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FOR HEALTH AND WELFARE

RESEARCH

Annu Näkki

Search for Susceptibility Genes in Osteoarthritis

Annu Näkki

SEARCH FOR SUSCEPTIBILITY GENES IN OSTEOARTHRITIS

ACADEMIC DISSERTATION

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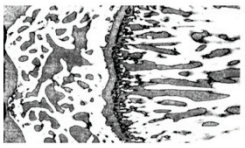

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“It's not that I'm so smart, it's just that I stay with problems longer.”
- Albert Einstein –

Omistettu Papalle

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ABSTRACT

Osteoarthritis (OA) is a complex disease characterized by the destruction of the articular cartilage of the joints. The main symptoms are pain and disability, caused most probably by secondary synovitis. Secondary manifestations of OA are considered to be bone changes, osteophytes, subchondral sclerosis, and bone cysts. The suspected risk factors for OA include genetic predisposition, gender (greater in females), old age, obesity (also in non-weight-bearing joints), previous injury and various physical loading conditions. Prevalence of the disease varies between approximately 8 % and 60 %, dependent on the joint, but also largely on the age of study subjects, the population and OA definition. The disease affects the cartilage, bone, and synovium, but the etiology is still mainly unknown. Even the initiating events and tissue are unknown, but mechanical wear and tear is considered as the prime candidate for the root cause of the disease.

The role of the genetic component in common skeletal diseases is well established but remains poorly understood. Heritability of hand and knee OA in women has been shown to range from 39 % to 65 %. Previous association studies have provided some evidence for genes affecting bone density, inflammation, composition and break down of the extracellular matrix. However, these results have been inconsistent.

The objective of the present study was to identify predisposing genes for OA using single nucleotide polymorphism (SNP) markers. The study consisted of hand, knee and hip OA cases (n = 1466) and controls (n = 4475). A set of 25 candidate genes were studied in carefully selected case-control, family and twin settings.

The objective of Study I was to pinpoint an OA-predisposing gene at the 2q11.2 region, which has previously been linked with hand OA using partially the same study sample (Leppävuori *et al.* 1999). In total 32 SNPs were genotyped in this region comprising 6 genes belonging to the interleukin 1 super-family. *IL1R1* was associated with hand OA ($p = 0.00091$, with a significance threshold of $p = 0.0021$).

The aim of Study II was to identify genetic variants in *MMP8* and *MMP9* genes that predispose to OA. Five different study cohorts including 1369 OA cases and 4445 controls were studied. Evidence for the role of *MMP8* in knee OA was observed in one study sample ($p = 0.0049$, with a significance threshold of 0.0057) without statistically significant replication. The main association finding was found in the

Finnish study sample for knee OA, while a similar tendency was observed in both the knee OA study sample of Spanish origin, and the hand OA sample of Finnish origin.

The aim of Study III was to identify variants predisposing to hip OA from a preselected set of 25 biologically interesting candidate genes. Hips of the study subjects had been analyzed using magnetic resonance imaging (MRI). Variants in the *COL9A2* and *COL10A1* genes showed suggestive association ($p = 0.0021$ and $p = 0.0015$, with significance threshold of 0.00073). The change in the same codon of the *COL9A2* has previously been shown to associate with a disc degeneration phenotype (Videman *et al.* 2009).

In conclusion, the results of this thesis support the theory that inflammatory factors, cartilage breaking factors and originally poor quality of collagen associate with OA. Validation of all these results in larger study populations with more accurate genetic analysis are still needed for any further conclusions.

Key words: osteoarthritis, gene, association, matrix metalloproteinase 8, interleukin 1 receptor 1, type IX collagen alpha 2

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TIIVISTELMÄ

Nivelrikko on monitekijäinen nivelruston rappeumasairaus. Sen pääasialliset oireet ovat todennäköisesti sekundaarisen synoviitin aiheuttamat kipu ja toiminnalliset nivelvaivat. Luunmuutoksia, osteofyyttejä, rustonalaisen luun skleroosia ja kystia pidetään sekundaarisina muutoksina. Perintötekijät, naissukupuoli, ikä, lihavuus (myös nivelissä, jotka eivät kuormitu painon vuoksi), aiemmat nivelvammat ja erilaiset fyysiset kuormitukset ovat nivelrikon riskitekijöitä. Taudin yleisyys vaihtelee 8 ja 60 %:n välillä riippuen tutkittavasta nivelestä, ikäryhmästä, väestöstä ja nivelrikon määritelmästä. Tauti vaikuttaa rustoon, luuhun ja nivelkalvoon, mutta taudin etiologia on vielä tuntematon. Jopa taudin alulle panevia tapahtumia tai varhaisia kudosmuutoksia ei vielä tunneta, mutta mekaanista kulutusta pidetään yhtenä tärkeimpänä tekijänä taudin synnyssä.

Geneettisten tekijöiden merkitys tuki- ja liikuntaelinsairauksissa on yleisesti tunnustettu, mutta huonosti ymmärretty. Geneettiset tekijät selittävät käsi- ja polvinivelrikosta 39 - 65 %. Aiemmat assosiaatiotutkimukset ovat osoittaneet luun tiheyteen, tulehdukseen, ruston koostumukseen ja hajotukseen liittyvien geenien vaikuttavan nivelrikkoon, mutta useat tulokset ovat edelleen ristiriitaisia.

Tämän väitöskirjatyön tavoitteena oli tunnistaa nivelrikolle altistavia geenejä. Tutkimus koostui käden, polven ja lonkan nivelrikkoa sairastavista henkilöistä (n = 1466) sekä näiden verrokkihenkilöistä (n = 4475). Yhteensä 25 ennalta valittua ehdokasgeeniä tutkittiin tarkoin valituissa tapaus-verrokki-, perhe- sekä kaksosasetelmissa.

Ensimmäisen tutkimuksen tavoitteena oli osoittaa kromosomialueelta 2q11.2 nivelrikolle altistava geeni. Alueen oli aiemmassa suomalaistutkimuksessa havaittu kytkeytyvän käden nivelrikkoon aineistossa, joka oli osittain päällekkäinen tämän tutkimuksen aineiston kanssa (Leppävuori *et al.* 1999). Yhteensä 32 SNPiä (yhden emäksen polymorfioita) analysoitiin tältä kuusi interleukiiniperheen geeniä sisältävältä alueelta. *IL1R1*-geenin todettiin assosioituvan käden nivelrikkoon ($p = 0.00091$, kun merkitsevyysraja oli $p = 0.0021$).

Toisessa tutkimuksessa syvennyttiin matriksi metalloproteinaasi 8 (*MMP8*) ja matriksi metalloproteinaasi 9 (*MMP9*) -geeneihin polven nivelrikossa. Tutkimuksessa käytettiin viittä eri tutkimusaineistoa, jotka sisälsivät 1369 nivelrikkoa sairastavaa and 4445 tervettä verrokkia. Näiden avulla löydettiin assosiaatio *MMP8*-geeniin ($p = 0.0049$, kun merkitsevyysraja oli 0.0057), mutta löydös ei toistunut muissa aineistoissa tilastollisesti merkittävällä tasolla. Päälöydös

tehtiin suomalaisessa polven nivelrikkoaineistossa, mutta myös suomalainen käden nivelrikkoaineisto ja espanjalainen polven nivelrikkoaineisto näyttivät samansuuntaista tulosta.

Kolmannen osatyön tavoitteena oli tutkia nivelrikolle altistavia variantteja 25:stä biologisen merkityksensä vuoksi ennalta valitusta ehdokasgeenistä. Tutkimushenkilöiden lonkkanivelet oli analysoitu magneettisella resonanssikuvantamisella (MRI). Kaksi varianttia, geeneissä kollageeni 9 alfa 2 (*COL9A2*) sekä kollageeni 10 alfa 1 (*COL10A1*), näyttivät altistavan lonkan nivelrikolle ($p = 0.0021$ and $p = 0.0015$, kun merkitsevyysraja oli 0.00073). Tulos ei ollut tilastollisesti merkitsevä. Saman kodonin muutoksen *COL9A2*-geenissä on aiemmin todettu altistavan selän välilevyrappeumalle (Videman *et al.* 2009).

Yhteenvetona voidaan todeta, että tämän väitöskirjatyön tulokset tukevat aiempaa teoriaa siitä, että tulehdukselliset ja rustoa hajottavat tekijät sekä jo alun alkaen huonolaatuinen kollageeni altistavat nivelrikolle. Nämä tulokset on kuitenkin validoitava laajemmissa aineistoissa ja geneettisiä analyysejä on tarkennettava varmempien johtopäätösten tekemiseksi.

Asiasanat: nivelrikko, geeni, assosiaatio, matriksi metalloproteinaasi 8, interleukiini 1 reseptori 1, tyypin IX kollageeni alfa 2

CONTENTS

ABBREVIATIONS

A2BP1	Ataxin-2 binding protein 1
ACAN AGC1	aggrecan
ADAM	a disintegrin and metalloproteinase domain-containing protein family
ADAM12	ADAM metallopeptidase domain 12
ADAMTS4	ADAM metallopeptidase with thrombospondin type 1 motif 4
ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif 5
ANP32A	acidic (leucine-rich) nuclear phosphoprotein 32 family, member A
Arg	arginine
ARHGAP25	Rho GTPase activating protein 25 isoform a
ASPN	asporin
BCAP29	B-cell receptor-associated protein 29
BMI	body mass index
BMPs	bone morphogenetic proteins
bp	base pair
BTNL2	butyrophilin-like 2 (MHC class II associated)
CALM1	calmodulin 1
CAPN7	calpain 7
CEP250	centrosomal protein 250 kDa
CEU	Utah residents with Northern and Western European ancestry from the CEPH collection
CI	confidence interval
CILP	cartilage intermediate layer protein
CMC	carpometacarpal
CNV	copy number variations
CNTNAP2	“contactin associated protein-like 2 gene
COG5	component of oligomeric golgi complex 5
COL1A1	type I collagen α1 chain
COL1A2	type I collagen α2 chain
COL2A1	type II collagen α1 chain
COL3A1	type III collagen α1 chain
COL5A1	type IV collagen α1 chain
COL6A4P1	collagen, type VI, alpha 4 pseudogene
COL9A1	type IX collagen α1 chain
COL9A2	type IX collagen α2 chain
COL9A3	type IX collagen α3 chain
COMP	cartilage oligomeric matrix protein
COX-2	cyclo-oxygenase-2
CPNE1	copine I
CRP	C-reactive protein
DD	disc degeneration
DIP	distal interphalangeal
DIO2	deiodinase, iodothyronine, type II
DIO3	deiodinase, iodothyronine, type III
DUS4L	dihydrouridine synthase 4-like (S. cerevisiae)
DVWA	double von Willebrand factor A domains
DZ	dizygote
ECM	extra cellular matrix

EDG2	endothelial differentiation gene 2
FRZB	frizzled-related protein
GDF5	growth and differentiation factor 5
GIST	genotype-IBD Sharing Test
Gln	glutamine
GOA	generalized OA
GPR22	G protein-coupled receptor 22
GWA	genome-wide association
GWL	genome-wide linkage
HA	hyaluronic acid, hyaluronan
HLA	human leukocyte antigen
HLA-DRA	major histocompatibility complex, class II, DR alpha
HLA-DRB1	major histocompatibility complex, class II, DR beta 1
HLA-DRB3	major histocompatibility complex, class II, DR beta 3
HLA-DRB4	major histocompatibility complex, class II, DR beta 4
HLA-DRB5	major histocompatibility complex, class II, DR beta 5
HLA-DQA1	major histocompatibility complex, class II, DQ alpha 1
HLA-DQB1	major histocompatibility complex, class II, DQ beta 1
hME	homogeneous MassEXTEND™
HPB1	official name *PBRM1*, polybromo 1
hs-CRP	high sensitive C-reactive protein
HW	Hardy-Weinberg
HWE	Hardy-Weinberg equilibrium
IBD	identity by descent
IBS	identity by state
IGF1	insulin-like growth factor 1
IDH1	isocitrate dehydrogenase 1 (NADP+), soluble
IL1A, IL-1α,	interleukin 1α
IL1RAcP, *IL1RAP*	interleukin 1 receptor accessory protein
IL1B, IL-1β,	interleukin 1β
IL1, IL-1,	interleukin 1
IL6, IL-6	interleukin 6
IL8, IL-8	interleukin 8
IL1R1	interleukin 1 receptor 1
IL1RL1	interleukin 1 receptor-like 1
IL1RL2	interleukin 1 receptor-like 2
IL1RN	interleukin 1 receptor antagonist (corresponding protein IL-1ra)
IL4R	interleukin 4 receptor
iNOS, *NOS2*,	nitric oxide synthase cytokine-inducible
JNK	c-Jun N-terminal kinase, formed by *JNK1* aka *MAPK8, JNK2* and *JNK3*
JSN	joint space narrowing
KL	Kellgren and Lawrence
LD	linkage disequilibrium
LOD	logarithm of odds
LRCH1	leucine-rich repeats and calponin homology (CH) domain containing 1
MAF	minor allele frequency
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
MATN3	matrilin 3
Mb	mega base
MCF	mitochondrial carrier family
MCF2L	MCF.2 cell line derived transforming sequence-like

MCP	metacarpophalangeal joints
MMP1 MMP-1	matrix metalloproteinase 1
MMP3 MMP-3	matrix metalloproteinase 3
MMP8 MMP-8	matrix metalloproteinase 8
MMP9 MMP-9	matrix metalloproteinase 9
MMP13 MMP-13	matrix metalloproteinase 13
MRI	magnetic resonance imaging
MZ	monozygote
NFκB	nuclear factor kappa beta
NGF	nerve growth factor family
NMR	nuclear magnetic resonance
NRP2	neuropilin 2 gene
NSAIDs	non-steroidal anti-inflammatory drugs
NTF3	neurotrophin 3
OA	osteoarthritis
OP	osteoporosis
OR	odds ratio
p38 MAPK	p38 mitogen-activated protein kinase
PCR	polymerase chain reaction
PGE2	prostaglandin E2
PIIANP	N-propeptide of collagen IIA
PIP	proximal interphalangeal
PLA2G4A	phospholipase A2, group IVA
PTGS2	prostaglandin-endoperoxide synthase 2
PRKAR2B	protein kinase, cAMP-dependent, regulatory, type II, beta
RA	rheumatoid arthritis
RBFOX1	RNA binding protein, fox-1 homolog
SD	standard deviation
SLRP	small leucine-rich proteoglycan
SMAD	(combination of SMA and mothers against decapentaplegic)
SMAD3	SMAD family member 3
Smurf2	SMAD specific E3 ubiquitin protein ligase 2
SNP	single nucleotide polymorphism
SNPSpD	Single Nucleotide Polymorphism Spectral Decomposition
TDT	transmission disequilibrium test
TFAP2B	transcription factor AP-2 beta
TGFB1, TGF- β1	transforming growth factor beta 1
TIMP	tissue inhibitor of metalloproteinase
TIP	thumb interphalangeal
TLR	Toll-like receptor
TNFA, TNF-α	tumor necrosis factor α
Trp	tryptophan
UQCC	ubiquinol-cytochrome c reductase complex chaperone
VDR	vitamin D receptor
VEGF	vascular endothelial growth factor
VNTR	variable number of tandem repeat

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I Näkki A*, Kouhia S*, Saarela J, Harilainen A, Tallroth K, Videman T, Battié MC, Kaprio J, Peltonen L, Kujala UM: Allelic variants of IL1R1 gene associate with severe hand osteoarthritis. *BMC Med Gen* 2010, 11:50.

II Näkki A, Rodriguez-Fontela C, A Gonzalez, Harilainen A, Leino-Arjas P, Heliövaara M, Tallroth K, Videman T, Kaprio J, Peltonen L, Saarela J, Kujala UM: MMP8 gene implies a role in osteoarthritis. *Manuscript soon to be submitted.*

III Näkki A, Videman T, Kujala UM, Suhonen M, Peltonen L, Battié MC, Kaprio J, Saarela J: Candidate gene association study of magnetic resonance imaging-based hip osteoarthritis (OA): Evidence for COL9A2 gene as a common predisposing factor for hip OA and lumbar disc degeneration. *J Rheumatol* 2011 38:747-52.

* These authors have contributed equally

This doctoral thesis includes components from the author's Master's thesis (Näkki 2009).

1 INTRODUCTION

Osteoarthritis (OA) is a degenerative joint disease with pain and disability as the main symptoms. The disease is common in the elderly, in fact it is so common that there seems to be a tendency towards thinking that aching joints are a part of normal aging, which to some extent may be true. However, OA changes in the joints represent wear and tear as a pathological process with heritable input. One theory behind the disease is that for some reason the cartilage of joints breaks down, and that the structural components of the cartilage initiate an auto-inflammatory reaction. This leads to a vicious circle of further degradation of the cartilage.

Even today the etiology of OA is mainly unknown. Researchers do not agree over where the disease originates; e.g. does it start from the cartilage of joints and progress to bone or vice versa. The complexity of the disease, with genetic and environmental factors acting together, the lack of knowledge on its etiology, and the usually slow disease progression, have made it difficult to develop disease-modifying medication. Analgetics and anti-inflammatory drugs are the main treatment in this disease. A true knowledge of the biological mechanisms behind OA is needed for further drug development.

Based on twin studies, it seems that about half of the variation in the occurrence of OA is due to genetics and half is caused by environmental factors (Spector *et al.* 1996; Neame *et al.* 2004). Previous genetic studies have concentrated on collagens and cartilage matrix degrading factors, since the main visual change in OA is the breakdown of joint cartilage. This is composed, to a large extent, of collagens that are maintained by cycles of breaking down and repair by chondrocytes, the main cell type of cartilage. Lately it has become clearer that cell signalling and inflammation additionally play a role in the pathomechanisms.

One reason for this lack of understanding, is that studies of musculoskeletal diseases are not as well financed in many countries, as studies of life-threatening diseases. However, the societal costs for musculoskeletal diseases are high. In Finland, OA causes more than 600,000 doctor visits per year (Häkkinen *et al.* 2006) with OA surgery causing costs of 84 Million € per year (Remes *et al.* 2007). Based on earlier studies (Mäkelä *et al.* 1993; Kiiskinen *et al.* 2005; Kaila-Kangas 2007), Heliövaara (2008) estimated the yearly costs of OA in Finland to be about 1000 Million €, including the direct costs of hospitalization and indirect costs such as working disability. Medical studies of OA are therefore an important investment that could decrease the cost this disease causes to society. The final aim of the studies is to stop the irreversible destruction of joints, or more importantly prevent the chain of

destructive events in the first place. Thus increase the quality of life of the patients, by lessening the pain this common disease causes to many of the elderly.

Exercise is one key element to keep the joints and the musculoskeletal system in good condition. This also affects the other way around, as functional joints are necessary for exercise, which in turn is vital to the prevention of many other common conditions, such as obesity, type II diabetes, and cardiovascular disease. A study by Nüesch *et al.* (2011) concluded that OA patients are at higher risk of death compared to the general population (standardized mortality ratio after 14 years of follow-up 1.55, 95% confidence interval (CI) 1.41 - 1.70). This effect was not due to factors like obesity or joint surgery, but the major risk factor for health was walking disability combined with the history of diabetes, cancer, or cardiovascular disease.

Lately, increasing knowledge of the human genome, improvements of genotyping technologies, and reduced genotyping costs, have made it possible to perform high throughput gene mapping studies. It is already possible to sequence the whole human genome, and soon such protocol will be achievable in a reasonable cost and time frame. Unfortunately, the complexity of many diseases makes it difficult to solve the entire underlying genetic background. In many diseases even the largest genome-wide studies, with tens of thousands of individuals and well defined family samples, have left a large part of the known disease heritability as a mystery. In OA, many study samples still wait to be analyzed with the genome-wide platforms, and many new causative gene variants are waiting to be found.

The aim of this thesis work was to investigate the role of several candidate genes in different types of OA, using twin, family and case-control settings. The study was initiated before the understanding of what is now fundamental knowledge. For example, the segmented structure of DNA was only detailed later through the HapMap project. Additionally, since this field evolves fast, the study designs used changed during the study.

2 REVIEW OF THE LITERATURE

2.1 Gene mapping of complex diseases

2.1.1 Variation in the genome

Nearly the entire human genome, $3.2x10^9$ base pairs, is similar in all humans. Only approximately 0.1 % of it differs between individuals. For the survival of a species living in a changing environment, the ability to develop changes in DNA over time is essential. Mutations can be advantageous, neutral or disease predisposing. Variation such as deletion, insertion, or substitution can occur in a single base pair or to larger segments of DNA. A single nucleotide polymorphism (SNP) is a sequence variation affecting only one base pair. Microsatellite markers (short tandem repeats, short sequence repeat) are repeat sequences of a few bases per repeat ([CCA]n), while minisatellites consist of dozens of base pairs, and copy number variations (CNVs) up to thousands of base pairs. In the repeat sequences, the amounts of repeats can differ between individuals (Lander *et al.* 2001; Venter *et al.* 2001; The International HapMap Consortium 2005).

2.1.2 Heritability

Heritability is defined as the proportion of phenotypic variability between individuals that is caused by genetic variance. Heritability is dependent on the population studied (Kempthorne *et al.* 1961). A trait with a high heritability, for example height, with a heritability of roughly 80 % indicates that 20 % can be attributed to environmental factors such as nutrition. (Silventoinen *et al.* 2003). Twins are usually studied for heritability estimates: on average monozygotic twins share 100 % and dizygotic twins 50 % of their genome. If both types of twins share their environmental factors, the monozygotic twins are more similar than dizygotic twins purely due to genetic factors (Kempthorne *et al.* 1961).

2.1.3 Linkage disequilibrium between markers and the HapMap project

Without the shuffling of recombination, all chromosomes would be inherited as a whole. Even if the chromosomes are under the mixing force of crossing over, markers close to each other are still more likely to be inherited together, and are therefore said to be in linkage disequilibrium (LD) with each other. An LD block can be considered as a region of low recombination, and the boundaries for these blocks have been shown to be similar across populations (Gabriel *et al.* 2002). The HapMap project was launched to determine the LD structure of the human genome. The idea was that an LD map of the whole genome would help geneticists to select relatively few informative tag SNPs for genotyping. A tag SNP would represent the surrounding region that is in high LD with it, and this SNP would therefore be the only one that needs to be genotyped in that specific region. All known variants in the region need not be genotyped in order to fully capture an LD block. In phase 1 of HapMap there were altogether 270 individuals with European, Nigerian, Japanese and Chinese backgrounds genotyped. The original goal was to genotype more than one million SNPs in these samples (The International HapMap Consortium 2003; 2005).

The LD blocks harbor different haplotypes, which are allele combinations of SNPs. Only about 3 – 5 common (> 5 %) haplotypes explain about 90 % of one LD block. Thus, it seems that only about 6 – 8 tag SNPs are needed to identify these different common haplotypes in one LD block (Gabriel *et al.* 2002).

The HapMap tag SNP method has been criticized over its ability to fully cover areas studied in the chromosomes. Some SNPs could be missed even when genotyping tag markers in high LD with them. Additionally, the LD structure might not be identical between case and control groups if one is looking at a functional SNP (Terwilliger *et al.* 2006).

