OA[b]									
Model 1	918/2533	1.36 (1.19-1.68)	<0.001	561/1647	1.42 (1.19-1.68)	<0.001	357/886	1.23 (0.98-1.555)	0.07
Model 2		1.20 (1.04-1.39)	0.012		1.23 (1.02-1.48)	0.029		1.13 (0.89-1.43)	0.33

Abbreviations: n.events/N = mortality events during the follow up/ total number of participants used in the analysis. Hazard Risk with corresponding 95% confidence intervals (95%CI) from multivariate Cox proportional hazards models. P values were calculated by using two sided Wald tests. a. Participants with radiographic hip or knee osteoarthritis (OA). b. Participants with radiographic definition of osteoarthritis and pain in the same joint. Model 1: adjusted for age, gender, cohort study. Model 2: adjusted for age, gender, cohort study, smoking, diabetes, cardiovascular disease, dementia, BMI, walking disability, analgesics-use, and functional limitations.

Association of OA with Comorbidities, Clinical Risk Factors and effect on Mortality risk

Figure 2 shows the association between OA and comorbidities after gender, cohort and age adjustment. Participants with hip or knee osteoarthritis had more disability and had more functional limitations. Other diseases and risk factors that strongly associated with osteoarthritis were: dementia (OR:2.05, CI:1.35-3.13), being overweight (BMI higher than 26, OR: 1.81, CI: 1.59-2.06) and diabetes mellitus (OR: 1.36, CI: 1.11-1.666). The use of analgesics was also significantly increased in the osteoarthritis group (OR: 1.24, CI: 1.01-1.51). Low education level (SES), CVD and smoking were not associated with osteoarthritis. The association of osteoarthritis with mortality was attenuated after adjustment for comorbidities. The majority of this attenuation was due to adjustment for walking disability and functional limitations (Table 1-model 2, radiological osteoarthritis HR: 1.23, 95%CI: 1.10-1.3 and clinical-OA HR: 1.20, 95%CI: 1.04-1.39).
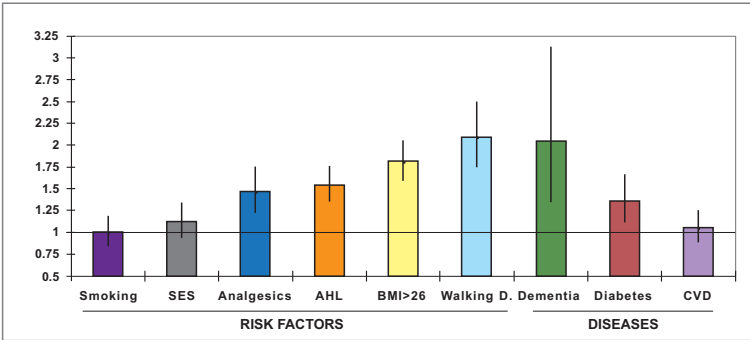
Figure 2 Comorbidities and Mortality-related Risk Factors in individuals with Hip or Knee Osteoarthritis Compared to Non-affected Individuals. osteoarthritis was defined as KL-score >= 2 in hip and/or knee. Values presented are Odd Ratios (OR) and 95% Confidence Intervals (CI). Comorbidities were analyzed in its relation with OA. Abbreviations: Cardiovascular Disease (CVD), Socio Economic Status (SES), Functional limitations in Activities of Household and Leisure (AHL), Body Mass Index (BMI). The associations shown were adjusted for age, gender and cohort study.

Cause-specific Mortality in Participants with OA

We subsequently examined cause-specific mortality risk and found that mortality risks were higher in participants with radiographic hip or knee OA than in participants without osteoarthritis for all specific causes of mortality (Table 2). The significant causes of mortality in subjects with hip or knee OA irrespective of gender and age were trauma, dementia, cardiovascular disease and others in order of effect-size (HR: 2.00, *P*=0.03; HR: 1.61, *P*=0.006, HR: 1.36, *P*=0.002 and HR: 1.36, *P*=0.01, respectively). There was a significant decrease in effect size for all specific causes of mortality after adjustment for

the comorbidities and risk factors previously described. Mortality risk due to trauma, infections and cardiovascular events were not significant after adjustment (Table 2, full adjusted model HR: 1.68, *P*=0.12, HR:1.24, *P*=0.36 and HR: 1.15, *P*=0.16 respectively). The attenuation of the HR for the specific causes of death was explained for a large part by walking disability and functional limitations. Mortality due to CV causes was partially explained by prevalent diabetes mellitus, analgesic use and walking disability in that order of importance. Prevalence cardiovascular disease was not a significant factor explaining mortality in participants with OA compared to participants without OA.

Table 2. Cause-specific mortality in participants with hip or knee osteoarthritis.

Cause of death	n.events(%)		Model 1		Model 2	
	No-OA	OA	Hazard Risks (95%CI)	*P* Value	Hazard Risk (95%CI)	*P* Value
Cardiovascular	238 (29)	241 (31)	1.37 (1.13-1.66)	0.001	1.16 (0.95-1.42)	0.14
Cancer	266 (32)	174 (22)	1.22 (1.00-1.49)	0.05	1.21 (0.99-1.49)	0. 07
Respiratory	36 (4)	25 (3)	1.19 (0.70-2.04)	0.52	1.27 (0.73 -2.22)	0.40
Other causes	156 (19)	172 (22)	1.36 (1.08-1.72)	0.008	1.21 (0.95-1.55)	0.12
Dementia	63 (8)	90 (12)	1. 61 (1.15-2.26)	0.006	1.43 (1.00-2.05)	0.05
Trauma	18 (2)	31 (4)	2.00 (1.09-3.71)	0.03	1.68 (0.87-3.22)	0.12
Infections	44 (5)	46 (6)	1.39 (0.90-2.15)	0.14	1.25 (0.79-1.98)	0.34

Abbreviations: OA: Radiological hip or knee osteoarthritis, H.R: Hazard risks and, 95% Confidence Interval (95%CI) for cases of hip or knee osteoarthritis (OA) versus no-cases (No-OA). Model1: adjusted by age, gender and cohort study. Model 2: adjusted by age, gender, smoking, prevalent diabetes, cardiovascular disease, dementia, BMI, walking disability, analgesics-use, functional limitations and cohort study.

Analyses of Survival Time

Participants with hip or knee OA had a shorter survival time compared to participants without OA (Mean survival time difference: 2.11 years, SD: 0.15, Log rank test: *P*<0.001 for these differences in survival time). There were also differences in the survival time according to the joints affected with osteoarthritis (Figure 3). Participants with both hip and knee OA had the shortest survival time; approximately 3 years less lifetime compared with participants without OA. Participants with only hip osteoarthritis

or only knee osteoarthritis had 1.7 years and 0.84 years respectively shorter survival time compared to participants without osteoarthritis (Fig 3, *P* value of Log rank test<0.001).
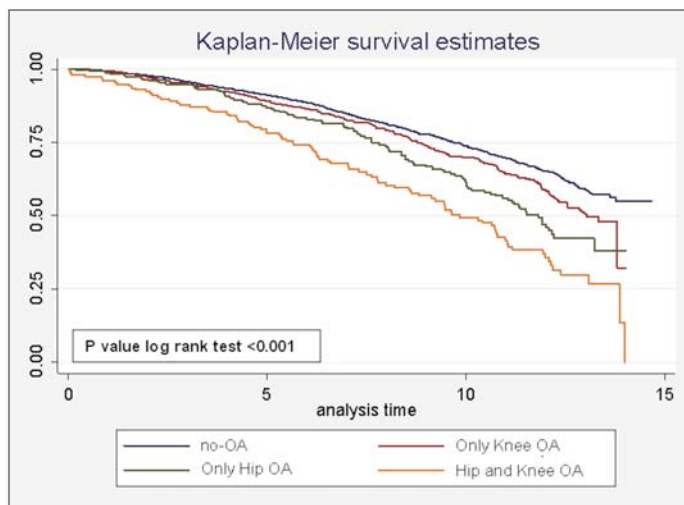
Figure 3   Kaplan and Meier Curves for the Mortality Risk of osteoarthritis According with Joints Affected. Comparison of cumulative survival in participants according to joints affected with radiological osteoarthritis (OA) at follow up. Log rank test P value <0.001 demonstrates significant differences between groups. Participants with hip and knee OA have a decreased survival time compared to controls (P value<0.001).

