SPINAL STENOSIS AND INTERVERTEBRAL DISC DISEASE

The role of sequence variations in collagen IX and XI, and inflammatory factors in spinal disorders

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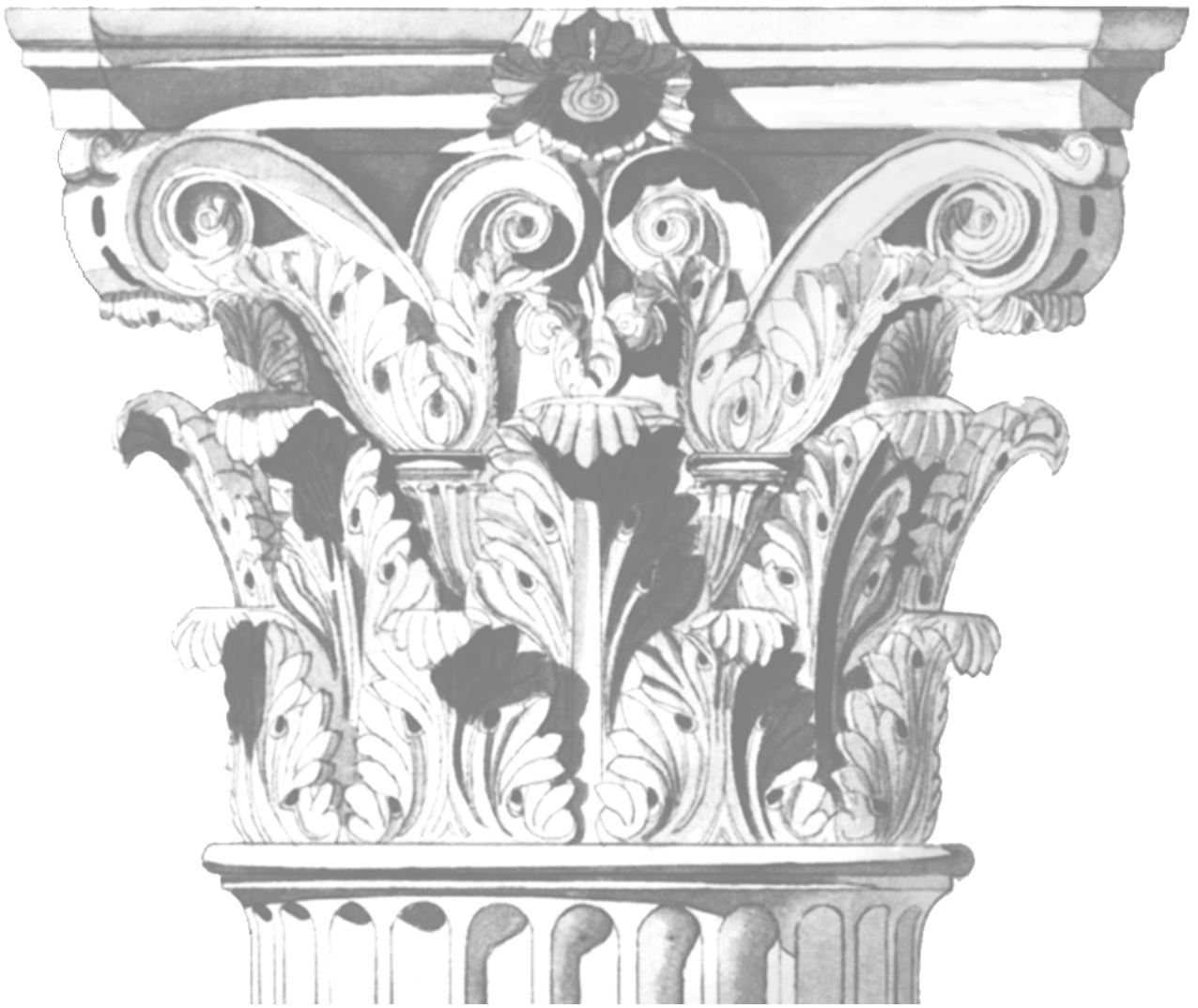

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Abstract

Genetic factors have been implicated to play a role in both degenerative lumbar spinal stenosis (LSS) and intervertebral disc disease (IDD). Sequence variations in the genes coding for collagen IX and inflammatory mediators have been indicated as risk factors for IDD.

Nine genes coding for intervertebral disc (IVD) collagens I, II, IX and XI and aggrecan (*AGC1*) were analyzed for sequence variations in 29 Finnish individuals with LSS. In addition, two polymorphisms in the vitamin D receptor gene and one in the matrix metalloproteinase-3 gene were studied. Study subjects were analyzed both clinically and radiologically. Results indicated an association between the *COL11A2* IVS6^{-4} a to t polymorphism and LSS ($p = 0.0016$). Moreover, the t/t genotype was found more often in the patient group compared to controls ($p = 0.0011$). A novel splicing mutation, likely resulting in the synthesis of a truncated protein, was identified in *COL9A2*.

Eight hundred four Chinese individuals were screened for the presence of the Trp2 and Trp3 alleles. The Trp2 allele was found in 20% of the individuals compared to the previously reported 5% in Finnish patients with IDD characterized by sciatica. The Trp2 allele was found to predispose to IVD degeneration and end plate herniations, increasing the risk by 2.4-fold from 40 to 49 years of age. In addition, the degeneration was worse in individuals with the Trp2 allele. The risk for annular tears was 4-fold greater in study subjects from 30 to 39 years of age who were Trp2 positive. Surprisingly, the Trp3 allele was absent even though it was found in about 9% of Finnish individuals.

One hundred fifty-five Finnish individuals with IDD characterized by sciatica were analyzed for sequence variations in four genes coding for inflammatory mediators IL1A, IL1B, IL6, and TNFA. In addition, sixteen polymorphisms in inflammatory mediator genes were analyzed. The results identified an association between sciatica and the E5^{+15}T>A polymorphism in IL6 ($p = 0.007$). A significant association was also seen in the IL6 haplotype analysis (-597 g>a, -572 g>c, -174 g>c and E5^{+15}T>A). The association of the GGGA haplotype with the disease was highly significant ($p = 0.0033$).

Keywords: collagen, inflammation, intervertebral disc disease, spinal stenosis

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Abbreviations

4Hyp	4-hydroxyproline
AGC1	aggrecan
AR	population attributable risk
bp	basepair
cDNA	complementary DNA
COL	collagenous domain
Col9a1	mouse α1(IX) collagen gene
COL9A2	human α2(IX) collagen gene
CMD	cartilage matrix deficiency
CSGE	conformation sensitive gel electrophoresis
CT	computed tomography
DDD	degenerative disc disease
FACIT	fibril-associated collagen with interrupted triple helices
Gly	glysine
HIZ	high intensity zone
IDD	intervertebral disc disease
IFNG	interferon gamma gene
IL	interleukin
IVD	intervertebral disc
kb	kilobase
LDD	lumbar disc disease
LSS	lumbar spinal stenosis
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
mRNA	messenger RNA
MS	multiple sclerosis
NC	noncollagenous
OA	osteoarthritis
OLF	ossification of the ligamentum flavum
OPLL	ossification of the posterior longitudinal ligament
OR	odds ratio

PCR	polymerase chain reaction
Pro	proline
RT-PCR	reverse transcriptase PCR
SNP	single nucleotide polymorphism
TNF	tumor necrosis factor
Trp	tryptophan
TR/TE	repetition time/echo time
VDR	vitamin D receptor
VNTR	variable number of tandem repeats
X	any amino acid
Y	any amino acid

List of original articles

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

I Noponen-Hietala N, Kyllönen E, Männikkö M, Ilkko E, Karppinen J, Ott J & Ala-Kokko L (2003) Sequence variations in the collagen IX and XI genes are associated with degenerative lumbar spinal stenosis. Ann Rheum Dis 62: 1208-1214.

II Jim JJT*, Noponen-Hietala N*, Cheung KMC*, Ott J, Karppinen J, Sahraravand A, Luk KDK, Yip S-P, Song Y-Q, Leong JCY, Cheah KSE, Ala-Kokko L & Chan D. The Trp2 allele of collagen IX is an age-dependent risk factor for the development and severity of intervertebral disc degeneration. Spine, in press.

III Noponen-Hietala N, Virtanen I, Karttunen R, Schwenke S, Jakkula E, Li H, Merikivi R, Ott J, Karppinen J & Ala-Kokko L (2005) Genetic vartions in *IL6* associate with intervertebral disc disease characterized by sciatica. Pain, 114:186-194.

*These authors contributed equally to this work. In addition, some unpublished data are presented.

Contents

1 Introduction

Intervertebral disc disease (IDD) is a common musculoskeletal disorder that is estimated to cause about 6% of populations' disability. IDD characterized by sciatica, a radiating pain along either leg, is usually caused by intervertebral disc (IVD) herniation. Lifting heavy loads, torsional stress, and motor vehicle driving have been identified as risk factors for this disease. However, recent evidence indicates that genetic factors may also play a role in IDD.

IVDs provide stability to the spine by anchoring the vertebral bodies to each other. They also allow movement between the vertebrae, giving the spine its flexibility. They are composed of three integrated tissues: the gelatinous nucleus pulposus, the laminated annulus fibrosus, and the cartilage end plate. Collagen I accounts for about 70% of the dry weight of the annulus, whereas the nucleus is rich in collagen II. The nucleus also contains small amounts of collagens IX and XI. In addition, collagen IX is found in small amounts in the annulus. Both the annulus and nucleus contain small amounts of collagen VI, and minor amounts of collagens III and V are present throughout the disc. A large proteoglycan, aggrecan, accounts for as much as 50% of the dry weight of the nucleus.

Collagen IX is a heterotrimeric protein composed of three chains, α1(IX), α2(IX), and α3(IX), encoded by *COL9A1*, *COL9A2*, and *COL9A3*, respectively. A Gln to Trp substitution in the α2 chain of collagen IX has been shown to be associated with IDD in the Finnish population. In addition, another study showed that an Arg to Trp substitution in the α3(IX) chain is a common risk factor for IDD in the Finnish population. Furthermore, an association between an intragenic aggrecan gene (*AGC1*) variable number of tandem repeat (VNTR) polymorphism and disc degeneration has been reported. The results of this study showed an overrepresentation of alleles with a small number of repeats in individuals with multilevel disc degeneration. In addition, an association between disc degeneration and polymorphisms in both the vitamin D receptor (*VDR*) and matrix metalloproteinase-3 (*MMP-3*) gene has been reported in some studies.

When the disc herniates, the adjacent nerve root may be compressed. Herniated IVD tissue has been shown to produce a number of proinflammatory mediators and several studies have suggested that the inflammatory factors are important contributors to sciatic pain.

Spinal stenosis is defined as the narrowing of the spinal canal, nerve root canals, or intervertebral foramina. It can be divided into congenital-developmental and acquired types. Congenital stenosis is due to a developmental narrowing of the spinal canal, whereas acquired or degenerative stenosis is usually caused by disc herniations and degenerative changes in the intervertebral disc, facet joints, and ligamentum flavum. Degenerative stenosis is a common clinically important spinal disorder, and it typically occurs in patients between the fifth and seventh decade of life with an incidence of 1.7% to 10%. The possible role of genetic factors in this disorder is supported by results from several observations including family and linkage studies. Ossification of the posterior longitudinal ligament (OPLL) is the leading cause of spinal stenosis in Japan. An association between OPLL and the *COL11A2* polymorphism ($IVS6^{-4}$ a>t) has been reported.

The purpose of this thesis is to increase the information concerning genetic factors in IDD and spinal stenosis. It describes the role of sequence variations in the genes coding for IVD proteins in degenerative spinal stenosis, and further supports the role of collagens IX and XI in spinal diseases. In addition, it strengthens the hypothesis that Trp2 acts as an important contributor to IDD. The pathogenesis of IDD also involves an inflammatory component. We showed here for the first time that the genetic variations in *IL6* are associated with IDD-related radiculopathy.

2 Review of the literature

2.1 Spine

2.1.1 Anatomy and function

The vertebral column is built up from alternating bony vertebrae and intervertebral discs that are connected by strong ligaments and supported by powerful musculotendinous masses. A typical vertebra is made up of an anterior, cylindrical body and a posterior arch composed of two pedicles and two laminae. The laminae are united to form a spinous process. (Netter 1987.)

The vertebral column typically consists of 33 vertebrae: 7 cervical, 12 thoracic, 5 lumbar, 5 sacral, and 4 coccygeal, but only the cervical, thoracic, and lumbar vertebrae are mobile. The five sacral vertebrae are fused to form the sacrum, and the four coccygeal vertebrae are fused to form the coccyx. The vertebral body is the anterior, more massive part of the bone that gives the vertebral column its strength and supports the body weight. Lumbar vertebrae have massive bodies because they support the largest body weight at the inferior end of the vertebral column. In the cervical and lumbar regions, facet joints bear some weight, sharing this function with the intervertebral discs. The vertebral column is stabilized by ligaments, muscles, intervertebral discs, and the shape of the vertebrae. (Moore & Dalley 1999.)

The body of each vertebra has a spongy medullary bone surrounded by bony cortex. The inferior and superior surfaces of the vertebral body are called end plates, which are covered by hyaline cartilage. The vertebral bodies are bordered by two major ligaments. The anterior longitudinal ligament is a broad band of fibers extending along the front and sides of the vertebral bodies (Borenstein *et al.* 1995) and is firmly united with the periosteum of the vertebral bodies, but is free over the intervertebral discs (Humzah & Soames 1988). The posterior longitudinal ligament runs along the posterior side of the vertebrae forming the anterior boundary of the spinal canal (Borenstein *et al.* 1995). The yellow ligaments (ligamentum flavum) run vertically from the lamina above to lamina

below, forming part of the posterior wall of the vertebral canal (Moore & Dalley 1999). Nerve roots exit from the spinal canal through the intervertebral foramina located inferiorly and superiorly between the pedicles. Anteriorly, the foramen is bordered by the intervertebral disc and vertebral body, and posteriorly by the lamina as well as the facet joint. (Borenstein *et al.* 1995.)