2.1.4 The 1000 Genomes Project

The aim of the 1000 Genomes Project, launched in 2008, was to sequence the genomes of 2,500 study subjects. Individuals of European, American, East Asian, South Asian and West African ancestry have been sequenced as part of the project (1000 Genomes, http://www.1000genomes.org/). The purpose is to gain deeper understanding of the human genome, especially the LD structure. The idea is to be able, in the future, to genotype one set of variants and impute the missing genotypes. Some basic information of the genome variation learned so far is that an individual differs from the reference sequence by 10,000-11,000 non-synonymous sites and by

10,000-12,000 synonymous sites. Each genome is heterozygous for 50-100 variants that cause inherited disorders. While new mutations occur in each individual with the rate of about 1.0×10^{-8} per base pair per generation (The 1000 Genomes Project Consortium 2010)

2.1.5 Association analysis

Association analysis is a statistical test of the co-occurrence of an allele with a phenotype. Association is commonly studied in a case-control setting of unrelated subjects (Ott *et al.* 2011). Association analysis is usually performed with SNP markers since they are more common than any other variation type. On average, there is 1 SNPs per 1 kb in the human genome. SNPs are also favorable markers because new mutations are rarer than in the mutation-susceptible repeat sequences (The International HapMap Consortium 2003; 2005). In addition to the case-control setting association can be studied using the transmission disequilibrium test (TDT). This tests if heterozygous parents transmit a certain allele to diseased offspring more often than the other allele. However, it uses only heterozygous parental genotypes, and it only detects linkage in the presence of population level association (Spielman *et al.* 1993). Additionally, programs that are able to analyze sib-pairs, families, cases and controls jointly have been developed. The analysis is more powerful with family-based data, and one can utilize all possible samples with a program combining different data sets than with programs only utilizing specific family structures or only the case-control setting (Hiekkalinna *et al.* 2011a; Hiekkalinna *et al.* 2011b). Haplotypes can also be used in the association analysis. However in haplotype association tests, estimations of the haplotypes have to be made (Ott *et al.* 2011).

2.1.6 Linkage analysis

Microsatellite markers have traditionally been used in linkage studies. They are more informative than SNPs since there are more alleles in microsatellite markers: Microsatellite markers are repeat sequences of different lengths whereas SNPs have typically only two alleles. Microsatellite markers are, however, rarer (Chapman *et al.* 1998). In linkage analysis it is possible to test if a chromosomal region co-segregates with the studied trait. Linkage analysis is based on the calculation of the recombination fraction (θ, theta), which is the proportion of meiotic events in which two loci are separated by recombination. Two loci close together are unlikely to have been separated from each other by crossing over and their recombination fraction is usually close to 0 (complete linkage). Two loci physically far apart are more likely to have been separated from each other, and therefore their

recombination fraction will be closer to 0.5 (no linkage). The θ can be calculated by dividing the number of recombinant progeny by the total number progeny. In computerized analysis, the likelihood of the two loci being linked (θ = 0) and the likelihood that the two loci are not linked (θ = 0.5) is calculated. The ratio of these likelihoods is called the odds of linkage and its logarithm is called the logarithm of odds of linkage (LOD score). The LOD scores are estimated for different values of θ, with the most likely value for θ being the one that gives the highest LOD score. When many families are used, the LOD scores of all the families are summed, with the LOD scores of 3 and -2 being used as criteria for the presence or exclusion of linkage, respectively (Morton 1955; Ott 1991).

Linkage analysis can be non-parametric (model free analysis) or parametric. Parametric analysis is a powerful analysis method when the inheritance model of a disease is known. Unfortunately, the inheritance model in complex diseases is often unknown (Morton 1955; Ott 1991).

2.1.7 Linkage vs. association

Linkage is a phenomenon of long chromosomal range whereas association has previously been used in narrowing a linkage finding to a smaller region. All humans have common ancestors if we go back far enough in time. Recombination over hundreds of years has made the shared chromosomal segments small between two randomly selected individuals. Population isolates are widely used in gene mapping studies with a common feature to them being their low immigration and emigration. Such isolates generally have a distribution over a relatively small area. One example of isolate linkage mapping is a type 2 diabetes study in Finnish population sub-isolate. A variant near the gene MODY3, on chromosome 12q24, was associated with the disease ($p = 2x10^{-5}$) (Mahtani *et al.* 1996) which has previously been associated with early onset monogenic diabetes (Vaxillaire *et al.* 1995)

2.1.8 Genome-wide strategy

Previously in disease mapping, biologically interesting candidate genes were selected as study targets. Today, GWA studies with about 600.000 SNP markers throughout the genome have become the standard way of performing gene mapping studies. Exome sequencing and whole-genome sequencing are now becoming more common. Many genome-wide studies have revealed remarkable results, such as finding of previously unknown gene loci for type 2 diabetes (Saxena *et al.* 2007; Sladek *et al.* 2007; Steinthorsdottir *et al.* 2007) discussed in the following chapter, since no prior knowledge of disease traits is needed opposite to candidate gene

studies. But then again for example, too sparse marker maps are one reason for failure in whole genome-wide studies.

Genome-wide linkage

Traditionally, genome-wide linkage analysis has been used to find susceptibility loci for disease. A few hundred microsatellite markers throughout the human genome are chosen, with a spacing of approximately 10 mega bases (Mb). This should cover approximately 50 % of the genetic variance in the genome when assuming that the used markers have a heterozygocity of 75 % (Kruglyak 1997). For population-based association studies the microsatellite marker maps are too sparse. Half of the LD blocks are shown to be 22 kb in length or larger in African and African-American populations, and 44 kb or larger in European populations. Thus only about 1 % of the genome would be covered in population-based studies with the microsatellite markers (Gabriel *et al.* 2002). Since the linked regions are usually large, familial genome-wide linkage analysis is often followed by fine-mapping and candidate gene studies in which biologically relevant genes from the linked regions are selected for denser mapping, as in the study by Leppävuori *et al.* (1999) and Study I of this thesis.

Genome-wide association

Improved technologies and statistical analysis methods, combined with decreased costs, have made it possible to conduct extensive genome-wide association (GWA) studies. Illumina, Inc. (2009) sells GWA chips covering over 1.1 million variants including CNVs. Whilst, Affymetrix, Inc. (2009) have a similar product with 1.8 million markers.

One well known success story of GWA is type 2 diabetes. In these studies, the types of genes found and confirmed to affect the disease would not have otherwise been studied, such as the "solute carrier family 30 (zinc transporter), member 8" gene (*SLC30A8*) ($p = 6x10^{-8}$) (Saxena *et al.* 2007; Sladek *et al.* 2007) and the "CDK5 regulatory subunit associated protein 1-like 1" gene (*CDKAL1*) ($p = 4.1\times10^{-11}$, $p = 7.7x10^{-9}$) (Saxena *et al.* 2007; Steinthorsdottir *et al.* 2007).

It is accepted that disease predisposing variants found from case-control GWA studies have only low odds ratios for the studied trait, as was the case in the diabetes GWA findings. However, one could still argue that they provide important knowledge of disease etiology. Opponents claim that since the idea of the GWA is

based on the tag SNP method, the genome will not be fully covered and much will be missed in these studies (Terwilliger *et al.* 2006).

In conclusion, with hundreds of thousands of SNPs analyzed throughout the genome, no assumptions need to be made concerning the genomic location of causal variants, giving this method a major advantage over the candidate gene approach.

Whole-genome and exome sequencing

As part of the 1000 Genomes project (The 1000 Genomes Project Consortium 2011) whole-genome sequencing in complex diseases will reveal the entire base content of the human genome for the study subjects. The goal will then become the understanding of the role of the variants in humans, how they function in expression modification or even protein structure, which will be achieved with the help of other disciplines. As in other genotyping methods, sequence data quality and genome coverage are still challenges to be solved.

Today, exome-sequencing is in use for disease-mapping. As a proof-of-concept, Ng *et al.* (2009) sequenced four individuals suffering from a rare monogenic disease with a known causative gene (Freeman-Sheldon syndrome (FSS)) and eight HapMap individuals originating from three different population backgrounds as controls. The causative variant was found when the authors excluded all variants seen in the healthy individuals either in the study itself or in pre-existing SNP data bases. Only those genes in which all the affected individuals harbored a base mutation, either as an amino acid-changing or affecting exon-splicing, were included, but not necessarily the same variant in all cases.

Currently, the cost of sequencing one genome is $ 10,000 but it is expected to decrease to 10 % of the current cost in the near future (Patterson 2011). Private companies already sell exome-sequencing to consumers for less than $1000 (23andMe, Inc. Mountain View, CA, USA).

2.1.9 Challenges in gene mapping

A positive finding could indicate a true biologically meaningful discovery, e.g. that a disease-causing or predisposing variant has been identified. However, a positive finding could be caused by other factors as well. A variant in high LD with the predisposing one might have been found or the finding could have been made by chance. Similarly, although a negative finding can be caused by lack of relevance of the gene to the studied trait it can also be observed due to factors relating to the

research frame, such as a statistically low-powered study, or phenotypic or genotypic heterogeneity.

Population stratification

One of the challenges is population stratification, which occurs when the study population contains genetically different subsets. For example, if controls are selected from Northern Finland and the cases from Southern Finland, the difference in allele frequencies between cases and controls could be due to normal genetic difference between people from different areas of Finland, and not due to the differing disease status between the people studied (Jakkula *et al.* 2008). The situation may also be problematic if individuals are closely related since the basic χ^2 association test assumes individuals to be unrelated (Ott 1991).

Multiple testing

Type 1 error is typically caused by performing large number of tests, thus increasing the probability of discovering positive results by chance. A Bonferroni correction for multiple testing is the most common and conservative method to account for the number of tests performed. However, the studied gene variants are most often in high LD with each other, and therefore the tests performed with these markers are not independent, as is assumed by the Bonferroni correction. The LD between the markers reduces the number of truly independent tests. One method that takes this LD into consideration is the SNP Spectral Decomposition (SpD) method used also in this thesis work (Nyholt 2004; Li *et al.* 2005). Nowadays, a p-value threshold for genome-wide significance is considered to be $p < 5x10^{-8}$, which equals a Bonferroni correction for one million tests.

Replications

One of the most important aspects of genetic findings is their reproducibility in other samples and other populations. In the case of familial mutations, such replication in another population will not be possible. However, for common disease-predisposing findings replication is the best way to validate the finding. This was the case in studies of type 2 diabetes, for example the finding in *SLC30A8* was replicated, which confirmed that the finding is real and not caused by chance after multiple tests or population stratification (Saxena *et al.* 2007; Sladek *et al.* 2007).

Power

The power to detect an association depends on the methods used, the type of markers, population history, the age of the mutation, the sample size, mode of inheritance, and the phenotypic heterogeneity of the study sample (Chapman *et al.* 1998). According to the GWA study by The Wellcome Trust Case Control Consortium (2007) of seven common diseases in 14,000 cases and 3,000 shared controls, the odds ratios (OR) usually seen in common variants predisposing to complex diseases are between 1.2 - 1.5. This means that large sample sizes are needed to detect any effect and thus in many cases a negative finding does not exclude the role of a variant for a disease.

Gene coverage

When studying candidate genes, it is important to take into consideration how well all the genetic variance in a gene region is caught by the SNP panel genotyped. Even with statistically powerful sample sizes, negative findings cannot exclude the role of a gene if only a portion of the gene has been studied. The HapMap genotyping database is one tool for selecting tag SNPs for both candidate gene studies, as discussed earlier, (The International HapMap Consortium *et al.* 2003) and for GWA platforms. However, this has been criticized, since disease causing SNPs could be missed when only genotyping a tag marker in high LD with it (Terwilliger *et al.* 2006). This matter might be solved soon when whole-genome sequencing becomes more common.

Phenotyping

Besides careful planning of genotyping, one important aspect when planning, or interpreting, a study is the phenotyping. In many cases the studied trait is quantitative. Height is a good example of such a phenotype, and with a heritability of 80 % (Silventoinen *et al.* 2003) GWA studies have revealed several pathways affecting the trait, including genes in extracellular matrix and cancer traits ($p < 5x10^{-5}$) (Weedon *et al.* 2008). Many complex diseases are spectrums of quantitative traits (**Figure 1**). When dividing a population into cases and controls, one cut-off makes some of the cases to be phenotypically close to controls, as shown in part "a" of **Figure 1**. This would be the case if height phenotype would be divided into two groups: short and non-short. For rare diseases the use of population controls does not seem to prevent the association finding, as was stated by the The Wellcome Trust Case Control Consortium (2007).

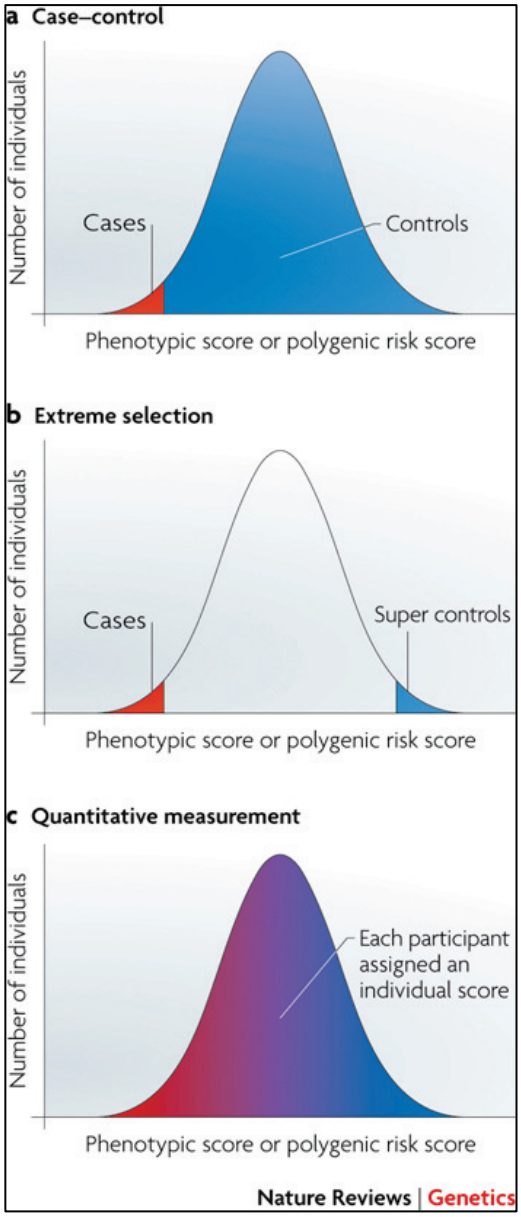

Figure 1. ***Many quantitative traits are dichotomized in genetic studies.*** *a) A cut-off between cases and controls lowers the statistical power. b) When selecting extreme phenotypes, the sample set can be used for only one study. c) Study of quantitative traits has statistically more power than dichotomous traits and one study sample can be used for studies of different phenotypes. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Genetics copyright 2009 (Plomin et al. 2009).*

When using quantitative measurements, the phenotype will correspond to biology as closely as possible, and therefore the power to detect, for example linkage, has been shown to be superior when compared to dichotomized traits (Duggirala *et al.* 1997). With a population-based sample of quantitative traits, one can use the same cohort for studies of different phenotypes, which can point to a network of related quantitative traits.

Based on the discussed studies on phenotyping, it is obvious that there is a need for quantitative phenotyping in OA. In OA, the diagnosis is made by a clinician and is therefore qualitative, since the diagnosis is usually made in order to assess the patient's need for pain medication, surgery, sick leave etc. Biologically this cut-off is not always justified and creates a bias within the study. The disease is more of a progress from mild to a possibly severe stage of OA, including different tissues and different biological events during the progression, discussed later.

Common and rare variants behind diseases

Usually the genetic models behind diseases are unknown. A hot topic discussed in gene mapping is whether common variants, that probably only have small effect sizes, or rare variants with larger effect sizes should be studied (**Figure 2**).

In the case of severe, heritable, early-onset diseases, common predisposing variants should not even exist due to evolutionary selection, unless there has been recent strong environmental changes predisposing to the disease. As OA is a late onset disease linked with the immune system, it could be possible that variants predisposing to OA today have been and still are useful, for example in microbial resistance or in bone health, and have thus become common.

One way of looking at the issue is to ask, which way is statistically, technically and financially possible and smart. As discussed previously, in the recent GWA studies performed, most of the common disease susceptibility variants found have only had a small effect on the disease, thus large study samples have been needed. Whereas, variants of low frequency and small effect sizes will not be detectable by population association studies, since only a handful of people will carry the disease predisposing variants. Detecting these rare variants in families is also difficult, since only some of the family members will be affected, even if carrying the rare allele. Finding common disease predisposing variants is dependent on high-throughput genotyping of large sample sets. Finding rare variants requires sequencing of a large number of carefully chosen cases followed by functional studies on the identified candidate variants. This kind of study approach has been shown to work even with a

handful of samples, when the studied trait is severe enough not to be expected to be seen in a population (Ng *et al.* 2009).

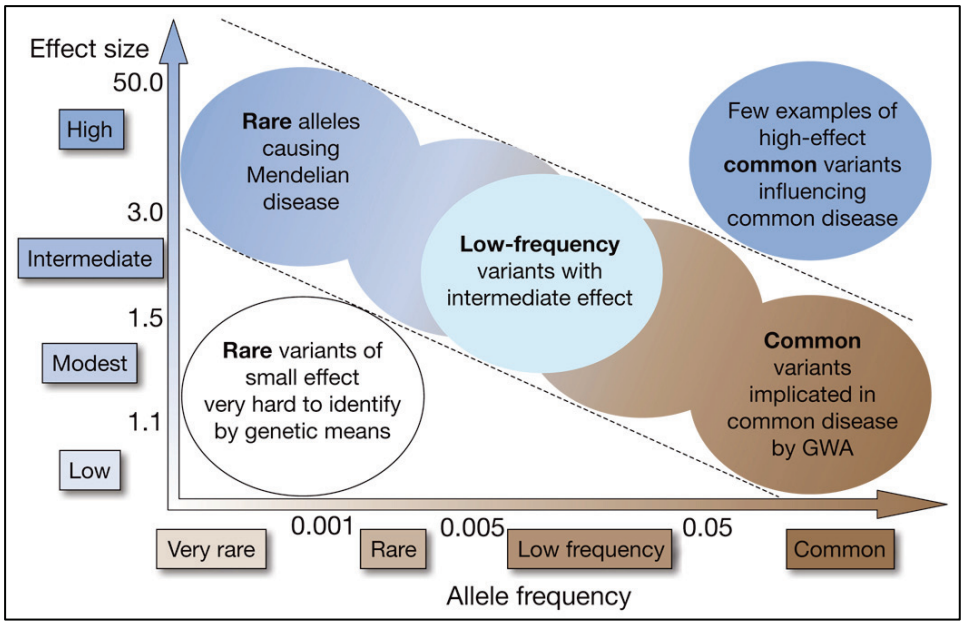

Figure 2. ***Effect size vs. allele frequency***. *Brown color: Common variants are shown to have low effect in complex diseases (Wellcome Trust Case Control Consortium 2007). Blue color: Rare alleles have been shown to cause Mendelian disorders such as the "RNA export mediator homolog (yeast)" gene (GLE1) causing lethal congenital contracture syndrome 1 (Nousiainen et al. 2008). Adapted by permission from Macmillan Publishers Ltd: Nature (Manolio et al. 2009) copyright 2009; Original image by McCarthy et al. (2008). Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Genetics, copyright 2008.*

More importantly we should be asking, which way is biologically sensible to look for variants predisposing to common complex diseases. Based on the initial 1000 Genomes data (The 1000 Genomes Project Consortium 2010), it was concluded that a maximum third of complex trait associations are likely to be caused by common coding variation. Whilst the majority of the contribution of common variants in complex diseases is assumed to be regulatory in nature. Moreover, based on a recent study by Zhu *et al.* (2011) comparing the phenotypic effect of rare and common alleles, it seems that rare variants are more likely to be functional than the common ones. If an allele had a frequency of more than 10 % it was less likely to be located in functional genomic regions, compared to alleles with a frequency of less than 10 %. However, this phenomenon was only seen in the common situation where the

major allele is the ancestral allele and the minor allele is the derived allele. The authors concluded that variants that should be enriched for positive selection are actually common through genetic drift, not through positive selection. The main selective pressure is purifying selection and the main part of the functional variation in our genome is caused by mutation-selection balance.

These findings might lead the geneticists back to studying large families and isolates in which the disease predisposing alleles are more likely shared between phenotypically similar individuals, as opposed to population based cohorts.

Missing heritability

Recently, a lot has been discussed about missing heritability. This refers to the issue that so far in studies of complex diseases, even the most extensive GWA studies in several large population have only been able to explain a small proportion of the heritability that have been discovered through twin studies (Wellcome Trust Case Control Consortium 2007). Thus rare, as yet unstudied, variants, rare Mendelian traits and well-genotyped families are promising candidates for future studies. All in all, it seems that different approaches are needed in order to solve the entire genetic background of complex diseases.

The complex nature of the challenges

There is a cross-link between the challenges of power and selecting a study population. When increasing the sample size for statistical power, one might also increase the allelic and phenotypic heterogeneity. As described by Terwilliger and Weiss (1998): “If there is substantial allelic heterogeneity then as one increases the sample size, the number of different disease-predisposing alleles (each with their own independent haplotype of nearby marker alleles) may likewise increase, and thus there may never be much power even with complete ascertainment of the entire human population.” (Reprinted with permission from Elsevier Ltd, Copyright 1998).

Another cross-link is between power, in terms of selecting common enough markers, and the biological background of complex diseases. Common SNP markers have not fully explained the familiality of many diseases, which could mean that the rare variants play the major role in many diseases (Wellcome Trust Case Control Consortium 2007). If this turns out to be the case, most of the meaningful markers have been selected out of GWA studies in order to gain statistical power.

The reality probably lies somewhere between these scenarios: GWA studies in large cohorts with many different traits have shown that there are common alleles that can be found, and some of the findings would not have been done if we only proceeded in an hypothesis based manner (Saxena *et al.* 2007; Sladek *et al.* 2007; Steinthorsdottir *et al.* 2007; Wellcome Trust Case Control Consortium 2007).

Population sub-isolates will probably be the way to go during the next decade, since in isolates the genetic heterogeneity is smaller and thus it is likely that individuals with similar outcome share the same potentially rare genetic factors.

2.2 Osteoarthritis

OA is a degenerative joint disease common in the elderly. The disease affects the bone, cartilage and synovium (**Figure 3**).