DISCUSSION

Results of this study on 4,733 individuals indicate that subjects with hip or knee OA (radiological or clinically defined) have a higher overall mortality. Mortality risk was increased for those with longer disease duration, suggesting that the negative effect of OA on survival is cumulative. Additionally, subjects with OA had more comorbidities than subjects without OA. Diabetes, dementia and other mortality-related risk factors as walking disability and functional limitations were independent mortality-related factors that partially explained the higher mortality in subjects with OA. Subjects with both joints affected: knee and hip, are the most vulnerable group and dye on average three years earlier than subjects without OA.

We here demonstrated that mortality risk for individuals with OA differ between those who received a joint replacement and those who did not. Subjects with OA who received total joint replacements had nearly 20% lower mortality risk, close to significant. This has been reported before, which indicates a truly different mortality hazard for individuals receiving a TJR compared to the rest of the individuals suffering from OA (15). This finding may be due to confounding by indication. In general, individuals that

receive a TJR are considered healthier and with greater life expectancy than the average general population, (15) the latest confirmed by our results. Joint replacement therapy is generally done in healthy low risk patients who are less affected by chronic diseases (16, 17). Thus, there is no surprise that OA patients who are elected for joint replacement have a better life expectancy compared to the general population (15, 18, 19).

Excess of mortality in participants with hip or knee OA was present for all specific causes, being more pronounced for trauma, dementia, infections and cardiovascular causes. The relation of OA with dementia as cause of death in subjects was previously reported in a single study (9). In our study, the majority of deaths in this group were due to Alzheimer's disease (86%, result not shown). Interestingly, a recent investigation using a mice model suggested that OA might exacerbate Alzheimer disease pathology.(20) However, the underlying biological mechanism is not yet clear. More studies will be needed to further elucidate this association between OA and dementia.

Regarding cardiovascular fatal events, there are previous reports that associate OA with CVD and cardiovascular mortality (9, 21-24). NSAID use, inflammatory factors, decrease in physical activity and disability have been suggested as possible explanatory factors for this relation (9). In this study diabetes, analgesics use, functional limitations and walking disability were important factors associated with OA, explaining the variation in the mortality risk due to cardiovascular causes in subjects with OA. Diabetes has recently been shown to be a predictor of severe OA independent of BMI and age (3). In this study, subjects with OA were more affected by diabetes than subjects without OA. Additionally, our results suggest that diabetes might have an important role in survival of subjects with OA. Research in this area is needed to elucidate the metabolic component that is involved in pathogenesis of OA. Prevalent cardiovascular disease did not explain the higher CV mortality in participants with OA. It might be possible that other cardiometabolic morbidities not included in the analysis would explain the rest of the CV mortality. It is known by previous publication (Hoeven TA et al.) that atherosclerosis is associated with knee OA. Atherosclerosis between other factors would explain the increased mortality due to CV causes in participants with OA. In conclusion, a significant part of mortality risk in participants with OA is due to consequences of OA (disability and analgesics use). Another part is due to diabetes, BMI (in those with knee OA only) and possibly other factors not included in this study.

Functional limitation was a covariate that explained also part of the mortality risk due to infectious, lung diseases and other causes. This variable comprised four questions that evaluate the ability to perform house-chores and social activities outside house as

shopping and travelling. This type of functional limitations, might be an adequate proxy to evaluate the severity and prognosis of the disease reflecting the impact of OA not only on the affected joints but, on the overall functionality of the affected subjects. Disability was a predictor of mortality not only for cardiovascular but also for traumatic causes. Disability itself is considered as an important risk factor for mortality independent of age and concomitant diseases in the same individual and it is known that disability it is also a risk factor for other adverse events related to a higher mortality risk (25).

We found a stronger association of OA with mortality in females than in males. There are important sex differences in OA and also in their relation with metabolic endpoints. OA is more prevalent, severe and symptomatic, with higher rates of progression in females (26-28). Women with OA have more pain and poorer joint function that translate in major limitations (29, 30). This could explain the higher mortality risk due to OA in women.

This is the first population-based study analysing the relation between mortality and OA, considering their possible causes and the effect in this relation of the disease duration. Previous reports recruited patients from hospitals, general practices or with joint pain and then they compared the mortality rates to that of the general population statistics affecting generalizability of their findings. Additionally, in other studies definitions of OA were not stringent; only based on the presence of possible osteophytes and/or only based on radiological criteria (8, 9, 31).

Our study has also some limitations. We estimated the disease duration using as reference the radiographic assessment at baseline and follow up. We cannot point out the exact duration of the disease (based on first symptom or first radiological evidence). We used multiple imputations to handle missing data on covariates. We compared these results with those obtained only in individuals with complete data, finding similar results. In spite that we analysed important comorbidities and risk factors, data on other comorbidities (such as depression) were not collected at the time of radiographic assessments and therefore were not included. Therefore, we cannot exclude the possibility that other comorbid conditions here not included might explain part of the association of OA with mortality. Finally, participants who were included in this study were those who were able to visit the research center for radiographic examination. Indeed, subjects who did not undergo radiographic examination had a higher disability index than those that visited the research center. This might introduce a health bias towards underestimation of the effect of OA.

In conclusion, we found a strong positive association between hip and/or knee OA and mortality due to all causes with the exception of cases of joint replacement where the association is inverse. The association was partially explained by the increased prevalence of comorbidities and risk factors as diabetes, dementia, high BMI, walking disability and functional limitations in this population.

The results from this study have implications for the management of patients with OA. Subjects with hip and/or knee OA should be targeted in programs for prevention of physical and functional decline and control of chronic diseases. Additionally, subjects with OA will benefit of alternative therapies for pain control beyond analgesics. Therefore, these results might also have consequences on policies, guidelines and treatment of subjects affected by OA.

Acknowledgements

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APPENDIX

Clinical assessment

For this study we used information at baseline that was collected between 1990-1993 for RS-I and 2000-2002 for RS-II and approximately six and 4 years follow up respectively. We analysed the presence of hip pain (joint complaints of right/left hip during the last month), activities of daily living based on the Instrumental Activities of Daily Living scale and lower limb disability assessed with a modified version of the Stanford Health Assessment Questionnaire (32, 33). For the purpose of this study, two items were considered: Ability to walk outdoors on flat ground for both cohorts (at baseline and follow-up) and ability in functional Activities of Household and Leisure (AHL), for practical purpose called functional limitations along the manuscript. Ability in AHL was evaluated using four different questions: Are you able to run errands and shop? Are you able to get in and out of a car? Are you able to travel? Are you able to do chores such as vacuuming or housework? These questions including walking outdoors were scored as follows; 0= without difficulty, 1= with some difficulty, 2= with much difficulty and 3= unable to do (needs help). Other methodological details of the definitions are described elsewhere (34).

We also used information about smoking (current and former vs. never), use of analgesics (in the last month: yes/no) and education level as a proxy of socioeconomic status (SES). Height and weight were measured at baseline examination with the subject in a standing position with indoor clothing without shoes and BMI was calculated (kg/m^2) and included as linear variable or categorical (BMI>26 kg/m^2).

Other comorbidities were also assessed; specifically myocardial infarction, dementia and type 2 diabetes mellitus. Myocardial infarction was verified by cardiologist, general practitioner or ECG. Diabetes mellitus was defined as use of antidiabetic medication and/or abnormal fasting glucose and/or abnormal oral glucose tolerance test; a nonfasting or postload glucose level of 11.1 mmol/L or over was considered abnormal. Dementia was assessed as a three-phase approach: brief cognitive test, neurological testing and diagnosis confirmation through detailed examination or medical records (35). All these variables were collected at baseline and during the follow up period.

Radiographic assessment and OA definition

Radiographic hip OA was defined as definite Joint Space Narrowing (JSN) and at least a possible femoral osteophyte and knee OA as possible JSN and at least a definite osteophyte, i.e. a score of 2 or more in one or both joints according to Kellgren and Lawrence (KL≥=2). Inter-rater agreements for KL score (Kappa coefficients) were 0.74, 076 and 0.71, 0.68 for hip and knee respectively in RS-I and RS-II.

