Understanding the role of Transforming Growth Factor Beta signalling and Epigenomics in Osteoarthritis

by

©Erfan Aref-Eshghi

A thesis submitted to the School of Graduate Studies

In partial fulfilment of the requirements for the degree of

Doctor of Philosophy in Medicine - Human Genetics

Discipline of Genetics, Faculty of Medicine

Memorial University of Newfoundland

St. John's, Newfoundland and Labrador, Canada

May 2016

Abstract

Osteoarthritis (OA) is the most common form of arthritis with a high socioeconomic burden, with an incompletely understood etiology. Evidence suggests a role for the transforming growth factor beta (TGF-ß) signalling pathway and epigenomics in OA. The aim of this thesis was to understand the involvement of the TGF-ß pathway in OA and to determine the DNA methylation patterns of OA-affected cartilage as compared to the OA-free cartilage.

First, I found that a common SNP in the *BMP2* gene, a ligand in the Bone morphogenetic protein (BMP) subunit of TGF-ß pathway, was associated with OA in the Newfoundland population. I also showed a genetic association between *SMAD3* - a signal transducer in the TGF-ß subunit of the TGF-ß signalling pathway - and the total radiographic burden of OA. I further demonstrated that *SMAD3* is over-expressed in OA cartilage, suggesting an over activation of the TGF-ß signalling in OA. Next, I examined the connection of these genes in the regulation of matrix metallopeptidase 13 (*MMP13*) - an enzyme known to destroy extracellular matrix in OA cartilage - in the context of the TGF-ß signalling. The analyses showed that *TGF-ß, MMP13,* and *SMAD3* were overexpressed in OA cartilage, whereas the expression of *BMP2* was significantly reduced. The expression of *TGF-ß* was positively correlated with that of *SMAD3* and *MMP13*, suggesting that TGF-ß signalling is involved in up-regulation of *MMP13*. This regulation, however, appears not to be controlled by *SMAD3* signals, possibly due to the involvement of collateral signalling, and to be suppressed by BMP regulation in healthy cartilage, whose levels were reduced in end-stage OA.

In a genome-wide DNA methylation analysis, I reported CpG sites differentially methylated in OA and showed that the cartilage methylome has a potential to distinguish between OA-affected

and non-OA cartilage. Functional clustering analysis of the genes harbouring differentially methylated loci revealed that they are enriched in the skeletal system morphogenesis pathway, which could be a potential candidate for further OA studies. Overall, the findings from the present thesis provide evidence that the TGF-ß signalling pathway is associated with the development of OA, and epigenomics might be involved as a potential mechanism in OA.

Acknowledgements

I would like to thank, first and foremost, my supervisor, Dr. Guangju Zhai, who gave me the opportunity to work under his supervision and to enjoy being a member of the research team in the Newfoundland Osteoarthritis Study (NFOAS). I also appreciate the support and advice I received from my supervisory committee, Drs. Andrew Furey and Guang Sun, particularly for their inputs into this thesis project. I would also like to remember my previous co-supervisor, Dr. Roger Green, who sadly and unexpectedly passed away in August 2015. Roger, you will be missed, and I will never forget the assistance I received from you in various steps of this project.

To everyone who assisted me with this work and helped in solving the problems at different stages of the project. Special thanks to Ming Liu, Patricia Harper, Yuhua Zhang, Edward Yaskowiak, Hongwei Zhang, Zoha Rabie, Michelle Simms, and all the staff at the operating rooms of the Health Sciences and St. Clare's Mercy Hospitals, who directly and indirectly contributed to this work.

To all my friends in St. John's: thanks for helping me not feel lonely as a new-comer. Special thanks to Atefeh and Justin, who filled my spare time with adventures, and to Shirin and Uzair, whom I shared with lots of memories over the course of my stay in St. John's. Thanks to all the Feild Hall residents who made me feel like living in a great family in all these years far from home, and thanks to everyone else with whom I shared coffee, had a small talk and made friend or acquaintance in St. John's, those who made this experience unique.

And finally, to my parents and younger sister: thanks for your continued support and hearing your son's concerns from thousands of kilometres away. I could not have done this without you.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

A disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS4)
Activin A receptor type II-like 1 (ALK1)
Aggrecan (AGC1)
Alpha-1 subunit of collagen type IV gene (COL4A1)
Anabolic molecule collagen, type IX, alpha 1 (COL9A1)
Aneurysm osteoarthritis syndrome (AOS)
Anterior cruciate ligament transaction model (ACLT)
Asporin gene (ASPN)
Basic local alignment search tool (BLAST)
Body mass index (BMI)
Bone morphogenetic protein (BMP)
Bone morphogenetic protein 2 (BMP2)
British Columbia (BC)
Camurati-Engelmann disease (CED)
Carpometacarpal joint (CMC)
Collagen, type XI, alpha 2 (COL11A2)
Computational tomography (CT)
Deoxyribonucleic acid (DNA)
Deoxyribose nucleoside triphosphate (dNTP)
Differentially methylated region (DMR)
Distal interphalangeal joint (DIP)
Erythrocyte sedimentation rate (ESR)
Extracellular matrix (ECM)
Gene ontology (GO)
Generalized osteoarthritis (GOA)
Genome-wide association study (GWAS)
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)
Growth differentiation factor 5 (GDF5)
Health research ethics authority (HREA)
Homeobox (HOX)
Hyaluronic Acid (HA)
Joint space narrowing (JSN)
Kellgren and Lawrence scaling (K&L)
Local health integration network (LHIN)
Magnetic resonance imaging (MRI)
Matrix metallopeptidase 13 (MMP13)
Metacarpophalangeal joint (MCP)
Micro RNA 128 (miR-128)
Mitochondrial DNA (mtDNA)
Mitogen-activated protein kinase (MAPK)
Mothers Against Decapentaplegic Homolog 3 (SMAD3)
National center for biotechnology information (NCBI)
Newfoundland and Labrador (NL)

Newfoundland Osteoarthritis Study (NFOAS)
Nitric oxide synthase (NOS)
Non-steroidal anti-inflammatory drugs (NSAIDs)
Ontario (ON)
Osteoarthritis (OA)
Osteophyte (OS)
Phosphoinositide 3-kinase (PI3K)
Polymerase chain reaction (PCR)
Proximal interphalangeal joints (PIP)
Relative quantification (RQ)
Ribonucleic acid (RNA)
Runt-related transcription factor 1&2 (RUNX1, RUNX2)
Single nucleotide polymorphism (SNP)
Spearman's rank correlation coefficient (rho)
Transforming growth factor beta (TGF-β)
Transforming growth factor beta 1 gene (TGFB1)
Type II collagen (COL2A1)
Ultrasonography (US)
United Kingdom (UK)
United States (US)
Western Ontario and McMaster osteoarthritis index (WOMAC)

Co-authorship Statement

This thesis consists of five published articles; I am the first author on all of them. I was involved in the study design, data collection (except for chapter 3), performing experiments, data analysis, and interpretation of the results. I participated in collecting specimens, patient interviewing and the consenting process. I had a role in the experimental component of the research including RNA/DNA extraction and quantitative PCR. Except for the data normalization in chapter 6, I performed all of the statistical and bioinformatics analyses. Finally, I prepared the initial draft of all of the manuscripts. The contributions of the co-authors on the manuscripts are as below:

Dr. Guangju Zhai conceptualized, designed, and oversaw the studies. Ms. Patricia Harper, Ms. Ming Liu, Dr. Andrew Furey, Dr. Glynn Martin, and Dr. Roger Green helped with the recruitment of study participants and sample collection, and commented on the final manuscripts. Dr. Kensuke Hirasawa and Mr. Seyd Babak Razavi-Lopez trained me in qPCR and gene expression and helped with *SMAD3* gene expression analysis and commented on the final draft of the manuscripts. Mr. Yuhua Zhang assisted with the data normalization in chapter six and reviewed the accuracy of the statistical analysis in chapter three. Dr. Ana M Valdes, Dr. Nigel Arden, Dr. Tim D Spector, and Dr. Deborah Hart were involved in the Chingford study and provided us with the de-identified data for Chapter three. Dr. Jules Doré, Dr. Guang Sun, and Dr. Proton Rahman provided guidance on the gene expression method and interpretation of the results and commented on the final manuscripts.

1

Introduction

1.1. An introduction to osteoarthritis (OA)

Osteoarthritis (OA), also known as degenerative joint disease, degenerative arthritis or osteoarthrosis, is the most common form of joint abnormality caused mainly by the gradual loss of articular cartilage, resulting in pain, stiffness, and limited range of motion, leading to functional disability in affected individuals [1]. It is highly frequent with a prevalence estimate of up to 20% worldwide [2]. Although knees, hips, hands and vertebral joints are the most common anatomical sites of involvement, any synovial joint could become affected by OA [3]. The cause of OA is not clearly understood. It appears that a combination of environmental, constitutional, and genetic factors play a role in its development and progression [3]. This chapter reviews OA definitions, its epidemiology, pathology and etiological factors, and presents the objectives and hypotheses of this dissertation.

1.1.1. Definition

The primary definitions of OA were based on the belief that OA is a normal consequence of aging, resulting from the gradual loss of hyaline cartilage due to tear and wear. Later investigations led to the separation of the process of normal aging from OA; and instead, OA was defined as a heterogeneous group of conditions resulting from multiple factors that lead to overlapping presentations caused by a variety of biochemical, hormonal and mechanical factors. The most recent standardized definition for OA has been suggested by the Osteoarthritis Research Society International (OARSI) in August 2015 as follows [4]:

> "Osteoarthritis is a disorder involving movable joints characterized by cell stress and extracellular matrix degradation initiated by micro- and macro-injury that activates

maladaptive repair responses including pro-inflammatory pathways of innate immunity. The disease manifests first as a molecular derangement (abnormal joint tissue metabolism) followed by anatomic, and/or physiologic derangements (characterized by cartilage degradation, bone remodeling, osteophyte formation, joint inflammation and loss of normal joint function), that can culminate in illness."

There have been attempts to classify OA diseases into specific diseases using the clinical and diagnostic measures such as the primary aetiology, the site of involvement, or the procedure used for the diagnosis [5]. The followings are some of the definitions of different subtypes of OA used in both research and clinical settings:

Primary or idiopathic OA [6] is an idiopathic outbreak of OA in a previously intact joint without any known predisposing aetiology related to OA, and could be further classified as localized and generalized OA.

Secondary OA [5], which occurs due to a known aetiology such as trauma, infection, congenital malformation, metabolic, endocrine, neurologic, and other medical conditions, has a known underlying cause. It is believed that a considerable portion of the primary OA will be eventually categorized as the secondary OA over time with the identification of the root factors of primary OA.

Symptomatic OA uses the clinical signs and symptoms of OA including pain, stiffness, range of motion, and the level of disability to define and classify OA. A patient with symptomatic OA presents with its clinical symptoms, from which, pain and stiffness of the joint are the most frequent ones [7]. Symptomatic OA and radiographic OA are not always perfectly associated with each other [8].

Radiographic OA is the presence of a joint abnormality in the radiography regardless of the existence of clinical presentations of OA. The traditional radiographic features used to define OA include joint space narrowing (JSN), osteophytes, subchondral sclerosis, cyst formation, and bone abnormalities. Although several grading systems are proposed for the classification of radiographic OA, Kellgren and Lawrence grading scale [9], which is mainly based on the JSN, osteophytes, and the sclerosis formation in the subchondral bone, is the most widely used method among both researchers and clinicians.

Osteoarthritis could involve more than one joint. The first recognition of a polyarticular form of OA was reported in 1805 by Haygarth [10]. In 1926, Cecil and Archer [11] associated this condition with Heberden's nodes - characteristic nodules in the distal interphalangeal joints described earlier by Heberden [12]. In 1952, Kellgren and Moore took advantage of the same definition and linked generalized OA (GOA) to the existence of Heberden's nodes in the hands [13]. however, there is no consensus on a universal definition regarding GOA. The American College of Rheumatology [14] defines GOA as the involvement of at least two joints in addition to the spine; while, based on Dougados criteria [15], the presence of OA in the spine besides either bilateral digital or bilateral knee OA is representative of GOA. Meanwhile, a preference for the involvement of multiple hand joints, a female predominance, node formation, and early inflammatory symptoms are amongst some of the most frequently agreed features of GOA [16, 17].

Recognition of GOA in patients has a practical value as it is suggestive of a systematic etiology and requires different therapeutic, diagnostic, and investigative measures than the localized form of OA. Similar concept extends to any subcategory of OA e.g. inflammatory OA or metabolic

OA. It should be kept in mind that these classifications are not complete, and further identification of OA aetiologies are expected to classify it into many underlying conditions.

1.1.2. Pathology of OA

Diarthrodial joints, in which the majority of OA involvements occur [18], have a similar anatomical structure: two subchondral bones covered with a layer of articular cartilage, which are surrounded by the synovial membrane and the joint capsule, and the area between them is filled with synovial fluid. Normal articular cartilage is composed of chondrocytes surrounded by an extracellular matrix mainly including collagen type II and proteoglycans. It has no blood vessels, nerves or lymphatics [19]. It can be compressed by 20% of its original height, which is essential for the protection of the underlying subchondral bone by distributing large loads, and reducing stress and friction in the joints [20]. Synovial fluid is produced by the cells from the synovial membrane (synoviocytes) and is mainly composed of hyaluronic acid (HA), - responsible for its viscoelasticity - and nutrients. As the joint moves, it is perfused in and out of the cartilage similar to the absorption and emission of water by a sponge, playing a great role in the compression of the cartilage and its nutrition [21].

The initiating mechanism in OA is supposed to be either a damage to normal cartilage by physical forces, or a failure due to a fundamentally defective cartilage [22]. Chondrocytes react to the injuries by releasing derivative enzymes and creating inadequate repair response. This is followed by a cascade of biochemical and inflammatory events that disrupt the dynamic equilibrium between synthesis and degradation of extracellular matrix components in the cartilage [23]. In early stages, due to the weakening of the collagen framework, an increase in the

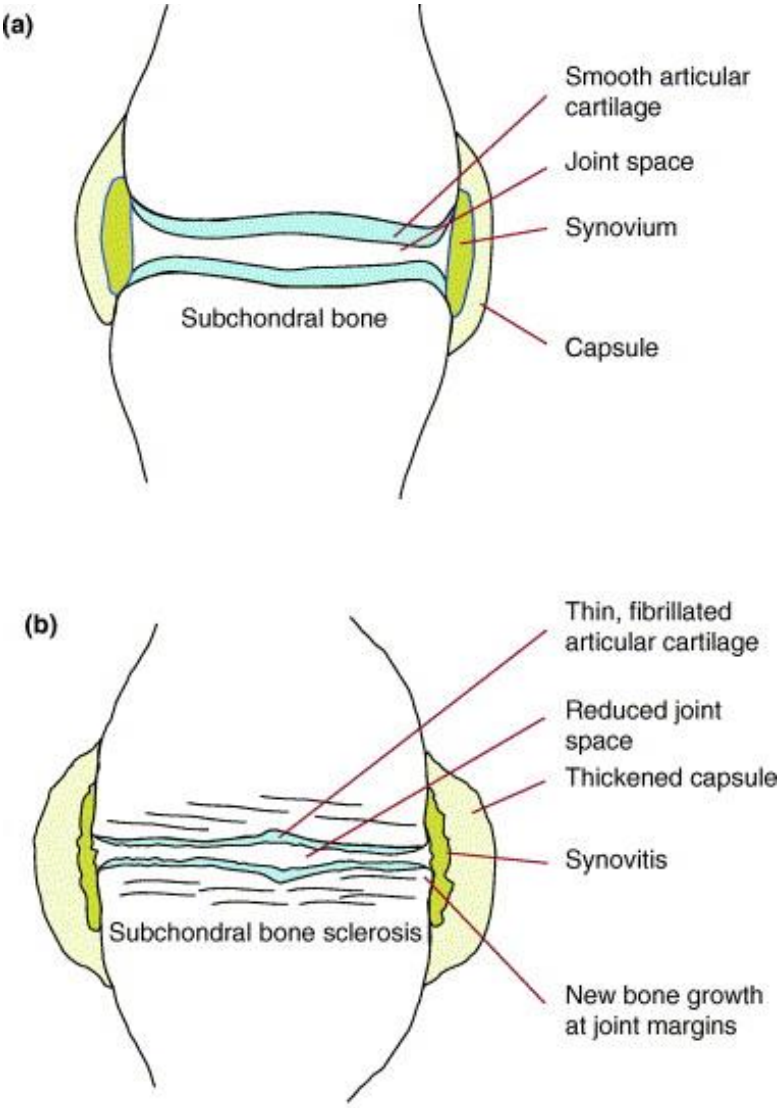
water content of cartilage occurs [24, 25]. This hydration subsequently dilutes the concentration of proteoglycans and the other main components of extracellular matrix [26], resulting in the loss of cartilage stiffness [27]. In other words, loosening of the collagen network and the loss of proteoglycans are the two most important molecular processes that occur in the osteoarthritic cartilage. In compensation for this loss, an increase in the synthesis of proteoglycan occurs, leadings to the growth in the volume of the cartilage, which is an effort by the chondrocytes to repair the cartilage damage [28]. This stage could last for years to decades, and the hypertrophic repair of the cartilage is its main characteristic. However, eventually, the proteoglycan in the cartilage decreases to a very low level, resulting in softening the cartilage and losing its elasticity, leading to attrition and diminution in the water content of the cartilage [29]. Over time the loss of cartilage results in the narrowing of the joint space.

Pathophysiologic changes also happen in other components of the joint [1]. After OA begins, the cartilage erosion continues until the exposure of the subchondral bone, resulting in the vascular invasion, increased thickness and an increased cellular population of the bone areas under pressure [30]. Osseous necrosis secondary to chronic compression of the bone or the intrusion of synovial fluid occurs in the traumatized bone, being represented as cyst degenerations ranging from 2 to 20 mm in diameter in the subchondral bone, which is also known as subchondral cyst or pseudocyst [31, 32]. They are covered with fibrous tissue and are filled with a gelatinous fluid. They can be connected to each other or the joint space through micro fractures in the bone. Irregular growth of new bony structures - osteophytes - also happens alongside the articular margin, in the non-pressurized segments of the joints. This is mainly due to vascularization of subchondral marrow, osseous metaplasia of synovial connective tissue, and ossifying cartilaginous protrusions [32], and is a remarkable pathological finding in radiography of OA

patients. These structures are represented as Heberden's nodes in the distal interphalangeal joints. Osteophytes, as well as the articular cartilage, could further break up, resulting in the intra-articular loose bodies in the joint which result in a state interpreted as joint locking by the patients [33]. These degraded particles could cause the release of collagenase and other enzymes, resulting in synovitis [34], which happens in approximately 20 to 50% of joints with OA [35]. The synovitis consists of mild infiltration of lymphocytes, few macrophages, immunoglobulin-producing plasma cells, and mast cells in synovial membrane [35, 36]. It does not express the classical features of inflammation. No immunoglobulin and complement inclusions exist in synovial cells [37], immunoblasts are absent in synovial fluid [38], the fluid white cell count is less than 2000 cells/ml – predominantly lymphocytes, and the complement levels in the fluid are normal [39].

OA changes do not remain restricted to the joint, and other surrounding components including ligaments, neuromuscular apparatus, articular capsule, and the neighbouring muscles could also undergo pathological changes. In Figure 1.1, a schematic representation of OA joint and a normal one is illustrated.

Figure 1.1- Schematic illustration of a normal joint versus an OA joint [40]



In the normal joint (a) the surface of the cartilage is smooth, and the joint space is broad, whereas in the osteoarthritic joint (b) the cartilage is thinned and degraded and the joint space narrowing is evident. Moreover, the subchondral bone is thickened, and its surface is uneven. New bone growth (osteophytes) is detectable at the margins of the joint; the synovium is inflamed, and the capsule can be thickened (Adapted from Peach *et al*. 2005 [40]).

1.1.3. Signs and symptoms of OA

At the early stages of the disease, OA is mainly asymptomatic and as the disease progresses clinical signs of OA gradually appear. The followings are the most common clinical manifestations of OA:

Pain is usually the first symptom that develops in OA joints. It aggravates with use and alleviates with rest. In later stages, it could happen during rest, at night in bed and does not alleviate with rest. The pain could also be described as a sharp, dull or snapping sensation by the patient, and could be obtained through both passive and active motion of the joint. Since there is no neural network in the cartilage, the origin of pain is related to the surrounding tissues such as subchondral bone, synovium, joint capsule, ligaments, and muscles [41].

Joint stiffness is a prominent sign of OA and especially occurs after any period of inactivity. It is common in the morning and usually lasts less than 30 minutes. It significantly decreases the range of motion in the joints and the ability of the patient to perform routine tasks [42].

Subluxation in the joints occurs in advanced OA and is described as a feeling of instability by the patient. It is usually accompanied by visible joint deformity. Heberden's and Bouchard's nodes, which appear as overgrown bony tissues similar to osteophytes in the distal interphalangeal (DIP) and proximal interphalangeal (PIP) joints, respectively, are a common finding in this stage of the disease. They usually begin after the age of 45 and affect women ten times more than men [43]. They could be either asymptomatic or painful and swollen. Involvement of the first carpometacarpal joint dramatically limits the patient's ability to use his/her hand.

Reduced range of motion is a common clinical finding in the advanced stages of OA. Potential causes include joint surface incongruity, an increase of pain in motion, muscle and capsular contracture, loose bodies in the joints, and other mechanical problems.

Another common sign of OA is crepitus in the joint movement. One could hear the sound of coarse crepitus (cracking and creaking) or it can be palpated by the medical examiner.

Muscle weakness, tenderness, deformity and joint swelling are of other OA signs. OA is not associated with fever, weight loss, anorexia, or severe muscle atrophy. In the case of polyarticular involvement, it has a tendency to affect joints asymmetrically, especially in the hands [17]. These features of OA distinguish it from inflammatory arthritis. Osteoarthritis of specific joints will have specific symptoms. For instance, OA in the vertebral area, in the case of deformity, might mimic lumbar disc disease syndromes; or the joint deformity by knee OA could lead to valgus or varus knee abnormalities.

To evaluate the clinical severity of the disease for epidemiological studies and clinical evaluations, several symptom severity scales (e.g. Western Ontario and McMaster Osteoarthritis Index [WOMAC]) are available, which are mainly based on the severity of symptoms including pain, stiffness, and functional disability.

Traditional studies of OA mostly have considered the clinical status of the disease in their investigations. However, it is shown that the use of endophenotypes of OA can improve the power, especially in genetic studies. Endophenotypes are measurable intermediate phenotypes of a condition that are closer to the function of the gene product or basic pathophysiology of the condition than the disease status, and thus, can significantly improve the power in gene finding

studies of complex traits [44]. Examples of endophenotypes in OA include cartilage characteristics, joint shape or clinical symptoms such as pain. A few examples of using these alternatives in genetic research exist that are discussed in "OA candidate gene studies" and "GWAS" sections of this chapter (sections 1.3.2.4 and 1.3.2.5).

1.1.4. Diagnosis

The diagnostic measures for OA rely on evaluating symptoms, physical examination, imaging, and laboratory testing.

In physical examination of the joints, signs and symptoms of OA including joint swelling and redness, visible joint malformations such as osteophytes, and the pattern of joint involvement should be thoroughly observed. The site of tenderness should be located by touching the joint area firmly. Shrug test is used to investigate the presence of crepitus in the joint [26]. Both passive and active movement of the joint is done to evaluate the range of motion [26].

Imaging is the next step in evaluating the susceptible joint. Although advanced imaging techniques such as Magnetic Resonance Imaging (MRI), computational tomography (CT), and ultrasonography (US) are available for the evaluation of joint, traditional radiography is still the gold standard for the diagnosis of OA. X-rays can reveal the presence of osteophytes, joint space narrowing, bone cysts and sclerosis. X-ray is also the most common method for assessing the need of surgical treatment in patients. Radiographic grading scales such as Kellgren and Lawrence grading system [45] have been proposed to determine the severity of OA (Table 1.1).

In early stages of OA development, X-ray could be normal as the involvement can be only limited to cartilage, which cannot be illustrated in X-ray.

In OA, no significant laboratory finding exists since the majority of routine tests including blood count, sedimentation rate, urine analysis, and biochemistries are normal. More accurate tests such as rheumatoid factor, thyroid function tests, and cellular antibody are negative but are used for the differential diagnosis. Arthrocentesis may be done on the joints with large effusions. In OA the synovial fluid is clear, and the white cell count is less than 10,000 per one-millimeter cube of fluid. Viscosity, Glucose, and protein levels are normal, and no crystals are observed. Degradation components of cartilage in synovial fluid, serum, and urine has been the focus of studies recently; however none of them are being used yet as a routine clinical diagnostic measure for OA [46].

Table 1.1- Kellgren and Lawrence grading system

Grade	Classification	Criteria
0	Normal	No feature of OA
1	Doubtful	Minute osteophyte, doubtful narrowing of joint space
2	Minimal	Definite osteophyte, absent or questionable narrowing of joint space
3	Moderate	Moderate osteophyte, definite narrowing of joint space, some sclerosis, possible deformity
4	Severe	Large osteophyte, marked narrowing of joint space, severe sclerosis, definite deformity

The American College of Rheumatology proposes the following criteria for the diagnosis of knee, hip, and hand OA:

***Osteoarthritis of the hand*:** The existence of hand pain in addition to at least three of the following four features [47]:

- Hard tissue enlargement of two or more of 10 selected joints (2nd and 3rd DIP, 3rd PIP, first carpometacarpal [CMC] joints)
- Deformity of at least two of the above ten selected joints
- Hard enlargement of two or more DIP joints
- Fewer than three swollen metacarpophalangeal (MCP) joints

***Osteoarthritis of the hip*:** The existence of hip pain in addition to at least two of the following three features [48]:

- Erythrocyte sedimentation rate (ESR) less than 20 mm/hour
- Femoral and/or acetabular osteophytes evident on X-ray
- Joint space narrowing evident on X-ray

***Osteoarthritis of the knee*:** The existence of knee pain in addition to at least three of the following six features [14]:

- Greater than 50 years of age
- Stiffness lasting less than 30 minutes
- Crepitus on active motion of the knee
- Bony tenderness
- Bony enlargement
- No palpable warmth

1.1.5. Management

Once the disease develops, it is almost impossible to stop or halt its progression, and there is no way to cure OA completely. Therefore, the main goal of OA treatment is the control of pain by decreasing inflammation in the joint, adjuvant control with analgesics, and physical and occupational therapy modalities [49]. There are nonpharmacologic, pharmacologic, and surgical interventions available to reach these goals.

Non-pharmacologic treatment

Physical and occupational therapy including exercise and physical modalities such as ice packs, splints, and braces have been shown to be effective in improving the functional abilities of OA patients and alleviating the pain and stiffness [50]. One study on knee OA suggests that exercise improves the range of motion and integrity of the supporting motion, which subsequently reduces the pain and improves the function [50]. Aerobic exercise programs have been shown to improve walking times, aerobic capacity, and decrease depression and anxiety in OA patients compared to those who only performed a range of motion exercises [51]. Physical activity is also helpful in losing weight in overweight individuals, which is shown to be beneficial in OA outcome, especially in women [52]. Several clinical trials also indicated a beneficial role of acupuncture in OA patients [53].

Pharmacologic Treatment

According to the guidelines by the American College of Rheumatology, the initial pharmacological treatment for hip and knee OA should start with Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) or an analgesic such as Acetaminophen [54]. A randomized,

crossover trial showed that 4 gram of Acetaminophen per day significantly reduced pain and improved function in patients with knee OA [55]. In the case of the progression of the disease and worsening of the pain, a short-term use of opioid-containing analgesics, including codeine and propoxyphene is suggested. There is no role for oral or intravenous corticosteroids in OA; however, evidence suggests that patients with a painful crisis of OA may benefit from an intra-articular injection of corticosteroids [56]. Intra-articular injection of hyaluronic acid derivatives [57], and the use of nutritional supplements containing glucosamine and chondroitin sulfate [58] are other suggested non-invasive treatments for OA.

Surgical intervention

The lack of response to medical therapy, the presence of moderate to severe pain and functional disability may recommend surgical interventions. Internal derangement of the knee OA could be treated with arthroscopic debridement. In the case of significant malalignment of the knee or hip, osteotomy might be indicated. At advanced stages of the disease, according to the radiographic and clinical evaluations, total joint arthroplasty or replacement is performed, which has been shown to improve the quality of life significantly in advanced OA patients [59].

Future outlook of OA treatment

Current practical therapeutic approaches for OA are only able to relieve the symptoms, and no method to stop the progression or cure the disease is available. Thus, most of the investigations are targeted toward the inhibition of the enzymes involved in the destruction of collagen. For instance, the use of Risedronate in Guinea Pig OA model has been shown to decrease chondrocyte loss and slow down X-ray progression [60]. Cartilage collagenase activity has been

suppressed by doxycycline in some animal studies, resulting in a reduction in the severity of OA [61]. Time-released transforming growth factor beta (TGF-ß) has repaired partial-thickness cartilage lesions in rabbit models [62]. Metalloproteinase inhibitors such as Tetracycline are also being tested in OA [63]. Other developments include tissue engineering using the transplantation of autologous chondrocytes after being cultured *in vitro* [64], and gene therapy for OA treatment. None of these approaches have gained value in routine clinical practice yet and are still being evaluated in research settings only.

1.2. Epidemiology

1.2.1. Prevalence and incidence of OA

Prevalence and incidence of OA are differentially reported according to the methodology used for OA definition, site of involvement, age, and the population. Knees, hips, and hands are the most common sites of involvement, and thus, the majority of epidemiological studies have focused on OA of these sites.

Prevalence of OA

Prevalence of OA as determined by radiological findings is quite high. In 1926, in a systematic autopsy study of 1,000 cases, almost universal cartilage damage in individuals over 65 was observed [65]. However, radiographic findings of OA in the general population are less frequent since the minimal pathological changes are not always evident in X-ray. The prevalence of radiographic OA increases dramatically by the age. A study on a large number of randomly selected residents of a Dutch village found that 10-20% of women by the age of 40 years present

with radiographic features of OA of the hand or feet while this figure rose to 75% in women aged 60-75 years [66]. Radiographic hip OA is less common than knee OA, and both knee and hand OA tend to be more common in women (Figure 1.2) [66].

Self-reported and symptomatic OA is more limited due to the late onset presentations of the pathologic OA changes, although they still correlate with age. A study in the UK [67] reported that 18.1% of patients over 55 years registered with the practice had a clinical diagnosis of knee OA. In contrast to radiographic OA, the prevalence of symptomatic hip OA is less common than knee OA.

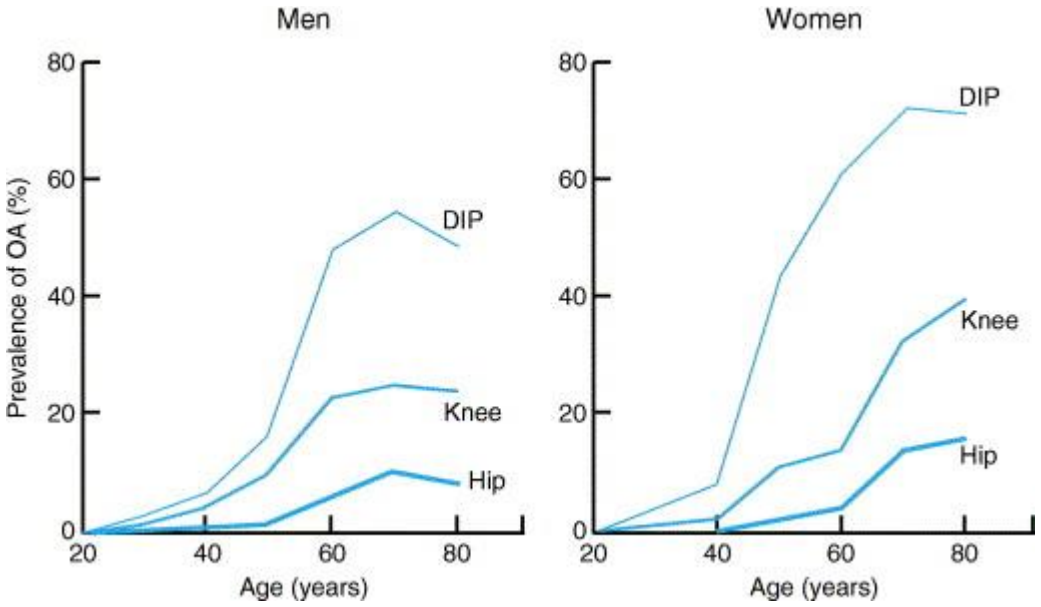
The prevalence of Osteoarthritis in Canada is sparsely reported. The 10-year prevalence of OA from 1991 to 2001 in British Columbia (BC) was reported as 10.8% through BC medical service plan [68]. Another study reported a prevalence of 14.7% among an Inuit population of the North West Territories in 1982 [69]. The prevalence in Ontario (ON) has been reported to be 7.5%-12.1% by the Local Health Integration Networks (LHINs) [70]. The 2011 report by the Arthritis Alliance of Canada estimated that 4.4 million people in Canada (10% of men and 15% of women) were living with OA in the year 2010, and this number was expected to reach over 10 million by the year 2040 [71].

Incidence of OA

Similar to the prevalence of OA, the incidence of OA highly correlates with age, gender, and the joints studied (Figure 1.3). In a population study from Rochester, Minnesota, OA incidence rates for hip and knee adjusted for age and sex was reported to be 47.3 per 100,000 person-years and 163.8 per 100,000 person-years, respectively [72]. Another study on the data from a health organization in the north-east US [73], reported that the incidence of hand, hip, and knee OA

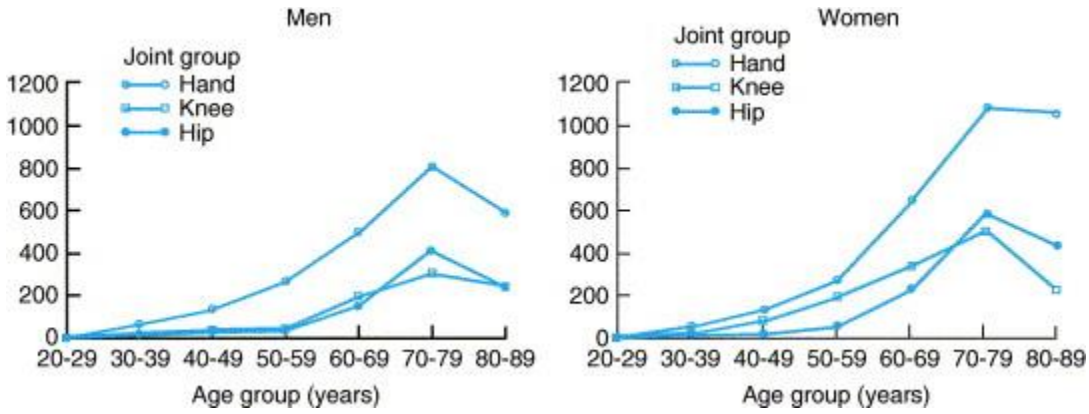
increases with age, especially after the age of 50, and women have a higher incidence rate than men. In both sexes and all joints the incidence rates decrease after the age of 80 years, which is hypothesized to result from a higher mortality rate before that age due to the increased comorbidities associated with OA [74, 75].

Figure 1.2- Estimates for the prevalence of radiographic osteoarthritis (OA) affecting the distal interphalangeal (DIP) joint, knee and hip in a large Dutch population sample



Adapted from Arden *et al*. 2006 [76]

Figure 1.3- Incidence of osteoarthritis by site and sex



Adapted from Arden *et al*. 2006 [76]

1.2.2. Socio-economic burden of OA

OA is one of the leading causes of disability and public health problems in developed countries and elsewhere. According to the report by Center for Disease Control and Prevention in 2010, OA-affected more than 27 million people in the US, imposed 11.1 million dollars on outpatient visits and 13.2 billion dollars on OA-related job absence [77]. In 2004, OA resulted in over 11 million physician and outpatient visits, 662,000 hospitalizations, and more than 632,000 total joint replacements, with accompanying hospital costs of $22.6 billion in the US [78]. In the National Health Interview Study [79], OA and related disorders just after heart disease and back disorder are the third leading chronic condition causing work limitation.

The disability caused by OA results in enormous costs in lost earnings, care, and hospitalization. Each year nearly 1 million years of human potentials are lost because of OA disability [78]. These burdens are increased by significant co-morbidities, which occur more commonly in OA patients, such as metabolic syndrome, diabetes, cardiovascular disease, and the adverse effects of NSAID therapy [74, 75]. The rise in the OA prevalence due to longer life expectancies and obesity epidemics is expected to add significantly to the burden of OA [80]. It is predicted that by the year 2030, 25% of adults in the United States will have physician-diagnosed arthritis [81], among which OA will be the most common form.

In Canada, arthritis as a whole affects more than 4.2 million Canadians (>10% of the population) and its prevalence has been expected to grow to 21-26% of the population by 2020. It is the most common chronic condition among Canadian women and the third among men [82]. More than 59% of Canadians with OA suffer from limitations in regular activities, twice as much as those with other chronic conditions [82]. More than 80% of hip replacement surgeries and 90% of knee

replacement surgeries are due to OA in Canada. The total impact of general arthritis has been estimated to be $33 billion each year, and it is estimated that the total cost spent on arthritis since 2010 will reach $233.5 billion by the end of 2015 [82]. Canadians with arthritis have a higher likelihood of developing other physical and mental conditions and are more prone to be in need of hospitalizations, which will add to the direct costs associated with OA [82]. The statistics about general arthritis can be well extended to OA as it is the most common form of arthritis.

1.3. Aetiology and risk factors

1.3.1. Environmental Factors

OA is a multifactorial disease, meaning that a variety of systemic factors (i.e. age, gender, hormone levels, genetics, and nutrition), intrinsic joint factors (i.e. anatomic variants, muscle weakness, misalignment, and joint laxity), and extrinsic risk factors (e.g. repetitive physical activities and obesity) interplay in both its development and progression [83, 84]. The main focus of this dissertation is to understand the genetic aetiology of OA; however, a brief overview of the major known non-genetic factors, which play a role in OA pathogenesis and interact with the genetic factors, should also be considered and are reviewed in the following section.

1.3.1.1. Age

The incidence and prevalence of OA are highly correlated with age, such that based on all reports the incidence of symptomatic OA exponentially increases with age in all joints [85, 86]. The National Health and Nutrition Examination Survey reports the prevalence of OA to be less

than 0.1 percent in those aged 25 to 34 years old versus a rate of over 80 percent in those older than 55 years [87]. Aging is thus considered as one of the strongest risk factors of OA. This does not, however, imply that OA is an inevitable disease of aging since it could also occur in some youth while some elderly live up to an advanced age with no signs of OA. The relationship between age and OA could be explained by a series of physiologic changes that occur during the process of aging, which predispose the joint to OA. These include impaired neuromuscular joint protective mechanisms [88], increased joint instability [89], and the rise in body mass index. The reconstruction abilities and resilience of cartilage decreases with age due to a reduced anabolic response to growth factors, thinning of the cartilage plate, reduction in the number of chondrocytes, and the physiological decrease in the extracellular matrix capacity to maintain water [90]. Nevertheless, the physiological changes in aging cartilage are different from those in OA cartilage, although they may predispose the joint to OA.

1.3.1.2. Sex

The age-related rise in the OA risk is more evident in females compared to males. Although the incidence rate of OA before the age of 50 is not broadly different between males and females, the prevalence and incidence of OA of the hand and knee more significantly increases in women compared to men after the age of 50 [91]. However, the rise in the frequency of hip OA remains steady in both sexes [92]. Overall, because the majority of OA-affected individuals are older than 50, OA appears to be more associated with female sex. The relative risk of developing OA for women compared to males has been estimated to be 2.6 after adjustment for age, weight, and smoking status [93]. In addition, women with OA tend to have more severe symptoms and faster

progression than men and they are more likely to undergo total joint replacement surgery [94]. It has been suggested that these differences might be explained by the role of sex hormones and menopause leading to estrogen deficiency [95]. However, the evidence for these hypotheses is not strong enough to explain the role of sex in OA development. Other studies suggest that male and female cartilage respond differentially to the sex hormones [96]. Kinney *et al*. showed that the chondrocytes from both males and females codes an estrogen receptor, although only female chondrocytes respond to estradiol supplementation [97]. On the other hand testosterone hormone has shown to have a receptor on both males' and females' chondrocytes, although it only responds to the hormone in males [98]. These observations may suggest that chondrocytes are constitutionally regulated in different ways among males and females, which could explain the differences in OA presentations in two sexes [96].

1.3.1.3. Obesity

Obesity is the strongest modifiable risk factor for OA [99], which has a variable role in different joints. It appears that it has the strongest effect on the knee OA. Development of knee OA occurs years earlier and progresses faster in obese individuals [100, 101], and there is evidence that the risk of knee OA decreases in low weight individuals [102]. The effect of obesity for hip OA, however, is less evident than knee OA [103]. Some studies have also indicated an increased risk of hand OA in obese individuals [104]. The apparent mechanism for the association of obesity with hip and knee OA is likely to be the result of excessive overloading of the knee and hip during weight bearing activities, beyond the tolerance of the cartilage and ligaments. However, metabolic factors associated with obesity, including circulating adipocytokines, adiposity-linked

glucose, lipid abnormalities, and chronic inflammation may also have an influence on the joints of overweight and obese individuals [105, 106, 107], and explain the moderate association of obesity with hand OA. Studies on mice suggest that OA is not merely related to body weight in mice; instead, a complex interaction of intrinsic and extrinsic factors associated with obesity may contribute to the incidence and severity of OA [108].

1.3.1.4. Physical activity, lifestyle, and occupations

The relationship between physical activity and OA is not entirely clear. Studies suggest that at least some types of repetitive joint usage contribute to OA development [109]. Individuals in occupations requiring a high physical activity including farmers, construction workers, and labourers have a higher prevalence of early onset OA [109, 110]. Repetitive lifting, carrying heavy objects, vibration, abnormal work postures, kneeling, squatting, climbing and continuously repeated movements are amongst the specific activities associated with OA [111]. These effects on OA risk could be amplified in individuals with excess body weight [112]. Studies also suggest a role for participation in competitive sports and vigorous exercise in OA [113]. In contrast, other studies reported individuals with life-long high levels of physical activities without OA development [114], and some studies found no overall associations between the activity level and OA [115]. Furthermore, more recent studies suggest that regular physical activity is not only harmful but also beneficial to joint health and prevents the development and progression of knee OA [116]. Vigorous activity has been associated with better joint health in people aged 50 to 79 [117], and women who walk regularly are less likely to have early signs of joint abnormalities, such as cartilage degeneration [118]. While imposing

higher workload to the joint might be the explanation for the harmful effect of physical activity, the increase in muscle mass and strength, and the decrease in body weight could also be the likely reason for the protective effects of regular exercise from degenerative changes of the joints. The outcome is also highly dependent upon the health of the joint. Neuro-anatomically normal joints are considered to be at risk of OA in both the absence of physical activity and repetitive high impact exercise but not recreational and low impact activity, whereas neuro-anatomically abnormal joints are at risk even upon exposure to low impact recreational activities [119, 120].

1.3.1.5. Injuries and joint abnormalities

Joint injuries including fractures and dislocations, meniscal and cruciate ligament tears of the knee impose a very high risk of later OA development to the injured joints [121]. This risk is not only caused by the direct trauma and injury but also the disruption of normal joint mechanics and load distributions within the joint. Although OA development may occur following the injury in any joints or individuals, studies suggest that chances are much higher in individuals who already have OA in other joints, or in those with other susceptibility factors to OA [122].

Abnormal loading distribution could be the result of congenital abnormalities such as slipped capital femoral epiphysis of the hip, acetabular dysplasia, leg perthes, developmental dysplasia of the hip. These abnormalities are associated with a very high risk of early development of OA in the affected joint [123].

Mechanical alignment of the knee, as indicated by the hip-knee-ankle is another important determinant of load distribution in the knee [124]. The proportion of weight-bearing load

transmission through the medial and lateral compartment of the knee is roughly adjusted. In varus and valgus mal-alignments, the prevalence of knee OA with medial and lateral compartment involvement is more frequent [125]. OA knees with a varus mal-alignment have a 3-4 fold increased risk of further joint space narrowing in the medial compartment, while OA knees with a valgus malalignment have a similar increased risk of narrowing of the lateral compartment joint space [126].

These forms of OA, which result from a previous injury or malalignment, are examples of secondary OA. In genetic studies, in particular this dissertation, the presence of this type of OA should be evaluated as an exclusion criterion.

1.3.2. Genetics

Genetic factors are implicated in the development, progression, and presentations of OA. As stated in the previous sections, OA occurs at different rates in various populations. While Caucasian populations tend to have higher frequencies of hip/knee OA and joint replacements due to OA, the incidence of hip OA is much less common in Asian and African populations, and also emigrants from these populations. The prevalence of hip OA has been reported to be as low as 1-4% among the black populations from Jamaica, South Africa, Nigeria, and Liberia [127], and OA of the hip is known to be a rare disorder in the Hong Kong Chinese population [128]. The prevalence of OA is much higher among the Caucasian population of Europe and America. As well, within the East Asians, the prevalence of OA tend to much higher in developed countries such as Japan as compared to less developed countries including Vietnam [129, 130]. Although the population genetic differences can account for the variation in OA prevalence, very

limited investigations have been performed to determine the effect of genetics on OA through estimating the prevalence of OA among Afro-Caribbean, West Indians, and East Asians who have migrated to Europe or America. Therefore, the main evidence for the involvement of genetics in OA comes from family based and twins studies. The evidence has led to the conduct of candidate gene and genome-wide studies in search of susceptibility loci. More recently epigenetic studies are further contributing towards the elucidation of the pathophysiology of OA. In the following sections, the current knowledge regarding genetic and epigenetic involvement in OA will be discussed.

1.3.2.1. Familial aggregation - family-based studies

The first mention of a hereditary component to OA dates back to 1881 when Charcot [131] commented that "Heberden's nodes are a hereditary disease that may appear in several members of the same family." In 1889, Duckworth [132] found that the nodes in a multigenerational family with a female predominance. In 1941, Stecher studied 74 individuals with multiple Heberden's nodes and concluded that the nodes were most likely the result of a single autosomal gene defect with a dominant inheritance in women and recessive in men [133]. In 1963, Kellgren *et al*. [134] examined the relationship of the nodes to OA at other sites. He reported that the nodes were associated with multiple joint OA, and called the condition nodal generalized OA. He also indicated that the condition was twice as common in the relatives of the affected individuals compared to the general population.

Familial aggregation studies are the primary measure in examining whether a complex trait runs in families. The risk ratio of a disease for a relative of an affected individual compared with that

of the general population is the measure used for this purpose [135]. The late onset of OA occurrence does not allow intergenerational family studies, and thus most family-based studies of OA have focused on estimating the risk ratio of the siblings of affected individuals. Since OA is a heterogeneous condition with the involvement of different sites, the use of specific radiographic/clinical criteria to determine the status of the disease remains an issue. As such, familial aggregation studies of OA conducted on a variety of radiographic (e.g. KL grading) and clinical (e.g. conduct of total joint replacement surgery) traits have led to variable risk ratios. Regardless of the criteria used, the results indicate a strong familial aggregation as compared to several autoimmune and metabolic conditions with high genetic impacts (Table 1.2).