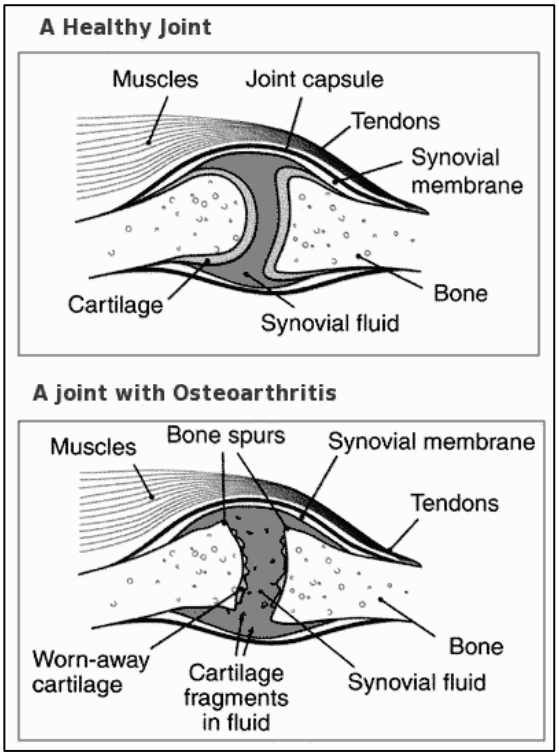

Figure 3. ***Normal joint and joint with osteoarthritic changes (Free ClipArt 2009)****. The OA joint suffers from a loss of cartilage, bone changes, osteophytes, subchondral sclerosis and bone cysts. Synovitis, joint space narrowing and subchondral bone sclerosis are erroneously not shown in the figure. http://cksinfo.com/medicine/page2.html.*

The etiology of the disease is, even today, not understood, but it is supposed that some unknown genetic factors play a role in its development. What is also known, is that OA can develop in an anatomically normal joint (primary OA), or in a joint suffering from injury, some other disease, or developmental defect such as congenital dislocation of the joint (secondary OA) (Pearson *et al.* 1962).

Autoimmune factors also play a role, linking the disease to other inflammatory diseases such as rheumatoid arthritis (RA). The heritability of radiographic hand and knee OA has been estimated to range between 39 % and 65 % (Spector *et al.* 1996; Neame *et al.* 2004).

2.2.1 Symptoms

Based on the criteria set by The American College of Rheumatology, pain, aching, and stiffness are the main symptoms of OA. Stiffness in the morning might occur for 30 minutes and there might be a mild inflammatory swelling. In rheumatoid arthritis the symptoms resemble OA, but for instance, the swelling and morning stiffness are more severe and last longer in RA (Altman *et al.* 1990; Altman *et al.* 1991; Kawasaki *et al.* 1998).

2.2.2 Kellgren and Lawrence classification

Diagnostics for OA have been discussed for a long time. Radiological classification of OA was invented by Kellgren and Lawrance (1957), and has been widely used ever since. The grading is between 0 and 4; 0 corresponding to a healthy joint and 4 corresponding to severe OA. The grading is based on visual x-ray observations of: ostophytes which could be described as formation of bony nodules; periarticular ossicles which are bone formations in an abnormal area of the joint; narrowing of the joint cartilage associated with sclerosis of the subchondral bone; small cystic areas with sclerotic wall situated usually in the subchondral bone; and altered shape of bone.

The controversy surrounding this classification is the relationship between the symptoms and the radiological assessment of this disease. The radiological changes are more common than the symptoms (see next chapter). The radiological changes predispose to the symptoms in the same joint. However, most people with the radiological changes do not have symptoms, such as pain and stiffness, at all. Moreover, patients suffering from pain in their spine can be totally normal in radiological grading (Lawrence *et al.* 1966).

2.2.3 Heritability and prevalence

MacGregor *et al.* (2000) reported heritability of radiographic hip OA to be 58 - 64 % in a UK population cohort of women. Zhai *et al.* (2007) estimated the heritability of radiological knee OA to be 69 - 80 % in a population-based study with twins in the UK. Similar heritability estimates, made based on radiological hand OA examinations in UK women, ranged between 48 - 67 % for radiological phenotype (Livshits *et al.* 2007).

The prevalence of the most common OA types vary approximately between 6 % (Zhang *et al.* 2001) and 48 % (Haara *et al.* 2003) depending mostly on the joint, but also largely on the age of the study subjects, the population, and definition of OA.

Hand and knee OA are common whilst hip OA is rare. The prevalence of radiological hand OA was 44 – 48 % in the Finnish population over the age of 30 years (Haara *et al.* 2003), and 27 % for radiological knee OA in a study of subjects under the age of 70 years (Felson *et al.* 1987). However, the prevalence of radiologic hip OA was 8 %, when standardized for age and sex in an Icelandic population over 35 years of age (Ingvarsson *et al.* 1999).

Women tend to have more OA compared to men. For symptomatic knee OA the prevalence was 6 % in men and 15 % in women (Zhang *et al.* 2001). In the case of hip OA there seems to be little or no difference between the genders, being 12 % for men and 10 % for women, in the Icelandic study (Ingvarsson *et al.* 1999).

Symptomatic OA is rarer than radiographic. The prevalence of radiographic knee OA in a population aged 60 years or over, was 22 - 43 %, whilst for symptomatic knee OA the prevalence was 6 - 15 % (Zhang *et al.* 2001).

Age is a significant factor. The prevalence of radiological hip OA being 2 % among individuals between the ages of 35-39 years and 35 % among individuals over the age of 85 years (Ingvarsson *et al.* 1999).

2.2.4 Risk factors predisposing to osteoarthritis

The most common known risk factors for OA are genetics factors, female sex, obesity, hard physical work, excessive repetitive motions, and previous joint trauma (Haara *et al.* 2003; Recnik *et al.* 2008).

Age and sex

Knee and hand OA prevalence is usually somewhat higher in females than in males (Zhang *et al.* 2001; Haara *et al.* 2003; Toivanen *et al.* 2010), which means that being female is a risk factor for OA. In a study by Toivanen *et al.* (2010), the odds ratio (OR) for OA in females was 1.7 (95 % confidence interval (CI) 1.0 - 3.1). OA is more common in older age groups than younger, with, for example, the OR for older age in hand OA being 1.8 (95 % CI 1.4 - 2.3) in the same study.

Obesity

One factor predisposing to OA is obesity. The role of obesity in non-weight bearing joints suggests a theory that systemic level pathways are playing a role in the disease. Muthuri *et al.* (2011) conducted a meta-analysis of 47 studies using 446,219 individuals originating from USA, Europe, Australia, North Africa, China, and Thailand, which are not individually discussed in the present thesis work. Obesity predisposed to knee OA with an overall odds ratio (OR) of 3.91 (95 % confidence interval (CI) 3.32 - 4.56), with all individual studies showing the same trend.

Furthermore, not only does obesity predispose to OA in weight bearing joints, but it also affects OA in non weight bearing joints, like the joints of the hands (Bagge *et al.* 1991; Cicuttini *et al.* 1996; Haara *et al.* 2003). A BMI of more than 35 kg/m^2 doubles the risk of hand OA, compaired to people with normal BMI (20.0 - 24.9 kg/m^2) (Haara *et al.* 2003). The prevalence of hand OA was found to especially increase in the presence of obesity, hypertension, and diabetes, mostly during younger age, in a cross-sectional study of a population based on 3585 individuals over the age of 55 years (Dahaghin *et al.* 2007). Additionally, it seems that the expression and protein levels of leptin produced by osteoblasts are increased in OA (Mutabaruka *et al.* 2010).

Physical loading

High work-related physical loading seems to have a negative effect on hand OA in women (Haara *et al.* 2003). Further, higher measured grip strength has been shown to predispose to PIP, MCP, and thumb base joint OA in men (Chaisson *et al.* 1999). It has even been shown that paralysis of hands protects from hand OA (Segal *et al.* 1998).

Similarly, many other studies show the unfavorable effect of too heavy loading in different joints. A study by Recnik *et al.* (2008) showed that an unfavorable

biomechanical constitution of the pelvis was associated with hip arthroplasty at younger age. While Toivanen *et al.* (2010) showed that the OR for knee OA, among individuals that are under heavy physical stress in their work, was 18.3 (95% CI 4.2 - 79.4), compared to the lightest physical stress category.

Traumatic injuries

Previous physical joint trauma is a well-known risk factor for OA. In the study by Toivanen *et al.* (2010) using the Mini-Finland Health Survey with Finnish participants, the odds ratio for knee OA was 5.1 (95 % CI 1.4 - 19.0) for previous knee trauma compared to a group without trauma. The part of the study examining the knee trauma was based on simple yes or no questionnaire.

2.2.5 Joint and cartilage

In a synovial joint, both endings of the two bones are covered with cartilage, which moderates the effect of weight and impact bearing on the bones. The cartilage neither contains nerves nor blood or lymphatic vessels, consisting mostly of extracellular matrix (ECM). ECM is both generated and broken down by the chondrocytes that constitute 1 – 10 % of the cartilage volume. The area around chondrocytes can be divided into different structural components of proteoglycans, link proteins, and hyaluronic acid (hyaluronan, HA). The matrix is formed by proteoglycans, collagens, and water (Huber *et al.* 2000). Due to the avascular nature of the cartilage, the chondrocytes change their metabolites with the surrounding system through diffusion to and from synovial fluid. Some of the deeper areas can also get nutrients from the subchondral bone (Arkill *et al.* 2008).

Proteoglycans and collagens are responsible for the elasticity of the cartilage. Collagens form a net, which counteracts the swelling pressure of a gel formed by water and proteoglycans (**Figure 4**). Type II collagen is the most abundant collagen in cartilage, but other types of collagens, such as type IX, participate by stabilizing the net (Huber *et al.* 2000).

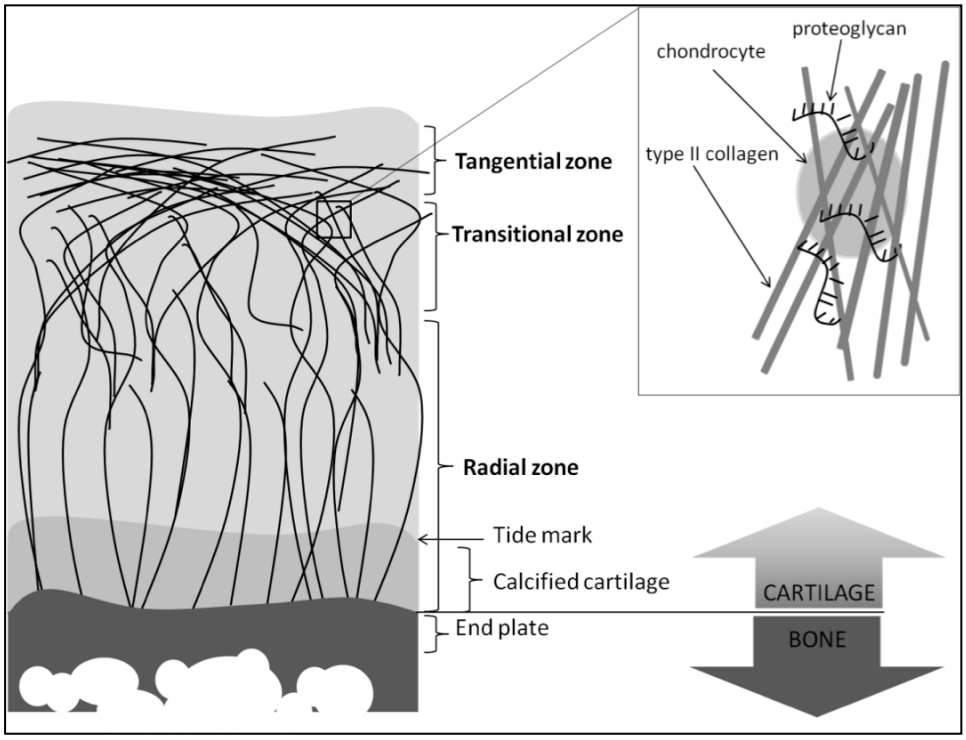

Figure 4. ***Type II collagen in different zones of articular cartilage and the organization of type II collagen, proteoglycans and chondrocytes in more detail.*** *The collagen net can be divided into three zones, where the uppermost tangential zone has a horizontal collagen fiber orientation. The transitional zone has more mixed and the radial zone contains mostly vertical collagen fibers. The deepest part of the radial zone is formed by calcified cartilage (Huber et al. 2000). Figure adapted from Knee joint surgery, www.kneejointsurgery.com/html/articular_cartilage/anatomy.html.*

Proteoglycans are formed when glycosaminoglycan chains attached to a protein core. Aggrecans are the most abundant proteoglycans and they can bind to HA in part via a link protein (**Figure 5**) (Heinegård *et al.* 2011). Aggrecans are rich in chondroitin sulfate and keratin sulfate, which comprise both sulfate and carboxyl residues. These residues cause high negative charge density, called the fixed charge density, in the ECM. This negative charge attracts positive mobile Na^+ ions that cause osmotic swelling pressure, and therefore draws water into the ECM. During loading, this balance is shifted, and some water is compressed out of the cartilage until a new balance is reached. When loading stops, the original balance is reached again as the water is absorbed back reinstalling the original water content. The ability of the cartilage to resist pressure is a complex system, which additionally includes characteristics such as electrostatic repulsion forces of the negative

residues, and friction between water and the ECM (Lai *et al.* 1991; Lu *et al.* 2008; Wang *et al.* 2008; Han *et al.* 2011).

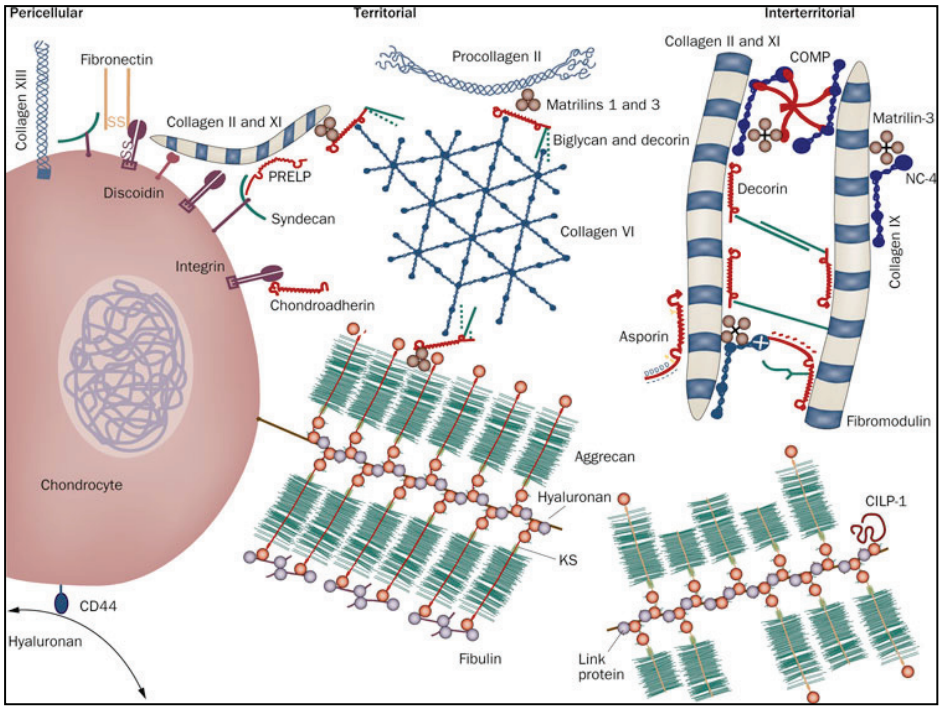

Figure 5. ***The different cartilage molecules and chondocyte.*** *Many non-collagenous proteins in cartilage attach, for example chondrocytes to collagen fibrils. Aggrecan is attached to hyaluronan via a link protein. Asporin, decorin, matrilins and cartilage oligomeric matrix protein are non-collagenous structural molecules of the cartilage (Heinegård et al. 2011). Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Rheumatology copyright 2011.*

Joint space is filled with synovial fluid. This decreases the friction between the moving bones, brings nutrients and oxygen to the cartilage, and transports metabolic waste away from the cartilage. Normally, the amount of synovial fluid in the knee joint is approximately 0.5 - 4.0 ml, under a pressure that is lower than normal atmospheric pressure (Kraus *et al.* 2007). Synovial fluid is produced from plasma by synoviocytes (fibroblast-like type B lining cells) (Wilkinson *et al.* 1992).

This joint structure is surrounded by both a joint capsule, which consists of an inner synovium containing blood vessels, lymphatics and nerves, and an outer fibrous lining and sub-lining membrane. These are then in turn enforced by ligaments and tendons that support the joint in its movements (Huber *et al.* 2000).

2.2.6 Osteoarthritic changes in the joint

Originally, OA was simply considered to represent a disease of mechanical joint wear, but nowadays it is known that an additonal complex molecular mechanism plays a role. According to one theory, OA represents an imbalance in the regenerative and degenerative processes of the cartilage. Nonetheless, even today it is not known if the disease starts from the cartilage and progresses to the bones, and other parts of the joint, or if this process occurs the reverse direction. Changes are seen in cartilage, bone, synovium and muscle as well as in motor programs and proprioception.

Cartilage

One basic feature of the disease progression is breakage of the cartilage ECM. Chodrocytes, are supposed to, at least in part, repair the damaged cartilage by first breaking down the damaged area, in order to subsequently repair it. At this point the cartilage might get thicker. In later stages, the repairing activity slows down and the volume of the cartilage decreases. The surfaces lose their smoothness, become fibrillated and fissures are formed. The factors that are breaking down the cartilage, e.g. collagenases, aggrecanases and other hydrolytic enzymes, are activated by cytokines, growth factors, inflammatory factors or mechanical factors. There are also factors inhibiting this breakdown. In OA, the subchondral bones of the bone endings get thicker, which might be caused by microfactures, due to excessive motion. The activity of bone forming cells called osteoblasts increases bone thickness. Resorption of bone tissue and the formation of cysts are seen. Additionally, reformation of the bone occurs as the result of ostephyte formation (Samuels *et al.* 2008; Martel-Pelletier *et al.* 2010).

Cartilage changes have been shown to be the initiating event in joint degeneration (Bennett *et al.* 1942). This finding has been later strengthened by, among others, Watters *et al.* (2007) who reported that mild cartilage damage precedes osteophyte development in the STR/Ort murine model. Meachim *et al.* (1965) concluded that the superficial layer of human articular cartilage is affected in aging, in a study performed using light microscopy. They showed that degenerative changes can occur in areas without fibrillation, changes described as fraying of the cartilage into long strands, causing lesions. They suggested that the other degenerative changes of the matrix precede the fibrillative changes. Later, among others, Kääb *et al.* (2003) showed, using rabbit knee joints, that the chondrocytes of the superficial layer are more affected than chondrocytes of the deeper layers, since their shape seems to change more under loading than the shape of the cells in deeper layers. Similarly, Chen *et al.* (2003) showed that the chondrocytes in the superficial layers of bovine

cartilage are in higher risk to die through necrosis than the ones in deeper zones, when predisposed to pressure. Type II collagen was degraded only in the superficial layer due to the loading and collagenase activity. Hollander *et al.* (1995) showed, similarly, in human articular cartilage samples that damage to the key component in articular cartilage, type II collagen, in aging and in OA joints occurs first in the upper mid zone, proceeding to the lower mid and deep zones with increasing degeneration, always starting around chondrocytes.

According to Lorenzo *et al.* (2004), cartilage goes through metabolic changes in very early stages of OA. The levels of non-collagenous molecules, like oligomeric matrix protein (COMP), fibronectin, and cartilage intermediate layer protein (CILP), were increased in cartilage during early OA when compared to both normal and late OA cartilage.

These findings suggest that the cartilage is affected first, with the bone changes occuring as a secondary phenomenon. However, larger systematic, proteomic, and expression studies at the molecular level are needed to confirm this hypothesis.

Inflammation and immune response

Originally, inflammation was thought to be a symptom of other joint diseases like RA. Today it is known that inflammation in synovial membranes is an important part of OA, even in moderate or mild disease stages. C-reactive protein (CRP) is slightly increased in knee OA patients (Spector *et al.* 1997). In patients undergoing joint replacement surgery, the inflammation occasionally even reaches the same level as in RA patients (Smith *et al.* 1997; Haywood *et al.* 2003). Inflammation probably also explains the discrepancy between symptoms and radiological findings.

Inflammation in the osteoarthritic synovium is associated with the production of proinflammatory cytokines, such as interleukin 1 alpha (IL-1α), interleukin 1 beta (IL-1β), and tumor necrosis factor (TNF-α) (Smith *et al.* 1997), and with the angiogenic growth factor called vascular endothelial growth factor (VEGF) (Haywood *et al.* 2003).

One promising theory connecting inflammation, cartilage breakdown and OA, is that the cartilage breakdown products might trigger auto-immune or auto-inflammatory reaction that further enhances the cartilage breakdown. Fibromodulin and other small leucine-rich proteoglycan (SLRP) family members can initiate the complement pathway (Sjöberg *et al.* 2005; Sjöberg *et al.* 2009). Many ECM proteins have been shown to act as antigens and form an immune complex (IC) with IgG in OA and RA joints, with their amount correlating with disease severity. Thus it is

possible that the mechanical factors that damage ECM result in a proinflammatory reaction (Monach *et al.* 2009). Moreover, it seems that damaged ECM fragments can trigger inflammatory responses by acting as ligands for Toll-like receptors (TLR) found in chondrocytes. Chondrocytes can, potentially, produce proinflammatory and algogenic molecules, thus they might be the initial source of factors responsible for the secondary inflammatory reaction and pain in the synovium (Konttinen *et al.* 2012).

COMP, found in OA and RA synovial fluid, and suggested to be an OA biomarker (Saxne *et al.* 1992), can initiate the alternative complement pathway (Happonen *et al.* 2010). A recent extensive study by Wang *et al.* (2011) confirmed the role of complement in OA. The gene expression and protein levels of complement proteins were higher in OA than in both normal synovial fluid and synovium. One complement member, membrane attack complex (MAC), was detected in the synovium and around chondrocytes of end-stage OA joints. Knockout mice deficient for C5 and C6 complement members were less prone to develop OA than controls. While knockout mice deficient for complement inhibitor CD59a were more prone to develop the disease. The complement cascade either ends up killing the chondrocytes or stimulates the cells to produce inflammatory and matrix-degrading enzymes (Wang *et al.* 2011).

To conclude, inflammation seems to be an essential element in OA. The complement pathway might be one of the key elements and the TLR-equipped chondrocytes might be the initiating cell-type in this process.

Obesity

Obesity is a significant risk factor for all OA types, with inflammation a part of the disease. Adipokines are one of the key regulators in OA (Koskinen *et al.* 2011). Interestingly, the STR/Ort mice that naturally develop OA, also have high serum total cholesterol, high serum triglyceride, hyperinsulinemia, insulin resistance, and low serum adiponectin (Uchida *et al.* 2009).

Bone

During progression of OA the shape of the bone ending starts to change, and bone marrow lesions appear. These are increased by physical loading and are associated with OA pain (Bennell *et al.* 2010a). Current knowledge cannot exclude the role of bone as the initiating tissue in OA. If there were any alterations in the bone endings, this would lead to increased pressure and stress in the corresponding area of the

cartilage. There is some evidence that osteoporosis (OP) might protect from OA, but opposing evidence has also been published, with disussicon about the relation of the two traits ongoing (Rechtman *et al.* 1954; Mäkinen *et al.* 2007).

Chondrocytes

Chondrocytes and their progenitors are the only cell types in cartilage, thus they are the main cellular elements that maintain the cartilage. Whatever it is that happens in cartilage in OA, it might be initiated by these cells, as mentioned earlier.