Baseline Characteristics	RS-I	RS-II
N. of participants	3308	1425
N. of events	1473	137
Mean follow up-time	9.6 (3.6)	5.7 (1.2)
Age mean (SD), y	72.7 (7.2)	68 (7.2)
Gender male N. (%)	1387 (42)	625 (44)
SES (Low educational level)	589 (18)	104 (7)
BMI mean (SD)	26.5 (3.7)	27.8 (4.1
Cardiovascular disease N (%)	584 (18)	109 (8)
Diabetes mellitus N (%)	308 (9)	191 (13)
Dementia (%)	108 (3)	18 (1)
Smoking current and former N (%)	602 (18)	194 (14)
Walking disability N (%)	426 (13)	261 (19)
Analgesics use N (%)	320 (10)	190 (13)
Difficulty in AHL (%)	784 (26)	401 (29)
Radiological Knee OA † (%)	1051 (32)	327 (23)
Radiological Hip OA † (%)	307 (9)	168 (12)
Hip and knee OA † (%)	99 (3)	34 (2)
Knee or hip pain (%)	1065 (57)	613 (67)

RS-I plot axis: Lower Mortality / Higher Mortality — 0.5 1.0 2.0 3.0

RS-II plot axis: Lower Mortality / Higher Mortality — 0.5 1.0 2.0 3.0 4.0

Appendix Figure 1 Clinical Characteristics of the Study Populations. Associations between characteristics at baseline and all cause mortality for Rotterdam study I (RS-I) and Rotterdam Study II (RS-II). Hazard ratios (HR) with corresponding 95% confidence intervals (95%CI) from multivariate Cox proportional hazards models in the complete data set for all covariates at baseline after adjustment for age and gender. P values were calculated by using two sided Wald tests. Values are mean and standard deviations (SD) or number of cases and percentages (%) for categorical variables. Age in years (y), BMI: Body Mass index, SES: Socio Economic Status according with educational level; risk are shown for lower education. † Individual without osteoarthritis in the joint(s) as reference category.

Chapter 6

General discussion

Hip osteoarthritis (OA) is a complex disease in which genetic and environmental factors play a role. In the previous chapters, new genes and risk factors for hip OA were identified and the burden of the disease was analyzed; increased fracture risk, more comorbidities and a decreased survival time are common outcomes for subjects with OA. The main findings and limitations of each study were previously discussed. In this chapter, the main points and considerations addressed in this thesis are presented and suggestions for future research are given.

Genetics of hip OA: defining the phenotype

Hip OA is a multifactorial disease in which diverse factors including genetics interact causing a process of change and deterioration of cartilage and subchondral bone and new bone formation on the cartilage edge. However, OA is a heterogeneous disease in which is possible to identify different sub-phenotypes, each of them with their own risk factors. For example, bone changes are not always present, which would allow defining different hip OA sub-phenotypes according with the bone response during OA. The genetic architecture of OA has also demonstrated complexity, as it does not follow a pattern of Mendelian inheritance while not many associated genetic variants have been identified. Therefore, a genome wide association study (GWAS) seems to be a good approach to identify new genes and genetic factors that might help to explain the genetic component of the disease and identify new biological pathways.

In a GWAS, the selection of a correct phenotype is critical for the interpretation of the study as well as for the power to find true significant and plausible results. Previous efforts using multiple dichotomous-composed definitions in GWAS of OA yielded few genomic loci. Reasons for this include, low power in the discovery phase of the GWAS and phenotypic heterogeneity. Phenotypic heterogeneity can substantially reduce power in GWAS [1] and it is clear that OA suffers from this as well. In OA, some cases only exhibit joint space narrowing and not osteophyte formation (as we show in chapter 4.1). A way to decrease phenotypic heterogeneity is through the use of intermediate phenotypes and endophenotypes. Endophenotypes are stable phenotypes that can be more reliably phenotyped and quantified and additionally, they might be closer to what is encoded in the DNA sequence. Minimal Joint Space Width (mJSW) is an endophenotype that represents cartilage thickness. The principal advantage of JSW as endophenotype is the focus on one structure of the joint (cartilage) and it is primarily "state-independent" (measurable in an individual whether or not illness is active). Additionally, gradual narrowing of the joint space width is part of the definition of OA and it is seen during the

progression of hip OA on radiographs with a very good reliability [2, 3]. The use of a definition based on the joint space width as endophenotype showed to be a successful approach to identify genes responsible for cartilage thickness (development and/or maintenance) and OA as it was presented in chapter 2. In addition, the use of a quantitative trait such as mJSW to discover signals implicated in hip OA has some advantages. Quantitative traits are more powerful than dichotomous traits especially when the accuracy of the measure is better for the quantitative trait [4]. Quantitative traits are traits with a continuous distribution in natural populations, with population variation often approximating a statistical normal distribution on an appropriate scale [5].

Unfortunately, studies in OA where DNA is available for subjects with data on diverse OA-phenotypes or endophenotypes are limited. The largest study so far of subjects included in a GWAS using mJSW as an endophenotype was presented in chapter 2.2, N=19,181. For OA, the maximum amount of subjects included in a study was presented in the arcOGEN study (a maximum of 14,883 cases and 53,947 controls) [6].

The use of endophenotypes has demonstrated to be successful also for other diseases as it is the case for osteoporosis using bone mineral density (BMD). With a similar sample size as the one used for mJSW in the discovery phase (N=19,195 for BMD and N=13,013 for mJSW), 20 loci were identified to be associated with BMD [7]. In average, are required 4 times more participants to identify the same amount of loci in GWAS of mJSW than in GWAS of BMD. Differences in methods used to measure BMD and mJSW, with more consensus and calibration in favour of the BMD measure, between other reasons might explain the differences in the relative number of identified loci [7].

Not all SNPs that are identified using an endophenotype are associated with the studied (end stage) disease. However, it is still a valid and successful approach to identify new signals and to understand the pathogenesis of the studied disease. Such is the case for the relation BMD-osteoporotic fractures. From a total amount of 56 SNPs associated with BMD at GWS level only 14 are associated with osteoporotic fracture risk (25%) [8]. The relation of mJSW and OA seems to follow a similar pattern; only some of the SNPs (3 out of 5) associated with mJSW are associated with hip OA (Chapter 2.2). However, the number of mJSW-associated SNPs is low to make a fair comparison with the findings in other studies as for example the GWAS on BMD.

The approach of using the endophenotype mJSW to identify genes for the end stage disease OA demonstrated to be successful not only to identify genes tat are relevant for

OA but, also to elucidate the role of variants that were previously found related to OA. Some of the SNPs that are approaching GWS level for mJSW (Chapter 2.2) are in linkage with SNPs in genes previously identified as associated with OA which allow inferring their relation. It was the case for the variant close to *SUPT3H* (rs10948155) and the variant from *ASTN2* (rs4837613), with respective variants rs10948172 and rs4836732 identified in the arcOGEN study for OA [6]. It indicates that the association with OA of the variant near of *SUPT3H* and *ASTN2* might be mediated by differences in cartilage homeostasis rather than bone changes or pain, which might be possibly suggested by the association of variants in *SUPT3H* with height or by the role of *ASTN2* in the central nervous system.

In addition to an increased sample size and studies in other ethnicities where new variants might be found, further studies in hip OA will need to use other specific endophenotypes (for example: hip geometry traits, sclerosis, cartilage regeneration, wound closure) to differentiate between the roles of bone, cartilage and other tissues implicated in the disease process.

From GWAS hit to gene

The success of GWAS identifying SNPs and genetic loci associated with a phenotype/ disease contrasts with the difficulties to confidently point to the implicated gene. Proving the involvement of an implicated gene is far from easy and does not follow automatically from identifying a SNP through a GWAS. A SNP identified using GWAS is likely part of a large region of linkage disequilibrium, thus making it difficult to correctly point out the SNP that have a biological link or is responsible for the association. This is also the case of intergenic SNPs or SNPs localized in a "gene desert area". It has been estimated that ~25% of the human genome consists of gene deserts, defined as long regions containing no protein-coding sequences and without obvious biological functions (9). There is not consensus about how far the identified SNP should be from the closest gene to be called gene desert area. Some authors have been using a distance equal or higher than 500 Kb as cut of point for the regions to be denominated gene desert (10).

There are different approaches to help in the identification of the responsible gene after the identification of a genetic locus by GWAS. At first it would be possible to check if the closest gene(s) is giving rise to a Mendelian disease with a phenotype resembling the GWAS phenotype or whether there are knock-out (KO) mouse models giving rise to a phenotype resembling the studied GWAS phenotype and therefore the phenotype might be observed in humans carrying the corresponding mutations [11].

Another approach is to see if the SNP, or an LD partner SNP, is correlated to expression of a nearby gene (so-called eQTL: expression Quantitative Trait Loci) [12]. SNPs that influence gene expression have been shown to be significantly enriched for GWAS associations (13-14). Other methods for identifying SNPs that overlap regulatory elements, such as transcription factor binding motif, have been also successfully used to identify specific loci that have a functional role (14-15).