Only limited flexion, extension, lateral bending, and rotation are possible between the vertebrae. Movements are freer in the cervical and lumbar regions compared to those in the thoracic region (Netter 1987). The mobility results primarily from the compressibility and elasticity of the intervertebral discs (Moore & Dalley 1999) and changes in the discs structure may cause pain and functional impairment.

2.1.2 Intervertebral disc

There are at least 25 IVDs between the adjacent surfaces of the vertebrae, uniting them from the cervical region to the sacrum. The disc has three integrated tissues: the gelatinous nucleus pulposus, the laminated annulus fibrosus, and the cartilage end plate (Humzah & Soames 1988, Borenstein *et al.* 1995). IVDs stabilize the spine, allow the movement between vertebrae giving the spine its flexibility, and absorb and distribute the loads applied to the spine (Buckwalter 1995). In health and maturity, the IVDs account for almost 25% of the length of the vertebral column. They are thinnest in the thoracic region and thickest in the lumbar region (Netter 1987).

With skeletal maturity, the IVDs lose the remaining blood vessels and become largely acellular (Buckwalter 1995). The main mechanism of nutrition transport is passive diffusion. The solutes can be changed with the blood vessels outside the disc and through the end plates (Urban *et al.* 1977). The outer annulus obtains nutrients from the blood vessels in the soft tissues around the annulus, whereas the inner annulus and nucleus rely on a path from the blood vessels of the vertebral body through the end-plate for their nutrition supply and the removal of wastes (Urban *et al.* 1977, Horner *et al.* 2001). New magnetic resonance imaging (MRI) techniques have made it possible to demonstrate the solute transport into the disc through the end plate (Bydder 2002).

2.2 IVD proteins

Collagens are a family of extracellular matrix proteins that play a role in maintaining the structural integrity of various tissues. Each collagen molecule is composed of three α chains characterized by a Gly-X-Y sequence that folds into a unique triple-helical structure.

The IVDs consist of a sparse population of cells and contain an abundant extracellular matrix of proteoglycans and collagens (Buckwalter 1995, Mirza & White 1995). The proportion of the different constituents varies between annulus, nucleus, and end plate (Table 1). Collagens account for 70% of the dry weight of the outer layer of the disc, the annulus fibrosus, but less than 20% of the dry weight of the inner part of the disc, the nucleus pulposus. In contrast, proteoglycans account for only a few percent of the dry

weight of the annulus, but up to 50% of the dry weight of the nucleus. Collagens give the tissue its strength, and the hydrated proteoglycans, the major structural component of which is aggrecan, give the disc its resilience to compression (Humzah & Soames 1988, Buckwalter 1995, Urban & Roberts 1995).

The annulus fibrosus is a complex of concentrically arranged lamellae of fibrocartilage. In the adult disc, collagen I accounts for about 70% of the dry weight of the annulus, but is virtually absent in the nucleus pulposus. In addition, the annulus contains about 10% of collagen VI and a small amount of collagen V, about 3%. The nucleus pulposus contains large concentrations of collagen II and proteoglycans (Eyre & Muir 1976). In the nucleus the concentration of collagen VI is more than 15%, while collagen XI is found in smaller amounts, about 3%. Both collagen IX and collagen III are found in small amounts throughout the disc. In addition, a variety of noncollagenous proteins exist in the disc. Elastin and other minor components are found in small amounts in both the annulus and nucleus pulposus.

The end-plates consist of a gel of hydrated proteoglycan molecules that is reinforced by collagen fibrils. They are considered to be the thin layer of hyaline cartilage that lies between the bone of the vertebral body and the soft tissue of the disc. The structure of the end plate is critical in maintaining the integrity of the disc. Proteoglycans, in particular, account for the transport of the solutes into and out of the disc. (Moore 2000.)

Those collagens that may play a role in IDD are presented in more detail below.

Table 1. Intervertebral disc collagens.

Collagen	Expression [a]	Amount of total collagen in AF [b]	Amount of total collagen in NP [b]
Collagen I	Most connective tissues, especially in dermis, bone, tendon, ligament	70%	-
Collagen II	Cartilage, vitreous humour	Small amount	Up to 80%
Collagen III	Same as collagen I, except absent in bone and tendon	Small amount	Small amount
Collagen V	Tissues containing collagen I	3%	-
Collagen VI	Most connective tissues	10%	>15%
Collagen IX	Cartilage, vitreous humour	1-2%	1-2%
Collagen XI	Catrilage, vitreous humour	-	3%

AF= annulus fibrosus. NP= nucleus pulposus. [a] Myllyharju & Kivirikko 2001. [b] Eyre 1988.

2.2.1 Collagens

2.2.1.1 Collagen biosynthesis

Collagen biosynthesis is a complex, multistage process that involves several posttranslational modifications, many of which are unique to collagens and a few other proteins with collagen-like amino acid sequences. The posttranslational modifications can

be regarded as taking place in two stages: intracellularly in the endoplasmic reticulum and extracellularly.

The fibril-forming collagens are first synthesized as large precursor molecules called procollagens. These molecules have propeptide extensions at both their amino- (N) and carboxy- (C) terminal ends. Intracellularly, specific enzymes, namely prolyl 4-hydroxylase and lysyl hydroxylase, hydroxylate the proline residues in the Y position to 4-hydroxyproline and the lysyl residues to hydroxylysine. In addition, some proline residues in Gly-Pro-4Hyp sequences are hydroxylated to 3-hydroxyprolines by prolyl 3-hydroxylase. 4-hydroxyproline is essential for maintaining triple helix stability at body temperature. Some of the hydroxylysine residues in the Y-position are further glycosylated with galactose or galactose and glucose. The formation of the triple helix is then propagated from the C-terminus towards the N-terminus in a zipper-like fashion. Stable triple helix formation requires the presence of glycine at every third residue, as well as 4-hydroxyproline in the Y position of a high proportion of the triplets. The correctly folded procollagen molecule is then secreted into the extracellular space. (For reviews, see Kielty *et al.* 1993, Kivirikko 1993, Prockop & Kivirikko 1995 and Myllyharju & Kivirikko 2001).

Extracellulary, a soluble procollagen is enzymatically processed to an insoluble collagen by the cleaving of the N- and C-propeptides by specific proteinases. This is the requirement for normal fibril formation. In contrast, non-fibrillar collagens, such as type IX, retain the C- and N-terminal noncollagenous domains in the mature molecule. Collagen molecules are then aggregated into fibrils and covalent crosslinks between the collagen molecules are formed. The crosslinks are formed either by disulphide bonds between cysteine residues or by lysyl or hydroxylysyl aldehydes formed in the reaction catalyzed by lysyl oxidase. (See Kielty *et al.* 1993, Kivirikko *et al.* 1993, Prockop & Kivirikko 1995 and Myllyharju & Kivirikko 2001).

2.2.1.2 Collagen II

Collagen II belongs to the class of fibrillar collagens. In adult tissues, it is found in cartilage, the vitreous body, intervertebral discs, and the inner ear. Collagen II is a homotrimer consisting of three identical α1(II) chains encoded by *COL2A1*. The gene consists of 54 exons and is about 31 kb in size (Ala-Kokko & Prockop 1990, Ala-Kokko *et al.* 1995). The chromosomal location of the human *COL2A1* gene is 12q13.11-q13.12 (Takahashi *et al.* 1990). The 5' end includes the sequence coding for an alternatively spliced exon 2A which codes for a 69-amino acid cysteine-rich domain in the N-propeptide of collagen II (Ryan & Sandell 1990). These forms have a distinct tissue distribution during chondrogenesis. The molecule including the cysteine-rich domain (IIA) is predominant in the prechondrogenic mesenchyme and differentiating chondrocytes, while the molecule excluding this domain (IIB) is expressed in differentiated chondrocytes (Nah & Upholt 1991, Sandell *et al.* 1991).

Defects in the collagen II sequence are known to cause a variety of cartilage diseases, such as hypochondrogenesis, spondyloepiphyseal dysplasia, and Stickler syndrome (Lee

et al. 1989, Vissing *et al.* 1989, Ala-Kokko *et al.* 1990, Ahmad *et al.* 1991, Vikkula *et al.* 1994).

2.2.1.3 Collagen IX

Collagen IX belongs to the fibril-associated collagens with interrupted triple helices (FACIT) family (Shaw & Olsen 1991). It is present in cartilage and ocular tissues. The collagen IX molecule is a heterotrimer of three genetically distinct α chains, α1, α2 and α3. Each of the three α chains consists of three collagenous domains (COL1, COL2 and COL3) that are flanked by four noncollagenous domains (NC1, NC2, NC3 and NC4). The central triple-helical domain is anchored to the surface of the type II collagen fibrils. The NC3 domain functions as a hinge, allowing the COL3 and NC4 domains to project away from the fibril surface (McCormick *et al.*1987, Bruckner *et al*. 1988, Vaughan *et al*. 1988). The molecule is covalently bound to the surface of collagen II fibrils in an antiparallel orientation. Covalent bonds are formed between the N-terminus of COL2 in all three chains of the collagen IX molecule and the N-telopeptide of collagen II molecules, as well as from an interior site in the COL2 domain of the α3(IX) to the α1(II) C-telopeptide (Eyre *et al*. 1987, Wu *et al*. 1992, Diab *et al*. 1996). In addition, it has been demonstrated that collagen type IX molecules are covalently crosslinked to other type IX molecules by bonds between the COL2 domains of α1(IX) or α3(IX) and the C-terminal NC1 domain of α3(IX) (Wu *et al*. 1992, Diab *et al*. 1996, Wu & Eyre 2003).

The α chains of collagen IX are encoded by the *COL9A1*, *COL9A2* and *COL9A3* genes. The chromosomal locations of *COL9A1*, *COL9A2* and *COL9A3* are 6q13, 1p33-p32.2, and 20q13.3, respectively (Warman *et al*. 1993, Warman *et al.* 1994, Tiller *et al.* 1998). The *COL9A2* and *COL9A3* genes consist of 32 exons, while the *COL9A1* gene consists of 38 exons (Pihlajamaa *et al*. 1998, Paassilta *et al*. 1999b). The six additional exons of the *COL9A1* gene code for the 243-amino acid globular NC4 domain that is formed only by the α1(IX) chain. The size of the *COL9A1* gene is about 90 kb, while the *COL9A2* and *COL9A3* genes are about 15 and 23 kb, respectively (Pihlajamaa *et al*. 1998, Paassilta *et al*. 1999b).

Collagen IX is also a proteoglycan. It has one chondroitin sulphate glycosaminoglycan side chain covalently linked to a serine residue in the attachment site, Gly-Ser-Ala-Asp, located in the α2(IX) NC3 domain (Bruckner *et al.* 1985, Huber *et al.* 1986, McCormick *et al.* 1987). However, collagen IX lacking an attached glycosaminoglycan along with the proteoglycan form are synthesized by human, bovine, and chicken cartilage in organ culture (Bruckner *et al.* 1988, Ayad *et al.* 1991). Two mRNA transcripts, a long and short form, of α1(IX) have been identified (Muragaki *et al.* 1990). In the ocular and embryonic tissues, collagen IX occurs in a form with a short α1(IX) chain lacking nearly all of the N-terminal globular domain. This short α1(IX) chain is transcribed from an alternative promoter located between exons 6 and 7 of the α1(IX) gene (Nishimura *et al*. 1989). The long form is expressed in hyaline cartilage and includes the globular N-terminal NC4 domain. A study shows that nucleus pulposus contains exclusively the short form of α1(IX). (Wu & Eyre 2003.)

The function of collagen IX is not well known. Based on the location of the collagen IX molecules on the surface of collagen II and the projection of the COL3 and NC4 domains away from the surface, collagen IX is thought to play a role in the intermolecular organization of cartilage and serve as a bridge between collagens and noncollagenous proteins in tissues. Moreover, animal (Nakata *et al.* 1993, Fässler *et al.* 1994, Hagg *et al.* 1998) and human studies (Muragaki *et al.* 1996, Holden *et al.* 1999, Paassilta *et al.* 1999a, Bönnemann *et al.* 2000, Lohiniva *et al.* 2000, Spayde *et al.* 2000, Czarny-Ratajczak *et al.* 2001, Annunen *et al.* 1999a, Paassilta *et al.* 2001) have provided evidence of collagen IX in the maintenance of tissue integrity.

2.2.1.4 Collagen XI

Collagen XI is a heterotrimer composed of the α1(XI), α2(XI), and α3(XI) chains (Burgeson & Hollister 1979, Morris & Bächinger 1987). The corresponding genes, *COL11A1*, *COL11A2*, and *COL2A1*, are located on chromosomes 1p21, 6p21.2, and 12q13.11-q13.12, respectively (Henry *et al.* 1988, Kimura *et al.* 1989, Takahashi *et al.* 1990). The α3(XI) chain is an overglycosylated variant of the α1(II) chain and is therefore encoded by the *COL2A1* gene (Eyre & Wu 1987). Collagen XI is expressed in cartilage, the ocular vitreous body, inner ear, and nucleus pulposus (Eyre & Wu 1987, Brewton & Mayne 1994).

Collagen XI, a fibril-forming collagen, is synthesized as a precursor procollagen molecule with N and C-terminal extensions. Unlike the major fibrillar collagens, collagen XI does not undergo complete cleavage of the N-terminal propeptide. Therefore, a significant globular N-terminal region remains uncleaved (Morris & Bächinger 1987). Collagen XI polymerizes with collagen II (Van der Rest & Bruckner 1993) in a way that the collagen XI molecules are located inside the collagen II fibrils (Mendler *et al.* 1989). There is evidence that collagen XI regulates the fibril diameter (Blaschke *et al.* 2000, Hansen & Bruckner 2003). This theory is supported by findings that collagen fibrils in human and mouse with collagen XI defects were found to be abnormally thick and disorganized (Seegmiller *et al.* 1971, Li Y *et al.* 1995, van Steensel *et al.* 1997, Li *et al.* 2001).