Table 1.2- Comparison of relative risk ratios reported for the siblings of affected individuals with OA and non-OA traits from different studies on various populations [136]

Trait	**Sibling recurrence risk ratio**
Non-OA traits	
Rheumatoid arthritis	5.00
Juvenile rheumatoid arthritis	15.00
Celiac disease	7.50–30.00
Obesity	1.60–1.91
Hyperglycemia	1.39–1.81
Type 2 diabetes	1.20–1.60
Hypertension	1.22–1.34
OA-related traits	
Total knee replacement	4.81
Anteromedial OA	3.21
Hip osteophytes grade 3	4.27
Total hip replacement	1.87–8.53
Hip KL grade ≥3	4.99
Hip Joint space width≤1.5 mm	5.07
Hand OA diagnosis	4.40
Hip OA diagnosis	3.90
Spine OA diagnosis	2.20
Hip and Spine OA diagnosis	4.70
Hip and Hand OA diagnosis	3.40

JSN radiographic progression of OA	3.00
Osteophyte radiographic progression of OA	1.50

Table 1.2 clearly shows that the effect is joint specific, and it is varied based on the type of trait and definition used or the population in which the study was done. The data presented proves that OA runs in families; however, this does not imply that the observed effect is entirely due to genetics since the familial aggregation also reflects the environmental factors that are shared within a family. The alternative method that is used to prove the genetic influence in OA is the application of classical twin studies.

1.3.2.2. Heritability - Twin studies

Classical twin studies compare the concordance of a trait among monozygotic twins to that of dizygotic twins. This method estimates the heritability, which is defined as the degree to which the genetic variations determine the population variability in the trait. The heritability of OA has been estimated for a variety of OA-related traits and joints after adjustment for OA risk factors. From such studies, the influence of genetics on radiographic markers of hand, hip and knee OA is reported to be from 39% to 65%. The heritability of the progression of OA has been estimated to be higher, 62% for osteophyte progression and 72% for JSN progression [137]. Similar to familial aggregation, the heritability also seems to be joint specific. For instance, the heritability estimate in the medial compartment of the knee is much higher than that in the lateral compartment [138]. Table 1.3 represents the heritability of a variety of OA-related traits estimated from different twin studies as compared to other complex human traits. As it can be seen, OA traits have heritability close to many other complex conditions with an established genetic influence such as type I diabetes.

Table 1.3- Heritability of OA-related traits compared to other complex traits [139,140]

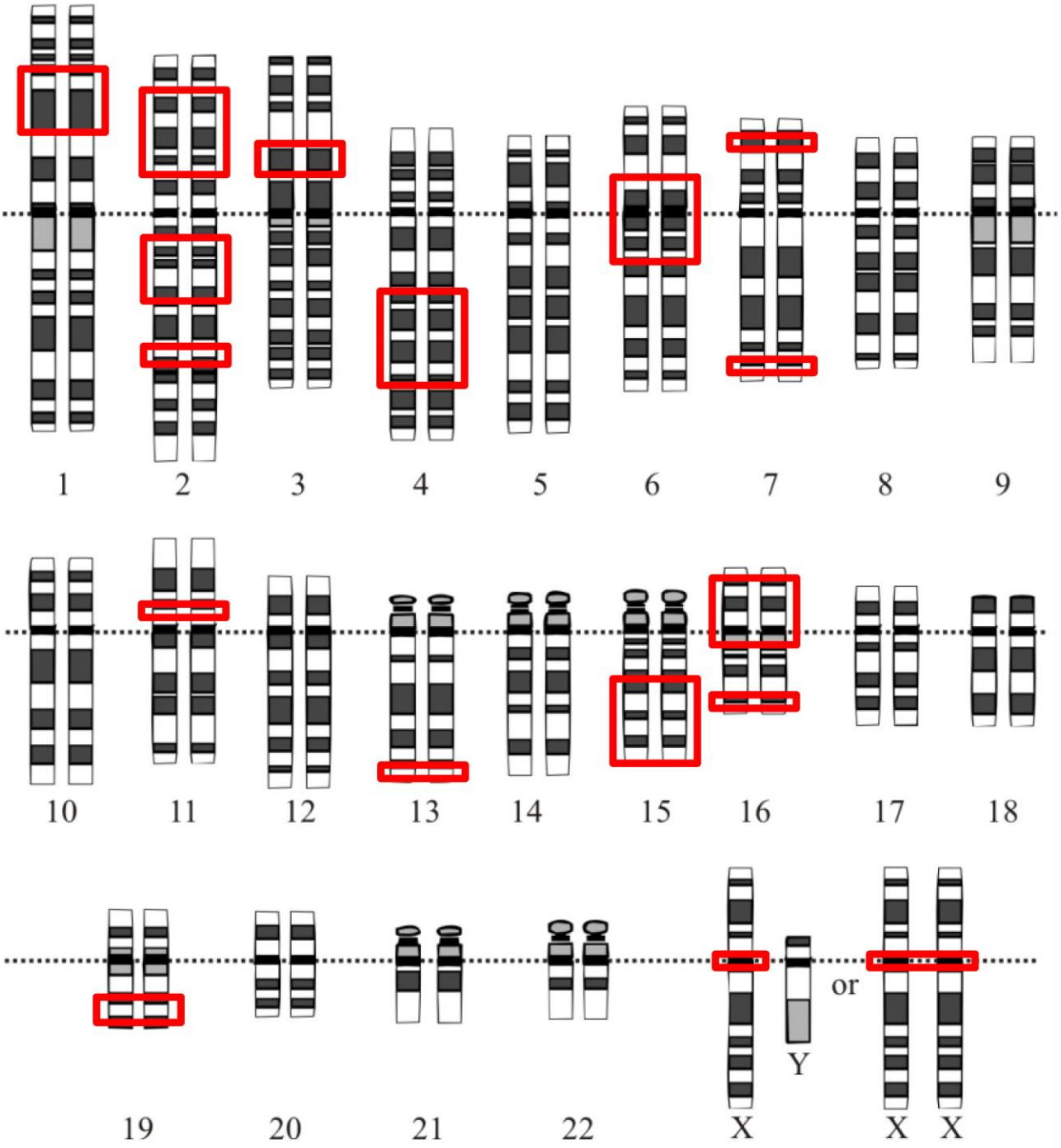
Trait	**Heritability**
OA-related traits	
Radiographic knee OA	39%
Radiographic hip OA	60%
Radiographic hand OA	59%
Femoral cartilage volume	61%
Tibial cartilage volume	76%
Patellar cartilage volume	66%
Change in medial cartilage volume	73%
Change in lateral cartilage volume	40%
Change in medial knee osteophyte grade	69%
Change in lateral knee osteophyte grade	33%
Change in knee JSN grade	74%
Lumbar spine OA	74%
Cervical spine OA	73%
Non-OA traits	
Obesity	70%
Leukemia	1%
Asthma	30%
Colon cancer	13%
Hypertension	30%
Migraine	53%

Type 2 diabetes	26%
Type 1 diabetes	88%
Schizophrenia	81%
Sexual orientation	60%
Height	55%-81%

1.3.2.3. Linkage analyses

The considerable estimation of heritability justified the search for OA genetic susceptibly loci. This search started from identifying the chromosomal regions linked to the segregation of OA in families. The method of choice in complex diseases is non-parametric linkage analysis which searches for the chromosomal segments that are shared more often than what is expected according to the Mendelian rules of inheritance among two relatives (most commonly sib-pairs) who share the trait. To date, five genome-wide linkage analyses have been performed on families with hand, hip or knee OA from the UK, Finland, Iceland, and the US. These analyses have found a number of relatively broad genomic intervals that may harbour OA susceptibility genes in chromosomes 2, 4, 6, 7, 11, 16, 19 and X [136, 141]. A meta-analysis of these reports has narrowed down these regions to 7q34–7q36.3, 11p12–11q13.4, 6p21.1–6q15, 2q31.1–2q34, and 15q21.3–15q26.1 as the most likely loci to harbor OA susceptibility genes [142]. Some of these intervals have been further analyzed using association and candidate gene studies. This has led to the discovery of genetic variants associated with OA in the interleukin 1 (*IL1*) gene cluster in Chr 2q11-q13, matrilin 3 (*MATN3*) in Chr 2p24.1, interleukin 4 receptor (*IL4R*) in Chr 16p12.1, secreted frizzled-related protein 3 (*FRZB*) in Chr 2q32.1, and bone morphogenetic protein 5 (*BMP5*) in chromosome 6p12.1 [143]. An ideogram of the regions identified through linkage analyses of OA is presented in figure 1.4.

Figure 1.4- Ideogram of the regions identified through genome-wide linkage analyses of OA



The red rectangles represent the identified regions.

1.3.2.4. Candidate gene studies

The influence of genetics on the development and progression of OA happens through several known biological pathways involved in the processes of the inflammatory response, bone remodeling, oxidative stress, and skeletal shape determination [141]. The genes involved in these processes, those reported being associated with other musculoskeletal conditions and those coding for the cartilage extracellular matrix (ECM) have a potential to be implicated in OA and have been studied in OA candidate gene searches. The number of reported genetic associations from such studies according to HuGE Navigator - a continuously updated source of genetic associations – is over 250 [144]. The majority of them have never been replicated or resulted from studies with low power, and thus are most likely spurious findings. Table 1.5 presents selected genetic association findings of OA along with their potential mechanism of involvement. Similar to twins and aggregation studies, the genetic associations reported here are site, sex, and population specific. The results of such reports need to be treated with caution since the majority of them have failed to produce replicable associations.

Some of the OA genetic association studies have benefited from using OA endophenotypes in gene hunting. An example would be a genetic association between variants in the *PACE4* gene and OA-related pain [145].

Table 1.5- Selected candidate genes with variants associated with OA and their potential function [136, 141, 144, 146]

Category	**Gene symbol**	**Gene name**	**Gene function related to OA**
Inflammation	*IL1*	Interleukin 1 gene cluster	Induces cartilage catabolism and protease activity
	IL6	Interleukin 6	Pro-inflammatory cytokine
	HLA	Human leukocyte antigen	Determination of the specificity of immune response
	IL10	Interleukin 10	Inhibition of IL1 synthesis
	ASPN	Asporin	Regulation of TGF-ß mediated chondrogenesis
ECM molecules	*MATN3*	Matrilin 3	Controlling interaction between collagen and Aggrecan
	COL2A1	Type II collagen	Major collagen found in cartilage
	COMP	Cartilage oligomeric matrix protein	Cartilage matrix macromolecule
	CILP	Cartilage intermediate layer protein	Regulation of cartilage matrix gene expressions through TGF-ß mediation
	BMP2	Bone morphogenetic protein 2	Growth factor involved in chondrogenesis and osteogenesis
Bone	*BMP5*	Bone morphogenetic protein 5	Regulation of articular chondrocyte

morphogenetic proteins			development
	GDF5	Growth differentiation factor 5	Regulation of growth and differentiation of bone and cartilage
	FRZB	Frizzled-related protein 3	Antagonist of Wnt signalling
Wnt-Frizzled Signalling	*LRP5*	Low-density lipoprotein receptor-related protein 5	Co-receptor of Wnt signalling in canonical beta-catenin pathway
	ADAM12	A disintegrin and metalloproteinase domain 12	Metalloproteinase involved in cell-cell fusion and osteoclast formation
Protease and their inhibitors	*MMP1*	Matrix Metallopeptidase 1	Cleaves cartilage matrix collagen
	AACT	Alpha1 antiproteinase antitrypsin	Inhibitor of matrix metallopeptidase
	OPG	Osteoprotegrin	Regulation of osteoclastogenesis
Chondrocyte differentiation	*ESR1*	Estrogen receptor alpha	Modulates proteoglycan degradation through metalloproteinase expression
	VDR1	Vitamin D receptor 1	Bone metabolism and remodeling
	DIO2	Type II iodothyronine deiodinase	Activation of thyroid hormone
	DIO3	Type III iodothyronine deiodinase	Activation of thyroid hormone
	ANP32A	Acidic nuclear phosphoprotein 32 family, member A	Tumor suppressor gene regulating apoptosis
Apoptotic Pathway, Mitochondrial damage	mtDNA variants	Mitochondrial haplogroups and mtDNA variants	Mitochondria-driven apoptosis

Among all of the reported associations, a variant in Growth Differentiation Factor 5 (*GDF5*) is the most replicated SNP by independent studies. GDF5 is one of the ligands in the bone morphogenetic pathway (BMP) of transforming growth factor beta signalling family. Miyamoto *et al*. first reported an association of rs143383 (T>C), located in the 5'UTR of *GDF5* with both knee and hip OA in a Japanese and Chinese population [147]. The T allele was shown to confer a greater risk to OA compared to the C allele with an odds ratio of 1.30 - 1.70. The same phenomenon was observed among Caucasians in studies from the UK and Spain, although with a lower effect size [148]. The risk allele is shown to be associated with a lower expression of the gene in both cell culture and mRNA extracted from human OA cartilage [149]. The variant has also been reported to be associated with a variety of skeletal conditions including several human skeletal dysplasia, congenital hip dysplasia, lumbar disk degeneration, the risk of bone fracture, and height [150]. A recent meta-analysis in 7,965 cases and 12,747 controls has reported a significant association between this variant and hip, knee, and hand OA [151]. The BMP pathway in the TGF-beta family will be discussed in further details in chapter five.

1.3.2.5. GWAS

Genome-wide association studies (GWAS) were made available as a consequence of the fulfilment of the Human Genome and HapMap projects in 2007. These studies are used to test the associations of genetic markers tagging to relatively small genomic intervals (LD blocks) that are expected to contain the causative genetic variants for complex traits. The first large scale OA genomic variant study tested 72,000 markers for hip OA and identified a variant in *LRCH1* in a Japanese population, which later failed to produce replicable associations [152]. Another study

using the testing of over 500,000 markers on knee OA identified an SNP in the 5'UTR of the *COX2* gene [153]. The product by this gene is involved in response to inflammation through the synthesis of prostaglandins. Another GWAS on hand OA reported rs716508 in the first intron of *A2BP1* (Ataxin 2 binding protein 1) in a UK population [154], which was later replicated in a Finnish population with radiographic osteoarthritis of the hands [155]. The gene codes for a transcription factor, mediating the neuron-specific splicing pattern of the calcitonin gene-related pre-mRNA.

The next GWAS was reported by the Rotterdam study in the Netherlands, where the testing of over 500,000 variants in 1,341 OA patients and 3,496 controls, with a replication sample of 15,000 OA patients and 40,000 controls, revealed an SNP tagging to Chr7q22 [156]. The significance of this region in OA is not entirely clear. The region is a haplotype block containing six genes, five of which are expressed in human cartilage. One of these, *HBP1*, is a suppressor of WNT signalling pathway and its expression levels are reported to be lower among carriers of the risk allele of the SNP. Among other variants reported in GWAS are SNPs in HLA class II/III, *BTNL2, DVWA,* and *DOT1L* genes [157]. The latter gene among these (*DOT1L*), was identified using the study of OA endophenotypes. It was found to be associated with cartilage thickness, as measured by joint space width on X-ray [158]. The other genes from this list are involved in immunologic response and regulation of WNT signalling.

The two largest GWAS conducted to date are those by the ARCOGEN study and the TREAT-OA consortium. The ARCOGEN study was conducted in two stages. In the first stage, 7,410 OA cases and 11,000 controls were examined, and the second stage was performed on 5,000 OA cases and 40,000 controls [157]. The completion of the two phases reported a total of nine

variants with genome-wide significant p-values located nearby the *MCFL2, GNL3/GLT8D1, TP63, SUPT3H/CDC5L, FILIP1/SENP6, ASTN2, KLHDC5/PTHLH, CHST11*, and *FTO* genes. The involvement many of these genes in OA is not understood. The signal from *FILIP1* is 360 kb away from *COL12A1*, a member of cartilage ECM. *PTH* and *CHST11* are reported to be expressed higher in OA chondrocytes. *TP63* is reported to be associated with facial morphology, and the deficient mice develop limb and craniofacial defects. *CDC5L* is in the same LD block as *RUNX2*, which is a transcription factor essential for osteoblast development and proper bone formation [157]. The GWAS by TREAT-OA consortium was performed on 11,000 hip OA and 67,000 controls and reported variants in *NCOA3,* which is known to have reduced expression in OA [157].

Genome-wide association studies have been criticized on the applicability of the findings. The identified signals do not necessarily represent functional SNPs, and the effect sizes are strikingly small. Studies on OA have led to the same conclusion that there is no variant with a large effect size, and the odds ratios range between only 1.10 to 1.30. OA genetic components seem to be a result of the accumulation and interaction of a vast number of genetic loci with small effect sizes. The present studies have not taken into account the rare variants, copy number variants, and genetic/environment interactions. As a result, the variants identified altogether represent a tiny portion of the OA heritability. Future studies accounting for these issues will likely determine a better understanding of OA genetic component.

1.3.2.6. Epigenetics

Epigenetics refers to the heritable changes in the DNA that are beyond the genetic code. They include methylation of CpG dinucleotides in the DNA, modification of histones, and regulatory RNAs. Epigenetics is increasingly being used to study complex traits such as OA. The rationale for studying epigenetics in OA is based on three criteria. 1) A lack of full concordance among monozygotic twins, suggests the involvement of post-zygotic DNA changes. 2) Variations in the expression of many of the regulatory components and extracellular matrix molecules in the OA cartilage could be caused by epigenetic regulations. 3) The epigenetic marks that escape reprogramming during gametogenesis may be responsible for a portion of missing heritability of OA, which has remained unexplained [159]. Despite these strong hypotheses, epigenetics is not extensively studied in OA.

The first epigenetic studies of OA focused on the DNA methylation changes in the promoter of the aberrantly activated degradative enzymes (e.g. *MMP3*, *MMP9*, *MMP13*, and *ADAMTS4*) and lower expressed cartilage molecules including collagen and Aggrecan genes. The summary of these studies suggests a hypermethylation of ECM genes (e.g. *COL9A1*, *ACAN*) and a lower methylation of some of the sites in the genes coding for degradative enzymes that are associated with their aberrant expression in OA chondrocytes [159]. These methylation changes were also shown to be triggered by treatment by inflammatory regulators including IL-1 and TNFα and hormones such as Leptin *in vitro* [159]. These studies were based on selected few CpG sites across the promoter and did not explore the effect of the intergenic and trans-regulatory elements on the gene expression, which could make them prone to bias. Among other studies on the OA DNA methylation changes are the demethylation of an enhancer element within the nitric oxide

synthase (*NOS*) gene, which is shown to increase transcription activity through an increased binding of the transcription factor NF-κB, resulting in the suppression of cartilage matrix synthesis [160]. DNA methylation can also modulate the effect of OA genetic susceptibility loci. For instance, the effect of the single nucleotide polymorphism (SNP) rs143383 in *GDF5*, the most replicated genetic association locus in OA, is thought to be caused by the variable methylation of the CpG dinucleotide created at the location of the SNP, leading to altered expression of the gene [161]. The handful of genome-wide methylation studies performed to date have also identified several potential candidate genes including *RUNX1*, *RUNX2*, *TGFB1*, *miR-128* and *COL11A2* [162], and emphasized the involvement of inflammation and immunity in OA pathogenesis [163].

Fewer studies have investigated the involvement of histone modifications and regulatory RNAs in OA. Chemical modification of histone residues at specific sites have shown to occur in OA. IL-1 treatment of human osteoarthritic chondrocytes is reported to increase H3K4 di- and tri-methylation of the promoters of cyclooxygenase 2 (*COX2*) and inducible nitric oxide (*iNOS*) genes and leading to their increased expressions [164]. The expression of these genes contributes to oxidative stress and increased protease activity in cartilage. Histone acetylation is removed by enzymes called histone deacetylases (HDACs) [165]. Several HDACs are upregulated in OA chondrocytes including HDAC1, HDAC2, and HDAC7. The first two are shown to repress the expression of collagen and Aggrecan while the latter one increases the expression of *MMP13* [165, 166]. Consistently, the inhibition of HDACs in a rabbit anterior cruciate ligament transaction model (ACLT) was followed by less severe cartilage erosion [167]. Altered expression of several micro RNAs is reported in OA, among which miR-140 has gained the most

attention in OA research. Its expression levels are increased during chondrogenesis, but reduced during OA progression [168].

miR-140 null mice are born normally, but within four months they present with craniofacial deformities, shorter long bones, and an early onset OA-like disease [168]. Genes targeted by miR-140 in mice models include *HDAC4* (a repressor of *RUNX2*), *CXCL12*, and *SMAD3*. In humans miR-140 has shown to decrease the expression of *ADAMTS5* and *LGFB5*, leading to a rise in Aggrecan production.

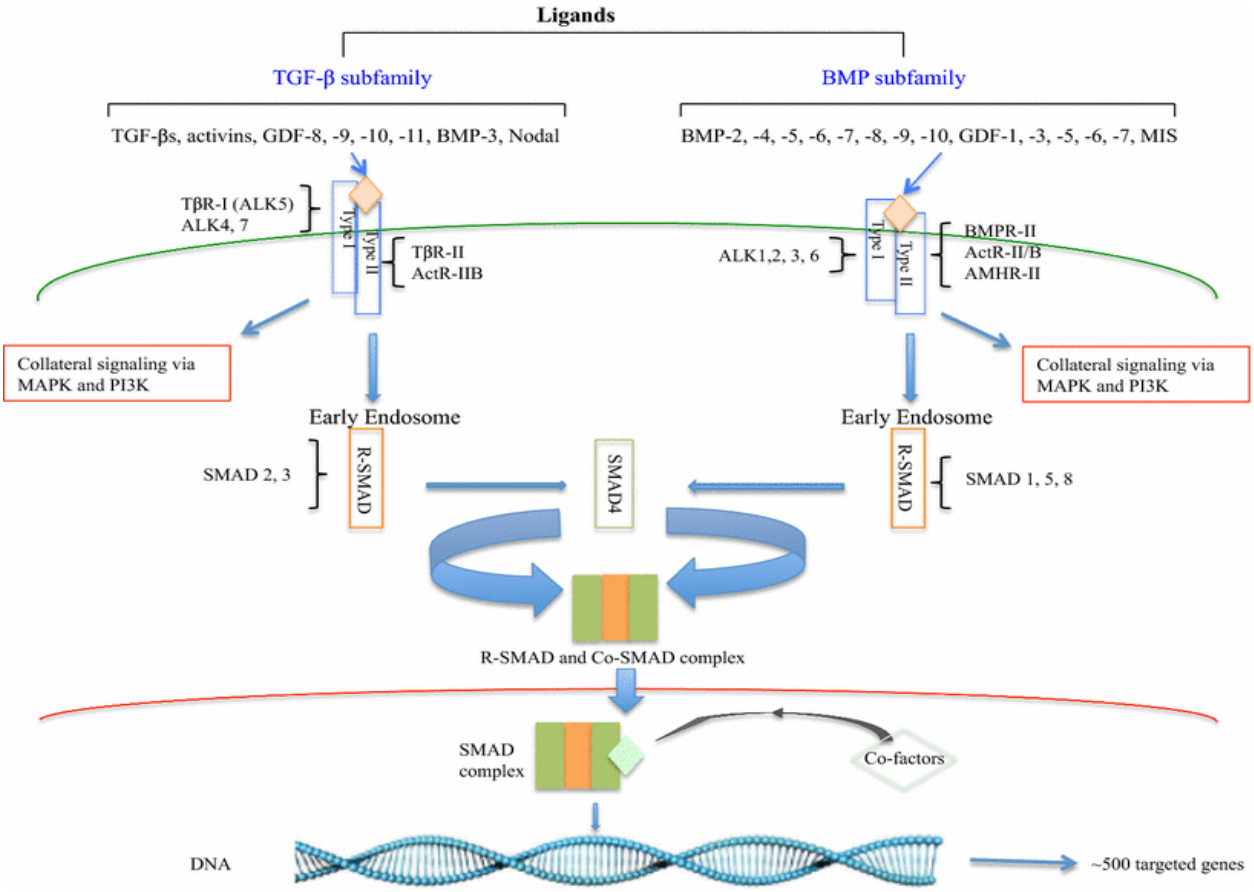
The knowledge on histone modification and regulatory RNA changes in OA is sparsely gained from few candidate gene studies and a large scale analysis is not yet performed. Understanding the epigenomic changes in OA will have a potential therapeutic value since the epigenetic marks have the ability to be modified through medication therapy.

1.4. Transforming growth factor-beta signalling pathway in osteoarthritis

Transforming growth factor-β (TGF-β) is an intracellular pathway consisting of over 30 proteins acting as receptors, ligands, and signal transducers. They have direct effect on cellular processes, including cell proliferation, recognition, differentiation, morphogenesis, apoptosis, tissue homeostasis, and regeneration through regulating the expression of a large set of nuclear genes [169]. The superfamily is composed of two subfamilies, TGF-β, and BMP, each having own distinct ligands, receptors, and signal transducers, activating distinct sets of target genes. The signalling is initiated by the binding of the ligand to the receptor on the cell surface, which activates the non-canonical pathway at the cell surface and the canonical pathway inside the cell

[170]. The signal in canonical pathway is generated through the phosphorylation of SMAD2 and SMAD3 (in TGF-ß pathway), and SMAD1, SMAD5 and SMAD8 (in BMP pathway). This leads to the formation of co-SMAD complex with SMAD4, which binds to other transcription factors in the nucleus and regulates gene expressions (Figure 1.5) [171].

Figure 1.5- Schematic illustration of TGF-ß superfamily (Adapted from Zhai *et al*. 2015 [172])



The alterations in the pathway have been reported to lead to several human diseases including OA [173]. A large number of studies on different components of TGF-β pathway report skeletal malformations in animals with the TGF-β genes deficiencies [174[. The findings of such studies suggest that the BMP signal pathway is required for early chondrogenesis while TGF-β signals are crucial for maintaining articular cartilage homeostasis by suppressing the process of terminal hypertrophic differentiation in chondrocytes [174]. The maintenance of cartilage function by TGF-β, however, is assumed to be related to a narrow range of bioactive TGF-β levels, concentrations below or above which may lead to aberrant alterations in TGF-β pathway, resulting in abnormal cartilage function [175]. In compliance with this, mice overexpressing SMURF-2, an E3 ubiquitin ligase known to inhibit TGF-β signalling, spontaneously develop an OA-like phenotype and have decreased levels of SMAD3 phosphorylation [176]. As well, TGF-β/SMAD3 signalling upregulation is shown to increase the expression of metalloproteinases in cartilage [177], and multiple intra-articular injections of TGF-β in mice joint also resulted in changes in articular cartilage with strong resemblance to both experimental and spontaneous mice OA [178].

A large number of the associated genes discovered in human studies are members of the TGF-β family. *GDF5*, harbouring the most replicated OA genetic variant association, encodes a ligand molecule to the BMP pathway. Polymorphisms in *BMP2, TGFB1*, Asporin, and *SMAD3* are also found to be associated with OA [146]. Point mutations in *SMAD3* [179, 180] have been reported to be the cause of the Aneurysm-Osteoarthritis Syndrome, a rare syndromic disease characterized with early-onset polyarticular OA, aneurysms of the main arteries, and several connective tissue disorders. Mutations in *TGFB1* are found in Camurati-Engelmann disease (CED), an autosomal dominant progressive diaphyseal dysplasia characterized by progressive cortical thickening and

subsequent sclerosis of the long bones [181]. TGFB isoforms and their receptor (ALK5) are reported to be downregulated in human OA cartilage [182]. In OA cartilage the expression of BMP receptor (ALK1) is positively correlated with the expression of *MMP13*, while the TGF-receptor (ALK5) expression is correlated with a higher level of cartilage matrix component, further highlighting the role of TGF-β pathway in cartilage maintenance [183]. Despite the numerous reports of the involvement of the pathway in OA, *in vivo* human studies of these genes are very limited. This thesis is one of the first examples of such studies in humans.

1.5. Hypotheses and Objectives

This thesis aimed to understand the etiology of OA regarding genetics and epigenomics with two main perspectives, namely the role of transforming growth factor beta and epigenetics in OA.

First, I hypothesized that the Newfoundland and Labrador population had a potential to detect robust genetic associations of OA. Although Canada, in general, has a diversity of cultures and ethnicities, the present NL population almost entirely originates from nearly 20,000 migrants from south-west England and the south of Ireland in the mid-1700s [184]. This founding population experienced a low level of in-migration over centuries, which resulted in NL being one of the few isolated Caucasian populations worldwide. This has resulted in lower population genetic diversity and longer linkage disequilibrium blocks, making it a unique source for studying the genetics of complex traits [184]. We aimed to replicate the previously reported genetic associations of OA in a cohort from the Newfoundland and Labrador population.

Second, I hypothesized that the *SMAD3* gene was associated with generalized OA. The rationale behind this hypothesis came from the recent report of a syndromic form of generalized OA,

called Aneurysm Osteoarthritis syndrome (AOS). Patients affected by this syndrome present with early onset generalized OA, aneurysms of main arteries and various connective tissue disorders. AOS was reported to be caused by eight missense mutations in *SMAD3* [179]. I hypothesized that because of the similarity in the phenotypes of the two conditions *SMAD3* could be a potential candidate for a generalized form of OA, and I aimed to examine the association of *SMAD3* common genetic variants with the OA involvement of multiple joints in a cohort from the Chingford study (UK).

Since the SNP association identified in the second project was located in a non-coding part of the gene, in the third project, I hypothesized that the association of *SMAD3* with OA could be through its expression regulation. The objective was to examine the difference in the expression of *SMAD3* in cases and controls and to test whether the expression was regulated by the promoter DNA methylation of the gene.

The results of the first three projects underpinned involvement of TGF-ß signalling pathway in OA. In the fourth project, I questioned the regulation of matrix degeneration by matrix metallopeptidase 13 (*MMP13*) in the context of TGF-ß signal transduction by SMAD3 and the effect of the BMP pathway. I aimed to examine the expression levels and pair-wise correlations of four genes in the pathway including *TGFB1* and *BMP2* as ligands, *SMAD3* as an intracellular mediator, and *MMP13* as a targeted gene in human cartilage tissues obtained from OA patients and healthy controls.

In the last project, I investigated the role of genome-wide DNA methylation changes in human OA cartilage. A genome-wide DNA methylation analysis in OA-free and OA-affected cartilage from human hips and knees using the Illumina Infinium HumanMethylation450 BeadChip was

conducted. I also examined the potential of the identified methylation sites in distinguishing OA-free and OA-affected cartilage in addition to identifying the pathways the differentially methylated genes are enriched in.

2

Attempt to replicate the published osteoarthritis associated genetic variants in the Newfoundland & Labrador Population

Erfan Aref-Eshghi[1], Proton Rahman[2], Hongwei Zhang[1], Glynn Martin[3], Andrew Furey[3], Roger Green[1], Guang Sun[2] and Guangju Zhai[1,4]

[1]Discipline of Genetics, [2]Discipline of Medicine, [3]Division of Orthopedics, Faculty of Medicine, Memorial University of Newfoundland, Canada

[4]Department of Twin Research & Genetic Epidemiology, King's College London, UK

A version of this chapter was published in:
J Orthop Rheumatol 2014; 1(3): 5.
doi: 10.13188/2334-2846.1000009

Abstract

Objective: Over 200 genes have been reported to be associated with osteoarthritis (OA), but most of them have not been replicated in an independent sample. Using the newly collected cohort from a genetically isolated population – the Newfoundland and Labrador population, I attempted to replicate 69 previously reported OA-associated SNPs.

Methods: A case-control study design was utilized in this study. Patients undergoing total hip/knee joint replacements due to severe OA were collected as cases. A group of healthy individuals with no evidence of OA was used as controls. Sixty-nine SNPs were genotyped either by Sequenom iPLEX Gold method or Illumina GWAS genotyping platform. The cross-reference was performed on both methods in a subset of samples for genotyping quality control. A logistic regression model was used to test for associations between the SNPs and OA.

Results: A total of 126 cases and 348 healthy controls were included in the final analysis. OA patients were on average nine years older than healthy controls ($p<0.0001$), but there was no difference in BMI. I was unable to replicate the previously reported associations. Two SNPs, rs2294995 (*COL9A3*), and rs1049007 (*BMP2*) showed an association with $p<0.05$, but the significance did not survive the Bonferroni multiple testing correction.

Conclusion: A lack of replication might be due to study design, complexity of OA, method of OA ascertainment, populations studied, or false positives in the original publications. A study with a larger sample is needed to confirm the association of two SNPs with OA.

Introduction

Osteoarthritis (OA) is the most common form of arthritis causing joint pain, stiffness, limited range of motion, joint deformity, and disability [185]. Its prevalence is on the rise due to population aging and increasing the prevalence of obesity. Knee, hip, hand, spine, and foot are the most affected joints while the greatest public health burden results from the hip and/or knee OA [186]. To date, there are no drugs available for rebuilding the damaged cartilage, nor is there a clear understanding of the pathogenesis of the disease. Total joint replacement therapy is the only choice for people with advanced OA. In the US alone, the total number of hip and/or knee joint replacement surgery due to OA is 350,000 each year [76], and the annual per person cost of those living with OA has been estimated to be around $5,700 [187]. Arthritis, mostly OA, costs $128 million per year in medical care and other indirect expenses including those resulting from work limitation and loss of productivity in the US [188].

Although the etiology of OA is not completely understood, it is believed that OA is a multifactorial condition developing and progressing as a result of a combination of different environmental and genetic factors [189]. The main non-genetic risk factors include age, gender, obesity, previous joint injury, and joint mal-alignments [190]. Evidence suggests a strong genetic component to OA. From twin studies this genetic influence has been estimated to be between 40% and 65% for knee, hip [191], and hand OA [192], and first-degree relatives of individuals

with spine, hand, hip, or polyarticular OA have a two- to three-fold increased risk of the disease [193, 194].

To date, nine Genome-Wide Association Studies (GWAS), along with a large number of candidate gene studies and linkage analyses, have been performed on OA. Although some OA-associated genes such as *GDF5* have been replicated in independent research, the majority of the studied loci yielded inconsistent results [195]. This might be due to the genetically heterogeneous nature of the disease or false positive findings in the initial studies. Genetically isolated populations have advantages for complex disease gene mapping because of their reduced genetic heterogeneity and extended LD [184]. The Newfoundland and Labrador population is a young, isolated founder population with a high degree of both genetic and cultural homogeneity exhibiting extended linkage disequilibrium and an increased kinship coefficient, which provides a unique source for investigating both single gene diseases and complex traits [196, 197]. The aim of the present study was to replicate previously reported OA-associated genes using this unique population.

Methods and Materials

Subjects

The study was part of the Newfoundland Osteoarthritis Study (NFOAS) that was initiated in 2011 and aimed at identifying novel genetic, epigenetic, and biochemical markers for OA. OA patients were recruited from those who underwent total knee or hip replacement surgery due to primary OA between Nov. 2011 to Jun. 2013 in St. Clare's Mercy Hospital and Health Science Centre General Hospital in St. John's, the capital city of Newfoundland and Labrador (NL)

province of Canada. A group of healthy people who do not have any evidence of either knee or hip OA was used as controls. The controls were selected from a previous genetic association study for type II diabetes and obesity. The controls completed a questionnaire regarding any ongoing symptoms, previous diagnosis and medication history. A rheumatologist also examined each subject. The controls were selected for this study if they had no musculoskeletal pain, a prior diagnosis of osteoarthritis, were not taking acetaminophen or NSAIDs and had a normal physical examination as it relates to the skeletal system. All cases and controls in this study were from NL. The study was approved by the Health Research Ethics Authority (HREA) of Newfoundland and Labrador (HREA11.311), and written consent was obtained from all the participants.

Height and weight measurements were obtained from the patient's hospital medical record. Body mass index (BMI) was calculated as weight in kilograms divided by squared height in meters. Age was calculated at the time of surgery or visit date.

Genotyping

HuGE Navigator [144], a continuously updated database in human genome epidemiology, indexes all of the genetic association studies for a given disease or trait. There were 231 genes reported to be associated with OA as of June 2013, only a few of which have been tested in replicating studies. Due to a limited budget, it was decided to replicate all the SNPs that were reported in OA GWAS studies plus some SNPs from candidate studies.

Blood samples were obtained from all study participants, and DNA was extracted by using a standard protocol. All OA cases were genotyped by Sequenom iPLEX Gold method [198]. Briefly, a 384-well plate chip on Sequenom platform using mass spectrometry was used for

genotyping. Each multiplex PCR was done using 30ng of DNA (n=1: 1.25X PCR buffer Roche, 2mM MgCl2 Roche, 0.5M dNTPs, 0.11uM PCR primer pool oligos ordered from IDT, 0.15U/µL Roche FastStart) and the amplification was done following this cycling protocol: [95°C 15min, 45x (95°C 20sec, 58°C 30sec, 72°C 60sec), 72c 3min]. The SAP reaction was done to clean the PCR product following by the single base extension reaction using mass-modified dideoxynucleotide terminators of an oligonucleotide primer which anneals immediately upstream of the polymorphic site of interest. After extension, the Salt Adduct Removal Step was run using 6mg of resin. The product was then spot on a Sequenom 384-well chip using a Nanodispenser and loaded onto the Mass Spectrometer for reading.

All the controls were previously genotyped using Illumina HumanHap550-Duo BeadChip at Centrillion Biosciences at Palo Alto, California. Cross-validation of genotyping quality was carried out on 31 controls that were genotyped by both Sequenom iPLEX Gold method and IlluminaHumanHap550-Duo BeadChip.

Statistical analysis

Distribution of age, gender and BMI was examined and tested between OA cases and controls by either Chi-squared test or Student t-test wherever appropriate. Concordance between the two genotyping methods was evaluated by calculating genotype concordance rate in those subjects who were genotyped by both methods. Hardy-Weinberg Equilibrium (HWE) test was performed for each of the SNPs by Chi-squared test and removed in the subsequent analysis if the p-value <0.05. Chi-squared test was utilized to test the association between each of the SNPs and disease status and logistic regression modeling was used to adjust for potential confounders including age, sex, and BMI since the cases and controls were different regarding these variables. The

significance level was set at the alpha level of 0.0007 after correcting for multiple testing with the Bonferroni method. All analyses were done using STATA/SE 11.2 (Stata Corp, College Station, Texas, USA) except for HWE test which was performed by PLINK version 1.07 [199].

Results

A total of 126 OA cases and 348 controls were included in this study. Among OA cases, 50 were males, and 76 were females, 42 had a total hip replacement, and 84 had total knee replacement due to primary OA. Controls (146 men and 202 women) were healthy subjects who did not have evidence of joint OA. Characteristics of the study participants are presented in Table 2.1. OA cases were significantly older than healthy controls and had a slightly higher BMI, which was not significant.

Table 2.1- Descriptive statistics of the study population

Variables	OA (n=126)	Healthy Controls (n=348)	P-value¶
Age (yr)	63.8±0.87	54.8±0.81	<0.0001
BMI (kg/m^2)	33.05±0.67	31.7±0.39	0.07
Sex (% female)	60.3%	58.1%	0.65

Figures stand for Mean ± SE unless stated. ¶P-value for comparison between OA cases and healthy controls

105 SNPs located in 71 genes were genotyped by Sequenom iPLEX Gold method for all cases and 52 healthy controls. Sixty-nine of these 105 SNPs were included in Illumina HumanHap550-Duo BeadChip and genotyped for all controls and therefore the subsequent analysis was focused on these 69 SNPs. 31 healthy controls were genotyped by both methods and were used to cross-validate the quality and accuracy between two genotyping methods. The genotype concordance rates for all 69 SNPs were 100%, indicating the comparability of the two genotyping methods.

Two SNPs were deviated from HWE and excluded from the subsequent analyses. Table 2.2 presents the results of the univariable and multivariable analyses of the association of each of the remaining 67 SNPs between OA and healthy controls. I found that the minor alleles of rs2294995 (located in *COL9A3*) and rs1049007 (located in *BMP2*) were associated with one-third reduced risk for OA, but the significance did not reach the Bonferroni correction for multiple testing.

In the joint specific analyses, three SNPs were associated with knee OA [*COL9A3* rs2294995 (OR:0.51, 95%CI: 0.32-0.8, P=0.004), *HFE* rs1799945 (OR:1.67, 95%CI: 1.06-2.6, P=0.025), and *PACE4* rs900414 (OR:0.63, 95%CI: 0.41-0.97, P=0.036)], and five SNPs with hip OA [*EDG2* rs10980705 (OR:0.4, 95%CI: 0.19-0.89, P=0.024), *IL1RN* rs315952 (OR:0.5, 95%CI: 0.26-0.93, P=0.03), *BMP2* rs1049007 (OR: 0.56, 95%CI: 0.33-0.95, P=0.03), *IL1RN* rs9005 (OR:1.69, 95%CI: 1.03-2.78, P=0.038), and *COX2* rs5277 (OR:1.74, 95%CI: 1-3.01, P=0.048)], but all the significance did not reach the Bonferroni multiple testing correction (Table 2.3).

Table 2.2- Results of the association tests for each SNP between OA cases and healthy controls*

Genetic variant		Risk Allele	Univariable		Multivariable		Risk Allele Freq.	
Gene symbol	**SNP**		**OR (95% CI)**	**P-value**	**OR (95% CI)**	**P-value**	**Controls**	**Cases**
BMP2	rs1049007 (A/G)	A	0.69 (0.50 - 0.94)	0.021*	0.64 (0.45 - 0.90)	0.011*	0.43	0.35
COL9A3	rs2294995 (A/G)	A	0.64 (0.46 - 0.90)	0.010*	0.65 (0.46 - 0.94)	0.021*	0.33	0.24
TLR8	rs5744080 (T/C)	T	1.20 (0.94 - 1.53)	0.130	1.31 (1.00 - 1.71)	0.040*	0.41	0.48
PACE4	rs900414 (A/G)	A	0.76 (0.54 - 1.06)	0.109	0.70 (0.48 - 1.01)	0.060	0.31	0.26
MTHFR	rs1801133 (A/G)	A	1.32 (0.98 - 1.78)	0.068	1.36 (0.98 - 1.89)	0.065	0.31	0.38
ADAM12	rs1871054 (A/G)	G	1.20 (0.90 - 1.61)	0.219	1.33 (0.96 - 1.85)	0.085	0.46	0.50
IL18	rs1946518 (A/C)	A	1.15 (0.85 - 1.55)	0.363	1.33 (0.95 - 1.85)	0.097	0.40	0.44
DIO3	rs945006 (A/C)	C	0.81 (0.52 - 1.26)	0.356	0.69 (0.43 - 1.13)	0.139	0.15	0.12
ADAM12	rs3740199 (C/G)	C	1.26 (0.95 - 1.68)	0.107	1.25 (0.91 - 1.70)	0.163	0.47	0.53
ADAM12	rs1044122 (A/G)	A	1.21 (0.88 - 1.67)	0.234	1.26 (0.89 - 1.78)	0.196	0.23	0.27
IL4R	rs2234895 (C/T)	T	0.70 (0.38 - 1.28)	0.244	0.65 (0.34 - 1.25)	0.199	0.08	0.06
PTGS2/COX-2	rs20417 (C/G)	G	1.23 (0.82 - 1.83)	0.319	1.33 (0.86 - 2.06)	0.201	0.15	0.18
ASTN2	rs4836732 (C/T)	C	1.19 (0.89 - 1.58)	0.243	1.22 (0.90 - 1.66)	0.208	0.48	0.53

HFE	rs1799945 (C/G)	C	1.30 (0.89 - 1.91)	0.178	1.30 (0.86 - 1.96)	0.214	0.15	0.19
DIO2	rs225014 (C/T)	T	1.27 (0.95 - 1.69)	0.108	1.22 (0.89 - 1.67)	0.214	0.36	0.42
IL6	rs1800797 (A/G)	A	0.78 (0.58 - 1.05)	0.098	0.82 (0.60 - 1.13)	0.228	0.41	0.35
IL6	rs1800796 (G/C)	G	0.70 (0.35 - 1.38)	0.310	0.60 (0.32 - 1.31)	0.230	0.05	0.04
GLTBD1	rs6976 (C/T)	C	0.89 (0.66 - 1.2)	0.447	0.82 (0.60 - 1.13)	0.230	0.41	0.38
IL1B	rs1143633 (A/G)	A	1.18 (0.88 - 1.58)	0.272	1.20 (0.87 - 1.64)	0.260	0.34	0.38
COX2	rs5277 (C/G)	G	1.14 (0.77 - 1.67)	0.518	1.26 (0.82 - 1.93)	0.285	0.15	0.17
IL1B	rs1143634 (C/T)	T	0.88 (0.63 - 1.25)	0.485	0.83 (0.57 - 1.19)	0.305	0.23	0.21
VDR	rs731236 (C/T)	T	1.16 (0.87 - 1.55)	0.302	1.17 (0.85 - 1.60)	0.329	0.42	0.46
IL1RN	rs315952 (A/G)	G	0.83 (0.59 - 1.15)	0.255	0.84 (0.59 - 1.19)	0.332	0.28	0.25
ESR1/alpha	rs2234693 (G/A)	G	0.80 (0.61 - 1.06)	0.130	0.87 (0.64 - 1.18)	0.370	0.46	0.40
ANP32A	rs7164503 (A/G)	G	0.92 (0.57 - 1.49)	0.737	0.79 (0.47 - 1.33)	0.375	0.11	0.10
HLA class II/III	rs7775228 (A/G)	G	1.20 (0.77 - 1.86)	0.414	1.22 (0.75 - 1.99)	0.417	0.11	0.13
DIO2	rs12885300 (A/G)	A	0.82 (0.60 - 1.12)	0.222	0.87 (0.62 - 1.22)	0.421	0.36	0.32
EDG2	rs10980705 (A/G)	G	0.82 (0.58 - 1.16)	0.256	0.86 (0.59 - 1.25)	0.427	0.23	0.19
CHST11	rs835487 (A/G)	A	0.84 (0.62 - 1.15)	0.280	0.87 (0.62 - 1.22)	0.431	0.36	0.32

IL1A	rs1800587 (A/G)	A	0.94 (0.69 - 1.30)	0.727	0.88 (0.62 - 1.25)	0.473	0.29	0.28
Matrilin3	rs8176070 (C/T)	C	0.95 (0.70 - 1.29)	0.755	0.89 (0.63 - 1.25)	0.496	0.35	0.34
SUPT3H/CDC5L	rs10948172 (A/G)	G	0.87 (0.63 - 1.20)	0.406	0.89 (0.63 - 1.26)	0.504	0.31	0.28
pTGS2/ PLA2G4A	rs4140564 (C/T)	C	1.56 (0.87 - 2.81)	0.137	1.24 (0.65 - 2.35)	0.515	0.05	0.08
LRCH1	rs912428 (A/G)	A	1.06 (0.74 - 1.52)	0.745	1.13 (0.77 - 1.68)	0.532	0.20	0.21
IL18R1/IL18RAP	rs2287037 (C/T)	T	1.05 (0.78 - 1.42)	0.743	1.11 (0.80 - 1.54)	0.539	0.35	0.36
IL1B	rs16944 (A/G)	A	1.03 (0.76 - 1.40)	0.835	1.11 (0.79 - 1.55)	0.543	0.34	0.35
TLR-3	rs3775296 (G/T)	T	1.03 (0.72 - 1.48)	0.868	1.12 (0.77 - 1.65)	0.549	0.18	0.18
IL4	rs2070874 (C/T)	T	0.89 (0.58 - 1.37)	0.598	0.87 (0.53 - 1.42)	0.566	0.13	0.12
MCF2L	rs11842874 (A/G)	G	0.87 (0.52 - 1.46)	0.592	0.86 (0.50 - 1.48)	0.580	0.09	0.08
CALM2	rs10153674 (A/G)	A	0.98 (0.65 - 1.48)	0.917	0.88 (0.56 - 1.39)	0.587	0.15	0.14
A2BP1	rs716508 (C/T)	T	1.12 (0.82 - 1.53)	0.461	1.09 (0.77 - 1.54)	0.615	0.31	0.34
FTO	rs8044769 (C/T)	C	0.83 (0.62 - 1.11)	0.208	0.92 (0.67 - 1.27)	0.621	0.48	0.43
HLA class II/III	rs10947262 (A/G)	A	1.20 (0.68 - 2.13)	0.530	1.16 (0.63 - 2.15)	0.632	0.06	0.07
TXNDC3	rs4720262 (C/T)	T	0.88 (0.64 - 1.22)	0.452	0.92 (0.65 - 1.30)	0.634	0.28	0.26
ADAMTS5	rs2380585 (A/G)	A	0.93 (0.65 - 1.34)	0.701	0.91 (0.62 - 1.36)	0.658	0.21	0.20

TLR-9	rs187084 (A/G)	G	1.01 (0.76 - 1.35)	0.928	0.93 (0.68 - 1.28)	0.673	0.41	0.41
IL1RN	rs419598 (A/G)	G	1.02 (0.74 - 1.39)	0.922	0.93 (0.66 - 1.31)	0.691	0.30	0.30
GDF5/ASPN	rs13301537 (C/T)	C	1.03 (0.75 - 1.42)	0.840	1.07 (0.76 - 1.52)	0.698	0.28	0.28
ESR2/beta	rs1256031 (C/T)	T	1.16 (0.86 - 1.56)	0.335	1.06 (0.77 - 1.47)	0.717	0.45	0.48
TRPV1	rs8065080 (A/G)	A	0.98 (0.72 - 1.32)	0.875	0.94 (0.68 - 1.31)	0.724	0.38	0.38
TNF-A	rs1800629 (A/G)	A	0.94 (0.64 - 1.39)	0.765	0.93 (0.61 - 1.41)	0.738	0.18	0.17
IL4R	rs1805013 (A/G)	A	1.09 (0.49 - 2.42)	0.838	0.87 (0.36 - 2.09)	0.760	0.03	0.04
AACT	rs4934 (C/T)	T	0.91 (0.68 - 1.21)	0.501	0.96 (0.70 - 1.31)	0.800	0.48	0.46
IL1R1	rs1465325 (C/T)	C	0.95 (0.65 - 1.40)	0.808	0.95 (0.62 - 1.44)	0.802	0.17	0.17
LEP	rs2060715 (A/G)	A	0.85 (0.63 - 1.13)	0.264	0.96 (0.70 - 1.32)	0.808	0.48	0.44
TP63	rs12107036 (A/G)	A	1.04 (0.78 - 1.38)	0.795	1.03 (0.76 - 1.42)	0.832	0.48	0.49
ESR1/alpha	rs2228480 (C/T)	T	0.89 (0.62 - 1.27)	0.513	0.96 (0.65 - 1.41)	0.835	0.22	0.20
NFKB1A	rs8904 (C/T)	C	0.96 (0.70 - 1.33)	0.820	0.97 (0.69 - 1.38)	0.871	0.31	0.31
HAPLN1	rs179851 (C/T)	C	0.92 (0.67 - 1.25)	0.588	1.03 (0.74 - 1.44)	0.873	0.32	0.31
LEP	rs12706832 (C/T)	T	0.86 (0.64 - 1.15)	0.303	0.97 (0.71 - 1.34)	0.875	0.47	0.44
IL1RN	rs9005 (C/T)	C	1.09 (0.80 - 1.49)	0.577	1.02 (0.73 - 1.43)	0.909	0.31	0.33

RAGE	rs2070600 (A/G)	A	1.39 (0.77 - 2.5)	0.280	1.04 (0.52 - 2.07)	0.921	0.05	0.07
COL2A1	rs2070739 (C/T)	T	0.92 (0.57 - 1.47)	0.720	0.98 (0.59 - 1.62)	0.922	0.11	0.10
UQCC	rs6087704 (A/G)	G	1.02 (0.75 - 1.37)	0.911	1.02 (0.73 - 1.41)	0.926	0.35	0.35
WISP1	rs2929970 (C/T)	C	0.99 (0.74 - 1.32)	0.952	0.99 (0.72 - 1.36)	0.958	0.49	0.49
ESR2/beta	rs1256049 (C/T)	T	0.69 (0.28 - 1.69)	0.418	0.98 (0.38 - 2.55)	0.973	0.03	0.02
GDF5	rs224329 (C/T)	T	1.01 (0.74 - 1.36)	0.964	1.00 (0.72 - 1.39)	1.000	0.34	0.34

*OR: Odds Ratio, CI: Confidence Interval. P-values smaller than 0.05 are indicated with an asterisk.