Pathways implicated in Hip OA and association with mJSW

From our genetic studies we observed several categories of proteins to be implicated in hip OA. These include transcription factors and signaling molecules which coordinate chondrocyte and osteoblast differentiation (endochondral bone formation) and finally help to determine the development of the hip joint including its size, orientation and height of the individual. This seems to be a plausible conclusion after the studies presented in Chapter 2 combined with published results from other studies [6, 16-18]. We observed associations between cartilage thickness and DNA variants near genes involved in joint and/or bone formation such as Wnt-signaling (*DOT1L*, chapter 2.1), *RUNX2* and Transforming Growth Factor Alpha (*TGFA*) among others (Chapter 2.2). Additionally, from other studies, the previously reported variants in Growth and differentiation factors *GDF5* and *SMAD* (receptors of TGF-beta signaling) have been associated with hip OA and/or height [6, 17,18].

GRAIL (Gene Relationships Across Implicated Loci (GRAIL)) was used to search for connectivity between genes near the SNPs previously reported as associated with hip OA at genome wide significant [6,16,19], and the SNPs reported in this thesis as associated with cartilage thickness (mJSW) discovered both through the use of a GWAS approach [20]. Only independent signals are included in a GRAIL analysis. Therefore, in this analysis, a total of 14 genomic regions (from 14 SNPs) were selected; 8 SNPs had been found associated with hip OA from previous studies (for more details see table 2, chapter 1) and the 6 signals reported in this thesis as associated with mJSW and or hip OA (Table 1, Chapter 2.2). GRAIL assesses the degree of relatedness among genes within regions that harbor predictive SNPs identified by GWAS, selecting the most connected gene that corresponds to 1 or more SNPs as the likely implicated gene. For each genomic region, a P-value is assigned by GRAIL based on he best candidate gene. The candidate gene reported by GRAIL (Table 1, second column) is the gene with the highest number of relationships to other associated genes in independent regions. GRAIL corrects its significance score for multiple hypothesis testing (by adjusting for the number of genes in the region), to assign a significance score to the region.

GRAIL offers a statistical approach based on connectivity described in literature to identify functionally related genes from across multiple disease regions that likely represent key disease pathways. GRAIL identifies the most significant gene as the likely candidate within a region identified by a GWAS hit. Therefore, not all genes reported by GRAIL coincide with the corresponding nearby genes for the identified associated SNPs reported by individual GWAS (Table 1). For each genomic region, a P value is assigned by GRAIL. From the set of genes reported by GRAIL as candidate for the SNPs reported associated with mJSW/cartilage thickness (Table 1), the genes with highest significant connections with others genes identified by GRAIL (according with the literature) were *PTHLH*, (reported by arcOGEN), *SULF2* (reported gene by Evangelou et al, 2013 was *NCOA3*), and *PAPPA* (the gene reported by arcOGEN was *ASTN2*) Which might be more suitable candidate for the association with hip OA because of his role in proliferative processes such as wound healing and bone remodeling (http://www.ncbi.nlm.nih.gov/refseq/). Additionally, Lango et al, identified also a SNP in the same region associated with height and they reported *PAPPA* as the gene responsible for the association [21].

Table 1. GRAIL data for selected individual SNPs of interest.

SNPs	CANDIDATE GENE REPORTED BY GRAIL	GENE REPORTED BY GWAS	GRAIL p-value	OA PHENOTYPE	REFERENCES
rs10492367	PTHLH	PTHLH	3×10^{-3}	Hip OA (KL or THR)	arcOGEN
rs6094710	SULF2	NCOA3	6×10^{-3}	Hip OA	TREAT-OA
rs4836732	PAPPA	PAPPA	0.04	THR–females	arcOGEN
rs11842874	F10	MCFL2	0.09	Hip OA (KL or THR)	Day-Williams et al
rs2862851	TGFA	TGFA	0.12	mJSW and Hip OA	This thesis
rs835487	CHST11	CHST11	0.14	THR	arcOGEN
rs10948172	RUNX2	RUNX2	0.15	Hip OA in males, mJSW	arcOGEN
rs2236995	SLBP	SLBP	0.150	mJSW	This thesis
rs12982744	AMH	DOT1L	0.22	mJSW and Hip OA	This thesis
rs11177	ITIH3	MUSTN1	0.33	THR or TKR	arcOGEN
rs10471753	PIK3R1	PIK3R1	0.24	mJSW	This thesis
rs496547	DDX6/	TREH	0.63	mJSW	This thesis
rs717433	HAO1	HAO1	0.73	mJSW	This thesis
rs9350591	MYO6	FILIP1	0.78	Hip or knee OA	arcOGEN

Abbreviations: minimal Joint Space Width (mJSW, hip osteoarthritis (hip OA). P-value >0.05 imply that the candidate gene reported by GRAIL has not significant connections with other genes within the study.

Connections between the genes are represented in the figure 1. The strongest connection between the genes identified by GRAIL was given between the Parathyroid hormone-like hormone (*PTHLH)* and other genes as *SULF2, RUNX2, TGFA, PIK3R1, PAPPA,* and *MUSTN1*. *PTHLH* regulates endochondral bone development and Sulfatase 2 (*SULF2),* act as co-receptors for numerous heparin-binding growth factor and cytokines [20]. Other gene with significant connections with the set of genes associated with mJSW and/or hip OA was *NCOA3* with connections with *PTHLH, AMH, NISCH* and *PAPPA*. In spite of significant connections with other genes of *NCOA3*, the best candidate gene reported by GRAIL for this SNP (rs6094710) was *SULF2* because it was more functionally related to the other signals included here in this analysis. GRAIL also assigns a select set of keywords using a text-based similarity metric supported on statistical text mining methods to suggest putative biological pathways [22]. Based on existing literature in PubMed till August 2012, GRAIL identified 20 keywords describing the functional connections of these loci. With these words it is possible to establish functional connections relevant in processes as development, growing and differentiation of bone and cartilage tissues. Between the first words from GRAIL are PTHrP and chondrotin- and heparan-sulfate. PTHrP is a protein member of the parathyroid hormone family that between other functions regulate bone development by maintaining the endochondral growth plate at a constant width act to mantein the proliferative state of chondrocytes and inhibit their maturation [23]. Chondroitin sulfate is an important structural component of cartilage and provides much of its resistance to compression [24]. Heparan sulfate is a sulfotransferase enzyme, co-receptor for growth factors, morphogenesis and adhesion of proteins. Heparan sulfate and chondroitin are both sulfated glycosaminoglycans which are produced by a biological sulfation reaction [25,26].

Understanding the role of the genes implicated in the development of the hip joint and OA development requires further research. The identified SNPs and suggested genes, which are implicated in bone and cartilage phenotypes, might have different roles in formation of articular cartilage, differentiation processes, maintaining and healing of bone and cartilage tissue that finally seems to have a direct effect on the risk of development of hip OA.
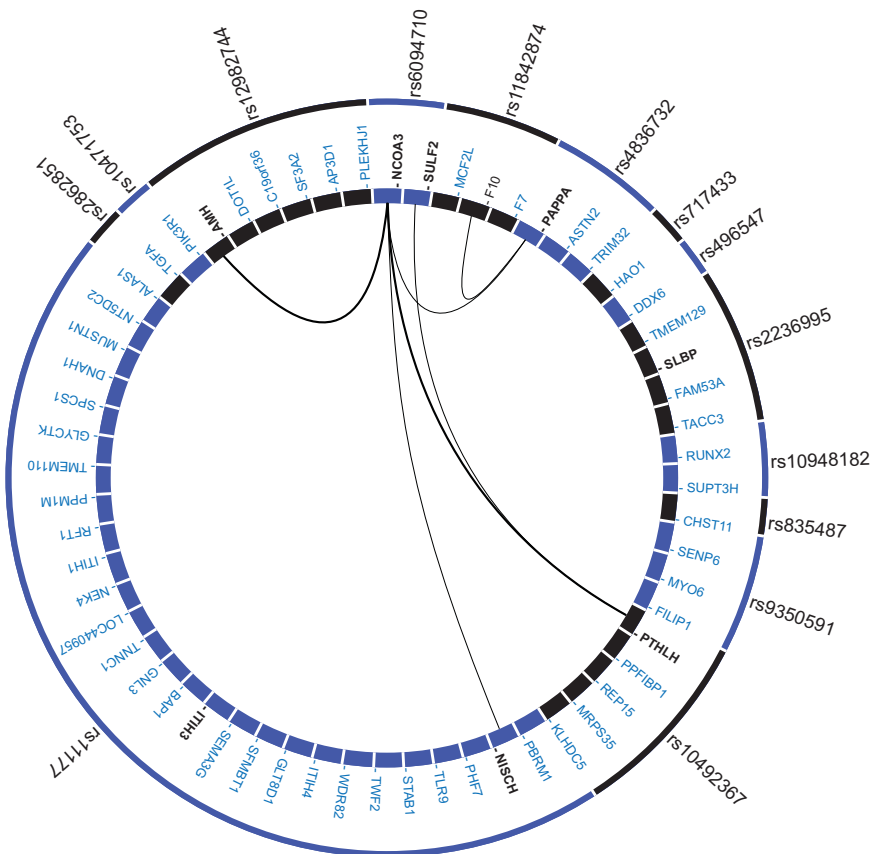
Figure 1 Graphical representation of the connections between the 14 selected SNPs associated with hip OA and/or mJSW with the corresponding genes using GRAIL. GRAIL analysis identified non-random connectivity (P<0.05) between associated genes (in bold). Only the genes with P-GRAIL <0.05 have connections displayed. The 20 keywords describing the functional connections of these loci in order of informativeness were: pthrp,chondroitin, sulfate, alpha, mullerian, factor, heparan, growth, histone, sulfation, sulfotransferase, hormone, loop, heavy, transforming, chains, parathyroid, bone, pregnancy and inhibitor.