Normal adult chondrocytes are not metabolically very active. In OA these cells go through maturation and express type X collagen. Endochondral ossification of growth plate chondrocytes during development resembles the maturation process of chondrocytes in OA. During this stage, chondrocytes proliferate and develop hypertrophy, and express type X collagen, which is typical in bone formation (Aigner *et al.* 1993). In OA, the cartilage ECM is being remodeled and mineralized, and blood vessels from the subchondral bone invade the cartilage (Bohr 1976), all of which are normal phenomenon in bone formation. In normal cartilage aging, the amount of chondrocytes decreases due to the lack of new chondrocytes and the cartilage becomes more fragile. The cells are not anchored as tightly as before to the ECM, and the amount of nutrients is decreased. The rate of maturation slows down and cytokines that are typical enhancers of OA are produced by the chondrocytes and synovial cells (Corvol 2000).

The important role of chondrocytes in OA has been suggested based on a mouse model. Using the STR/Ort mouse strain, it has been shown that chondrocyte cell death correlates with the progression of OA (Mistry *et al.* 2004). Regions of inactive chondrocytes appear in the cartilage, before any major histological evidence of degeneration appears (Altman 1981). Based on gene expression results reported by Watters *et al.* (2007), it seems that the default pathway of the mesenchymal cell differentiation is changing from adipogenesis towards osteogenesis in the OA mouse model. Therefore, as mentioned before, it is possible that chondrocytes are the initiative source for the inflammation and pain in the synovium.

Ligaments

All joint tissues seem to play a role, at least at some stage, in OA. Cruciate ligament collagen metabolism is upregulated in the STR/Ort mouse compared to controls. While at the same time, the anterior cruciate ligament is weaker in STR/Ort mice than in controls (Anderson-MacKenzie *et al.* 1999).

Biomechanics and physical stress

As discussed earlier, excessive physical stress predisposes to OA whilst an appropriate level of exercise can be protective (Haara *et al.* 2003; Hart *et al.* 2008; Recnik *et al.* 2008; Bennell *et al.* 2010a). One possibility, to clarify the beneficial effects of physical activity, could be that the chondrocytes get their nutrients through diffusion and convection from the surrounding synovial fluid more effectively. Increased use of the joint, pumping the fluids in and around cartilage, could improve the nutrition of the cartilage.

It is, however, still not entirely known if the benefits of physical stress are due to the loss of adipose tissue and the increase in cardiovascular health, or if the benefits are gained due to mild loading of the joints. This question is especially important in the case of hand OA, where physical activities like jogging or swimming could be beneficial due to the above mentioned process, whereas exercise loading hand joints could actually be harmful. The study mentioned earlier (Segal *et al.* 1998), in which hand joints unable to move were protected from OA, speaks for the possibility that even mild loading might be harmful and only the other outcomes of physical activity, i.e. weight loss and cardiovascular health, would confer beneficial factors. It is possible that the beneficial effects seen in the study are due to not using the joints, but it is also possible that they are due to not using the hand with too high a loading. These aspects need to be studied more and the results need to be replicated in larger study samples in order to draw further conclusions.

One interesting question about OA is: Why are all joints not affected by some slight OA? The ankle, wrist, elbow, and shoulder are usually unaffected by OA even though degeneration of the articular cartilage occurs. Especially fascinating is why the ankle is not affected by OA, even though it carries the same or even greater body mass than the hip or knee. The answer is not simple or fully known, but it seems to involve the cartilage, subchondral bone and the chondrocytes. First of all, the extracellular matrix of the cartilage in the ankle has been shown to contain more proteoglycan and less water than the cartilage of the knee. This makes the ankle cartilage stiff, and low in hydraulic permeability, thereby allowing the cartilage to endure high compressive forces better than the cartilage in the knee (Treppo *et al.* 2000). Second, the ankle chondrocytes do not seem to respond to catabolic factors as strongly as the chondrocytes of the knee, however, they do respond well to removal of IL-1 (Eger *et al.* 2002). Third, it seems that the degeneration of ankle cartilage does not increase the subchondral bone density, as opposed to what normally occurs in other joints affected by OA (Muehleman *et al.* 2002).

2.2.7 Medical treatment

There is no disease preventing treatment for OA. In practice, the first treatment in many cases is exercise and muscle strengthening, with weight loss an important factor in disease control.

Pain killing medicine, such as paracetamol, is the main medical treatment, along with some locally used pain killers. Non-steroidal anti-inflammatory drugs (NSAIDs) are also used to some extent, but long term use is limited by their gastrointestinal adverse effects. Cyclo-oxygenase-2 (COX-2) inhibitors, that are more specific, are also used as treatment. However, their possible cardiovascular adverse effects have been under debate. A meta-analysis of 145,373 participants using COX-2 inhibitors, NSAIDs or placebo was studied for cardiovascular events (Kearney *et al.* 2006). The authors concluded that COX-2 inhibitors increased the risk for severe vascular events compared to placebo (rate ratio 1.42, 95% CI 1.13 - 1.78; p = 0.003). Further, there seemed to be a similar kind of risk with NSAIDs, like ibuprofen and diclofenac, but a lower risk with naproxen. More studies are still needed for further conclusions.

Surgery can be used in the treatment of OA. The weight-bearing forces can be redirected with osteotomy. Joint replacement surgery is used in a progressed disease, when the pain and loss of function is considered to trouble everyday life. Based on research evaluating a total of 20 studies, there is systematic evidence that in hip OA, total hip replacement surgery remarkably increased the quality of life, usually already within the first 3 – 6 months after surgery (Towheed *et al.* 1996).

Future prospects in medical treatment

Currently, some of the molecules and pathways discussed in this thesis work have been under research for drug targets by medical companies (Steinmeyer *et al.* 2006). These targets have been widely studied, and their role in OA appears to be crucial. Inhibitors for catabolic enzymes are one interesting group. MMP inhibitors such as bisphosphanates and tetracycline are under research, since MMPs cleave the collagen network in cartilage. Doxycycline has already been shown to slow joint space narrowing in knee OA (Brandt *et al.* 2005). Inhibitors for "disintegrin and metalloproteinase with thrombospondin-like repeat" (ADAMTS) family have also been under research since ADAMTS break down aggracan (Yao *et al.* 2002). Cathepsin K is another potential drug target. It cleaves type II collagen at several sites under the acidic conditions that seem to be present in the OA joint. The gene expression of cathepsin K is elevated in OA cartilage, but it is also present in normal subchondral bone, in osteoclastas, and mononuclear cells (Konttinen *et al.* 2002).

Inhibitors for glycosidases are under research, since glycosidases cleave HA and proteoglycan sugar chains (Liu *et al.* 2001). The inflammation pathway is another study target. Recombinant interleukin-1 receptor antagonist (IL-1RA protein coded by *IL1RN* gene) which is already in use for RA, has raised interest as a possible treatment for OA, without success yet (Chevalier *et al.* 2005). It decreases OA symptoms in animal model (Bacconnier *et al.* 2009). Inhibiting some intracellular signal transduction pathways is also under investigation. Inhibition of mitogen-activated protein (MAP) kinase is of interest, since it enhances apoptosis and production of catabolic factors (Pelletier *et al.* 2003). While, inhibitors of nuclear factor-κB (NF-κB) are interesting, since it stimulates the production of cytokines chemokines, and proteins involved in the control of cell death (Tegeder *et al.* 2004).

2.2.8 Physical activity

It is widely accepted that physical exercise is a key-element in knee and hip OA pain management, and in the maintenance of joint function. In 1975, it was shown in a small study that 74 Finnish championship runners are not predisposed to hip OA compared to the normal population (Puranen *et al.* 1975). Later it has been widely accepted that low-impact physical exercise is beneficial for joints. As Keysor *et al.* (2011) concluded in their systematic study, which included the large collaborative consensus recommendation guidelines (Jordan *et al.* 2003; Roddy *et al.* 2005; Vignon *et al.* 2006; Zhang *et al.* 2008; Chodzko-Zajko *et al.* 2009; Zhang *et al.* 2010), that people with knee OA should perform both moderate low-impact activity and progressive resistive strengthening exercise.

OA patients should be educated about the positive outcomes of exercise (Roddy *et al.* 2005) as long as the activity is not painful and does not predispose to trauma, since they are two risk factors for OA (Vignon *et al.* 2006). Exercise in general and exercise of the hand muscles in hand OA are not widely studied. Therefore the usefulness of these exercises are more unclear than in knee and hip OA, but it is still recommended (Zhang *et al.* 2007).

Based on a study by Bennell *et al.* (2010b), the strengthening of hip muscles improved knee OA symptoms but did not affect medial knee load. Thus it is unlikely to have directly influenced the structural changes in joint tissues suffering from OA.

Based on the presented data, it seems that physical exercise either affects OA through metabolic pathways, possibly by decreasing the amount of adipose tissue and related hormones, or by increasing the exchange of nutrients and harmful metabolites of chondrocytes within the joint cavity. Exercise does not seem to affect the mechanical loading, at least not OA of the knee, but then again the supporting

tissues are probably strengthened. Could the effect of exercise in OA be related to the range of motion during movement? Discussion on the effectiveness of manual physical therapy including manipulation and stretching techniques is ongoing (Rhon 2008). Some evidence for its usefulness, even when added to the exercise therapy, has been shown in a small study of 100 individuals (OR 1.92, 95% CI 1.30 - 2.60) (Hoeksma *et al.* 2004). However, more knowledge is needed about the exact biological mechanism, at the molecular level, of the benefits of exercise in OA patients.

For knee and hip OA the positive effects of exercise is clear, yet the effect on hand OA is less studied, even though it would be biologically more interesting. More studies are needed to address the issue of what type of exercise hand OA patients should do: mainly hand joint exercises or other types of aerobic and strength exercises that are not directly related to the hand joints?

2.3 Genetic studies of OA

Previously, candidate gene studies have focused on genes affecting cartilage structure, such as asporin (*ASPN*) (Kizawa *et al.* 2005; Mustafa *et al.* 2005; Jiang *et al.* 2006; Shi *et al.* 2007). However, newer extensive GWA studies have the advantage of not requiring knowledge and hypothesis on the gene functions beforehand, although interpretation of the findings can be complicated afterwards. Recent large GWA studies have revealed entirely new study targets in OA. Next, I will discuss studies that show some of the most promising results in this field so far. I will classify them based on study type since, to some degree, the study approach tells about the reliability and broadness of the study.

It is still difficult to say, if the majority of the OA phenotype is caused by thousands of different familial mutations with large effect, or common population based alleles with small effects. Since OA is a late-onset disease and the phenotype is not life-threatening, even in the early-onset familial cases, evolutionary pressure has probably been very mild against the disease alleles. Since the disease is related to the inflammatory system, the disease-predisposing alleles might even have an advantageous effect in survival through some other trait possibly leading to high allele frequencies of the OA-risk alleles. In the case in the *MCF2L* gene predisposing to OA, the predisposing allele had a frequency over 90 % while the ancestral allele frequency was less than 10 % (Day-Williams *et al.* 2011).

2.3.1 Genome-wide association studies in OA

GWA studies do not require pre-formulated hypotheses of the genes or gene networks involved, but rely on systematic statistical assessment throughout the genome. So far eight GWA studies have been conducted in OA (Mototani *et al.* 2005; Miyamoto *et al.* 2008; Zhai *et al.* 2009; Kerkhof *et al.* 2010; Nakajima *et al.* 2010; Day-Williams *et al.* 2011; Evangelou *et al.* 2011; Panoutsopoulou *et al.* 2011). However, the coverage of genetic variation in the genome was low (<100.000 markers) in some of the studies (Mototani *et al.* 2005; Miyamoto *et al.* 2008). Four GWA studies in OA showed genome-wide significant associations ($p < 5x10^{-8}$) and will be discussed in more detail (**Table 1**).

Table 1. Genome-wide significant findings ($p<5x10^{-8}$) in OA. Modified from Näkki *et al.* (In press).

Chr.	Variant	Gene	RAF	p OR (95 % CI)	n (cases) n (controls) Phenotype Population	Ref.
3p24	rs7639618	*DVWA/ COL6A4P1*	0.64	$7.3x10^{-11}$ 1.43 (1.28–1.60)	1,399 2,141 Knee OA Asian	Miyamoto *et al.* 2008
6p21	rs10947262	*BTNL2*	0.58	$5.1x10^{-9}$ 1.31 (1.20-1.44)	1,879 4,814 Knee OA Asian, Caucasian	Nakajima *et al.* 2010
7q22	rs4730250	*DUS4L*	0.17	$9.2x10^{-9}$ 1.17 (1.11-1.24)	6,709 44.439 Knee OA Caucasian	Evangelou *et al.* 2011
13q34	rs11842874	*MCF2L*	0.93	$2.13x10^{-8}$ 1.17 1.11-1.23	19,041 24,504 Knee OA Hip OA Caucasian	Day-Williams *et al.* 2011

Chr = chromosome, Variant= rs number of the SNP, RAF = risk allele frequency, p = combined p-value of screening and replication; OR = odds ratio; n(cases) = number of cases; n(controls) = number of controls.

Further, there are differences in OA phenotyping between and even within studies. Susceptibility loci for OA seem to differ between populations, e.g. between Asian and European populations. It is possible that a SNP finding in one population does not replicate in another population since it tags different haplotypes in different populations.

Finding on chromosome 3p24: DVWA/COL6A4P1

An association between a marker in "double von Willebrand factor domain A" (*DVWA*) ($p = 7.3x10^{-11}$) and knee OA was observed by Miyamoto *et al.* (2008). The finding was later replicated ($p = 0.018$) by Valdes *et al.* (2009b). However, negative association for the studied variant has been published (Meulenbelt *et al.* 2009; Kerkhof *et al.* 2010). Of interest is that nearby regions on chromosomes 3p21 and 3p24 have shown suggestive evidence of linkage to hand OA in several studies with LOD scores of 2.7 (Hunter *et al.* 2004) and 2.8 (Livshits *et al.* 2007). DVWA interacts with β-tubulin, which has an essential role in protein trafficking and secretion of proteins (Miyamoto *et al.* 2008). The gene has lately been suggested to represent the *COL6A4P1* gene (Wagener *et al.* 2009). The *CAPN* gene is also located in the same LD block making it difficult to pinpoint the contributing gene.

Finding on chromosome 6p21: *BTNL2* in the HLA region

A variant in the butyrophilin-like 2 gene (*BTNL2*) in the HLA II locus has been associated with knee OA ($p = 2.43x10^{-8}$) (Nakajima *et al.* 2010), although, negative association in Chinese and Australian study samples have been published for the same variant (Shi *et al.* 2010). The region has shown suggestive evidence for linkage to OA (LOD = 2.1) (Loughlin *et al.* 1999). The region harbors several genes from the HLA class II group that are in high LD with each other, including *BTNL2, HLA-DRA, HLA-DRB5, HLA-DRB1, HLA-DQA1,* and *HLA-DQB1* (Nakajima *et al.* 2010). They all are expressed by antigen presenting cells (B lymphocytes, dendritic cells, macrophages) and play an important role by presenting peptides from extracellular proteins. The *BTNL2* gene negatively regulates T-cell activation (Arnett *et al.* 2007). The true causative variant remains unknown due to the LD in the region, but presumably the causative gene is tightly involved in the immune system.

Finding on chromosome 7q22: *DUS4L*

A GWA study by Kerkhof *et al.* (2010) identified a locus on 7q22 to be associated with clinical and/or radiological generalized OA ($p = 8x10^{-8}$). Later meta-analysis on Caucasian subjects, including the previous study sample, revealed association near the original finding, with the "dihydrouridine synthase 4-like" gene (*DUS4L*) ($p = 9.17\times10^{-9}$) and with the "B cell receptor associated protein 29" gene (*BCAP29*) ($p = 3.90\times10^{-8}$) (Evangelou *et al.* 2011). Although, this finding was not replicated in the Japanese and Chinese cohorts from the same study. The associated region harbors genes *DUS4L, COG5, BCAP29,* G protein-coupled receptor 22 (*GPR22*), "protein kinase, cAMP-dependent, regulatory, type II, β" (*PRKAR2B)*, and "HMG-box transcription factor 1" (*HPB1*). Again due to the high LD in the region, pinpointing the causative variant will be demanding, but some speculation about the eventual roles of these genes are discussed next.

GPR22-positive chondrocytes are present in the upper layers of the articular cartilage of mice knee joints with induced arthritis and absent from controls (Kerkhof *et al.* 2010). *COG5* is involved in formation of the Golgi-localized complex (Ungar *et al.* 2002). *BCAP29* is involved in B-cell signaling with the Bap29/31 complex seeming to play a role in the trafficking of MHC class I molecules (Paquet *et al.* 2004). *PRKAR2B* is a signaling molecule involved in many cellular functions. *HPB1* is involved in cell cycle and the Wnt pathway (Sampson *et al.* 2001).

Finding on 13q34: *MCF2L*

A marker in the "MCF.2 cell-line-derived transforming sequence-like" gene (*MCF2L*) showed association with knee and hip OA ($p = 2.13x10^{-8}$). There was only little LD outside the gene region (Day-Williams *et al.* 2011). The Mcf2l gene is expressed in rat cartilage. It regulates neurotrophin-3 (*NTF3*) -induced cell migration in Schwann cells (Liu *et al.* 2009). The *NTF3* is part of the nerve growth factor family (NGF) that could play a role in OA pain, since inhibiting this family seems to decrease joint pain and increase joint function (Lane *et al.* 2010).

Still only little is known with high certainty about the genetics behind OA. The 3p24, 6p21, 7q22, and 13q34 loci seem to have population level, genome-wide significance in OA. As in so many other complex diseases, the ORs in these findings vary only between 1.2 - 1.8. Proof about true causative genes and variants are in most cases still modest, due to the LD in the regions and the lack of extensive functional studies. However, the HLA region on 6p21 and the *MCF2L* gene are

highly likely candidates, suggesting pathways involved in the immune system and nerve growth.

2.3.2 Genome-wide linkage studies in OA

Family-based GWL studies and follow-up fine-mapping studies have discovered many possibly OA-predisposing loci (Leppävuori *et al.* 1999; Loughlin *et al.* 1999; Ingvarsson *et al.* 2001; Demissie *et al.* 2002; Loughlin *et al.* 2002b; Stefansson *et al.* 2003; Forster *et al.* 2004; Hunter *et al.* 2004; Loughlin *et al.* 2004; Southam *et al.* 2004; Greig *et al.* 2006; Lee *et al.* 2006; Mabuchi *et al.* 2006; Meulenbelt *et al.* 2006; Livshits *et al.* 2007; Min *et al.* 2007; Meulenbelt *et al.* 2008). Significant evidence of linkage has been found for thirteen different loci and OA, based on the linkage threshold guidelines of Lander and Kruglyak (1995) (LOD > 3.3). Some of the most significant linkage findings have been on chromosomal regions 2p23.3 (LOD = 4.4) (Stefansson *et al.* 2003), 2q33.3 (LOD = 6.1) (Meulenbelt *et al.* 2006), and 15q25.3 (LOD = 6.3) (Hunter *et al.* 2004). These findings will be discussed in more detail, **Table 2**.

Table 2. Results from OA linkage studies. Significant linkage findings are presented (LOD ≥ 3.3, bold font). In addition, suggestive linkage findings are presented (LOD ≥ 1.9, normal font) if they are on a region showing significant linkage in another study. In sib-pair studies, thresholds of LOD 3.6 and 2.2 are used respectively (Lander *et al.* 1995). Modified from Kämäräinen (2009) and Näkki *et al.* (In press).

Chr	LOD	Family/sibpair Method	Phenotype (details)	Country	Reference
2p23.3–p24.1	4.4	Families Aff. allele share	Hand (DIP/first CMC)	Iceland	Stefánsson *et al.* 2003 A
2p23.3	2.2	Families Multipoint	Hand (JSN)	USA	Demissie *et al.* 2002
2p12–p13.3	4.0	Twins Multipoint	Hand (TotKL, women)	UK	Livshits *et al.* 2007
2q33.3	6.1	Families Two-point	Generalized	Netherlands	Meulenbelt *et al.* 2006
4q31.3	3.3	Families Aff. allele share	Hand (DIP)	Iceland	Stefánsson *et al.* 2003
4q32.3	3.8	Twins Multipoint	Hand (TotKL)	UK	Livshits *et al.* 2007
6p12	4.6	Families Two-point	Hip (THR, female)	UK	Loughlin *et al.* 2002b
8p23.2	4.3	Family Multipoint	Biomarker (PIIANP)	Afr.Am / Nat.Am.	Chen *et al.* 2010
9q34.2–q34.3	4.5	Twins Multipoint	Hand (DIP)	UK	Livshits *et al.* 2007
12q21.33–q22	3.9	Twins Multipoint	Hand (DIP)	UK	Livshits *et al.* 2007 B
13q22.1	3.6	Family Multipoint	Hip	Japan	Mabuchi *et al.* 2006
15q25.3	6.3	Families Multipoint	Hand (First CMC)	USA	Hunter *et al.* 2004
19q13.2	4.3	Twins Multipoint	Hand (TotKL)	UK	Livshits *et al.* 2007
20p13	3.7	Families Multipoint	Hand (DIP, women)	USA	Hunter *et al.* 2004

In the case that several findings were made in the same region and with overlapping study samples, only one study is presented, the one with the highest logarithm of odds (LOD) score, even if the study was a finemapping study. Family/sibpair = familial or sib-pair study sample; Method = linkage analyses used; Aff. allele share = affected only allele sharing method; Multipoint = multipoint linkage analysis; Two-point = two-point linkage analysis; DIP = distal interphalangeal; CMC = carpometacarpal; JSN = joint space narrowing; generalized = generalized OA; TotKL = Kellgren Lawrence score sum for both hands; OST = osteophyte; PIP = proximal interphalangeal; TIP = thumb interphalangeal; Association finemapping ($p < 0.05$) in the region: A = *MATN3* (Stefansson *et al.* 2003), B = intergenic (Evangelou *et al.* 2011).

Linkage region of 2p23.3-p24.1 harbors *MATN3*

A chromosomal region on 2p23.3–p24.1 has shown linkage with hand OA in two studies, yielding LOD scores of 4.4 (Stefansson *et al.* 2003) and 2.2 (Demissie *et al.* 2002). A possible disease causing gene *MATN3* was finemapped, but the mutation found did not fully explain the linkage (Stefansson *et al.* 2003). The same variant might also predispose to disc degeneration (p = 0.02) (Min *et al.* 2006). Matrilins are ECM proteins. *MATN3* expression has been shown to be upregulated in the OA cartilage of humans (Pullig *et al.* 2002), and to increase the expression of genes encoding TNF-α, IL-1β, IL-6, IL-8, iNOS, and COX-2 in chondrocytes obtained from human OA cartilage (Klatt *et al.* 2009).

Linkage region of 2q33.3 harbors *FRZB*

A locus on 2q33.3 showed significant evidence of linkage to generalized osteoarthritis (LOD = 6.1) (Meulenbelt *et al.* 2006), with similar evidence gained in a meta-analysis (p = 0.03, 10,000 permutations) (Lee et al (2006) involving several other studies (Chapman *et al.* 1999; Stefansson *et al.* 2003; Hunter *et al.* 2004). Frizzled-related protein (*FRZB*) has been suggested to be the predisposing gene in several studies (p = 0.007) (Loughlin *et al.* 2004), (p = 0.02) (Min *et al.* 2005), (p = 0.01) (Lane *et al.* 2006), (p = 0.04) (Valdes *et al.* 2007). The function of *FRZB* in OA might be due to its role in wingless (Wnt) signaling, which is involved in, among others, skeletal metabolism, joint patterning in embryogenesis, and plays a role in apoptosis (James *et al.* 2000).