Table 2.3- Results of the joint specific multivariable association tests for each SNP*

Genetic variant		**Risk Allele**	**Knee OA** (N=84)		**Hip OA** (N=42)	
Gene symbol	**SNP**		**OR (95% CI)**	**P-value**	**OR (95% CI)**	**P-value**
COL9A3	rs2294995 (A/G)	A	0.51 (0.33 - 0.81)	0.004	1.12 (0.68 - 1.85)	0.664
HFE	rs1799945 (C/G)	C	1.67 (1.07 - 2.63)	0.025	0.99 (0.51 - 1.92)	0.981
PACE4	rs900414 (A/G)	A	0.64 (0.41 - 0.98)	0.040	0.90 (0.52 - 1.53)	0.687
IL1B	rs1143633 (A/G)	A	1.39 (0.98 - 1.99)	0.067	1.00 (0.61 - 1.64)	0.997
ADAM12	rs1871054 (A/G)	G	1.41 (0.97 - 2.04)	0.069	1.04 (0.64 - 1.70)	0.865
ASTN2	rs4836732 (C/T)	C	1.37 (0.96 - 1.96)	0.086	0.90 (0.55 - 1.46)	0.664
TLR8	rs5744080 (T/C)	T	1.29 (0.96 - 1.72)	0.087	1.02 (0.68 - 1.51)	0.934
SUPT3H/CDC5L	rs10948172 (A/G)	G	0.73 (0.49 - 1.09)	0.122	1.31 (0.81 - 2.12)	0.269
ADAM12	rs3740199 (C/G)	C	1.31 (0.92 - 1.86)	0.131	1.33 (0.83 - 2.14)	0.230
BMP2	rs1049007 (A/G)	A	0.74 (0.51 - 1.09)	0.132	0.57 (0.34 - 0.96)	0.033
IL6	rs1800797 (A/G)	A	0.77 (0.53 - 1.10)	0.154	0.88 (0.55 - 1.41)	0.583
pTGS2/ PLA2G4A	rs4140564 (C/T)	C	1.55 (0.84 - 2.88)	0.164	0.40 (0.09 - 1.68)	0.210
IL18	rs1946518 (A/C)	A	1.28 (0.88 - 1.86)	0.19	1.17 (0.72 - 1.90)	0.514

GDF5	rs224329 (C/T)	T	0.78 (0.53 - 1.14)	0.198	1.37 (0.86 - 2.20)	0.188
DIO3	rs945006 (A/C)	C	0.70 (0.40 - 1.20)	0.205	0.76 (0.38 - 1.54)	0.449
PTGS2/COX-2	rs20417 (C/G)	G	1.38 (0.84 - 2.27)	0.206	1.46 (0.77 - 2.76)	0.246
IL4R	rs2234895 (C/T)	T	0.61 (0.28 - 1.33)	0.21	0.91 (0.37 - 2.26)	0.846
GLTBD1	rs6976 (C/T)	C	0.82 (0.56 - 1.19)	0.286	1.11 (0.68 - 1.81)	0.680
IL1A	rs1800587 (A/G)	A	0.81 (0.54 - 1.21)	0.299	1.04 (0.63 - 1.72)	0.876
UQCC	rs6087704 (A/G)	G	0.83 (0.57 - 1.21)	0.332	1.34 (0.83 - 2.14)	0.230
IL6	rs1800796 (G/C)	G	0.69 (0.32 - 1.49)	0.341	0.43 (0.11 - 1.67)	0.221
ADAM12	rs1044122 (A/G)	A	1.21 (0.82 - 1.78)	0.344	1.11 (0.65 - 1.87)	0.708
DIO2	rs12885300 (A/G)	A	0.84 (0.57 - 1.23)	0.363	0.92 (0.56 - 1.50)	0.734
ESR1/alpha	rs2234693 (G/A)	G	0.85 (0.60 - 1.21)	0.375	0.91 (0.57 - 1.45)	0.687
GDF5/ASPN	rs13301537 (C/T)	C	1.19 (0.80 - 1.76)	0.389	0.84 (0.48 - 1.47)	0.540
WISP1	rs2929970 (C/T)	C	0.86 (0.60 - 1.24)	0.426	1.22 (0.76 - 1.96)	0.419
IL1RN	rs9005 (C/T)	C	0.86 (0.58 - 1.28)	0.461	1.69 (1.030 - 2.78)	0.038
IL1B	rs1143634 (C/T)	T	0.86 (0.57 - 1.30)	0.477	0.80 (0.46 - 1.39)	0.428
LRCH1	rs912428 (A/G)	A	1.17 (0.76 - 1.80)	0.477	1.08 (0.60 - 1.94)	0.805

CHST11	rs835487 (A/G)	A	0.88 (0.60 - 1.28)	0.489	0.84 (0.51 - 1.37)	0.481
FTO	rs8044769 (C/T)	C	0.88 (0.62 - 1.27)	0.508	0.98 (0.61 - 1.58)	0.931
TNF-A	rs1800629 (A/G)	A	1.17 (0.74 - 1.85)	0.509	0.61 (0.30 - 1.27)	0.191
TXNDC3	rs4720262 (C/T)	T	1.13 (0.77 - 1.66)	0.524	0.56 (0.31 - 1.02)	0.057
COL2A1	rs2070739 (C/T)	T	1.20 (0.68 - 2.13)	0.529	0.87 (0.39 - 1.95)	0.743
HLA class II/III	rs10947262 (A/G)	A	1.26 (0.60 - 2.62)	0.542	1.78 (0.74 - 4.27)	0.197
IL1RN	rs315952 (A/G)	G	1.12 (0.77 - 1.65)	0.55	0.50 (0.27 - 0.93)	0.030
LEP	rs12706832 (C/T)	T	0.90 (0.63 - 1.28)	0.552	1.32 (0.84 - 2.08)	0.232
MTHFR	rs1801133 (A/G)	A	1.12 (0.78 - 1.60)	0.552	1.29 (0.80 - 2.08)	0.304
ANP32A	rs7164503 (A/G)	G	0.85 (0.48 - 1.50)	0.564	0.83 (0.39 - 1.78)	0.634
CALM2	rs10153674 (A/G)	A	0.86 (0.51 - 1.45)	0.572	1.02 (0.52 - 1.98)	0.960
IL1R1	rs1465325 (C/T)	C	1.13 (0.72 - 1.79)	0.595	0.87 (0.45 - 1.69)	0.688
EDG2	rs10980705 (A/G)	G	1.11 (0.74 - 1.68)	0.609	0.42 (0.20 - 0.89)	0.024
ESR2/beta	rs1256049 (C/T)	T	0.77 (0.27 - 2.16)	0.613	0.76 (0.19 - 2.95)	0.686
ADAMTS5	rs2380585 (A/G)	A	0.91 (0.60 - 1.38)	0.654	0.65 (0.35 - 1.20)	0.168
MCF2L	rs11842874 (A/G)	G	0.87 (0.47 - 1.63)	0.669	0.99 (0.46 - 2.16)	0.982

LEP	rs2060715 (A/G)	A	0.93 (0.66 - 1.33)	0.704	1.18 (0.75 - 1.86)	0.485
HLA class II/III	rs7775228 (A/G)	G	1.11 (0.65 - 1.91)	0.706	1.15 (0.58 - 2.27)	0.686
ESR1/alpha	rs2228480 (C/T)	T	0.92 (0.59 - 1.43)	0.709	0.90 (0.49 - 1.66)	0.740
DIO2	rs225014 (C/T)	T	1.07 (0.74 - 1.53)	0.726	1.37 (0.85 - 2.22)	0.193
TP63	rs12107036 (A/G)	A	1.06 (0.75 - 1.50)	0.74	0.95 (0.60 - 1.51)	0.836
IL1RN	rs419598 (A/G)	G	0.94 (0.64 - 1.38)	0.751	1.38 (0.83 - 2.29)	0.214
IL4	rs2070874 (C/T)	T	1.08 (0.64 - 1.83)	0.778	0.58 (0.24 - 1.38)	0.221
VDR	rs731236 (C/T)	T	1.05 (0.74 - 1.50)	0.778	1.29 (0.81 - 2.06)	0.276
HAPLN1	rs179851 (C/T)	C	0.95 (0.65 - 1.39)	0.801	1.13 (0.69 - 1.85)	0.620
Matrilin3	rs8176070 (C/T)	C	0.95 (0.66 - 1.38)	0.803	0.79 (0.47 - 1.31)	0.354
TLR-3	rs3775296 (G/T)	T	0.96 (0.60 - 1.51)	0.845	1.42 (0.82 - 2.45)	0.213
NFKB1A	rs8904 (C/T)	C	0.97 (0.65 - 1.44)	0.869	1.03 (0.62 - 1.70)	0.920
ESR2/beta	rs1256031 (C/T)	T	1.02 (0.72 - 1.46)	0.893	1.15 (0.72 - 1.84)	0.549
COX2	rs5277 (C/G)	G	0.97 (0.58 - 1.61)	0.899	1.74 (1.01 - 3.02)	0.048
AACT	rs4934 (C/T)	T	0.98 (0.69 - 1.41)	0.932	0.94 (0.59 - 1.48)	0.778
TLR-9	rs187084 (A/G)	G	1.01 (0.71 - 1.44)	0.947	1.08 (0.68 - 1.73)	0.735

IL18R1/IL18RAP	rs2287037 (C/T)	T	0.99 (0.69 - 1.43)	0.953	0.97 (0.6 - 1.56)	0.893
A2BP1	rs716508 (C/T)	T	0.99 (0.67 - 1.46)	0.961	1.09 (0.67 - 1.78)	0.728
RAGE	rs2070600 (A/G)	A	1.01 (0.49 - 2.1)	0.968	0.72 (0.24 - 2.14)	0.557
TRPV1	rs8065080 (A/G)	A	0.99 (0.69 - 1.43)	0.972	0.74 (0.45 - 1.22)	0.240
IL4R	rs1805013 (A/G)	A	1.01 (0.39 - 2.6)	0.982	0.65 (0.15 - 2.87)	0.567
IL1B	rs16944 (A/G)	A	1 (0.68 - 1.47)	0.992	1.46 (0.88 - 2.42)	0.140

*OR: Odds Ratio, CI: Confidence Interval.

Discussion

The study is one of the few efforts on the replication of the previously identified genetic variants in OA. Advantages of the current study include using a genetically homogeneous population and advanced OA cases. A smaller population genetic heterogeneity and the use of advanced OA cases are believed to increase the study power in OA genetic association studies. This is because advanced OA cases are expected to carry more risk alleles, and population homogeneity is thought to reduce genetic diversity and make it easier to identify the specific SNPs associated with complex traits.

Although I was unable to replicate any of the SNPs that were previously reported to be associated with OA with a stringent significance level, two SNPs showed a potential to be associated with OA. One is rs2292995 located in *COL9A3*, which is a gene coding for the structural components of the articular cartilage. The gene has been associated with multiple epiphyseal dysplasia type 3 [200], and primary OA [201]. The second SNP is rs1049007 located in *BMP2*, which is involved in TGF-β signalling pathway that has been implicated in OA [175]. Because the associations for these SNPs did not reach the statistical significance after the Bonferroni correction was applied, they warrant further investigation in a larger cohort.

The join specific analyses were performed to replicate potential joint specific associations. Considering the small sample size in this analysis, the results should be regarded as exploratory. The two replicated SNPs from the *BMP2,* and *COL9A3* genes were present among those SNPs reaching the threshold of alpha<0.05 but in different joints. This shows that the association of these genes with OA can be joint specific, and the future replication studies may rather be performed on the individuals affected with the respective joints only.

SNP rs143383 located in *GDF5* gene is by far the most replicated SNP that is associated with OA. It was initially discovered in the Asian population [147], followed by replication studies in European populations [149], and several meta-analyses confirmation [202, 203]. The SNP was not included in the Illumina GWAS genotyping platform, but a proxy SNP, rs224329, which has r2=0.92 with rs143383 was used instead. However, I could not detect any association between rs224329 and OA. Small sample size in the current study might be a possible explanation, but the results are the same as in the recent large GWAS on OA performed in the UK population [204], in which over 7,410 OA cases and 11,009 unrelated population controls were included. The NL population is shown to have ancestry admixture of almost entirely British and Irish populations, and its genetic structure resembles those populations [205]. Considering the genetic similarity between the NL and British populations may suggest that the *GDF5*-OA association is population specific.

Data on the replication of previously reported OA-associated genes are limited. GOAL study [206] utilized a large number of symptomatic radiographic knee or hip OA and controls to replicate 68 variants in 12 genes including *IL1A, IL1B, IL1RN, IL4R, IL6, COL2A1, ADAM12, ASPN, IGF1, TGFB1, ESR1,* and *VDR*, but they did not replicate any of these associations. All the genetic variants they studied were included in this study. Similarly, these SNPs were not associated with OA in this sample; neither did they in the large genome-wide meta-analysis [207].

Failure in the replication of previously reported genetic associations is common to the point that less than 5% of the reported associations could be replicated in an independent study [208], which along with other factors has raised criticism on the usefulness of association studies [209]. The reasons for the failures are numerous: initially reported associations might be false positives

or replications might be false negative findings resulting from a biased sampling, hidden or uncorrected population stratification, small sample sizes. The association might only hold true for the population in which the association was reported due to the differences and specificity of LD patterns across different populations. The complexity of multifactorial traits is another issue. The effect of multiple genetic variants and heterogeneity means that the presence of all of the risk alleles together may not be required for the disease to develop; therefore, distribution of risk alleles in two groups of cases from different or even the same population might be different from each other. All these factors are aggravated in the study of OA due to the age dependency and the lack of a unique method for the ascertainment of the study subjects used by different investigators.

The reasons mentioned above could partially explain why it was not possible to replicate the examined SNPs in this study. Also, this study has been limited by some factors: sample size is relatively small. Considering this sample size and assuming minor allele frequency of 35% in controls, the study has 80% power only to detect an OR of 1.8 or above at an alpha level of 0.05. The minimum detectable OR would be 2.5 if the significance level were defined at 0.0007 after taking into account multiple testing. However, the study power is maximized by the optimal case-control ratio and using extreme severe OA cases. It was recently shown that NL population has slightly subtle population stratification [205], which might lead to false negatives.

In conclusion, we were unable to replicate the previously reported OA-associated genes, but two SNPs showed suggestive associations with plausible biological mechanisms. Further studies are needed to test this hypothesis.

3

SMAD3 is associated with the total burden of radiographic osteoarthritis: the Chingford study

Erfan Aref-Eshghi[1], Yuhua Zhang[1], Deborah Hart[2], Ana M. Valdes[2], Andrew Furey[3], Glynn Martin[3], Guang Sun[4], Proton Rahman[4], Nigel Arden[5], Tim D Spector[2], Guangju Zhai[1,2]

[1]Discipline of Genetics, [3]Division of Orthopaedics, [4]Discipline of Medicine, Faculty of Medicine Memorial University of Newfoundland, NL, Canada

[2]Department of Twin Research & Genetic Epidemiology, King's College London, UK

[5]Musculoskeletal Epidemiology and Biobank, University of Oxford, UK

A version of this chapter was published in:
PLoS ONE 2014; 9(5): e97786.
doi: 10.1371/journal.pone.0097786

Abstract

Background: A newly described syndrome called Aneurysm-Osteoarthritis Syndrome (AOS) was recently reported. AOS presents with early-onset osteoarthritis (OA) in multiple joints, together with aneurysms in major arteries, and is caused by rare mutations in *SMAD3*. Because of the similarity of AOS to idiopathic generalized OA (GOA), I hypothesized that *SMAD3* is also associated with GOA and tested the hypothesis in a population-based cohort.

Methods: Study participants were derived from the Chingford study. Kellgren-Lawrence (KL) grades and the individual features of osteophytes and joint space narrowing (JSN) were scored from radiographs of hands, knees, hips, and lumbar spines. The total KL score, osteophyte score, and JSN score were calculated and used as indicators of the total burden of radiographic OA. Forty-one common SNPs within *SMAD3* were genotyped using the Illumina HumanHap610Q array. Linear regression modelling was used to test the association between the total KL score, osteophyte score, and JSN score and each of the 41 SNPs, with adjustment for patient age and BMI. Permutation testing was used to control the false positive rate.

Results: A total of 609 individuals were included in the analysis. All were Caucasian females with a mean age of 60.9 ± 5.8. I found that rs3825977, with a minor allele (T) frequency of 20%, in the last intron of *SMAD3*, was significantly associated with total KL score (β= 0.14, Ppermutation= 0.002). This association was stronger for the total JSN score (β= 0.19, Ppermutation= 0.002) than for total osteophyte score (β= 0.11, Ppermutation= 0.02). The T allele

is associated with a 1.47-fold increased odds for people with 5 or more joints to be affected by radiographic OA (Ppermutation= 0.046).

Conclusion: I found that *SMAD3* is significantly associated with the total burden of radiographic OA. Further studies are required to reveal the mechanism of the association.

Introduction

Osteoarthritis (OA) is the most common form of arthritis in the elderly, characterized pathologically by focal areas of damage to the articular cartilage centered on load-bearing joints. It is associated with the new bone formation at the joint margins (osteophytes), changes in the subchondral bone, variable degrees of mild synovitis, and thickening of the joint capsule [1] which lead to the presentation of pain, stiffness, and disability. Its prevalence—already high—is increasing due to population aging and the increase in obesity. Eighty percent of individuals over 75 years of age have radiographic OA changes in at least one of their joints [76]. According to a report from the Arthritis Community Research & Evaluation Unit in April 2010, the prevalence of self-reported and physician-diagnosed OA in individuals over age 45 ranged from 2.3%-11% in the low/middle-income countries to 8%-16% in the USA [70]. In the same year, it affected 27 million people in the USA, imposing a burden of over 11 million dollars on outpatient visits and over 13 billion dollars on OA-related job absence [77]. Half of all adults will develop symptomatic OA of the knee at some points in their lives [210].

OA is a multifactorial disease whose etiology is incompletely understood. It is believed that a number of different environmental and genetic factors interact in its initiation and progression [189]. Evidence suggests that genetic factors play a major role in OA, although they may be site- and sex- specific. From twin studies, this genetic influence has been estimated to be between 40% and 65% on hand and knee OA [192]. First-degree relatives of individuals with spine, hand, hip, or polyarticular OA have a two- to three-fold increased risk of the disease [193, 194]. The nature of the genetic influence in OA is still unclear, but it is likely to involve a combination of effects on structure (i.e. collagen), alterations in cartilage, bone metabolism, and inflammation [211]. Although the genetic influence on OA was recognized more than 130 years ago [131], genetic variants identified so far account only for a small fraction of its heritability [212]. This may reflect several factors including the heterogeneous nature of the disease, the tendency to use less severe phenotypes in genetic searches and the reliance on underpowered studies [213]. Generalized OA—a subtype of primary OA—is characterized by the involvement of multiple joints, and is believed to have a stronger genetic component than individual joint OA [214]. However, genetic data on generalized OA are limited.

Recently, a new syndrome called Aneurysm-Osteoarthritis Syndrome (AOS) was reported [179]. Patients with AOS present with early-onset OA affecting multiple joints including feet/ankle, hand/wrist, knee, hip, facet joints, uncovertebral joints and also exhibit degeneration of the intervertebral discs [179, 180]. Eight rare mutations in the *SMAD3* gene (Similar to Mothers Against Decapentaplegic type 3) were identified as responsible for AOS in eight unrelated families. Subsequent studies reported additional *SMAD3* mutations [215, 216] and also a CNV (copy number variant) [217] linked to AOS. The *SMAD3* gene encodes a protein that belongs to the SMAD protein family, that are downstream mediators of the transforming growth factor beta

(*TGF-β*) signalling pathway [218], which inhibits terminal hypertrophic differentiation of chondrocytes and is essential for maintaining the integrity of articular cartilage [218, 219]. This regulatory pathway also stimulates osteogenesis and bone formation [220].

SMAD3 knock-out mice develop a degenerative joint disease similar to human OA [221]. Although a few studies on *SMAD3* and single-joint OA have been reported, no data are available regarding the role *SMAD3* plays in generalized OA. Because of the similarity with AOS, in which multiple joints are also affected, I hypothesized that the *SMAD3* gene played a role in idiopathic generalized OA. I tested this hypothesis in a large population-based cohort of individuals who had a radiographic assessment of multiple joints.

Methods and Subjects

Subjects

The study subjects were women aged 43-67 years at baseline (1988–1989) who were participating in the Chingford Study, a prospective population-based study of OA and osteoporosis. The Chingford Study cohort comprises 1,003 women derived from the register of an extensive general practice in North London, who are similar to the UK population for most demographic variables [222].

Height, weight, and details of concomitant diseases, operations and medications were recorded for all subjects. DNA was extracted from blood by standard phenol or salting-out methods. At both baseline and ten years later, all subjects completed a standardized medical history questionnaire.

Ethics

The Guys & St Thomas' Trust and the Waltham Forest Trust ethics committees approved the Chingford study protocol. Written consent was obtained from all participants. The current study was a secondary analysis of de-identified data from the Chingford Study; thus, no further ethics approval was needed.

Radiography

Plain films of all joints were obtained from a standard posteroanterior view at baseline and again 9-11 years later. The distal interphalangeal (DIP), proximal interphalangeal (PIP) and first carpometacarpal (CMC) joints of the thumbs, the knee- and hip-joints, as well as four lumbar spinal joints (L1-L5), were assessed for radiographic OA according to the Kellgren & Lawrence (KL) score using a 0-4 scale [223]. Joint Space Narrowing (JSN) and osteophyte characteristics were each scored on a 0-3 scale using a standard atlas [224]. All radiographs were independently assessed by two trained observers (DJ Hunter and DJ Hart). In cases of disagreement, a third adjudicator was used. The intra- and inter-observer reproducibility of the scoring measured on a subgroup of 50 hands had a Kappa statistic of approximately 0.68 for all sites and features.

For the current study, the most recent radiographic and demographic data were used, including cross-sectional radiographic data for hip, spine, knee and hand from years 8, 9, 10, and 11 and the age and body mass index (BMI) from the 8th year of the study. All patients were visited at year 8 when the demographic information was collected. Due to the schedule of the radiology department, different joints were assessed in different years (between year 8 and year 11). Total KL score, osteophyte, and JSN scores were used as indicators of the total burden of radiographic OA, which was calculated by summing up the individual scores of each joint. Total radiographic

scores have been used by researchers in clinical, biomedical, and genetic studies of OA [214, 225] as an indicator of the total burden of OA. In addition, individuals were evaluated for the criteria required for a diagnosis of generalized OA. To this end, joints were defined as being affected by OA if the KL score was ≥2. OA of either the DIP or PIP joint groups was defined as the presence of OA in at least two of the relevant joints. A diagnosis of GOA was based on the definition used by Cooper *et al*. [17]. Fourteen joints or joint groups were considered: the four lumbar joints together with the left and right knee, hip, DIP group, PIP group and thumb CMCs. GOA was defined as the presence of OA in at least five of these 14 joints. Those with fewer than five joints affected were designated as controls.

Genotyping

The samples were genotyped using the Illumina HumanHap610Q array. The normalized intensity data was used by the Illuminus calling algorithm [226] to assign genotypes. No calls were assigned if an individual's most likely genotype was called with a posterior probability threshold of less than 0.95. Sample exclusion criteria were: (i) sample call rate <98%, (ii) heterozygosity across all SNPs ≥2 s.d. from the sample mean; (iii) evidence of non-European ancestry as assessed by PCA comparison with HapMap3 populations; (iv) observed pair-wise IBD probabilities suggestive of sample identity errors; (v) SNP exclusion criteria included (i) Hardy-Weinberg p-value<10^{-6}, assessed in a set of unrelated samples; (ii) MAF<1%, assessed in a set of unrelated samples; (iii) SNP call rate <97% (SNPs with MAF≥5%) or <99% (for 1%≤MAF<5%). For the current study, I retrieved genotype data for all 41 SNPs within the *SMAD3* gene which were available on the array after consideration of exclusion criteria.

Statistics

Since the distribution of the total KL, JSN, and osteophyte scores was skewed, a logarithmic transformation was performed to approximate a normal distribution. Subsequent analyses were performed on the log-transformed values. A linear regression model, testing for an additive genetic model, was used to test the association between each of the 41 candidate SNPs and the total KL, JSN, and osteophyte scores individually. A logistic regression model was used to test the association between each of the 41 SNPs and the total radiographic scores. Potential confounders such as age and BMI were considered in both models. All SNP associations with $p<0.05$ in the initial analyses were subject to permutation testing to control the false positive rate. The permutation method is well established as a robust approach for obtaining empirical significance levels while minimizing Type I errors [227, 228], and has been used to correct for multiple testing in genetic association studies [229]. Because of the infinite permutation with this study's sample size, a Monte Carlo permutation procedure was used, and the phenotype labels were reshuffled 10,000 times. The permutation-based p-value was calculated as the proportion of the statistic on all the reshuffled data sets greater than the observed statistic [229]. In permutation testing, it is assumed that if the first data is true, the shuffled data should also be true. The generated permutation p-value represents the probability of deviation from this assumption. This significance level was defined as a permutation-based p-value of less than 0.05 in this study. All analyses were conducted using STATA/SE 11.2 (Stata Corp, College Station, Texas, USA).

Results

All study subjects were Caucasian females. Radiographic data for spine, hips, knees and hand joints were available for 796, 794, 614, and 687 individuals, respectively. The age and BMI data were available for 843 participants with a mean age of 61.2 ± 5.8 and a mean BMI of 26.7 ± 4.7. Total KL, osteophyte, and JSN scores were available for 609, 603, and 607 individuals respectively. As expected, patients with GOA—defined as having 5 or more joints affected—were, on average, older than those with fewer than five affected joints, and also had a higher BMI (Table 3.1). The frequency of subjects with a different number of affected joints is presented in Table 3.2.

Forty-one common SNPs within the *SMAD3* gene were genotyped and passed quality control. They were scattered randomly throughout the *SMAD3* gene, but none was located in exons (Figure 3.1). The average pairwise R^2 between SNPs was 0.07.

I found that SNP rs3825977 was significantly associated with all phenotypes analyzed, *viz.* total KL, osteophyte and JSN scores. The differences in these traits among individuals with different genotypes are presented in Figures 3.2-3.4.

Table 3.1- Descriptive statistics of the study population

	GOA (n=247)	**Controls (n=360)**	**P-Value**
Age	64.21±0.34	58.71±0.3	P<0.0001
BMI	27.50±0.26	26.02±0.2	P<0.0001

Figures are mean ± SD, and Student's T-test was used for the comparison

Table 3.2- Frequency of patients with a different number of joints affected.

Number of joints affected	**Frequency (%)**
0	43 (7.06%)
1	62 (10.18%)
2	83 (13.63%)
3	93 (15.27%)
4	80 (13.14%)
5	51 (8.37%)
6	64 (10.51%)
7	38 (6.24%)
8	36 (5.91%)
9	22 (3.61%)
10	15 (2.46%)
11	12 (1.97%)
12	9 (1.48%)
13	1 (0.16%)
Total	609 (100%)

Figure 3.1- Distribution and LD pattern of 41 genotyped SNPs in *SMAD3* gene

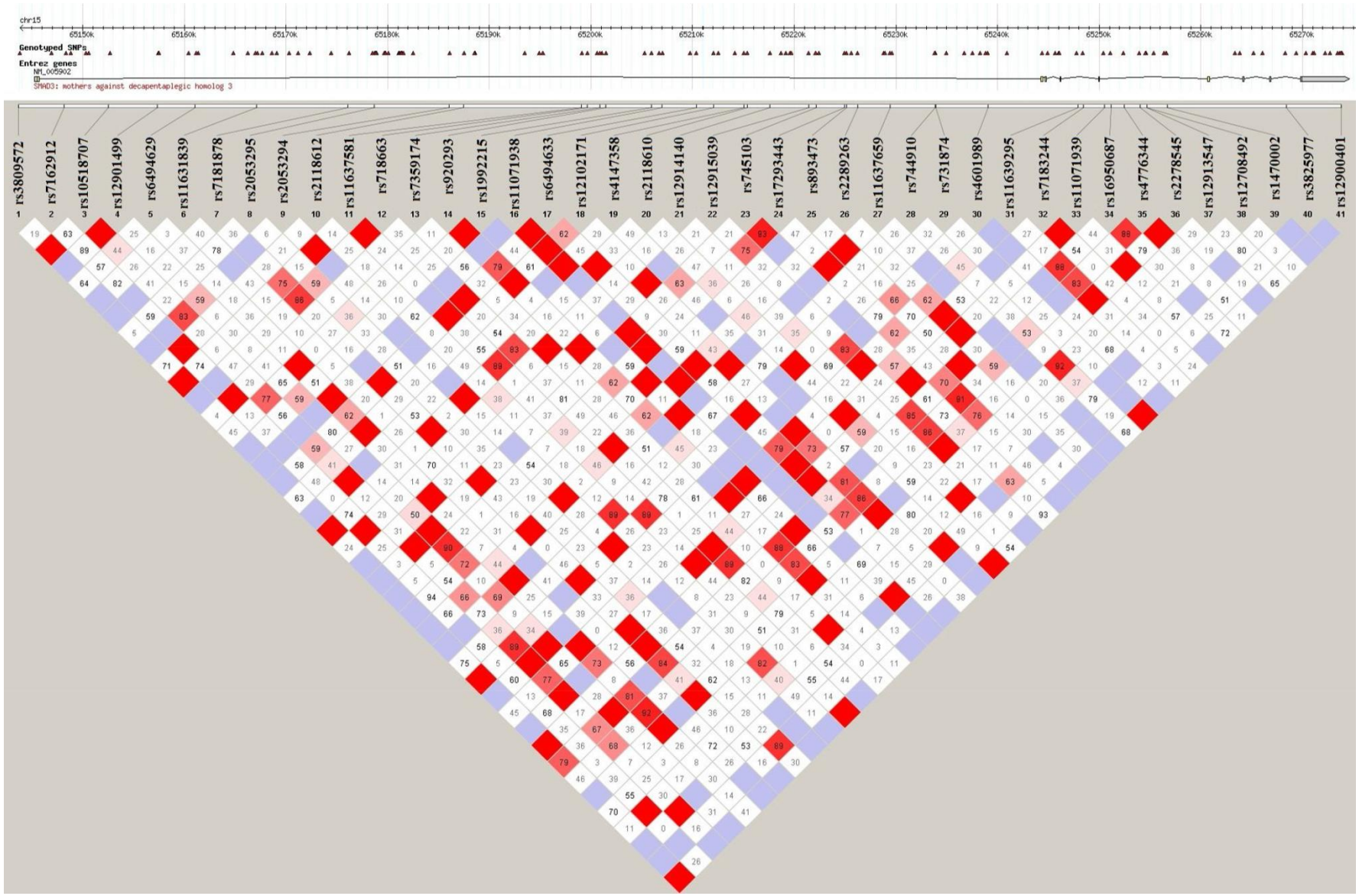
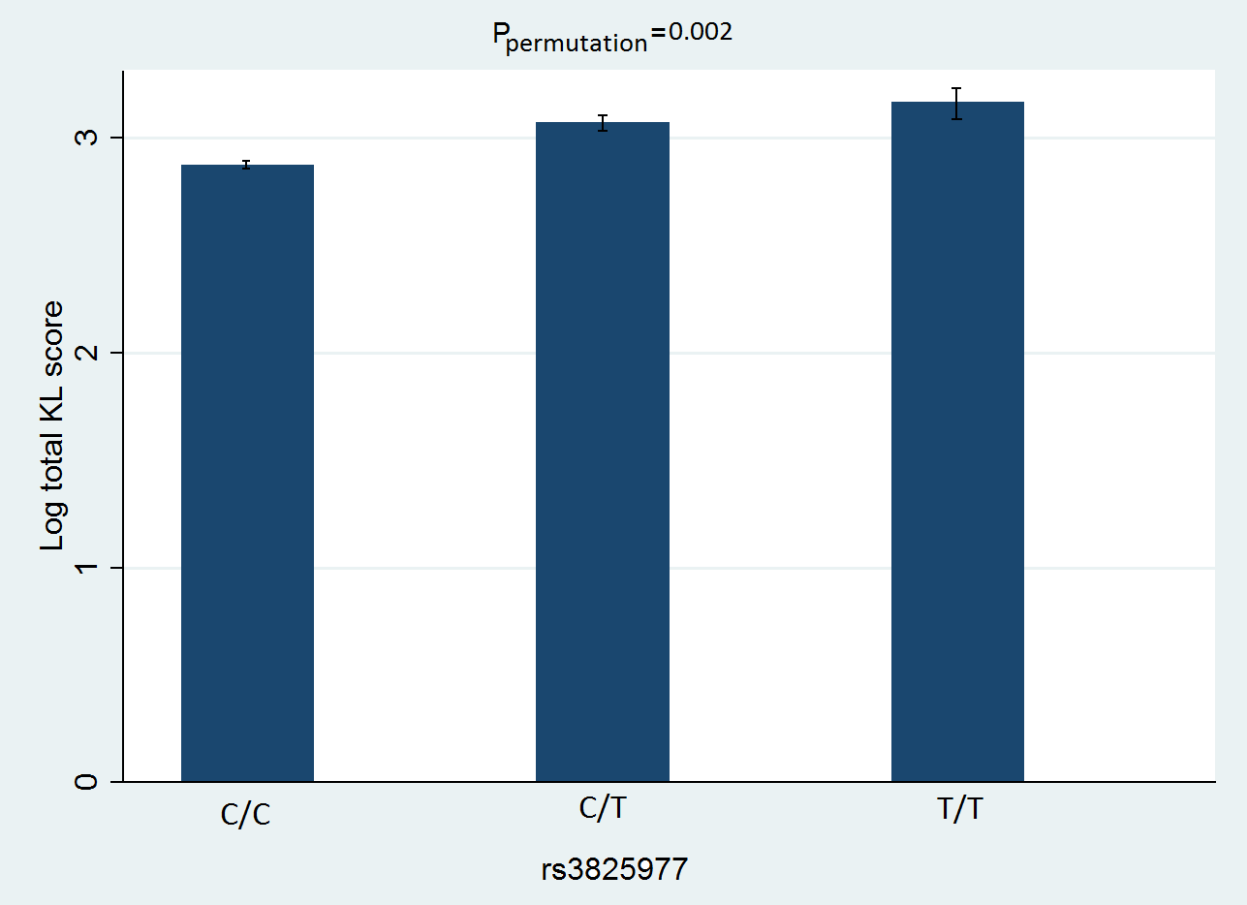
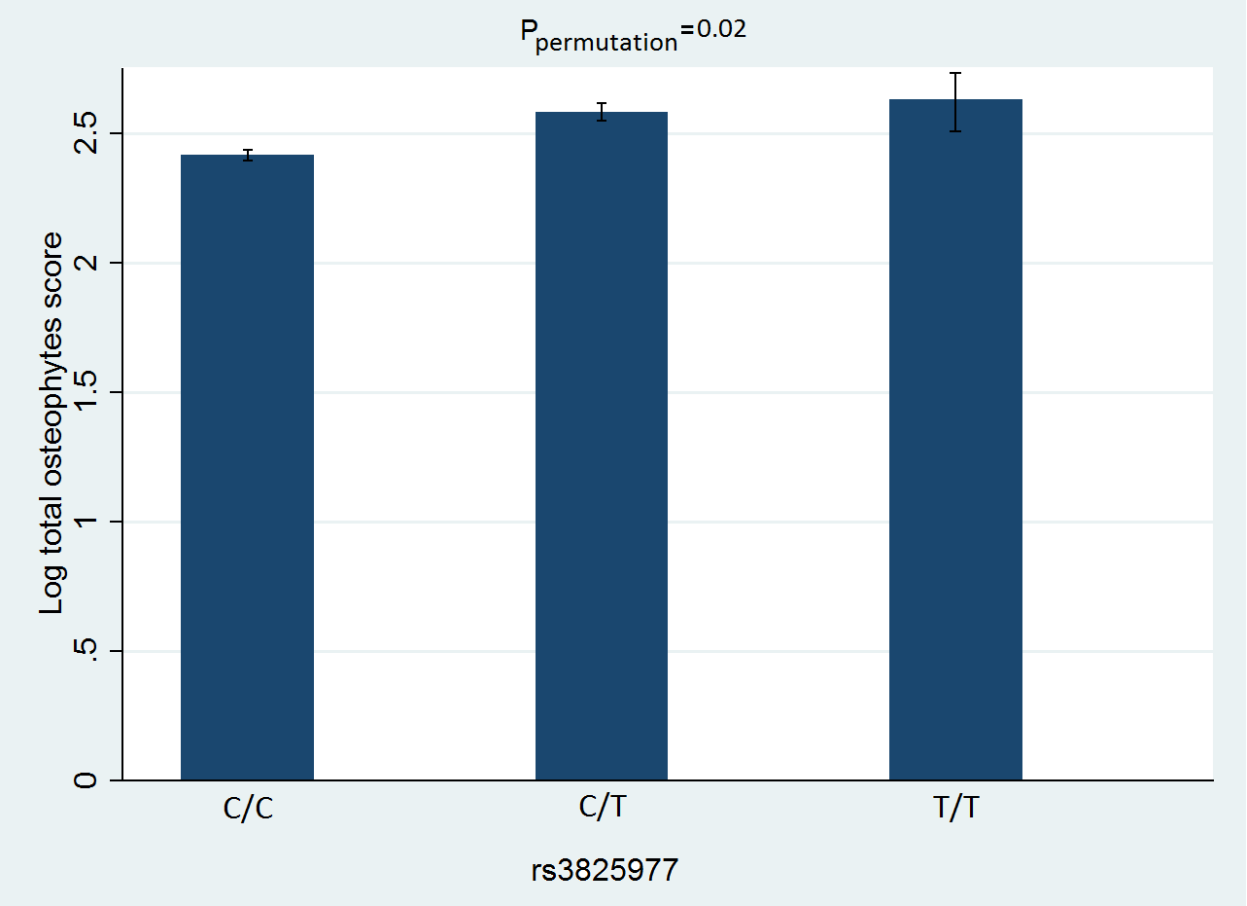


Figure 3.2- Total KL and genotypes of rs3825977



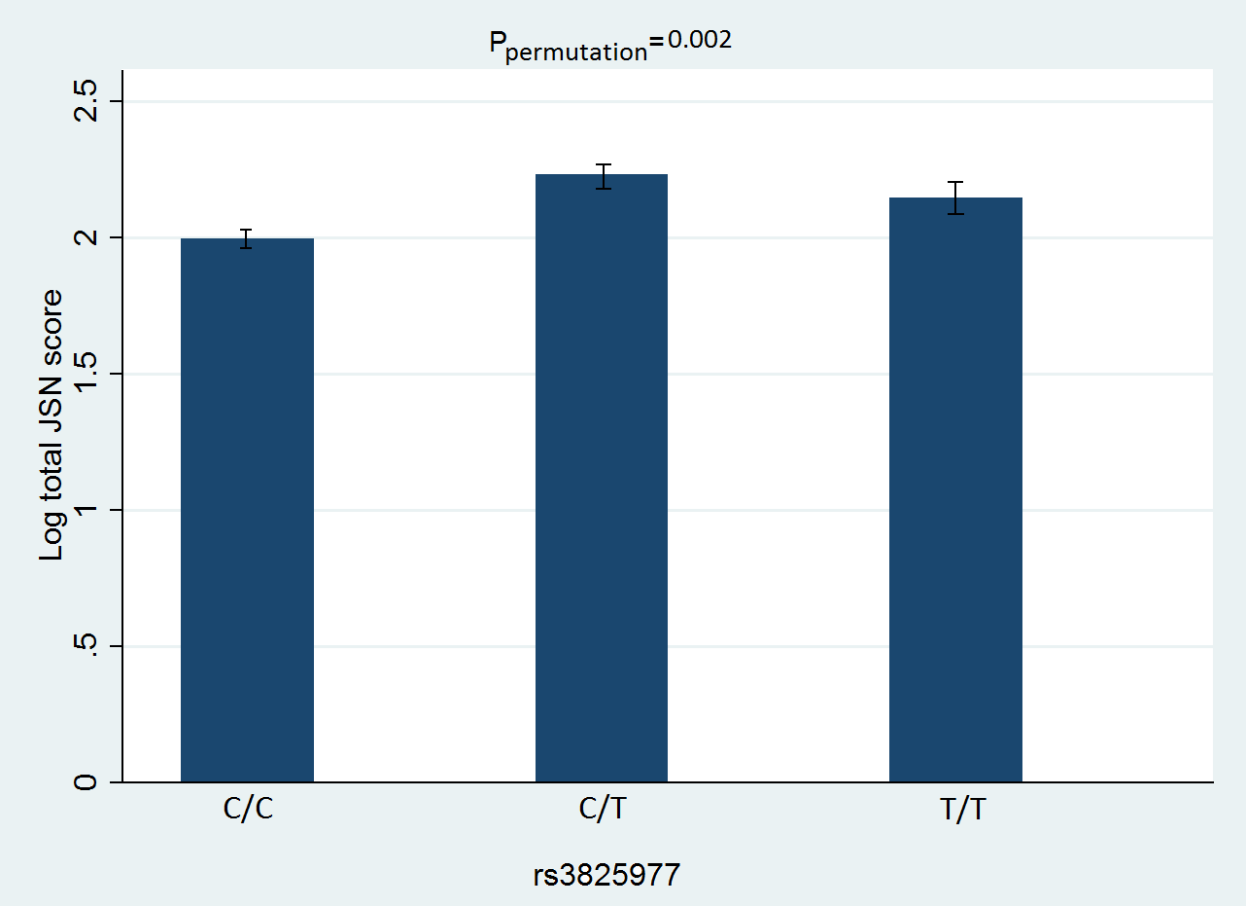
Error bars indicate Standard Error of the mean

Figure 3.3- Total osteophyte and genotypes of rs3825977



Error bars indicate Standard Error of the mean

Figure 3.4- Total JSN and genotypes of rs3825977



Error bars indicate Standard Error of the mean

After adjustment for age and BMI, the minor (T) allele of rs3825977—with 20% allele frequency—was associated with a 0.14 increase in log total KL score (95% CI 0.04-0.20, $P_{permutation}$= 0.002). The association is stronger for log total JSN score with β = 0.19 (95%CI 0.07-0.31, $P_{permutation}$= 0.002) than for log total osteophyte score with β= 0.11 (95%CI 0.01-0.20, $P_{permutation}$= 0.02). Two other SNPs—rs6494629 and rs2118612—were significant for only total osteophyte score in the univariate analysis but not in a multivariate analysis. All the results of univariate and multivariate linear regression analyses for total KL, osteophyte and JSN scores for all 41 SNPs are presented in Tables 3.3-3.5, respectively.