Hip Geometry

From the studies presented in Chapter 3 and previous studies on the relation between hip geometry and OA, it is possible to conclude that there is a connection between hip geometry differences and the risk for hip OA. Bone is a dynamic tissue that is subject to a continuous process of remodelling as response to different physical demands (activity, load), hormonal, neurological factors, and aging among others. Therefore, it is difficult to determine whether geometry differences/disadvantages are causal factors of hip OA in older individuals, since during aging other risk factors might have a role on both

remodelling and OA. However, recent studies show that variation in acetabular coverage (dysplasia and protrusio acetabuli) in children and impingement in young adults are predisposing to hip OA [27]. This indicates that these alterations are present very early in life, probably at birth. Additionally, according with our results it seems that such differences remain until old age and have a significant predictive value for hip OA (Chapter 3).

Not much is known about the genetic factors that are responsible for differences in hip geometry and whether they are relevant for the risk of OA. Considering the role of hip geometry in OA and hip fracture, it is possible to hypothesize that some of the genes responsible for geometry differences might be relevant for the risk of OA and fractures. This aspect was not explored in this thesis, but it will be relevant to further analyse. There are few GWAS performed on measures of hip bone geometry including cortical thickness, buckling ratio (BR), cross-sectional area, femoral neck-shaft angle (NSA), the width of the femoral neck at the narrowest point (NW), femoral neck length, and proximal femur size in EU populations. Genome-wide significant associations with NW were found for SNPs located on chromosomes 1p13.2 (*RAP1A*) and 18q11.2 (*OSBPL1A*) [28]. Genome-wide significant associations with femoral neck shaft angle (NSA) were found for SNPs located on chromosome 2q11.2 (*TBC1D8*) [28]. A polymorphism within the *RTP3* gene was associated with BR in both EU and Chinese populations [29]. It is unknown whether these SNPs are associated with OA. Possibly, there will be many more genetic variants associated with geometric traits that might be relevant for bone and cartilage tissue and OA but these remain to be demonstrated.

Atrophic versus osteophytic OA types: differences in bone tissue and fracture risk

Hip OA can be classified according to the absence or presence of osteophyte formation and cartilage loss, expressed as degree of narrowing of the joint space on a radiograph. The formation of osteophytes is strongly associated with higher bone mineral density (BMD), not only on the joint affected with OA but also systemically. In this thesis, the relation between BMD, hip OA and lumbar disc degeneration (Chapter 4) was studied. It is interesting that BMD in subjects with the osteophytic-OA phenotype was also higher in areas as skull (Chapter 4.1 and 4.2), where BMD is less subject to changes due to aging and environmental influences (as physical activity). The fact that individuals that display the atrophic form of OA, i.e, without new bone formation, also have a lower systemic BMD suggests that individuals that form osteophytes have a better "bone-forming capacity" than people who do not. Subjects with osteophytic OA also have a

wider femoral neck, and greater bone strength than subjects with atrophic OA or those without any OA-feature as we have shown in chapter 4.1. It is plausible that these bone-differences between OA- sub-phenotypes, BMD and geometry are already there before to development of OA.

Differences in BMD seem to be not only systemically but also localized (Chapter 3.2). The superior part of the femoral head and other areas of the hip also have increased BMD at initial stages of OA and are predictive of progression of hip OA compared to the contralateral without-OA side (Chapter 3.2).

Besides, narrowing of the joint space alone (atrophic OA), without osteophytes, might indicate some degree of osteopenia/osteoporosis possibly accompanied with bone-fragility that translates in higher risk for osteoporotic fractures as shown in chapter 4.1. However, for lumbar disc degeneration (LDD), the relation between disc degeneration and fractures seems to be different. Osteophytes are very common in subjects with radiographic evidence of lumbar disc degeneration and those subjects also have a higher systemic BMD than subjects without LDD. Nevertheless, males with LDD are at higher risk for osteoporotic fractures including clinical vertebral fractures (Chapter 4.2). It is known that most of the fractures occur in men with normal BMD and clinical vertebral fractures are particularly common in oldest men. We did not have data on other bone properties or markers of bone metabolism to clarify this association. Considering that the study on the association of osteoporotic fractures and LDD (Chapter 4.2) is the first realized in males, our findings require further corroboration by other population-based studies.

There is not much information about the quality of bone tissue in subjects affected by OA and LDD. It is known that high BMD not always coincides with other measures of bone properties such as a Trabecular-Bone Score (TBS). TBS is a gray-level texture measurement that is applicable to DXA images measuring the mean rate of local variation of gray levels in 2D-projection images. The correlation between BMD and TBS is lower than expected [30, 31] while not many studies have been performed in males. Other measures of bone properties as TBS might give more information about the characteristics in favor of resistance of bone tissue to fractures that might differ in individuals affected by OA. Until now there are no studies analyzing TBS and BMD in populations affected with hip OA and LDD. According to our results presented in Chapter 4, we hypothesize that there are differences in other bone properties related to fracture risk between individuals affected and non-affected by OA, even in those with "normal"-BMD.

OA: a heavy burden for affected individuals

At the starting point of this thesis, OA was already recognized as a disease strongly associated with disability, especially walking disability and decreasing the years of productive live. In chapter 5 we show that individuals with OA had an increased mortality risk compared to those with the same characteristics without OA. The mortality risk was especially elevated in those individuals that already had OA for a longer period (>4 years). It is also of concern that individuals with hip or knee OA have a very limited participation in functional activities in and outside home that include personal and leisure activities. Thus the impact of OA on general health is dramatic and it is finally reflected in a higher mortality. Health professionals therefore need to be more aware of the serious health impairment that the subjects with hip or knee OA are suffering. Contingency plans needs to be carefully elaborated to hamper the negative effects of OA on disability and functionality. Subjects with OA need to be targeted in preventive programs for chronic diseases in the elderly. Monitoring important daily life activities could be a good preventive factor for morbidity and survival.

Suggestions for future research

Based on the finding presented in this thesis there are several research topics that will deserve further investigation:

Genetics:
With current GWAS approaches in OA we have identified several genetic factors but they explain only a modest part of the total phenotypic variation and a modest part of the genetic variation. The total genetic variation to be explained is indicated by the heritability (discussed in chapter 1.3) and ranges from 40 to 60% for hip OA and from 51–85% for knee-cartilage volume, being not yet estimated for mJSW at the hip joint [32]. The limited explained variance is due to the current focus on common variants and a heterogeneous disease phenotype. Some suggestions to identify more variants that might explain the genetic component of the disease are given below. GWAS with a higher number of participants or studying rare variants with novel techniques that have recently become available such as next generation sequencing. Family-based genetic studies using GWAS and exome sequencing with very clear and straightforward OA phenotypes and endophenotypes like the one here analyzed (mJSW) are needed. The use of linkage analysis on large families identified with co-inheritance of OA followed by whole exome sequencing of interesting regions might detect mutations that cosegregate with OA.

Stratification according to etiological factors. Future studies for example will have to consider gender and previous trauma not only for finding associated genetic variants but also to determine the predictive value of the genetic markers. Predictive markers might have a different value according with their relation with healing of cartilage/bone tissue and might interact with gender between other possible explanations. Additionally, there might be specific genetic variants related to inherent properties of the bone formation/ response and OA. Possibly, atrophic hip OA should be studied as a separate phenotype to give insight in the role of differences in bone density and other bone properties as etiological factors in the risk for OA.