COL11A1 and *COL11A2* are composed of 68 and 66 exons, respectively (Vuoristo *et al.* 1995, Lui *et al.* 1996a, Annunen *et al.* 1999b). Both the α1(XI) and α2(XI) chains undergo alternative splicing in the N-terminal propeptide. Alternative splicing has been detected in two of the α1(XI) chain exons, IIA and IIB. IIA encodes for highly acidic 39 amino acids, whereas IIB encodes 49 amino acids and is highly basic. Transcripts were found to contain either exon IIA, IIB, or neither of them. (Zhidkova *et al.* 1995.) Alternative splicing of the α2(XI) chain includes exons 6, 7, and 8, which code for part of the acidic subdomain. The isoform including exons 6-8 has been found in non-chondrogenic tissues, whereas the isoform lacking these exons is predominant in cartilage. Human fetal tissues have been shown to express several splice variants of the α2(XI) mRNA. (Tsumaki & Kimura 1996, Lui *et al.* 1996b.)

2.2.2 Aggrecan

Aggrecan (*AGC1*), a large proteoglycan, is one of the major structural components of the nucleus pulposus, binding to hyaluronan and link protein to form huge aggregates (Doege *et al.* 1991, Hardingham & Fosang 1992, Roughley & Lee 1994). The *AGC1* gene is located on chromosome 15q26.1 (Korenberg *et al.* 1993). *AGC1* consists of a large core protein, to which about 30 keratan sulphate (KS) chains and over 100 chondroitin sulphate (CS) chains are attached, forming a "bottle brush" structure. The KS domain includes a hexameric sequence that is repeated 11 times, whereas the serine-glycine-containing CS domain includes a sequence of 19 amino acids reiterated 19 times (Doege *et al.* 1991). The molecule is flanked by two globular domains in the N-terminus (GI and G2) and one in the C-terminus (G3). The G1 domain at the N-terminal end of the molecule binds to hyaluronan. This interaction is stabilized by link protein. The strong negative charge of the side chains draws water from the surrounding areas and therefore, *AGC1* is responsible for the high water content of the disc, and plays a critical role in its load carrying function. (Doege *et al.* 1991, Hardingham & Fosang 1995).

2.3 Intervertebral disc disease

2.3.1 General

The specific factors causing intervertebral disc disease (IDD) remain unknown. Abnormalities in the annulus fibrosus are known to be the initiating factors in the degenerative process that results in disc herniation. The annulus fibrosus generally expands at the expense of the nucleus pulposus. Degeneration begins early in life and is the consequence of a variety of factors, as well as normal ageing (Buckwalter 1995). Herniation of the nucleus pulposus also often leads to a decrease in the disc height and disc degeneration (Matsui *et al.* 1998). On the other hand, disc degeneration can occur without herniation (Buckwalter 1995). Disc degeneration is considered as one of the underlying factors for low-back pain (Salminen *et al.* 1999). However, disc degeneration can also be seen in asymptomatic individuals (Borenstein *et al.* 2001).

IDD is a common musculoskeletal disorder and about 6% of the populations' disability has been estimated to be caused by this disease (Heliövaara *et al.* 1987, Frymoyer 1992). IDD characterized by intervertebral disc herniations, which cause sciatica, a radiating pain along either leg, is also referred to as lumbar disc syndrome or lumbar disc disease (LDD) (Heliövaara 1987). IDD characterized by sciatica can occur before the age of 20, but it is most likely to occur in the fourth and fifth decades of life (Frymoyer 1988, Heliövaara 1989, Ala-Kokko 2002). Lifting heavy loads, torsional stress and motor vehicle driving have been identified as risk factors for IDD. There is, however, evidence from family and twin studies that genetic factors also have an influence in IDD (Varlotta *et al.* 1991, Matsui *et al.* 1992, Battié *et al.* 1995a, Battié *et al.* 1995b, Sambrook *et al.*

1999). In addition, studies have shown that tryptophan alleles in α2(IX) and α3(IX) can predispose to IDD (Annunen *et al.* 1999a, Paassilta *et al.* 2001).

2.3.2 Symptoms and radiological findings

When the nucleus herniates, the adjacent nerve root may be compressed. Sciatica, a myotomal pain radiating along either leg, is the most charasteristic symptom of a herniated intervertebral disc and can be caused by either mechanical compression of the nerve root or as a result from an inflammatory irritation of it (Saal *et al.* 1990, Kang *et al.* 1996, Takahashi *et al.* 1996, Brisby *et al.* 2002). In some rare cases, sciatica can also be caused by tumors, infections or arteriovenous malformation, but its most common cause is disc herniation.

MRI and computed tomography (CT) are noninvasive imaging methods used to study IVD herniations and other abnormalities. However, only MRI provides information on the physicochemical changes occuring with age or a degenerating disc before alteration of the disc contour. The clinical significance of these methods can be determined only by correlating the radiological findings with the person´s medical history, physical examination, and symptoms. Studies show that even in the case of a strong presumptive clinical diagnosis of a lumbar disc herniation, not all patients have a herniated disc in the radiological examinations (Gallucci *et al.* 1995, Modic *et al.* 1995). In contrast, disc abnormalities are common in asymptomatic subjects (Boden *et al.* 1990, Boos *et al.* 1995) and therefore, a correlation between the radiological and clinical findings is essential in determining the accurate diagnosis. In addition, the degree of the disc displacement does not correlate with the severity of the subjective symptoms among sciatic patients (Karppinen *et al.* 2001a), nor do the findings from MRI predict the development or duration of the low back pain (Borenstein *et al.* 2001).

In a recent study on rats, no changes in spontaneous behavior were seen after slight mechanical deformation or experimental disc incision, leading to herniation. However, the combination of these two caused increased focal pain (Olmarker *et al.* 2002). In contrast, another study indicated that disc disruption passing into the outer layers of the annulus without deformation of the outer wall, is as frequently associated with lower extremity pain as are discs with more severe disruption deforming the outer annular wall (Ohnmeiss *et al.* 1997). Mechanical failure of the annulus can be seen as high-intensity zonal (HIZ) lesions or as radial tears (Aprill & Bogduk 1992).

2.3.3 Environmental and constitutional factors

Several environmental and constitutional factors have been implicated to play a role in sciatica, low back pain, and disc degeneration.

Frequent lifting, postural stress, and occupational factors such as truck driving or physical and psychosocial demands have been identified as risk factors for disc degeneration, low back pain, and sciatica (Frymoyer *et al.* 1980, Damkot *et al.* 1984, Heliövaara 1989, Manninen *et al.* 1995, Hartvigsen *et al.* 2001, Kerr *et al.* 2001). There

are studies, however, in which occupational driving was not found to be a risk factor for disc degeneration (Battié *et al.* 2002).

Smoking has been shown to be a possible risk factor for sciatica (Kelsey *et al.* 1984, An *et al.* 1994), disc degeneration (Frymoyer 1988, Battié *et al.* 1991), and low back pain (Frymoyer *et al.* 1980, Heliövaara *et al.* 1991). In addition, the relative risk for tall men (>179 cm) was 2.3 and for tall women (>169) 3.7, in developing a disc herniation compared to those at least 10 cm shorter (Heliövaara 1987). Men seem to be at greater risk for lumbar disc herniation than women (Heliövaara *et al.* 1987). A significant association has also been indicated between atheromatous lesions in the abdominal aorta and low back pain (Kurunlahti *et al.* 1999). Large waist circumference was found to increase the risk for both low back pain and intervertebral disc herniation (Lean *et al.* 1998). Psychological factors such as anxiety and depression are more frequent in subjects with low back pain than in those who are asymptomatic (Frymoyer *et al.* 1980, Heliövaara 1989). However, conflicting results have been reported regarding the association of some environmental and constitutional factors with IDD.

2.3.4 Intervertebral disc degeneration

2.3.4.1 Age-related changes

With increasing age, the discs undergo changes in structure, composition, and volume (Buckwalter 1995). The degenerative changes include fissures in the annulus fibrosus and loss of water in the nucleus pulposus (Hormel & Eyre 1991, Buckwalter 1995, Urban & Roberts 1995).

Most of the changes occur in the nucleus pulposus where the amount of viable cells and the concentration of proteoglycans and water decrease. Mechanisms of the age-related changes are thought to include a decline in nutrition, a decreasing concentration of viable cells, cell senescence, the posttranslational modification of matrix proteins, accumulation of degraded matrix macromolecules, and fatigue of the matrix (Buckwalter 1995). Studies show that the biosynthesis of proteoglycans by the nucleus pulposus of the adult disc increases progressively toward the sacrum (Taylor *et al.* 2000). A recent study shows histologic evidence of the detrimental effect of a diminished blood supply on the end plate, resulting in tissue breakdown beginning in the nucleus pulposus and starting in the second decade of life (Boos *et al.* 2002). MRI studies reveal both an increase in signal density (Sether *et al.* 1990) and a decrease in disc height (Battié *et al.* 1995b, Pfirrmann *et al.* 2001) with increasing age. Macroscopically the disc tissue gradually changes in color from white in the young to yellow brown in the elderly (Hormel & Eyre 1991). With age, all discs develop similar age-related changes, but the rate and extent varies within the same person and among people. Several studies have shown, however, that genetic factors also play a role in the development of disc degeneration in humans (Battié *et al.* 1995a, Battié *et al.* 1995b, Harris *et al.* 1997, Matsui *et al.* 1998, Videman *et al.* 1998, Kawaguchi *et al.* 1999, Sambrook *et al.* 1999, Takahashi *et al.* 2001).

2.3.4.2 Radiological classifications

Plain radiography is an uncertain method to detect changes in the IVDs. Radiography only provides information about the disc height and the end plates. However, no other abnormalities such as IVD herniations can be seen in plain radiography. MRI is a non-invasive, reliable and widely used method to study changes in the IVDs. Disc degeneration can be seen as a decrease in disc height and a loss of signal on T2-weighted images (Modic *et al.* 1988).

Several grading systems have been developed for disc degeneration. Probably the most used is the Schneiderman's classification. In this classification, grade 0 indicates no signal change, grade 1, a slight decrease in signal intensity in the nucleus pulposus, grade 2, a generalized hypointense nucleus and grade 3, a hypointense nucleus with disc space narrowing. (Schneiderman *et al.* 1987.) Another, recently developed grading system for IVD degeneration is based on the distinction between nucleus and annulus, disc height, signal intensity, and disc structure. A homogenous bright white structure is classified as grade I (normal), whereas a homogenous white structure with possible horizontal bands is grade II. A nonhomogenous structure with no horizontal bands but with a distinction between the annulus and nucleus is classified as a grade III disc. If, in addition to grade III, the disc has no distinction between the annulus and nucleus, it is graded as grade IV. A disc is classified as grade V when, in addition to grade IV, the disc space has collapsed. (Pfirrmann *et al.* 2001.)

Several studies indicate that radial tears in the IVDs precede disc degeneration and cause sciatic pain (Yu *et al.* 1988, Osti *et al.* 1992, Ohnmeiss *et al.* 1997). In one study, discoloration of the nucleus pulposus, dimished disc height, and decreased signal intensity were found to be associated with a radial tear of the annulus fibrosus (Yu *et al.* 1989a). Furthermore, a study on rabbits showed that an annular laceration of an IVD results in disc degeneration (Anderson *et al.* 2002).

A positive correlation has also been found between end plate degeneration and disc degeneration (Kokkonen *et al*. 2002). Contrary to earlier findings (Nguyen-Minh *et al*. 1997), a recent study shows evidence of increased solute transport in the mildly degenerated disc (Bydder 2002). IVD herniations through the end plate, Schmorl's nodes, are defined as areas of end plate irregularities (Takahashi *et al.* 1995). It is hypothesed that end plate degeneration predisposes to Schmorl's nodes.

2.3.5 Spinal stenosis

Spinal stenosis is defined as the narrowing of the spinal canal, nerve root canals, or intervertebral foramina. It can be congenital or acquired (Arnoldi *et al.* 1976).

Symptoms can be unilateral or bilateral and typically include pseudoclaudication, low back pain, numbness, weakness, and pain on extension of the lumbar spine, and are generally releaved by flexing on the lumbosacral spine. The correlation of radiological with clinical findings is crucial for establishing the correct diagnosis. (Hall *et al.* 1985, Spengler 1987, Porter 1996, Spivak 1998.)

Congenital stenosis is due to an idiopathic developmental narrowing of the spinal canal. It is typically associated with hypochondroplasia or achondroplasia and genetic factors are known to cause this rare disease. (Wynne-Davies *et al.* 1981, Francomano & Muenke 2002.) While congenital spinal stenosis is rare, degenerative stenosis is one of the most common clinically important spinal disorders in the ageing population (Spivak 1998). Lumbar spinal stenosis (LSS) usually affects individuals over 50 years of age with a prevalence from 1.7% to 10% (Casey *et al.* 2001, Sheehan *et al.* 2001). Degenerative stenosis is usually caused by IVD herniation or degenerative changes in the IVD facet joints and ligamentum flavum, but can also be caused by spondylolisthesis, tumors, Paget's disease, or ossification of the posterior longitudinal ligament (OPLL) of the spine (Arnoldi *et al.* 1976, Matsunaga *et al.* 1997, Koga 1998, Spivak 1998).

2.4 Genetic factors in IDD

2.4.1 Family and twin studies

The results of several studies have suggested that IDD, including either disc degeneration, disc herniation or sciatica or a combination of these, has a strong genetic background.

Twin studies suggest that genetic influence explains up to 77% and heavy leisure time physical load only 2% of the variability in disc degeneration for the lower lumbar region (Battié *et al.* 1995a, Battié *et al.* 1995b, Sambrook *et al.* 1999). Developing a disc herniation before the age of twenty-one was found to be approximately five times greater in patients who had a positive family history compared to those with a negative family history (Varlotta *et al.* 1991). Genetic predisposition to an early-onset disc herniation has also been shown in other studies, in contrast to environmental factors which were not found to play a significant role in the development of the disease during young age (Matsui *et al.* 1992, Scapinelli 1993). Furthermore, family studies demonstrate the role of genetic factors in degenerative disc disease (Simmons *et al.* 1996, Matsui *et al.* 1998).

2.4.2 Collagen IX tryptophan alleles

A study indicates that a heterozygous substitution of Trp for either Gln326 or Arg326 in the COL2 domain in the α2(IX) chain (Trp2 allele) is associated with IDD characterized by sciatica in the Finnish population (Annunen *et al.* 1999a). The Trp2 allele was found in six of the 157 probands, but in none of the 174 controls. In addition, closer studies of the families of four original probands revealed that all family members who had inherited the allele had IDD characterized by intervertebral disc herniations. These findings are further supported by another study where the individuals with the Trp2 allele were found to be at greater risk for developing an IVD herniation compared to controls (Wrocklage *et al.* 2000). In addition, individuals with the Trp2 allele were found to be more flexible and

more often tended to have a radial tear in a nonherniated disc than the controls (Karppinen *et al.* 2002).