A larger chromosomal region on 2q12-q34 has been linked to different OA types in several studies (Leppävuori *et al.* 1999; Loughlin *et al.* 2000; Loughlin *et al.* 2002a; Loughlin *et al.* 2002b; Hunter *et al.* 2004; Meulenbelt *et al.* 2006), but the findings have not been statistically significant. An association with interleukin 1 receptor 1 (*IL1R1*) in the same region is shown in Study I of the present thesis.

Linkage region of 15q25

Significant evidence for linkage has been found between a region on 15q25 and hand OA (Hunter *et al.* 2004) (LOD = 6.25). Further, a trend was seen in a meta-analysis by Lee et al (2006) (p = 0.04, 10,000 permutations), which included the original study sample (Chapman *et al.* 1999; Stefansson *et al.* 2003; Hunter *et al.* 2004). A predisposing variant is still unknown.

For some of the most promising linkage findings presented above, association fine mapping has revealed potential causative candidates, but in most cases the proof is still modest and the linkage regions are large. The candidate genes neither show genome-wide significance ($p < 5x10^{-8}$) nor fully explain the original linkage (Stefansson *et al.* 2003; Loughlin *et al.* 2004; Min *et al.* 2005; Lane *et al.* 2006; Valdes *et al.* 2007). Thus more evidence is needed for any further conclusions.

2.3.3 Candidate gene studies in OA

Next I will discuss the variants that have been shown to associate with OA in independent candidate gene studies with replication. Only the finding for the *GDF5* is genome-wide significant ($p = 1.8x10^{-13}$) (Miyamoto *et al.* 2007), however, the other studies are interesting with regards to future efforts to unravel OA genetics. Based on familial studies, it seems that rare, familial mutations might have an important role in the disease, additionally bringing collagens into the picture.

GDF5 on 20q11 showed association with OA in several candidate gene studies

One recently found OA gene is growth and differentiation factor 5 (*GDF5*). The SNP rs143383 has been shown to associate with hip and knee OA in Asian populations ($p = 1.8x10^{-13}$) (Miyamoto *et al.* 2007), and with knee OA in a European population ($p = 9.4x10^{-7}$) (Evangelou *et al.* 2009). *GDF5* is a member of the transforming growth factor- β (TGF- β) superfamily and is related to the bone morphogenetic proteins (BMPs) that induce cartilage and bone formation, and stimulate de novo synthesis of ACAN protein (Erlacher *et al.* 1998). Mutations in the *GDF5* gene cause skeletal alterations both in humans (Thomas *et al.* 1996) and mice (Storm *et al.* 1994). TGF-β is a pleiotropic cytokine and growth factor that has important anabolic effects on chondrocytes. It can stimulate type II collagen synthesis and down-regulate cartilage-degrading enzymes. It also has a role in decreasing the IL-1 induced suppression of proteoglycan synthesis.

ASPN on 9q22 showed association with OA in several candidate gene studies

The association of allele D14 of the asporin gene (*ASPN*) with hand, knee or hip OA has been shown in Japanese, Chinese and Caucasian individuals ($0.0008 < p < 0.02$) (Kizawa *et al.* 2005; Mustafa *et al.* 2005; Jiang *et al.* 2006; Shi *et al.* 2007). *ASPN* is a member of the ECM SLRP family, which is a group of proteins that bind to other proteins in the extra cellular matrix. *ASPN* can inhibit TGF-β signaling with the D14 allele showing greater inhibition than the other alleles (Kizawa *et al.* 2005).

DIO2, SMAD3 and *ANP32A* as candidate genes

Some other interesting associations have been seen between *DIO2,* on 14q31, and hip OA ($p = 2.02 \times 10^{-5}$) (Meulenbelt *et al.* 2008), and with the *SMAD3*, 15q22, ($p < 7.5 \times 10^{-6}$) (Valdes *et al.* 2010) and "acidic (leucine-rich) nuclear phosphoprotein 32 family member A14" genes (*ANP32A*) to hip OA ($p < 3.8 \times 10^{-4}$) (Valdes *et al.* 2009a).

Collagens coding genes

Previously, two biologically interesting and widely studied groups of genes in OA genetics were cartilage genes and cartilage degrading genes. As one hypothesis for OA related to pathogenesis is based on an imbalance between degradation and repair of the cartilage (Ala-Kokko *et al.* 1990; Horton *et al.* 1998; Tetlow *et al.* 2001; Kirk *et al.* 2003; Alizadeh *et al.* 2005; Jakkula *et al.* 2005). Compared with the recent extensive GWA gene mapping studies, the older studies were conducted with smaller sample sizes, fewer markers and candidate genes, and without replication. However, many of the studies were performed in families, identifying family-specific mutations. Many of these studies were used as data sources when candidate genes were selected for the three studies presented in this current thesis work, thus they are now shortly presented.

One important group of structural genes of relevance for joints and joint diseases are the collagens. **Table 3** summarizes the types and locations of some of the collagens most important to the joint and cartilage.

Type II collagen is the most common collagen in articular cartilage, but collagen types III, VI, IX, X, XI, XII and XIV all contribute to the mature matrix. In developing cartilage, the basic collagen network is a composite of collagens II, IX and XI. Many collagen proteins are formed by several alpha chains coded by their own gene (Kuivaniemi *et al.* 1997; Eyre 2002).

Table 3. Collagen types, structures of the collagens and collagen expressing tissues based on Eyre (2002) and the National Center for Biotechnology Information (NCBI) database.

Type	Structure	Tissue
I	Fibrillar	Connective tissues, bone, cornea, dermis and tendon
II	Fibrillar	Eye, main component of cartilage
III	Fibrillar, associated with type I collagen	Skin, lung, uterus, intestine and the vascular system, smaller amounts in many tissues rich in type I collagen
V	Fibrillar	Low abundance, tissues containing type I collagen; closely related to type XI collagen
VI	Beaded-filament forming collagen	Cartilage, the major structural component of the microfibrils
IX	Fibril associated with interrupted triple helices (FACIT)	Articular cartilage, eye, tissues containing type II collagen; located on the surface of type II collagen containing fibrils
X	Homotrimer, network-forming collagen	Hypertrophic chondrocytes during endochondral ossification, cartilage
XI	Fibrillar; the α3(XI) chain is encoded by the gene for type II procollagen	Cartilage
XII	Fibril associated with interrupted triple helices (FACIT)	Cartilage, eye
XIV	Fibril associated with interrupted triple helices (FACIT)	Cartilage

A fibril-forming collagen is a long, thin rod and contains three polypeptide chains called the α chains. These chains are wrapped into highly ordered triple helixes that are made possible by every third amino acid being glycine, the amino acid with the smallest side chain (Kuivaniemi *et al.* 1997).

Variants in many of the collagen genes have previously been associated with OA, including *COL1A1* (Lian *et al.* 2005), *COL2A1* (Hämäläinen *et al.* 2008), *COL9A1* (Alizadeh *et al.* 2005), and *COL9A3* (Ikeda *et al.* 2002). Rare familial variants in the genes coding for collagens II and XI have been identified to predispose to OA (Ala-

Kokko *et al.* 1990; Jakkula *et al.* 2005) and to a familial OA-like disease spectrum (*COL2A1*) (Su *et al.* 2008). Exprssion studies have shown that the expression of *COL2A1, COL1A2, COL3A1* and *COL5A1* increases in OA cartilage in a naturally occurring OA in canines (Clements *et al.* 2006)

Aggrecan

One important gene in cartilage structure is aggrecan (*ACAN)*. An association between a *VNTR* polymorphism in *ACAN* and OA has been observed (Horton *et al.* 1998; Kirk *et al.* 2003), with the same *VNTR* region in *ACAN* also being associated with disc degeneration (Kawaguchi *et al.* 1999; Solovieva *et al.* 2007). As mentioned earlier, *ACAN* is an important part of cartilage, responsible in part for its elasticity. A functional mouse study with the STR/Ort model showed that aggrecan gene transcription is elevated in early osteoarthritis (Gaffen *et al.* 1997).

Matrix metalloproteinases (*MMPs*)

Matrix metalloprotinases (MMP) are a group of extracellular matrix breaking enzymes, neutral endoproteinases. MMP-1, MMP-8 and MMP-13 are the best known enzymes to break down fibrillar collagens. MMP-1 best breaks type III collagen, MMP-8 type I collagen and MMP-13 type II collagen. MMP-3 and MMP-10 break down proteoglycans. MMPs are inhibited by tissue inhibitors of metalloproteinases (TIMPs). Factors like the IL-1β pathway (Stern *et al.* 2003; Riyazi *et al.* 2005; Moxley *et al.* 2007; Kanoh *et al.* 2008) have been shown to affect MMP-8 expression. MMP-8 has low expression in normal chondrocytes but is up-regulated by IL-1β (Cole *et al.* 1996), and there seems to be co-expression between the two (Tetlow *et al.* 2001).

Macrophages, fibroblasts and chondrocytes synthesize MMP-1 and MMP-13 proteins in the presence of inflammation. The *MMP1*, *MMP3*, *MMP8* and *MMP13* genes are expressed in OA cartilage (Tetlow *et al.* 2001). Additionally, the expression of *MMP13* when stimulated by IL-1 and TNF-α has been shown to increase OA of joints in an animal model (Clements *et al.* 2006).

Interleukins

Inflammation in OA joints is one widely studied topic, with somewhat consistent results. The role of the interleukin-1 gene family cluster on chromosome 2q12-13 has been suggested in hip, knee, or hand OA in Study I of the present thesis in

addition to other studies (Moos *et al.* 2000; Loughlin *et al.* 2002a; Meulenbelt *et al.* 2004; Solovieva *et al.* 2009). These findings have not been confirmed, including those of Study I in the present thesis work. The evidence is at its most convincing for the widely studied *IL1B* and *IL1RN* genes that cause and inhibits inflammation repectively. These genes show suggestive association with knee and hip OA ($p = 0.006$ for haplotype; 5000 knee OA cases, 9000 controls) (Kerkhof *et al.* 2011).

IL-1 and TNF-α inhibit chondrocyte production of type II collagen – the most important collagen type in cartilage. IL-1 and TNF-α are both activators of signaling pathways of c-Jun N-terminal kinase (JNK), p38 MAP kinase (p38 MAPK) and NF-κB. All three lead to the inhibition of the *COL2A1* gene (Robbins *et al.* 2000; Seguin *et al.* 2003). Excessive mechanical stress might activate these pathways. Matrix metalloproteinases (MMPs 1, 3, 8, and 13), IL-1β, and TNF-α are present in the superficial zone of OA cartilage. This zone also harbors chondrocyte clusters and degenerative matrix changes (Tetlow *et al.* 2001). Cytokines increase the expression of these matrix metalloproteinases, as well as some aggrecanases (Goldring *et al.* 2008). Interleukin 6 (IL-6) is a pleiotropic pro-inflammatory cytokine and its expression is increased by tissue inflammation in OA cartilage, serum and synovial fluid (Kaneko *et al.* 2000). It has been shown through a knockout mouse model that OA is more severe if IL-6 is not present compared to wild-type mice (de Hooge *et al.* 2005). As for many other genes discussed here, strong evidence for disease gene variants is still missing.

2.3.4 Biomarkers and expression studies

For a broader view on OA, I will shortly discuss biomarker and gene expression studies. Since OA has an inflammatory feature there are probably molecules affecting OA that could be seen in a systemic level. Biomarkers also form an interesting study subject in scientific studies and potential diagnostic tools. The expression and biomarker studies presented here are all significantly affected either by a small number of studied samples, a small number of studied genes, or a small number of studied biomarkers. Thus either the risk of false positive findings is high or the study has not systematically covered numerous possible factors affecting OA. In many studies both these limitations are present and the results should be interpreted with caution.

Genome-wide expression profiling has been performed by Karlsson *et al.* (2010), among others. This group studied the gene expression profile of healthy ($n = 5$) and osteoarthritic human cartilage ($n = 5$), and revealed that expression was significantly changed for many different groups of genes. The study had the capacity to analyze over 47, 000 transcripts. Among many other genes, the expression differed between

the two groups for cytokines (such as tumor necrosis factors, TNFs), chemokines (like IL-8), enzymes (such as MMPs), growth factors (like IGFs), matrix components (like collagen type I), and others (such as HLA-DQA1). In another study based on candidate gene expression analysis, *SOX9* ($p < 0.001$) and *ACAN* ($p < 0.001$) seemed to be down-regulated in osteochondral samples of OA patients ($n = 20$) compared to controls ($n = 8$) (Brew *et al.* 2010). Additionally, locally expressed glycosidases seem to play a role in OA as well as in RA. These determine the final structures of glycans (Pasztoi *et al.* 2009).

The expression of genes involved in the development and function of connective tissue, and in lipid metabolism has been shown to be most significantly up-regulated during disease progression in the STR/Ort mouse joints (Watters *et al.* 2007).

In a serum-based metabolomic study of OA, the ratio of two branched-chain amino acids, valine, leucine or isoleucine, to histidine was associated with the disease (Zhai *et al.* 2010). Chen *et al.* (2008) performed a candidate biomarker study using human blood samples from an extended family with no known specific disease ($n = 287$). Three of the studied biomarkers showed some level of heritability (heritability > 43 %) and were also associated with OA ($p < 0.05$): HA, COMP and type IIA collagen N-propeptide (PIIANP). PIIANP is a marker of a fetal form of collagen II which is also produced in OA cartilage. The COMP molecule binds to the collagenous structure in cartilage and can initiate the alternative complement pathway (Happonen *et al.* 2010). HA has a role in cartilage structure as mentioned earlier and also plays an important role in the synovial fluid.

3 AIMS

The aim of this thesis study was to investigate the role of 25 structural, degenerative and inflammatory candidate genes in OA using SNPs. The purpose was to find gene variants that predispose to OA. Three different study settings were used: hand OA families and healthy controls, a case-control setting for knee OA, and a twin pair study with an MRI based hip OA phenotype.

The specific aims of this thesis were:

I) To identify an OA-predisposing gene for hand OA on the 2q11.2 region. In a previous study, the genomic region of 2q11.2 showed linkage to hand OA (Leppävuori *et al.* 1999). The aim was now to identify the OA-predisposing gene variant in that region.

II) To study if *MMP8* and *MMP9* genes predispose to OA. Levels of proMMP-8 and proMMP-9 have been shown to be higher in the serum of OA patients than in controls (Tchetverikov *et al.* 2004). The aim was now to analyze the *MMP8* and *MMP9* genes in knee OA study samples, in order to find out if there are genetic variants in these genes that predispose to knee OA.

III) To study the role of *ACAN*, *COL*, *MMP* and *IL* candidate gene groups in hip OA. In a previous study, 25 candidates from the *ACAN*, *COL*, *MMP* and *IL* gene groups which were selected based on their previously identified biological relevance, were studied for disc degeneration traits in a twin study sample (Videman *et al.* 2009). The aim was now to analyze the same SNP panel in the same study population with a hip OA phenotype using MRI-visible bone changes.

4 MATERIALS AND METHODS

4.1 Study samples

The study design was approved by the Ethical Committees of the National Public Health Institute, Helsinki, Finland (currently National Institute for Health and Welfare), the Department of Public Health at the University of Helsinki, Helsinki, Finland, the Hospital District of Helsinki and Uusimaa, Helsinki, Finland, the Faculty of Rehabilitation Medicine at the University of Alberta, Edmonton, Canada, and the Ethical Committee for Clinical Research of Galicia, Spain. The project numbers for the ethical approvals are 131/2006 (H, N, I); 9/270598 (B, D); 37/96, 2/96 (A); 407/E3/2000 (E, F, K, L); 140/E3/2004, 344/E3/2000 (C,J); 2008/063 (G, M), letters indicate the study sub-sample referred to, see **Table 4**.

The study sample was composed of 5941 subjects (1466 OA cases and 4475 controls) for the study of OA of hand, knee, and hip OA (**Table 4**).

Table 4. The number of cases and controls in studies I-III.

Study samples	*vs.*	Tot.	Ma	Fe	Study		
Cases							
Hand OA cases							
A) Radiological[a]		104	42	62	**I**	II	
B) Clinical[b]		28	5	23			
C) Clinical [HBCS]		524	169	355		**II**	
Knee OA cases							
D) Radiological[b]		113	27	86	I	**II**	
E) Clinical discovery cases [H2000]		75	25	50		**II**	
F) Clinical verification cases [H2000]		302	102	200		II	
G) Joint replacement patient [Spanish]		254	50	204		II	
Hip OA cases							
H) MRI based		94	94	0			**III**
Controls							
I) Clin. non-OA	A+B	436	277	159	**I**	II	
J) Clin. non-hand OA [HBCS]	C	970	490	480			
K) Clin. non knee/hip OA [H2000]	D+E	895	415	480		**II**	
L) Clin. non knee/hip OA [H2000]	F	1700	761	939		II	
M) Clinical non-knee OA[Spanish]	G	449	290	159		II	
N) MRI based non-hip OA[c]	H	251	251	0			**III**
In total		5941					

a = Familial study sample, of which 85 were unrelated. In addition, 34 healthy family members were used in the analyses, but are not shown in the table; b = 28 individuals were part of clinical hand osteoarthritis (OA) cases and radiological knee OA cases; c = MRI based hip OA phenotype; 226 individuals from this group were also part of the control group I; A-N = Study samples are referred in the text with the letter; *vs.* indicates which control and case groups were compared with each other; Ma = male, Fe = female; bold font = main analysis; normal font = validation analysis; HBCS = Helsinki Birth Cohort Study; H2000 = Health 2000 study; MRI = magnetic resonance imaging.

4.1.1 Cases

Radiological familial hand OA study subjects and clinical hand OA subjects

Families and individuals with hand OA were collected from the Mini-Finland Health Survey (Kärkkäinen 1985), the Finnish twin-cohort study (Kujala *et al.* 1999), the Rheumatism Foundation Hospital (Heinola, Finland) (Vikkula *et al.* 1993), and the ORTON Orthopaedic Hospital (Helsinki, Finland). In total 168 study subjects were collected. Altogether, 134 of them were affected, of which 85 were unrelated. DIP OA status was mainly radiologically examined (Studies I and II) (**Table 4**, A).

The DIP OA phenotype studied was based on radiological Kellgren classification (Kellgren 1963) with individuals of stage 3-4/4 DIP OA considered as affected. Subjects were invited to participate in a hand x-ray examination, if they were recorded as having hand OA based on a clinical evaluation as part of the Mini-Finland Health Survey, (Kärkkäinen 1985), if they were recorded as having a physician-diagnosed hand OA based on a questionnaire from the Finnish twin-cohort study in 1995 (Kujala *et al.* 1999), or identified through the Rheumatism Foundation Hospital (Heinola, Finland) (Vikkula *et al.* 1993) and the ORTON Orthopaedic Hospital (Helsinki, Finland). Other family members were included as affected individuals if they had a DIP OA score of 2-4/4. All other family members were classified as unknown. The average age at grading of hand X-rays was 62 years (SD 9 y).

In addition, 28 clinically diagnosed hand OA cases (**Table 4**, B) were included which were also included in the knee OA study group (**Table 4**, D). For clinical diagnostics, see Haara *et al.* (2003). Subjects with clinical or radiological rheumatoid arthritis (RA) were excluded.

Verification sample: Clinical hand OA study subjects

Subjects from the Finnish Helsinki Birth Cohort Study were studied to verify any association results in Study II (Barker *et al.* 2005) (**Table 4**, C). They were all above the age of 56 years and their hand joints were visually evaluated for Heberden's nodes. If at least one DIP joint was affect, the individual was ranked as affected.

Radiological knee OA study subjects

In total 113 subjects with radiological knee OA were analyzed in Studies I and II (**Table 4**, D). They suffered from primary bilateral knee OA severe enough to fulfill the criteria for knee arthroplasty: pain, walking disability and radiologically stage 3-4/4 osteoarthritic changes according to KL classification (Kellgren *et al.* 1957). OA symptoms began at a mean age of 52 years (SD 12 years), with the mean age at first arthroplasty being 67 years (SD 8 years). The study was initiated by reviewing 852 patient reports of hospital visits for knee OA, between 1994 and 2001, to the ORTON Orthopaedic Hospital (Helsinki, Finland). Of these, 113 subjects had not had a major knee trauma, and volunteered to participate in the study by providing a DNA sample. Of the 113 individuals, 28 had severe physician-diagnosed hand OA and were also included in the hand OA study sample. In total 110 of the 113 patients were used as part of the initial discovery sample in Study II.

Clinical discovery and verification knee OA cases

Clinician diagnosed knee OA cases and controls were analyzed in Study II. The cases were from a subset of the Finnish Health 2000 cohort (2118 individuals) ascertained for a matched case–control study for metabolic syndrome (Perttilä *et al.* 2009) and genotyped using the Illumina HumanHap 610 k GWA chip (Illumina, Inc., San Diego, CA, USA). Of the 2118 genotyped individuals, 75 had clinician diagnosed probable or definite knee OA, based on physical status, symptoms, and medical history, and were over the age of 50 years. Radiographic assessment of OA was not included in this study (**Table 4**, E). Individuals suffering from seropositive or -negative RA or having unknown RA status, were excluded. Cases with radiological knee OA (**Table 4**, D) were combined with this group.

For verification of any association finding in Study II, the remaining Finnish Health 2000 study sample was genotyped. This study sample included 302 clinical knee OA cases selected using the same criteria as in the discovery phase (**Table 4**, F).

Verification sample: Joint replacement knee OA cases

In total, 254 Spanish knee OA cases were used in a replication analysis of Study II (**Table 4**, G). Cases had undergone knee joint replacement surgery and had severe primary OA based on a rheumatologists evaluation. Exclusion criteria were inflammatory, infectious, traumatic or congenital joint pathology, and lesions due to crystal deposition or osteonecrosis (Rodriguez-Lopez *et al*. 2007).

Hip OA cases

The hip OA study sample was part of the Finnish Twin Cohort called the Twin Spine study (Videman *et al.* 1998; Kaprio *et al.* 2002; Battié *et al.* 2007). The original Twin Spine study consisted of 588 Finnish monozygotic or dizygotic male twins aged 35-70 years. The twin pairs were selected for the Twin Spine study based on discordance for specific exposures to low back pain and disc degeneration, such as exercise participation, occupational driving, heavy material handling, and sedentary work. The twins did not necessarily suffer from back problems.