Phenotypes. Along this thesis different approaches were explored to measure hip geometry: predefined geometry parameters (e.g, neck width) and Active Shape Modelling (ASM). The use of predefined geometry measures might be the option as endophenotype for genetic studies in OA and osteoporosis, in favour of efficiency, reproducibility, validation and interpretation needed in a GWAS, compared to ASM. Geometry as previously explained, might be a useful approach to identify variants important not only for fracture risk but also to hip OA. It will also be interesting to analyze the genetic factors that contribute to the differences in hip geometry that as we presented in chapter 3.1 have a role in the progression of hip OA.

In this thesis we focused on methods based on DXA scans to measure different bone properties relevant to hip OA and other bone diseases as LDD. However, other techniques are suitable to study bone tissue in population affected by OA. For example the use of TBS and studies with markers of bone/cartilage metabolism will give insight on the cause of increased fracture risk associated with OA. Additionally, this will give insight in the role of the bone tissue in the progression of OA.

mJSW demonstrated to be an important endophenotype for hip OA because it is a continuous trait, normally distributed in population, relatively easy to measure and with good reproducibility. It might be possible that genetic variants associated with cartilage thickness are site- and joint-dependent therefore GWAS is advised to be done for specific sites of the hip joint and for other joints. It might also be a successful approach for finding genetic associated variants for knee OA, that might be not only relevant for cartilage thickness of the knee joint but also for knee OA-risk. Similarly, studying genetics of JSW at other joints such as hand and vertebral joints might result in new variants associated with the disease.

This thesis focused on hip OA and it has been seen that variants for hip OA are not associated to knee OA and vice-versa. The genetics of OA seems to be joint-specific although variants in common for the disease at different joints might exist given the systemic nature of the disease. Clustering joints affected by OA should be based on similarities of the disease at different sites. For example, clustering knee and hand OA seems logical, since they share a similar strong risk factor (BMI) [33].

Other approaches to study mJSW more effectively is to examine younger populations. Studying this endophenotype in children will help to discriminate the genes that are relevant for cartilage development from those relevant only to cartilage loss that might occur in joint diseases in elderly.

It will be of crucial importance to determine the biological mechanism behind the discovered genetic loci that we present in Chapter 2. Although many of the SNPs/loci have good nearby candidate genes (such as *RUNX2* and *TGFA)*, it remains to be proven whether these genes are actually the culprit genes underlying the associations. Analysis of the expression in joint tissue and functional characterization of *TGFA*, *RUNX2*, *ASTN2, SLBP* will result in better understanding of the implicated signalling pathways and how they interact during the disease. This will give better insight and might be opening possibilities for future treatment of the disease.

Re-defining hip OA. Advances in the understanding of hip OA might benefit of a quantitative definition as is was the case for osteoporosis and BMD. In spite of the importance of bone phenotypes in OA as it was mention along this thesis and based on individual differences in bone response during the disease process, I would suggest narrowing of the joint space measure in mm as a good approach for defining the disease that deserves to be analyzed. However, reference standards from different young populations stratified by gender and adjusted by height with cut off values. Therefore, consensus on radiographic protocols to measure mJSW and narrowing of the Joint space are needed to be able to establish some parameters. Finally, considering different etiological risk factors might improve the benefit on future treatments focusing for example in cartilage repair, bone or hip geometry alterations pointing to a more personalized medicine.

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General discussion

Chapter 7

Summary / Samenvatting

SUMMARY

Osteoarthritis (OA) is the most prevalent joint disease that principally affects articular cartilage and bone tissue. It is a leading cause of chronic disability, because of its symptoms, pain, stiffness and limitation in joint movement. OA is regarded as a complex disease that is not completely understood. There is still lack of information in different areas including its pathophysiology, risk factors and genetic predisposition. Lack of knowledge about OA has been reflected in absence of effective treatments.

Between the most important risk factors for OA, age is considered the strongest predictor of the disease. However, other factors that have been associated with OA might have an important role in the disease and prognosis of individuals affected including their survival. They include height, bone mineral density, elderly comorbidities and genetic factors.

The aims of this thesis were to identify genetic and bone-related risk factors for hip OA and to determine the osteoporotic fracture and mortality risk in subjects affected by the disease. The majority of studies described in this thesis were conducted within the Rotterdam Study, a population based cohort study from the Netherlands. Genetic studies were often done in collaboration with other large studies on OA from Netherlands and abroad. **Chapter 1** gave an introduction of OA focusing on the hip joint, embryological joint development, geometry, bone related aspects and generalities about hip OA.

In **chapter 2**, genome-wide association studies on cartilage thickness and hip OA were presented. **Chapter 2.1**, using a GWAS approach we describe the association of a common variant from *DOT1L* gene with cartilage thickness and hip OA. We additionally presented functional studies on *DOT1L*, demonstrating a role in chondrogenic differentiation and adult articular cartilage through influence on the Wnt Signaling. In **chapter 2.2**, we identified five genome-wide significant (GWS) variants localized in or close to *TGFA, SUPT3H, RUNX2, PIK3R1*, *DOT1L*. The variants were associated with hip OA except for *PIK3R1*. Another variants were suggestive for association with cartilage thickness; they are localized in or close to *SLBP, TREH and HAO1* being *SLBP* the most significant for hip OA.. We found a significant connectivity between the genes associated with cartilage thickness, principally *TGFA* and *RUNX2* using GRAIL. Pathways where these genes are implicated are the EGF receptor signaling pathway (TGFA), TGF-beta signaling pathway (TGFB2), Insulin/IGF pathway protein kinase B signaling and PI3 kinase pathway between others, according to results of a special software that can identify functional connections between genes **(Chapter 2.2)**.

Deregulation of these genes and mechanisms might result in an increased risk for OA. Further studies on the functionality of these variants will improve our understanding of cartilage, bone tissue and will open the possibility of using them as potential pharmacological targets. **Chapter 2.3** using a different approach (bivariate analysis) and taking benefit of the mutual correlation that exists between cartilage thickness and height (~20%) found genetic variants in common for these traits: *DOT1L, SUPTH3, ASTN2* (previously identified associated with OA and height), and identified significant variants for cartilage thickness in other genes previously associated only with height: *DYM, FBXW11, TEAD1, IGF2BP3* and *IER3*. The variants showed independent effect on cartilage and height, suggesting a pleiotropic effect of these genes. A role of these genes in endochondral ossification process is plausible and verification of these findings will be pursued.

Chapter 3 describes the results of the prediction of hip OA (incident and progression) using two bone related factors. In **chapter 3.1** using two different methods, we quantified the variations in hip geometry in subjects without OA at baseline and we demonstrated how certain aspects of geometry contribute to the prediction of hip OA on top of the known clinical risk factors, age, gender, height and initial OA changes measured by Kellgren and Lawrence score. Hip geometry had a moderate but still significant contribution in the prediction of OA. **Chapter 3.2** studied the contribution of DXA based parameters of the proximal femur and femoral head on the prediction of progression of hip OA measured as narrowing of joint space width (>= 20%) or total joint replacement (progressors). Bone mineral density (BMD) and bone mineral content (BMC) values from areas close to the joint space including superior part of femoral head were higher in the group of progressors. Femoral head and femoral neck width were also bigger in the group of progressors. Differences in area, BMD/BMC of the superior part of femoral head, major trochanter, intertrochanteric between affected and unaffected hip were significant contributors in the prediction of progression of OA.

The complexity in the relation between BMD and OA is evidenced in **chapter 4**. Hip OA can be classified according with the principal phenotypic characteristics of OA: narrowing and osteophytes. Atrophic, hypertrophic and normotrophic hip OA types were analyzed. Subjects with OA characterized by osteophytes (hypertrophic/normotrophic) demonstrated higher BMD not only at places near to the affected joint but also systemic. Geometrical and BMD changes as those evidenced in chapter 3 were also found in participants with different osteophytic types of OA. Besides, subjects with atrophic hip OA characterized by degradation of articular cartilage without evidence of osteophytes showed a systemically lower BMD and higher risk for osteoporotic fractures that was not

explained by the lower BMD **(chapter 4.1)**. Subjects with lumbar disc degeneration (LDD), characterized by disc space narrowing and in the majority of cases multiple osteophytes have a higher BMD than those subjects without LDD. In spite of higher BMD, we found that male subjects with LDD had a higher risk for osteoporotic fractures, principally clinical vertebral fractures **(chapter 4.2)**.