Yet another Trp allele, Trp3, has been found to increase individual's risk for IDD (Paassilta *et al.* 2001). The Arg103 to Trp substitution that is located in the COL3 domain of the α3(IX) chain was found in about 24% of the Finnish patients, but only in 9% of the Finnish controls. These results indicated that Trp3 does not itself cause the disease, but is a common risk factor for it. In a study on Southern European patients with IDD, 8.6% of the patients were found to have at least one Trp3 allele when compared to 4.6% in controls (Kales *et al.* 2004). None of the individuals in this study had the Trp2 allele. Results from a radiological study showed that the Trp3 allele is associated with Scheuermann's disease and intervertebral disc degeneration (Karppinen *et al.* 2003a). Scheuermann's disease is a condition affecting either the thoracic or both thoracic and lumbar spine. The findings include an increase in thoracic kyphosis, the wedging of the vertebral bodies, and Schmorl's nodes (Lowe 1990). Individuals with the Trp3 allele have also been found to have an increased risk for disc degeneration if they were persistently obese (Solovieva *et al.* 2002).

The amino acid tryptophan is rarely found in collagenous domains, and there are no Trp residues in the collagenous domains of human or mouse collagen IX (Muragaki *et al.* 1990, Perälä *et al.* 1993, Brewton *et al.*1995, Perälä *et al.* 1994, Rokos *et al.* 1994). There are several mechanisms by which the Trp substitution could affect to the disease. Being the most hydrophobic amino acid, Trp may influence on the formation or the stability of the collagen triple helix. Moreover, it could affect the interactions between collagens IX and II or inhibit the action of lysyl oxidase, which catalyzes crosslink formation (Kielty *et al.* 1993). The Trp2 substitution is located only three amino acid residues from the covalent crosslink between the α3(IX) chain and collagen II. A recent study has indicated that the Trp residues do not affect the assembly or amount of collagen IX produced in embryonic and fetal human cartilage. Both Trp2 and Trp3 allelic products were incorporated into the crosslinked fibrillar network and the cartilage appeared normal. Any pathological consequences are therefore likely to be long-term and indirect rather than from misassembly of the matrix (Matsui *et al.* 2003). However, similar analyses have not been done on intervertebral disc tissue.

2.4.3 AGC1 polymorphism

A human-specific variable number of tandem repeat (VNTR) polymorphism in the CS-domain has been reported (Doege *et al.* 1997). Thirteen alleles have been identified with the repeat numbers ranging from 13 to 33. Moreover, an association between this polymorphism and lumbar disc degeneration has been implicated (Kawaguchi *et al.* 1999). Findings showed an overrepresentation of alleles with a small number of repeats in subjects with multilevel disc degeneration, suggesting an association between this polymorphism and disc degeneration. However, no association was found between the number of alleles and disc herniation. These results imply that the aggrecan molecules with a lesser number of CS chains have a poorer capacity for hydrating the disc and will thus predispose to degeneration.

2.4.4 VDR polymorphisms

Two intragenic polymorphisms, T to C in exon 9 (*Taq*I) and T to C at the translation initiation codon (*Fok*I) in the vitamin D receptor (*VDR*) gene, have been implicated to be associated with disc degeneration.

A *Taq*I polymorphism at position 352 from the start of the translation has been found to be associated with low bone mineral density, early osteoarthritis (OA) of the knee, lumbar disc degeneration, annular tears, and disc herniation (Spector *et al.* 1995, Keen *et al.* 1997, Jones *et al.* 1998, Videman *et al.* 1998, Videman *et al.* 2001, Kawaguchi *et al.* 2002). Another study, however, showed no association between bone mineral density and this polymorphism (Hustmyer *et al.* 1994).

Another polymorphism, *Fok*I, at position 2 from the start of translation in the *VDR* gene, has been shown to be associated with disc degeneration, bulging, and disc height (Videman *et al.* 1998). Allele 'F' indicates the presence of nucleotide C and 'f' indicates the presence of nucleotide T. The presence of nucleotide C results in the absence of the first translation initiation codon and the use of the second methionine codon located three amino acids downstream (Gross *et al.* 1996, Harris *et al.* 1997). Disc degeneration was found to be more severe in those with the ff genotype, compared to those with the FF genotype.

2.4.5 Matrix metalloproteinases

The majority of extracellular matrix degradation is mediated by matrix metalloproteinases (MMPs) and therefore, they have been suggested to have an important role in disc degeneration.

Eighteen MMPs have been identified. Human intervertebral discs have been shown to contain at least MMPs 1, 2, 3, 7, 8, 9, and 13 (Roberts *et al.* 2000). MMPs 1, 8, and 13 are able to cleave the triple-helical part of the fibrillar collagens I, II, and III. MMP-3 (stromelysin-1) has the ability to degrade proteoglycans and collagens II and IX. It also recruits macrophages that activate other MMPs. Therefore, a catabolic cascade can be generated from the activation of perhaps only one enzyme. Osteoarthritic cartilage has been shown to contain elevated levels of MMP-3 compared to normal cartilage. (Goupille *et al.* 1998.)

Studies show that in prolapsed disc, there may be an excess of, or abnormal forms of, degradative enzymes, leading to the weakening of the annulus fibrosus and susceptibility to posterior or posterolateral prolapse (Goupille *et al.* 1998). In addition, an annular laceration to an IVD caused a marked upregulation of MMPs 1, 9, and 13 in rats (Anderson *et al.* 2002).

A study of the in vivo production of MMPs by herniated lumbar IVD tissue from the patients undergoing surgery for persistent radicular pain, in comparison with IVDs of the patients undergoing surgery for scoliosis and traumatic burst fractures, showed increased levels of MMP activity in the patients with IVD herniation (Kang *et al*. 1996). A common 5a/6a polymorphism in the promoter region of the human *MMP-3* gene has been identified (Ye *et al*. 1995) and it was reported to be an important regulator of *MMP-3*

gene expression, with the 5a allele having twice as much promoter activity as the 6a allele (Ye *et al*. 1996). A study indicated that elderly individuals having the 5a/5a or 5a/6a genotype are at greater risk of IVD degeneration than those with the 6a/6a genotype (Takahashi *et al.* 2001).

2.4.6 Collagens XI and VI in LSS

Even though no mutations have been reported in degenerative LSS, there is some evidence that genetic factors may contribute to it. Varughese and Quartey reported four brothers with lumbar disc herniation associated with narrowing of the spinal canal, possibly implicating genetic factors in this disorder as well (Varughese & Quartey 1979). In addition, genetic factors have been indicated in OPLL, which is a leading cause of spinal stenosis in the Japanese population (Koga *et al.* 1998, Maeda *et al.* 2001).

A linkage between OPLL and the HLA locus on chromosome 6p was found in the Japanese patients. This locus contains two candidate genes, *COL11A2* and the retinoid X receptor β (*RXRβ*), which are possibly involved in bone formation. Screening of the candidate genes for mutations did not identify any disease-causing mutations, but provided evidence of an association between OPLL and the *COL11A2* IVS6^{-4} t allele. The frequency of the IVS6^{-4} t allele was found to be 86% in probands compared with 74% in controls, suggesting that the t allele may predispose to the disease. The IVS6^{-4} a allele is associated with a different splice pattern from the t allele, with exon 6 being skipped in its presence. The region containing exons from 6 to 8 is an acidic subdomain presumably exposed to the surface and is thought to interact with other extracellular proteins. Therefore, the removal of exon 6 may have functional consequences that affect the putative interactions between collagen XI and other extracellular matrix proteins. (Koga *et al.* 1998, Maeda *et al.* 2001.) A recent study indicates *COL6A1* on chromosome 21 as the locus for OPLL. Haplotype analysis with three SNPs in *COL6A1* identified a p-value of 0.0000007 (Tanaka *et al.* 2003).

2.5 Animal models

2.5.1 Collagen II animal models

To study the role of collagen II in tissues, two transgenic mouse lines with deletions in the triple-helical domain and two with glycine substitutions have been generated. The phenotypes caused by these mutations are usually perinatally lethal chondrodysplasias characterized by dwarfism, a short snout, cleft palate, disorganized growth plates, and delayed mineralization of bone. (Vandenberg *et al.* 1991, Garofalo *et al.* 1991, Metsäranta *et al.* 1992, Savontaus *et al.* 1997.)

Transgenic mice that lacked a large central region of the *Col2a1* gene containing 12 of the 52 exons developed a phenotype characterized by chondrodysplasia with dwarfism,

short and thick limbs, a short snout, a cranial bulge, cleft palate, and delayed mineralization of bone (Vanderberg et al. 1991). In the microscopic examination of the cartilage, the organization and density of the collagen fibrils were found to be poorer when compared to the wild type mice. Comparison of mice ranging in age from 1 day to 15 months revealed that the evidence of chondrodysplasia decreased with age. Instead, the older mice were found to develop degenerative changes in the articular cartilage, resembling OA (Helminen et al. 1993).

Mouse models with both overexpression and total inactivation of collagen II have been generated. When collagen II was overexpressed, the mice died at birth but did not exhibit cleft palate, abnormal cranial features, or other skeletal deformities. However, the length of the long bones was slightly decreased and microscopically the cartilage fibers were abnormally thick. This led to the discussion of whether the imbalance in the amount of collagen XI disrupted the mechanism that controls the diameter of the collagen II fibrils. (Garofalo *et al.* 1993.) In contrast, total inactivation of the *Col2a1* gene led to severe perinatally lethal chondrodysplasia (Li SW *et al.* 1995). At the age of one month, the heterozygous mice were characterized by shorter limbs, skulls, and spines. In addition, they were found to have more irregular vertebral end plates with disturbed calcification. The glycosaminoglycan concentration in the annulus fibrosus, end plates, and vertebral bone was also lower than in the controls. The changes, except the length of the bones, were compensated by the age of 15 months. (Sahlman *et al.* 2001.) It is not currently known if collagen II plays a role in human disc disease. However, findings from the animal studies suggest that collagen II acts as an important factor in cartilage and bone formation.

2.5.2 Collagen IX animal models

The effect of the mutations in collagen IX has been studied with the help of animal models. Three mouse lines harboring a collagen IX mutation have been generated. Mice expressing a truncated α1(IX) chain developed OA which was the most severe in the knee joints. In addition, the mice homozygous for the mutation showed early onset progressive OA associated with mild dwarfism, spine involvement, and eye abnormalities. Fibrils in transgenic mice were found to be thinner than those in controls (Nakata *et al.* 1993). During a long-term follow-up, the transgenic mice were found to develop intervertebral disc degeneration which was more advanced than the degeneration seen in the controls. In addition, fissures in the annulus and IVD herniations were seen in the transgenic mice (Kimura *et al.* 1996). Knockout mice lacking the entire α1(IX) chain have also been generated (Fässler *et al.* 1994). Homozygous mutant mice appeared normal at birth but developed a severe degenerative joint disease with age. A mouse line with overexpression of the NC4 domain induced osteoarthritis analogous to human OA (Haimes *et al.* 1995).

2.5.3 Collagen XI animal models

Two mouse lines harboring a collagen XI mutation have been reported. Autosomal recessive chondrodysplasia (cho) in mice is characterized by short limbs with abnormal metaphyses, a short snout, short mandible, protruding tongue, and cleft palate. They die perinatally probably because of soft tracheal rings. Microscopic examination of the cartilage revealed disorganization of the growth plates and abnormally thick collagen fibrils. (Seegmiller *et al.* 1971, Li Y *et al.* 1995.) Linkage between the cho locus and *Col11a1* was identified, and studies revealed a frame shift mutation 570 bp downstream of the translation initiation codon, causing a premature translation termination codon and the absence of the α1(XI) chain.

A knockout mouse line was generated by homologous recombination between exons 27 and 28 of the *Col11a2* gene (Li *et al.* 2001). Translation of the full-length *Col11a2* chain was unable to occur because of the presence of premature termination codons within the insertion. Homozygous mice lacking the α2(XI) chains had a smaller body size, receding snouts, and deafness. In addition, chondrocytes in growth plates of all long bones were disorganized. The phenotype was consistent with the phenotype of the human chondrodysplasia, otospondylomegaepiphyseal dysplasia (OSMED) (Vikkula *et al.* 1995, Melkoniemi *et al.* 2000). OSMED is a recessive disorder typically caused by loss of function mutations in the *COL11A2* gene (Melkoniemi *et al.* 2000).

2.5.4 Cmd mice

Mouse cartilage matrix deficiency (cmd) is an autosomal recessive disorder characterized by a short trunk, limbs, tail, and snout, as well as cleft palate (Rittenhouse *et al.* 1978). Homozygous mice die just after birth due to respiratory failure, while heterozygotes appear normal at birth.

Biochemical and immunofluorescent techniques on homozygous cmd mice revealed normal amounts of type II collagen, but reduced amounts of the cartilage proteoglycan, aggrecan (Kimata *et al.* 1981). DNA sequencing of *Agc1* identified a 7-bp deletion in exon 5 resulting in a severely truncated molecule (Watanabe *et al.* 1994). Even though the heterozygous mice appeared normal at birth, within 19 months of age they developed a spinal misalignment, leading to a spastic gait and death (Watanabe *et al.* 1997). Radiographs of the heterozygous mice identified a lordosis in the cervical spine and kyphosis in the thoraco lumbar spine. Heterozygous mice were found to live no longer than nineteen months, whereas all the wild-type mice included in the study lived for more than two years. Histological examination revealed a high incidence of herniation and degeneration of IVDs. In heterozygous mice, the disc herniations explain their spastic gait disturbance that results in death due to starvation. No pathological changes were seen in the facet joints, indicating that the primary lesion lies in the IVDs (Watanabe *et al.* 1997).