The precise phenotype in the present study was MRI-based calcified structure changes that were also visible in radiographs. Imaging of the hips was performed using a Siemens Vision 1.5 T, scanning both hips at the same time using a body matrix coil. Analysis of the scans was done using a Siemens MRI workstation at the Puijon Magneetti Oy company (Kuopio, Finland). The grading scale was based on Li et al (1988) and Kellgren and Lawrence (1957). The trait was dichotomized so that individuals with both hip joints normal according to a MRI hip image grade of 0 were considered as healthy, while individuals with at least small local osteophytes in caput or acetabulum (grades 1-4) in at least one hip were considered as affected, yielding a phenotype allowing for early-onset OA. One randomly selected individual from each monozygotic pair and both twins from the dizygotic pairs were included in the genetic analyses. Based on the dichotomization criteria, 94 individuals were classified as affected with hip OA (Study III) (**Table 4**, H).

4.1.2 Controls

Clinical non-OA controls

The first set of control subjects (n = 436, aged 35-70) (Studies I and II) (**Table 4**, I) were selected from the Finnish twin cohort (Kaprio *et al.* 2002). Initially, 210 unrelated individuals were selected from the twin sample using the inclusion criteria of being born between 1938 and 1941, responded to a questionnaire during the years 1996-1997, gave DNA samples for analysis, and that both twins reported that neither them nor their mother, father or any other sibling had physician diagnosed OA or RA. Second, 226 individuals were selected from the Twin Spine study that was used in the hip OA study (III). Subjects with physician-diagnosed OA without imaging were excluded, and only one twin from a pair was included.

Verification sample: Clinical non-hand OA controls

Controls originating from the Finnish Helsinki Birth Cohort Study were also involved in the study (Barker *et al.* 2005) (**Table 4**, J). They were above the age of 56 years and had visually healthy and symptomless finger joints (n=970).

Clinical non-knee/hip OA controls in discovery and verification

From the Health 2000 study, a total of 895 individuals were over 50 years of age and did not have clinically diagnosed probable or definite knee or hip OA. These individuals were used as knee OA controls (Study II) (**Table 4**, K). Individuals suffering from seropositive or -negative RA or having an unknown RA status were excluded.

In total, 1700 controls were selected using exclusion of the criteria used for the clinician diagnosed OA criteria in the discovery study sample. These were used in verification analysis and were genotyped using a different genotyping platform (**Table 4**, L).

Verification sample: Clinical non-knee OA controls

Altoghter 449 individuals from Spain, older than 55 years of age and undergoing pre-operative work-up for elective surgeries other than joint surgery, were used as knee OA controls in the verification analysis of Study II (**Table 4**, M). They did not have OA based on clinical diagnosis.

Clinical non-hip OA controls

In total, 251 hip OA controls (Study III) (**Table 4**, N) were selected from the individuals participating in the hip OA study of the Finnish Twin Spine study sample. The controls had healthy hip joints based on the MRI grading.

4.2 Marker selection and genotyping

4.2.1 Marker selection

A total of 166 SNPs in 25 structural, degenerative and inflammatory candidate genes were selected for Study III. The studied genes were *ACAN, COL1A1, COL1A2, COL2A1, COL3A1, COL5A1, COL5A2, COL9A1, COL9A2, COL9A3, COL10A1, COL11A1, COL11A1, MMP3, MMP8, MMP9, MMP13, IL1R1, IL1R2, IL1RL1, IL1RL2, IL18R1, IL1RAP, IL1A* and *IL1B*. The criteria for SNP selection was, a minimum heterozygosity of 20 % in the Caucasian population and 10 kb spacing between SNPs. Two different databases were used to select SNPs. Initially, the SNPper program was used (Snpper CHIP Bioinformatics Tools © 2001-2008, University of Florida, Gainesville Florida, USA; http://snpper.chip.org (Riva *et al.* 2001; 2002; 2004)), which utilizes the UCSC Human Genome Browser database (University of California, Santa Cruz Santa Cruz, California, USA; http://genome.ucsc.edu (Lander *et al.* 2001; Kent 2002; Fujita *et al.* 2010)). Secondly, the SeattleSNPs database was used (SeattleSNPs. NHLBI Program for Genomic Applications, SeattleSNPs, Seattle, WA, USA; http://pga.gs.washington.edu). Five of the 166 SNPs were selected based on previous association findings: rs7533552 in *COL9A2* (Annunen *et al.* 1999) (Noponen-Hietala *et al.* 2003), rs2857401 flanking *COL1A1* (Garcia-Giralt *et al.* 2002), rs1800012 in *COL1A1* (Grant *et al.* 1996), rs412777 in *COL1A2* (Suuriniemi *et al.* 2003), and rs9277933 in *COL11A2* (Maeda *et al.* 2001).

The same *IL1* SNPs analysed in Study I were studied in Study III. For Study II, tag SNPs for *MMP8* and *MM9* genes were selected as presented in the Illumina HumanHap 610 GWA chip (Illumina, Inc., San Diego, CA, USA).

4.2.2 DNA extraction and genotyping of the study samples

DNA was extracted from peripheral blood leukocytes using standard methods of phenol DNA extraction, salt precipitation extraction, silica-membrane-based purification (DNA Blood Maxi Kit, Qiagen, Venlo, The Netherlands) and Chemagen MSMI magnetic bead technology (PerkinElmer, Inc. Waltham, MA, USA).

Genotype data was obtained using the following methods: the homogeneous MassEXTEND™ (hME) and iPLEX Gold platforms in the Sequenom MassARRAY system (Sequenom Inc., San Diego, California, USA), the Microarray based allele

specific primer extension method (Silander *et al.* 2005), by capillary sequencing (ABI3730xl DNA Analyzer, BigDye Terminator v3.1 Cycle Sequencing Kit, Life Technologies Corp., Carlsbad, CA, USA), the Illumina HumanHap 610 GWA chip (Illumina, Inc., San Diego, CA, USA), and the SNaPshot Multiplex Kit (Applied Biosystems, Foster City, CA, USA) with the Abi-Prism 3130xl Genetic Analyzer (Applied Biosystems).

The Sequenom MassARRAY system is discussed in more detail here, since the other methods were run, at least partly, through a core-facility.

The Sequenom MassARRAY system

The 99 SNPs in studies I and III were genotyped using hME and iPLEX Gold technologies on the Sequenom MassARRAY platforms (Sequenom Inc., San Diego, California, USA) as recommended by the manufacturer with additional quality control steps (Silander *et al.* 2003). These systems are based on allele-specific primer extension and separation of different alleles, by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Sequenom Inc. 2002; Sequenom Inc. 2005).

Assays were designed into multiplexes using the SpectroDESIGNER 1.3 (Sequenom Inc., San Diego, California, USA) and MassARRAY Assay Design v3.1 (Sequenom Inc., San Diego, California, USA) programs. These design PCR and extension primers, arranging the SNPs into multiplexes, of 2-6 SNPs for the hME technology, and 13-20 SNPs for the iPLEX technology. Genotyping of the samples was done in 384 well plates and the mass spectrums were analyzed by the SpectroTYPER RT 2.0 software (Sequenom Inc., San Diego, California, USA) for the hME technology and by the MassARRAY Typer software when using the iPLEX technology (Sequenom Inc., San Diego, California, USA).

Prior to OA sample genotyping, the SNPs that comprised a multiplex for the hME technology were validated by genotyping them singularly on 12 test samples. In order to determine that the SNPs are polymorphic in the studied population, and to confirm that the assays performed acceptably. Next, the hME and iPLEX multiplexes were optimized. This is perfomed by genotyping them as multiplexes on test samples, so that adjustments to the amounts of PCR and extension primers for each SNP assay in the multiplex can be made. For the hME system, 23 test samples and 1 empty well was used while for the iPLEX system 47 test samples and 1 empty well was used.

Sample plates included 8 empty wells and 8 duplicate samples for quality control. In the hME technology, all genotypes were manually read using the spectrograms of the SpectroTYPER RT 2.0 software (Sequenom Inc., San Diego, California, USA). SNP was excluded from the study if; there were alleles in empty sample wells, the allele peaks of the SNPs were consistently low (less than 2, when normally between 3 - 120), the allele peaks were significantly lower compared to extension primer peaks (less than 20 % of the height of extension primer), or if the allele pattern of heterozygous samples were inconsistently variable skewed (other allele peak was approximately 50 % higher in some of the samples and 50 % lower in others). Afterwards, the clustering option of the MassARRAY Typer software was used to verify the reliability of the hME assays.

In the iPLEX technology, MassARRAY Typer was used to perform genotype reading. Mainly the genotype clustering was used to assess the reliability of the SNP assays, while spectrograms were used as supportive information. Genotypes were clustered according to their signal intensity and their signal intensity ratio to the extension primer peak intensity. Outlier genotypes were set to unknown. Also, if more than half of the assays were unsuccessfully readable in a well, all genotypes of that well were set to missing. In the iPLEX technology observing allele spectrums in empty wells is not an uncommon phenomenon, therefore only if an assay showed alleles in all of the empty wells was it excluded from the study.

Additionally, 30 trio families consisting of mother, father and child, were added to the sample plates for quality control purposes. The trio samples were used to test if any of the SNP alleles were transmitted from the parents to the child against the Mendelian laws of inheritance using the PedCheck 1.1 Program. In a case of several families showing inheritance against the Mendelian laws, the SNP was excluded (O'Connell *et al.* 1998).

Only SNPs with a call rate over 90 % were included in the association analysis. Deviation from the Hardy-Weinberg (HW) equilibrium was tested using Pearson's chi-square test (Purcell *et al.* 2007). SNPs passing the quality control in validation, optimization, and sample genotyping were taken into the association analyses.

Capillary sequencing

Two SNPs in the *COL9A2* gene (Study III) were genotyped by sequencing the region harboring the SNPs, since they were both in the same codon (ABI3730xl DNA Analyzer, BigDye Terminator v3.1 Cycle Sequencing Kit, Life Technologies Corp., Carlsbad, CA, USA). Sequence chromatograms were manually read. Two empty wells in each 96 well plate were used as a quality control.

Illumina HumanHap 610 GWA chip

Two genes, *MMP8* and *MMP9*, were selected for analysis (Study II). Genotypes for the controls in the Finnish knee OA discovery study sample (**Table 4**, E, K) were obtained from the Illumina HumanHap 610 GWA chip (Illumina, Inc., San Diego, CA, USA). The quality control criteria used for exclusion were; MAF less than 1 %, genotyping success rate less than 95 % for a marker or individual, HWE less than p = 10^{-6}, piHat value more than 0.1 (on a scale 0-1), and no discrepencies between reported and genetic gender. Chromosome Y and mitochondrial DNA were not a part of this study.

The remaining portion of the genotypes were obtained using The Sequenom MassARRAY iPLEX system as described above (**Table 4**, D, F, L) or with the SNaPshot Multiplex Kit (Applied Biosystems, Foster City, CA, USA) and Abi-Prism 3130xl Genetic Analyzer (Applied Biosystems) (**Table 4**, G, M).

4.3 Statistical analyses

To analyze which of the selected candidate genes play a role in OA, association analyses were performed in all three studies. The LD pattern of the studied areas, gene tagging, and haplotypes were also studied.

4.3.1 Heritability

Heritability estimates were performed in the twin sample (Study III) (Professor Jaakko Kaprio's laboratory, Department of Public Health, University of Helsinki). First, polychoric correlations for MZ and DZ twins were calculated separately and then heritability was estimated using the Mx software (Neale, M.C., Virginia Commonwealth University, Richmond, Virginia, USA). Quantitative genetic models allowing for additive genetic, common, and unique environmental variance in the liability were used.

4.3.2 Statistical power

Statistical power calculations were done in Study I, in order to evaluate the power to detect similar association in another study sample as was detected in Study I. The aim was also to calculate the amount of cases required for another study sample to

have 90 % power of detecting a similar association as in Study I (Purcell *et al.* 2003).

4.3.3 Gene tagging

The Tagger program (de Bakker *et al.* 2005) was used in order to reveal what percentage of the ungenotyped SNPs in the studied gene region are captured by the used SNP panels (Studies II and III). The program is based on HapMap data (The International HapMap Consortium *et al.* 2003). An r^2 threshold of 50 % and 80 % between two markers was used (Study III and II respectively). In other words, when a SNP is in r^2 correlation of more than 80 % with another SNP, the SNPs are tagged by one another and are representing similar genotypic information

4.3.4 Correction for multiple testing

After obtaining the genotype data, and due to forthcoming multiple testing, the next step was to evaluate p-value threshold for 5 % significance in the association analysis (Studies I-III). The Single Nucleotide Polymorphism Spectral Decomposition (SNPSpD) method (Nyholt 2004) with modifications by Li and Ji (Li *et al.* 2005) was used to calculate the p-value threshold for 5 % significance. This method took into account the LD between studied SNPs and thus is not as conservative as performing Bonferroni correction.

4.3.5 Association analysis

SNP association analysis

A χ^2 test was conducted using either Plink (Purcell *et al.* 2007) or Haploview (Barrett *et al.* 2005). If 5 or less individuals carried a genotype, either in the case or in the control group, the 2-Tail Fisher's exact test ((Agresti 1992) © Øyvind Langsrud) was used instead. The analysis methods assume indivduals to be unrelated. Therefore only one person per family was included in the familial (Study I) and in the twin study samples (Study III).

For all three studies (I-III), familial association was calculated using the Pseudomarker program 0.9.7 beta, which can utilize combined data of individual cases, sib pairs, families and healthy controls and separate the linkage evidence from evidence for association (Hiekkalinna *et al.* 2011a; Hiekkalinna *et al.* 2011b). Both

dominant and recessive modes of inheritance were used. The association was calculated taking into account the possible presence of linkage.

In Study II, the meta-analysis option of Cochran-Mantel-Haenszel was used in Plink to assess the overall effect size of the SNP combining different populations and phenotypes (Purcell *et al.* 2007).

LD structure and haplotype association analysis

The Haploview program (Barrett *et al.* 2005) was used to determine the pair-wise LD between SNPs (r^2 and D' value) within the studied gene regions (Studies I-III), and to identify LD block structures using the r^2 measure and the solid spine of LD algorithm implemented in the Haploview program. D' allows correlation between markers with different allele frequencies (Lewontin 1964). The solid spine of LD defines the block in such a manner that the first and last marker in a block are in strong LD with all markers between them, but the intermediate markers need not to be in LD with each other (Barrett *et al.* 2005).

In Study I, haplotype association analysis was performed using the Haploview program (Barrett *et al.* 2005) for the haplotypes containing the best individual associated SNPs. The SNPs located in *IL1R1* formed one LD block based on the r^2 measure. Haplotypes were also formed for a larger SNP panel forming one LD block based on the "solid spine of LD" measure.

To calculate odds ratios (OR), individual haplotypes for each study subjects were estimated using the Phase2 program (Stephens *et al.* 2001; Stephens *et al.* 2003). A minimum probability of $\geq$ 60 % was requested for each haplotype. Alleles with a frequency of over 5 % in the control sample were each analyzed separately with the less common haplotype alleles being grouped together. Hardy-Weinberg equilibrium was calculated for the tested haplotypes to confirm the correctness of the haplotype allele distribution ($p > 0.05$).

5 RESULTS AND DISCUSSION

5.1 *IL1R1* associated with hand OA (I)

The *IL1* gene region was originally selected for the study due to a suggestive linkage finding at chromosome 2q12 (Z = 2.34 at recombination fraction 0) in a previous DIP OA study by Leppävuori *et al.* (1999) with a partly overlapping study sample to Study I. Further, other findings have shown evidence for the relevance of this region in knee and hip OA (Moos *et al.* 2000; Meulenbelt *et al.* 2004; Smith *et al.* 2004). Of the 16 genes in the 2 Mb linkage region, the *IL1* gene family of six genes was selected for further study, based on their biological relevance and the fact that the *IL1R1* gene is located at the highest Z_{max} linkage peak. The *IL1R2, IL1R1, IL1RL2, IL1RL1, IL18R1,* and *IL18RAP* genes are located in a 470 kb cluster on chromosome 2q12-14.

IL-1 is a pleiotropic cytokine involved in inflammatory and immune responses. A complex of either IL-1α or IL-1β in combination with IL-1R1 and IL-1RAcP activates NF-κB (Bomsztyk *et al.* 1990; Beg *et al.* 1993). This pathway leads to the inflammatory and immune response (Sen *et al.* 1986).

In Study I, a total of 168 familial subjects were genotyped, of which 134 were mainly radiologically diagnosed to have DIP OA. Additionally, 435 healthy controls were included in the study. In total, 32 SNPs were analyzed (**Table 4** A, B, I).

5.1.1 Significant association with SNP rs2287047

The *IL1R1* region was fine-mapped in the current association study to reveal variants underlying hand OA. The Pseudomarker program (Hiekkalinna *et al.* 2011a; Hiekkalinna *et al.* 2011b) was used for family based association analysis. The $\chi 2$ and Fisher's exact tests were used to confirm the finding in unrelated individuals excluding one individual from each per twin (85 hand OA cases, 435 controls). Altogether 32 SNPs were analyzed. Association with severe hand OA was concentrated on the region harboring the long promoter and coding region of the *IL1R1* gene (**Table 5**).

Association was seen for SNP rs2287047 to severe hand OA (OR for AA vs. GG = 0.16, 95% CI = 0.06 - 0.45; OR for AA vs. AG = 0.26, 95% CI = 0.09 - 0.73;

protective A allele being the minor allele; p = 0.00091 dominant mode of inheritance). This association was statistically significant after correction for multiple testing. The p-value threshold that corresponds to significance at the 5 % level was 0.0021, based on SNPSpD (Nyholt 2004; Li *et al.* 2005). Based on this results, the *IL1R1* gene region was studied further.

Table 5. Single SNP association results between *IL1R1* gene area and hand OA.

Gene	SNP id	MAF		p-value	
		Controls	Hand OA	χ^2	Dominant
IL1R1	rs1465325	0.195	0.119	0.022[a]	0.005
	rs956730	0.260	0.204	0.140[a]	0.026
	rs3917225	0.396	0.309	0.043	0.078
	rs2287047	0.249	0.159	0.013[a]	**0.00091**
	rs3771200	0.411	0.453	0.312	0.539
IL1RL2	rs2241132	0.158	0.141	0.644[a]	0.491
	rs870684	0.378	0.394	0.690	0.781
	rs1922290	0.377	0.420	0.304	0.495
	rs1922295	0.379	0.400	0.613	0.696
	rs1997502	0.353	0.318	0.375	0.681
	rs2302612	0.119	0.157	0.199[a]	0.505

a = Fisher's exact test; Dominant = association p-value with dominant model of the Pseudomarker program (Hiekkalinna *et al.* 2011a, Hiekkalinna *et al.* 2001b).

For confirmation of the original linkage observed, linkage of the region was analyzed using the Pseudomarker program (Hiekkalinna *et al.* 2011a; Hiekkalinna *et al.* 2011b) in the hand OA families. SNP rs956730 showed the strongest evidence for linkage to hand OA (LOD = 1.34, p = 0.006, two-point linkage, dominant model).

5.1.2 Haplotype tagged by rs1465325 associated with hand OA

The LD block of the promising region was studied in controls using the Haploview program (Barrett *et al.* 2005). Associated SNPs seemed to be in LD with each other (D' LD ≥ 94 %; r^2 LD ≥ 34 %) (**Figure 6**). Next, SNPs in LD with each other were selected for haplotyping.

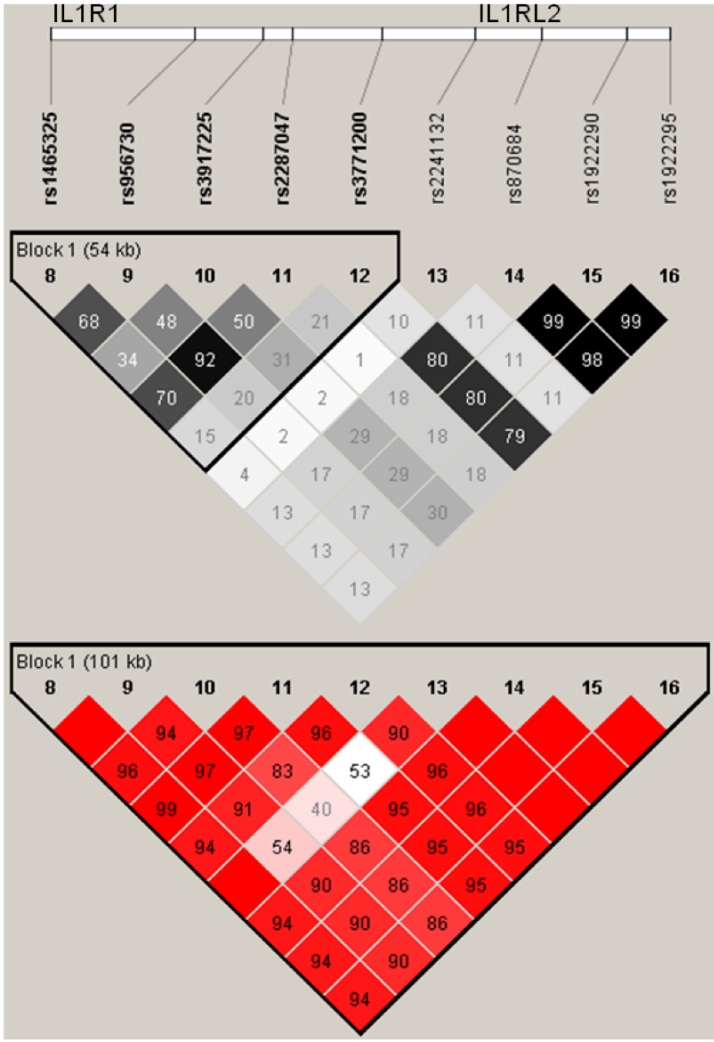

Figure 6. ***The LD block structure of IL1R1 and IL1RL2 genes in the control sample.*** *Above the r^2 measure of LD, below the D' measure of LD. The pair-wise LD is shown as different shadings of grey (r^2) and red (D'). The LD blocks selected for haplotyping are marked with black triangle (Barrett et al. 2005).*

Haplotypes were analyzed to gain further understanding of the allelic background in the *IL1R1* area. First, SNPs in the *IL1R1* gene were selected for haplotyping (rs1465325, rs956730, rs3917225, rs2287047, rs3771200; D' LD > 83 %, r^2 > 15 %). Haplotype CAAAG was observed to be the protecting allele as it was rarer in cases (0.104) than in controls (0.188) [OR for "heterozygous for CAAAG" vs. "homozygous for others" = 0.54; 95% CI = 0.41 to 0.72; p = 0.0079 based on the

Phase2 (Stephens *et al.* 2001; Stephens *et al.* 2003) and the Haploview programs (Barrett *et al.* 2005)].

Table 6. *IL1R1* haplotype frequencies and the p-value for the association (one haplotype versus all other haplotypes) based on the Haploview program (Barrett *et al.* 2005).

Haplotype	Allele frequencies		p-value
	Controls	Hand OA[a]	
TGGGA	0.381	0.440	0.147
TGGGG	0.218	0.212	0.865
CAAAG	**0.188**	**0.104**	**0.0079**
TGAGG	0.115	0.123	0.772
TAAAG	0.053	0.048	0.796

a = one individual per family

Secondly, the solid spine of LD algorithm, as used in the Haploview program, was used to select SNPs for confirmatory haplotype association analysis (Barrett *et al.* 2005). SNPs rs1465325, rs956730, rs3917225, rs2287047, rs3771200, rs2241132, rs870684, rs1922290, and rs1922295 were used (D' LD ≥ 94 % for rs1465325 with other markers; D' LD ≥ 86 % for rs1922295 with other markers). Based on this confirmatory analysis, the CAAAGCTGC haplotype seemed to protect from hand OA as it was rarer in the hand OA cases (0.101) than in controls (0.190) (p = 0.0058).