Chapter 5 presents a comprehensive analysis of the comorbidities and impact of hip and knee OA in the survival on individuals affected. Participants from the first two cohorts from the Rotterdam Study were included in the analyses. Seven different causes of mortality were considered. Clinical and radiological OA was associated with higher overall mortality due to all causes. Participants with hip and/or knee OA have more comorbidities; Diabetes, dementia, overweight, walking disability and difficulty in activities of daily life were more frequent in subjects affected by OA and partially explained the higher mortality risk. Prevention and control of these chronic diseases in subjects with OA become relevant. In the treatment of OA, policies and guidelines should target the negative consequences of the disease, improving functionality, decreasing disability, controlling and preventing comorbidities in the population affected.

Finally, **chapter 6** offers a general discussion with the most relevant and controversial aspects of this thesis and give suggestion for future research in the area.

SAMENVATTING

Osteo artrose (OA), in de volksmond ook wel artrose genoemd, is de meest voorkomende gewrichtsaandoening die voornamelijk het articulaire kraakbeen en bot aantast. Het is een belangrijke oorzaak van chronische invaliditeit vanwege de pijn, stijfheid en bewegingsbeperking die het gevolg zijn van deze ziekte. OA wordt beschouwd als een zogenaamde "complexe ziekte", waarvan we weten dat zowel genen als omgeving een rol spelen, maar we begrijpen de initiatie en proces van de ziekte nog niet. Er is nog steeds een gebrek aan informatie in verschillende gebieden, waaronder de pathofysiologie, risicofactoren en genetische aanleg. Gebrek aan kennis over OA heeft tot gevolg dat er geen effectieve behandelingen zijn voor deze ziekte. Leeftijd wordt beschouwd als een van de belangrijkste risicofactoren voor OA. Andere factoren die zijn geassocieerd met OA en de prognose voor het erger worden van de ziekte zijn lengte, botmineraaldichtheid, comorbiditeit en genetische factoren.

Het doel van dit proefschrift is om genetische en bot-gerelateerde risicofactoren voor heupartrose en osteoporotische fracturen en het bijbehorende sterfterisico vast te stellen. De meerderheid van de studies beschreven in dit proefschrift werden uitgevoerd binnen de Rotterdam Studie, een populatie-gebaseerd cohort onderzoek in Nederland. Genetische studies werden vaak uitgevoerd in samenwerking met andere grote studies over OA afkomstig uit Nederland en het buitenland.

Hoofdstuk 1 geeft een inleiding op de embryologische ontwikkeling van gewrichten, geometrie en bot-gerelateerde aspecten van artrose, heupartrose in het bijzonder.

In **hoofdstuk 2**, werden genoom-wijde associatiestudies (GWAS) naar kraakbeen dikte en heupartrose gepresenteerd. **Hoofdstuk 2.1**, beschrijft de identificatie van de associatie van een vaak voorkomende variant in het *DOT1L* gen met kraakbeen dikte en heupartrose. Ook werden functionele studies van *DOT1L* gepresenteerd, waaruit blijkt dat het gen een rol speelt in chondrogene differentiatie en volwassen gewrichtskraakbeen via beïnvloeding van het Wnt-siganlerings systeem. In **hoofdstuk 2.2**, identificeerden we vijf genoomwijde significante (GWS) varianten in of dicht bij *TGFA, SUPT3H, RUNX2, PIK3R1, DOT1L*. De varianten waren geassocieerd met heup OA, behalve PIK3R1. Andere varianten waren suggestief voor associatie met kraakbeen dikte; ze zijn gelokaliseerd in of dicht bij *SLBP, TREH* en *HAO1*. *SLBP* is het belangrijkste voor heupartrose. We vonden significante verbanden tussen de geïdentificeerde genen door middel van speciale software die functionele verbanden kan identificeren. Pathways waarin deze genen zijn betrokken zijn de EGF-receptor signalering (*TGFA*), TGF-beta

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signalering (*TGFB2*), insuline / IGF-route, proteïne kinase B signalering en PI3 signalering (hoofdstuk 2.2). Verdere studies naar de functionaliteit van deze varianten zullen ons begrip van kraakbeen en bot verbeteren en wellicht mogelijkheid bieden om ze te gebruiken als potentiële farmacologische doelen. **Hoofdstuk 2.3** met behulp van bivariate analyse, welke gebruik maakt van de onderlinge samenhang die bestaat tussen kraakbeen dikte en lengte (~20%), werden genetische varianten gevonden die correleerden met verschillen in lengte en kraakbeendikte. Daarbij werden reeds eerder varianten gevonden (*DOT1L, SUPT3H, ASTN2*), maar er werden ook nieuwe variaties gevonden waarvan nog niet bekend was dat ze iets te maken hadden met kraakbeen: variaties in of vlakbij de genen *DYM, FBXW11, TEAD1, IGF2BP3* en *IER3*. De varianten vertoonden onafhankelijke effecten op kraakbeen en lengte, wat pleiotrope effecten van deze genen suggereert. Een rol van deze genen in het endochondrale ossificatieproces is plausibel en verificatie van deze bevindingen zullen worden nagestreefd.

Hoofdstuk 3 beschrijft de resultaten van de voorspelling van heupartrose (incidentie en progressie) met twee bot-gerelateerde factoren. In **hoofdstuk 3.1** werd met behulp van twee verschillende methoden variatie in heup geometrie gekwantificeerd bij personen zonder OA bij aanvang van de studie. Vervolgens zagen we hoe bepaalde aspecten van geometrie bijdragen aan de voorspelling van heupartrose bovenop de bekende klinische risicofactoren: leeftijd, geslacht, lengte en eerste OA veranderingen gemeten door de Kellgren en Lawrence score. Heup geometrie had een matig sterke, maar nog steeds belangrijke bijdrage in de voorspelling van OA. **Hoofdstuk 3.2** bestudeerde de bijdrage van DXA parameters van de proximale femur en heupkop aan het voorspellen van progressie van heupartrose. Botmineraaldichtheid (BMD) en botmineraalgehalte (BMC) waarden in gebieden dicht bij het kraakbeen met inbegrip van het bovenste deel van de heupkop waren hoger in de groep van progressors. Femurkop en femurhals breedte waren ook groter in de groep van progressors. Verschillen in oppervlakte, BMD / BMC van het superieure deel van de heupkop, trochanter major en intertrochanterisch dragen significant bij aan het voorspellen van progressie van OA.

Zoals te zien is in **hoofdstuk 4** is de relatie tussen BMD en OA complex. Heupartrose kan worden ingedeeld in een aantal groepen aan de hand van fenotypische kenmerken van OA: kraakbeenverlies en vorming van osteofyten. Atrofische, hypertrofische en normotrofische heupartrose werden geanalyseerd. Personen met OA gekenmerkt door vorming van osteofyten (hypertrofisch/normotrofisch) hadden hogere BMD niet alleen op plaatsen nabij het aangetaste gewricht maar ook systemische. Veranderingen in geometrie en BMD zoals die uit hoofdstuk 3 werden ook gevonden in deelnemers met

verschillende osteophytische types van OA. Daarnaast hadden patiënten met atrofische heupartrose, gekenmerkt door afbraak van gewrichtskraakbeen zonder bewijs van osteofyten, een systemisch lagere BMD en een hoger risico op osteoporotische fracturen, wat niet werd verklaard door lagere BMD (**hoofdstuk 4.1**). Personen met lumbale discus degeneratie (LDD), gekenmerkt door tussenwervelschijfruimte vernauwing en in de meeste gevallen meerdere osteofyten, hebben een hogere BMD dan personen zonder LDD. Ondanks een hogere BMD hadden mannelijke personen met LDD een hoger risico op osteoporotische fracturen, voornamelijk klinische en vertebrale fracturen (**hoofdstuk 4.2**).

Hoofdstuk 5 presenteert een uitgebreide analyse van de comorbiditeit en de impact van heup en knie artrose op de overleving. De relatie tussen het hebben van artrose en mortaliteit werd bestudeert in de Rotterdam, daarbij werden zeven veelvoorkomende doodsoorzaken beschouwd. Klinische en radiologische artrose waren geassocieerd met hogere mortaliteit, dit was te zien voor alle onderzochte doodsoorzaken. Deelnemers met heup- en / of knie OA hebben meer comorbiditeit; diabetes, dementie, overgewicht, problemen met lopen en moeilijkheden bij de activiteiten van het dagelijks leven kwamen vaker voor. Dit verklaarde deels het hogere sterfterisico. Preventie en bestrijding van deze chronische ziekten bij personen met artrose is relevant. In de behandeling van artrose, het beleid en richtlijnen moeten richten op de negatieve gevolgen van de ziekte, het verbeteren van de functionaliteit, het verminderen van beperking en bestrijding en preventie van cormorbiteit in de getroffen bevolking.