2.6 Inflammation and IDD

Herniated IVD tissue has been shown to produce a number of cytokines such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor α (TNF-α). IL-1 and TNF-α have been indicated as the predominant proinflammatory and catabolic cytokines involved in the initiation and progression of cartilage destruction. IL-1 and TNF-α are also potent stimulators of mesenchymal cells, such as osteoclasts and chondrocytes, that release tissue-destroying MMPs. In addition, they inhibit the production of tissue inhibitors of metalloproteinases (TIMPs) by synovial fibroblasts. (Choy & Panayi 2001, Goldring 2001.) Those inflammatory mediators that may play a role in IDD are presented in more detail below.

2.6.1 Inflammatory mediators

The interleukin 1 gene cluster on chromosome 2q14 contains three related genes within a 360 kb region, *IL1A*, *IL1B* and *IL1RN*, which encode the proinflammatory cytokines IL-1α and IL-1β as well as their receptor antagonist IL-1RA, respectively (Dinarello 1996). IL-1 is mostly produced by monocytes and magrophages but also by endothelial cells, B cells, and activated T cells. IL-1 is known to act in close relation with TNF-α. The blocking of TNF-α in culture diminished the amounts of IL-1, IL-6, and IL-8, while the blocking of IL-1 with IL-1RA blocked IL-6 and IL-8, but had no affect on TNF-α (Butler *et al.* 1995). This suggests that TNF-α may act through IL-1 (Feldmann *et al.* 2001). IL-1 is also known to stimulate the production of MMPs (Bunning *et al.* 1986) which are thought to play a role in IDD. Polymorphisms in IL-1B may be associated with gastric cancer, periodontitis, rheumatoid arthritis, and bowel disease (Eastgate *et al.* 1988, Cantagrel *et al.* 1999, Galbraith *et al.* 1999, Nemetz *et al.* 1999, El-Omar *et al.* 2000). Moreover, an association between the IL-1 gene cluster and OA has been implicated (Leppävuori *et al.* 1999, Moos *et al.* 2000, Loughlin *et al.* 2002), as well as between the IL-1RA VNTR polymorphism and inflammatory bowel disease (Vijgen *et al.* 2002). Recent studies suggest the association of low back pain and disc degeneration with the polymorphisms in the *IL1* locus (Solovieva *et al*. 2004a, Solovieva *et al*. 2004b).

TNF-α is a soluble 17-kd protein whose gene is located on chromosome 6p12.3. It is produced by monocytes, macrophages, B cells, T cells, and fibroblasts and is known to increase the amount of some interleukins such as IL-1 and IL-6. Polymorphisms in *TNFA* have been shown to be associated with several diseases that have an inflammatory component such as multiple sclerosis (MS), rheumatoid arthritis, helicobacter pylori-associated gastric ulcers, and asthma (Allcock *et al.* 1999, Chagani *et al.* 1999, Kunstmann *et al.* 1999, Ozen *et al.* 2002). Another cytokine, IL-6, is produced by T cells, monocytes, macrophages and fibroblasts, and one of its functions is the activation of T cells. IL-1α, TNF-α, and IL-6 have been shown to induce the production of prostaglandin E2, which causes pain or enhances sensitivity to other pain-producing substances (Ferreira *et al.* 1973, Dayer *et al.* 1985). A polymorphism in IL-6 may be associated with juvenile chronic arthritis (Fishman *et al.* 1998). IL-2 is an immunoregulatory cytokine produced by activated T cells. Polymorphisms in IL-2 and interleukin-4 receptor α (IL-

4RA) have been found to increase individual's risk for MS and atopy, respectively (Matesanz *et al.* 2001, Hershey *et al.* 1997).

While some cytokines initiate and maintain the inflammatory process, IL-10 and IL-4 have been shown to cooperate to inhibit the production of inflammatory mediators. IL-10 is produced by monocytes, macrophages, B cells, and T cells. It inhibits the production of several cytokines, including IL-1, IL-6, and TNF-α. However, it is thought that the increase in the amount of IL-10 may be insufficient to suppress inflammation in diseases with an inflammatory component (Katsikis *et al.* 1994). In addition, it has been reported that after tissue injury the amount of IL-1β, IL-6, and TNF-α increases more rapidly compared to IL-10 (Okamoto *et al.* 2001). The polymorphism in the IL-10 gene has been shown to be related to ulcerative colitis and MS. Moreover, an association with the IL-10 haplotype and juvenile rheumatoid arthritis has been reported. (Crawley *et al.* 1999, Bidwell *et al.* 2001.) IL-4 is produced by CD4+ type helper T cells and it inhibits the activation of type 1 helper T cells. It also decreases the production of IL-1, IL-6, and TNF-α and inhibits cartilage damage. In a study on patients with rheumatoid arthritis, IL-4 was found to inhibit the production of IL-1 and increase the expression of IL-1RA, both of which should decrease inflammation (Chomarat *et al.* 1995). An association between a rare allele of IL-4 VNTR polymorphism and protection against joint destruction in rheumatoid arthritis has been published (Buchs *et al.* 2000). IL-4 has been shown to downregulate collagenases and therefore protect cartilage collagen (Cawston *et al.* 1999).

Interferon gamma (*IFNG*) is a 34-kd peptide secreted by T-lymphocytes and by natural killer cells. It has a crucial role in the regulation of expression during the immune response. Several polymorphisms in the *INFG* gene have been indicated, and studies suggest that some of these polymorphisms are associated with different amounts of *IFNG* secretion (Pravica *et al.* 1999). A microsatellite polymorphism in the first intron of the *IFNG* gene has been shown to be associated with susceptibility to and the severity of rheumatoid arthritis (Khani-Hanjani *et al.* 2000).

2.6.2 Role of inflammatory mediators in IDD

Disc herniation causes pain by mechanical compression but also by chemical stimulation. Herniated IVD tissue has been shown to produce a number of proinflammatory cytokines (Saal *et al.* 1990, Kang *et al.* 1996, Takahashi *et al.* 1996, Kang *et al.* 1997, Ahn *et al.* 2002). Some patients with a large herniation have no radicular symptoms, whereas some patients with no evidence of disc herniation have severe radiculopathy (Halperin *et al.* 1982, Boden *et al.* 1990). Another study indicated that disc disruption passing into the outer layers of the annulus, without deformation of the outer wall, was as frequently associated with lower extremity pain as were discs with a more severe disruption deforming the outer annular wall (Ohnmeiss *et al.* 1997). Thus, it is possible that the inflammatory factors are the main mediators of pain sensation in cases without obvious mechanical compression of the sciatic nerve.

A study has been performed to determine if murine disc-derived cells are capable of secreting cytokines IL-1β, IL-6, TNF-α, and IL-10. The results concluded that cultured cells derived from intact murine disc have a trace or no basal secretion of IL-1β, but were capable of secreting significant amounts of IL-1β after stimulation with

lipopolysaccharide. In addition, the cells were found to have a basal secretion of IL-6 and IL-10 which was significantly increased after stimulation. (Rand *et al.* 1997.) It has also been shown that herniated IVDs spontaneously produce MMPs, nitric oxide (NO), IL-6, and prostaglandin E2 (Kang *et al.* 1996, Takahashi *et al.* 1996, Burke *et al.* 2002). Application of IL-1β, IL-6, or TNF onto the dorsal root of rats statistically decreased the neural activity after three hours of each cytokine application. Moreover, recent findings show that the application of nucleus pulposus to nerve roots or dorsal ganglion can induce an inflammatory irritation without mechanical compression (Takebayashi *et al.* 2001, Aoki *et al.* 2002). One study indicated that the concentration of *INFG* was higher in the herniated disc than in the contained disc (Park *et al.* 2002).

These findings demonstrate that these substances might contribute to the sensation of pain (Özaktay *et al.* 2002).

2.6.3 Anti-inflammatory treatment

Non-steroidal anti-inflammatory drugs (NSAIDs) are often used to treat individuals with low back pain and sciatica. The anti-inflammatory effect of NSAIDs acts through the inhibition of prostaglandin synthesis (Vane & Botting 1998). Prostaglandins are important in the regulation of inflammation, pain, and fever induction. It has been shown that herniated IVDs spontaneously produce prostaglandins (Kang *et al.* 1996). When treating acute low back pain, a statistically significant favor of NSAID over a placebo has been indicated by several studies (Szpalski & Hayez 1994).

Corticosteroids are known to decrease the amount of inflammation in tissues. A study has implicated that patients who underwent a surgery for IVD herniation and received a metyhylprednisolone treatment had a statistically shorter hospital stay compared to the individuals who were treated with a placebo or bupivacaine only (Glasser *et al.* 1993). In addition, it was reported that epidural corticosteroid injection continued to decrease the pain in some cases 3 and 6 months after the original treatment when compared to the control group (Karppinen *et al.* 2001b).

In another study, TNF-α inhibitors (etanercept and infliximab) prevented the reduction of nerve conduction velocity and seemed to limit nerve fiber injury and intraneural edema formation (Olmarker & Rydevik 2001). A recent study indicates interesting results for the treatment of sciatica with an anti-cytokine therapy. Ten patients with severe sciatica were treated with an intravenous infusion of a monoclonal antibody against TNF-α, infliximab, and compared to historical controls. Infliximab infusion produced a rapid decrease in subjective symptoms (Karppinen *et al.* 2003b.), the favorable effect being maintained over the one-year follow-up period (Korhonen *et al*. 2004). These results further support the role of inflammation in sciatica.

3 Outlines of the present research

IDD is a common disease, but its pathogenesis is not well understood. Constitutional and environmental factors, such as height, motor vehicle driving, and smoking, have been indicated as risk factors for the disease. However, several recent studies have suggested that genetic factors play an important role in IDD. IDD characterized by sciatica is caused by intervertebral disc herniation that leads to sciatica, a radiating pain along the leg. It is also thought that in addition to mechanical compression, inflammatory factors also play a role in the development of sciatica.

Degenerative spinal stenosis is common in the elderly and is usually caused by disc degeneration or herniation. Genetic factors in many genes, such as the vitamin D receptor, aggrecan, and matrix metalloproteinase 3, have been linked to disc degeneration. A mutation in *COL11A2* has been found to be associated with OPLL, which is the major cause for spinal stenosis in the Japanese population.

Collagen IX is a minor collagen expressed in cartilage and intervertebral discs. It has been shown to play a role in IDD in humans and mice. The Trp2 and Trp3 alleles in collagen IX are risk factors for IDD in the Finnish population. However, they have not been studied earlier in other populations.

Based on these earlier findings, it was hypothesized that there are additional genetic factors behind these two common diseases. The object of this research was to study the role of genetic factors in these spinal diseases. The aims of the study were:

1. to analyze patients with spinal stenosis for mutations in the genes coding for several intervertebral disc proteins,
2. to analyze the frequency of the Trp2 and Tp3 alleles in the Chinese population and correlate the genetic and spinal MRI findings, and
3. to study the role of genetic variations in the genes coding for several inflammatory mediators in IDD characterized by sciatica.

4 Materials and methods

4.1 Study subjects (I-III)

Twenty-nine unrelated Finnish patients (14 male and 15 female) of ages ranging from 42 to 72 years with symptoms consistent with LSS were included in the study.

The symptoms consisted of self-reported claudication, stenotic symptoms extending to the lower extremities upon extension of the lumbar spine, or numbness or weakness of the lower extremities. Clinical and radiological examinations were performed on all patients to confirm the degenerative nature of the disease. In addition, a blood sample was taken from all the patients for the isolation of genomic DNA. The control set consisted of 56 Finnish individuals of ages ranging from 22 to 72 years with no history of back problems. An additional control set for the *AGC1* VNTR polymorphism study consisted of 153 Finnish individuals from 40 to 45 years of age without a history of back problems.

Eight hundred four volunteers of Southern Chinese origin aged from 18 to 55 years were recruited from the general population with an open invitation. All study subjects underwent an MRI examination and blood sampling.

One hundred fifty-five unrelated Finnish individuals with a history of at least three weeks of unilateral discogenic sciatica radiating from the back to below the knee were included in the study. The study subjects were part of a randomized controlled trial of periradicular infiltration for sciatica (Karppinen *et al.* 2001b). The age of the patients ranged from 19 to 78 years. A blood sample was taken from all the study subjects and clinical and radiological examinations were performed. Study subjects were re-evaluated clinically and radiologically three years after the enrollment. The control group consisted of 179 Finnish individuals, university staff and students, aged from 20 to 69 years. No data were collected of their possible musculoskeletal symptoms.

4.2 Clinical and radiological examination (I-III)

The individuals with LSS were examined clinically and radiologically. The neurological examination consisted of an evaluation of the motor reflexes in the lower extremities. Peripheral pulses were examined to exclude vascular claudication. The MRI examinations performed with a 1.5 T scanner (Signa, GE Medical systems) consisted of T2-weighted sagittal fast spin echo images with a repetition time/echo time (TR/TE) of 4000/95 ms and a slice thickness of 4 mm. T1-weighted (580/15 ms) sagittal spin echo images and transaxial T2-weighted (6000/105 ms) fast spin echo images were taken, also with a slice thickness of 4 mm. The transaxial MRI images consisted of scans through the L2 to S1 interspaces, and the CT images (Hi Speed Advantage, GE Medical Systems) of scans through the same interspaces with a slice thickness of 4 mm. MRI scans were obtained for all the patients and CT scans for 25 of the 29 patients.

The MRI examinations for the Chinese study subjects were carried out at the Jockey Club MRI Engineering Center using a 0.2 T Profile open MRI system (General Electric Medical System, Milwaukee, WI). Sagittal T2-weighed Fast Spin Echo sequences (TR = 3000 ms, TE = 92 ms, slice thickness = 5 mm) were acquired with a built-in flexible body coil.

Clinical and radiological examinations were performed on the Finnish study subjects with sciatica. The clinical examination included the straight-leg-raising test, assessment of lumbar flexion by the modified Schober measure, tendon reflexes, and evaluation of motor and sensory deficits (Karppinen *et al.* 2001b). Patients reported their leg and back pain using a 100-mm visual analog scale (VAS), disability by the Oswestry scale, and quality of life by the Nottingham Health Profile (Karppinen *et al*. 2001a, Karppinen *et al*. 2001b). The MRI examinations were carried out through the L2-S1 interspaces with a 1.5 T scanner (Signa, GE Medical systems) to detect the IVD abnormalities. It consisted of sagittal images with a TR/TE of 4000/95 ms and axial images with a repetition time/echo time of 640/14 ms.