Table 3.3- Univariate and multivariate linear regression for total KL score and each SNP

	Univariate Analysis		Multivariate Analysis	
SNP	**Coefficient (95% CI)**	**P-value**	**Coefficient (95% CI)**	**P-value**
rs3825977	0.178 (0.068 - 0.288)	0.002	0.143 (0.048 - 0.238)	0.003
rs6494629	-0.085 (-0.171 - 0.001)	0.053	-0.051 (-0.125 - 0.023)	0.179
rs745103	-0.052 (-0.137 - 0.034)	0.237	-0.05 (-0.123 - 0.024)	0.183
rs718663	-0.069 (-0.274 - 0.137)	0.513	-0.102 (-0.278 - 0.075)	0.259
rs11637659	-0.054 (-0.166 - 0.059)	0.351	-0.058 (-0.155 - 0.039)	0.241
rs2118612	-0.091 (-0.206 - 0.024)	0.119	-0.058 (-0.157 - 0.041)	0.253
rs16950687	0.044 (-0.051 - 0.14)	0.358	0.039 (-0.043 - 0.121)	0.348
rs11631839	0.05 (-0.037 - 0.136)	0.257	0.034 (-0.04 - 0.109)	0.365
rs12900401	-0.115 (-0.318 - 0.088)	0.266	-0.079 (-0.254 - 0.096)	0.374
rs893473	-0.06 (-0.176 - 0.056)	0.307	-0.043 (-0.143 - 0.057)	0.400
rs744910	-0.027 (-0.115 - 0.061)	0.547	-0.028 (-0.104 - 0.048)	0.477
rs1470002	0.069 (-0.023 - 0.16)	0.143	0.026 (-0.053 - 0.106)	0.513
rs2278545	-0.042 (-0.188 - 0.103)	0.567	-0.04 (-0.165 - 0.086)	0.535
rs11071939	-0.082 (-0.245 - 0.081)	0.323	-0.047 (-0.187 - 0.093)	0.513
rs2118610	0.016 (-0.07 - 0.103)	0.711	0.023 (-0.053 - 0.098)	0.558
rs11637581	0.012 (-0.08 - 0.104)	0.799	0.025 (-0.055 - 0.104)	0.537
rs2053295	0.068 (-0.063 - 0.2)	0.307	0.036 (-0.078 - 0.149)	0.537
rs12914140	-0.056 (-0.231 - 0.118)	0.525	-0.051 (-0.201 - 0.099)	0.506
rs920293	0.042 (-0.101 - 0.186)	0.562	0.037 (-0.088 - 0.161)	0.563
rs4601989	0.055 (-0.052 - 0.163)	0.314	0.026 (-0.067 - 0.119)	0.585

rs6494633	0.015 (-0.072 - 0.102)	0.742	0.022 (-0.054 - 0.098)	0.566
rs2053294	0.059 (-0.073 - 0.191)	0.382	0.031 (-0.083 - 0.145)	0.595
rs12915039	0.031 (-0.075 - 0.138)	0.563	0.024 (-0.068 - 0.116)	0.608
rs4147358	-0.037 (-0.145 - 0.071)	0.503	-0.021 (-0.115 - 0.072)	0.654
rs7183244	-0.033 (-0.12 - 0.054)	0.455	-0.018 (-0.093 - 0.057)	0.645
rs7359174	-0.078 (-0.217 - 0.06)	0.267	-0.025 (-0.144 - 0.094)	0.679
rs2289263	0.04 (-0.044 - 0.123)	0.352	0.013 (-0.059 - 0.086)	0.723
rs17293443	-0.007 (-0.11 - 0.096)	0.897	-0.016 (-0.105 - 0.072)	0.722
rs3809572	-0.008 (-0.141 - 0.124)	0.900	-0.02 (-0.134 - 0.095)	0.734
rs12913547	0.08 (-0.029 - 0.189)	0.151	0.017 (-0.078 - 0.111)	0.732
rs7181878	-0.038 (-0.124 - 0.049)	0.390	-0.011 (-0.086 - 0.064)	0.772
rs11071938	0.007 (-0.087 - 0.100)	0.884	-0.01 (-0.091 - 0.071)	0.803
rs11639295	0.016 (-0.080 - 0.112)	0.748	-0.011 (-0.094 - 0.072)	0.795
rs1992215	0.011 (-0.083 - 0.104)	0.825	-0.009 (-0.09 - 0.072)	0.82
rs12901499	0.001 (-0.087 - 0.088)	0.989	-0.006 (-0.081 - 0.07)	0.886
rs731874	-0.02 (-0.110 - 0.070)	0.658	0.005 (-0.074 - 0.083)	0.907
rs12708492	-0.001 (-0.091 - 0.088)	0.974	0.004 (-0.073 - 0.081)	0.912
rs4776344	-0.04 (-0.172 - 0.091)	0.547	-0.004 (-0.118 - 0.11)	0.945
rs7162912	-0.008 (-0.103 - 0.088)	0.876	0.002 (-0.08 - 0.084)	0.962
rs12102171	-0.005 (-0.123 - 0.112)	0.932	0.002 (-0.1 - 0.103)	0.976
rs10518707	0.01 (-0.078 - 0.098)	0.819	-0.001 (-0.077 - 0.075)	0.981

N: Number of individuals with genotyping data for each SNP, CI: Confidence Interval

Table 3.4- Univariate and multivariate linear regression for total osteophytes score and each SNP

	Univariate Analysis		**Multivariate Analysis**	
SNP	**Coefficient (95% CI)**	**P-value**	**Coefficient (95% CI)**	**P-value**
rs3825977	0.143 (0.031 - 0.255)	0.012	0.106 (0.01 - 0.201)	0.03
rs2118612	-0.119 (-0.235 - -0.003)	0.044	-0.089 (-0.188 - 0.01)	0.078
rs6494629	-0.094 (-0.181 - -0.007)	0.034	-0.062 (-0.137 - 0.012)	0.100
rs12914140	-0.103 (-0.28 - 0.074)	0.253	-0.101 (-0.252 - 0.049)	0.187
rs16950687	0.048 (-0.049 - 0.144)	0.333	0.044 (-0.038 - 0.126)	0.296
rs3809572	-0.046 (-0.18 - 0.088)	0.499	-0.058 (-0.172 - 0.056)	0.320
rs11637581	0.023 (-0.071 - 0.116)	0.635	0.034 (-0.046 - 0.113)	0.405
rs2053295	0.083 (-0.05 - 0.215)	0.222	0.047 (-0.066 - 0.161)	0.411
rs2053294	0.076 (-0.058 - 0.209)	0.268	0.045 (-0.069 - 0.16)	0.435
rs7359174	-0.1 (-0.24 - 0.039)	0.159	-0.047 (-0.166 - 0.072)	0.436
rs11631839	0.042 (-0.046 - 0.129)	0.350	0.029 (-0.045 - 0.104)	0.442
rs745103	-0.028 (-0.115 - 0.058)	0.520	-0.029 (-0.102 - 0.045)	0.444
rs11637659	-0.024 (-0.138 - 0.09)	0.683	-0.033 (-0.13 - 0.064)	0.501
rs12900401	-0.116 (-0.323 - 0.091)	0.270	-0.06 (-0.236 - 0.117)	0.507
rs7183244	-0.038 (-0.126 - 0.051)	0.403	-0.022 (-0.097 - 0.053)	0.569
rs12708492	0.018 (-0.072 - 0.108)	0.691	0.023 (-0.053 - 0.1)	0.549
rs1470002	0.06 (-0.033 - 0.153)	0.208	0.021 (-0.058 - 0.101)	0.600
rs2278545	-0.031 (-0.179 - 0.117)	0.679	-0.033 (-0.158 - 0.093)	0.612
rs12901499	-0.014 (-0.102 - 0.075)	0.763	-0.021 (-0.097 - 0.054)	0.583
rs2118610	0.015 (-0.073 - 0.103)	0.737	0.019 (-0.056 - 0.095)	0.619

rs6494633	0.013 (-0.075 - 0.101)	0.768	0.019 (-0.057 - 0.095)	0.621
rs893473	-0.045 (-0.162 - 0.071)	0.446	-0.027 (-0.127 - 0.072)	0.589
rs7181878	-0.04 (-0.127 - 0.048)	0.376	-0.017 (-0.092 - 0.058)	0.664
rs718663	-0.004 (-0.21 - 0.202)	0.970	-0.035 (-0.21 - 0.14)	0.694
rs12913547	0.086 (-0.025 - 0.197)	0.127	0.02 (-0.075 - 0.115)	0.677
rs4601989	0.049 (-0.06 - 0.159)	0.374	0.02 (-0.073 - 0.113)	0.670
rs920293	0.031 (-0.115 - 0.176)	0.678	0.026 (-0.099 - 0.15)	0.686
rs10518707	-0.001 (-0.09 - 0.089)	0.986	-0.014 (-0.09 - 0.062)	0.724
rs1992215	0.002 (-0.093 - 0.097)	0.970	-0.014 (-0.095 - 0.067)	0.737
rs12102171	0.014 (-0.105 - 0.133)	0.822	0.017 (-0.084 - 0.118)	0.741
rs11071939	-0.056 (-0.221 - 0.108)	0.500	-0.024 (-0.164 - 0.116)	0.736
rs17293443	-0.002 (-0.107 - 0.103)	0.969	-0.013 (-0.102 - 0.076)	0.767
rs744910	-0.009 (-0.099 - 0.08)	0.837	-0.012 (-0.088 - 0.064)	0.757
rs2289263	0.036 (-0.049 - 0.121)	0.401	0.011 (-0.061 - 0.084)	0.759
rs11071938	0.001 (-0.094 - 0.096)	0.991	-0.012 (-0.094 - 0.069)	0.768
rs4147358	-0.023 (-0.132 - 0.086)	0.677	-0.01 (-0.103 - 0.082)	0.826
rs731874	-0.034 (-0.125 - 0.057)	0.465	-0.007 (-0.085 - 0.071)	0.861
rs7162912	-0.002 (-0.098 - 0.095)	0.972	0.007 (-0.075 - 0.09)	0.862
rs4776344	-0.042 (-0.175 - 0.091)	0.537	-0.009 (-0.123 - 0.104)	0.874
rs12915039	0.008 (-0.101 - 0.116)	0.891	0.006 (-0.087 - 0.099)	0.896
rs11639295	0.029 (-0.069 - 0.126)	0.563	0.001 (-0.082 - 0.084)	0.988

N: Number of individuals with genotyping data for each SNP, CI: Confidence Interval

Table 3.5- Univariate and multivariate linear regression for total JSN score and each SNP

	Univariate Analysis		Multivariable Analysis	
SNP	**Coefficient (95% CI)**	**P-value**	**Coefficient (95% CI)**	**P-value**
rs3825977	0.222 (0.094 , 0.35)	0.001	0.187 (0.066 , 0.309)	0.003
rs11637581	0.058 (-0.05 , 0.166)	0.292	0.068 (-0.033 , 0.17)	0.186
rs12914140	-0.124 (-0.327 , 0.08)	0.233	-0.126 (-0.317 , 0.066)	0.199
rs745103	-0.063 (-0.163 , 0.037)	0.215	-0.058 (-0.152 , 0.036)	0.227
rs12900401	-0.148 (-0.387 , 0.092)	0.226	-0.136 (-0.361 , 0.09)	0.237
rs2118612	-0.096 (-0.23 , 0.039)	0.163	-0.075 (-0.201 , 0.052)	0.246
rs17293443	-0.052 (-0.172 , 0.068)	0.394	-0.063 (-0.176 , 0.05)	0.276
rs4601989	0.084 (-0.042 , 0.21)	0.192	0.06 (-0.059 , 0.18)	0.319
rs1470002	0.09 (-0.017 , 0.197)	0.100	0.051 (-0.05 , 0.153)	0.322
rs7359174	-0.112 (-0.274 , 0.049)	0.172	-0.076 (-0.229 , 0.076)	0.326
rs2118610	0.034 (-0.067 , 0.135)	0.505	0.041 (-0.055 , 0.138)	0.398
rs6494633	0.032 (-0.07 , 0.133)	0.541	0.04 (-0.057 , 0.136)	0.420
rs4147358	-0.061 (-0.187 , 0.064)	0.338	-0.048 (-0.167 , 0.071)	0.430
rs12102171	-0.056 (-0.193 , 0.082)	0.426	-0.049 (-0.178 , 0.081)	0.460
rs12708492	0.034 (-0.07 , 0.138)	0.516	0.035 (-0.063 , 0.133)	0.482
rs731874	-0.059 (-0.165 , 0.046)	0.269	-0.036 (-0.136 , 0.065)	0.487
rs7162912	0.022 (-0.09 , 0.134)	0.698	0.037 (-0.069 , 0.142)	0.497
rs6494629	-0.047 (-0.148 , 0.053)	0.357	-0.029 (-0.125 , 0.066)	0.551
rs718663	-0.043 (-0.282 , 0.195)	0.722	-0.067 (-0.291 , 0.157)	0.557
rs2053295	0.055 (-0.098 , 0.209)	0.478	0.042 (-0.102 , 0.187)	0.566

rs11071939	-0.077 (-0.269 , 0.114)	0.426	-0.052 (-0.232 , 0.128)	0.568
rs2278545	0.037 (-0.133 , 0.208)	0.667	0.036 (-0.125 , 0.196)	0.661
rs2289263	0.044 (-0.053 , 0.142)	0.374	0.02 (-0.073 , 0.112)	0.677
rs2053294	0.036 (-0.119 , 0.192)	0.644	0.027 (-0.119 , 0.174)	0.715
rs12915039	0.031 (-0.094 , 0.156)	0.624	0.021 (-0.097 , 0.139)	0.726
rs11637659	-0.013 (-0.145 , 0.119)	0.849	-0.02 (-0.144 , 0.105)	0.755
rs16950687	0.023 (-0.089 , 0.134)	0.689	0.015 (-0.09 , 0.12)	0.779
rs744910	-0.008 (-0.111 , 0.095)	0.879	-0.013 (-0.11 , 0.085)	0.798
rs12913547	0.067 (-0.061 , 0.195)	0.307	0.015 (-0.106 , 0.136)	0.809
rs4776344	-0.013 (-0.167 , 0.142)	0.873	0.016 (-0.13 , 0.162)	0.83
rs11639295	0.034 (-0.079 , 0.147)	0.558	0.011 (-0.096 , 0.117)	0.843
rs1992215	0.009 (-0.101 , 0.118)	0.875	-0.01 (-0.114 , 0.093)	0.846
rs11071938	0.007 (-0.103 , 0.116)	0.905	-0.01 (-0.114 , 0.094)	0.850
rs7181878	-0.018 (-0.119 , 0.083)	0.728	-0.003 (-0.099 , 0.093)	0.948
rs893473	-0.014 (-0.149 , 0.121)	0.839	-0.004 (-0.131 , 0.123)	0.956
rs11631839	0.009 (-0.092 , 0.11)	0.860	-0.002 (-0.097 , 0.093)	0.968
rs7183244	-0.016 (-0.119 , 0.087)	0.758	0.002 (-0.096 , 0.099)	0.970
rs920293	-0.004 (-0.172 , 0.164)	0.965	0.003 (-0.156 , 0.162)	0.971
rs12901499	0 (-0.103 , 0.103)	0.998	-0.001 (-0.098 , 0.096)	0.977
rs3809572	0.004 (-0.151 , 0.16)	0.958	0.001 (-0.145 , 0.148)	0.985
rs10518707	0.006 (-0.098 , 0.109)	0.911	0.001 (-0.097 , 0.098)	0.988

N: Number of individuals with genotyping data for each SNP, CI: Confidence Interval

Furthermore, I categorized the study participants into two groups: one with ≥5 joints affected (GOA) and one with <5 joints affected and examined the association of each group with each of the 41 SNPs. I found that the T allele of rs3825977 was significantly associated with a 1.47-fold increased risk of GOA (95% CI 1.02-2.1, $P_{permutation}$= 0.046) after adjustment for age and BMI (Table 3.6). All results of the associations with each of the 41 SNPs are presented in Table 3.7.

Table 3.6- Association between GOA and rs3825977

	Multivariable Analysis			MAF	
Variables	**OR (95% CI)**	**P-value**	**$P_{permutation}$**	**Cases**	**Controls**
rs3825977 (T vs. C allele)	1.47 (1.02 - 2.1)	0.037	0.046	0.23	0.17
Age (per year)	1.20 (1.16 - 1.25)	<0.0001	-	-	-
BMI (per kg/m^2)	1.09 (1.04 - 1.15)	<0.0001	-	-	-

Logistic regression was used. MAF: Minor allele frequency, OR: Odds Ratio, CI: Confidence interval

Table 3.7- Univariate and multivariate logistic regression for GOA and each SNP

	Univariate Analysis		Multivariable Analysis		MAF	
SNP	**OR (95% CI)**	**P-value**	**OR (95% CI)**	**P-value**	**Controls**	**GOA**
rs3825977	1.48 (1.08 - 2.03)	0.016	1.47 (1.02 - 2.1)	0.037	0.17	0.23
rs12914140	0.62 (0.36 - 1.05)	0.076	0.53 (0.28 - 0.98)	0.044	0.08	0.05
rs11637581	1.2 (0.93 - 1.57)	0.166	1.36 (1.00 - 1.84)	0.050	0.28	0.32
rs745103	0.82 (0.64 - 1.05)	0.116	0.77 (0.58 - 1.02)	0.068	0.47	0.42
rs2118612	0.71 (0.5 - 0.99)	0.044	0.71 (0.48 - 1.05)	0.087	0.20	0.15
rs7359174	0.67 (0.44 - 1.01)	0.057	0.7 (0.44 - 1.14)	0.153	0.13	0.09
rs3809572	0.86 (0.59 - 1.26)	0.438	0.75 (0.48 - 1.18)	0.212	0.13	0.11
rs2278545	0.79 (0.51 - 1.2)	0.269	0.74 (0.45 - 1.2)	0.224	0.11	0.09
rs893473	0.8 (0.57 - 1.12)	0.197	0.79 (0.53 - 1.18)	0.247	0.19	0.16
rs2118610	1.1 (0.86 - 1.41)	0.451	1.18 (0.89 - 1.58)	0.250	0.48	0.51
rs11071938	0.93 (0.71 - 1.21)	0.579	0.83 (0.61 - 1.14)	0.254	0.31	0.30

rs6494633	1.09 (0.85 - 1.4)	0.496	1.18 (0.88 - 1.57)	0.269	0.48	0.50
rs11071939	1.14 (0.72 - 1.82)	0.568	1.34 (0.79 - 2.29)	0.279	0.07	0.08
rs1992215	0.94 (0.72 - 1.23)	0.659	0.84 (0.62 - 1.15)	0.285	0.31	0.30
rs6494629	0.8 (0.62 - 1.02)	0.075	0.86 (0.65 - 1.14)	0.300	0.51	0.45
rs17293443	0.92 (0.69 - 1.24)	0.600	0.87 (0.62 - 1.23)	0.434	0.24	0.23
rs7162912	0.91 (0.7 - 1.2)	0.521	0.9 (0.65 - 1.23)	0.492	0.37	0.35
rs744910	0.93 (0.72 - 1.2)	0.590	0.91 (0.68 - 1.21)	0.502	0.49	0.47
rs1470002	1.03 (0.8 - 1.34)	0.804	0.9 (0.66 - 1.22)	0.506	0.37	0.38
rs731874	0.99 (0.77 - 1.29)	0.96	1.1 (0.82 - 1.48)	0.524	0.30	0.30
rs12900401	0.76 (0.42 - 1.37)	0.358	0.8 (0.4 - 1.61)	0.536	0.06	0.04
rs11637659	0.93 (0.67 - 1.29)	0.665	0.89 (0.62 - 1.29)	0.551	0.19	0.18
rs2289263	1.02 (0.8 - 1.29)	0.893	0.92 (0.7 - 1.22)	0.573	0.47	0.48
rs12901499	0.98 (0.76 - 1.25)	0.846	0.92 (0.69 - 1.23)	0.576	0.48	0.47
rs11631839	1.11 (0.87 - 1.42)	0.414	1.07 (0.81 - 1.42)	0.629	0.46	0.49
rs10518707	1 (0.78 - 1.28)	0.993	0.93 (0.7 - 1.25)	0.637	0.48	0.48
rs718663	0.96 (0.53 - 1.74)	0.902	0.85 (0.43 - 1.69)	0.645	0.05	0.05
rs920293	1.11 (0.74 - 1.67)	0.625	1.11 (0.69 - 1.78)	0.664	0.09	0.10
rs4776344	0.95 (0.65 - 1.39)	0.806	1.07 (0.7 - 1.66)	0.749	0.13	0.13
rs7183244	0.91 (0.71 - 1.17)	0.463	0.95 (0.72 - 1.27)	0.754	0.41	0.39
rs2053294	1.14 (0.78 - 1.66)	0.503	1.06 (0.69 - 1.64)	0.794	0.12	0.13
rs12102171	1.02 (0.73 - 1.43)	0.905	1.04 (0.71 - 1.53)	0.845	0.16	0.16
rs11639295	1.11 (0.85 - 1.46)	0.447	1.03 (0.75 - 1.41)	0.851	0.29	0.31
rs7181878	0.94 (0.73 - 1.2)	0.612	1.02 (0.77 - 1.36)	0.867	0.50	0.48

rs4601989	1.07 (0.79 - 1.46)	0.653	0.97 (0.69 - 1.38)	0.881	0.2	0.22
rs4147358	0.95 (0.69 - 1.29)	0.733	0.98 (0.68 - 1.4)	0.895	0.21	0.2
rs12915039	1.01 (0.75 - 1.38)	0.925	0.98 (0.69 - 1.4)	0.911	0.22	0.22
rs16950687	1.03 (0.79 - 1.35)	0.827	1.02 (0.75 - 1.39)	0.911	0.28	0.28
rs12708492	0.98 (0.76 - 1.27)	0.905	0.99 (0.74 - 1.33)	0.958	0.48	0.48
rs12913547	1.19 (0.87 - 1.63)	0.266	0.99 (0.69 - 1.42)	0.965	0.19	0.22
rs2053295	1.11 (0.76 - 1.61)	0.582	1.01 (0.66 - 1.55)	0.965	0.13	0.14

OR: Odds Ratio, CI: Confidence Interval, MAF: Minor allele frequency

Discussion

In the present study, I demonstrate a significant association of SNP rs3825977—located in the last intron of *SMAD3*—with the total burden of radiographic OA. This SNP is more strongly associated with total JSN score than with total KL score or osteophyte score, suggesting that the potential mechanism for the association is more likely through cartilage loss rather than osteophyte formation. The same SNP has previously been reported as associated with increased breast cancer risk for *BRCA2* mutation carriers [230]. Although the possible effect of the SNP on *SMAD3* function is still unclear, it is believed that the effects on both breast cancer and generalized OA susceptibility are mediated through the *TGF-β* signalling pathway [231].

None of the other SNPs in this study were found to be associated with OA. This might be explained by the LD pattern of the gene as shown in figure 3.1. Since none of the tested SNPs are in high LD with each other, it can be presumed that the mechanism of association for the specific SNP identified here is independent of the other potential associated SNPs within the gene.

Data on the associations between the *SMAD3* gene and GOA are limited and, to my knowledge, no genetic or genome-wide association study has been performed on GOA. A study by Yao *et al.* [232] was the first to report a connection between *SMAD3* and OA. This paper described a missense mutation located in the linker region of the SMAD3 protein which resulted in an increased expression of matrix metalloproteinase (MMP) 2 and 9 in the serum of one OA mutation carrier compared to MMP expression in other OA patients and controls. Another study by A. Valdes and colleagues [233] reported the association of a variant in the *SMAD3* gene with hip and knee OA. In that study, the frequency of the major (G) allele of rs12901499—located in

the first intron of *SMAD3*—was increased in patients undergoing hip or knee replacement as compared to controls. A recent study by Jiang Liying *et al.* [234] found this SNP was also associated with hand and knee OA in a northeast Chinese population. However, I did not observe a significant association with rs12901499, which is not in LD with rs3825977 (R^2= 0.01). This may have resulted from the different methods used for the definition and classification of OA in this study and the previous studies which used either end-stage OA (requiring total joint replacement) or symptomatic OA, neither of which is necessarily concordant with radiographic OA [235]. Alternatively, one or both of these SNPs may be non-functional but rather in LD with causal variants in the gene that were not typed in these studies. It is possible that causal variants exist in the vicinity of rs3825977, and in fact, a query using HaploReg v4.1 database (BROAD institute, Cambridge, Massachusetts, US) found eight SNPs in the same region with an R^2>0.8 with rs3825977. Therefore, a fine mapping approach using the sequence data will likely determine the possible causative variant with the strongest association in the last intron of *SMAD3*. The database also predicted that the SNP makes a transcription factor binding motif change between AP-2 and EBF. The possible effect of this change on the gene regulation will have to be determined through functional studies.

Cartilage homeostasis depends on a balance between the catabolic and anabolic activities of chondrocytes being controlled by numerous cytokines and growth factors. *TGF-β* is an important molecule that plays a critical role in the development, growth, maintenance and repair of articular cartilage by modifying the metabolism of the chondrocyte. Deregulation of *TGF-β* signalling and responses have been shown to be involved in OA [176]. The SMAD family proteins, including SMAD3, are important intracellular signals in the *TGF-β* pathway [219]. Another possible mechanism by which SMAD3 acts to maintain cartilage homeostasis is by

inducing the expression of type II collagen and repressing *MMP-13*. A recent study by Chen and colleagues [236] showed that *SMAD3* (FL/FL) mice were severely deficient in both type II collagen and Aggrecan due to the proteolytic activity of *MMP-13*, which is usually down-regulated by *TGF-β* signals mediated through *SMAD3*.

There are some limitations in the study. All the participants were female, which limits the generalizability. Given its unknown function, it is not clear whether the associated SNP is causal. The SNP has to be tested in a replicating study to determine the reliability of this finding. The findings are merely obtained from radiographic data which does not necessarily represent the clinical presentations, and thus, the results may not be directly applicable to symptomatic OA.

Conclusions

I demonstrated that the *SMAD3* gene was associated with the total burden of radiographic OA. As a marker, it has a potential in identifying those with increased risk of OA, thus permitting earlier joint-preserving intervention. It also has potential as a molecular target for developing new OA drugs.

4

SMAD3 is up-regulated in human osteoarthritic cartilage independent of the promoter DNA methylation

Erfan Aref-Eshghi[1], Ming Liu[1], Seyd Babak Razavi-Lopez[2], Kensuke Hirasawa[2], Patricia E. Harper[1], Glynn Martin[3], Andrew Furey[3], Roger Green[1], Guang Sun[4], Proton Rahman[4], Guangju Zhai[1,5]

[1]Discipline of Genetics, [2]Division of Biomedical Science, [3]Division of Orthopedics,

[4]Disicpline of Medicine, Faculty of Medicine, Memorial University of Newfoundland, St. John's, NL, Canada

[5]Department of Twin Research & Genetic Epidemiology, King's College London, London, UK

A version of this chapter was published in:
J Rheumatol 2016; 43(2):388-94.
doi:10.3899/jrheum.150609

Abstract

***Objectives*:** To compare the *SMAD3* gene expression between human osteoarthritic and healthy cartilage and to examine whether the expression is regulated by the promoter DNA methylation of the gene.

Methods: Human cartilage samples were collected from patients undergoing total hip/knee joint replacement surgery due to primary osteoarthritis (OA) or hip fractures as controls. DNA/RNA was extracted from the cartilage tissues. Real-Time Quantitative PCR was performed to measure gene expression, and Sequenom's EpiTYPER was used to assay DNA methylation. Methylation and expression assays were used in the same population. Mann-Whitney U test was utilized to compare the methylation and expression levels between OA cases and controls. Spearman's rank correlation coefficient was calculated to examine the association between the methylation and gene expression.

Results: A total of 58 OA patients (22 males, 36 females; mean age 64±9 years) and 55 controls (12 males, 43 females; mean age 79±10 years) were included in the study. *SMAD3* was expressed on average 83% higher in OA cartilage than controls (p=0.0005). No difference was observed for the DNA methylation levels of the four CpG sites in the *SMAD3* promoter region between OA cases and controls. No correlation was found between the *SMAD3* expression and the promoter DNA methylation.

Conclusions: This study demonstrates that *SMAD3* is significantly over-expressed in OA. This over-expression, however, cannot be explained by the DNA methylation in the promoter segment that was studied. The results suggest that TGF-β/SMAD3 pathway may be over activated in OA cartilage and has potential in developing targeted therapies for OA.

Introduction

Osteoarthritis (OA), affecting 250 million people worldwide, is the most common form of arthritis [237]. It presents with joint pain, stiffness, joint deformity, and disability [185], and imposes a high socio-economic burden on societies [238]. Despite the high prevalence and socioeconomic burden, the pathogenesis of OA remains elusive [239, 240]. The evidence is accumulating to suggest that "Similar to Mothers Against Decapentaplegic type 3 (*SMAD3*) gene" plays a role in the development of OA [176].

SMAD3 is one of the important intracellular signal transducers of the transforming growth factor beta (TGF-β) signalling pathway, which is known to play a critical role in the development, homeostasis, and repair of the cartilage [241]. A lack of TGF-β/SMAD3 signalling activity is suggested to contribute to OA development. *SMAD3* deficient chondrocytes exhibit a decreased TGF-β activity and an enhanced inappropriate terminal maturation [242]. Mice overexpressing SMURF-2, an E3 ubiquitin ligase known to inhibit TGF-β signalling, spontaneously develop an OA-like phenotype and have decreased levels of SMAD3 phosphorylation [176]. Consistent with these findings is that the *SMAD3* knocked-out mice develop a degenerative joint disease similar to human OA [221]. These mice are severely deficient in both type II collagen and Aggrecan as a result of an increased proteolytic activity of matrix metalloproteinase 13 [236]. Aligned with

this, a patient with knee OA was found to have a missense mutation in the linker region of the SMAD3 protein and an elevated serum level of matrix metalloproteinase (MMP-2 and MMP-9) [232].

Eight missense point mutations in *SMAD3* [179, 180] have been reported to be the cause of the Aneurysm-Osteoarthritis Syndrome, a rare syndromic disease characterized with early-onset polyarticular OA, aneurysms of the main arteries, and several connective tissue disorders. A single nucleotide polymorphism (SNP) mapping to the first intron of *SMAD3* was reported to be involved in the risk of both hip and knee OA in European populations [233], and in a northeast Chinese population [234]. Recently, I found that an SNP located in the last intron of *SMAD3* was significantly associated with the total burden of radiographic OA [243]. Given the location of this SNP in the gene, it is more likely that the effect of the *SMAD3* on later-onset OA is regulatory. We, therefore, undertook this study to investigate whether gene expression of *SMAD3* is different between OA-affected and healthy cartilage and whether the different expression is due to the promoter DNA methylation changes.

Methods

Subjects

The study was part of the ongoing Newfoundland Osteoarthritis Study (NFOAS) that was initiated in 2011, aiming at identifying novel genetic, epigenetic, and biochemical markers for OA [244, 245]. OA patients were recruited from those who underwent total knee or hip joint replacement due to severe primary OA between November 2011 and December 2013 in St. Clare's Mercy Hospital and Health Science Centre General Hospital in St. John's, the capital city

of Newfoundland and Labrador (NL), Canada. Healthy controls were recruited in the same hospitals from those who underwent hemiarthroplasty of the hip due to hip fracture with no evidence of OA. OA diagnosis was made based on the American College of Rheumatology criteria [14, 48] and the judgement of the attending orthopaedic surgeons. The pathology report on the removed cartilage was reviewed for all subjects to ensure the accuracy of the diagnosis and the status of any cartilage degeneration in the controls. The consent rate of the study was 90%. The study protocol was approved by the Health Research Ethics Authority (HREA) of Newfoundland and Labrador (HREA11.311), and written consent was obtained from all the participants.

Demographics and anthropometrics

Demographic information was obtained by a self-administered questionnaire with the help of the research staff if necessary. Anthropometric data including height and weight was retrieved from their hospital admission charts and medical records, and body mass index (BMI) was calculated by dividing weight in kilograms by squared height in meters. Age was calculated at the time of the surgery.

DNA/RNA isolation

Four pieces (~200mg each) of cartilage tissues were retained from either tibial plateau or femoral heads during the surgery. The samples were then flash-frozen and stored in liquid nitrogen until the experiment. DNA and RNA were extracted from the same piece of the cartilage tissue to avoid sampling bias. Up to 200mg frozen cartilage tissue was transferred to the homogenizing cylinder together with 1 ml TRIzole lysis reagent and 200µl guanidine thiocyanate and homogenized using a cryogenic mill (Spex Freezer Mill, model 6770, Metuchen, New Jersey,

USA) with the following parameters: two cycles of 2 minutes grinding at maximum frequency with 10 minutes cooling down between grinding cycles. The homogenate was then transferred to a new 2ml RNase-free tube and incubated for 5 min at room temperature. Then, 200µl chloroform was added, and the mix was vortexed vigorously, before being incubated for 2-3 min, followed by centrifugation at 12,000xg at -4°C for 15 min. Following centrifugation, the sample separated into 3 phases: the aqueous phase containing RNA, the interphase, and the organic phase containing DNA. RNeasy Lipid Tissue Mini Kit (Qiagen, Venlo, the Netherlands) was then used for extracting total RNA from the aqueous phase according to the manufacturers' protocol. The DNA was extracted using Phenol-Chloroform method from the interphase and organic phase.

SMAD3 expression measurement

Complementary DNA (cDNA) synthesis from the extracted RNA was done using Maxima H Minus First Strand cDNA Synthesis Kit (K1682, Vilnius, Lithuania). One hundred ng of RNA from each sample primed with 0.5 µl of random primer was denatured at 65°C for 5 minutes and chilled on ice before addition of a reverse-transcription solution containing 2µl of 5x buffer, 0.5 µl Ribolock, 1 µl of 10mM dNTPs mix (Invitrogen, California, USA), and 0.5µl Maxima polymerase in a final volume of 20µl. The cDNA-synthesis reaction was performed at 42 °C for 60 minutes and followed by 5 minutes at 70°C. One µl of the converted cDNA was subject to quality control by PCR amplification of the *SMAD3* and *GAPDH* genes followed by agarose gel electrophoresis.

Expression quantification of *SMAD3* was performed using ABI 7900HT Fast Real-Time PCR System on 96-well plate. *GAPDH* was used as an internal reference gene to normalize the

relative quantification of the targeted gene - *SMAD3*. *GAPDH* and *SMAD3* amplification primers were designed using NCBI primer-blast tool for the shortest isoforms of the genes, and the sequences were blasted in NCBI BLAST tool to ensure 100% coverage of all of the isoforms as well as minor similarly to other genomic sequences. Primers were validated using a 4-point dilution series of two random cDNA samples. The primer efficiencies were found to be within the acceptable range, i.e. 106% and 110% for *GAPDH* and *SMAD3*, respectively. Table 4.1 presents the primer sequences used and the size of PCR products. qPCR was then performed in triplicate using 5µl of cDNA, 10µl SYBR Green (Power SYBR® Green PCR Master Mix, Applied Biosystems, 4367659), and 0.4µl of forward and reverse primers in a final volume of 20 µl. Cycling conditions were: 95˚C for 10 min, 95˚C for 15 sec, and 60˚C for 1 min, repeated for 45 cycles, followed by melt-curve analysis. One of the control samples was selected as calibrator and the relative quantification (RQ) of *SMAD3* expression in each sample was calculated as fold changes in relation to the calibrator using the Livak method [246] The average of the calculated RQ values in every group was used as fold change in the expression levels between the comparison groups.

SMAD3 promoter methylation assay

Bisulfite conversion of DNA was conducted using the EpiTect Bisulfite Kit (Qiagen, Venlo, the Netherlands). Briefly, 50 ng of genomic DNA in 2 µl water was mixed with 38 µl RNase-free water, 85 µl Bisulfate Mix, and 15 µl DNA protect water in a final volume of 140 µl. The conversion reaction and DNA clean-up were conducted according to the manufacturer's instructions.

SMAD3 promoter DNA methylation was quantified using the Sequenom EpiTYPER platform [247]. Primers for the target region were designed using EpiDesigner (www.epidesigner.com, Sequenom) and the PCR target sequence was tested with program RSeqMeth implemented in R. Table 4.1 presents the primer details. The converted DNA was subject to PCR amplification, SAP (shrimp alkaline phosphatase) treatment, in vitro RNA transcription, base specific cleavage (MassCleave sites in figure 4.5), and analysis on mass spectrometer according to the manufacturer's protocol. The generated mass signals were translated into quantitative DNA-methylation levels (beta-values ranging 0-1) by MassARRAY EpiTYPER Analyzer software. On every bisulphite plate, standard DNA samples with 0%, 50%, 100% methylations were included as controls for the technical steps of the experiment.

Statistics

Descriptive statistics were summarized using either mean or percentage and comparisons between OA cases and controls were performed using the student's T-test or chi-squared test wherever appropriate. Non-parametric Mann-Whitney U test was utilized to compare the gene expression and methylation levels between OA cases and controls and a non-parametric regression model was used to adjust for potential confounders. Spearman's rank correlation coefficient (*rho*) was calculated to examine the relationship between the promoter DNA methylation and gene expression. All the statistical analysis was conducted using STATA/SE 11.2 (Stata Corp, College Station, Texas, USA). The significance level was defined as α level of 0.05.

Table 4.1- Primers used in qPCR and EpiTyper experiments

	Primer sequence (5'>3')	Product size
SMAD3 reverse primer - qPCR	GGCTCGCAGTAGGTAACTGG	91 bp
SMAD3 forward primer - qPCR	GCATGGACGCAGGTTCTCC	
GAPDH reverse primer - qPCR	TCGCCCCACTTGATTTTGG	106 bp
GAPDH forward primer - qPCR	GCAAATTCCATGGCACCGT	
SMAD3 reverse primer - EpiTyper	CAGTAATACGACTCACTATAGGGAGAAGGCTTCCAACCATTAAAAAATAACCAAAA	208 bp
SMAD3 forward primer - EpiTyper	AGGAAGAGAGAAAGGATTTGAATTATAGGAGGATAG	

Results

A total of 113 study participants were included in the study, 58 (11 knee OA and 47 hip OA) of whom were categorized as OA cases and 55 as healthy controls (hip fracture patients). Overall, 69% of the participants were female, and 31% were male. Controls were on average 15 years older than OA cases ($p<0.0001$) and had a lower BMI than OA cases ($p<0.0001$). Table 4.2 presents the characteristics of the study population. Pathological examination of the joint cartilage confirmed all the OA cases. It also confirmed that 21 controls had healthy cartilage, but the other 34 controls had age-related minor degenerative changes. The gene expression experiment was performed on 38 patients with OA (32 hips and 6 knees) and 28 healthy controls, and the methylation analysis was conducted for 49 patients with OA (38 hips and 11 knees) and 51 controls. Since 52 subjects had data on both methylation and expression, they were included in the methylation-expression correlation analysis.

Table 4.2- Characteristics of the study population

	Cases (n=58)	Controls (n=55)	P-value
Age (yrs.)	64.2 ± 10.2	79.3 ± 9.4	<0.0001
BMI (kg/m2)	31.7 ± 0.9	23.5 ± 0.8	<0.0001
Sex (females; %)	62%	78%	0.06

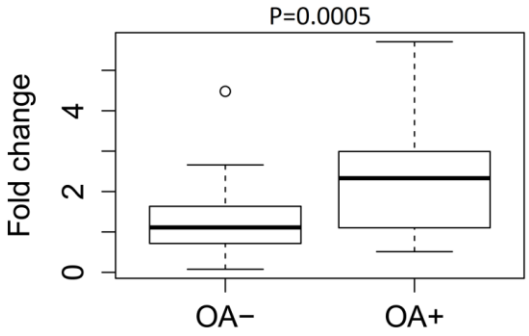
Values are expressed as mean ± standard deviation unless indicated otherwise.

SMAD3 expression in cartilage

I first examined the *SMAD3* expression between the controls with intact healthy cartilage, and those with minor age-related degeneration and found no difference among the two groups. The average RQ values were 1.25 ± 1.05 and 1.35 ± 0.69 for the two groups (p= 0.37), respectively. We, therefore, combined these two groups together and used them as controls in the subsequent analyses.

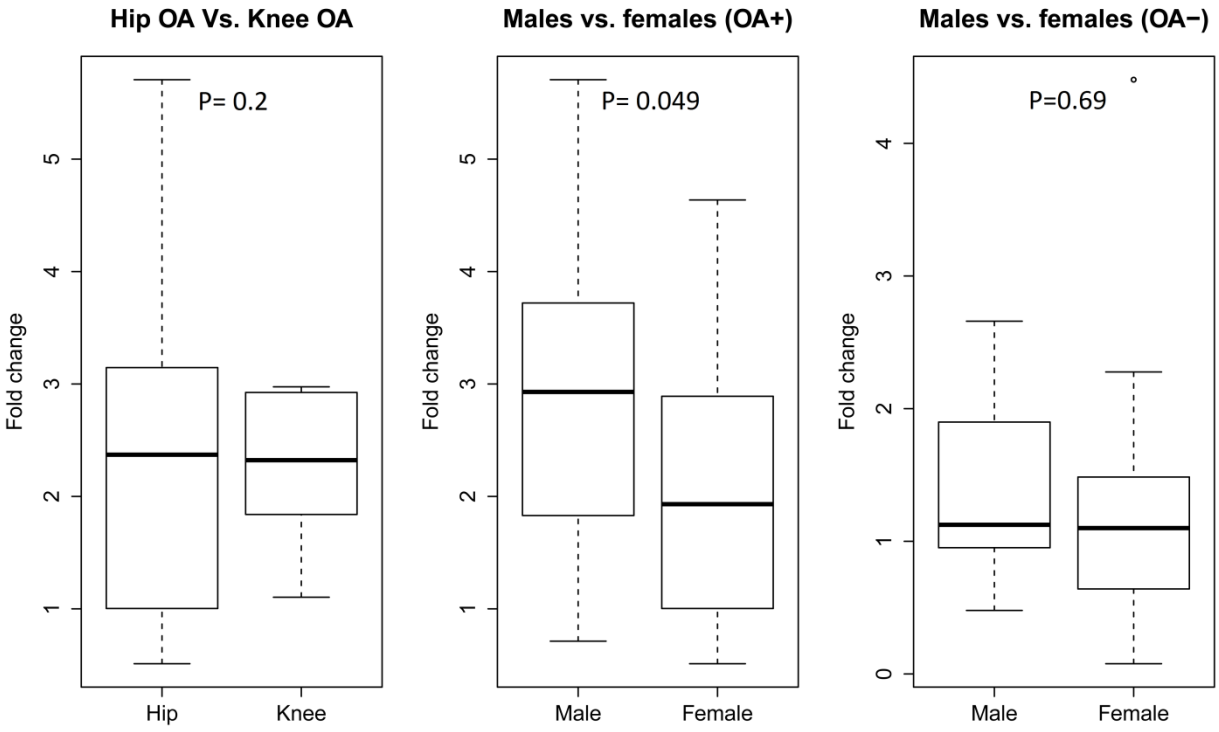
The mean RQ values of the *SMAD3* were 2.37 ± 1.30 in OA cartilage and 1.30 ± 0.89 for controls. This represents 83% increased expression of *SMAD3* in OA cartilage compared to controls (p=0.0005) (Figure 4.1). Similar results were observed when analyses were done separately for knee and hip OA (72% increase for knee OA, p= 0.01; 84% increase for hip OA, p= 0.001), but no difference was found between knee OA and hip OA (p= 1.00) (Figure 4.2).

Figure 4.1- Relative quantification of *SMAD3* expression in human cartilage between OA cases and controls



Mann-Whitney U test was used for the comparisons.

Figure 4.2- *SMAD3* expression between different joints and genders



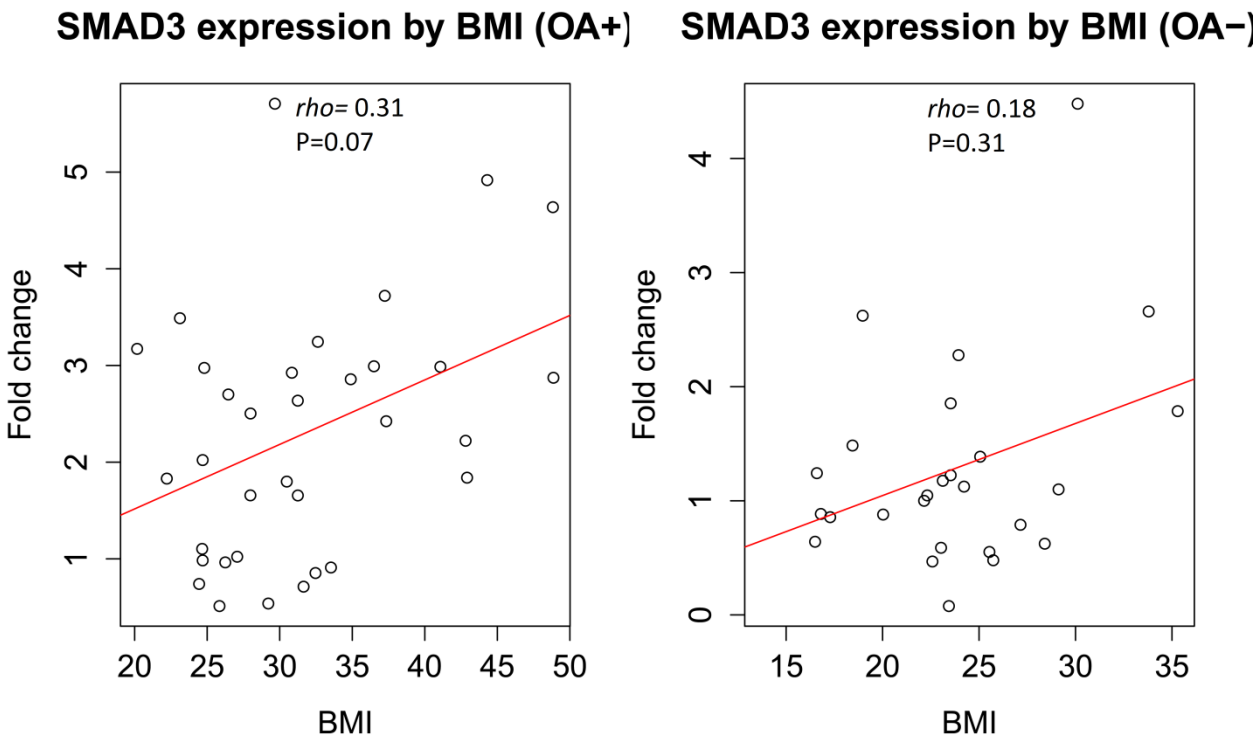
Mann-Whitney U test was used for the comparisons.

I found that *SMAD3* expression was not associated with age and BMI in either OA or healthy cartilage (all $p>0.07$). However, I found that females tended to have a lower expression than males, but only significant in OA cartilage ($p=0.05$) (Figures 4.2 - 4.4). The significant difference in the *SMAD3* expression between OA cases and controls remained after adjustment for sex using non-parametric regression ($p=0.001$).

Figure 4.3- *SMAD3* expression by age among cases and controls

Spearman's rank correlation coefficient was used.