The rs1465325 SNP appears to tag the protective allele (OR CT vs. TT = 0.59, CI = 0.45 to 0.78; p = 0.022).

5.1.3 Power in the knee OA sample

Based on the Genetic power calculator (Purcell *et al.* 2003), at least 503 knee OA cases would have been required to have 90% power to detect this association, when assuming a similar effect size as observed in the family-based severe hand OA, with an allele frequency of 0.84 and prevalence of 7 %. The power to detect association with the 113 knee OA cases used in Study I was only 12 %.

The effect size of the studied SNP (rs2287047) might have been an over-estimate due to the limited sample size. Since, assuming an effect size typically seen in complex diseases (risk ratios for a single SNP 1.15 - 1.20), approximately 4000

cases are required to detect similar association with 80% power (allele frequency of 0.75 and prevalence of 10 %).

5.1.4 The role of *IL1R1* in OA

To conclude, association was detected between severe hand OA and four SNPs in the *IL1R1* gene. The strongest evidence for association was observed with the rs2287047 SNP (p = 0.00091) located in intron 1 of the *IL1R1* gene. The other three associated SNPs are located in the long promoter region of the gene.

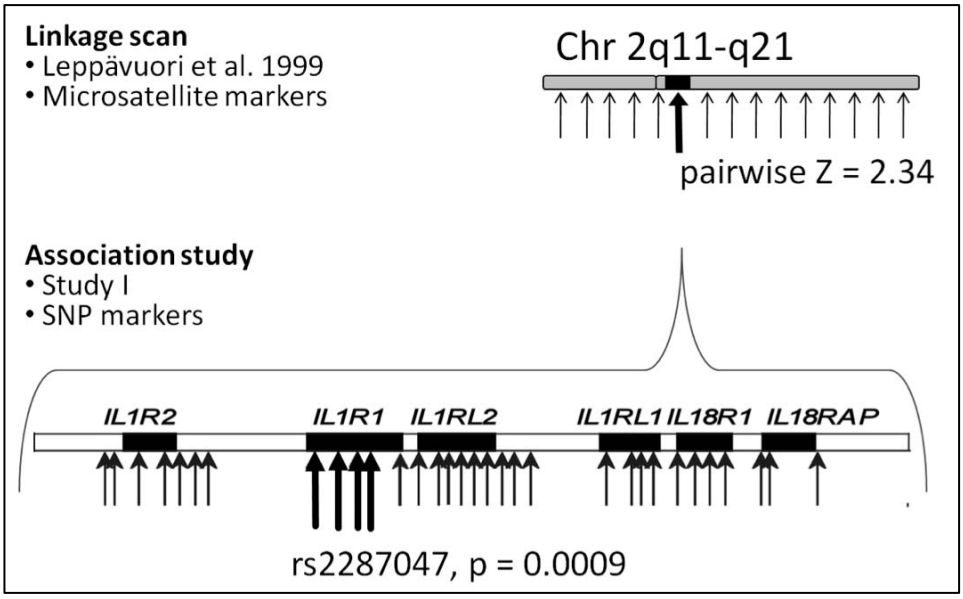

Figure 7. ***Flowchart of the two studies on IL1R1 and hand OA.*** *Suggestive evidence of linkage between hand OA and chromosome 2q11-q21 was observed in a genome-wide scan by Leppävuori et al. (1999). Association fine-mepping was performed in an extended hand OA study sample in Study I and variants in IL1R1were observed to "associate" with hand OA.*

One hypothesis for the role of this finding in OA is that the associated SNP region affects the gene expression of *IL1R1* gene. Altered expression levels of *IL1R1* could further affect NF-κB signaling, resulting in elevated expression levels of other inflammatory components leading to an increased tendency to inflammatory reactions in hand joints. However, the *IL1R1* gene variants could affect OA in a number of other ways, due to the complexity of NF-κB signaling.

IL-1β binds to the IL-1R1 protein that is widely expressed in different tissues on the cell surface. The roles of the *IL1* gene family members in this pathway are presented in **Figure 8**. The IL-1 – IL-1R1 – IL1RAcP complex starts the pathway, leading to the phosphorylation of inhibitory κB (IκB) with the help of inhibitory κB kinase (IKK). The release of IκB enables NFκB to promote the expression of corresponding genes, including *IL1A* and *IL1B*. The *IL1RN* gene codes for the IL-1Ra protein which inhibits the binding of IL-1α and IL-1β to their receptor IL-1R1. (O'Neill *et al.* 1998; Debets *et al.* 2001; Subramaniam *et al.* 2004; Towne *et al.* 2004; Roman-Blas *et al.* 2006).

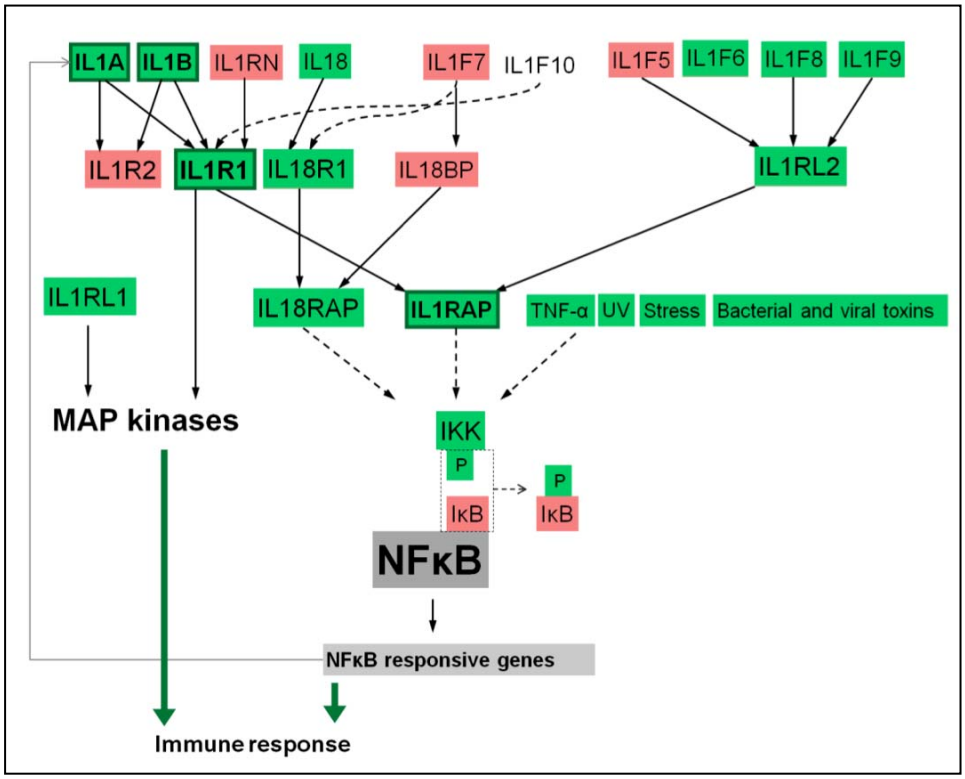

Figure 8. ***IL1 gene family memebers and activation of NFKB.*** *Green color indicates a proinflammatory and red color an anti-inflammatory molecule (O'Neill et al. 1998; Debets et al. 2001; Subramaniam et al. 2004; Towne et al. 2004; Roman-Blas et al. 2006).*

The role of the IL-1 pathway in OA has been previously studied. Firstly, the production of the IL-1β (by the *IL1B* gene) and the IL-1Ra (by the *IL1RN* gene) proteins was shown to be high in OA patient blood samples stimulated with LPS when compared to control blood samples stimulated with LPS (Riyazi *et al.* 2005).

Secondly, a study by Fernandes *et al.* (1999) showed that injection of the IL-1Ra (*IL1RN*) plasmid gene, into menisectomized rabbit knee, reduced the progression of OA. Thirdly, anakinra, a recombinant IL-1Ra protein, has been shown to decrease the symptoms in erosive OA patients (Bacconnie *et al.* 2009). However the results have not been unequivocal (Chevalier *et al.* 2009), this is possibly due to the short half life of anakinra (a few hours) and thus therapeutically too low concentrations were attained in the study (Martel-Pelletier *et al.* 2009) or because of a low affect in OA. Anakinra was approved for use in the treatment of RA by the European Commission European union in 2002.

The four associated SNPs of this study were located in the non-coding region of *IL1R1*. Three of them were in the long, highly polymorphic promoter region (rs1465325, rs956730, rs3917225) (Smith *et al.* 2004). It is possible that the studied SNPs, or other SNPs in high LD with the studied ones, affect the expression of the *IL1R1* gene or other genes in the same region. Three SNPs (rs1465325, rs3917225, rs2287047) co-localized with predicted transcription factor binding sites. In all three cases the conserved allele matched with the consensus sequence of a transcription factor binding site, while the other allele changed the site either to another transcription factor binding site or caused a loss of the binding site (Computational Biology Research Center CBRC, http://www.cbrc.jp/index.eng.html).

The role of the pathway from IL-1β to NF-κB activation is important for microbial defense. Mice deficient in IL-1β are more susceptible to several microbial infections compared to wild type mice (Netea *et al.* 2010). Survival from microbial infections has been, and still is, likely to be one of the many key issues for survival of a species. In comparison, joint wellbeing in later age is probably one of the least important characteristics for evolutionary survival.

Study I was the first study to show an association between *IL1R1* and OA. The *IL1R1* gene did not show significant association with knee OA, possibly due to either lack of importance in knee OA or lack of power in the knee OA study group. The *IL1R1* gene has been associated with other diseases like AIDS progression (Do *et al.* 2006) and endometriosis (D'Amora *et al.* 2006).

5.2 *MMP8* associated with knee OA

The aim of Study II was to investigate if there were allelic variants of *MMP8* and *MMP9* genes that would associate with knee OA. A Finnish discovery sample of 185 knee OA cases, with either clinical (n=75) or radiological (n=110) diagnosis, and 895 controls were analyzed (**Table 4**, D, E, K). Based on the results, one SNP in *MMP8* gene was further genotyped in a Finnish knee OA verification sample of 302 clinical knee OA cases and 1700 controls (**Table 4**, F, L) and in a Spanish knee OA verification sample of 254 knee OA cases and 449 controls (**Table 4**, G, M). Furthermore, a Finnish hand OA verification sample 1, consisting of 132 familial radiological hand OA cases and 435 controls, was analyzed (**Table 4**, A, I) as along with a second Finnish hand OA verification sample, consisting of 524 visually evaluated hand OA cases and 970 controls (**Table 4**, C, J). Meta-analysis was performed analyzing all knee OA and hand OA cases as two separate phenotypes and jointly as one OA phenotype.

5.2.1 Significant SNP association with only one study sample

Altogether, 13 tag SNPs were studied in the *MMP8* and *MMP9* genes and analyzed using Plink (Purcell *et al.* 2007). The main finding of Study II was for the SNP rs1940475 located in the *MMP8* gene (**Table 7**). The initial finding was observed with the Finnish knee OA discovery sample (OR = 0.721 95 % CI 0.575 - 0.906; p = 0.0049). A similar trend was seen in the Finnish knee OA verification sample, the Spanish knee OA verification sample, and the Finnish hand OA verification samples, but without statistical significance. Meta-analysis combining all study samples yielded a p-value of 0.028 (OR = 0.904, 95 % CI 0.827 - 0.989) (1369 OA cases, 4445 controls). The p-value threshold for 5 % significance was 0.0057 for the initial finding (Nyholt 2004; Li *et al.* 2005). The effect was stronger in the Finnish study sample than in the Spanish.

The same magnitude of significance was seen with another SNP (OR = 0.710, 95 % CI 0.562 - 0.895; p = 0.004; rs3765620). Both SNPs are non-synonymous. The SNP rs1940475 changes a positively charged OA protecting lysine to a negatively charged OA predisposing glutamic acid (Lys87 to Glu), while the SNP rs3765620 changes a protective polar threonine to a predisposing non-polar isoleucine (Thr32 to Ile). For both SNPs, the predisposing allele was the common, ancestral allele.

Table 7. Results of an association analysis on *MMP8* in the Finnish knee OA discovery sample.

SNP (allele)	Freq ctrls	Freq cases	χ^2 p-value	OR	L95	U95
rs12274992 (T)	0.051	0.074	0.088	1.470	0.942	2.293
rs3740938 (A)	0.074	0.068	0.688	0.913	0.587	1.422
rs2012390 (C)	0.205	0.186	0.417	0.888	0.666	1.183
rs1940475 (T)	0.493	0.413	0.0049	0.721	0.575	0.906
Finnish knee OA verification sample	0.473	0.454	0.371	0.924	0.776	1.099
Spanish knee OA verification sample	0.462	0.461	0.966	0.995	0.800	1.239
Knee OA META			0.039	0.884	0.787	0.994
Finnish hand OA verification sample 1	0.484	0.428	0.187	0.798	0.571	1.116
Finnish hand OA verification sample 2	0.474	0.465	0.639	0.965	0.830	1.121
Hand OA META			0.332	0.934	0.814	1.072
OA META			**0.028**	**0.904**	**0.827**	**0.989**
rs7123682 (C)	0.129	0.159	0.132	1.271	0.930	1.738
rs3765620 (G)	0.446	0.363	0.004	0.710	0.562	0.895
rs7943404 (G)	0.378	0.431	0.060	1.245	0.990	1.565
rs10895354 (C)	0.129	0.159	0.125	1.277	0.934	1.746

(allele) = allele for which the allele frequencies are shown; OA = osteoarthritis; META = meta-analysis of study samples above; Freq = allele frequency; ctrls = controls; OR = odds ratio; L95 = lower 95 % confidence interval; U95 = upper 95 % confidence interval.

5.2.2 *MMP8* region is in one LD block

The LD block structure was analyzed using the Haploview program (Barrett *et al.* 2005). Controls of the Finnish knee OA discovery sample were used in this analysis. The D' measure takes into account the differences in allele frequencies allowing strong correlation for mutations occurring at different times throughout evolution. The *MMP8* gene is located in one LD block based on the D' measure. The two SNPs associating with knee OA are in high LD with each other (r^2 = 82 %).

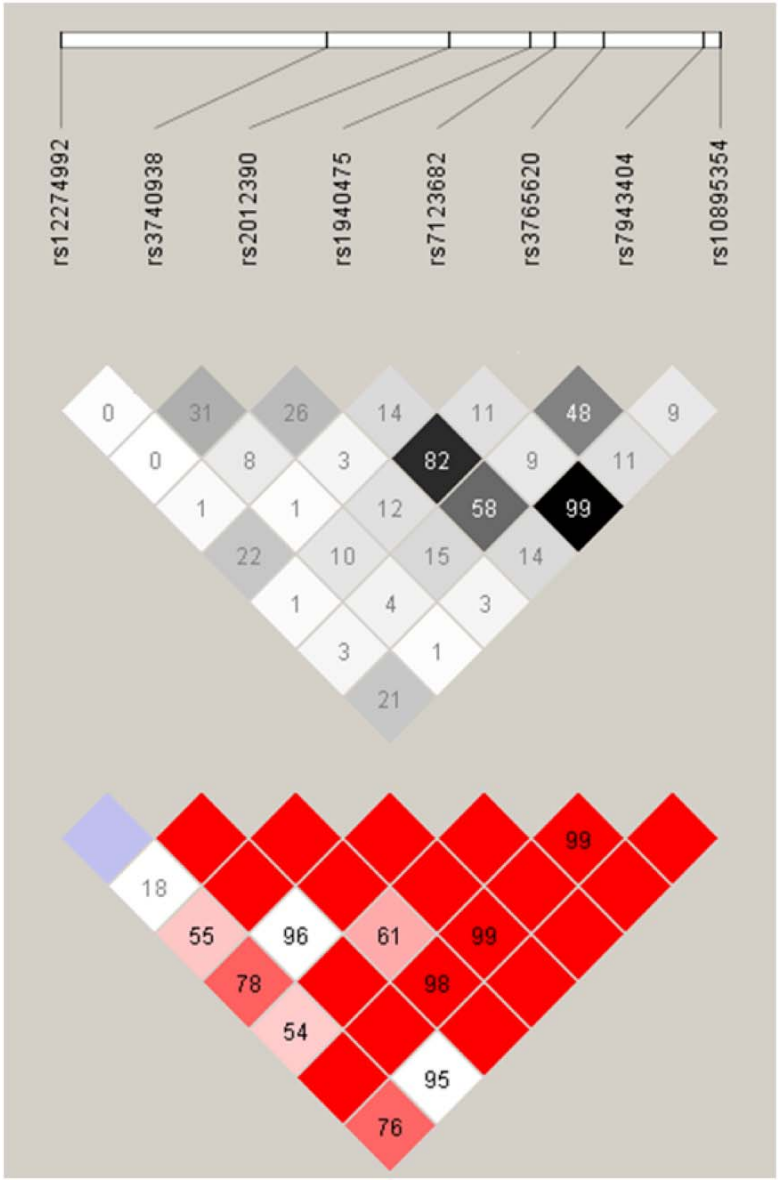

Figure 9. ***The LD block structure of the MMP8 gene in the Finnish controls of the Finnish discovery knee OA sample.*** *Above the r^2 measure of LD, below the D' measure of LD. The pair-wise LD is shown as different shadings of grey (r^2) and red (D') (Barrett et al. 2005).*

5.2.3 The role of MMP8 in OA

The MMP-8 protein (neutrophil collagenase, collagenase 2) cleaves collagens type I, II, III, other ECM compounds, and non-structural molecules (Barksby *et al.* 2006). A major source of MMP-8 is the neutrophils (Lazarus *et al.* 1968), which are essential cells in acute response to pathogens. Neutrophils appear in inflamed tissues within minutes to hours from contact with a pathogen. A mesenchymal form of the MMP-8 molecule is expressed by chondrocytes following a proinflammatory stimulus (Barksby *et al.* 2006), rheumatoid synovial fibroblasts and human endothelial cells (Hanemaaijer *et al.* 1997), osteoblastic progenitors, differentiated osteoblasts, osteocytes, and chondrocytes in the growth plate (Sasano *et al.* 2002), all of which might explain the connection between *MMP8* and OA.

The role of MMP-8 in OA has been suggested by the following studies: Increased expression of MMP-8 is associated with increased cleavage of type II collagen in OA in human osteoarthritic articular cartilage. *MMP8* mRNA was present in OA cartilage but absent from normal cartilage (Shlopov *et al.* 1997; Chubinskaya *et al.* 1999) and the levels were higher in chondrocytes obtained from areas surrounding OA lesion compared to other areas of the cartilage (Shlopov *et al.* 1999). The expression is not however, elevated in the synovium (Davidson *et al.* 2006). A similar trend was seen in cartilage immunostaining (Tetlow *et al.* 2001). MMP-8 levels were higher in blood of OA patients compared to controls (Tchetverikov *et al.* 2004).

TNF-α increases MMP-8 expression (Stannus *et al.*; Shlopov *et al.* 1999; Tetlow *et al.* 2001; Guler-Yuksel *et al.* 2010; Riepl *et al.* 2010). Inhibition of MMP-8 could be an important target in the prevention of the development of OA. Tetracycline (Suomalainen *et al.* 1992), doxycycline (Lauhio *et al.* 1994; Shlopov *et al.* 1999), and Ro 32-3555 (Trocade) (Lewis *et al.* 1997) could be potential treatments for OA due to their ability to decrease the levels of MMP-8. However, the important role of MMP-8 in other pathways including cancer related pathways (Balbin *et al.* 2003; Palavalli *et al.* 2009) raises the question of possible risks associated with this type of OA treatment.

5.3 *COL9A2* and *COL10A1* in hip OA

The heritability of the studied hip OA phenotype was 72 % (95% CI 49–87 %). Altogether 99 SNPs in 25 candidate genes were studied. The studied genes were *ACAN*, collagen genes (*COL1A1, COL1A2, COL2A1, COL3A1, COL5A1, COL5A2, COL9A1, COL9A2, COL9A3, COL10A1, COL11A1* and *COL11A1*), matrix metalloproteinase genes (*MMP3, MMP8, MMP9* and *MMP13*), and interleukin and interleukin receptor genes (*IL1A, IL1B IL1R1, IL1R2, IL1RL1, IL1RL2, IL18R1,* and *IL1RAP*). In total, 94 hip OA cases and 251 controls were analyzed (Table 4, H, N).

The Tagger program was used to analyze, how well the genetic variance in the studied regions, was tagged by the studied SNPs. Coverage (r^2 = 50 %) was 42 % on average, varying from 0 % to 80 % (*COL9A2*).

5.3.1 Suggestive evidence for association

The main association findings concentrated on the *COL9A2* and *COL10A1* genes ($p < 0.01$) (**Table 8**). None of the findings reached the 5 % threshold for significance that was set at 0.00073, based on SNPSpD analysis (Nyholt 2004; Li *et al.* 2005).

Table 8. Association of SNPs in *COL9A2* and *COL10A1* genes with hip OA as analyzed using the Pseudomarker program (Hiekkalinna *et al.* 2011a; Hiekkalinna *et al.* 2011b) and χ^2.test. The significance threshold was 0.00073.

Gene	SNP id	MAF		p-value	
		Controls	Hip OA	χ^2	Dominant
COL9A2	rs449541	0.340	0.289	0.287	0.158
	rs364281	0.397	0.386	0.817	0.920
	rs12077871	0.010	0.014	1.000[a,b]	0.215
	rs7533552	0.130	0.308	0.0021[a,c]	0.003
COL10A1	rs549332	0.434	0.507	0.157	0.179
	rs1064583	0.396	0.306	0.066	0.030
	rs3812111	0.394	0.297	0.053	0.024
	rs568725	0.383	0.250	0.006	0.0015

a = Fisher's exact test; b = Trp versus combined Arg and Gln alleles; c = combined Arg and Trp alleles versus Gln; Dominant = dominant mode in the familial association analysis performed with Pseudomarker (Hiekkalinna *et al.* 2011a; Hiekkalinna *et al.* 2011b).

Three SNPs in moderate LD with each other ($r^2 \geq 60$ %) showed a trend towards association in *COL10A1* (p = 0.0015, dominant mode of inheritance, the Pseudomarker program (Hiekkalinna *et al.* 2011a; Hiekkalinna *et al.* 2011b)).

This was the first time when a common SNP (rs7533552, alternative name rs2228564) in the *COL9A2* gene, on chromosome 1p34.2, was identified to associate with hip OA (p = 0.003). What makes this finding especially interesting, is a previous Finnish association finding between the same locus and disc degeneration (the Trp allele) in the same study sample (Videman *et al.* 2009).