Hoofdstuk 6 tenslotte biedt een algemene discussie met de meest relevante en controversiële aspecten van dit proeftschrift en geeft suggesties voor toekomstig onderzoek in het gebied.

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List of publications
& PhD portfolio

LIST OF PUBLICATIONS

Papers published in indexed journals

1.   Campos-Obando N, **Castaño-Betancourt MC**, Oei L, Franco OH, Stricker BH, Brusselle GG, Lahousse L, Hofman A, Tiemeier H, Rivadeneira F, Uitterlinden AG, Zillikens MC. Bone mineral density and chronic lung disease mortality: the rotterdam study. J Clin Endocrinol Metab. 2014 May;99(5):1834-42.

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15. 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. Kerkhof HJ, Bierma-Zeinstra SM, **Castaño-Betancourt MC**, de Maat MP, Hofman A, Pols HA, Rivadeneira F, Witteman JC, Uitterlinden AG, van Meurs JB. Ann Rheum Dis. 2010 Nov;69(11):1976-82.

16. Dual energy x-ray absorptiometry analysis contributes to the prediction of hip osteoarthritis progression. **Castaño-Betancourt MC**, Linden JC, Rivadeneira F, Rozendaal RM, Zeinstra SM, Weinans H, Waarsing JH. Arthritis Res Ther. 2009;11(6):R162.

PhD Portfolio

Name:	Martha C. Castaño Betancourt
Erasmus MC Department:	Internal Medicine – Genetic
Research school:	Laboratory
	NIHES/MolMed
PhD period:	August 1st, 2008 – August 1st, 2013
Promotor:	Prof. dr. André G. Uitterlinden
Supervisor:	Dr. Joyce B.J. van Meurs

Name	Year	Workload
1. PhD training		
Master Genetics Epidemiology	2011-2012	72.5 ECTS
(Inter) national Conferences		
- 13th World congress of the Osteoarthritis Research Society International - Rome, Italy	2008	Sept 18-21
- 14th World congress of the Osteoarthritis Research Society International - Montreal, Canada	2009	Sept 10-13
- Nederlandse Vereniging voor Calcium en Bot- stofwisseling – Zeist, NL	2009	Nov -12-13
- 37th European Symposium on Calcified Tissues -, Glasgow, Scotland	2010	June 26-30
- 15th World congress of the Osteoarthritis Research Society International - Brussels, Belgium	2010	Sept 23-26

List of publications & PhD portfolio

- Nederlandse vereniging voor Calcium en Bot- Stofwisseling-Zeist, NL	2010	Nov 11-12
- International Conference: "From DNA to phenotype" stofwisseling - Zeist , NL	2011	May 11
- 38th European Symposium on Calcified Tissues - ECTS. Athens-Greece	2011	May 07-11
- European Society for Human Genetics Nuremberg, Germany	2012	June 23-26
- 18th World congress of the Osteoarthritis Research Society International - Philadelphia, USA	2013	April 18-21

Seminars and Workshops

- Workshop writing grant Proposals. Molmed, Rotterdam, Netherlands	2008	Jan 21
- Regression Analysis	2009	1 Nov
- SNP's and Human Diseases	2009	1 week
- Seminar Netherlands consortium for Healthy aging(NCHA). Delft-Netherlands	2010	Nov 2
- TREAT-OA Symposium: Breaking boundaries in OA - London, UK	2010	Feb 1
- Joint Valorisation Workshop NCHA-Rotterdam	2010	Mar 10
- Writing Grant Proposals	2010	Jun 2
- "Nederlandse taal" course level B1-B2 (upper intermediate)	2010	15 days
- R (statistical package). Rotterdam-NL	2010	Nov 22-26
- Symposium on "DNA variation to phenotype" Rotterdam-Netherlands	2011	Mar -9-11
- Netherlands consortium for health aging (NCHA) Rotterdam, NL	2011	Mar 14-15
- Mendelian Randomization	2011	1 week
- Netherlands consortium for health aging NCHA. Rotterdam, NL	2011	Sept 5
- Research management course for PhDs	2012	2 days
- Annual MolMed Day. Rotterdam, NL	2013	13 Feb

Presentations <u></u>

Presentation	Year	Type
- "Dual energy X-ray absorptiometry contributes to the Prediction of hip osteoarthritis". OARSI	2008	Poster
- "Higher lumbar spine bone mineral density and osteophytosis at baseline increase the risk of hip OA progression". OARSI	2009	Poster
- "Higher lumbar spine bone mineral density and osteophytosis at baseline increase the risk of hip OA progression". Wettenschappendagen	2009	Poster
- "Higher lumbar spine bone mineral density and osteophytosis at baseline increase the risk of hip OA progression". NVCB	2009	Oral
- "Contribution of statistical shape model and predefined geometry parameters in prediction of hip osteoarthritis". Wetenschapsdagen, Antwerpen	2010	Poster
- "Bone and osteoarthritis: geometry shape and genetics". NCHA	2010	Oral
- "BMD and DXA-derived geometry parameters differ according to type of hip osteoarthritis". ECTS	2010	Oral
- "Atrophic type of hip OA increases the risk of OP fractures and decreases survival time for fragility fractures". OARSI	2010	Poster
- "Do Predefined geometry measures and statistical shape Models predict hip OA progression". OARSI	2010	Poster
- "Lumbar disk degeneration increases the risk for incident vertebral fractures despite higher lumbar spine BMD". OARSI	2010	Oral
- "Sistemically higher bone mineral density does not protect lumbar disc degeneration cases for vertebral and osteoporotic fractures". *NVCB*	2010	Oral
- "Genetic variation near the GDF5 region gene is associated to ECTS with different aspects of hip geometry"	2011	Poster
- "Contribution of statistical shape model and predefined geometry parameters in prediction of hip osteoarthritis". ECTS	2011	Poster
- "Contribution of statistical shape model and predefined geometry parameters in prediction of hip osteoarthritis". NCHA	2011	Oral

List of publications & PhD portfolio

- Relevance of Minimal Joint Space for hip osteoarthritis and genetic variants that contribute to explain it. NCHA — 2011 — Oral

- Genome wide association and functional studies identify the DOT1L gene to be involved in cartilage thickness and hip osteoarthritis. ESHG — 2012 — Oral

- "Association of hip and knee osteoarthritis" With Mortality". OARSI — 2013 — Poster

- "Osteoarthritis and mortality : meta-analysis of two prospective cohorts". Wetenschappen dagen — 2013 — Poster

- "Association of hip and knee osteoarthritis With Mortality". MolMed day — 2013 — Poster

LIST OF ABBREVIATIONS

AHL: Activities of household and leisure

AIC: "An information criterion", it is a measure of the goodness of fit of an estimated statistical model. It is a tool for model selection.

AUC: Area under the curve

BMC: Bone mineral content

BMD: Bone mineral density

BMI: Body mass index

CVD: Cardiovascular disease

DXA: Dual energy X-ray absorptiometry

DDXA: Difference in DXA measurements within the hips of each subject.

DK-L: Difference in KL score within the hips of each subject

DSN: Disc space narrowing

ECG: Electro-cardiogram

FN: Femoral neck

FOI: Foramen obturator index

FPR: False positive rate

GWAS: Genome-Wide Association Study

GWS: Genome-Wide Significant

HAL: Hip axis length

HR: Head radius

H.R.:   Hazard ratio

K-L: Kellgren and Lawrence Score

IPI: Isquio-pubic index

JSN: Joint space narrowing

JSW: Joint space width

LDD: Lumbar disc degeneration

MAF: Minor allele frequency/ Modelled allele frequency

MJS: Minimal joint space

NL: Neck length

NN: Narrow neck

NSA: Neck shaft angle

NSAID: Non-steroidal anti-inflamatory drugs.

NW:	Neck width
OA:	Osteoarthritis
OP:	Osteoporosis/osteoporotic
OPH:	Osteophyte(s)
OR:	Odds ratio
PGP:	Predefined geomtry measures
PW:	Pelvic Width
QC:	Quality control
QQ-Plot:	Quantile-quantile plot
RefSeq:	The Reference Sequence collection
ROC:	Receiver Operator Characteristic (curves)
RS:	Rotterdam Study
SE:	Standard error
SES:	Socio-economic status
SS:	Spherical sector
SSM:	Statistical Shape Model/Modeling
TI:	Triangular index
TPR:	True positive rate
THR:	Total Hip Replacement
VFx:	Vertebral fracture