Figure 4.4- *SMAD3* expression by BMI among cases and controls

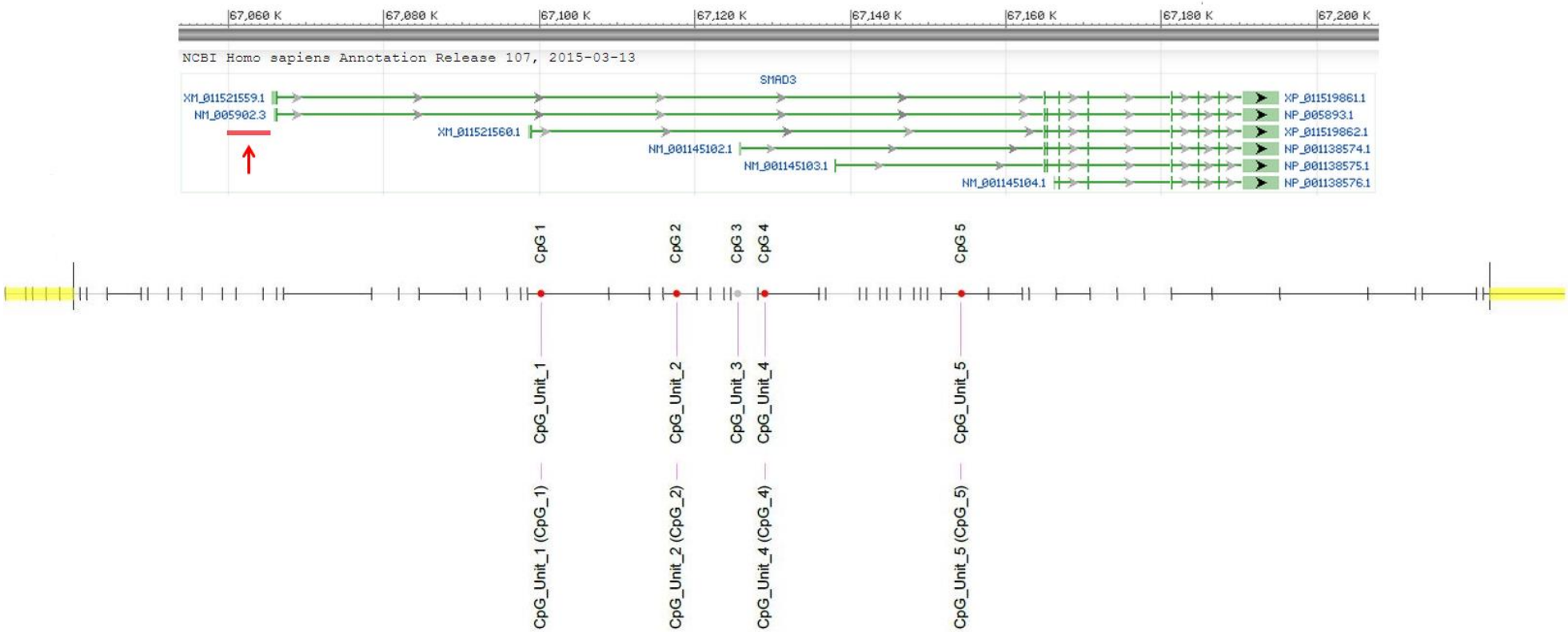


Spearman's rank correlation coefficient was used.

DNA methylation in the SMAD3 promoter region

The upstream 600bp sequence of the first exon of the longest isoform of *SMAD3* (Figure 4.5) was retrieved from the Ensemble genome browser (ENST00000327367) and copied into the EpiDesigner [247], from which a 208bp region was identified as optimal for the experiment design, containing the largest number of detectable CpG sites. The identified sequence was subsequently blasted in UCSC blast and confirmed to be part of the active promoter. It contained 5 CpG sites, 4 of which were successfully assayed by the EpiTyper [247]. These four sites are located 413bp, 442bp, 455bp, and 475bp upstream of the first exon of *SMAD3*, respectively (Figure 4.5). The methylation levels at these four CpG sites were similar between OA cases and controls (Figure 4.6) (all $p>0.05$). The same results were observed when the analyses were done for knee OA and hip OA, respectively ($p>0.05$). I also calculated the Spearman's rank correlation coefficients between each CpG site and the *SMAD3* expression and found no correlations (Table 4.3, Figure 4.7) (all $p>0.05$).

Figure 4.5- Location of the four CpG sites in the promoter region of *SMAD3*



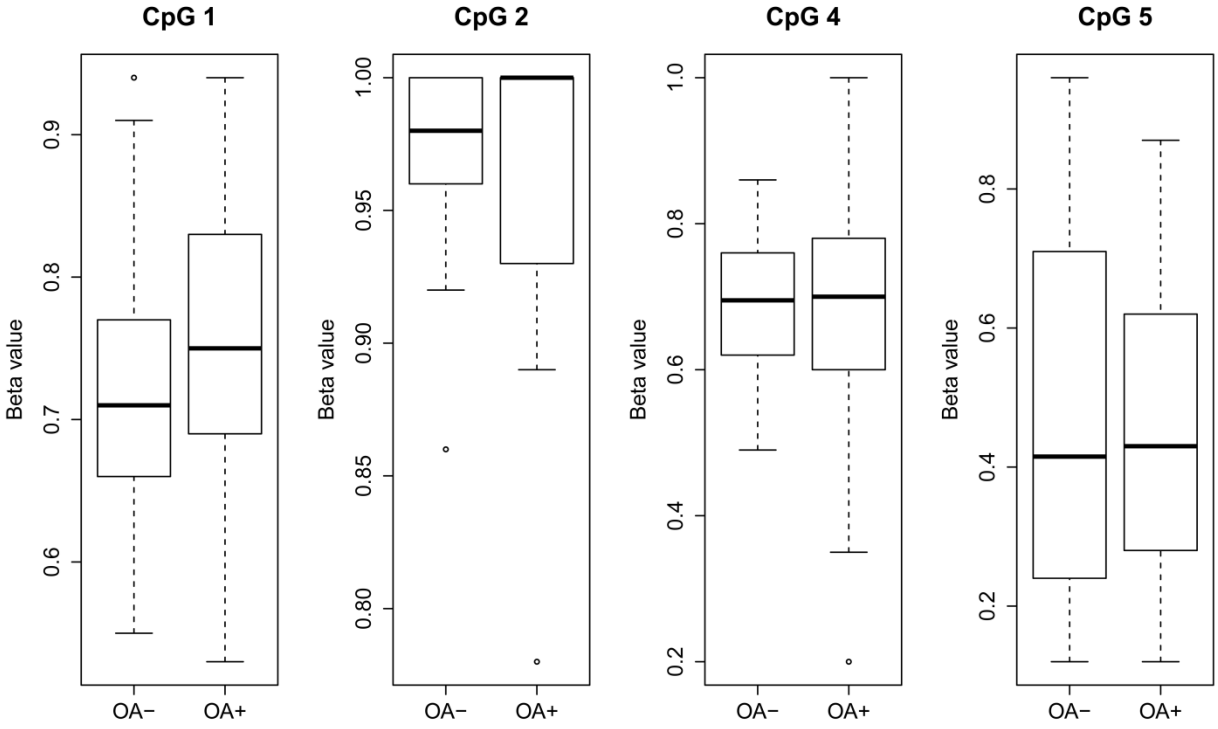
The DNA segment within the 600bp region (red arrow) upstream of the first exon of SMAD3 was the targeted region for the methylation assay using EpiTyper. A 208bp segment within the region (lower horizontal line) containing 5 CpG sites was amplified, of which 4 CpG sites were successfully assayed by the EpiTyper (red dots) and one was not analyzed (gray dot). The breaks represent the fragmentation in MassCLEAVE reaction sites. Locations of the CpG sites on Ensembl-Havana GENCODE gene set (release 22): CpG1: chr15:67,062,688; CpG2: chr15:67,062,708; CpG4: chr15:67,062,721; CpG5: chr15:67,062,750

Table 4.3- Spearman correlation coefficient between methylation levels and gene expression

	CpG 1	CpG 2	CpG 4	CpG 5
Controls (n=22)	-0.14	-0.31	0.18	-0.06
OA (n=30)	0.04	-0.20	0.11	-0.01
Combined (n=52)	0.12	-0.02	0.16	0.01

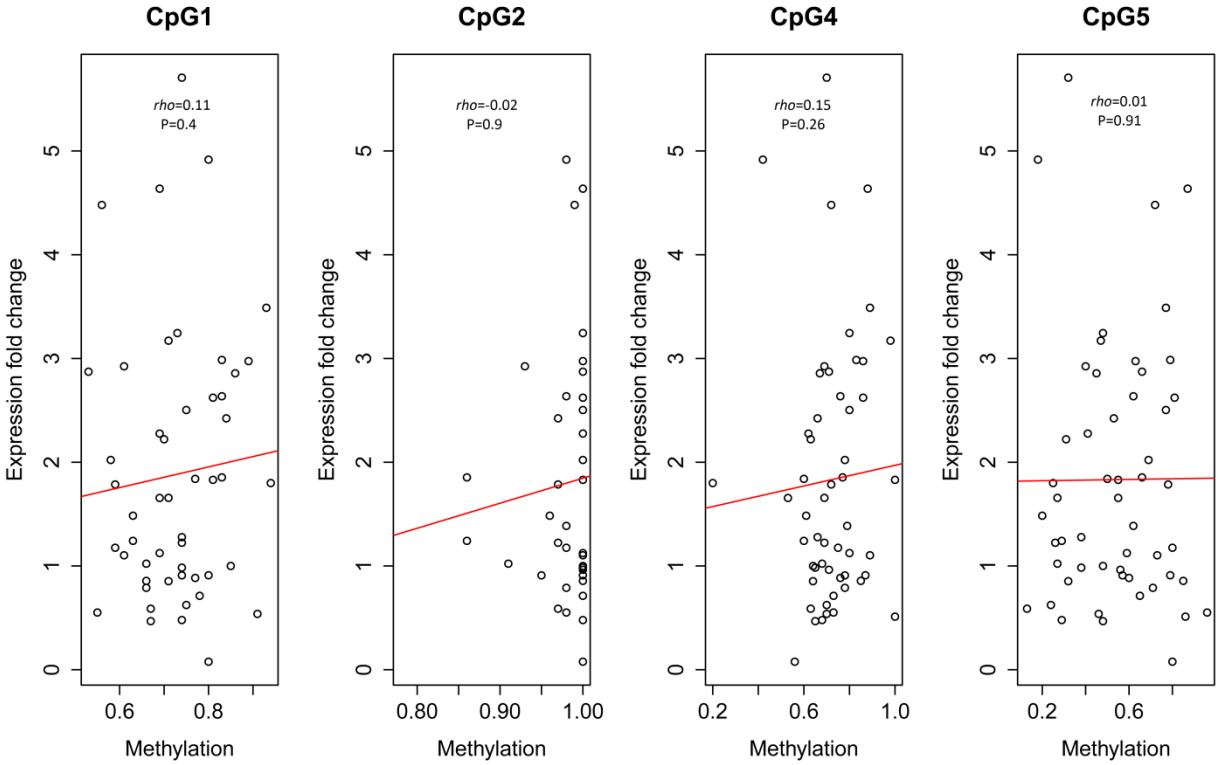
P-value for all tests >0.05

Figure 4.6- Methylation levels of the four CpG sites in the *SMAD3* promoter in OA cases and healthy controls



None of the comparisons was significant (all p>0.05). Mann-Whitney U test was used for the comparisons.

Figure 4.7- *SMAD3* gene expression by methylation of the four CpG sites



Spearman's rank correlation coefficient was used.

Discussion

To the best of my knowledge, this is the first study investigating *SMAD3* gene expression and its promoter DNA methylation in human osteoarthritic and healthy cartilage tissues. I found that *SMAD3* was significantly overexpressed in the osteoarthritic cartilage compared to the healthy cartilage. The over-expression is independent of the DNA methylation in the *SMAD3* promoter region and appears not to be joint specific.

SMAD3 is one of the intracellular mediators of TGF-β signalling pathway, which is involved in diverse cellular processes including proliferation, differentiation, migration and apoptosis, as well as extracellular matrix (ECM) synthesis and degradation [248, 249]. Its activity is essential to the maintenance of the cartilage [250]. The signalling suppresses the catabolic effects of IL-1 and TNF-α on cartilage degradation and prevents the degradation of ECM molecules through enhancing the production of protease inhibitors, such as tissue inhibitors of metalloproteinase (TIMP) [251]. Decreased phosphorylation of SMAD3 was previously observed during the OA progression of murine models of OA [252, 253], and it was also shown that *SMAD3* knocked out mice develop OA-like features [221]. These observations indicate that a lack of TGF-β/SMAD2/3 signalling activity is involved in the development of OA, particularly early onset OA [221]. Thus, it was expected that *SMAD3* expression is lowered in OA cartilage. In the current study, however, I observed that *SMAD3* was significantly over-expressed in OA cartilage.

The observed paradox can be interpreted in lines of two possible mechanisms happening during OA development. First, the over activity of TGF-β pathway could be indicative of an attempt in the cartilage to repair the damage occurred during the process of OA development. OA initiation

is thought to be caused by an attempt in the cartilage to repair an initial cartilage injury [254, 255]. The response results in overproduction and accumulation of collagen and proteoglycans, leading to cartilage swelling and breakage, subchondral bone cavity formation and osteophytes growth, and finally the involvement of the whole joint and OA presentation [254, 255].

The second possible explanation could be related to the hypothesis that only a narrow range of bioactive TGF-β levels can maintain cartilage health, and any concentrations below or above this range may lead to aberrant alterations in TGF-β pathways, resulting in abnormal cartilage function [256]. In line with this hypothesis, multiple intra-articular injections of TGF-β in mice joint led to changes in articular cartilage with a strong resemblance to both experimental and spontaneous mice OA [178]. Enhanced expression of *TGFβ1* and *TGFβ3* was detected in developing osteophytes and articular cartilage during murine experimental osteoarthritis, and the inhibition of endogenous TGF-β prevented osteophyte formation [252]. Increased activity of TGF-β was also observed in other joint tissues. High concentration of active TGF-β1 in the mice subchondral bone was reported to initiate osteoarthritic changes in the bone and cartilage [257]. Induced expression of *TGFβ1* from the synovial lining layers resulted in OA-like changes in the murine knee joint including hyperplasia of synovium and chondro-osteophyte formation [258]. Increased activity of TGF-β can also enhance the expression of cartilage degradative enzymes such as matrix metallopeptidase 13 (MMP13). A study showed that TGF-β can up-regulate the levels of *MMP13* in normal cartilage *in vitro* and mimic the *in situ* distribution of the increased MMP13 in both OA and rheumatoid arthritis affected cartilage [259]. This phenomenon has also been observed in other tissues. Activation of TGF-β/SMAD3 pathway enhances *MMP13* expression in squamous carcinoma cells [260], breast cancer cells [261], human gingival

fibroblasts [262], and osteoblastic cells [263]. These results favor this hypothesis, but require further studies to investigate *SMAD3* and *MMP13* simultaneously in human joint tissues.

It is not clear yet what causes an increased *SMAD3* expression in OA cartilage. DNA methylation is thought to regulate gene expression. I examined the correlation between a portion of the promoter DNA methylation and *SMAD3* expression but found no correlation. A recent study by Raine *et al*. [264] examined whether the expression of *SMAD3* in OA cartilage was correlated with rs12901499, an SNP reported to be associated with OA [233]. They found no correlation between this SNP and *SMAD3* expression in OA cartilage but identified another SNP, rs8031440, located at 3'UTR, to be associated with the expression of *SMAD3*. The SNP was weakly associated with OA. The study also found that *SMAD3* expression in knee OA cartilage was different from hip OA cartilage, which is in contrast to what I found in the current study. The reason for this discrepancy is not clear. It may have resulted from the differences in the cohort characteristics such as age and sex between this study and theirs. The study by Raine *et al*. [264] did not include healthy control cartilage, making the interpretation of their results difficult.

It should also be considered that potential confounders may bias the results since a previous study reported that females had lower *SMAD3* expression than males [265]. Consistently, I also found that females had lower expression of *SMAD3* in cartilage than males. However, the significant difference in *SMAD3* expression between OA-affected cartilage and controls was not altered after adjustment for sex, indicating sex can not explain the observed association. I also examined the effects of age and BMI on *SMAD3* expression and found there was no significant association. However, there was a trend of decrease in *SMAD3* expression with increasing age in OA cases only and an increase with BMI in both OA cases and controls (Figure 4.3). It is

possible that this trend might become significant with a larger sample size. Further studies with a large sample size are needed to rule out the confounding effects of age and BMI on the observed association.

The strength of the current study is the use of human cartilage rather than animal models or cultured cells, thus having a direct application to OA patients. I extracted DNA and RNA from the same sample and minimized the bias in examining the correlation between DNA methylation and gene expression due to differential sampling. However, I only measured mRNA expression level, which may not reflect the corresponding protein levels as well as their phosphorylated SMAD3 isoforms. This is particularly of importance since the function of the SMAD3 protein depends on not only its expression levels but also its phosphorylation status. In addition, it is notable that the small sample size was small, and a chance of false-positive findings should be considered in this regards. Further, only four CpG sites in the *SMAD3* promoter were investigated, and I cannot rule out the association with DNA methylation at other CpG sites in the gene. Also, DNA methylation levels are known to be dynamic in cells, and change on a timely basis and thus the levels in this study may represent the situation and the time the samples were obtained. However, I assume that the same situation applies to both cases and the controls in this study, and the main differences that I am observing in this study are because of the differences in the disease status of the subjects, not the constitutional variability of DNA methylation. I captured the promoter region of the longest isoform of the *SMAD3* gene while there are several different isoforms of the gene and possibly multiple promoters, and I may have missed the isoform-specific CpG sites. Obtaining cartilage tissue samples from healthy individuals is near impossible ethically, and I used cartilage samples from hip fracture patients as controls, which may not necessarily represent true healthy cartilage. However, I examined the

pathology reports and confirmed the healthy status of those control cartilage samples. OA patients were all at end-stage of the disease. Thus, the findings may not be related to OA initiation and progression. Lastly, I only studied cartilage tissue, limiting the generalizability of the findings to other joint tissues.

In conclusion, I demonstrated that *SMAD3* was over-expressed in osteoarthritic cartilage independent of the promoter DNA methylation, suggesting TGF-β/SMAD3 pathway may be over activated in OA cartilage, which may have a potential for developing targeted therapies for OA.

5

Overexpression of *MMP13* in human osteoarthritic cartilage is associated with the SMAD-independent TGF-β signalling pathway

Erfan Aref-Eshghi[1], Ming Liu[1], Patricia E. Harper[1], Jules Doré[2], Glynn Martin[3], Andrew Furey[3], Roger Green[1], Proton Rahman[4], Guangju Zhai[1,5]

[1]Discipline of Genetics, [2]Division of Biomedical Science, [3]Division of Orthopedics, [4]Discipline of Medicine, Faculty of Medicine, Memorial University of Newfoundland, St. John's, NL, Canada

[5]Department of Twin Research & Genetic Epidemiology, King's College London, London, UK

A version of this chapter was published in:
Arthritis Res Ther 2015; 17 (1): 1-8.
doi: 10.1186/s13075-015-0788-x

Abstract

Introduction**:** *In vitro* and animal model of osteoarthritis (OA) studies suggest that TGF-β signalling is involved in OA, but human data is limited. I undertook this study to elucidate the role of TGF-β signalling pathway in OA by comparing the expression levels of *TGFB1* and *BMP2* as ligands, *SMAD3* as an intracellular mediator, and *MMP13* as a targeted gene between human osteoarthritic and healthy cartilage.

Methods: Human cartilage samples were collected from patients undergoing total hip/knee joint replacement surgery due to primary OA or hip fractures as controls. RNA was extracted from the cartilage tissues. Real-time quantitative PCR was performed to measure gene expression. Mann-Whitney U test was utilized to compare the expression levels of *TGFB1*, *BMP2, SMAD3* and *MMP13* in the human cartilage between OA cases and controls. Spearman's rank correlation coefficient (*rho*) was calculated to examine the relationship between the expression levels of the four genes studied, and non-parametric regression was used to adjust for age, sex, and BMI.

Results: A total of 32 OA cases (25 hip OA and 7 knee OA) and 21 healthy controls were included. The expression of *TGFB1*, *SMAD3*, and *MMP13* were on average 70%, 46%, and 355% higher, whereas the expression of *BMP2* was 88% lower, in OA-affected cartilage than that of controls (all $p<0.03$), respectively, but no difference was observed between hip and knee OA (all $p>0.4$). The expression of *TGFB1* was correlated with the expression of *SMAD3* (*rho*=0.50, p=0.003) and *MMP13* (*rho*=0.46 p=0.007) in OA-affected cartilage and the significance remained after adjustment for age, sex, and BMI. The expression of *BMP2* was

negatively correlated with both *TGFB1* (*rho*=-0.50, p=0.02) and *MMP13* (*rho*=-0.48, p= 0.02) in healthy cartilage, but the significance was altered after adjustment for the covariates. There was no correlation between the expression of *SMAD3* and *MMP13*.

Conclusions: These results demonstrate that *MMP13* expression is associated with an increased expression of *TGFB1* in OA-affected cartilage, possibly through SMAD-independent TGF-β pathway. Furthermore, TGF-β/SMAD3 is over-activated in OA cartilage; yet, the consequence of this over-activation remains to be established.

Introduction

Osteoarthritis (OA), the most common rheumatic condition, is primarily a disease of articular cartilage and subchondral bone [1]. It presents with joint pain, stiffness, deformity, and joint failure at advanced stage [185], and imposes a high socio-economic burden on society [266]. Although the pathogenesis of OA remains elusive, mounting evidence suggests that transforming growth factor β (TGF-β) signalling plays a role in the development of OA [172].

TGF-β signalling is involved in diverse cellular processes including proliferation, differentiation, migration, and apoptosis, as well as extracellular matrix (ECM) synthesis and degradation [249]. It plays a critical role in the development, homeostasis, and repair of the cartilage [172]. Population-based association studies have identified genetic variants in different components of TGF-β signalling to be associated with OA. A single nucleotide polymorphism (SNP; T29>C) in the transforming growth factor beta 1 (*TGFB1*) gene was reported to be associated with the

incidence of spinal osteophyte formation in a Japanese population [267]. SNPs rs2278422, and rs8179181, located in the 6th intron of *TGFB1*, have been associated with knee and hip OA susceptibility in a British Caucasian population [206]. Camurati–Engelmann disease (CED), which presents with long bone osteosclerosis, is caused by mutations in *TGFB1*, which lead to elevated TGF-β1 activity [181].

The Asporin gene (*ASPN)* has been shown to inhibit TGF-β signalling-mediated syntheses of cartilage-specific extracellular matrix components, such as type II collagen (COL2A1) and Aggrecan (AGC1) in chondrocytes [268]. The Aspartic acid (D) repeat polymorphism in *ASPN* has been associated with OA in Asian populations [268, 269] .When compared to common *ASPN* D-13 allele, the D-14 allele was found to be overrepresented in knee and hip OA patients, relative to healthy controls [268], resulting in greater inhibitory effects on TGF-β induced expression of *AGC1* and *COL2A1* [270]. Additionally, the *ASPN* variant, rs13301537, was recently reported to contribute to knee OA risk in the Chinese Han population [271].

Apart from *TGFB1*, other components of the TGF-β signalling pathways have also reported being associated with OA. Growth differentiation factor 5 (*GDF5*), a member of the TGF-β superfamily, has been associated with OA in Asian and European populations [147, 272]. The risk allele (T) in SNP rs143383 (T/C), located in the promoter of *GDF5*, was found to confer lower *GDF5* transcription activity both *in vitro* and in human cartilage [147, 149]. The SNP is located in the promoter of the gene, and its risk allele is shown to be associated with a reduced expression of the gene [140, 142]. Polymorphisms in the *GDF5* gene have also been associated with other skeletal disorders such as congenital hip dysplasia, Hunter-Thompson-type acro mesomelic dysplasia, type C brachydactyly, and Grebe-type chondrodysplasia [172, 273].

In an attempt to replicate OA associated loci in the Newfoundland and Labrador population, I previously reported an association between SNP rs1049007 located in the bone morphogenetic protein 2 (*BMP2*) gene and OA [274]. *BMP2* is also a member of the TGF-β superfamily. Given the SNP is a synonymous polymorphism, what the relationship between the SNP and OA remains to be discovered. Mutations in Mothers Against Decapentaplegic Homolog 3 (*SMAD3*), one of the intracellular mediators of TGF-β signalling, are known to cause the Aneurysm-Osteoarthritis Syndrome, presenting with early-onset polyarticular OA [179]. In my previous study, I found an SNP in the last intron of *SMAD3* to be associated with the total burden of radiographic OA [243], although the exact mechanism for the association needs to be established.

However, most of these studies focused on a single gene at a time. Given that those genetic variants are not functional, how these genes exert their effect on OA remain to be investigated. We, therefore, undertook the current study to elucidate the role of TGF-β signalling pathway in OA by simultaneously examining the expression levels and pair-wise correlations of four genes including *TGFB1* and *BMP2* as ligands, *SMAD3* as an intracellular mediator, and matrix metallopeptidase 13 (*MMP13)* as a targeted gene in human cartilage tissues obtained from OA patients and healthy controls.

Methods

Subjects

The study was part of the ongoing Newfoundland Osteoarthritis Study (NFOAS) that was initiated in 2011, aiming at identifying novel genetic, epigenetic, and biochemical markers for

OA [245]. OA patients were recruited from those who underwent total knee or hip joint replacement due to primary OA between November 2011 and December 2013 in St. Clare's Mercy Hospital and Health Science Centre General Hospital in St. John's, the capital city of Newfoundland and Labrador (NL), Canada. Healthy controls were recruited from the same hospitals from those who underwent hemiarthroplasty of the hip due to fractures of the femoral neck but did not have evidence of hip OA based on their hip X-ray data which were further confirmed by pathological examination on the removed femoral head cartilage. OA diagnosis was made based on the American College of Rheumatology criteria [14, 48], and the judgement of the attending orthopaedic surgeons. The pathology reports on the removed cartilage were reviewed for all subjects to ensure the accuracy of the diagnosis and the status of any cartilage degeneration in the controls. The study was approved by the Health Research Ethics Authority (HREA) of Newfoundland and Labrador (HREA11.311), and written consent was obtained from all study participants. The consent rate was 90%.

Demographics and anthropometrics

Demographic information was obtained by a self-administered questionnaire with the help of the research staff, if necessary. Anthropometric data including height and weight was retrieved from their hospital admission and medical records and body mass index (BMI) was calculated by dividing weight in kilograms by squared height in meters. Age was calculated at the time of the surgery.

RNA isolation

Four pieces (~200mg each) of cartilage tissues were retained from either the tibial plateau or femoral heads during the surgery. The samples were then flash-frozen and stored in liquid nitrogen until the experiment. Up to 200mg frozen cartilage tissue was transferred to the homogenizing cylinder with 1 ml TRIzol lysis reagent and 200µl guanidine thiocyanate and homogenized using a cryogenic mill (Spex Freezer Mill, model 6770, Metuchen, NJ, USA) with the following parameters: two cycles of 2 minutes grinding at maximum frequency with 10 minutes cooling down between grinding cycles. The homogenate was then transferred to a new 2ml RNase-free tube and incubated for 5 min at room temperature. RNeasy Lipid Tissue Mini Kit (Qiagen, Venlo, Netherlands) was used for extracting total RNA from the aqueous phase as per the manufacturers' protocol.

Gene expression measurement

Gene expression experiment was conducted as described in Chapter 4. The same primer set was used for *SMAD3*. Primers for *TGFB1*, *MMP13,* and *BMP2* are shown in Table 5.1.

Table 5.1- Primers used in qPCR experiments

	Primer sequence (5'>3')	Product size
SMAD3 reverse primer	GGCTCGCAGTAGGTAACTGG	91 bp
SMAD3 forward primer	GCATGGACGCAGGTTCTCC	
TGFB1 reverse primer	CTCAATTTCCCCTCCACGGC	114 bp
TGFB1 forward primer	TCCTGGCGATACCTCAGCAA	
MMP13 reverse primer	AGGTAGCGCTCTGCAAACTGG	92 bp
MMP13 forward primer	AGCTGGACTCATTGTCGGGC	
BMP2 reverse primer	CTTGCGCCAGGTCCTTTGAC	111bp
BMP2 forward primer	CCACCATGGTCGACCTTTAGGA	
GAPDH reverse primer	TCGCCCCACTTGATTTTGG	106 bp
GAPDH forward primer	GCAAATTCCATGGCACCGT	

Statistics

Descriptive statistics were summarized using either mean or percentage and comparisons between OA cases and controls were performed using Student's t-test or Chi-square test wherever appropriate. Mann-Whitney U test was utilized to compare gene expression levels between OA cases and controls. Spearman's rank correlation coefficient (*rho*) was calculated to examine the relationship between the expression levels of the four genes studied, and a non-parametric regression model was used to adjust for potential confounding factors including age, sex, and BMI. The association between each of the genes studied and the covariates including age, sex, and BMI was also examined. All the statistical analysis was conducted using STATA/SE 11.2 (Stata Corp, College Station, Texas, USA). The significance level was defined as at α level of 0.05 [275].

Results

A total of 53 study participants were included in the study: 32 (7 knee OA and 25 hip OA) OA cases and 21 healthy controls. This cohort combination is different from the one used in Chapter 4. All of them were Caucasians. 70% of the study participants were females, and 30% were males. Controls were on average 12 years older than OA cases ($p=0.0002$) and had a lower BMI than OA cases ($p<0.0001$). Table 5.2 presents the characteristics of the study population.

Table 5.2- Characteristics of the study population

	Controls (n=21)	OA (n=32)	P
Age (yrs)	76.45 ± 10.93	64.30 ± 10.43	0.0002
BMI (kg/m^2)	23.79 ± 1.03	32.25 ± 1.35	<0.0001
Sex (% females)	76	66	0.4

Values are expressed as mean ± standard deviation unless indicated otherwise.

Gene expression differences between OA and controls

Pathological reports on the cartilage confirmed the status of all the OA cases. It also found that 14 controls had healthy intact cartilage, but 7 other controls had age-related minor degenerative changes in their cartilage. I, therefore, compared the differences in the expression of the four genes between the healthy intact cartilage samples and those with age-related minor degenerative changes in the controls. I found that there was no difference (all $p>0.2$; Table 5.3). Then, I compared the expression of the four genes between hip and knee OA cases; again, I found no difference (all $p>0.13$; Table 5.3). Consequently, I combined hip OA and knee OA together and compared them with the data from all 21 controls.

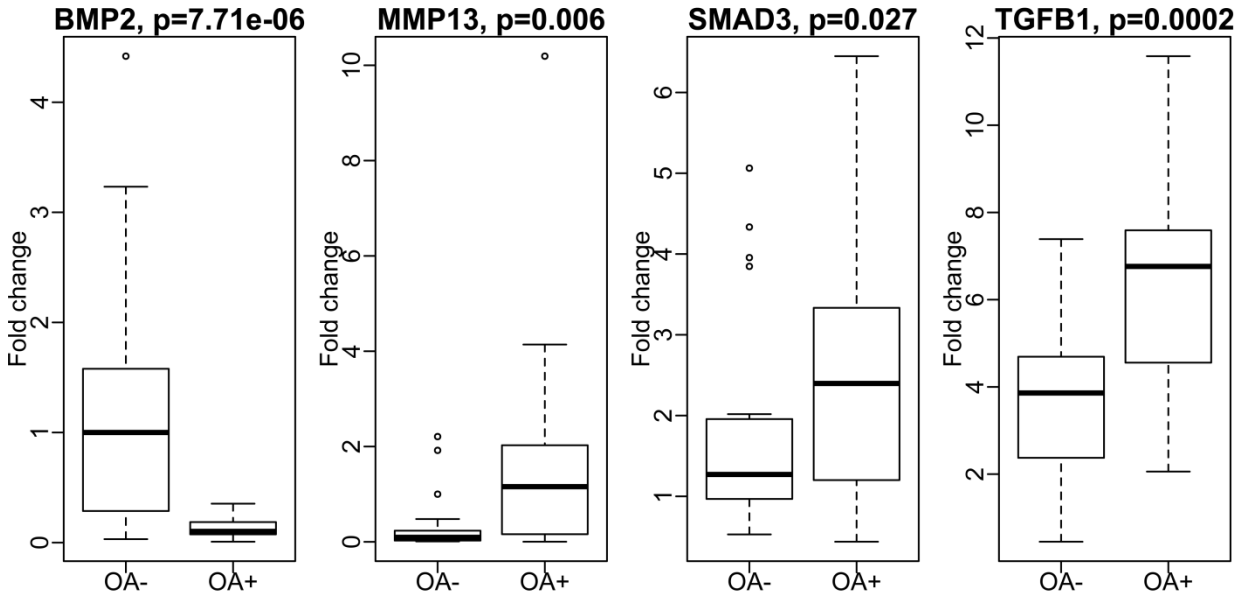
Table 5.3- Gene expression comparison between hip vs. knee OA, and intact healthy cartilage vs. cartilage with minor degeneration in controls

Gene	Hip OA (n=7)	Knee OA (n=25)	P	Healthy controls (n=14)	Controls with minor degeneration (n=7)	P
TGFB1	6.40 ± 2.16	5.71 ± 2.51	0.42	3.35 ± 1.74	4.36 ± 1.89	0.20
SMAD3	2.55 ± 1.59	2.62 ± 0.76	0.56	1.84 ± 1.46	1.60 ± 1.14	1.00
MMP13	1.74 ± 2.21	0.90 ± 0.74	0.53	0.31 ± 0.53	0.41 ± 0.80	0.82
BMP2	0.15 ± 0.02	0.08 ± 0.01	0.13	1.17 ± 0.31	1.23 ± 0.46	0.65

Figures are RQ mean ± standard deviation

I found that all four genes were expressed in OA-affected and healthy cartilage. The expression of *TGFB1*, *SMAD3* and *MMP13* was on average 70%, 46%, and 355% higher, whereas the expression of *BMP2* was 88% lower, in OA-affected cartilage than that in controls (all $p<0.03$), respectively (Figure 5.1).

Figure 5.1- Comparison of the expression levels of *BMP2*, *TGFB1*, *SMAD3*, and *MMP13* in human cartilage between OA cases and controls



The expressions of *TGFB1, BMP2,* and *MMP13* were not associated with age, sex, and BMI either in OA cases or controls (all p > 0.09). However, I found that the expression of *SMAD3* was correlated with age (*rho*= -0.35, p= 0.05) and BMI (*rho*= 0.38, p= 0.03). *SMAD3* was also expressed higher in females than in males (48% higher, p=0.04). These differences were only observed in individuals with OA but not between controls.

Relationship between TGFB1, BMP2, SMAD3, and MMP13 expression

I found that the expression of *TGFB1* was significantly correlated with the expression of *SMAD3* (*rho*= 0.50, p= 0.003) and *MMP13* (*rho*= 0.46, p= 0.007) in OA-affected cartilage but not in healthy cartilage (Table 5.4). The significance became even stronger after adjustment for age, sex, and BMI (p=0.002 and p<0.0001, respectively).

I also found that the expression of *BMP2* was negatively correlated with both *TGFB1* (*rho*=-0.50, p=0.02) and *MMP13* (*rho*=-0.48, p= 0.02) in healthy cartilage but not in OA-affected cartilage (Table 5.4). However, the significances were altered after adjustment for age, sex, and BMI.

I found there was no correlation between the expression of *SMAD3* and *MMP13* either in OA-affected cartilage or controls (Table 5.4).

Table 5.4- Spearman's correlation coefficients (*rho*) between the expression of *TGFB1*, *BMP2*, *SMAD3*, and *MMP13* in OA-affected and healthy cartilage, respectively

	Controls (N=21)	OA (N=32)
TGFB1 & *SMAD3*	*rho*=0.07, p=0.7	*rho*=0.50, p=0.003
TGFB1 & *MMP13*	*rho*=0.28, p=0.2	*rho*=0.46, p=0.007
SMAD3 & *MMP13*	*rho*=0.05, p=0.83	*rho*=0.15, p=0.390
BMP2 & *TGFB1*	*rho*=-0.50, p=0.02	*rho*=0.24, p=0.210
BMP2 & *MMP13*	*rho*=-0.48, p=0.03	*rho*=0.17, p=0.350

Discussion

To the best of my knowledge, this is the first study of using human cartilage samples to demonstrate a significant association between the expression of *TGFB1* and *MMP13*, suggesting TGF-β signalling pathway switches its protective role in normal cartilage observed from *in vitro* studies [172], to a damaging factor in advanced OA, possibly through SMAD-independent TGF-β pathway.

Evidence from animal models of OA indicates that increased expression of *TGFB1* is involved in OA development. Multiple intra-articular injections of TGF-β in mice joint results in changes to articular cartilage with a strong resemblance to both experimental and spontaneous mice OA [178]. High concentrations of active *TGFB1* in the mice subchondral bone is reported to initiate osteoarthritic changes in the bone and cartilage [257], and induced expression of *TGFB1* from the synovial lining layers results in OA-like changes in the murine knee joint including hyperplasia of synovium and chondro-osteophyte formation [258]. Data from human joint tissue, however, are limited. Pombo-Suarez *et al.* [276] studied cartilage samples obtained from 11 patients with hip OA and 11 patients with a femoral neck fracture and found that all three *TGFB* isoforms including *TGFB1* were significantly and highly expressed in osteoarthritic cartilage. My results are consistent with theirs, demonstrating a 70% increase in *TGFB1* expression in OA-affected cartilage. Since I only measured the mRNA expression of *TGFB1,* these results may not reflect the corresponding protein levels. Pombo-Suarez *et al.* [276] found that the increased mRNA levels of *TGFB* isoforms was in relation to an increased percentage of TGF-β positive staining chondrocytes, indicating that mRNA expression of *TGFB* isoforms is well correlated to their protein levels. However, Wu *et al.* [182] performed a proteomic analysis of articular cartilage from 7 knee OA and 7 healthy controls and found a 16 fold decreased protein

expression of *TGFB1* in OA cartilage, suggesting the effect of *TGFB1* in OA may be joint specific. I included cartilage samples from both knee and hip OA patients but did not find any difference in the mRNA expression of these three genes. The reason for the discrepancy between these results and the Wu's [182] is unclear. However, possible reasons leading to false positives include sampling bias due to different population sources, control cartilage of unspecified origin, and the utilization of less stringent significance level (raw p-value <0.03) given the large number of proteins (n=814) examined in their study. Furthermore, apart from TGF-β1, no other protein involved in the TGF-β signalling was found to be significantly different, indicating caution should be used in interpreting their results.

Verdier *et al*. [277] reported that expression levels could vary, based on the OA stage and the level of involvement. In the immunohistochemical analysis of cartilage tissues obtained from six hip OA patients and four controls, TGF-β1 staining was increased in slightly altered areas, reduced in more degraded cartilage, but markedly increased in the osteophytes, suggesting *TGFB1* may take part in the hypertrophic stage of the OA process. Unfortunately, I do not have cartilage severity data to assess the distribution of *TGFB1* expression in different layers of cartilage.

The consequence of increased *TGFB1* activity is unknown. *In vitro* studies showed that activity of TGF-β sub-pathway had a protective role in articular cartilage [4]. However, Pombo-Suarez *et al.* [28] found that none of the expression levels of the three isoforms of *TGF-β* were correlated with the expressions of main proteins in human cartilage, i.e. *COL2A1* and *AGC1*, suggesting the expected role of TGF-β pathway is altered in human OA cartilage. Moldovan *et al.* [259] found that TGF-β can upregulate the levels of *MMP13* in cultured cartilage explants and cause a mimicking of the *in situ* distribution of the increased *MMP13* observed in both OA- and

rheumatoid arthritis affected cartilage. My results are consistent with theirs with a strong correlation between expressions of *TGFB1* and *MMP13* in OA-affected cartilage, suggesting TGF-β switches from a protective role observed from *in vitro* studies to a damaging factor in OA-affected cartilage. A similar phenomenon has also been reported for other tissues including squamous carcinoma [260], breast cancer [261], human gingival fibroblasts [262] and osteoblasts [263].

MMP13 is a major enzyme targeting cartilage for the degradation of types II, IV, and IX collagen, proteoglycan, osteonectin and perlecan [278]. Its overexpression has been shown to be related to cartilage destruction among both human OA patients and animal models of OA [239]. It seems that TGF-β signalling regulates expression of *MMP13* through SMAD-dependent pathway in squamous carcinoma cells [260], and in human gingival fibroblasts [262]. In mice, primary chondrocytes TGF-β signals through SMAD3 rapidly repress *MMP13* expression but induce its expression in the absence of SMAD3 [236]. Alternatively, TGF-β has been described as increasing *MMP13* expression in osteoblast cells through a combination of SMAD-dependent and SMAD-independent pathways [263]. In the current study, I found there was no correlation between *SMAD3* and *MMP13* expression in either normal or OA cartilage, suggesting that the association between *TGFB1* and *MMP13* expression in OA-affected cartilage is primarily through the SMAD-independent pathway.

TGF-β receptors can exert their effect through collateral signalling via mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) proteins [172]. The biochemical blockade of MAPK pathway abolishes TGF-β induction of *MMP13* in human breast cancer cell lines [261], and the inhibition of the MAPK pathways reduces *TGFB1*-stimulated *MMP13* expression in the rat osteosarcoma cell line (UMR 106-01) [263], favouring the SMAD-independent

pathway for enhanced *MMP13* expression in OA cartilage. The regulation might also occur through other mechanisms including inflammatory factors. It is reported that TGF-β1 treatment increases the expression of pro-inflammatory cytokines, including interleukin 1 (IL-1) and metalloproteinase-1 in synovial fibroblasts from rheumatoid arthritis and normal individuals [279], and IL-1 secretion by chondrocytes has shown to stimulate *MMP13* expression and cartilage degradation in OA [280]. This data also showed that the increased expression in *MMP13* was disproportional to the increased *TGFB1* expression, suggesting other factors may also play a role in increasing *MMP13* expression in OA cartilage. Blaney Davidson *et al.* showed that an increase in activin A receptor type II-like 1 (*ALK1*) expression (BMP pathway receptor) was associated with elevated *MMP13* expression in human osteoarthritic cartilage [281], suggesting BMP sub-pathway may also be involved in the regulation of *MMP13* expression in human cartilage. In the current study, I found that *BMP2* expression was negatively associated with both *TGFB1* and *MMP13* expression in healthy cartilage, suggesting that *BMP2* can inhibit *MMP13* expression either directly or indirectly, but this inhibitory effect disappeared because of the reduced *BMP2* expression in OA-affected cartilage. However, the significant correlation between *BMP2* and *TGFB1/MMP13* was altered after adjustment for potential confounding factors. Sample size might be the blame. I conducted a *posthoc* power calculation using data on *SMAD3* which had the smallest effect size in this study. For the given sample size and the observed effect size, I had 100% study power. An independent study is needed to confirm the effect of BMP pathway in the regulation of *MMP13* expression.

Although *SMAD3* appeared not to be associated with *MMP13* expression in this study, its expression was highly correlated with *TGFB1* and was increased in OA cartilage compared to controls, suggesting that TGF-β/SMAD3 signalling is also over activated in OA. This enhanced

activity may indicate a reparative response by chondrocytes to the cartilage damage resulting from OA progression, through TGF-β/SMAD3 signalling. While TGF-β signals through Smad1/5/8 route are shown to lead to deleterious cartilage response, the signals through SMAD2/3 are mainly protective, which indicates that TGF- β/SMAD3 signalling is essential for the cartilage maintenance [282]. In line with this, decreased phosphorylation of SMAD3, an indication of decreased signalling activity, has been reported during OA progression of murine models of OA [252, 253], and *SMAD3* knockout mice have shown to develop OA-like features in their joints [221]. Further studies are needed to elucidate the consequence of the over activity of TGF- β/SMAD3 pathway.

The strength of the current study is the use of human cartilage rather than animal models or cultured cells, thus having a direct application to OA patients. However, I only studied cartilage tissue, limiting the generalizability of the findings to other joint tissues involved in OA. mRNA expression levels may not reflect the corresponding protein levels, but previous studies found that mRNA levels of *TGFB1* were well correlated with its protein levels [260], suggesting this is not a concern. However, this study is cross-sectional, and I cannot conclude a causal relationship. In the end, it is notable that the small sample size of the study did not allow for clear interpretations of the effect of confounding variables such as age, sex, and BMI on the expression correlations and changes.

Conclusions

I demonstrated that *TGFB1* switched its protective role as observed using *in vitro* studies to a damaging factor in human OA cartilage, leading to an increased expression of *MMP13*, possibly

through the SMAD-independent pathway. Further, I found that TGF-β/SMAD3 pathway was also over activated, but the consequence needs to be established.

6

Genome-wide DNA methylation study of hip and knee cartilage reveals embryonic organ and skeletal system morphogenesis as major pathways involved in osteoarthritis

Erfan Aref-Eshghi[1], Yuhua Zhang[1], Ming Liu[1], Patricia E. Harper[1], Glynn Martin[2], Andrew Furey[2], Roger Green[1], Guang Sun[3], Proton Rahman[3], Guangju Zhai[1,4]

[1]Discipline of Genetics, [2]Division of Orthopedics, [3]Disicpline of Medicine, Faculty of Medicine, Memorial University of Newfoundland, St. John's, NL, Canada

[4]Department of Twin Research & Genetic Epidemiology, King's College London, London, UK

A version of this chapter was published in:
BMC Musculoskelet Disord 2015; 16(1): 287.
doi: 10.1186/s12891-015-0745-5

Abstract

Objectives: To describe the genome-wide DNA methylation changes in hip and knee osteoarthritis (OA) and identify novel genes and pathways involved in OA by comparing the DNA methylome of the hip and knee osteoarthritic cartilage tissues with those of OA-free individuals.

Methods: Cartilage samples were collected from hip or knee joint replacement patients either due to primary OA or hip fractures as controls. DNA was extracted from the collected cartilage and assayed by Illumina Infinium HumanMethylation450 BeadChip array, which allows for the analysis of >480,000 CpG sites. Student T-test was conducted for each CpG site and those sites with at least 10% methylation difference and a p-value <0.0005 were defined as differentially methylated regions (DMRs) for OA. A sub-analysis was also done for hip and knee OA separately. DAVID tool v6.7 was used for the functional annotation clustering of the DMR genes. Clustering analysis was done using multiple dimensional scaling and hierarchical clustering methods.

Results: The study included five patients with hip OA, six patients with knee OA and seven hip cartilage samples from OA-free individuals. The comparisons of hip, knee and combined hip/knee OA patients with controls resulted in 26, 72, and 103 DMRs, respectively. The comparison between hip and knee OA revealed 67 DMRs. The overall number of the sites after considering the overlaps was 239, among which 151 sites were annotated to 145 genes. One-fifth of these genes were reported in previous studies. The functional annotation clustering of

the identified genes revealed clusters significantly enriched in skeletal system morphogenesis and development. The analysis revealed significant difference among OA and OA-free cartilage, but less difference between hip OA and knee OA.

Conclusions: I found that a number of CpG sites and genes across the genome were differentially methylated in OA patients, a remarkable portion of which seem to be involved in potential etiologic mechanisms of OA. Genes involved in skeletal developmental pathways and embryonic organ morphogenesis may be a potential area for further OA studies.

Introduction

Osteoarthritis (OA), affecting 250 million people worldwide, is the most common form of arthritis [237]. It is characterized by gradual loss of articular cartilage and subchondral bone changes, presents with joint pain, stiffness, joint deformity and disability [185], and imposes a great socio-economic burden on societies, mainly as a result of hip and/or knee involvement [283].