The predisposing G allele changes a codon of a non-polar amino acid, glutamine (Gln326), to a positively charged arginine (Arg), or to tryptophan (Trp) when together with a rare T allele in the SNP next to it (rs12077871). The locus was sequenced with capillary sequencing to define the genotypes of both variants and, therefore, to reveal the amino acid for each individual. Eleven individuals carried a Trp allele as a combination of C/T heterozygote for SNP rs12077871 (1st base) together with A/G heterozygote or G/G homozygote for SNP rs7533552 (2nd base). This indicated that the minor alleles T and G of the two studied SNPs were inherited together (D' LD = 100 %, r^2 LD = 4 %, calculated using the Haploview program (Barrett *et al.* 2005) (**Table 9**) (**Figure 10**).

Table 9. The *COL9A2* finding in hip OA in Study III. The number of different amino acid combinations in unrelated hip OA cases and in controls for the Gln326 codon that contains two SNPs.

1st base rs12077871	2nd base rs7533552	3rd base	Corresponding amino acid	Controls (n)	Controls (%)	Hip OA (n)	Hip OA (%)
C	A	G	Gln	96	67.1	36	49.3
C	A	G	Gln				
C	A	G	Gln	41	28.7	28	38.4
C	G	G	Arg				
C	A	G	Gln	3	2.1	1	1.4
T	G	G	Trp				
C	G	G	Arg	3	2.1	7	9.6
C	G	G	Arg				
C	G	G	Arg	0	0.0	1	1.4
T	G	G	Trp				

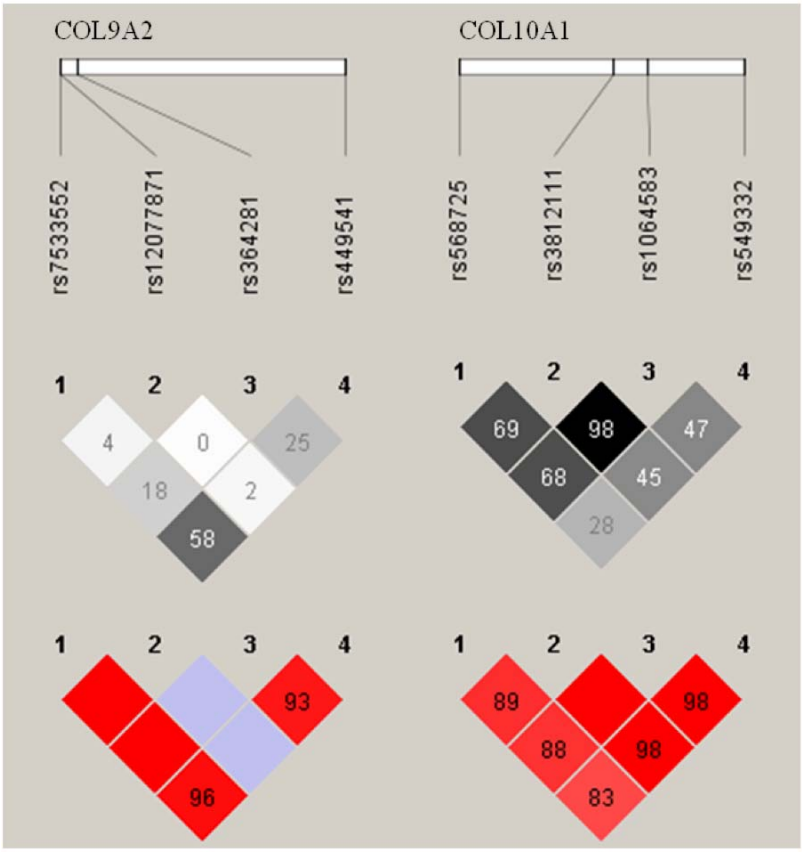

Figure 10. ***The LD patterns of the studied SNPs in COL9A2 and COL10A1 genes according to the Haploview program (Barrett et al. 2005)****. Above the* r^2 *measure of LD, below the D' measure of LD. The pair-wise LD is shown as different shadings of grey (*r^2*) and red (D') (Barrett et al. 2005).*

The Odds ratio was calculated by comparing amino acid counts of the wild-type Gln allele with the OA predisposing Arg or Trp alleles between the hip OA cases (45 predisposing alleles, 101 wild-type alleles) and controls (50 predisposing alleles, 236 wild-type alleles). The disease OR for carrying either of the predisposing variant alleles Arg or Trp versus the wild-type allele Gln was 2.10 (95% CI 1.66–2.67; p = 0.0021, Fisher's exact test). It seemed that the Trp allele was not associated with hip OA when compared with Arg and Gln alleles combined, but due to the limited number of individuals carrying the Trp allele, conclusion on its independent role cannot be drawn.

5.3.2 The role of *COL9A2* and *COL10A1* in OA

The hip OA predisposing variant in *COL9A2* (**Figure 11**) seemed to have a pleiotropic effect. The same predisposing G allele (Arg^{326} or Trp^{326}) associated with lumbar disc degeneration (p = 0.036 after 1000 permutations; disc bulging in discs L1-L4) in a previous study of the same twin sample (Videman *et al.* 2009). In addition, a similar trend was seen in a German population. The Arg amino acid was less frequent in controls than in patients suffering from "lumbar disc disease" and in those surgically treated for intervertebral disc herniation. The association did not, however, reach statistical significance (Knoeringer *et al.* 2008).

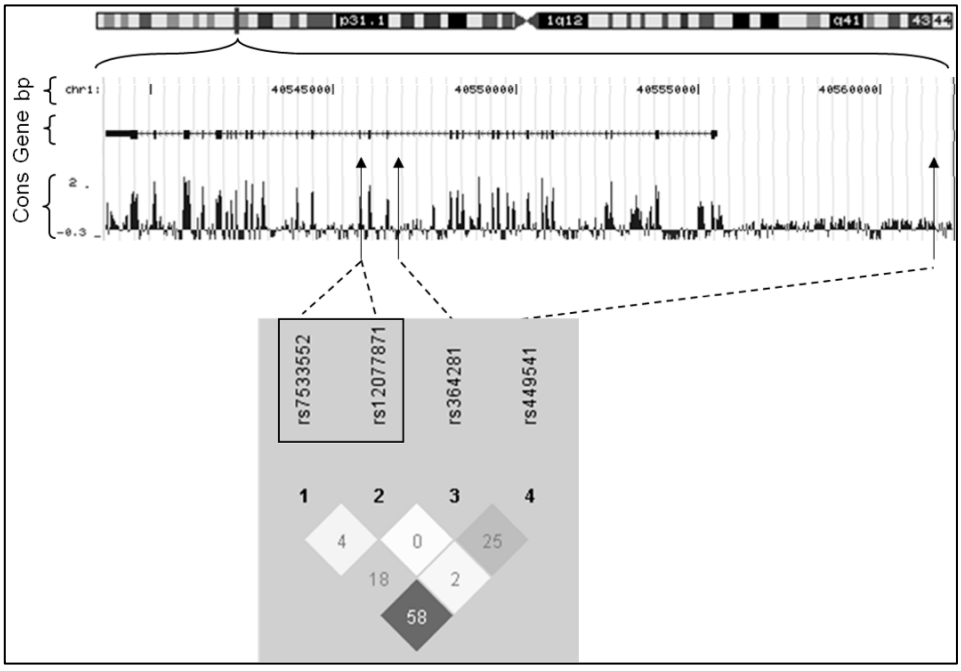

Figure 11. ***Location of the variants in COL9A2 gene on chromosome 1p34.2***. *bp = base pair location coordinates; Gene = the COL9A2 gene, exons are marked as boxes; Cons = conservation on the area between mammals on a scale (+2)-(-3); LD is shown as the r^2 measure. Modified from UCSC Genome Browser on Human Mar. 2006 Assembly and Haploview (Barrett et al. 2005).*

The rare amino acid change Gln^{326} to Trp ("the Trp2 allele") has been associated with intervertebral disc disease with associated sciatica in Finns (Annunen *et al.* 1999). The Trp2 allele has also been shown to play a role in a degenerative lumbar spinal stenosis in Finns (Noponen-Hietala *et al.* 2003). Finnish patients carrying the Trp2 allele have been shown to be more flexible according to the modified Schober

measure, and they more often have a radial tear in a non-herniated disc compared to controls (Karppinen *et al.* 2002). Surprisingly, the Trp2 allele seems to be relatively common in the Southern Chinese population, occurring in 20 % of the population. Where it is an age-dependent risk factor for the development and severity of a "degenerative disc disease" phenotype (associated with it in the age group of 40-49 years), for the development of anular tears (associated with it in the age group of 30-39 years), and Schmorl's nodes (associated with the age grouop of 40-49 years) (Jim *et al.* 2005). Additionally, the Trp2 allele was shown to associate with disc degeneration in Japanese patients less than 40 years of age (Higashino *et al.* 2007). The Trp2 allele was associated with the deteriorating mechanical properties of the nucleus pulposus as early as adolescence, when no other signs of disc degeneration were present (Aladin *et al.* 2007).

Of special importance to the present study, is that the SNP rs7533552, in the *COL9A2* gene and moderately associating with hip OA, is likely to be an actual disease causing variant rather than a marker in high LD with such a variant. As discussed earlier, type IX collagen contains sequences that form triple-helical domains and plays a role in balancing the type II collagen by forming cross-links with it. Based on the present results, it can be hypothesized that a change from glutamine to either arginine or tryptophan affects the triple helical structure, thereby weakening the cartilage of the hip and intervertebral discs. However, based on the PolyPhen protein structure prediction program, only the change from glutamine to tryptophan was predicted as possibly damaging (Division of Genetics, Department of Medicine, Brigham and Women's Hospital – Harvard Medical School, Cambridge, MA, USA; http://genetics.bwh.harvard.edu/pph/).

Other variations in *COL9A2* gene have been shown to associate with intrafamilial phenotypic diversity in multiple epiphyseal dysplasia (Takahashi *et al.* 2006). The *COL9A2* gene encodes one (alpha 2) of the three alpha chains of type IX collagen, which is usually found in tissues containing type II collagen. Type IX collagen plays a role in load bearing cartilages by organizing and stabilizing the network of collagen fibrils. Deficiency in α1(IX) chains leads to a dysfunction of type IX collagen. However, it was shown that type IX collagen is not essential for formation of the fibril network, instead the protein is required for long term tissue stability (Hagg *et al.* 1997). The other two alpha chains in type IX collagen have also been associated with disc disease and OA, the alpha 1 chain displayed an association with disc degeneration in a previous study on twins (Videman *et al.* 2009) and it has been associated with hip OA.

Three neighboring SNPs showed some level of association with hip OA in the *COL10A1* gene on chromosome 6q22.1 (Figure 12). The most significant p-value in the *COL10A1* gene was obtained for SNP rs568725 (p = 0.0015). The SNP

rs1064583, which showed only a modest association with hip OA (p = 0.0245), is located in an exon and changes the amino acid methionin to threonine (Met27 to Thr). Interestingly, there is another gene, Homo sapiens 5'-nucleotidase domain containing 1 (*NT5DC1*) in the same location, but the direction of this gene is opposite to that of the *COL10A1*.

The *COL10A1* gene (**Figure 12**) encodes three alpha 1 chains of the type X collagen homotrimer. It is expressed by hypertrophic chondrocytes during endochondral ossification, and can thus be considered as a marker for new bone formation in articular cartilage (Shen 2005). Type X collagen was found in the osteoarthritic cartilage of the hip, but was absent from normal adult cartilage (Boos *et al.* 1999).

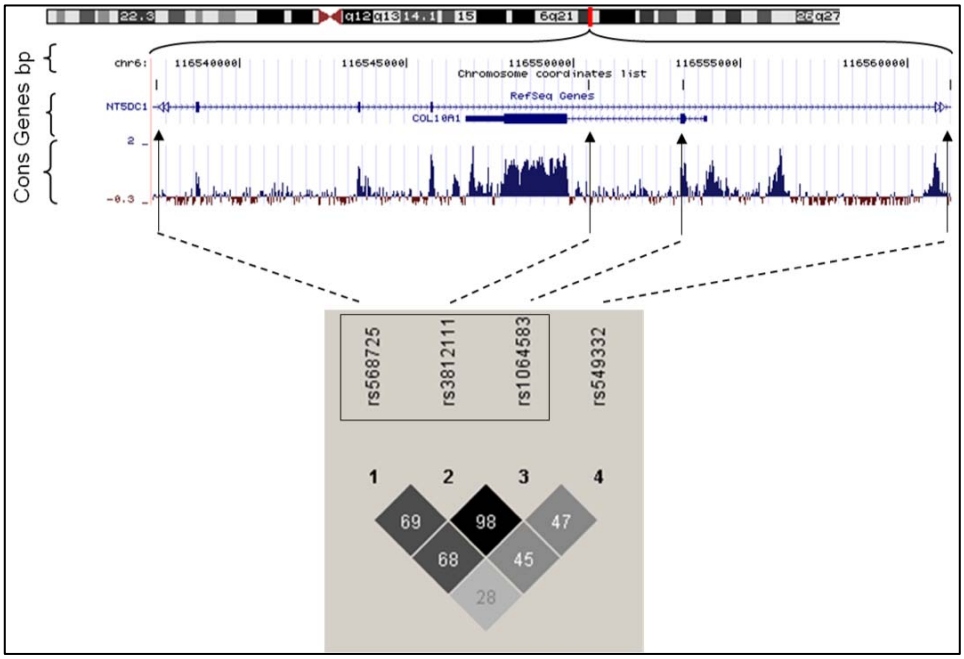

Figure 12. ***Location of the variants in COL10A1 gene on chromosome 6q22.1****. Bp = base pair location coordinates; Gene = genes in the area, boxes represent the exons and continuous line represent the introns; Cons = conservation on the area between mammals on a scale (+2)-(-3); LD is shown as the* r^2 *measure. Modified from UCSC Genome Browser on Human Mar. 2006 Assembly and* Haploview *(Barrett et al. 2005).*

5.4 Limitations of the study

The three most significant limitations of the present thesis work were, the limited sample sizes, lack of replication, and the insufficient marker coverage of the genes studied.

5.4.1 Sample size

A small sample size reduces the power for the detection of SNPs of low-effect, which are likely to be typical in complex diseases. In the present thesis work, the number of individuals studied, and especially the number of individuals affected, was not high enough for sufficient power to detect low-effect variants, or to exclude the relevance of a gene to a phenotype. For instance in Study I, the required number of cases was 500 to have 90 % power for detection of the observed association signal, which, further, might have been an over-estimate (OR = 0.26). Genome-wide association signals of $p < 5x10^{-8}$ with typical ORs of about 1.25 (Wellcome Trust Case Control Consortium 2007) would require 6000 cases and 6000 controls for detection (Hirschhorn *et al.* 2005). The small sample size, therefore, increases the risk for false positive findings.

5.4.2 Phenotyping

Most disease phenotypes cannot be biologically divided into groups of healthy and affected individuals, but the phenotypes are usually continuous quantitative traits, as discussed earlier. In the present study, the populations were divided into two groups, healthy and affected, based on their OA grade or clinical evaluation. Some individuals in these two different groups might still be biologically very similar to each other. A pure quantitative trait could have given a better starting point for the study. However, developing a reliable quantitative measurement for OA, that is also heritable, is still a challenge.

Additionally, the grading of the hip OA using MRI was originally performed for the disc degeneration study, and only individuals imaged with the method were included in the present study. There were a few individuals excluded that had gone through joint replacement surgery, and thus, were unable to participate in the MRI grading due to the metallic object now in their body. Moreover, severe forms of OA might significantly limit physical activities such as walking, and, therefore, such individuals may not participate in these studies.

5.4.3 Gene coverage

The SNP panels in studies I and III were designed before the availability of the HapMap genotyping database (The International HapMap Consortium *et al.* 2003) and thus the tagging SNP method was not used. Without such knowledge of the LD structure of the genome, revealed by the HapMap project, the SNPs were selected to cover the studied genes in terms of equal physical distance. The number of SNPs and their locations was later found not to be optimal.

Furthermore, a large number of SNPs were excluded from the study during the strict quality control stage. Therefore, the SNP panels in these studies did not cover the genes sufficiently, and variants affecting the disease may have been missed. On the other hand, the tagging system based on HapMap linkage disequilibrium data has been criticized, since a disease causing SNP could be missed even when genotyping a tag marker in high LD with it (Terwilliger *et al.* 2006).

5.4.4 Studied population

The population structure of the study subjects was not taken into consideration when planning the study. However, both hand and knee OA study samples were collected from nationwide cohorts or from national hospitals specialized in rheumatic diseases. Recently published data shows that subjects originating from different parts of Finland are heterogeneous in their genetic background, even if the total amount of variance is lower than in other European populations (Jakkula *et al.* 2008). However, the GWA data of the Finnish Health 2000 sample showed relatively homogenous genetic background between the clinical knee OA cases and controls (**Table 4**, K, E), with a p-value greater than 0.1 for the difference between cases and controls for the 10 most significant principal components describing the genetic heterogeneity of the two groups.

5.4.5 Lack of replication

For validation of a finding the results should be replicated in a larger sample set to verify association with OA. Until then, the presented results should only be considered as a possible starting point for future studies. Future GWA and whole-genome sequencing studies will cover the studied areas and, therefore, should shed more light on these findings.

5.4.6 Statistical significance of the findings

In the present study, association analyses were performed using the χ^2 test and the Pseudomarker program's dominant and recessive models. Performing the association test in three different ways increases the number of statistical tests performed. However, these tests are still highly dependent on each other. That is why they were not corrected for separately, which would have decreased the p-value threshold of significance. Further, some of the SNPs were in high LD with each other. These factors were taken into account in the correction of multiple testing. With some of the SNPs considered as one test and the tests of different inheritance models also considered as one test.

Today, the emerging technology of whole-genome sequencing in large study samples enables positive findings with p-values of several magnitudes larger in size. A genome-wide significance level of $5x10^{-8}$ is not obtainable with the sample sizes used here or with the number of SNPs studied. The current findings should be considered as an implication of a possible association, and therefore as a base for future studies.

5.4.7 Biological meaning of the findings

Clinically and diagnostically, these findings are still far from practical applications in terms of medical treatment for OA. Evidence for an eventual role of the *IL1R1* variant in OA is needed, and similarly protein structure analysis corresponding to the *MMP8* and *COL9A2* findings are needed.

6 CONCLUSIONS AND FUTURE PROSPECTS

In this thesis, association between OA and SNPs in several biologically relevant candidate genes was analyzed. Association was observed between hand OA and the *IL1R1* gene ($p = 0.00091$, with a significance threshold of $p = 0.0021$), knee OA and the *MMP8* gene ($p = 0.0049$, with a significance threshold of 0.0057), and between hip OA and the *COL9A2* and *COL10A1* genes ($p = 0.0021$ and $p = 0.0015$, with a significance threshold of 0.00073). However, due to the small sample sizes and the moderate strength of the findings, these results need to be replicated in larger sample sets in order to verify their true importance in the etiology and pathogenesis of OA. Today, with the commonly used GWA methods and large sample sets of tens of thousands study subjects, p-value thresholds of $p < 5x10^{-8}$ are considered as statistically significant, with newer technologies that will perform whole-genome sequencing likely to decrease this limit further. Compared to the newer knowledge and studies, which are possible to conduct today, the current findings are of moderate value. However, it is important to emphasize the fact that the association with the *ILR1* gene was found in a region previously indicated in a linkage study on the same study sample. While, the finding for *MMP8* showed a similar trend in several study samples, even if not in a statistically significant manner, and the codon in the *COL9A2* gene had associated with disc degeneration in a previous study (Videman *et al.* 2009).

For the association findings presented in this thesis work for the *IL1R1*, *MMP8* and *COL9A2* genes, it is important to replicate them in other larger study samples, including both Finnish cohorts and cohorts of other ethnic backgrounds.

In general, extending the studied samples with new samples would be of use. Another way to proceed could be by collecting a new large OA study sample with known common ancestors from an isolated population, so as to perform exome or whole-genome sequencing in order to find rarer familial variants. A cost effective study approach would be through utilizing large population based cohorts that are currently being deep-sequenced, exome-sequenced or genotyped with GWA chips. Besides clinical and/or radiological data, applying known OA biomarker data from whole blood samples could yield interesting results. However, choosing reliable OA biomarkers might be a difficult task. However, on the other hand, their biological reliability would likely be higher than the symptom based clinical phenotype.
Moreover, an expression study assessing the role of the four associated SNPs in the expression of *IL1R1* gene itself, and other *IL1* genes, would be useful. Detailed protein structure analyses of type IX collagen and MMP-8 for the different forms, according to the associated SNPs, would also gain important information about the

true biological meaning of these findings. Based on these results, the region of *IL1R1* is currently undergoing deep sequencing in two pools of 8 and 10 individuals, selected based on their carrier status for the GG or GA genotype of the SNP rs2287047.

When these projects were initiated, one hypotheses of OA was that the disease is caused by defective protein structure of the cartilage. Another point of view was that there is an imbalance between the cartilage break down and repair by chondrocytes. Additionally, based on disease mapping of different complex traits, it is now known that in addition to common variants predisposing to common diseases, it is likely that rare variants also contribute to common diseases, with rare variants being more likely to be functional than a common one (Zhu *et al.* 2011).

With the newer technology it has been possible to perform studies without delimiting the study to a few *a priori* ideas and this will help OA research to a new level. Based on large GWA studies, it seems that both the immune system (Nakajima *et al.* 2010) and nerve growth (Day-Williams *et al.* 2011) are factors contributing to the disease. On the other hand, the role of chondrocytes is especially interesting in the development of OA. These cells are the only cells to maintain the cartilage, and while doing so they use the synovial fluid both as a source for nutrients and as a sewer system for metabolites (Corvol 2000). Another interesting view of OA relates it to obesity and cardiovascular diseases (Uchida *et al.* 2009).

All in all, it has been known for decades that radiological OA changes and OA symptoms correlate relatively poorly. This speaks for the theory that both structural proteins of the joint in addition to the inflammatory system are involved. One can have degenerative changes without inflammation and painful symptoms. On the other hand, some individuals with very few radiological changes develop very painful symptoms in early stages. Cartilage breakdown products can trigger a pro-inflammatory reaction (Monach *et al.* 2009) and immunological genes have been shown to associate with OA (Nakajima *et al.* 2010). This could indicate that some individuals have genetically the kind of host defense system that tends to react too keenly to cartilage breakdown particles and molecules. If this is the case, then also evolutionary this too keen immune system has probably had significant advantage in, for example, the defense against different microbial attacks.

Finally, even with all the presented studies and results in this thesis work, scientists are still not even unanimous whether the disease progress initiates from the cartilage, bone or synovium. Even though we have come far since the earliest studies, we can still neither confirm nor discard the hypothesis of Bennett (1942), Collins (1947) and their co-workers who concluded that the OA changes initiate from the cartilage surface and proceed to deeper layers, causing later the changes to

the bone. We still need more systematic large gene mapping studies and large familial studies, combined with proteomics, lipidomics, and genome-wide expression profiling, for a better understanding of the disease.

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Helsinki, February 3, 2012
Annu Näkki

8 ELECTRONIC DATABASES

Databases and programs

1000 Genomes, http://www.1000genomes.org/

International HapMap Project, http://www.hapmap.org/

Mx program, Neale, M.C., Virginia Commonwealth University, Richmond, Virginia, USA; http://www.vcu.edu/mx/.

National Center for Biotechnology Information (NCBI), http://www.ncbi.nlm.nih.gov/

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