4.3 Radiological classification (I-II)

The degree of stenosis, degeneration, and end plate changes were evaluated from the MRI scans. Stenosis was graded as 0 (no stenosis), grade 1 (mild to moderate) if the sagittal diameter of the dural sac at the disc level was from 5 to 10 mm, and grade 2 (severe) if the diameter was < 5 mm. Degeneration was evaluated from the T2-weighted sagittal scans and classified as 0 (no signal changes), 1 (slight decrease in signal intensity in the nucleus), 2 (hypointense nucleus pulposus with normal disc height), and 3 (hypointense nucleus with disc space narrowing). End plate changes were graded according to the Modic's criteria (Modic *et al.* 1988). Ossification of the ligamentum flavum (OLF) was evaluated from the CT scans on the basis of (1) hyperdense calcified areas in the ligamentum and OPLL in terms of a local calcification on the CT and MRI scan and (2) a hypointense hypertrophic band at the posterior edge of the vertebral body on the T2-weighted sagittal MRI scan. All the MRI and CT scans were read by two experienced physicians blinded to the results of the genetic analysis and to the clinical

history and physical status of the patients. Those responsible for the clinical assessments were also blinded to the results of the genetic analysis.

DDD was diagnosed on the basis of signal changes in the intervertebral discs of the lumbar spine and graded using Schneiderman's classification (Schneiderman *et al.* 1987), in which grade 0 indicates no signal change, grade 1, a slight decrease in signal intensity in the nucleus pulposus, grade 2, a generalized hypointense nucleus, and grade 3, a hypointense nucleus with disc space narrowing. The presence of any disc herniations, annular tears, and end plate herniations (Schmorl's nodes) was also noted. Annular tears were identified as areas of high signal intensity within the posterior annulus surrounded by a dark rim, but no distinction was made between these and radial tears. Schmorl's nodes were defined as areas of end plate irregularities. (Aprill *et al.* 1992, Yu *et al.* 1989b, Takahashi *et al.* 1995.) All the MRI scans were analyzed by two experienced physicians blinded to the results of the genetic analysis and patients' clinical history. Any disagreements were settled by consensus. For association analysis, individuals with five grade-0 discs of the lumbar spine were classified as DDD- (normal), marking the absence of degeneration, and all others were classified as DDD+ subjects for the presence of degeneration. The DDD+ subjects were further subdivided into "moderate to severe" and "mild" groups. The "moderate to severe" group consisted of individuals with one or more levels of grade-3 disc(s) and individuals with two or more levels of grade-2 disc(s). All other DDD+ individuals were allocated to the "mild" group.

4.4 Screening for sequence variations in the genes coding for IVD proteins and inflammatory mediators (I-III)

Genomic DNA extracted from the blood samples was used as a template for PCR. The individuals with LSS were screened for sequence variations in the exons and exon boundaries of *COL1A1*, *COL1A2*, *COL2A1*, *COL9A1*, *COL9A2*, *COL9A3*, *COL11A1*, *COL11A2* and *AGC1*. The *COL9A1*, *COL9A2*, and *COL9A3* genes were analyzed in a subset of 100 randomly selected study subjects in a study of the Chinese population. Fourty-eight out of the 155 individuals with sciatica were screened for sequence variations in the genes coding for *IL1A*, *IL1B*, *IL6*, and *TNFA*. If a sequence variation was found, 48 control samples were analyzed for the variations. In case of a difference in the frequency of a sequence variation between the patients and the controls, the rest of the samples were analyzed for the presence of the variations. The primers were designed so that they flanked about 50 bp of both the 5' and 3' ends of the exons. (See Table 2.)

PCR amplifications were carried out in a volume of 20 µl containing 20 to 40 ng of genomic DNA, 5 to 10 pmol of each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 0.5-1.0 U Ampli*Taq* Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA). The conditions, after an initial denaturation at 95ºC for 10 min, were 34 cycles of 30 s at 95ºC, 30 s at 50 to 68ºC and 30 s at 72ºC, followed by a final extension at 72ºC for 5 min. The PCR products were denatured at 95ºC for 4 min, followed by annealing at 68ºC for 30 min to generate heteroduplexes for conformation sensitive gel electrophoresis (CSGE). An aliquot of 5 µl was used to check the concentration and quality of the PCR

products by agarose gel electrophoresis. From 0.1 to 0.5 µl of each PCR product was loaded on CSGE. CSGE can detect PCR products with heterozygous mutations.

CSGE was performed with a 1 mm gel in a standard DNA sequencing apparatus. The gel composition was 15% of 1,4-bis(acrolyol)piperazine (BAP) to acrylamide, 10% ethylene glycol, 15% formamide, 0.1% ammonium persulphate (APS) and 0.07% N,N,N',N'-tetramethylethylenediamine (TEMED) in 0.5 x TTE buffer (44mM Tris – 14.5 mM taurine – 0.1 mM EDTA, pH 9.0), according to previously published protocols (Ganguly *et al.* 1993, Ganguly & Prockop 1995, Körkkö *et al.* 1998). The electrophoresis was performed at 40 W for 8 hours. After the electrophoresis, the gels were stained with SYBR Gold nucleic acid gel stain (Molecular Probes, Eugene, USA) and photographed with a high quality charge-coupled device (CCD) camera (Fotodyne or UVP).

Table 2. Number of patients, radiological methods used and genes/polymorphisms analyzed in the studies.

Study number	Patients (n)	Radiological assessments	Genes analyzed	Polymorphisms analyzed
I	29	MRI, CT	*COL1A1, COL1A2, COL2A1, COL9A1, COL9A2, COL9A3, COL11A1, COL11A2, AGC1*	*MMP-3*: -1171Δa[a]; *VDR*: 352 T>C[b], 2 T>C
II	804	MRI	*COL9A1, COL9A2, COL9A3*	*COL9A2*: Trp2 (exon 19), E21^{+9} G>A, IVS27^{-61} c>a, IVS30^{+17} g>a; *COL9A3*: Trp3 (exon 5)
III	155	MRI	*IL1A, IL1B, IL6, TNFA*	*IL1A*: -889 c>t; *IL1B*: -31 t>c, IVS4^{-64} g>a, 3954 C>T; *IL1RN*: VNTR (IVS2); *IL2*: 114 G>T; *IL4*: VNTR (IVS3); *IL4R*: 1902 G>A; *IL6*: -597 g>a, -572 g>c, -174 g>c, 15 T>A (exon 5), 132 C>T (exon 5); *IL10*: -1082 g>a; *TNFA*: -308 g>a; *IFNG*: microsatellite

[a] From the start of transcription.

4.5 Analysis of the *VDR* and *MMP-3* polymorphisms (I)

Two previously identified polymorphisms in *VDR* and one in *MMP-3* were analyzed.

After PCR amplification, a *VDR* gene polymorphism at position 352 from the start of translation was analyzed by the *Taq*I restriction enzyme, and another polymorphism, T to C at the translation initiation codon of the *VDR* gene, by the *Fok*I restriction enzyme (Saijo *et al.* 1991, Morrison *et al.* 1994). A region of the *MMP-3* promoter containing a

5a/6a polymorphism was amplified by PCR (Ye *et al.* 1995) and the alleles were determined by sequencing.

4.6 RT-PCR (I)

Total RNA was extracted from the Epstein-Barr virus-transformed lymphoblasts of patient 23. The cDNA synthesis was carried out using the Superscript First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA), followed by amplification with two sets of primers. The first set of the primers corresponded to exons 20 and 30 of the *COL9A2* gene. The second primer pair was nested, and corresponded to exons 24 and 29. The amplification was carried out in a volume of 25 µl containing 1µl of the cDNA, 7.5 pmol of each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 1.5 U Ampli*Taq* Gold DNA polymerase. The conditions, after an initial denaturation at 95ºC for 12 min, were 34 cycles of 30 s at 95ºC, 30 s at 60ºC, and 30 s at 72ºC, followed by a final extension at 72ºC for 8 min. The products were analyzed on agarose gels and by sequencing.

4.7 Southern blotting (I)

A probe for the *AGC1* VNTR in exon 12 was generated by PCR as earlier described (Doege *et al.* 1997). The PCR was carried out with the Advantage –GC Genomic PCR kit (Clontech, Palo Alto, CA, USA) with 0.5 M of GC-melt mix. The conditions, after an initial denaturation at 95ºC for 1 min, were 30 cycles of 30 s at 94ºC, 1 min at 65ºC, and 2 min at 68ºC, followed by a final extension of 5 min at 68ºC. The PCR product was analyzed on an agarose gel, purified from the gel (Qiaex II Gel Extraction Kit, Qiagen, Valencia, CA, USA), cloned, and sequenced using the ABI PRISM 377 Sequencer (Applied Biosystems, Foster City, CA, USA). The clone was digested with EcoRI, and the insert was purified from the gel and labeled with 32p-dCTP using the Rediprime II DNA Labelling System (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

The VNTR polymorphism was analyzed by Southern hybridization (Doege *et al.* 1997). Five µg of genomic DNA was digested with *Hae*III and separated on a 1.2% agarose gel for 20 h at 45 V. A molecular weight marker, the 100bp DNA Step Ladder (Promega, Madison, WI, USA), was added to each gel. The size of one repeat was 57 bp. The number of repeats for each sample was estimated from the sizes of the fragments observed in Southern analysis.

4.8 Analysis of the Trp2 and Trp3 alleles (II)

Exons 19 and 5 of the *COL9A2* and *COL9A3* genes, respectively, containing the previously described Trp alleles were amplified by PCR.

The Trp2 allele was analyzed by sequencing from all study individuals. All the probands who proved positive for the Trp2 allele were analyzed for three additional polymorphisms in *COL9A2*: E21^{+9} G>A, IVS27^{-61} c>a, and IVS30^{+17} g>a. The other polymorphisms were analyzed by restriction enzymes.

The Trp3 allele was identified by CSGE. The products that contained a single band were mixed together with another product that was known to contain no Trp3 allele, denatured at 95ºC for 4 min, followed by annealing at 68ºC for 30 min to identify the possible homozygous mutations. The Trp3 alleles were identified as typical double bands on CSGE.

4.9 Genotyping of the polymorphisms in the inflammatory mediator genes (III)

Sixteen polymorphisms in ten genes coding for the inflammatory mediators were genotyped from all the study subjects. The regions containing the variations were amplified by PCR and analyzed by restriction enzyme digestion, sequencing, or agarose gel electrophoresis. The microsatellite marker in *INFG* was analyzed using fluorescent markers and sequencing. (Table 3)

Table 3. Methods for analyzing the inflammatory mediators.

Gene	Variation	Detection method
IL1A	-889 c>t	*Nco*I
IL1B	-31 t>c	*Alu*I
	IVS4^{-64} g>a	Sequencing
	3954 C>T	Sequencing
IL1RN	VNTR	Agarose gel
IL2	-114 g>t	*Mwo*I
IL4	VNTR	Agarose gel
IL4R	1902 G>A	*Pvu*I
IL6	-597 g>a	Sequencing
	-572 g>c	Sequencing
	-174 g>c	*Nla*III
	E5^{+15} T>A	Sequencing
	E5^{+132} C>T	Sequencing
IL10	-1082 g>a	Agarose gel[a]
INFG	microsatellite	Genotyping
TNFA	-308 g>a	Sequencing

[a]allele-specific primers

4.10 Sequencing (I-III)

The PCR products were sequenced with an ABI PRISM 377 or 3100 sequencer using the BigDye Terminator Sequencing Kit (Applied Biosystems). Sequencing was performed if the sample showed a double band on CSGE or to confirm the results from the restriction enzyme analysis. The Trp2 allele and some variations in the inflammation mediators were analyzed only by sequencing (see Table 3).

Prior to sequencing, the samples were treated with exonuclease I (10U/µl) and shrimp alkaline phosphatase (1U/µl) in a volume of 5 µl containing 0.5 µl of both EXO and SAP and 2 µl of both the PCR product and dH_2O. The mixture was incubated at 37°C for 15 min followed by 15 min at 85°C. Five pmol of sequencing primer was added. Some PCR products were purified from the agarose gel and cloned prior to sequencing.

4.11 Statistical analysis (I-III)

To test the equality of the allele frequencies between the LSS probands and controls, chi-square tests were carried out for 2 x 2 tables of allele counts. Haplotype frequencies for four marker loci, *COL11A2* IVS6^{-4}, *VDR* (*Fok*I and *Taq*I) and *MMP-3* polymorphisms, were estimated with the snphap program (Chiano & Clayton 1998) and a likelihood ratio test was employed to test for differences in these frequencies between the probands and controls.

As disc degeneration is known to be related to age, the Chinese study subjects were stratified into four groups by age. The association between the Trp2 allele and pathological changes in the disc (degeneration, herniations, annular tears, and Schmorl's nodes) was studied by assessing the association in each age group separately by chi-square statistics and an odds ratio computed from a 2x2 table with rows. The effects of the four 2×2 tables were combined by the Mantel-Haenszel procedure (Armitage & Berry 1988), which enabled the analysis of two independent sources of association between Trp2 and the disease, overall allele frequency differences (expressed as odds ratio), and heterogeneity of the odds ratios between age groups.

To test the equality of the allele and/or genotype frequencies between the individuals with sciatica and controls, tests for trends were carried out for 2 x 3 tables of genotype counts (Armitage & Berry 1987). For those loci with more than one polymorphism, haplotype frequencies were estimated with the snphap program and a likelihood ratio test was employed to test for differences in these frequencies between the patients and controls (Chiano & Clayton 1998). In addition, the patients were divided into four different subgroups based on the clinical findings: 1) operated/not operated; 2) number of previous sciatica episodes (0 to 1, >1 sciatica episodes); 3) straight-leg-raising test results of the index sciatica episode (<50º, 50-70º, >70º); and 4) durations of the index episode (3-4 weeks, 1-2 months, >2 months). Differences in genotype frequencies were tested between each of these four category types by carrying out 4 × 15 chi-square tests (15 markers). For the marker *INFG* with a repeat polymorphism, we applied one-way ANOVA to see whether the mean number of repeats was different between sub-classifications.