OA is a multifactorial condition arising from the combination and interaction of natural and environmental factors [284]. Age, gender, obesity, previous joint injury, mal-alignments, and genetics are known to be of the major risk factors for OA; yet, the etiology of OA remains incompletely elucidated [285]. In pathology, an imbalance between catabolism and anabolism of the molecules in the cartilage extracellular matrix is a major finding in OA [286]. Since these changes are suggested to result from an altered gene expression related to epigenetic

modifications of the OA candidate genes [240], it is hypothesized that epigenetic changes in chondrocytes could be a key factor in OA pathogenesis [287]. DNA methylation is by far the most extensively studied epigenetic regulator in complex diseases, and it has long been thought that its changes plays a key role in the onset and progression of complex diseases by linking the genetic and environmental risk factors [288].

To date, only a few studies have been undertaken to examine the role of DNA methylation in OA. Candidate gene studies have shown the upregulation of catabolic factors of Matrix metallopeptidase 9 (*MMP9)*, Matrix metallopeptidase 13 (*MMP13)*, Leptin receptor (*LEPR)*, and A disintegrin and metalloproteinase with thrombospondin motifs 4 (*ADAMTS4)* [240], as well as the down-regulation of anabolic molecule collagen, type IX, alpha 1 (*COL9A1)* [289], caused by the promoter hypo- and hyper- methylations of the corresponding genes. Demethylation of an enhancer element within the nitric oxide synthase (*NOS*) gene is shown to increase transcription through elevated binding of the transcription factor NF-κB, which leads to suppression of the synthesis of cartilage matrix [290]. DNA methylation can also modulate the effect of OA genetic susceptibility loci; for instance, the effect of single nucleotide polymorphism (SNP) rs143383 in *GDF5* -- the most replicated genetic association locus in OA - is thought to be caused by the methylation level variability of the CpG dinucleotide created at the location of the SNP, leading to altered expression of the gene [291]. The handful of genome-wide methylation studies performed to date have also identified several potential candidate genes including runt-related transcription factor 1&2 (*RUNX1*, *RUNX2)*, transforming growth factor beta 1 (*TGFB1)*, micro RNA 128 (*miR-128)* and collagen, type XI, alpha 2 *COL11A2* [292], suggesting the involvement of inflammation and immunity in OA pathogenesis [163]. Despite the invaluable information

obtained about the pathogenesis of complex diseases from epigenetic studies, the area still remains as one of the least investigated fields in OA research.

In the present study, I conducted a genome-wide DNA methylation analysis in OA-free and OA-affected cartilage from human hips and knees using the Illumina Infinium HumanMethylation450 BeadChip in the hope of providing novel insights into the pathogenesis and treatment of OA.

Methods

Samples and patients' information

The study was part of the ongoing Newfoundland Osteoarthritis Study (NFOAS) that was initiated in 2011, aiming at identifying novel genetic, epigenetic, and biochemical markers for OA [244, 245]. OA patients were recruited from those who underwent total knee or hip joint replacement due to primary OA between November 2011 and December 2013 in St. Clare's Mercy Hospital and Health Science Centre General Hospital in St. John's, the capital city of Newfoundland and Labrador (NL), Canada. OA-free controls were recruited in the same hospitals from those who underwent hemiarthroplasty of the hip due to hip fracture with no evidence of OA. OA diagnosis was made based on the American College of Rheumatology criteria [14, 48] and the judgement of the attending orthopaedic surgeons. Cartilage samples were collected from the articular surfaces of the tibial plateau or femoral head where the OA lesion occurred. The pathology report of the cartilage following the surgery was reviewed for all subjects to ensure the consistency of the diagnosis and the status of cartilage degeneration among the control subjects.

Demographic information was obtained by a self-administered questionnaire with the help of the research staff if necessary. Anthropometric data including height and weight was retrieved from their hospital admission and medical records and body mass index (BMI) was calculated by dividing weight in kilograms by squared height in meters. Age was calculated at the time of the surgery.

DNA extraction

Four pieces (~200mg each) of cartilage tissues were retained from either tibial plateau or femoral heads during the surgery. The samples were then flash-frozen and stored in liquid nitrogen until the experiment. Up to 200mg frozen cartilage tissue was transferred to the homogenizing cylinder together with 1 ml TRIzole lysis reagent and 200µl guanidine thiocyanate and homogenized using a cryogenic mill (Spex Freezer Mill, model 6770, Metuchen, New Jersey, USA) with the following parameters: two cycles of 2 minutes grinding at maximum frequency with 10 minutes cooling down between grinding cycles. The homogenate was then transferred to a new 2ml RNase-free tube and incubated for 5 min at room temperature. Then, 200µl chloroform was added, and the mix was vortexed vigorously, before being incubated for 2-3 min, followed by centrifugation at 12,000xg at -4°C for 15 min. Following centrifugation, the sample separated into 3 phases: the aqueous phase containing RNA, the interphase, and the organic phase containing DNA. The DNA was extracted using Phenol-Chloroform method from the interphase and organic phase.

DNA methylation profiling

DNA methylation assay was conducted using the HumanMethylation 450 Bead-Chip microarray (Illumina, San Diego, California, USA), which analyzes the methylation status of 485,000

methylation sites throughout the genome, covering 99% of RefSeq genes at an average of 17 CpG sites per gene across the 5-UTR, gene promoter regions, first exon, gene body, and 3-UTR, and covering 96% of University of California, Santa Cruz-defined CpG islands and their flanking regions. Briefly, DNA is first bisulfite converted, which results in unmethylated cytosines being converted to uracils, whereas methylated cytosines are not converted. The bisulfite-converted DNA is amplified, fragmented and hybridized to the arrays. For each CpG site, methylation levels are measured by probes attached to beads, one each for unmethylated and methylated DNA, followed by an allele-specific base extension that includes a fluorescent label. Different labels are used for the T (unmethylated) or C (methylated) alleles. The array is fluorescently stained and scanned, and the intensities of the unmethylated and methylated bead types are measured. DNA methylation values, described as "beta values (β)", are recorded for each locus in each sample and represent the ratio of the intensity of the methylated bead type to the combined locus intensity. The array has several features that make it a powerful option for genome-wide DNA methylation profiling: (i) multi-sample format allows for interrogation of 12 samples on a single BeadChip i.e. it is high-throughput and cost-effective; (ii) low sample input (500ng genomic DNA); (iii) reproducibility (>0.98 between technical replicates) [293, 294]. The genome-wide DNA methylation data is available at http://www.ncbi.nlm.nih.gov/projects/geo/ with accession number GSE73626.

Statistical analysis

R packages minfi (version 1.6.0) [295] and minfiData (version 0.3.4) [296] were used in R version 3.1.2 to convert signal intensity data into methylation data. Beta values were calculated as M/(M+U), where M represents the fluorescent signal of the methylation probe and U accounts

for the methylation signal of the unmethylated probe. The β values range from 0 (no methylation) to 1 (100% methylation) [297].

For the purpose of quality control, CpG probes with detection p-value above 0.01, those located in sex chromosomes and at SNPs, and those with deviation from bimodal distribution were removed from further analysis. To eliminate the difference caused by two type design probes (type I and type II), beta-mixture quantile normalization (BMIQ) method [298] was used to normalize the raw methylation level data.

To identify DMRs, the average beta values were compared between the groups of interest using a student T-test assuming equal variances [299]. Given the small sample size, I was not able to correct for multiple testing as none of the tests reached the strict threshold of genome-wide significance ($10e^{-6}$). Instead, to minimize false positives, I reported the loci with at least 10% methylation difference and p-value ≤ 0.0005. I believe this is an acceptable threshold given the small sample size, to obtain the trends of change and not necessarily genuine associations in this analysis. The identified loci were examined using a linear regression model to determine if they were associated with age. Genomic annotation of DMRs was carried out using the Infinium HumanMethylation450 BeadChip annotation file (http://www.illumina.com).

Gene ontology analysis

DAVID bioinformatics database functional tool [300] was used to identify the enriched gene ontology (GO) terms. Gene symbols were used as input for the analysis. Medium classification stringency was used. Enriched GO terms with a Bonferroni corrected p-value of less than 0.05 were reported.

Phenotype clustering

Hierarchical clustering and multiple dimensional scaling were performed on all individuals after genome-wide methylation distance reduction. The distance was calculated between samples taking into account the genome-wide methylation levels of each sample. Then a matrix containing the pairwise distances between the samples was created. Using singular value decomposition, the matrix was transformed into three matrixes, two of which were orthogonal (U and V) and one was diagonal (D). A new matrix was generated using the multiplication of V and D, containing 12 rows representing each sample and 24 columns representing each dimension. Every two dimensions were plotted against each other. Clustering was further illustrated using heat maps of the top 800 loci with the greatest variations in methylation levels across the entire study population after the same methodology of dimension reduction. To obtain these 800 sites, the cross-population variance for each CpG site was calculated. The clustering was performed for the top 200, 400, 600, 800, and above sites with the highest variance. The top 800 samples resulted in the best visual grouping of the three phenotypes while the numbers beyond this figure did not change the pattern.

Ethics statement

The study protocol was approved by the Health Research Ethics Authority (HREA) of Newfoundland and Labrador (HREA11.311), and a written informed consent was obtained from all the participants.

Results

Subjects

The study included cartilage samples from 5 patients with hip OA, six patients with knee OA, and 7 OA-free hip controls. All subjects were females. The OA-free population were on average about ten years older than the affected group and had a lower BMI. Table 6.1 shows the characteristics of the study population.

Table 6.1- Characteristics of study population

	OA-free Hip	Knee OA	P	Hip OA	P
Age	78.2±11.6	65.3±10.6	0.06	64.4±13.8	0.09
BMI	26.0±4.6	34.6±8.3	0.03	32.3±9.5	0.15

Figures are Mean± standard deviation; the p-values were obtained using student's T-test between OA-affected and OA-free individuals.

Differentially methylated loci

A total of 384,266 CpG sites were included in the final analysis after quality control. A total of 72, 26, and 103 CpG sites was identified from the comparison of knee OA, hip OA, and combined knee/hip OA versus hip controls, respectively. The comparison of hip and knee OA resulted in 67 DMR CpG sites. After removing the overlaps between these analyses, a total of 239 CpG sites showed more than 10% difference in β values among the comparison groups with all $p < 0.0005$. Methylation levels of these sites were not associated with age. Almost half of the sites (53%) showed hypomethylation and the remainders represented hypermethylation in OA compared to controls. This was, however, reversed among the sites with the highest methylation difference, since most of them were hypomethylated in OA as seen in Table 6.2. Table 6.3 presents the β value differences in each comparison. Among the reported sites, 151 sites were annotated to 145 genes; 119 of the sites are located in CpG islands; 79 sites are located in enhancers, 46 in regions with regulatory features, and 28 in DNAse Hypersensitivity sites. From the sites annotated to genes, the majority (46%) were located in gene bodies, 11% were in 5'UTR, 11% were in 3'UTR, 5% were in the first exon, and 27% were located within 1,500 bp upstream the transcription start site. Table 6.2 shows the DMRs with β value differences above 15% between knee/hip OA and OA-free cartilage. The complete list of the DMRs is presented in Table 6.3.

Table 6.2- Top CpG sites differentially methylated in knee/hip OA compared to OA-free cartilage

CpG	Δβ	P-value	Gene symbol	UCSC location group	UCSC island group	Enhancer
cg22669656	-0.22	0.0004	*PGS1*	Body		Yes
cg11905061	0.21	0.0004	*AGAP1*	Body	S_Shore	
cg27390206	-0.21	0.0002	*BLMH*	Body		Yes
cg09140531	-0.21	0.0004				Yes
cg14223856	-0.21	0.0005				Yes
cg13688786	-0.20	0.0003	*MYO18A*	Body		Yes
cg22022821	-0.20	0.0002				
cg10340048	-0.20	0.0002				Yes
cg02464866	-0.20	0.0004			N_Shore	Yes
cg05033952	-0.20	0.0001			N_Shore	
cg19629120	-0.19	0.0005	*EFCAB6*	3'UTR		
cg04973183	-0.19	0.0004				Yes
cg00150785	-0.19	0.0004			N_Shore	Yes
cg12027254	-0.19	0.00004	*TNRC6C*	Body		Yes
cg14068309	-0.19	0.0002	*EIF2B1*	3'UTR		
cg13556934	-0.18	0.0003			N_Shelf	Yes
cg14022778	-0.18	0.0004	*FHAD1*	Body		
cg02017450	0.18	0.0004				Yes
cg23074762	-0.18	0.0002	*CHSY1*	Body		Yes

cg04228742	-0.18	0.0002				
cg22203890	-0.18	0.0005				
cg17611936	0.18	0.0003	*PRKAG2*	Body		
cg07107113	-0.17	0.0004	*FBLIM1*	5'UTR	S_Shore	
cg12582728	-0.17	0.0004				Yes
cg07404223	-0.17	0.0002				Yes
cg25002179	-0.16	0.0005	*STARD13*	5'UTR;Body;TSS1500		Yes
cg17025149	-0.16	0.0001				Yes
cg26043955	-0.16	0.0004				Yes
cg26919145	-0.16	0.0002	*LDLRAD3*	Body		Yes
cg09425279	-0.16	0.0003			N_Shelf	
cg06712559	0.16	0.0004	*AGRN*	Body	Island	Yes
cg02099390	-0.16	0.0002	*OSBPL10*	Body		Yes
cg11805414	-0.16	0.0005				Yes
cg04038680	-0.16	0.0002	*SHISA9*	Body		Yes
cg25341923	-0.15	0.0001	*KRTAP4-7*	TSS1500		
cg14728071	-0.15	0.00004	*MLLT10*	3'UTR		
cg03667871	-0.15	0.0004	*NEK7*	TSS1500	N_Shore	
cg13258453	-0.15	0.0002				Yes
cg23010507	-0.15	0.0001			S_Shelf	Yes
cg12158488	-0.15	0.0002				Yes

Δβ: difference in methylation value between sample groups (OA cartilage - intact); UCSC: University of California, Santa Cruz; 5'-UTR: 5'-untranslated region; N: North; S: South; Shore: immediate surroundings of CpG islands; Shelf: distant surroundings of CpG islands; TSS 200: within 200 bp of transcription start site. TSS 1500: within 1500 bp of transcription start site.

Table 6.3- CpG sites differentially methylated in knee/hip OA compared to healthy cartilage

CpG	Gene	Region	Difference in Beta values			
			Hip/knee OA vs. OA free cartilage	Knee OA vs. OA free cartilage	Hip OA vs. OA free cartilage	Hip OA vs. Knee OA
cg27394794	*A1CF*	Body	-0.103021			
cg04573661	*AASDH*	TSS1500		-0.205279		
cg03213833	*ADARB2*	Body		-0.286976		
cg11905061	*AGAP1*	Body	0.210268			
cg06712559	*AGRN*	Body	0.159346			
cg12899423	*ALX4*	Body	0.147695	0.182835		
cg19894728	*ATXN7L1*	Body	-0.114155			
cg23730617	*B3GALNT1*	TSS1500				-0.185146
cg25764534	*BIN3*	Body		0.104192		
cg27390206	*BLMH*	Body	-0.209088			
cg27143664	*C14orf38*	Body	-0.130948			
cg23276912	*C1orf212*	TSS1500		-0.113101		
cg10916651	*C1QA*	TSS200				0.108015
cg08166362	*C3orf37*	Body				-0.112695
cg22199118	*C8orf34*	5'UTR;1st Exon	-0.104984			
cg13551505	*CACNA2D4*	Body	-0.101028	-0.112074		
cg23327859	*CCNT1*	TSS1500				-0.117283
cg27106290	*CDH12*	TSS200		-0.182634		
cg10303842	*CDH12*	5'UTR;1st Exon		-0.105454		
cg20429981	*CDK11B;LOC728661*	Body;5'UTR				0.132189
cg23074762	*CHSY1*	Body	-0.181762			
cg01413281	*CHUK*	TSS1500		-0.210765		
cg05516020	*CLCN7*	Body	-0.120175			
cg26066361	*CLEC7A*	1st Exon				0.111099
cg06551161	*CLP1;CLP1*	TSS1500				-0.218961

cg12138483	*CLPP*	TSS1500	-0.132099			
cg01487542	*CMAH*	TSS200;Body				0.136506
cg10908116	*COL4A1*	Body			-0.188257	
cg16524108	*COQ6;FAM161B*	TSS1500;Body		-0.135545		
cg27318087	*CPPED1*	Body		-0.16637		
cg17641876	*CPT1B;CHKB*	TSS1500;Body			-0.1261	
cg27410679	*CRHR1*	Body				0.139596
cg01462727	*CYP24A1*	Body		-0.308772		
cg07054208	*DCDC2*	TSS1500				-0.134915
cg19931902	*DEFB129*	TSS1500	-0.123362			
cg20728490	*DNTT*	5'UTR;1stExon	-0.109343	-0.129115		
cg11969108	*DPP6*	Body	-0.125924			
cg19629120	*EFCAB*	3'UTR;3'UTR	-0.194821			
cg14068309	*EIF2B1*	3'UTR	-0.185456			
cg16324018	*ELMOD3; RETSAT*	TSS1500;Body		-0.10651		
cg14671809	*ERC2*	3'UTR		0.21432		
cg17279365	*ESRRG*	5'UTR				0.54009
cg22357390	*ETV6*	Body	-0.106365			
cg11562401	*FAM19A5*	Body		-0.140515		
cg07107113	*FBLIM1*	5'UTR	-0.172251			
cg14022778	*FHAD1*	Body	-0.182901			
cg04988917	*FKSG83*	TSS1500		-0.136044		
cg19177125	*GATA6*	Body				-0.158489
cg06679990	*GLI1*	Body	-0.125178			
cg00362680	*GLI3*	Body	-0.109003			
cg20148127	*GLIS1*	5'UTR		-0.131564		
cg17154975	*GPR133*	Body	-0.131627			
cg07785447	*GSC*	Body				0.143192
cg01163842	*GSC*	Body				0.227057
cg04387592	*GUCA1A*	5'UTR	-0.125863		-0.141964	

cg24974365	*HAND2*	Body				0.345116
cg26729101	*HBQ1*	TSS1500		-0.158386		
cg26495711	*HDAC4*	Body	-0.13355			
cg04625975	*HNRNPA3P1*	Body				0.110269
cg01593673	*HOXB3*	5'UTR		-0.147136		
cg22660542	*HOXC8*	TSS1500				0.168632
cg23685155	*HOXC9*	Body				0.284029
cg02773086	*HOXD3*	TSS1500		0.30235		0.344425
cg01293179	*HOXD8*	Body				-0.219926
cg15991405	*HOXD9*	Body		-0.135009		-0.228901
cg12969193	*HOXD9*	Body				-0.245435
cg06150772	*HRNBP3*	5'UTR	-0.122901	-0.140676		
cg19442493	*HRNBP3*	5'UTR			-0.101717	
cg19815720	*HTR3C*	TSS200	-0.121236	-0.103268		
cg27027427	*IFIT3*	Body;TSS1500			-0.134842	
cg14340103	*IL21*	TSS1500			-0.107352	
cg15152331	*ITGB6*	Body		0.107484		
cg18942298	*JPH2*	Body	-0.144872			
cg01575590	*KCNQ2*	Body	-0.121138			
cg17022362	*KCNQ2*	Body	-0.136883			
cg25341923	*KRTAP4-7*	TSS1500	-0.152813			
cg20634798	*LCE3A*	1stExon				0.201253
cg26135325	*LCE3A*	1stExon				0.196904
cg26919145	*LDLRAD3*	Body	-0.163533			
cg03050981	*LEPR*	5'UTR	-0.136643	-0.155669		
cg25788513	*LOC100188947*	Body		-0.103142		
cg23343309	*LOC150185*	TSS1500		-0.116973		
cg07676709	*LOC404266;HOXB6*	Body		-0.13161		
cg16770054	*MAD1L1*	Body				0.102319
cg26537478	*MEIS1*	Body				0.442717

cg05877497	*MEIS1*	Body				0.537868
cg11362604	*MEIS2*	Body				0.286791
cg03951374	*MEIS2*	1stExon;Body;5'UTR				0.101942
cg14728071	*MLLT10*	3'UTR	-0.152616			
cg18637380	*MTHFD1*	TSS1500	-0.128176			
cg13688786	*MYO18A*	Body	-0.204580			
cg00729885	*NBEA*	Body		0.36108		
cg03667871	*NEK7*	TSS1500	-0.152465	-0.16889		
cg12044531	*NIN*	3'UTR	-0.149347			
cg26489750	*NLRP2*	3'UTR	-0.123049			
cg24307499	*NLRP2*	Body		-0.123286		
cg07429087	*NMUR2*	3'UTR		-0.104922		
cg12986700	*NPFFR2*	5'UTR;Body				0.210622
cg16170380	*OR11A1*	1stExon	-0.110280			
cg12080717	*OR11L1*	1stExon	-0.103754			
cg20867746	*OR51S1*	TSS1500	-0.105627			
cg02099390	*OSBPL10*	Body	-0.159326			
cg05986505	*OSR2*	TSS1500		0.264363		
cg27319188	*PACS1*	Body				0.183381
cg05477457	*PALLD*	Body		0.154197		
cg08189448	*PAPPA*	TSS200				0.189692
cg04462132	*PARK2*	Body			-0.120193	
cg10629004	*PAX1*	3'UTR			0.212762	
cg11164441	*PDE6B*	Body			-0.105268	
cg11320144	*PDYN*	TSS1500		-0.105703		
cg23975251	*PEX14*	Body			-0.149433	
cg03068346	*PFKP*	Body			-0.109451	
cg22669656	*PGS1*	Body	-0.220716			
cg03803861	*PLCXD3*	TSS1500	0.114606			
cg09050331	*PRDM14*	TSS1500		0.145683		

cg17611936	*PRKAG2*	Body	0.176367	0.220172		
cg12304937	*PRKAR1B*	Body	-0.115834			
cg16197388	*PSG3*	3'UTR	-0.1227	-0.142669		
cg19741675	*PSORS1C1*	5'UTR			-0.102053	
cg01394116	*RBM22*	TSS1500		-0.164379		
cg21090457	*ROBO2*	Body	-0.149873	-0.151843		
cg00424286	*RPS6KA2*	Body				0.104957
cg07902192	*RUNX2*	Body	-0.143582			
cg00442802	*SAA3P*	TSS1500	-0.144992			
cg09699193	*SEMA5A*	5'UTR		-0.161465		
cg04038680	*SHISA9*	Body	-0.155409			
cg18074184	*SLC10A4*	TSS1500				-0.13289
cg14995160	*SLC18A2*	3'UTR			-0.103794	
cg20388916	*SLCO2A1*	Body		0.121495		
cg26059632	*SPRR2A*	TSS1500	-0.118555			
cg10661163	*ST7OT4;ST7OT1;ST7*	TSS1500;Body				-0.100255
cg25002179	*STARD13*	5'UTR;Body;TSS1500	-0.1638			
cg18847227	*SUMF1*	TSS1500				-0.132954
cg23083424	*SYNPO2L*	TSS200	-0.119664			
cg22378919	*TBX15*	5'UTR				0.155047
cg06884495	*TDRD9*	TSS200				-0.121617
cg02937313	*THAP1*	TSS1500		-0.168846		
cg13314965	*TM4SF19*	TSS1500	-0.134876			
cg12027254	*TNRC6C*	Body	-0.185722			
cg11559731	*TOX*	TSS1500			0.10719	
cg20071744	*TRAPPC9*	Body				0.110648
cg16422492	*TRIM72;PYDC1*	Body;TSS200		-0.102686		
cg22081905	*TRPM5*	Body				0.104541
cg11937920	*UACA*	Body;TSS1500	-0.149382	-0.160089		
cg18889780	*UCHL1*	TSS1500		0.109206		

cg09305680	*UTP23*	TSS1500				0.270613
cg19907796	*VAX2*	Body	0.126749		0.146154	
cg02774935	*YPEL1*	TSS1500	0.117408			
cg06545761	*ZCCHC14*	3'UTR			-0.135392	
cg16727006	*ZCCHC14*	Body			-0.142668	
cg23238231	*ZNF521*	Body				-0.215518
cg00150785			-0.18856			
cg01478628			-0.124007			
cg01549315			0.141595	0.159916		
cg02017450			0.182854			
cg02097429			-0.115893	-0.120603		
cg02464866			-0.201143			
cg02555944			-0.100254			
cg04228742			-0.181463			
cg04973183			-0.194445	-0.218185		
cg05033952			-0.200003			
cg06410273			-0.147566			
cg07404223			-0.166132			
cg08189043			-0.101672			
cg08236537			-0.129884			
cg09140531			-0.208274			
cg09318857			-0.103781			
cg09425279			-0.163148			
cg09932730			-0.112782	-0.133961		
cg10201735			-0.108855			
cg10340048			-0.203169			
cg11351841			-0.10315			
cg11805414			-0.157269			
cg12158488			-0.151087			
cg12399687			-0.133118		-0.144144	

cg12582728			-0.167141			
cg12760319			-0.120773	-0.154492		
cg13258453			-0.152225		-0.199005	
cg13397166			-0.131556			
cg13556934			-0.183866			
cg13607993			-0.110454			
cg14223856			-0.208172			
cg15272641			-0.1204			
cg15609373			-0.146004			
cg15994519			-0.108552			
cg17025149			-0.163611	-0.190251		
cg21249771			-0.138605			
cg22022821			-0.204191		-0.265695	
cg22203890			-0.176764			
cg23010507			-0.151239			
cg23153661			-0.124432			
cg24757553			0.12996			
cg26043955			-0.163536		-0.202862	
cg26196162			-0.129839			
cg14592399				0.109712		
cg24138867				-0.169935		
cg25843866				0.16252		
cg17171786				0.140878		
cg21776682				0.113997		
cg24415066				0.378201		
cg12754260				-0.140132		-0.152867
cg16138557				-0.131028		
cg13441058				0.134108		
cg07536144				0.163068		
cg08018143				-0.186449		

cg15372218				-0.12534		
cg23348270				0.277439		0.304979
cg11246774				-0.184875		
cg09817024				0.175003		0.175612
cg14356225				-0.351866		
cg08873424				0.167794		
cg15275309				-0.110718		-0.1184
cg00333870				-0.1351		
cg04234597					-0.115774	
cg05334656					0.226141	
cg05573844					0.142033	
cg05552543					-0.117198	
cg27403071					-0.108052	
cg04588138						0.100713
cg10481584						0.104582
cg26469220						-0.143677
cg03913423						-0.14046
cg04517282						0.179701
cg22855900						0.143314
cg14671000						-0.151115
cg01519253						0.208831
cg01419670						-0.149344
cg09889228						-0.119094
cg10111328						-0.259716
cg12547939						0.100249
cg14132364						0.173478
cg26399903						0.120465
cg02622133						0.105884
cg25941985						-0.28141
cg10536898						-0.238791

cg01637125						0.128925
cg02166394						0.250803
cg03787282						0.118125
cg19506686						-0.214439

All p-values≤0.0005; the cell numbers represent the differences between the mean beta values for every two comparison groups.

Functional annotation clustering of the differentially methylated loci

The annotation clustering was conducted for the 145 genes. Table 6.4 shows those GO terms with a Bonferroni corrected p-value<0.05. The most significant terms are related to skeletal and embryonic organ system development and homeobox (HOX). By increasing the classification stringency, the GO terms were classified into 31 clusters, among which only two yielded significant Bonferroni corrected p-values including Embryonic and skeletal system development and HOX genes (enrichment scores 6.48 and 5.52, respectively). The analysis was repeated after the removal of 33 genes which were only identified in the comparison of knee OA and hip OA, to concentrate further on the main objective of the study, i.e. the differences between OA-affected and OA-free cartilage. Similar to the previous clustering, the GO terms included skeletal and embryonic system development, but no HOX genes.

Table 6.4- Enrichment clustering of the differentially methylated genes

Term	Gene count	% of the total genes entered	Genes in each pathway from the results	Fold enrichment	Bonferroni P-value
GO:0048705~skeletal system morphogenesis	14	9.6	*GSC, TBX15, PAX1, GLI3, HOXB3, HOXD9, HOXC8, HOXD8, HOXC9, OSR2, HOXD3, HOXB6, ALX4, RUNX2*	14.96	8.39E-09
GO:0048704~embryonic skeletal system morphogenesis	10	6.9	*HOXD9, HOXB3, GSC, OSR2, TBX15, HOXC9, HOXD3, HOXB6, ALX4, GLI3*	21.00	9.89E-07
GO:0001501~skeletal system development	17	11.7	*CYP24A1, GSC, TBX15, GLI3, PAX1, GLI1, HOXD9, HOXB3, HDAC4, HOXC8, HOXD8, HOXC9, OSR2, HOXD3, HOXB6, ALX4, RUNX2*	6.38	8.98E-06
GO:0048562~embryonic organ morphogenesis	12	8.3	*HOXD9, HOXB3, GSC, OSR2, TBX15, HOXC9, HOXD3, HOXB6, VAX2, ALX4, GLI3, GLI1*	10.80	1.43E-05
GO:0048706~embryonic skeletal system development	10	6.8	*HOXD9, HOXB3, GSC, OSR2, TBX15, HOXC9, HOXD3, HOXB6, ALX4, GLI3*	15.54	1.55E-05
GO:0003002~regionalization	13	8.9	*GSC, VAX2, PAX1, GLI3, GLI1, HOXB3, HOXD9, HOXC8, HOXC9, HOXD8, HOXD3, HOXB6, ALX4*	7.91	9.34E-05
GO:0043565~sequence-specific DNA binding#	20	13.8	*GSC, ESRRG, VAX2, MEIS1, GLI3, GLI1, HOXD9, HOXB3, HDAC4, HOXC8, HOXD8, MEIS2, HOXC9, HAND2,*	4.03	1.02E-04

			GATA6, *HOXD3*, *HOXB6*, *THAP1*, *ALX4*, *ETV6*		
GO:0048568~embryonic organ development	12	8.3	*HOXD9*, *HOXB3*, *GSC*, *OSR2*, *TBX15*, *HOXC9*, *HOXD3*, *HOXB6*, *VAX2*, *ALX4*, *GLI3*, *GLI1*	8.35	2.05E-04
Homeobox#	12	8.3	*HOXD9*, *HOXB3*, *HOXC8*, *GSC*, *HOXD8*, *MEIS2*, *HOXC9*, *HOXD3*, *HOXB6*, *VAX2*, *ALX4*, *MEIS1*	6.96	3.14E-04
GO:0007389~pattern specification process	14	9.6	*GSC*, *VAX2*, *PAX1*, *GLI3*, *GLI1*, *HOXB3*, *SEMA5A*, *HOXD9*, *HOXC8*, *HOXD8*, *HOXC9*, *HOXD3*, *HOXB6*, *ALX4*	6.27	3.57E-04
SM00389:HOX#	12	8.3	*HOXD9*, *HOXB3*, *HOXC8*, *GSC*, *HOXD8*, *MEIS2*, *HOXC9*, *HOXD3*, *HOXB6*, *VAX2*, *ALX4*, *MEIS1*	5.65	5.84E-04
IPR017970:Homeobox, conserved site#	12	8.3	*HOXD9*, *HOXB3*, *HOXC8*, *GSC*, *HOXD8*, *MEIS2*, *HOXC9*, *HOXD3*, *HOXB6*, *VAX2*, *ALX4*, *MEIS1*	6.62	6.07E-04
IPR001356:Homeobox#	12	8.3	*HOXD9*, *HOXB3*, *HOXC8*, *GSC*, *HOXD8*, *MEIS2*, *HOXC9*, *HOXD3*, *HOXB6*, *VAX2*, *ALX4*, *MEIS1*	6.54	6.87E-04
IPR012287:Homeodomain -related#	12	8.3	*HOXD9*, *HOXB3*, *HOXC8*, *GSC*, *HOXD8*, *MEIS2*, *HOXC9*, *HOXD3*, *HOXB6*, *VAX2*, *ALX4*, *MEIS1*	6.46	7.77E-04
GO:0009952~anterior/posterior pattern formation#	10	6.9	*HOXD9*, *HOXB3*, *HOXC8*, *HOXD8*,	8.55	0.002

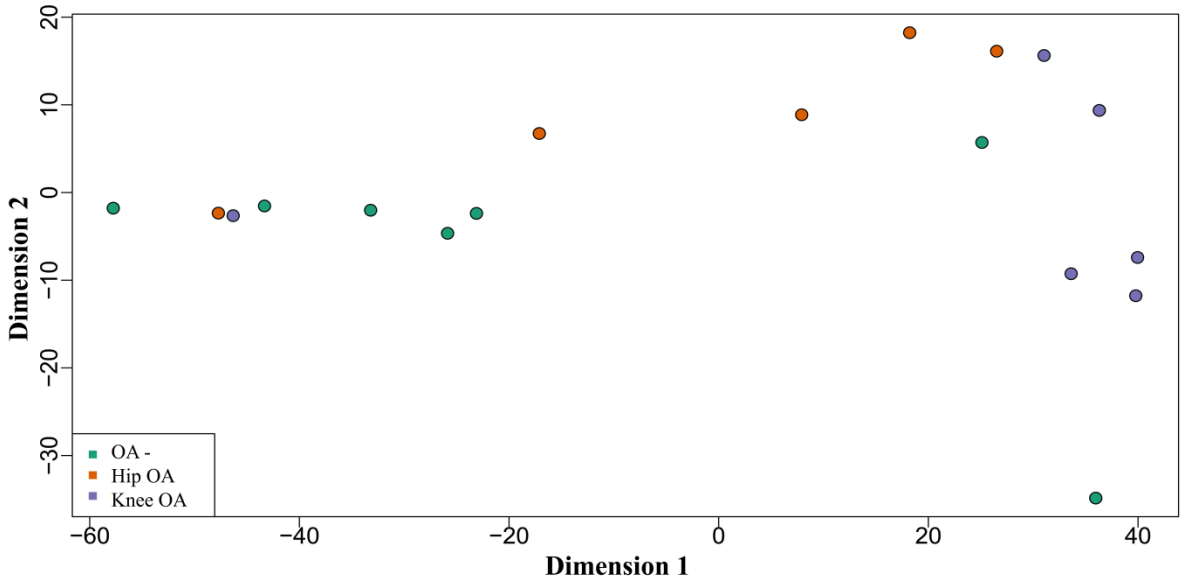
			HOXC9, *HOXD3*, *HOXB6*, *ALX4*, *PAX1*, *GLI3*		
DNA-binding region:Homeobox#	10	6.9	*HOXD9*, *HOXB3*, *HOXC8*, *GSC*, *HOXD8*, *HOXC9*, *HOXD3*, *HOXB6*, *VAX2*, *ALX4*	7.34	0.004
IPR001827:Homeobox protein, antennapedia type, conserved site#	5	3.4	*HOXB3*, *HOXC8*, *HOXD8*, *HOXD3*, *HOXB6*	26.69	0.01
GO:0043009~chordate embryonic development#	13	8.9	*GSC*, *TBX15*, *PAX1*, *GLI3*, *HOXB3*, *HOXD9*, *HOXC9*, *OSR2*, *HAND2*, *GATA6*, *HOXD3*, *HOXB6*, *ALX4*	4.70	0.02
GO:0009792~embryonic development ending in birth or egg hatching#	13	8.9	*GSC*, *TBX15*, *PAX1*, *GLI3*, *HOXB3*, *HOXD9*, *HOXC9*, *OSR2*, *HAND2*, *GATA6*, *HOXD3*, *HOXB6*, *ALX4*	4.66	0.02
GO:0003700~transcription factor activity#	21	14.5	*TBX15*, *GSC*, *ESRRG*, *VAX2*, *MEIS1*, *GLI3*, *GLI1*, *HOXD9*, *HOXB3*, *HOXC8*, *HOXD8*, *MEIS2*, *HOXC9*, *HAND2*, *GATA6*, *HOXD3*, *MLLT10*, *HOXB6*, *ALX4*, *ETV6*, *RUNX2*	2.64	0.02
Developmental protein#	17	11.7	*GSC*, *ZNF521*, *VAX2*, *MEIS1*, *PAX1*, *GLI1*, *HOXD9*, *HOXB3*, *SEMA5A*, *HOXC8*, *HOXD8*, *HOXC9*, *HAND2*, *HOXD3*, *HOXB6*, *ROBO2*, *ALX4*	3.06	0.03

All of the GO terms above were clustered into one annotation cluster with an overall enrichment score of 3.95. #The GO terms were only significant before the removal of the genes differentially methylated between hip OA and knee OA.

Clustering of hip OA, knee OA, and OA-free cartilage

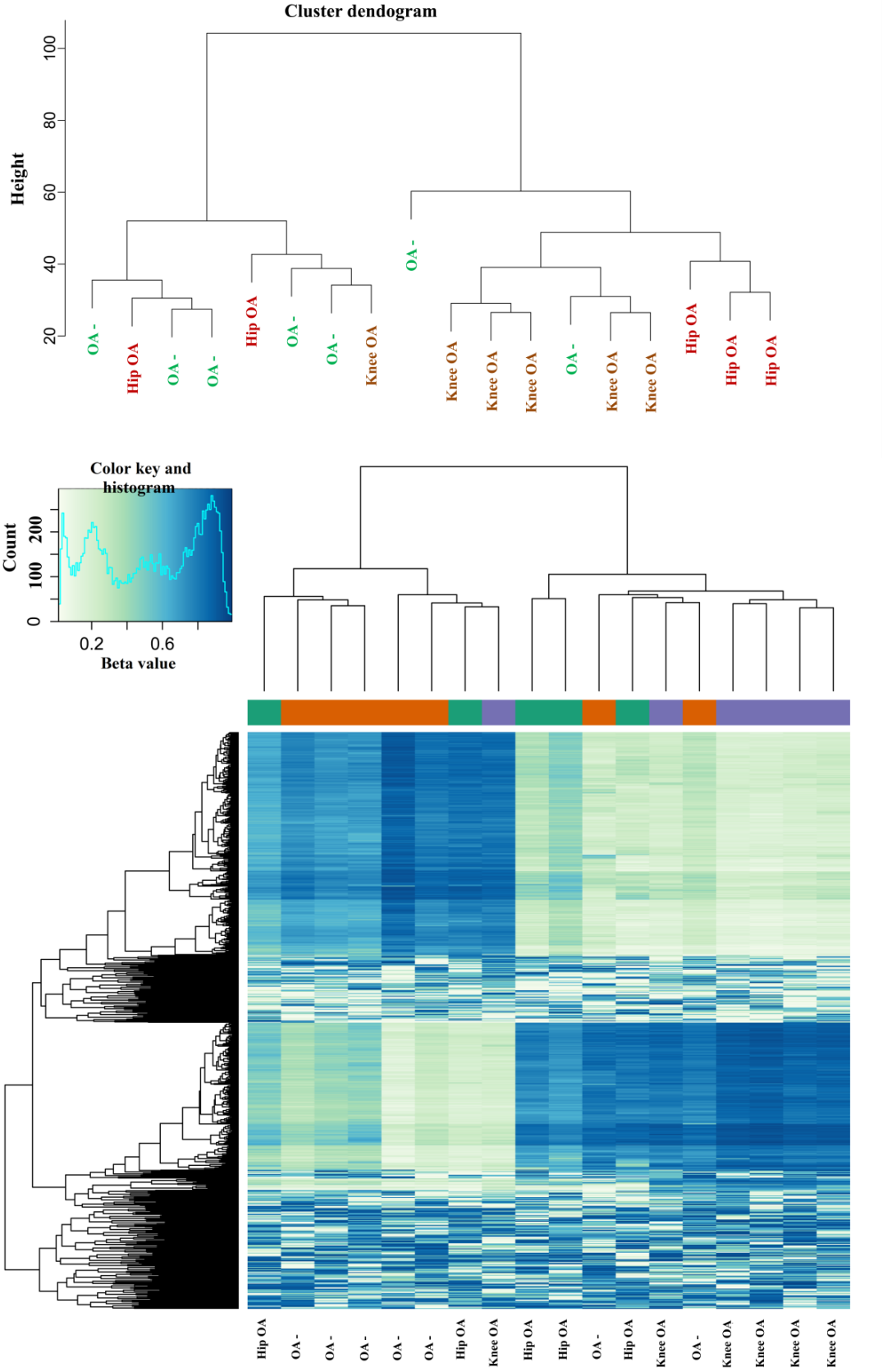
I used multiple dimensional scaling and hierarchical clustering to classify the three phenotypes in the study, i.e. hip OA, knee OA, and OA-free hip cartilage. Due to the small sample size, the classification was not perfect; however, some trends were observed which are worthy of consideration. As it is seen in the plots (Figure 6.1), samples from each phenotype tend to cluster together, although a few outliers exist. Overall, OA-free hip cartilage samples tend to be different from hip OA and knee OA samples. Although hip OA and knee OA samples are very close together, the similarity to OA-free hip cartilage is more seen in hip OA rather than knee OA samples. Scaling beyond the 2^{nd} dimension was not informative (not shown). Similar patterns are observed from the heat map and dendrogram as shown in Figure 6.2.

Figure 6.1- Multiple dimensions scaling of hip OA, knee OA, and OA-free hip cartilage



Similarities between hip OA, knee OA, and OA-free cartilage, drawn from log-spectral decompositions for each subject as represented in the two-dimensional space by multiple dimensional scaling (MDS). Each dot represents one sample. Colors represent the type of involvement and the site samples obtained. X- and Y- axes represent the first and the second dimension reductions.

Figure 6.2- Hierarchical clustering and heat map of hip OA, knee OA, and OA-free controls*



*Top: Cluster dendrogram was created using the genome-wide information; Bottom: Heat map shows the top 800 CpG sites with the most variation across hip OA, knee OA, and OA-free hip cartilage samples. Rows represent CpG sites. Columns represent samples. Dark blue indicates hypermethylation and light blue/white indicates hypomethylation.

Discussion

Our study is one of the few reports on the status of genome-wide methylation of DNA from OA-free and OA-affected human cartilage. I found a number of CpG sites differentially methylated in hip and knee OA, identified the pathways enriched in the sites, and attempted to classify hip and knee OA and OA-free cartilage according to their genome-wide DNA methylation profiling.

The majority of the CpG sites I identified were novel and only about one-fifth of them was reported by the previous epigenetic studies of OA (Table 6.5) [163, 292, 301, 302]. Similarly, most of the genes differentially methylated were not known to play a role in OA, although several of them were previously reported as candidate genes to OA or other bone metabolic conditions. They include cg07902192 in *RUNX2*, involved in the regulation of matrix metallopeptidase 13 in OA cartilage [303], cg03050981 in *LEPR*, associated with knee OA [304], bone marrow density and bone hemostasis [305], cg05516020 in *CLCN7*, associated with bone marrow density [306], cg17279365 in *ESRRG*, associated with multiple bone disease phenotypes [307], cg14340103 in *IL21*, being upregulated in synovial biopsies of rheumatoid arthritis patients [308], cg18637380 in *MTHFD1*, associated with response to osteosarcoma chemotherapy [309], cg10629004 in *PAX1*, associated with congenital scoliosis [310], and cg10908116 in the alpha-1 subunit of collagen type IV gene (*COL4A1*).

Table 6.5- The genes and CpG sites mutually reported between this study and previous epigenome-wide studies of OA

CpG	UCSC reference gene name
cg05877497	*MEIS1*
cg11362604	*MEIS2*
cg09305680	*UTP23*
cg24974365	*HAND2*
cg23685155	*HOXC9*
cg23348270	
cg00729885	*NBEA*
cg24415066	
cg02773086	*HOXD3*
cg22378919	*TBX15*
cg01163842	*GSC*
cg04625975	*HNRNPA3P1*
cg02464866	
cg22199118	*C8orf34*
cg07785447	*GSC*
cg05334656	
cg03913423	
cg07676709	*LOC404266; HOXB6*
cg01462727	*CYP24A1*
cg13556934	
cg14671000	
cg23238231	*ZNF521*
cg10629004	*PAX1*
cg05573844	
cg11562401	*FAM19A5*
cg09889228	
cg06551161	*CLP1*
cg27106290	*CDH12*
cg03213833	*ADARB2*
cg01293179	*HOXD8*
cg10111328	
cg10536898	
cg22660542	*HOXC8*
cg26537478	*MEIS1*
cg16324018	*ELMOD3;RETSAT*
cg19177125	*GATA6*
cg01593673	*HOXB3*

cg16197388	*PSG3*
cg25941985	
cg19506686	
cg01519253	

Although most of the DMR genes in this study were of unknown significance in OA, the functional analysis revealed their enrichment in relevant pathways; i.e. skeletal and embryonic organ system development and homeobox. The latter was only found from the genes differentially methylated in the comparison of knee OA and hip OA methylation. Since homeobox genes are responsible for the body segmentation procedure and specification of lower limbs from upper limbs, it is likely that the DMRs from the knee OA and hip OA comparison represent the processes required for body segment specifications rather than the underlying genetic difference between hip OA and knee OA. Knee and hip cartilage are primarily one tissue but located in different body segments. It is very likely that the epigenomic differences between them be minimal. Since they are located in different segments of the body, chondrocytes or their predecessors must have obtained different modifications of homeobox genes during organogenesis, and these modifications have remained unchanged until an advanced age. Consistent with this, the same gene ontology term has also been reported by Den Hollander *et al*. who made a comparison between genome-wide methylation of articular cartilage DNA from hip OA and knee OA [301]. The small number of genome-wide methylation studies of OA have reported inflammation and immunity [163, 302], transforming growth factor beta signalling [311], and developmental pathways [312]. This study, however, points out the involvement of skeletal system development in OA, which is in accordance with the findings of Delgado-Calle *et al*. who reported the enrichment of genes associated with the development of the appendicular skeleton and limb morphogenesis in a genome-wide methylation study of femoral bone [313]. In this process, the anatomical and physical structures of the skeleton are generated and organized. Skeletal shape, which is tightly regulated by genetics [314], is suggested as a possible mechanism for the influence of genetics in OA incidence. An abnormal center-edge angle and

acetabular dysplasia are shown to be associated with an increased risk of hip OA [315], and a significant difference in the shape of the intercondylar notch between the OA and non-OA individuals is reported [316]. Wnt signalling and bone morphogenetic proteins are among the pathways involved in OA, which also control skeletal development in animal models, and it is suspected that their mechanism of action in OA could be due to their effect on skeletal shape [136].

My clustering analysis of the three phenotypes in the study shows that OA-affected cartilage has a trend of distinct methylation profiling compared to OA-free cartilage. The hip OA and knee OA clustering, however, is not perfect and is suggesting that although hip OA and knee OA could have different epigenomic landscape, they are very similar to each other. The only minor differences observed in hip OA and knee OA might only be joint specific differences that could have been observed if OA-free knee and hip cartilage were studied. Den Hollander *et al.* performed the only comparison of the hip OA and knee OA [310], who successfully grouped hip OA and knee OA into separate clusters. The main conclusion in that study was based on the OA-affected cartilage, and similar to this study the major pathways they identified enriched in the DMRs between hip OA and knee OA, was homeobox, which strengthens the hypothesis that the observed differences in hip and knee OA might be due to differences in the joints rather than the disease status.

My study is limited by several factors. Due to technical issues I studied a small sample size, and as the result, none of the DMRs reported reached Bonferroni corrected significance. The cases and controls were different in age, which might influence the status of DNA methylation. The controls were selected from the population of patients with possible osteoporosis who may not necessarily represent healthy cartilage epigenome. In addition, I did not have information on the

pathological scoring of the OA joints, which could partially explain why the clustering was not perfect. Due to financial issues, I did not validate the methylation levels using alternative methods such as pyrosequencing and did not perform functional experiments to add more to the mechanism of involvement. These limitations will likely be tackled by future studies attempting to replicate and further studying these findings. Despite these limitations, the trends observed in the study are informative and add to the current knowledge on the pathogenesis of OA.

Conclusion

Through a genome-wide methylation study of OA-free and OA-affected human cartilage, I was able to identify a number of CpG sites with methylation changes in OA. I also reported that genes involved in skeletal system morphogenesis are differentially methylated in OA and might be candidate genes for further OA studies. I found a small difference between the overall landscapes of hip OA and knee OA; however, OA-free hip cartilage samples had a trend towards differentiation from the OA-affected ones. These findings shed light on the pathophysiology of OA and can pave the road for further research in the field.

7

Discussion, limitations, and conclusion

7.1. General discussion and concluding remarks

OA is a highly prevalent and seriously disabling disease with a significant burden at both the individual and population levels. This has led to a surge of research in delineating its underlying cause to understand its pathogenesis further in the hope of developing new therapeutic measures for OA. The current thesis has added to the growing body of literature on OA by attempting to replicate the OA associated genetic loci in the Newfoundland and Labrador (NL) population, studying the role of transforming growth factor beta (TGF-ß) signalling pathway in OA patients, and investigating the genome-wide DNA methylation changes in OA.

First, I attempted to replicate the previously reported OA genetic loci using the NL population, a homogenous population with a recent ancestry which makes it ideal for testing the robustness of genetic associations (Chapter 2). The majority of the SNPs that I tested did not show a significant association in the NL population. This might have resulted from several factors: 1) many of the reported SNP associations in complex disease are false positives and replication rates are generally low; 2) since most associated SNPs are thought to be in linkage disequilibrium (LD) with the functional loci and the LD patterns vary across populations, it is very likely that associations do not replicate in different populations; and 3) the definitions of the phenotypes can be varied in different studies. This analysis, however, resulted in the association of two SNPs located in *COL9A3* and *BMP2*, the latter being a member of TGF-ß signalling family. This was of interest as a recent report of a syndromic form of OA had highlighted the *SMAD3* gene, another member of the TGF-ß pathway. My study found that, indeed, a polymorphism in *SMAD3* is involved in generalized OA (Chapter 3). A non-coding SNP located in the last intron of *SMAD3* was found to be significantly associated with the total radiographic burden of OA obtained from the radiographic assessment of the common joints of OA

involvement; namely, hips, knees, hands, and spine. Given the position of this SNP, I assumed that its mechanism of action is quantitative and examined the expression changes of the gene in OA-affected and healthy cartilage (Chapter 4). The results showed higher *SMAD3* mRNA levels in the affected cartilage, suggesting a higher activity of TGF-ß signalling pathway in advanced stages of OA. While the cause of this up-regulation is not clear, my study found that the overexpression of *SMAD3* in OA-affected cartilage was not due to the promoter DNA methylation of the part of the gene promoter studied.

Since the initial findings of the first three projects were signifying the role of TGF-ß signalling in OA pathogenesis, I aimed to understand the connection of the genes I had studied, i.e. *SMAD3* and *BMP2*, in the disease processes, e.g. regulation of matrix metallopeptidase, in the context of the TGF-ß pathway (Chapter 5). My analyses showed that, indeed, the TGF-ß pathway is overactivated in end-stage OA cartilage, which results in up-regulation of *MMP13*, the major enzyme destructing the collagen matrix and leading to cartilage loss in OA. This regulation, however, was found to be not controlled through *SMAD3* signalling, and to be suppressed by BMP regulation in healthy cartilage, which has reduced levels in end-stage OA.

This showed that the pathway was differentially regulated in healthy and OA cartilage. However, it is not clear whether this change, characterized by a lower activity of the BMP pathway and a lack of suppression of destructive signalling by the TGF-ß/MMP13 route, is the cause or consequence of OA, or whether it is directly related to OA at all, only occurring as a by-product of other pathological events during the course of OA development. A longitudinal study design would better address this question. If it is proved to be a causal factor in OA development, the outcome would be promising for designing new OA therapies by specifically targeting the TGF-

ß pathway to reverse this dysregulation, which would result in lower matrix metalloproteinase levels and cartilage degradation.

This thesis also contributed to the limited amount of knowledge about the DNA methylation changes in OA. I investigated the genome-wide methylation levels of cartilage DNA among hip and knee OA and OA free hip cartilage (Chapter 6). I reported CpG sites differentially methylated in OA and showed that the cartilage methylome has a potential to distinguish between OA-affected and non-OA cartilage. Functional clustering analysis of the genes harbouring differentially methylated loci revealed that they are enriched in skeletal system morphogenesis pathway, which could be a potential candidate for further OA studies. One of the ontology terms among these pathways was "limb morphogenesis". This may indicate that the utilized DNA might have contained elements of bone DNA besides cartilage DNA. As obtaining cartilage DNA was extremely a difficult task and those cartilage samples with bone contamination yielded a better result than pure specimens, it is likely that this hypothesis is relevant, but it has to be evaluated by replication studies. There was almost no overlap between the genes found in the epigenomic study of OA and those from other chapters. The explanation for this would be the small sample size in the epigenomic study, which did not allow for identifying all of the potential genes involved in OA. Also, it should be considered that the mechanism of involvement for DNA methylation can be different from DNA sequence variants, and thus they may not necessarily be overlapping.

In conclusion, the current work has shed light on some of the least investigated aspects of human studies of OA including epigenomics of OA and the role of the TGF-ß pathway in OA. The strength of my study is the use of human OA cartilage from end-stage OA patients, whereas the majority of the similar data available previously were obtained from animal models. The

ultimate goal of OA research is to understand OA pathogenesis, and through which, to develop new therapeutic measures for this disabling devastating disease. I am confident that these findings have made us one step closer to this objective.

7.2. Limitations

Despite the useful findings in my study, this work has several limitations that need to be considered while interpreting the results. The main issue is regarding the sample size and representativeness of the samples. Since it is tough to obtain healthy human cartilage specimens, the control population of this study was selected from patients with femoral hip fracture - most commonly due to osteoporosis - whose samples may not necessarily represent healthy cartilage. It is likely that the molecular mechanisms underlying osteoporosis are in effect in those cartilage samples, which could enhance/conceal false/true differences between the OA cartilage and real healthy cartilage, and predispose the results to bias. However, my control samples were examined by pathologists and determined to be not different histologically from healthy cartilage samples. The sample size in the replication study (Chapter 2) was not sufficient to detect associations with small effect size typically expected from complex genetic studies, and it is possible that some genetic associations are missed in my study. This has to be addressed by replicating the study in a larger cohort.

Other issues will regard the complexity of OA and classification of the disease. OA is not a cartilage disease only, and it affects the whole component of the joint including the synovial membranes, subchondral bones, and surrounding muscles. This is while my investigations were only limited to the cartilage (Chapters 4-6). In addition, I did not investigate gene regulations

beyond the mRNA levels (e.g. protein levels and modifications which are considered to be closer to the phenotype, i.e. OA). The OA population who took part in the study were selected from the end stage group who were undergoing total joint replacement surgery, and the result of the study could not represent the earlier stages of the disease (Chapters 2, 4-6). The diagnosis of OA was based on clinical guidelines (except for Chapter 3), whereas alternative strategies to detect OA, such as radiography, are commonly used by other researchers, leading to a common source of variability in the findings of my study and those of others. It is worthy of consideration that in none of the chapters endophenotypes or deep phenotyping of OA was taken into account in the analyses, as no information on these important subjects had been obtained from the participating patients.

7.3. Future directions

The work in this thesis is a step towards future investigations in the field of genomics and epigenomics of OA. Aside from the fact that the findings of this thesis will have to be replicated and confirmed in independent studies, several other hypotheses remain to be tested.

In the first project, two SNPs showed a trend of association with OA in the NL population. The blood sample size in NFAOS has reached 550 by now, which could provide a greater power for conducting a replication study. The *SMAD3* SNP that was identified in the Chingford cohort (Chapter 3) may also be tested to find whether it confers an association in the NL population. Given I found a differential expression in OA and healthy cartilage for *SMAD3,* the effect of that SNP on the gene expression regulation can be tested ideally using a gene expression reporter assay in chondrocytes. Other experiments which can likely add more to the regulation of *SMAD3*

in OA is measuring its protein levels as well as the phosphorylation levels of the SMAD3 protein as an indicator for the activity of the TGF-ß/SMAD3 signalling. It is also of interest to examine the relationship between the components of TGF-ß pathway and the expression of the extracellular molecules in collagen including collagen and Aggrecan. Currently, Dr. Zhai's lab is conducting an experiment on human cartilage samples obtained from OA joints to examine the effect of treatment with TGF-ß and BMP proteins on the regulation of other components of the pathway and cartilage matrix genes expressions. This will likely further expand my understanding of the TGF-ß pathway in OA development.

The last topic that remains to be further investigated is the role of the genes involved in skeletal system morphogenesis in OA. My epigenomic study (Chapter 6) together with a few other studies support the role of these genes in OA, whereas the area remains to be investigated. Functional studies will likely determine the effect of the methylation sites in those genes on gene expressions and association studies will identify the genomic variations within those regions that are associated with OA. Investigations of other fields that can proceed from this thesis include quantitative trait loci, genome-wide gene expressions, histone modifications, and DNA methylations using high-throughput technologies and the study of rare genomic variants and copy number variations, none of which have by far been investigated in OA genomics. Other factors that can be used for unraveling the etiology of OA, is the use of gene-gene and gene-environment interaction studies, as well as the use of endophenotypes and deep phenotyping to enhance the study power in detecting OA candidate genes. To my best of knowledge, the latter (deep phenotyping) has never been used in OA research despite its obvious potential in a heterogeneous condition like OA.

References

1. Paitzker K. Pathology of osteoarthritis. In: Osteoarthritis. Edited by K Brandt, M Doherty, L.S. Lohmander, 2nd edn. Oxford: Oxford University Press; 2003: 49-58.

2. Litwic A, Edwards MH, Dennison EM, Cooper C. Epidemiology and burden of osteoarthritis. Br Med Bull 2013; 105:185-99.

3. National Collaborating Centre for Chronic Conditions. Osteoarthritis: national clinical guideline for care and management in adults. London: Royal College of Physicians; 2008.

4. Kraus VB, Blanco FJ, Englund M, Karsdal MA, Lohmander LS. Call for standardized definitions of osteoarthritis and risk stratification for clinical trials and clinical use. Osteoarthritis Cartilage 2015; 23(8):1233-41.

5. McAlindon D. Osteoarthritis: definitions and criteria. Ann Rheum Dis 1989; 48: 531-532.

6. Peyron J G. The epidemiology of osteoarthritis. In: Moskowitz R W, Howell D S, Goldberg V M, Mankin H S, eds. Osteoarthritis: diagnosis and management. Philadelphia and London: Saunders, 1984: 9-27.

7. Hutton CW. Osteoarthritis: the cause not result of joint failure? Ann Rheum Dis 1989; 48(11): 958-961.

8. Cubukcu D, Sarsan A, Alkan H. Relationships between Pain, Function and Radiographic Findings in Osteoarthritis of the Knee: A Cross-Sectional Study. Arthritis 2012; 2012:984060.

9. Kellgren JH, Lawrence JS. Radiological assessment of osteo-arthrosis. Ann Rheum Dis 1957; 16(4): 494–502.

10. Haygarth J. Clinical History of Diseases. London: Gadell and Davies; 1805.

11. Cecil RL, Archer BH. Classification and Treatment of Chronic Arthritis. JAMA 1926; 87:741-746.

12. Heberden W. Commentaries on History and Cure of Diseases. London: T Payne; 1802.

13. Kellgren JH, Moore R. Generalized osteoarthritis and Heberden's nodes. British Medical Journal 1952; 1(4751): 181–187.

14. Altman R, Asch E, Bloch D, Bole G, Borenstein D, et al. Development of criteria for the classification and reporting of osteoarthritis. Classification of osteoarthritis of the knee. Diagnostic and Therapeutic Criteria Committee of the American Rheumatism Association. Arthritis and rheumatism 1986; 29(8):1039-1049.

15. Dougados M, Nakache JP, Gueguen A. Criteria for generalized and focal osteoarthritis. Rev Rhum Engl Ed 1996; 63:569-75.

16. Egger P, Cooper C, Hart DJ, et al. Patterns of joint involvement in osteoarthritis of the hand: the Chingford Study. The Journal of Rheumatology 1995; 22(8): 1509-1513.

17. Cooper C, Egger P, Coggon D, et al. Generalized osteoarthritis in women: pattern of joint involvement and approaches to definition for epidemiological studies. The Journal of Rheumatology 1996; 23(11): 1938–1942.

18. Mow VC, Ratcliffe A, Woo SL. Biomechanics of diarthrodial joints (Vol. 1). Springer Science & Business Media. Chicago; 2012

19. Muir H. The chondrocyte, architect of cartilage. Biomechanics, structure, function and molecular biology of cartilage matrix macromolecules. Bioessays 1995; 17, 1039–1048

20. Buchanan, WW, Smith FC. Understanding the normal joint: a basis for diagnosis, Canad J Diagnosis 1995; 12:45–53.

21. Ghadially FN. Fine structure of joints, in: The Joints and Synovial Fluid, Vol. 1, L. Sokoloff (Ed.), pp. 105–168. Academic Press, New York; 1978.

22. Mow VC, Ratcliffe A, Poole AR. Cartilage and diarthrodial joints as paradigms for hierarchical materials and structures. Biomaterials 1992;13(2):67-97.

23. Poole AR. An introduction to the pathophysiology of osteoarthritis. Front Biosci 1999; 4:D662-70.

24. Maroudas A. Balance between swelling pressure and collagen tension in normal and degenerative cartilage, Nature 1976; 260: 808–809.

25. Muir H. Current and future trends in articular cartilage research and osteoarthritis, in: Articular Cartilage Biochemistry, K. E. Kuettner, R. Schleyenbach and R. Hascall (Eds). Raven Press, New York; 1986: 423.

26. Bullough PC. The pathology of osteoarthritis, in: Osteoarthritis Diagnosis and Medical/ Surgical Management, 2nd Edition, R. W. Moskowitz, D. S. Howell, V. M. Gouldberg and H. J. Mankin (Eds) 1992; 39–69. W B Saunders, Philadelphia.

27. Kempson GE, Spivey CJ, Swanson SA V, et al. Patterns of cartilage stiffness on normal and degenerate human femoral heads. J Biomech 1971; 4: 597–609.

28. Adams ME, Brandt KD. Hypertrophic repair of canine articular cartilage in osteoarthritis after anterior cruciate ligament transaction. J Rheum 1991; 18: 428–435.

29. Mankin HJ, Mow VC, Buckwalter JA, et al. Form and function of articular cartilage, in: Orthopaedic Basic Science, S. Simon (Ed.) 1994: p. 1. American Academy of Orthopaedic Surgeons, Chicago.

30. Radin EL, Paul IL. Response of joints to impact loading. I. In vitro wear. Arthritis Rheum 1971; 14(3):356-62.

31. Landells JW. The bone cysts in osteoarthritis. J Bone Jt Surg 1953; 35B:643-649.

32. Rhaney K, Lamb DW. The cysts of osteoarthritis of the hip. A radiological and pathological study. J Bone Joint Surg 1955; 37B: 663–675.

33. Evans CH, Mears DC, McKnightr JL. A preliminary ferro graphic study of the wear particles in human synovial fluid. Arthritis Rheum 1981; 24: 912-918.

34. Evans CH. Cellular mechanisms of hydrolysis enzyme release in proteoglycan, Semin. Arthritis Rheum 1982; 11 (Suppl. 1): 93–95.

35. Cooper NS, Soren A, McCain C, et al. Diagnostic specificity of synovial lesions, Human Pathology 1981; 12: 314–328.

36. Dean G, Hoyland JA, Denton J, et al. Mast cells in the synovium and synovial fluid in osteoarthritis,Brit. J Rheumatol 1993; 32: 671–675.

37. Kinsella TD, Baum J, Ziff M. Studies of isolated synovial lining cells of rheumatoid and non rheumatoid synovial membranes, Arthritis Rheum 1970; 13: 734–753.

38. Eghtedari AA, Bacon PA, Collins A. Immunoblasts in synovial fluid and blood in the rheumatic diseases, Ann. Rheum Dis 1980; 39: 318–322.

39. Bunch TW, Hunder GG, McDuf e FC, et al. Synovial fluid complement determination as a diagnostic aid in inflammatory joint disease, Mayo Clin Proc 1974; 49:715–720.

40. Peach CA, Carr AJ, Loughlin J. Recent advances in the genetic investigation of osteoarthritis. Trends Mol Med 2005; 11(4):186-91.

41. Klippel JK. Primer on the Rheumatic Diseases, Arthritis Foundation Publication. Edition 12; 2001.

42. Johnston SA. Osteoarthritis: joint anatomy, physiology, and pathobiology. Veterinary Clinics of North America: Small Animal Practice 1997; 27(4): 699-723.

43. Szoeke CEI, Cicuttini FM, Guthrie JR, Clark MS, Dennerstein L. Factors affecting the prevalence of osteoarthritis in healthy middle-aged women: data from the longitudinal Melbourne Women's Midlife Health Project. Bone 2006; 39(5): 1149-1155.

44. Gottesman II, Gould TD. The endophenotype concept in psychiatry: etymology and strategic intentions. American Journal of Psychiatry 2003; 160: 636–645.

45. Altman RD, Hochberg M, Murphy WA, Wolfe F, Lequesne M. Atlas of individual radiographic features in osteoarthritis. Osteoarthritis Cartilage 1995; 3(Supplement A): 3-70.

46. Rousseau J Ch, Garnero P. Biological markers in osteoarthritis. Bone 2012; 51: 265-277.

47. Altman R, Alarcón G, Appelrouth D, Bloch D, Borenstein D, Brandt K, et al. The American College of Rheumatology criteria for the classification and reporting of osteoarthritis of the hand. Arthritis Rheum. 1990; 33(11):1601-10.

48. Altman R, Alarcón G, Appelrouth D, Bloch D, Borenstein D, Brandt K, Brown C, Cooke TD, Daniel W, Feldman D, et al. The American College of Rheumatology criteria for the classification and reporting of osteoarthritis of the hip. Arthritis Rheum. 1991; 34(5):505-14.

49. Felson D, et al. Osteoarthritis: new insights. Part 2: treatment approaches. Ann Intern Med 2000; 133(9):726-37.

50. Puett DW, Griffin MR. Published trials of nonmedicinal and noninvasive therapies for hip and knee osteoarthritis. Ann Intern Med. 1994; 121(2):133-40.

51. Minor MA, Hewett JE, Webel RR, Anderson SK, Kay DR. Efficacy of physical conditioning exercise in patients with rheumatoid arthritis and osteoarthritis. Arthritis Rheum 1989; 32:1396–405.

52. Bennell KL, Hinman RS. A review of the clinical evidence for exercise in osteoarthritis of the hip and knee. Journal of Science and Medicine in Sport 2011; 14(1): 4-9.

53. Kaptchuk RJ. Acupuncture: theory, efficacy, and practice. Ann Intern Med 2002; 136(5):374-83.

54. American College of Rheumatology Subcommitteee on Osteoarthritis Guidelines. Recommendations for the medical management of osteoarthritis of the hip and knee: 200 update. Arthritis Rheum 2000; 43(9):1905-15.

55. Amadio P, Cummings DM. Evaluation of acetaminophen in the management of osteoarthritis of the knee. Curr Ther Res 1983; 34:59–66.

56. Freidman DM, Moore ME. The efficacy of intra-articular steroids in osteoarthritis: a double-blind study. J Rheumatol 1980; 7: 850–6.

57. Wobig M, Dickhut A, Maier R, Vetter G. Viscosupplementation with hylan G-F 20: a 26-week controlled trial of efficacy and safety in the osteoarthritic knee. Clin Ther 1998; 20(3):410-23.

58. Reginster JY, et al. Long-term effects of glucosamine sulphate on osteoarthritis progression: a randomised, placebo-controlled clinical trial. Lancet 2001; 357(9252):251-6.

59. uckwalter JA, Lohmander S. Operative treatment of osteoarthritis. Current practice and future development. J Bone Joint Surg [Am] 1994; 76:1405–18.

60. Meyer J, Farmer R, Prenger MC. Risedronate but not alendronate slows disease progression in the guinea pig model of primary osteoarthritis. J Bone Miner Res 2001; SA472: 5305. [Abstract]

61. Yu LP Jr, Smith GN Jr, Brandt KD, Myers SL, O'Connor BL, Brandt DA. Reduction of the severity of canine osteoarthritis by prophylactic treatment with oral doxycycline. Arthritis Rheum 1992; 35(10):1150-9.

62. Andre F. Steinert, Ulrich Nöth, and Rocky S. Tuan. Concepts in Gene Therapy for Cartilage Repair. Injury 2008; 39(Suppl 1): S97–113.

63. Ryan ME, Greenwald RA, Golub LM. Potential of tetracyclines to modify cartilage breakdown in osteoarthritis. Curr Opin Rheumatol 1996; 8(3):238-47.

64. Brittberg M, Lindahl A, Nilsson A, et al. Treatment of deep cartilage defects with autologous chondrocyte transplantation. N Engl J Med 1993; 331(14):889-95.

65. Heine J. Arthritis deformans. Vfrchow's Arch Path Anat 1926; 260: 521-663.

66. van Saase JL, van Romunde LK, Cats A et al. Epidemiology of osteoarthritis: Zoetermeer survey. Comparison of radiological osteoarthritis in a Dutch population with that in 10 other populations. Ann Rheum Dis 1989; 48(4): 271–280.

67. Jordan K, Sawyer S, Coakley P, Smith H, Cooper C, Arden NK. The use of conventional and complementary treatements for knee osteoarthritis in the community. Rheumatology (Oxford); Rheumatology (Oxford) 2004; 43(3):381-4.

68. Arden N, Nevitt MC. Osteoarthritis: Epidemiology. Best Pract Res Clin Rheumatol 2006; 20: 3-25.

69. Kopec JA, Rahman MM, Berthelot J, et al. Descriptive epidemiology of osteoarthritis in British Columbia, Canada. Journal of Rheumatology 2007; 34(2):386-393.

70. Oen K, Postl B, Chalmers IM, et al. Rheumatic diseases in an Inuit population. Arthritis Rheum 1986; 29(1):65-74.

71. Arthritis community research & evaluation unit, Toronto Western Research Institute (2010) Prevalence of Arthritis and Rheumatic Diseases around the World, A Growing Burden and Implications for Health Care Needs. Available from: http://www.modelsofcare.ca/pdf/10-02.pdf (Accessed Sept. 2015)

72. The arthritis alliance of Canada. The impact of arthritis in Canada: today and over the next 30 years. Available from: http://www.ergoresearch.com/wp-content/uploads/2012/04/Impact%20on%20arthrisis%20in%20Canada_Today%20and%20over%20the%20next%2030%20years.pdf (accessed October 2015)

73. Wilson MG, Michet Jr CJ, Ilstrup DM, Melton III LJ. Idiopathic symptomatic osteoarthritis of the hip and knee: a population-based incidence study. Mayo Clinic Proceedings 1990; 65(9): 1214–1221.

74. Oliveria SA, Felson DT, Reed JI, et al. Incidence of symptomatic hand, hip, and knee osteoarthritis among patients in a health maintenance organization. Arthritis Rheum 1995; 38(8): 1134–1141

75. Puenpatom RA, Victor TW. Increased prevalence of meta-bolic syndrome in individuals with osteoarthritis: an analysis of NHANES III data. Postgrad Med 2009; 121(6):9-20.

76. Sowers M, et al. Knee osteoarthritis in obese women with cardiometabolic clustering. Arthritis Rheum 2009; 61(10): 1328-36.

77. Center for disease control and prevention. National Public Health Agenda for Osteoarthritis. 2010, Available from: http://www.cdc.gov/arthritis/docs/OAagenda.pdf (accessed August 2015)

78. United States Bone and Joint Decade. The burden of musculoskeletal diseases in the United States. Rosemont, IL:American Academy of Orthopaedic Surgeons; 2008.

79. Stoddard SF, Jans L, Ripple J, Kraus L. Chartbook on Work and Disability in the United States. In Washington, DC: U.S. National Institute on Disability and Rehabilitation Research 1998; InfoUse report.

80. Ackerman IN, Osborne RH. Obesity and increased burden of hip and knee joint disease in Australia: results from a national survey. BMC Musculoskelet Disord 2012; 213:254.

81. Hootman JM, Helmick CG. Projections of US prevalence of arthritis and associated activity limitations. Arthritis Rheum 2006; 54: 226-9.

82. The arthritis society: Arthritis in Canada, facts and figures. 2015; Available from: http://arthritis.ca/understand-arthritis/arthritis-facts-figures (accessed Sept. 2015)

83. Reijman M, Pols HA, Bergink AP, Hazes JM, Belo JN, Lievense AM, Bierma-Zeinstra SM. Body mass index associated with onset and progression of osteoarthritis of the knee but not of the hip: the Rotterdam Study. Ann Rheum Dis 2007; 66:158–162.

84. Reijman M, Hazes JM, Pols HA, Bernsen RM, Koes BW, Bierma-Zeinstra SM. Role of radiography in predicting progression of osteoarthritis of the hip: prospective cohort study. BMJ 2005; 330:1183.

85. Felson DT, Zhang Y, Hannan MT, et al. The incidence and natural history of knee osteoarthritis in the elderly. Arthritis and Rheumatism 1995; 38: 1500–1505.

86. Quintana JM, Arostegui I, Escobar A, Azkarate J, Goenaga JI, Lafuente I. Prevalence of knee and hip osteoarthritis and the appropriateness of joint replacement in an older population. Archives of internal medicine 2008; 168(14): 1576-1584.

87. Hartz AJ, Fischer ME, Bril G, Kelber S, Rupley D Jr, Oken B, Rimm AA. The association of obesity with joint pain and osteoarthritis in the HANES data. J Chronic Dis 1986; 39(4):311-9.

88. Newman AB, Haggerty CL, Goodpaster B, et al. Strength and muscle quality in a well-functioning cohort of older adults: the health, aging and body composition study. Journal of the American Geriatrics Society 2003; 51: 323–330.

89. Sharma L, Lou C, Felson DT, et al. Laxity in health and osteoarthritic knees. Arthritis and Rheumatism 1999; 42: 861–870.

90. Loeser RF, Shakoor N. Aging or osteoarthritis: which is the problem? Rheumatic Diseases Clinics of North America 2003; 29: 653–673.

91. Oliveria SA, Felson DT, Reed JI et al. Incidence of symptomatic hand, hip, and knee osteoarthritis among patients in a health maintenance organization. Arthritis and Rheumatism 1995; 38: 1134–1141.

92. Ledingham J, Dawson S, Preston B, et al. Radiographic progression of hospital referred osteoarthritis of the hip. Ann Rheum Dis 1993; 52: 263–267.

93. Davis MA, Ettinger WH, Neuhaus JM, Hauck WW. Sex differences in osteoarthritis of the knee. The role of obesity. Am J Epidemiol 1988; 127(5):1019.

94. Maillefert JF, Gueguen A, Monreal M, Nguyen M, Berdah L, Lequesne M, Mazieres B, Vignon E, Dougados M. Sex differences in hip osteoarthritis: results of a longitudinal study in 508 patients. Ann Rheum Dis 2003; 62(10):931.

95. Cauley J, Kwoh C, Egeland G et al. Serum sex hormones and severity of osteoarthritis of the hand. The Journal of Rheumatology 1993; 20:1165–1170.

96. Linn S, Murtaugh B, Casey E. Role of sex hormones in the development of osteoarthritis. PM&R. 2012;4(5):S169-73.

97. Kinney RC, Schwartz Z, Week K, Lotz MK, Boyan BD. Human articular chondrocytes exhibit sexual dimorphism in their responses to 17β-estradiol. Osteoarthritis and cartilage. 2005;13(4):330-7.

98. Raz P, Nasatzky E, Boyan BD, Ornoy A, Schwartz Z. Sexual dimorphism of growth plate prehypertrophic and hypertrophic chondrocytes in response to testosterone requires metabolism to dihydrotestosterone (DHT) by steroid 5-alpha reductase type 1. Journal of cellular biochemistry. 2005;95(1):108-19.

99. Felson DT, Lawrence RC, Dieppe PA, et al. Osteoarthritis: new insights. Part 1: the disease and its risk factors. Annals of Internal Medicine 2000; 133: 635–646.

100. Spector TD, Hart DJ, Doyle DV. Incidence and progression of osteoarthritis in women with unilateral knee disease in the general population: the effect of obesity. Ann Rheum Dis 1994; 53(9):565–568.

101. Cooper C, Snow S, McAlindon TE, et al. Risk factors for the incidence and progression of radiographic knee osteoarthritis. Arthritis and Rheumatism 2000; 43(5): 995–1000.

102. Felson DT, Zhang Y, Anthony JM, et al. Weight loss reduces the risk of symptomatic knee osteoarthritis in women. Annals of Internal Medicine 1992; 116: 535–539.

103. Karlson EW, Mandl LA, Aweh GN, et al. Total hip replacement due to osteoarthritis: the importance of age, obesity, and other modifiable risk factors. The American Journal of Medicine 2003; 114: 93–98.

104. Oliveria SA, Felson DT, Cirillo PA, et al. Body weight, body mass index, and incident symptomatic osteoarthritis of the hand, hip, and knee. Epidemiology 1999; 10: 161–166.

105. Hart DJ, Doyle DV, Spector TD. Association between metabolic factors and knee osteoarthritis in women: the Chingford study. The Journal of Rheumatology 1995; 22: 1118–1123.

106. Spector TD, Hart DJ, Nandra D, et al. Low-level increases in serum creative protein are present in early osteoarthritis of the knee and predict progressive disease. Arthritis and Rheumatism 1997; 40: 723–727.

107. Collins KH, Hart DA, Reimer RA, Seerattan RA, Herzog W. Response to diet-induced obesity produces time-dependent induction and progression of metabolic osteoarthritis in rat knees. J Orthop Res; doi: 10.1002/jor.23103

108. Griffin TM, Guilak F. Why is obesity associated with osteoarthritis? Insights from mouse models of obesity. Biorheology 2008;45(3-4):387-98.

109. Buckwalter JA, Ine NE, Gordon SL. Exercise as a cause of osteoarthritis. In Osteoarthritic Disorders. Kuettner KE, Goldberg VM, Editors 1995. American Academy of Orthopaedic Surgeons, Rosemont IL1995; 405-417.

110. Palmer KT. Occupational activities and osteoarthritis of the knee. Br Med Bull 2012; 102:147-70.

111. Heliovaara M, Makela M, Impivaara O, Knekt P, Aroma A, Sievers K. Association of overweight, trauma and workload with coxarthrosis: a health survey of 7,217 persons. Acta Orthop Scand 1993; 64(5):513-518.

112. Coggon D, Croft P, Kellingray S, et al. Occupational physical activities and osteoarthritis of the knee. Arthritis and Rheumatism 2000; 43(7): 1443–1449.

113. Imeokparia RL, et al. Physical activity as a risk factor for osteoarthritis of the knee. Ann. Epidemiology 1994; 4(3):221-230.

114. Buckwalter JA. Activity vs. rest in the treatment of bone, joint and soft tissue injuries. Iowa Ortho J 1995; 15:2942.

115. Hannan MT, Felson DT. Anderson JJ, Naimark A. Habitual physical activity is not associated with knee osteoarthritis: the Framingham study. J Rheum 1993; 20(4) :704-709.

116. Semanik PA, Chang RW, Dunlop DD. Aerobic activity in prevention and symptom control of osteoarthritis. PM R 2012; 4(5 Suppl):S37-44.

117. Racunica TL, Teichtahl AJ, Wang Y, Wluka AE, English DR, Gile GG, et al. Effect of physical activity on ar¬ticular knee joint structures in community-based adults. Arthritis & Rheumatism 2007; 57: 1261-1268.

118. Ding C, Jones G, Wluka A, Cicut¬tini F. What can we learn about osteoarthritis by studying people from the healthy to those with early disease? Curr Opin Rheumatol 2010; 22(5):520-7.

119. Felson DT, Niu J, Clancy M, Sack B, Aliabadi P, Zhang Y. Effect of recreational physical activities on the development of knee osteoarthritis in older adults of different weights: the Framingham Study. Arthritis Rheum. 2007;57(1):6.

120. Buckwalter JA. Osteoarthritis and articular cartilage use, disuse, and abuse: experimental studies. J Rheumatol Suppl 1995; 43:13.

121. Gelber AC, Hochberg MC, Mead LA, et al. Joint injury in young adults and risk for subsequent knee and hip osteoarthritis. Annals of Internal Medicine 2000; 133: 321–328.

122. Doherty M, Watt I, Dieppe P. Influence of primary generalized osteoarthritis on development of secondary osteoarthritis. Lancet 1983; 2(8340): 8–11.

123. Harris WH. Etiology of osteoarthritis of the hip. Clinical Orthopaedics and Related Research 1986; 213: 20–33.

124. Andriacchi TP. Dynamics of knee malalignment. The Orthopedic Clinics of North America 1994; 25: 395– 403.

125. Felson DT, McLaughlin S, Goggins J, et al. Bone marrow edema and its relation to progression of knee osteoarthritis. Annals of Internal Medicine 2003; 139: 330–336.

126. Sharma L, Song J, Felson DT, et al. The role of knee alignment in disease progression and functional decline in knee osteoarthritis. The Journal of the American Medical Association 2001; 286: 188–195.

127. Felson DT, Zhang Y. An update on the epidemiology of hip and knee osteoarthritis with a view to prevention. Arthritis Rheum 1998; 41(8): 1343-1355.

128. Hoaglund F T, Yau A C M. Wong W L. Osteoarthritis of the hip and other joints in southern Chinese in Hong Kong: incidence and related factors. Bone Joint Surg [AM] 1973; 55: 545-57

129. Yoshimura N. Epidemiology of osteoarthritis in Japan : the ROAD study. Clin Calcium 2011;21(6):821-5

130. Ho-Pham LT, Lai TQ, Mai LD, Doan MC, Pham HN, Nguyen TV. Prevalence of radiographic osteoarthritis of the knee and its relationship to self-reported pain. PloS one. 2014; 10;9(4):e94563.

131. Charcot J. The New Sydenham Society; Clinical lectures on senile and chronic diseases. London 1881.

132. Duckworth D. A treatise on gout. London: Charles Griffin and Company; 1889:71.

133. Stecher RM. Heberden's nodes: heredity in hypertrophic arthritis of the finger joints. Am J Med Sci 1941; 201:801.

134. Kellgren JH, Lawrence JS, Bier F. Genetic factors in generalized osteoarthritis. Ann Rheum Dis 1963; 22:237-55.

135. Guo S-W. Sibling recurrence risk ratio as a measure of genetic effect: caveat emptor! Am J Hum Genet 2002; 70 (3): 818–819

136. Valdes AM, Spector TD. The contribution of genes to osteoarthritis. Med Clin North Am 2009; 93(1): 45-66.

137. Zhai G, Hart DJ, Kato BS, et al. Genetic influence on the progression of radiographic knee osteoarthritis: a longitudinal twin study. Osteoarthritis Cartilage 2007; 15 (2): 222-225

138. Zhai G, Ding C, Stankovich J, et al. The genetic contribution to longitudinal changes in knee structure and muscle strength: a sibpair study. Arthritis Rheum 2005; 52: 2830-2834

139. Valdes AM. Genetic Markers of Osteoarthritis. Current Rheumatology Reviews 2010; 6(4): 257-267.

140. Heritability (2015) SNPedia. Available from: http://www.snpedia.com/index.php/Heritability last accessed: 30th August 2015

141. Valdes AM, Spector TD. Genetic epidemiology of hip and knee osteoarthritis. Nature Reviews Rheumatology 2011; 7(1), 23-32.

142. Lee YH, Rho YH, Choi SJ, Ji JD, Song GG. Osteoarthritis susceptibility loci defined by genome scan meta-analysis. Rheumatol Int 2006; 26 (11): 996–1000.

143. Loughlin J. Polymorphism in signal transduction is a major route through which osteoarthritis susceptibility is acting. Curr Opin Rheumatol 2005; 17 (5): 629–633

144. Yu W, Gwinn M, Clyne M, Yesupriya A, Khoury MJ. A Navigator for Human Genome Epidemiology. Nat Genet 2008; 40: 124-125.

145. Malfait AM, Seymour AB, Gao F, Tortorella MD, Le Graverand-Gastineau MP, Wood LS, Doherty M, Doherty S, Zhang W, Arden NK, Vaughn FL. A role for PACE4 in osteoarthritis pain: evidence from human genetic association and null mutant phenotype. Annals of the rheumatic diseases. 2012;71(6):1042-8.

146. Reynard LN, Loughlin J. The genetics and functional analysis of primary osteoarthritis susceptibility. Expert Rev Mol Med 2013; 15: e2.

147. Miyamoto Y, Mabuchi A, Shi D, Kubo T, Takatori Y, Saito S, Fujioka M, Sudo A, Uchida A, Yamamoto S et al. A functional polymorphism in the 5' UTR of GDF5 is associated with susceptibility to osteoarthritis. Nature genetics 2007; 39(4):529-533.

148. Valdes AM, Spector TD, Doherty S, Wheeler M, Hart DJ, Doherty M. Association of the DVWA and GDF5 polymorphisms with osteoarthritis in UK populations. Ann Rheum Dis. 2009; 68(12):1916-20.

149. Southam L, Rodriguez-Lopez J, Wilkins JM, Pombo-Suarez M, Snelling S, Gomez-Reino JJ, Chapman K, Gonzalez A, Loughlin J: An SNP in the 5'-UTR of GDF5 is associated with osteoarthritis susceptibility in Europeans and with in vivo differences in allelic expression in articular cartilage. Human molecular genetics 2007; 16(18):2226-2232.

150. Ikegawa S. The genetics of common degenerative skeletal disorders: osteoarthritis and degenerative disc disease. Annu Rev Genomics Hum Genet, 2013; 14: 245-256.

151. Zhang R, Yao J, Xu P, Ji B, Luck JV, Chin B, Lu S, Kelsoe JR, Ma J. A comprehensive meta-analysis of association between genetic variants of GDF5 and osteoarthritis of the knee, hip and hand. Inflamm Res. 2015; 64(6):405-14.

152. Drazen JM, Phimister EG. Publishing genomewide association studies. N Engl J Med 2007; 357 (5): 496

153. Spector TD, Reneland RH, Mah S, et al. Association between a variation in LRCH1 and knee osteoarthritis: a genome-wide single-nucleotide polymorphism association study using DNA pooling. Arthritis Rheum 2006; 54 (2): 524–532

154. Zhai G, van Meurs JB, Livshits G, Meulenbelt I, Valdes AM, Soranzo N, et al. A genome-wide association study suggests that a locus within the ataxin 2 binding protein 1 gene is associated with hand osteoarthritis: the Treat-OA consortium. J Med Genet 2009; 46(9): 614-6.

155. Hämäläinen S, Solovieva S, Vehmas T, Luoma K, Leino-Arjas P, Hirvonen A. Genetic influences on hand osteoarthritis in Finnish women--a replication study of candidate genes. PLoS One 2014; 9(5): e97417.

156. Kerkhof HJ, Lories RJ, Meulenbelt I, Jonsdottir I, Valdes AM, et al. A genome-wide association study identifies an osteoarthritis susceptibility locus on chromosome 7q22. Arthritis Rheum 2010; 62(2):499-510.

157. Panoutsopoulou K, Zeggini E. Advances in osteoarthritis genetics. J Med Genet 2013; 50(11): 715-24.

158. Betancourt MC, Cailotto F, Kerkhof HJ, Cornelis FM, Doherty SA, Hart DJ, Hofman A, Luyten FP, Maciewicz RA, Mangino M, Metrustry S. Genome-wide association and functional studies identify the DOT1L gene to be involved in cartilage thickness and hip osteoarthritis. Proceedings of the National Academy of Sciences. 2012;109(21):8218-23.

159. Roach HI, Aigner T. DNA methylation in osteoarthritic chondrocytes: a new molecular target. Osteoarthritis and cartilage 2007; 15 (2): 128–137

160. De Andres MC, Imagawa K, Hashimoto K, Gonzalez A, Roach HI, Goldring MB, et al. Loss of methylation in CpG sites in the NF-κB enhancer elements of inducible nitric oxide synthase is responsible for gene induction in human articular chondrocytes. Arthritis Rheum 2013; 65: 732–42.

161. Reynard LN, Bui C, Canty-Laird EG, Young DA, Loughlin J. Expression of the osteoarthritis-associated gene GDF5 is modulated epigenetically by DNA methylation. Hum Mol Genet 2011; 20:3450–60.

162. Jeffries MA, Donica M, Baker LW, Stevenson ME, Annan AC, Humphrey MB, James JA, Sawalha AH. Genome-wide DNA methylation study identifies significant epigenomic changes in osteoarthritic cartilage. Arthritis Rheumatol 2014; 66(10):2804-15.

163. Rushton MD, Reynard LN, Barter MJ, Refaie R, Rankin KS, Young DA, Loughlin J. Characterization of the cartilage DNA methylome in knee and hip osteoarthritis. Arthritis Rheum 2014; 66(9): 2450-2460.

164. El Mansouri FE, Chabane N, Zayed N, et al. Contribution of H3K4 methylation by SET-1A to interleukin-1-induced cyclogenase 2 and inducible nitric oxide synthase expression in human osteoarthritic chondrocytes. FASEB J 2009; 23:3539-52.

165. Hong S, Derfoul A, Pereira-Mouries L, Hall DJ. A novel domain in histone deacetylase 1 and 2 mediates repression of cartilage-specific genes in human chondrocytes. FASEB J 2009; 23:3539–52.

166. Higashiyama R, Miyaki S, Yamashita S, et al. Correlation between MMP-13 and HDAC7 expression in human knee osteoarthritis. Mod Rheumatol 2010; 20:11–7.

167. Chen WP, Bao JP, Hu PF, Feng J, Wu LD. Alleviation of osteoarthritis by trichostatin A, a histone deacetylase inhibitor, in experimental osteoarthritis. Mol Biol Rep 2010; 37(8): 3967-72.

168. Barter MJ, Bui C, Young DA. Epigenetic mechanisms in cartilage and osteoarthritis: DNA methylation, histone modifications and microRNAs. Osteoarthritis Cartilage 2012; 20(5):339-49.

169. Shi Y, Massague J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 2003; 113(6):685–700

170. Massague J. TGFbeta signalling in context. Nat Rev Mol Cell Biol 2012; 13(10):616-630.

171. Mehra A, Wrana JL. TGF-beta and the Smad signal transduction pathway. Biochem Cell Biol. Biochimie et biologie cellulaire 2002; 80(5):605–622

172. Zhai G, Dore J, Rahman P. TGF-beta signal transduction pathways and osteoarthritis. Rheumatol Int 2015; 35: 1283-1292

173. Massague J, Blain SW, Lo RS. TGFβ signaling in growth control, cancer, and heritable disorders. Cell 2000; 103(2):295–309

174. Song B, Estrada KD, Lyons KM. Smad signaling in skeletal development and regeneration. Cytokine Growth Factor Rev 2009; 20(5–6):379–388.

175. Finnson KW, Chi Y, Bou-Gharios G, Leask A, Philip A. TGF-b signaling in cartilage homeostasis and osteoarthritis. Front Biosci (Schol Ed) 2012; 1(4):251-68.

176. Wu Q, Huang JH, Sampson ER, Kim KO, Zuscik MJ, O'Keefe RJ, Chen D, Rosier RN. Smurf2 induces degradation of GSK-3β and upregulates β-catenin in chondrocytes: a potential mechanism for Smurf2-induced degeneration of articular cartilage. Exp Cell Res 2009; 315(14): 2386-98.

177. Tardif G, Pelletier JP, Fahmi H, Hum D, Zhang Y, Kapoor M, Martel-Pelletier J. NFAT3 and TGF-β/SMAD3 regulate the expression of miR-140 in osteoarthritis. Arthritis Res Ther 2013; 15(6):R197

178. van Beuningen HM, Glansbeek HL, van der Kraan PM, van den Berg WB. Osteoarthritis-like changes in the murine knee joint resulting from intra-articular transforming growth factor-β injections. Osteoarthr Cartil 2000; 8(1):25–33.

179. Van de Laar IM, Oldenburg RA, Pals G, Roos-Hesselink JW, De Graaf BM, et al. Mutations in SMAD3 cause a syndromic form of aortic aneurysms and dissections with early-onset osteoarthritis. Nat Genet 2011; 43:121e6.

180. van de Laar IM, van der Linde D, Oei EH, Bos PK, Bessems JH, Bierma-Zeinstra SM, et al. Phenotypic spectrum of the SMAD3-related aneurysms–osteoarthritis syndrome. J Med Genet 2012; 49(1): 47-57.

181. Kinoshita A, Saito T, Tomita H, Makita Y, Yoshida K, Ghadami M, et al. Domain-specific mutations in TGFB1 result in Camurati–Engelmann disease. Nat Genet 2000; 26(1):19–20.

182. Wu J, Liu W, Bemis A, Wang E, Qiu Y, Morris EA, Flannery CR, Yang Z. Comparative proteomic characterization of articular cartilage tissue from normal donors and patients with osteoarthritis. Arthritis Rheum 2007; 56(11):3675–3684.

183. Blaney Davidson EN, Remst DF, Vitters EL, van Beuningen HM, Blom AB, Goumans MJ, van den Berg WB, van der Kraan PM. Increase in ALK1/ALK5 ratio as a cause for elevated MMP-13 expression in osteoarthritis in humans and mice. J Immunol 2009; 182(12):7937–7945.

184. Rahman P, Jones A, Curtis J, Bartlett S, Peddle L, Fernandez BA, et al. The Newfoundland population: a unique resource for genetic investigation of complex diseases. Hum Mol Genet 2003; 15: R167-72.

185. Kean WF, Kean R, Buchanan WW. Osteoarthritis: symptoms, signs and source of pain. Inflammopharmacology 2004; 12: 3-31.

186. Guccione AA, Felson DT, Anderson JJ, Anthony JM, Zhang Y, et al. The effects of specific medical conditions on the functional limitations of elders in the Framingham Study. Am J Public Health 1994; 84: 351-358.

187. Maetzel A, Li LC, Pencharz J, Tomlinson G, Bombardier C. The economic burden associated with osteoarthritis, rheumatoid arthritis, and hypertension: A comparative study. Ann Rheum Dis 2004; 63: 395-401.

188. National and State Medical Expenditures and Lost Earnings Attributable to Arthritis and Other Rheumatic Conditions—United States. MMWR 2003; 56: 4-7. Available from http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5601a2.htm (accessed Sept. 2015)

189. Felson DT. An update on the pathogenesis and epidemiology of osteoarthritis. Radiol Clin North Am 2004; 42: 1-9.

190. Hunter DJ, March L, Sambrook PN. Knee osteoarthritis: the influence of environmental factors. Clin Exp Rheumatol 2002; 20: 93-100.

191. MacGregor AJ, Antoniades L, Matson M, Andrew T, Spector TD. The genetic contribution to radiographic hip osteoarthritis in women: results of a classic twin study. Arthritis Rheum 2000; 43: 2410-2416.

192. Spector TD, Cicuttini F, Baker J, Loughlin J, Hart D. Genetic influences on osteoarthritis in women: a twin study. BMJ 1996; 312: 940-944.

193. Hirsch R, Lethbridge-Cejku M, Hanson R, Scott WW Jr, Reichle R, et al. Familial aggregation of osteoarthritis: data from the Baltimore Longitudinal Study on Aging. Arthritis Rheum 1998; 4: 1227-1232.