5 Results

5.1 Spinal stenosis (I)

Twenty-nine Finnish probands with LSS were screened for mutations in *COL1A1*, *COL1A2*, *COL2A1*, *COL9A1*, *COL9A2*, *COL9A3*, *COL11A1*, *COL11A2*, and *AGC1*. In addition, three polymorphisms previously shown to associate with DDD were analyzed, two of these in the *VDR* and one in the *MMP-3* gene. All probands underwent an MRI examination to confirm the degenerative nature of the disease.

5.1.1 Clinical and radiological findings

All probands had a self-reported limitation in walking distance (range 50-3000 m) and at least one other stenotic symptom (pain or numbness of the lower extremities upon extension of the lumbar spine, or numbness or weakness in the lower extremities). Clinically, seven probands had bilateral defects in tendon reflexes in the lower extremities. None of the probands had objective muscle weakness or peripheral pulse abnormalities. Twelve probands had undergone a decompressive operation. In addition, three individuals had previously been operated on for a herniated nucleus pulposus and were found to have stenosis at a different lumbar level.

Radiological evaluation of the lumbar region indicated that every proband except one who had undergone decompressive surgery for stenosis prior to inclusion in the study had stenosis at least at one level. Severe disc degeneration (grade 2 or 3) was also observed in every proband at least at one lumbar level. Twenty probands had grade 2 end plate degeneration according to Modic's criteria, six had OPLL, and eight were found to have OLF.

5.1.2 Screening of the genes coding for IVD collagens

Screening of the *COL1A1*, *COL1A2*, *COL2A1*, *COL9A1*, *COL9A2*, *COL9A3*, *COL11A1* and *COL11A2* genes revealed four interesting changes.

The *COL11A2* IVS6^{-4} t allele, previously shown to be associated with OPLL, which is the main cause of spinal stenosis in the Japanese (Maeda *et al.* 2001), was found more often in study subjects than in controls. The frequency of the t allele was 93.1% in the probands compared to 72.3% in controls ($p = 0.0016$). When the t/a and a/a genotypes were combined, no statistically significant differences were seen between the patients and controls. However, the t/t genotype was found more often in the patient group compared to controls ($p=0.0011$). Using the population's attributable risk (Armitage & Berry 1988), it is estimated that 20% of the affected subjects in the population are attributable to the t/t genotype. Another polymorphism, exon 6^{+28} A>G, also reported previously, was found to be in complete linkage disequilibrium with the IVS6^{-4} a>t polymorphism. The IVS6-4 a allele has previously been shown to result in the skipping of exon 6. We confirmed this finding by performing an RT-PCR analysis on total lymphoblast RNA from a proband that was homozygous for the IVS6-4 t allele. The analysis revealed only one fragment which contained sequences for exons 6 (78 bp) and 7 (63 bp). In addition to the inclusion of exon 6, we showed here that exon 8 was skipped in the presence of the t allele.

Analysis revealed one proband with the Trp2 allele, while four probands had the Trp3 allele. One of the probands with Trp3 was homozygous for the sequence variation. In addition, a novel mutation resulting in a premature stop codon in the *COL9A2* gene was found. To study the possible splicing effects, total RNA was isolated from the lymphoblasts of the proband with the mutation and analyzed by RT-PCR. Analysis identified two products, about 550 bp and 460 bp, with the primer set for exons 23-30, and about 430 bp and 340 bp with the primer set for exons 24-29. Sequencing indicated that the product contained only the wild-type sequence, while the larger product contained the wild-type sequence and sequences for the entire intron 26. This a to c mutation in IVS26^{-2} of *COL9A2* prevented the splicing of intron 26 and lead to the insertion of 88 extra nucleotides, and thus of 21 new amino acids, followed by a premature stop codon.

Several other polymorphisms in the genes analyzed were identified, but they were found in equal frequencies in the controls and were therefore likely to be neutral.

5.1.3 Identification of mutations in AGC1

Exons 2 to 19 of *AGC1* were analyzed. Analysis revealed several polymorphisms which were all found in controls as frequently as in the probands, which gave reason to believe that they are not associated with the disease.

The VNTR polymorphism in exon 12, previously shown to be associated with multilevel disc degeneration (Kawaguchi *et al.* 1999), was analyzed by Southern blotting. Alleles with fewer repeats have been shown to be associated with DDD. However, in our study, no association was found between the length of the VNTR region and the severity of spinal stenosis.

5.1.4 MMP-3 and VDR polymorphisms

Two intragenic polymorphisms, T to C in exon 9 (*Taq*I) and T to C at the translation initiation codon (*Fok*I) in the *VDR* gene, as well as one in the *MMP-3* gene were analyzed. *Fok*I restriction enzyme digestion of exon 2 of *VDR* showed that the frequency of the f allele was 0.41 in the probands and 0.32 in the controls. This difference was not significant, giving a p-value of 0.24. The allele frequencies for the *Taq*I polymorphism likewise did not differ significantly between the proband and control groups. The 5a/5a and 5a/6a genotypes of the *MMP-3* promoter region previously shown to associate with DDD (Takahashi *et al.* 2001) were not found to be associated with the disease in our study. The distribution of the 5a and 6a alleles was equal in the patient and control groups.

5.2 Collagen IX Trp alleles and IDD in the Chinese study subjects (II)

5.2.1 Radiological findings

Five hundred twenty-four (65.2%) individuals had additional disc abnormalities, including 261 disc herniations, 178 annular tears, and 85 Schmorl's nodes. The proportion of individuals with annular tears increased with age, while disc herniations were the most common in the group under 30-years of age. No apparent age association was found for Schmorl's nodes.

5.2.2 Analysis of COL9A1, COL9A2 and COL9A3

The *COL9A1*, *COL9A2*, and *COL9A3* genes were screened for mutations in 100 individuals. The analysis did not identify any putatively disease-associated mutations.

Analysis of exon 19 of *COL9A2* showed that the Trp2 allele was present in 160 of the 804 subjects (19.9%), and 12 individuals were homozygous for the allele. Surprisingly, the Trp3 allele was absent, while it has been reported to be found in about 24% of the Finnish patients with sciatica and in about 9% of the Finnish controls (Paassilta *et al.* 2001). To verify that the allele frequencies represented a random population, 100 anonymous blood bank samples and 100 university students were screened. In both sets, the Trp2 allele was present at 20% and the Trp3 allele was absent, thus suggesting that the data set is representative of the general Chinese population of Southern China.

5.2.3 Association between radiological findings and Trp2

The Trp2 allele was found to result in a 4-fold increase in the risk of annular tears at 30-39 years of age and a 2.4-fold increase in the risk of DDD and end plate herniations at 40-49 years of age. Moreover, the individuals with Trp2 had a tendency for more severe disc degeneration than non-carriers.

5.2.4 Haplotypes

Two polymorphisms, $E21^{+9}$ G>A and $IVS30^{+17}$ g>a, have been shown to coexist with the Trp2 allele in the Finnish population (Annunen *et al.* 1999a). As these were not found in any Trp2-negative individuals, it was thought that the individuals with the Trp2 allele had inherited the same ancestral haplotype. Recently, another polymorphism, $IVS27^{-61}$ c>a, that coexists with the three other sequence variations in the Finnish probands, giving the haplotype Trp-A-a-a, was identified (Noponen-Hietala & Ala-Kokko, unpublished data). This was found to be the most common haplotype among the Chinese, even though four others were also found to exist with the Trp2 allele. No difference was found between the haplotypes and the prevalence of DDD.

5.3 Inflammatory mediators and sciatica (III)

5.3.1 Clinical and radiological findings

All subjects had had unilateral sciatic pain for three weeks to six months radiating from the back to below the knee (dermatomes L4, L5, and S1). The straight-leg-raising test was positive (≤ 60°) in 59% of the subjects. At the baseline, the mean intensity of the leg pain of sciatica patients was 73 mm on a 100-mm VAS scale (SD 18 mm), the intensity of back pain 56 mm (SD 25 mm), and disability on the Oswestry scale 43% (SD 15%). IVD herniation - contained, non-contained or sequestered - was present in 86% of patients during the 3-year follow-up period. At the time of the 3-year follow-up period, 29% of the subjects had undergone an operation for herniated disc.

5.3.2 Analyzing the polymorphisms in the genes coding for the inflammatory mediators

The promoter regions, exons, and exon boundaries of *IL1A*, *IL1B*, *IL6*, and *TNFA* were analyzed for mutations in 48 patients. When a sequence variation was found, 48 control samples were analyzed for the variations. In case of a difference in the frequency of a

sequence variation between the patients and controls, the rest of the samples were also analyzed for the presence of the variation.

Twelve sequence variations were detected of which several were located in the promoter regions of the genes (Table 4). Two of the four exonic variations (3954 C>T in *IL1B* and $E5^{+132}$ C>T in *IL6*) did not result in a change of an amino acid encoded. The remaining two exonic variations resulted in an amino acid substitution; $E5^{+21}$ G>T in *IL1A* changed a codon GCA for Ala to a codon TCA for Ser, and $E5^{+15}$ T>A in *IL6* that changed a codon GAT for Asp to GAA for Glu. None of the twelve variations were putatively disease-causing, and they were found both in the 48 patients and controls. Allele frequencies of two of the variations, $E5^{+21}$ G>T in *IL1A* and -511 t>c in the promoter of *IL1B*, were determined by sequencing the 48 patient and control samples. Because no differences were found, the rest of the patients were not analyzed for these variations. The remaining 10 variations were genotyped from all the patients and controls.

5.3.3 Genotype comparison in four clinical subtypes

To ascertain whether the patient group was heterogeneous regarding the four clinical criteria (operated/not operated, number of sciatica episodes, straight-leg-raising test results and duration of sciatic episodes), differences in genotype frequencies between each of these four categories were tested. The smallest p-value obtained was $p = 0.0056$ for *IL1B* (C3954T polymorphism) versus "classification" (1 against >1 sciatica episodes). All other p-values exceeded 0.01. However, because a total of 64 statistical tests were carried out, the smallest significance level after adjustment for multiple testing turned out to be $64p = 0.36$. That is, we did not find any significant genotype differences between the various clinical categories tested and were therefore able to consider the patient group as a homogeneous entity suitable for further genetic analysis.

Table 4. Sequence variations identified in IL1A, IL1B, IL6, and TNFA.

Gene	Region	Variation
IL1A	Promoter	-889 c>t
	Exon 5	+21 G>T
IL1B	Promoter	-511 t>c
	Promoter	-31 t>c
	Intron 4	-64 g>a
	Exon 5	3954 C>T
IL6	Promoter	-597 g>a
	Promoter	-572 g>c
	Promoter	-174 g>c
	Exon 5	+15 T>A
	Exon 5	+132 C>T
TNFA	Promoter	-308 g>a

5.3.4 Allele frequencies

In addition to these ten polymorphisms, six previously identified polymorphisms in the inflammatory mediator genes were analyzed (see Table 3). Significant differences were observed in the allele frequencies between the patients and controls for the *IL6* polymorphism $E5^{+15}$ T>A (p=0.007). In addition, the -597 g>a and -174 g>c polymorphisms in *IL6* showed suggestive evidence for association although, after correction for multiple testing, they were not formally significant. Differences in allele frequencies in the other polymorphisms analyzed were not statistically significant between the cases and controls.

In addition, a difference was observed in the $E5^{+15}$ T>A genotype frequencies. AA or AT genotypes were found in 13 out of 133 patiens (9.8%) when compared to only 3 out of 124 controls (2.4%) (p=0.011). The corresponding attributable risk AR for the presence of the A allele was 7.5% (95% CI=1.6-13.1%) and OR 4.4 (1.2-15.7).

5.3.5 Haplotype frequencies

Haplotype frequencies for the five *IL1* marker loci (-889 c>t in *IL1A*, -31 t>c, $IVS4^{-64}$ g>a and 3954 C>T in *IL1B*, and VNTR in *IL1RN*) were tested using various combinations of the SNPs. No significant differences were observed in the haplotype frequencies. Similar analysis was performed for the five *IL6* marker loci. No significant differences were observed when all five polymorphisms (-597 g>a, -572 g>c, -174C g>c, $E5^{+15}$ T>A and $E5^{+132}$ C>T) were used to generate haplotypes. However, when four SNPs (-597 g>a, -572 g>c, -174 g>c, and $E5^{+15}$ T>A) were tested, a significant difference in the haplotype frequency was observed (p=0.012). Six of the possible sixteen haplotypes were observed, with the GGGA haplotype being more frequently found in the patients (p=0.011; OR=4.8 (95% CI=1.6-14.5). To evaluate the attributable risk, haplotype pairs (genotypes) were assigned to the individuals. The GGGA/GGGA or GGGA/other genotypes were overrepresented in the patients. This corresponded to an OR of 5.4 (1.5-19.2). The association of GGGA with disease was highly significant (p=0.0033), and the associated AR was 6.8% (1.9-11.5%).

6 Discussion

6.1 Genetic factors in degenerative spinal stenosis

Degenerative lumbar spinal stenosis (LSS) is a common disease usually affecting individuals over 50 years of age. A number of genetic factors have been implicated in disc herniation, disc degeneration, and OPLL, which are the leading causes of degenerative stenosis (Koga *et al.* 1998, Maeda *et al.* 2001, Ala-Kokko 2002).

We investigated possible associations between the previously identified genetic factors and degenerative LSS, and also analyzed nine genes encoding intervertebral disc matrix proteins for sequence variations. Twenty-nine unrelated Finnish individuals with radiologically confirmed LSS were included in the study. All underwent a clinical and an MRI examination.

Previous studies have indicated that genetic factors play a role in LSS. OPLL is a common cause for LSS in the Japanese, and a linkage between OPLL and chromosome 6p has been reported (Koga *et al.* 1998). This region contains at least two candidate genes, *COL11A2* and the retinoid X receptor β, *RXRβ*. The *COL11A2* IVS6^{-4} a to t polymorphism has previously been shown to be associated with OPLL in the Japanese population (Maeda *et al.* 2001). In the current study, the frequency of the IVS6^{-4} t allele was found to be 93.1% in the patients with LSS compared to 72.3% in the controls ($p=0.0016$). In addition, we found that the t/t genotype was more common in the patient group compared to the controls ($p=0.0011$). It is estimated that 20% of the affected subjects in the population are attributable to the t/t genotype. Thus, our study suggests that *COL11A2* plays a role also in spinal stenosis that is not associated with OPLL.