194. Riyazi N, Meulenbelt I, Kroon HM, Ronday KH, Hellio le Graverand MP, et al. Evidence for familial aggregation of hand, hip, and spine but not knee osteoarthritis in siblings with multiple joint involvement: the GARP study. Ann Rheum Dis 2005; 64: 438-443.

195. Lafeber FP, van Spil WE. Osteoarthritis year 2013 in review: biomarkers; reflecting before moving forward, one step at a time, Osteoarthritis Cartilage 2013; 21: 1452-1464.

196. Rahman P, Jones A, Curtis J, Bartlett S, Peddle L, et al. The Newfoundland population: a unique resource for genetic investigation of complex diseases. Hum Mol Genet 2003; 13: 1287.

197. Kristiansson K, Naukkarinen J, Peltonen L. Isolated populations and complex disease gene identification. Genome Biol 2008; 9: 109.

198. Gabriel S, Ziaugra L, Tabbaa D. SNP genotyping using the Sequenom MassARRAY iPLEX platform. Curr Protoc Hum Genet ;2009 Chapter 2:Unit 2.12.

199. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, et al. PLINK: a toolset for whole-genome association and population-based linkage analysis. Am J Hum Genet 2007; 8: 559-575.

200. Jackson GC, Marcus-Soekarman D, Stolte-Dijkstra I, Verrips A, Taylor JA, et al. Type IX collagen gene mutations can result in multiple epiphyseal dysplasia that is associated with osteochondritis dissecans and a mild myopathy. Am J Med Genet 2010; 152A: 863-869.

201. Ikeda T, Mabuchi A, Fukuda A, Kawakami A, Ryo Y, et al. Association analysis of single nucleotide polymorphisms in cartilage-specific collagen genes with knee and hip osteoarthritis in the Japanese population. J Bone Miner Res 2002; 17: 1290-1296

202. Liu J, Cai W, Zhang H, He C, Deng L. Rs143383 in the growth differentiation factor 5 (GDF5) gene significantly associated with osteoarthritis (OA)-a comprehensive meta-analysis. Int J Med Sci 2013; 10: 312-319.

203. Hao SW, Jin QH. Association between the +104T/C polymorphism in the 5′UTR of GDF5 and susceptibility to knee osteoarthritis: a meta-analysis. Mol Med Rep 2013; 7: 485-488.

204. Zeggini E, Panoutsopoulou K, Southam L, Rayner NW, Day-Williams AG, et al. Identification of new susceptibility loci for osteoarthritis (arcOGEN): a genome-wide association study. Lancet 2012; 380: 815-823

205. Zhai G, Zhou J, Woods MO, Green JS, Parfrey P, Rahman P, Green RC. Genetic structure of the Newfoundland and Labrador population: founder effects modulate variability. European Journal of Human Genetics. 2015, 1-8.

206. Limer KL, Tosh K, Bujac SR, McConnell R, Doherty S, et al. Attempt to replicate published genetic associations in a large, well-defined osteoarthritis case-control population (the GOAL study). Osteoarthritis Cartilage 2009; 17: 782-789.

207. Evangelou E, Kerkhof HJ, Styrkarsdottir U, Ntzani EE, Bos SD, et al. A meta-analysis of genome-wide association studies identifies novel variants associated with osteoarthritis of the hip. Ann Rheum Dis 2014; 73(12):2130-6.

208. Hirschhorn JN, Lohmueller K, Byrne E, Hirschhorn K. A comprehensive review of genetic association studies. Genet Med 2004; 4: 45-61.

209. Couzin-Frankel J. Major heart disease genes prove elusive. Science 2010; 328: 1220-1221

210. Murphy L, Schwartz TA, Helmick CG, Renner JB, Tudor G, et al. Lifetime risk of symptomatic knee osteoarthritis. Arthritis & Rheumatism 2008; 59(9):1207-1213

211. Valdes AM, Spector TD. Genetic epidemiology of hip and knee osteoarthritis. Nat Rev Rheumatol 2011; 7(1):23-32.

212. Meulenbelt I. Osteoarthritis year 2011 in review: genetics. Osteoarthritis and Cartilage 2012; 20:218-222.

213. Loughlin J. Genetics of osteoarthritis. Current Opinion in Rheumatology 2011; 23(4):479-483.

214. Bijkerk C, Houwing-Duistermaat JJ, Valkenburg HA, Meulenbelt I, Hofman A, et al. Heritabilities of radiologic osteoarthritis in peripheral joints and of disc degeneration of the spine. Arthritis Rheum 1999; 42(8):1729-35.

215. Wischmeijer A, Van Laer L, Tortora G, Bolar NA, Van Camp G, et al. Thoracic aortic aneurysm in infancy in aneurysms-osteoarthritis syndrome due to a novel SMAD3 mutation: further delineation of the phenotype. Am J Med Genet A 2013; 161A(5):1028-35.

216. Regalado ES, Guo DC, Villamizar C, Avidan N, Gilchrist D, et al. Exome sequencing identifies SMAD3 mutations as a cause of familial thoracic aortic

aneurysm and dissection with intracranial and other arterial aneurysms. Circ Res 2011; 109(6):680-6.

217. Hilhorst-Hofstee Y, Scholte AJ, Rijlaarsdam ME, van Haeringen A, Kroft LJ, et al. An unanticipated copy number variant of chromosome 15 disrupting SMAD3 reveals a three-generation family at serious risk for aortic dissection. Clin Genet 2013; 83(4):337-44.

218. Finnson KW, Chi Y, Bou-Gharios G, Leask A, Philip A. TGF-b signaling in cartilage homeostasis and osteoarthritis. Front Biosci (Schol Ed) 2012; 4:251-68.

219. Alvarez J, Serra R. Unique and redundant roles of Smad3 in TGF-beta-mediated regulation of long bone development in organ culture. Dev Dyn 2004; 230(4):685-99.

220. Chen G, Deng C, Li YP. TGF-β and BMP signaling in osteoblast differentiation and bone formation. Int J Biol Sci 2012; 8(2):272-88

221. Yang X, Chen L, Xu X, Li C, Huang C, Deng CX: TGF-beta/Smad3 signals repress chondrocyte hypertrophic differentiation and are required for maintaining articular cartilage. J Cell Biol 2001; 153(1):35-46.

222. Hart DJ, Spector TD. The relationship of obesity, fat distribution and osteoarthritis in the general population: the Chingford Study. J Rheumatol 1993; 20: 331–5.

223. Kellgren JH, Lawrence JS. Atlas of standard radiographs of arthritis; The epidemiology of chronic rheumatism. Oxford: Blackwell Scientific Publications 1963.

224. Burnett S, Hart DJ, Cooper C, Spector TD. A radiographic atlas of osteoarthritis. London: Springer Verlag 1994.

225. Livshits G, Kato BS, Zhai G, Hart DJ, Hunter D, et al. Genomewide linkage scan of hand osteoarthritis in female twin pairs showing replication of quantitative trait loci on chromosomes 2 and 19. Ann Rheum Dis 2007; 66(5):623-7.

226. Teo YY, Inouye M, Small KS, Gwilliam R, Deloukas P, et al. A genotype calling algorithm for the Illumina BeadArray platform. Bioinformatics 2007; 23(20):2741-6

227. Good P. A practical guide to resampling methods for testing hypotheses. New York: Springer-Verlag 1994.

228. Doerge RW, Churchill GA. Permutation tests for multiple loci affecting a quantitative character. Genetics 1996; 142: 285-94.

229. Knüppel S, Rohde K, Meidtner K, Drogan D, Holzhütter HG, et al. Evaluation of 41 candidate gene variants for obesity in the EPIC-Potsdam cohort by multi-locus stepwise regression. PLoS ONE 2013; 8(7): e68941.

230. Walker LC, Fredericksen ZS, Wang X, Tarrell R, Pankratz VS, et al. Evidence for SMAD3 as a modifier of breast cancer risk in BRCA2 mutation carriers. Breast Cancer Res 2010; 12(6): R102.

231. Tang B, Vu M, Booker T, Santner SJ, Miller FR, Anver MR, Wakefield LM. TGF-β switches from tumor suppressor to prometastatic factor in a model of breast cancer progression. The Journal of clinical investigation. 2003;112(7):1116-24.

232. Yao JY, Wang Y, An J, Mao C, M Hou, et al. Mutations analysis of the SMAD3 gene in human osteoarthritis. European journal of Human Genetics 2003; 11(9):714-7.

233. Valdes AM, Spector TD, Tamm A, Kisand K, Doherty SA, et al. Genetic variation in the SMAD3 gene is associated with hip and knee osteoarthritis. Arthritis and rheumatism 2010; 62(8):2347-52.

234. Liying J, Yuchun T, Youcheng W, Yingchen W, Chunyu J, et al (2013) A SMAD3 gene polymorphism is related with osteoarthritis in a Northeast Chinese population. Rheumatology international 2013; 33(7), 1763-1768.

235. Hannan MT, Felson DT, Pincus T. Analysis of the discordance between radiographic changes and knee pain in osteoarthritis of the knee. J Rheumatol 2000; 27(6):1513-7

236. Chen CG, Thuillier D, Chin EN, Alliston T. Chondrocyte-intrinsic Smad3 represses Runx2-inducible matrix metalloproteinase 13 expression to maintain articular cartilage and prevent osteoarthritis. Arthritis Rheum 2012; 64(10):3278-89.

237. Vos T, Flaxman AD, Naghavi M, Lozano R, Michaud C, Ezzati M, et al. Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and injuries 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet. 2012; 380:2163-96.

238. Woolf AD, Pfleger B. Burden of major musculoskeletal condition—bone and joint decade 2000–2010. Bull World Health Org 2003; 81:646-56.

239. Roach HI. The complex pathology of osteoarthritis: even mitochondria are involved. Arthritis & Rheumatism 2008; 58: 2217-2218.

240. Zhai G, Aref-Eshghi E. Biomarkers for osteoarthritis: investigation, identification, and prognosis. Curr Biomark Find 2012; 2: 19-28.

241. Shen J, Li S, Chen D. TGF-β signaling and the development of osteoarthritis. Bone Res 2014; 2. pii: 14002.

242. Li TF, Dowish M, Zuscik MJ, Chen D, Schwarz EM, Rosier RN, Drissi H, O'Keefe RJ. Smad3-deficient chondrocytes have enhanced BMP signaling and accelerated differentiation. J Bone Miner Res 2006; 21: 4-16

243. Aref-Eshghi E, Zhang Y, Hart D, Valdes AM, Furey A, Martin G, et al. SMAD3 Is Associated with the Total Burden of Radiographic Osteoarthritis: The Chingford Study. PLoS One 2014; 9: e97786.

244. Aref-Eshghi E, Rahman P, Zhang H, Martin G, Furey A, Green R, et al. Attempt to replicate the published osteoarthritis-associated genetic variants in the Newfoundland& Labrador Population. J Orthopedics Rheumatol 2014; 1: 5.

245. Zhang W, Likhodii S, Zhang Y, Aref-Eshghi E, Harper PE, Randell E, et al. Classification of osteoarthritis phenotypes by metabolomics analysis. BMJ open. 2014; 4: e006286.

246. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25:402-408.

247. Ehrich M, Nelson MR, Stanssens P, Zabeau M, Liloglou T, Xinarianos G, et al. Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry. Proc Natl Acad Sci USA 2005; 102: 15785-15790.

248. Wu MY, Hill CS. TGF-β superfamily signaling in embryonic development and homeostasis. Dev Cell 2009; 16: 329-43.

249. Wharton K, Derynck R. TGF-β family signaling: novel insights in development and disease. Development 2009; 136: 3691-7.

250. Van der Kraan PM, Blaney Davidson EN, Van den Berg WB. A role for age-related changes in TGF-β signaling in aberrant chondrocyte differentiation and osteoarthritis. Arthritis Res Ther 2010; 12: 201.

251. Van der Kraan PM, Blaney Davidson EN, Blom A, Van den Berg WB. TGF-β signaling in chondrocyte terminal differentiation and osteoarthritis: modulation and integration of signaling pathways through receptor-Smads. Osteoarthritis Cartilage 2009; 17: 1539-45.

252. Blaney Davidson EN, Vitters EL, Van der Kraan PM, Van den Berg WB. Expression of transforming growth factor-β (TGF-β) and the TGF-β signalling molecule Smad-2P in spontaneous and instability-induced osteoarthritis: role in cartilage degradation, chondrogenesis and osteophyte formation. Ann Rheum Dis 2006; 65:1414-1421.

253. Blaney Davidson EN, Scharstuhl A, Vitters EL, Van der Kraan PM, Van den Berg WB. Reduced transforming growth factor-β signaling in cartilage of old mice: role in impaired repair capacity. Arthritis Res Ther 2005; 7: R1338-47.

254. Harrison MH, Shajowicz F, Trueta J. Osteoarthritis of the hip: a study of the nature and evolution of the disease. J Bone Joint Surg Br 1953; 35: 598–626.

255. Lajeunesse D. The role of bone in the treatment of osteoarthritis. Osteoarthritis Cartilage 2004; 12 :S34-8.

256. Finnson KW, Chi Y, Bou-Gharios G, Leask A, Philip A. TGF-b signaling in cartilage homeostasis and osteoarthritis. Front Biosci (Schol Ed) 2012; 1:251-68.

257. Zhen G, Wen C, Jia X, Li Y, Crane JL, Mears SC, et al. Inhibition of TGF-β signaling in mesenchymal stem cells of subchondral bone attenuates osteoarthritis. Nat Med 2013; 19: 704–712.

258. Bakker AC, van de Loo FA, van Beuningen HM, Sime P, van Lent PL, van der Kraan PM, et al. Overexpression of active TGF-β1 in the murine knee joint: evidence for synovial-layer-dependent chondro-osteophyte formation. Osteoarthritis Cartilage 2001; 9: 128-36.

259. Moldovan F, Pelletier JP, Hambor J, Cloutier JM, Martel-Pelletier J. Collagenase-3 (matrix metalloprotease 13) is preferentially localized in the deep layer of human arthritic cartilage in situ: in vitro mimicking effect by transforming growth factor beta. Arthritis Rheum 1997; 40:1653-61.

260. Leivonen SK, Ala-Aho R, Koli K, Grénman R, Peltonen J, Kähäri VM. Activation of Smad signaling enhances collagenase-3 (MMP-13) expression and invasion of head and neck squamous carcinoma cells. Oncogene 2006; 25:2588-600.

261. Selvamurugan N, Kwok S, Partridge NC. Smad3 interacts with JunB and Cbfa1/Runx2 for transforming growth factor-beta1-stimulated collagenase-3 expression in human breast cancer cells. J Biol Chem 2004; 279:27764-73.

262. Leivonen SK, Chantry A, Hakkinen L, Han J, Kahari VM. Smad3 mediates transforming growth factor-beta-induced collagenase-3 (matrix metalloproteinase-13) expression in human gingival fibroblasts. Evidence for cross-talk between Smad3 and p38 signaling pathways. J Biol Chem 2002; 277:46338-46.

263. Selvamurugan N, Kwok S, Alliston T, Reiss M, Partridge NC. Transforming growth factor-beta 1 regulation of collagenase-3 expression in osteoblastic cells by cross-talk between the Smad and MAPK signaling pathways and their components, Smad2 and Runx2. J Biol Chem 2004; 279:19327-34.

264. Raine EV, Reynard LN, van de Laar IM, Bertoli-Avella AM, Loughlin J. Identification and analysis of a SMAD3 cis-acting eQTL operating in primary osteoarthritis and in the aneurysms and osteoarthritis syndrome. Osteoarthritis Cartilage 2014; 22: 698-705.

265. Busque L, Belisle C, Provost S, Giroux M, Perreault C. Differential expression of SMAD3 transcripts is not regulated by cis-acting genetic elements but has a gender specificity. Genes Immun 2009; 10:192-6.

266. Cross M, Smith E, Hoy D, Nolte S, Ackerman I, Fransen M, Bridgett L, Williams S, Guillemin F, Hill CL et al. The global burden of hip and knee osteoarthritis: estimates from the global burden of disease 2010 study. Ann Rheum Dis 2014; 73(7):1323-1330.

267. Yamada Y, Okuizumi H, Miyauchi A, Takagi Y, Ikeda K, Harada A. Association of transforming growth factor beta1 genotype with spinal osteophytosis in Japanese women. Arthritis Rheum 2000; 43(2):452-460.

268. Kizawa H, Kou I, Iida A, Sudo A, Miyamoto Y, Fukuda A, Mabuchi A, Kotani A, Kawakami A, Yamamoto S et al: An aspartic acid repeat polymorphism in asporin inhibits chondrogenesis and increases susceptibility to osteoarthritis. Nat Genet 2005; 37(2):138-144.

269. Jiang Q, Shi D, Yi L, Ikegawa S, Wang Y, Nakamura T, Qiao D, Liu C, Dai J. Replication of the association of the aspartic acid repeat polymorphism in the asporin gene with knee-osteoarthritis susceptibility in Han Chinese. Journal of human genetics 2006; 51(12):1068-1072.

270. Chapman K, Takahashi A, Meulenbelt I, Watson C, Rodriguez-Lopez J, Egli R, Tsezou A, Malizos KN, Kloppenburg M, Shi D et al. A meta-analysis of European and Asian cohorts reveals a global role of a functional SNP in the 5' UTR of GDF5 with osteoarthritis susceptibility. Hum mol genet 2008; 17(10):1497-1504.

271. Liang W, Gao B, Xu G, Weng D, Xie M, Qian Y. Association between single nucleotide polymorphisms of asporin (ASPN) and BMP5 with the risk of knee osteoarthritis in a Chinese Han population. Cell biochemistry and biophysics 2014; 70(3):1603-1608.

272. Williams FM, Popham M, Hart DJ, de Schepper E, Bierma-Zeinstra S, Hofman A, Uitterlinden AG, Arden NK, Cooper C, Spector TD et al. GDF5 single-nucleotide polymorphism rs143383 is associated with lumbar disc degeneration in Northern European women. Arthritis Rheum 2011; 63(3):708-712.

273. Southam L, Rodriguez-Lopez J, Wilkins JM, Pombo-Suarez M, Snelling S, Gomez-Reino JJ, Chapman K, Gonzalez A, Loughlin J: An SNP in the 5'-UTR of GDF5 is associated with osteoarthritis susceptibility in Europeans and with in vivo differences in allelic expression in articular cartilage. Human molecular genetics 2007; 16(18):2226-2232.

274. Aref-Eshghi E, Rahman P, Zhang H, Martin G, Furey A, Green R, Sun G, Zhai G: Attempt to replicate the published osteoarthritis-associated genetic variants in the Newfoundland & Labrador Population. J Orthopedics Rheumatol 2014; 1(3):5.

275. Rothman KJ. No adjustments are needed for multiple comparisons. Epidemiology 1990; 1(1):43-46.

276. Pombo-Suarez M, Castano-Oreja MT, Calaza M, Gomez-Reino J, Gonzalez A. Differential upregulation of the three transforming growth factor beta isoforms in human osteoarthritic cartilage. Ann Rheum Dis 2009; 68(4):568-571.

277. Verdier MP, Seite S, Guntzer K, Pujol JP, Boumediene K: Immunohistochemical analysis of transforming growth factor beta isoforms and their receptors in human cartilage from normal and osteoarthritic femoral heads. Rheumatol Int 2005; 25(2):118-124.

278. Shiomi T, Lemaitre V, D'Armiento J, Okada Y. Matrix metalloproteinases, a disintegrin and metalloproteinases, and a disintegrin and metalloproteinases with thrombospondin motifs in non-neoplastic diseases. Pathology international 2010; 60(7):477-496.

279. Cheon H, Yu SJ, Yoo DH, Chae IJ, Song GG, Sohn J. Increased expression of pro-inflammatory cytokines and metalloproteinase-1 by TGF-beta1 in synovial fibroblasts from rheumatoid arthritis and normal individuals. Clinical and experimental immunology 2002; 127(3):547-552.

280. Mengshol JA, Vincenti MP, Brinckerhoff CE. IL-1 induces collagenase-3 (MMP-13) promoter activity in stably transfected chondrocytic cells: requirement for Runx-2 and activation by p38 MAPK and JNK pathways. Nucleic Acids Res 2001; 29(21):4361-4372.

281. Blaney Davidson EN, Remst DF, Vitters EL, van Beuningen HM, Blom AB, Goumans MJ, van den Berg WB, van der Kraan PM. Increase in ALK1/ALK5 ratio as a cause for elevated MMP-13 expression in osteoarthritis in humans and mice. J Immunol 2009; 182(12):7937-7945.

282. van der Kraan PM. Age-related alterations in TGF beta signaling as a causal factor of cartilage degeneration in osteoarthritis. Bio-medical materials and engineering 2014; 24(0):75-80.

283. Guccione AA, Felson DT, Anderson JJ, Anthony JM, Zhang Y, Wilson PW, et al. The effects of specific medical conditions on the functional limitations of elders in the Framingham Study. Am J Public Health 1994;84:351-8.

284. Felson DT. An update on the pathogenesis and epidemiology of osteoarthritis. Radiol Clin North Am 2004; 42:1-9.

285. Hunter DJ, March L, Sambrook PN. Knee osteoarthritis: the influence of environmental factors. Clin Exp Rheumatol 2002; 20:93-100.

286. Martel-Pelletier J, Boileau C, Pelletier JP, Roughley PJ. Cartilage in normal and osteoarthritis conditions. Best Pract Res Clin Rheumatol 2008; 22:351-84.

287. Reynard LN, Loughlin J. Genetics and epigenetics of osteoarthritis. Maturitas 2012; 71:200-4.

288. Rodriguez-Paredes M, Esteller M. Cancer epigenetics reaches mainstream oncology. Nat Med 2011; 17:330–9.

289. Imagawa K, de Andrés MC, Hashimoto K, Itoi E, Otero M, Roach HI, et al. Association of Reduced Type IX Collagen Gene Expression in Human Osteoarthritic Chondrocytes With Epigenetic Silencing by DNA Hypermethylation. Arthritis Rheumatol 2014; 66: 3040-51.

290. De Andres MC, Imagawa K, Hashimoto K, Gonzalez A, Roach HI, Goldring MB, et al. Loss of methylation in CpG sites in the NF-κB enhancer elements of inducible nitric oxide synthase is responsible for gene induction in human articular chondrocytes. Arthritis Rheum 2013; 65:732-42.

291. Reynard LN, Bui C, Canty-Laird EG, Young DA, Loughlin J. Expression of the osteoarthritis-associated gene GDF5 is modulated epigenetically by DNA methylation. Hum Mol Genet 2011; 20:3450-60.

292. Jeffries MA, Donica M, Baker LW, Stevenson ME, Annan AC, Humphrey MB, et al. Genome-wide DNA methylation study identifies significant epigenomic changes in osteoarthritic cartilage. Arthritis Rheumatol 2014; 66:2804-15.

293. Dedeurwaerder S, Defrance M, Calonne E, Denis H, Sotiriou C, Fuks F. Evaluation of the Infinium Methylation 450K technology. Epigenomics 2011; 3: 771-784.

294. Bibikova M, Barnes B, Tsan C, Ho V, Klotzle B, Le J M, et al. High density DNA methylation array with single CpG site resolution. Genomics 2011; 98(4), 288-295.

295. Aryee MJ, Jaffe AE, Corrada-Bravo H, Ladd-Acosta C, Feinberg AP, Hansen KD, Irizarry RA. Minfi: a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. Bioinformatics 2014; 30(10): 1363-1369.

296. Hansen KD, Aryee M, Timp W. minfiData: Example data for the Illumina Methylation 450k array. R package version 0.7, 1.

297. Du P, Zhang X, Huang CC, Jafari N, Kibbe WA, Hou L, Lin SM. Comparison of Beta-value and M-value methods for quantifying methylation levels by microarray analysis. BMC bioinformatics 2010; 11(1):587.

298. Teschendorff AE, Marabita F, Lechner M, Bartlett T, Tegner J, Gomez-Cabrero D, et al. A beta-mixture quantile normalization method for correcting probe design bias in Illumina Infinium 450 k DNA methylation data. Bioinformatics 2013; 29:189-96.

299. Laird PW. Principles and challenges of genome-wide DNA methylation analysis. Nature Reviews Genetics 2010; 11:191-203.

300. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. Nature Protoc 2009; 4:44-57.

301. den Hollander W, Ramos YF, Bos SD, Bomer N, van der Breggen R, Lakenberg N, et al. Knee and hip articular cartilage have distinct epigenomic landscapes: implications for future cartilage regeneration approaches. Ann Rheum Dis 2014; 73:2208-12.

302. Fernández-Tajes J, Soto-Hermida A, Vázquez-Mosquera ME, Cortés-Pereira E, Mosquera A, Fernández-Moreno M, et al. Genome-wide DNA methylation analysis of articular chondrocytes reveals a cluster of osteoarthritic patients. Ann Rheum Dis 2014; 73:668-77.

303. Wang X, Manner PA, Horner A, Shum L, Tuan RS, Nuckolls GH. Regulation of MMP-13 expression by RUNX2 and FGF2 in osteoarthritic cartilage. Osteoarthritis Cartilage 2004; 12:963-73.

304. Ma XJ, Guo HH, Hao SW, Sun SX, Yang XC, Yu B, et al. Association of single nucleotide polymorphisms (SNPs) in leptin receptor gene with knee osteoarthritis in the Ningxia Hui population. Yi Chuan 2013; 35:359-64.

305. Ye XL, Lu CF. Association of polymorphisms in the leptin and leptin receptor genes with inflammatory mediators in patients with osteoporosis. Endocrine 2013; 44:481-8.

306. Pettersson U, Albagha OM, Mirolo M, Taranta A, Frattini A, McGuigan FE, et al. Polymorphisms of the CLCN7 gene are associated with BMD in women. J Bone Miner Res 2005; 20:1960-7.

307. Elfassihi L, Giroux S, Bureau A, Laflamme N, Cole DE, Rousseau F. Association with replication between estrogen-related receptor gamma (ESRRgamma) polymorphisms and bone phenotypes in women of European ancestry. J Bone Miner Res 2010; 25:901-11.

308. Ettinger R, Kuchen S, Lipsky PE. Interleukin 21 as a target of intervention in autoimmune disease. Ann Rheum Dis 2008; 67 Suppl 3:iii83-6.

309. Windsor RE, Strauss SJ, Kallis C, Wood NE, Whelan JS. Germline genetic polymorphisms may influence chemotherapy response and disease outcome in osteosarcoma: a pilot study. Cancer 2012; 118:1856-67.

310. Fei Q, Wu ZH, Yuan SM, Wang H, Zhou X, Liu Z, et al. Association of PAX1 gene polymorphisms with susceptibility to congenital scoliosis in Chinese Han population. Zhonghua Yi Xue Za Zhi 2008; 88:2597-602.

311. Jeffries MA, Donica M, Baker LW, Stevenson ME, Annan AC, Humphrey MB, et al. Genome-wide DNA methylation study identifies significant epigenomic changes in osteoarthritic cartilage. Arthritis Rheumatol 2014; 66:2804-15.

312. Moazedi-Fuerst FC, Hofner M, Gruber G, Weinhaeusel A, Stradner MH, Angerer H, et al. Epigenetic differences in human cartilage between mild and severe OA. J Orthop Res 2014; 32:1636-45.

313. Delgado-Calle J, Fernández AF, Sainz J, Zarrabeitia MT, Sañudo C, García-Renedo R, et al. Genome-wide profiling of bone reveals differentially methylated regions in osteoporosis and osteoarthritis. Arthritis Rheum. 2013;65:197-205.

314. Wagner EF, Karsenty G. Genetic control of skeletal development. Curr Opin Genet Dev 2001; 11:527–532

315. Lane NE, Lin P, Christiansen L, Gore LR, Williams EN, Hochberg MC, et al. Association of mild acetabular dysplasia with an increased risk of incident hip osteoarthritis in elderly white women: the Study of Osteoporotic Fractures. Arthritis Rheum 2000; 43:400-404.

316. Shepstone L, Rogers J, Kirwan JR, Silverman BW. Shape of the intercondylar notch of the human femur: a comparison of osteoarthritic and non-osteoarthritic bones from a skeletal sample. Ann Rheum Dis 2001; 60:968-973.

Appendix

MEMORIAL UNIVERSITY
Faculty of Medicine

Discipline of Genetics
Health Sciences Centre
St. John's, NL Canada A1B 3V6
Tel: 709 777-6807 Fax:709 777-7497
www.med.mun.ca

Consent to Take Part in Research

TITLE: Newfoundland Osteoarthritis Study (NFOAS)

INVESTIGATOR(S): Drs. G. Zhai, A. Furey, G. Martin, P. Rahman, R. Green, and G. Sun.

You have been invited to take part in a research study. It is up to you to decide whether to be in the study or not. Before you decide, you need to understand what the study is for, what risks you might take and what benefits you might receive. This consent form explains the study.

The researchers will:

discuss the study with you
answer your questions
keep confidential any information which could identify you personally
be available during the study to deal with problems and answer questions

If you decide not to take part or to leave the study, your normal treatment will not be affected.

Introduction/Background:
You have a form of arthritis called osteoarthritis. Many people in Newfoundland and other places have arthritis, but we still don't know much about what causes it. We know that what you inherit from your parents plays a part because if you have a close relative with arthritis, you are more likely to get the disease yourself. You inherit genes (made up of DNA) from both your parents. As part of this study we will be examining DNA from osteoarthritis patients and comparing it with DNA from those who do not have arthritis.

2. Purpose of study:
We will recruit about 1000 people who have osteoarthritis and 1000 people who don't have, and be looking to see how the genes you inherit from your parents contribute to the cause of arthritis. We will also look to see whether small changes to these genes within the joints themselves can make a difference.

3. Description of the study procedures and tests:
You are going to have a knee or hip joint replaced because of arthritis in the joint. You will get exactly the same treatment whether you decide to join our research study or not.

If you join the study we will ask you for some extra tubes of blood (up to 4 extra tubes) when you have your routine blood collection. From this blood we will prepare your DNA (the material that makes up your genes) that we will store in a freezer. We will test the DNA from hundreds of arthritis patients to find changes that could be involved in causing arthritis.

We will ask you to complete a questionnaire about your general medical history plus a short form just about your arthritis. We will help to complete the questionnaires, if you would like.

During your joint surgery we will be keeping small samples of the cartilage, subchondral bone, synovial membrane, and of the fluid inside the joint, which are normally thrown away. This material may also be tested for its genetic content.

We also ask your consent to access your medical records, both now and as long as this study continues (at least 5 years). This lets us compare the symptoms you have or may develop, and when you get them, to any genetic changes we may find.

4. Length of time:
Completing the questionnaires should take about 30 minutes.
There is nothing else for you to do.

5. Possible risks and discomforts:
Bruising/discomfort after blood sample.

6. Benefits:
It is unlikely that this study will benefit you personally. We hope that our findings may help in preventing arthritis in the future or in finding better treatments.

7. Liability statement:
Signing this form gives us your consent to be in this study. It tells us that you understand the information about the research study. When you sign this form, you do not give up your legal rights. Researchers or agencies involved in this research study still have their legal and professional responsibilities.

8. What about my privacy and confidentiality?
Protecting your privacy is an important part of this study. Every effort to protect your privacy will be made. However it cannot be guaranteed. For example we may be required by law to allow access to research records.
When you sign this consent form you give us permission to

Collect information from you
Collect information from your health record
Share information with the people conducting the study
Share information with the people responsible for protecting your safety

Access to records
The members of the research team will see health and study records that identify you by name. Other people may need to look at the study records that identify you by name. This might include the research ethics board. You may ask to see the list of these people. They can look at your records only when one of the research team is present.

Use of records
The research team will collect and use only the information they need for this research study.

This information will include your
date of birth
sex
medical conditions
medications
the results of tests and procedures you had before and during the study
information from study interviews and questionnaires

Your name and contact information will be kept secure by the research team in Newfoundland and Labrador. It will not be shared with others without your permission. Your name will never appear in any report or article published as a result of this study.

Information collected for this study will be kept for at least five years after the end of the study.

If you decide to withdraw from the study, the information collected up to that time will continue to be used by the research team. It may not be removed. This information will only be used for the purposes of this study. At any time, you may ask that your DNA and any other specimens be destroyed.

After your part in this study ends, we may continue to review your health records to check that the information we collected is correct.

Information collected and used by the research team will be stored by Dr. Zhai who is the person responsible for keeping it secure**.**

Your access to records
You may ask the study doctor or researcher to see the information that has been collected about you.

9. Questions:
If you have any questions about taking part in this study, you can meet with the investigator, Dr. Guangju Zhai, 709-864-6683 who is in charge of the study at this institution.

Or you can talk to someone who is not involved with the study, but can advise you on your rights as a participant in a research study. This person can be reached through: Health Research Ethics Authority (HREA) at 709-777-6974 or email**: info**@hrea.ca

Future use of DNA or other samples:

In order to preserve a valuable resource, your DNA and other samples may be stored at the end of this research project. It is possible that these samples may be used in a future research project. **Any future research would first have to be approved by a Research Ethics Board (REB).**

Please tick **one** of the following two options:

	I agree that my samples can be used for an approved research project without contacting me again, but **only if my name* cannot be linked, in any way, to the samples.**
	Under no circumstances may my samples be used for future research. **My samples must be destroyed at the end of this present project**.

*Includes name, MCP number or any other identifying information.

The DNA and other samples from this study will be stored in St. John's, NL for an indefinite period of time.

At any time, you may ask that your DNA and any other specimens be destroyed. To do this please call Dr. Zhai at (709) 864-6683, contact anyone else on the research team, or contact the Office of the HREA at 709-777-6974 or email: info@hrea.ca

To protect your samples, we will ask that you confirm your request in writing before we destroy them.

After signing this consent you will be given a copy.

Signature Page

Study title: Genetics of Osteoarthritis in Newfoundland

Name of principal investigator: Dr. G. Zhai

To be filled out and signed by the participant:

Please check as appropriate:

I have read the consent form.	Yes { }	No { }
I have had the opportunity to ask questions and to discuss this study.	Yes { }	No { }
I have received satisfactory answers to all of my questions.	Yes { }	No { }
I have received enough information about the study.	Yes { }	No { }
I have spoken to _____ and he/she has answered my questions	Yes { }	No { }
I understand that I am free to withdraw from the study at any time without having to give a reason without affecting my future care	Yes { }	No { }
I understand that it is my choice to be in the study and that I may not benefit.	Yes { }	No { }
I agree that the study doctor or investigator may read the parts of my hospital records which are relevant to the study.	Yes { }	No { }
I agree to take part in this study.	Yes { }	No { }

_____ _____

Signature of participant Date

_____ _____

Signature of witness Date

<u>To be signed by the investigator or person obtaining consent</u>

I have explained this study to the best of my ability. I invited questions and gave answers. I believe that the participant fully understands what is involved in being in the study, any potential risks of the study and that he or she has freely chosen to be in the study.

_____ _____

Signature of investigator/person obtaining consent Date

Telephone number: _____

MEMORIAL UNIVERSITY
Faculty of Medicine

Discipline of Genetics
Health Sciences Centre
St. John's, NL Canada A1B 3V6
Tel: 709 777-6807 Fax:709 777-7497
www.med.mun.ca

Genetic Study of Osteoarthritis in the Newfoundland Population

General Questionnaire

Date form completed: (dd/mm/yyyy) ☐☐ / ☐☐ / ☐☐☐☐

Instruction for completing the questionnaire:

Please answer all questions to the best of your ability (leave blank if unknown).

Please write in block letters using the boxes where provided.

Use a black/blue pen.

Cross out any mistakes & write correct answers just below the relevant boxes.

Indicate your response by filling in the box next to the most appropriate answer or by writing clearly in the boxes or space provided.

Your answers will be completely confidential.

Self administered: ☐

Research assistant administered: ☐

Name and address

Surname

Given name

Title

Maiden Name (if applicable)

Address

Province

Postal code

Date of Birth (dd/mm/yyyy)

/ /

Place of Birth
City/Town

Province/Country

Gender: Male ☐ Female ☐

MCP number:

Section 1: Demographics

1. Ethnic: White ☐ Black ☐ Other ☐, please specify ☐

2. Height: ☐☐☐ cm

3. Weight: ☐☐☐ kg

4a. Smoker: yes (current) ☐ no ☐ ex-smoker ☐

4b. If Yes, how many cigarettes do/did you smoke a day? ☐☐☐

5a. In the past 4 weeks approximately how many units of alcohol did you drink per week?

(1 unit = 1 glass of wine/½ pint of beer /1 shot of sprit)? ☐☐☐

5b. Do you think your drinking habits in the last 4 weeks reflect your typical drinking habit? Yes ☐ no, less than usual ☐ no, more than usual ☐

6a. How heavy were you when you were born?

☐☐☐ grams or ☐☐ lbs ☐☐ ozs

6b. If weight unknown, were you Light ☐ Average ☐ Heavy ☐

6c. Were you born prematurely (more than 1 week early) Yes ☐ No ☐

7a. How heavy were you at age 20 yrs? ☐☐☐ kg

7b. How heavy were you at age 50 yrs? ☐☐☐ kg

For women only:

8. At what age did your period start? ☐☐

9. At what age did your period stop? ☐☐

Section 1: Demographics (continued)

10a. Have you had a hysterectomy (removal of the womb)? Yes ☐ No ☐

10b. If Yes, how old were you? ☐☐

10c. Did the hysterectomy include removal of the ovaries?

Yes ☐ No ☐ or Unknown ☐

11. Have you ever taken an oral contraceptive pill? Yes ☐ No ☐

12a. Have you ever taken hormone replacement therapy? Yes ☐ No ☐

12b. If Yes, how long in total did you take it for?

Less than 3 months ☐

3 to 12 months ☐

1 to 5 years ☐

Longer than 5 years ☐

13. How many live births have you had? ☐☐

Section 2 - Occupation

14a. What was your current/last occupation (job title)? ☐

14b. In what industry did you carry out this occupation (eg farming, shipyard, car factory, shoe shop, hospital, insurance office)? ☐

14c. Number of years in job: ☐☐

15a. What was the main occupation that you held for the longest period of time (job title)? ☐

15b. In what industry did you carry out this occupation (eg farming, shipyard, car factory, shoe shop, hospital, insurance office)? ☐

15c. Number of years in job: ☐☐

For your main occupation in an average working day, did you:

16. Sit for more than two hours in total? Yes ☐ No ☐ Don't know ☐

17. Stand or walk for more than two hours in total? Yes ☐ No ☐ Don't know ☐

18. Kneel for more than one hour in total? Yes ☐ No ☐ Don't know ☐

19. Squat for more than one hour in total? Yes ☐ No ☐ Don't know ☐

20. Drive for more than 4 hours in total? Yes ☐ No ☐ Don't know ☐

21. Walk more than 2 miles in total? Yes ☐ No ☐ Don't know ☐

Section 2 – Occupation (continued)

22. In the course of your work how often on average did you lift or carry weights of 10 kg or more?

Never ☐

Less than once per week ☐

1 to 10 times per week ☐

More than 10 times per week ☐

23. In the course of your work how often on average did you lift or carry weights of 25kg or more (Equivalent to half a bag of cement)

Never ☐

Less than once per week ☐

1 to 10 times per week ☐

More than 10 times per week ☐

Section 3 – Medical history (1)

Please list in the box below all medication that the patient is currently taking:

Have you **EVER** been told by a Doctor or other health professional that you have **ANY** of the following conditions (please tick all that apply to you):

Cardiology

24. Congenital Heart Disease ☐
25. Coronary Heart Disease ☐
26. Heart Attack ☐
27. Hypertension (high blood pressure) ☐
28. High Blood Pressure in Pregnancy ☐
29. Angina ☐
30. High Cholesterol ☐
31. Deep Vein Thrombosis ☐
32. Varicose Veins ☐
33. Pulmonary Embolism ☐

Immunology/Chest Medicine

34. Asthma ☐
35. Hayfever ☐
36. Eczema ☐
37. Sinusitis ☐

Gastroenterology/Endocronology

38. Heartburn ☐
39. Irritable Bowel Syndrome ☐
40. Crohn's ☐
41. Diabetes ☐

Neurology/Psychiatry

42. Dyslexia ☐
43. Clinical Depression ☐
44. Anxiety/Stress Disorder ☐
45. Epilepsy ☐
46. Stroke ☐
47. Motion Sickness ☐
48. Migraine ☐

Section 3 – Medical history (2)

Have you **EVER** been told by a Doctor or other health professional that you have **ANY** of the following conditions (please tick all that apply to you):

Oncology/Cancers

49. Breast Cancer ☐

50. Colon Cancer ☐

51a. Skin Cancer ☐

if yes, was it:

51b. Melanoma ☐

51c. Basal Cell Carcinoma ☐

51d. Squamous Cell Carcinoma ☐

Rheumatology

52. Gout ☐

53. Paget's Disease ☐

54. Bunions ☐

55. Frozen Shoulder ☐

56. Osteoporosis ☐

57. Carpal Tunnel ☐

58. Tennis Elbow ☐

59. Golfer's Elbow ☐

Dermatology/Skin

60. Acne (that caused scarring) ☐

61. Viral Warts ☐

62. Cold Sores ☐

Hearing

63. Hearing Loss ☐

64. Tinnitus (ringing in ears) ☐

Opthalmology/Eyes

65. Glaucoma ☐

66. Cataract ☐

67. Myopia (short sightedness) ☐

68. Age-related Macular Degeneration (AMD) ☐

Urology

69. Incontinence (leak urine) ☐

70. Polycystic ovary syndrome ☐

Section 3 – Medical history (3)

Please answer the following questions by ticking the appropriate box:

71a. Have you ever lost the use of an arm, leg, vision, or ability to speak?

Yes ☐ No ☐

71b. If Yes, how long for : less than 24 hours ☐ or more than 24 hours ☐

72a. Do you usually bring up phlegm from your chest in winter? Yes ☐ No ☐

72b. Do you usually bring up phlegm on most days for at least 3 months a year?

Yes ☐ No ☐

73a. Have you had heartburn or acid regurgitation in the last year? Yes ☐ No ☐

73b. If Yes, how many times have you had heartburn/acid regurgitation in the last year?

Less than once a month ☐

About once a month ☐

Once a week or more ☐

74a. Have you been bothered by recurrent headaches? Yes ☐ No ☐

74b. If Yes, do you still have recurrent headaches? Yes ☐ No ☐

74c. If Yes, are your most troubling headaches

One sided ☐

Accompanied by sensitivity to light/noise ☐

4 to 72 hours in duration if untreated ☐

Section 3 – Medical history (4)

Please answer the following questions by ticking the appropriate box:

75. Since turning 16 have you ever fractured or broken a bone? Yes ☐ No ☐

If Yes, please tick which of the following bones you have fractured or broken

Wrist ☐ Arm ☐ Ribs ☐ Hip ☐ Ankle ☐ Vertebra ☐ Other ☐

76. In the past 3 months have you had pain in your back on most days?

Yes ☐ No ☐

If Yes, does this pain typically radiate to either leg? Yes ☐ No ☐

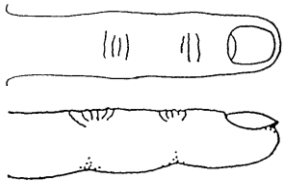
77. In the past 3 months have you had any pain in any part of your body lasting at least 24 hours? Yes ☐ No ☐

Section 4 – Nodal status

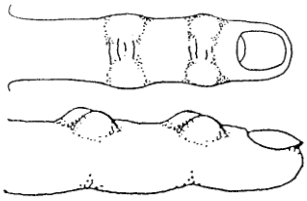
We are interested in knowing whether you have any finger nodes. These sometimes relate to arthritis at the hand and other joints. A finger node is a firm, bobbly swelling on the back of the finger joint.

For example:

A finger **without** nodes:



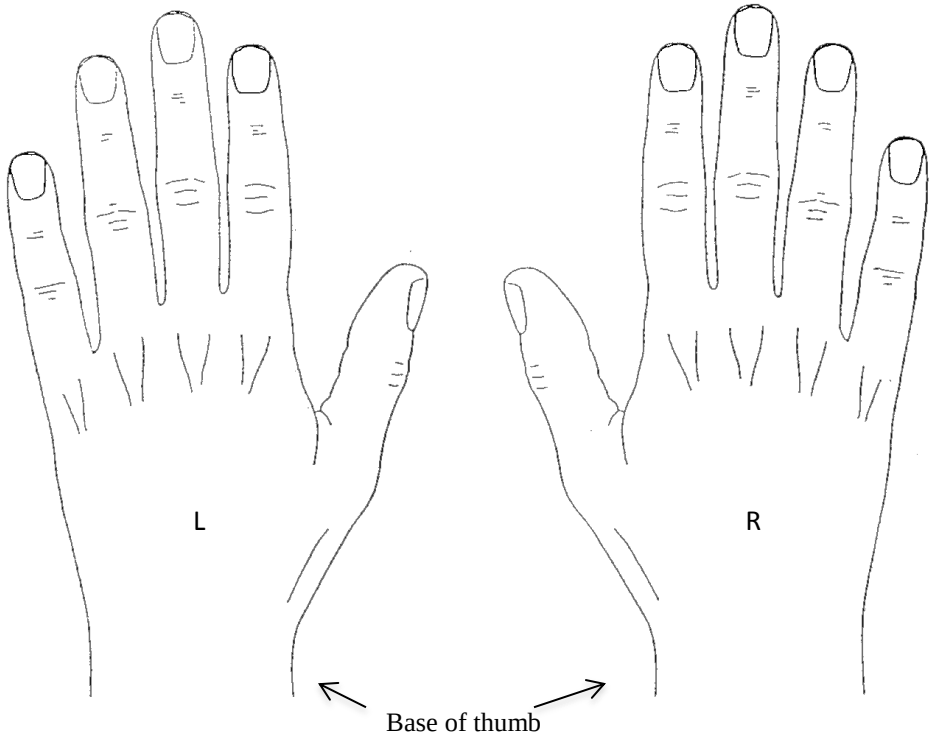
A finger **with** nodes:



When you meet with the research assistant, please look at your hands and then answer the following questions:

78a. Do you think you have any nodes/swellings on your hands? Yes ☐ No ☐

If Yes, for each hand please circle the finger joint(s) where you have these nodes. (You may circle several joints).



Section 4 – Nodal status (continued)

78b. If Yes, at what age did the nodes first develop? ☐☐

78c. How many nodes do you have on the: left hand ☐☐

right hand ☐☐

79. Which hand do you write with? Left ☐

Right ☐

80a. Have you suffered from pain in the fingers for most days for at least one month?

Yes ☐ No ☐

80b. If Yes, at what age did you first develop 'significant' pain in your fingers? ☐☐

81. Do you have pain in the base of your thumb (as arrow on drawing)?

Yes ☐ No ☐

Section 5 – Family History of Osteoarthritis

82. Does/did your mother suffer from osteoarthritis of the knee/hip?

Yes ☐ No ☐ Don't know ☐

If Yes, has/did your mother had/have a total joint replacement of the knee/hip?

Yes ☐ No ☐ Don't know ☐

83. Does/did your father suffer from osteoarthritis of the knee/hip?

Yes ☐ No ☐ Don't know ☐

If Yes, has/did your father had/have a total joint replacement of the knee/hip?

Yes ☐ No ☐ Don't know ☐

84. Does/did your brothers/sisters suffer from osteoarthritis of the knee/hip?

Yes ☐ No ☐ Don't know ☐

If Yes, has/did your brothers/sisters had/have a total joint replacement of the knee/hip? Yes ☐ No ☐ Don't know ☐