COL11A2 exons 6 to 8 are known to undergo complex alternative splicing. Earlier findings have indicated that the IVS6^{-4} a allele results in a different splicing pattern from the t allele, with exon 6 being skipped in its presence (Maeda *et al.* 2001). In addition to the inclusion of exon 6, we showed that exon 8 was skipped in the presence of the t allele. Since any nucleotide can be found at position -4 in the acceptor splice site (Cartegni *et al.* 2002), it is not clear through which mechanism the IVS6^{-4} polymorphism affects the splicing. Even though screening of the exons and exon boundaries of *COL11A2* did not result in the identification of any other potentially disease-associated changes, it is

possible that there is another, yet unidentified mutation in the non-coding region of the gene that is in complete linkage disequilibrium with the IVS6^{-4} polymorphism.

The Trp2 and Trp3 alleles have previously been described as risk factors for IDD in the Finnish population (Annunen *et al.* 1999, Paassilta *et al.* 2001). In our study, one of the individuals had the Trp2 allele and four had the Trp3 allele. One individual was homozygous for the Trp3 allele. It is not certain, through which mechanism the Trp alleles predispose to the disease. Tryptophan is the most hydrophobic amino acid, rarely found in the normal structure of collagens. In fact, in there are no Trp residues in the collagenous domains of collagen IX in man or mouse (Muragaki *et al.* 1990, Perälä *et al.* 1993, Brewton *et al.*1995, Perälä *et al.* 1994, Rokos *et al.* 1994). In addition, the Trp2 residue is located only three amino acid residues from the covalent crosslink between the α3(IX) chain and collagen II. As the most hydrophobic amino acid, Trp could also have an effect on the formation or the stability of the triple helix. However, a recent study showed that the Trp residues do not affect the assembly or amount of collagen IX in embryonic and fetal human cartilage. Both Trp2 and Trp3 allelic products were incorporated into the crosslinked fibrillar network and the cartilage appeared normal. This finding suggests that the pathological consequences are most likely to be long-term and indirect (Matsui *et al.* 2003). IVD herniations and IDD are among the factors that predispose to LSS. It is therefore possible that the Trp alleles also increase the risk for LSS. However, it is unlikely that Trp alleles alone would cause LSS. Other factors, genetic or environmental, together with the Trp alleles are likely to be needed for the development of LSS.

The analysis also revealed a heterozygous a to c mutation at position -2 in IVS26 of the *COL9A2* gene. The mutation prevented the splicing of intron 26 and resulted in a premature translation termination codon in IVS26 88 nucleotides downstream of the exon. This mutation is likely to lead to the synthesis of a truncated α2(IX) chain that lacks the C-terminal end of the COL2 domain and the entire NC2, COL1, and NC1 domains. The formation of the collagen triple helix is known to occur from the C-terminal end towards the N-terminal end. Since the truncated α2(IX) chain lacks the C-terminus, required for collagen triple helix assembly, that mutation is likely to lead to a reduced amount of collagen IX in the tissue. These findings further support the role of collagen IX in spinal disorders.

6.2 Association between Trp2 allele and IDD in the Chinese population

IDD characterized by sciatica is a common musculoskeletal disorder affecting about 5% of individuals (Heliövaara *et al.* 1987). Several environmental and constitutional factors have been implicated as risk factors for IDD. However, family and twin studies have shown that genetic factors may explain up to 77% of the variability of disc degeneration (Battié *et al.* 1995a, Battié *et al.* 1995b, Sambrook *et al.* 1999).

Tryptophan alleles in collagen IX have been indicated to be predisposing factors for IDD characterized by sciatica in the Finnish population (Annunen *et al.* 1999a, Paassilta *et al.* 2001). The Trp2 allele in α2(IX) was found in 3.8% of the Finnish patients and in

none of the controls (Annunen *et al.* 1999a). In a previous study, the α3(IX) Trp3 allele was found in about 24% of the Finnish patients and in about 9% of the Finnish controls (Paassilta *et al.* 2001).

Our study group consisted of 804 unrelated Chinese individuals. Surprisingly, the Trp2 allele was found in 20% of the general population while the Trp3 allele was absent. This finding gave us a great opportunity to study the correlation between the Trp2 allele and MRI findings. A hundred randomly selected samples from the local blood bank were also analyzed to assure that our sample set represented the general population. The Trp2 allele was found in 20% of the blood bank samples, giving us the reason to believe that our study group represented the general population. All individuals included in the study underwent an MRI examination. The MRI scans were graded by two readers blinded to the genetic results.

We found that in the Chinese population, the Trp2 allele was associated with a 4-fold increase in the risk of annular tears at 30 to 39 years of age and a 2.5-fold increase in the risk of DDD and end-plate herniations (Schmorl's nodes) at 40 to 49 years of age. In addition, the individuals with the Trp2 allele had more severe disc degeneration compared to those who were Trp2 negative. A previous study on rabbits showed that an annular laceration of IVD resulted in disc degeneration (Anderson *et al.* 2002). In addition, several studies have indicated that radial tears in the IVDs precede disc degeneration and cause sciatic pain (Yu *et al.* 1988, Osti *et al.* 1992, Ohnmeiss *et al.* 1997). Our findings further support this hypothesis.

Collagen IX is a heterotrimer of the α1(IX), α2(IX) and α3(IX) chains encoded by the *COL9A1*, *COL9A2*, and *COL9A3* genes, respectively (Pihlajamaa *et al.* 1998, Paassilta *et al.* 1999b). It is located on the surface of collagen II and part of the molecule projects away from the surface of collagen II. The function of collagen IX is not well known but it is thought to act as a mediator between collagen II and other extracellular matrix proteins. Several studies on humans and mice have indicated that collagen IX has an important role in maintaining tissue integrity (Nakata *et al.* 1993, Fässler *et al.* 1994, Kimura *et al.* 1996, Muragaki *et al.* 1996, Holden *et al.* 1999, Paassilta *et al.* 1999a, Czarny-Ratajczak *et al.* 2001). Consistent with these observations, it is possible that Trp as the most hydrophobic amino acid interferes with the interactions between collagen IX and other matrix proteins.

Studies concerning the Trp alleles had earlier been done only in the Caucasian population and this is the first time that Trp alleles were studied in the Asian population. Our study is also the largest to date to compare the genetic factors to radiological findings using MRI and the candidate gene approach. Our findings further strengthen the hypothesis that the Trp2 allele acts as a predisposing factor for IDD and end plate changes such as Schmorl's nodes. The end plates consist of a gel of hydrated proteoglycan molecules that is reinforced by collagen fibrils, including collagen IX. In a previous radiological study, the individuals with the Tpr3 allele were found to have a greater risk for Scheuermann's disease and disc degeneration (Karppinen *et al*. 2003a). In addition, a positive correlation has been reported between end plate degeneration and disc degeneration (Kokkonen *et al.* 2002).

6.3 Role of *IL6* in IDD characterized by sciatica

Several studies have indicated that in addition to mechanical compression, inflammation plays an important role in IDD characterized by sciatica. It has been shown that pro-inflammatory cytokines, such as IL-1, IL-6, and TNF-α, are produced at the site of IVD herniation (Kang *et al.* 1996, Takahashi *et al.* 1996, Burke *et al.* 2002). It has been reported that disc disruption passing into the outer layers of the annulus, without deformation of the outer wall, was as frequently associated with lower extremity pain as were discs with more severe disruption deforming the outer annular wall (Ohnmeiss *et al.* 1997). In addition, the application of nucleus pulposus to nerve roots or dorsal ganglion can induce an inflammatory irritation without mechanical compression (Takebayashi *et al*. 2001, Aoki *et al*. 2002).

Several studies have suggested that inflammatory mediators play a role in this disease, and previous studies have shown an association between low back pain and disc degeneration with the polymorphisms in the *IL1* locus; -889 c>t in *IL1A*, 3954 C>T in *IL1B*, and 1812 G>A in *IL1RN* (Solovieva *et al*. 2004a, Solovieva *et al.* 2004b). However, a possible association between inflammatory mediators and IDD characterized by sciatica has not been studied.

The role of sequence variations in several inflammatory mediator genes was investigated here in 155 Finnish individuals with IDD characterized by sciatica and 179 controls. The study did not show evidence for the role of structural mutations in the major pro-inflammatory cytokine genes, *IL1A*, *IL1B*, *IL6*, and *TNFA*, in the pathogenesis of IDD and sciatica. Nevertheless, association analysis provided support for a link between the *IL6* sequence variations -597 g>a, -174 g>c, and $E5^{+15}$ T>A, and the disease. The g alleles of -597 and -174 polymorphisms and the A allele of the $E5^{+15}$ polymorphism were overrepresented in the patient group. These findings were interesting because the g allele of the -174 g>c variation has been previously shown to be associated with the increased expression and plasma levels of IL-6 (Fishman *et al*. 1998, Yucesoy *et al.* 2003). The $E5^{+15}$ T>A polymorphism changed an amino acid encoded, from Asp to Glu. It is possible, although not very likely, that this substitution results in major functional or structural consequences because both amino acids are acidic and structurally related.

A study by Terry *et al*. (2000) demonstrated that genetic polymorphisms in *IL6* exhibit a co-operative influence on transcriptional regulation of the gene. Specifically, they showed that four sequence variations in the *IL6* promoter, -597 g>a, -572 g>c, -373AnTn, and -174 g>c, influence *IL6* transcription through a complex interaction determined by the haplotype, with the g alleles of the three markers being associated with an increase in the transcription of *IL6*. In addition to $E5^{+15}$ T>A, three of the markers tested in the study by Terry *et al*. (2000), -597 g>a, -572 g>c, and -174 g>c, were used here to generate haplotypes. In our study the haplotype GGGA was associated with the phenotype. It is thus possible that this haplotype is also associated with the increased production of IL-6 (Fishman *et al*. 1998, Terry *et al*. 2000). IL-6 together with IL1-α, IL1-β, and TNF-α are among the most potent pro-inflammatory and catabolic cytokines known and are released as a consequence of cell or tissue injury (Choy and Panayi 2001, Goldring 2001, Benoist 2002). It has been demonstrated that IL-6 is produced at the site of a lumbar disc herniation (Kang *et al*. 1996, Takahashi *et al*. 1996, Burke *et al*. 2002). As a consequence

of the increased pro-inflammatory cytokine production, herniated discs produce matrix metalloproteinases, nitric oxide, and prostaglandin E2, which have been shown to mediate pain or to enhance sensitivity to other pain-producing substances (Kang *et al*. 1996, Takahashi *et al*. 1996).

IDD characterized by sciatica is a common disorder usually affecting individuals from 40 to 50 years of age, and it can keep people out of work for a long time. In approximately 10% of patients, low back pain persists for more than 6 weeks (Frymoyer 1988). About 5 to 20% of individuals with IDD-related sciatica need surgical intervention (Heliövaara *et al.* 1987, Deyo *et al.* 1990, Frymoyer *et al.* 1992), but even higher figures have been reported in another study (Balague *et al.* 1999). Several studies suggest that inflammatory mediators have an important role in the process of sciatic pain. Corticosteroids are well-known anti-inflammatory substances that have been in clinical use for a long time in the treatment of inflammatory diseases such as rheumatic disorders. Several recent studies indicate that corticosteroids are also useful in the treatment of sciatica (Glasser *et al.* 1993, Buchner *et al.* 2000, Byrod *et al.* 2000, Karppinen *et al.* 2001b, Papagelopoulos *et al.* 2001). In addition, TNF-α inhibitors have recently been suggested to be useful in the treatment of sciatica. They were found to reduce the nerve conduction velocity and to limit nerve fiber injury and intraneural edema (Olmarker & Rydevik 2001). In another study, TNF-α monoclonal antibody, infliximab, was shown to decrease the pain by 50% at 1 hour after infusion. At two weeks, 60% of the patients treated with infliximab were painless compared to 16% in the control group. (Karppinen *et al.* 2003b.) TNF-α has been shown to be one of the most important pro-inflammatory mediators (Olmarker & Larsson 1998) and it is known to act in a close relationship with IL-1. The blocking of TNF-α in culture diminished the amounts of IL-1, IL-6, and IL-8, while the blocking of IL-1 blocked IL-6 and IL-8 but had no affect on TNF-α (Butler *et al*. 1995).

In the light of the earlier findings, anti-inflammatory substances have potential in the treatment of IDD characterized by sciatica in the future. As this treatment is very expensive, it is important to learn to select the individuals who would benefit most from the anti-inflammatory treatment. Our study is the first one to indicate a genetic association between sciatica and inflammatory mediators. The finding that sequence variations in the *IL6* gene are associated with IDD characterized by sciatica supports the role of IL-6 in the process of sciatic pain. This finding may have an impact on identifying patients who could benefit from treatment with anti-inflammatory substances.

6.4 Conclusion

Degenerative spinal stenosis and IDD are common clinically important spinal disorders. Several environmental factors have been reported to play a role in the complex etiology of IDD. However, the role of genetic factors in these diseases is strongly supported by family and linkage studies.

The findings of this thesis further support the hypothesis that genetic factors have an important role in the development of degenerative spinal stenosis and IDD. Genetic variations in the collagen IX and XI genes were found to be associated with degenerative

spinal stenosis. Our studies also showed that the collagen IX Trp2 allele acts as a predisposing factor for disc degeneration in an age-dependent manner in the Chinese population. The Trp2 allele was present in 20% of the population and was associated with a 4-fold increase in the risk of developing annular tears at the age from 30 to 39 years, and a 2.4-fold increase in the risk of developing DDD and end plate herniations at the age from 40 to 49 years. The Trp2 allele has earlier been shown to be a risk factor for IDD in the Finnish population. Our results indicate that Trp2 acts as a predisposing factor for IDD also in another ethnic population.

Inflammation has been suggested to be one of the underlying factors for sciatic pain and tissue destruction. The role of inflammation is supported by the usefulness of anti-inflammatory substances in the treatment of sciatica. This hypothesis is further supported by our finding that certain *IL6* haplotypes predispose to sciatica. It is not well understood, however, through which mechanisms the inflammation contributes to sciatic pain and destruction.

The findings of this thesis provide new information on the etiology and pathogenesis of disc diseases. However, further studies are needed in order to develop more specific tools for the treatment of these diseases and the development of more efficient preventive means.

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