GENETIC DEFECTS OF COLLAGEN XI

The role of a minor cartilage collagen in chondrodysplasias, oral cleft defects and osteoarthrosis

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Abstract

Collagen XI is a minor component of articular cartilage collagen fibrils together with collagen IX. They are in close functional relationship with the major cartilage collagen II. Collagen XI has been suggested to play a role in regulating the diameter of collagen II fibrils. Together these collagens form a supportive framework in the extracellular matrix. Besides articular cartilage, these three collagens can also be found in the vitreous body of the eye, the intervertebral disc, the inner ear and in various tissues during embryonic development.

As the major cartilage collagen, collagen II has been studied quite extensively. Several syndromes ranging from lethal to milder ones have been shown to result from collagen II gene defects. Far less is known about defects in genes coding for the minor cartilage collagens, IX and XI. By identifying mutations in the coding genes and observing the resulting phenotypes, the function and importance of these genes start to unravel.

The goal of this study was to provide more information about collagen XI. As a quantatively minor cartilage component, it is a good candidate for mild disease phenotypes. Collagen XI gene mutations have been shown to cause relatively mild phenotypes, such as Stickler and Marshall syndromes and non-syndromic hearing loss.

Seven families with a recessive chondrodysplasia, otospondylomegaepiphyseal dysplasia (OSMED), were analysed for mutations in COL11A2. This study showed that OSMED is typically caused by the absence of the α2(XI) chains. Sixty-two patients with isolated Robin sequence, cleft palate or micrognathia were analysed for COL11A2 gene mutations. Six unique nucleotide changes were found that are likely to associate with the phenotype. The results showed that collagen XI gene defects can play a role in the etiology of oral clefting, but are not common causes of these phenotypes. Altogether 72 unrelated osteoarthrosis (OA) patients and one family with OA were analysed for mutations in genes coding for collagens II, IX and XI. Eighteen percent of them were found to have a unique sequence variation. An association analysis of OA patients failed to reveal any common predisposing alleles in these genes.

Keywords: cartilage, cleft, collagen, mutation, osteoarthritis, osteochondrodysplasias

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Abbreviations

α1(XI)	collagen XI α1 chain, and other collagen polypeptide chains accordingly
bp	base pair
C-	carboxy-
CD	chondrodysplasia
cDNA	complimentary DNA
CP(O)	cleft palate (only)
CL/P	cleft lip/palate
COL11A1	human gene for α1 (XI) chains, and other human collagen genes accordingly
Col11a1	mouse gene for α1 (XI) chain
COMP	cartilage oligomeric matrix protein
CSGE	conformation-sensitive gel electrophoresis
ECM	extracellular matrix
FACIT	fibril-associated collagens with interrupted triple helices
Gly	glycine
N-	amino-
NC	noncollagenous
nt	nucleotide
ND	not determined
MED	multiple epiphyseal dysplasia
MIM	Mendelian Inheritance in Man
mRNA	messenger RNA
OA	osteoarthtosis
OSMED	otospondylomegaepiphyseal dysplasia
PARP	proline arginine rich peptide
PCR	polymerase chain reaction
(P)RS	(Pierre) Robin sequence
RT-PCR	reverse transcriptase PCR
SED	spondylopepiphyseal dysplasia
SNP	single nucleotide polymorphism

WZS	Weissenbacher-Zweymüller syndrome
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 Melkoniemi M, Brunner HG, Manouvrier S, Hennekam R, Superti-Furga A, Kääriäinen H, Pauli RM, van Essen T, Warman ML, Bonaventure J, Miny P & Ala-Kokko L (2000) The autosomal recessive disorder otospondylomegaepiphyseal dysplasia (OSMED) is associated with loss of function mutations in the COL11A2 gene. Am J Hum Genet 66: 368-377.

II Melkoniemi M, Koillinen H, Männikkö M, Warman ML, Pihlajamaa T, Kääriäinen H, Rautio J, Hukki J, Stofko JA, Cisneros GJ, Krakow D, Cohn DH, Kere J & Ala-Kokko L (2003) Collagen XI sequence variations in non-syndromic cleft palate, Robin sequence and micrognathia. EJHG 11: 265-270.

III Jakkula E, Melkoniemi M, Kiviranta I, Lohiniva J, Räinä SS, Warman ML, Ahonen K, Kröger H, Göring HHH & Ala-Kokko L. The role of sequence variations within the genes encoding collagen II, IX and XI in non-syndromic, early-onset osteoarthritis. Accepted for publication.

Contents

1 Introduction

Collagens are proteins located in the extracellular matrix (ECM). Their main function is to maintain the structural integrity of tissues. Collagens are especially abundant in cartilage, bone, skin, tendons and ligaments. There are several different collagen types, all having their special function and location. Cartilage is a highly specialised form of connective tissue and is widely distributed throughout the body. Collagen types that are present in cartilagenous tissues include collagen II, V, VI, IX, X and XI. Collagen II is the major cartilage collagen and the most abundant protein in hyaline cartilage. Collagens IX and XI are minor components in hyaline cartilage.

Both collagen II and XI form fibrils in the ECM and are closely connected. Collagen XI is thought to be located inside collagen II macrofibrils and regulate the macrofibril diameter. Collagen XI is encoded by three genes, COL11A1, COL11A2 and COL2A1. The third gene also encodes collagen II. Defects in these genes have been shown to result in phenotypes ranging from mild, such as non-syndromic hearing loss, to severe phenotypes that lead to early lethality.

As the major collagen in cartilage, collagen II has been studied more extensively than the two minor ones, collagens IX and XI. In April 2004, a web portal Medline search with the term ‘collagen II’ gave 9781 articles, whereas the same search for collagen XI gave only 430 articles. This work was initiated to obtain more information about collagen XI. Based on the existing information on the consequences of collagen II, IX and XI mutations in humans and mice, the approach of screening collagen XI for mutations in relatively mild cartilage phenotypes was used. Identifying the underlying genetic defects in disease will help to understand the function and importance of a protein. This information should be very helpful for genetic counselling, and is necessery for the development of targeted treatments. The future holds a promise for effective gene therapy, a targeted treatment for patients with genetic disorders.

2 Review of the literature

2.1 Collagens

The most abundant proteins in human body, collagens, are connective tissue proteins. They are present in most tissues and tissues such as bone, tendon, skin and cartilage are especially rich in collagens. The main function of collagen is to maintain the structural integrity of tissues. Collagens also play a role in early development and organogenesis, cell attachment, chemotaxis, platelet aggregation and filtration through basement membranes. (See Kivirikko 1993).

Collagens comprise a large family of proteins. This superfamily includes at least 27 proteins and the number of known family members is increasing constantly. Also more than twenty other proteins are known to have collagen-like domains, but are not called collagens. Every collagen has at least one triple helical part. (See Kivirikko 1993, Prockop & Kivirikko 1995, Myllyharju & Kivirikko 2001, Myllyharju & Kivirikko 2004).

The collagen triple helix is formed from three polypeptide chains, called α chains, which are each coiled into a left-handed helix. These three α chains are coiled around each other to form a right-handed superhelix. Every third amino acid of each α chain is glycine, the smallest amino acid, which is placed in the middle of the collagen triple helix. It is essential that every third amino acid is glycine because the space inside the triple helix is restricted and any other amino acid in this position would cause an interruption in the helix. The formula for collagenous domains in α chains is therefore Gly-X-Y. The triple helix conformation also requires the presence of proline and 4-hydroxyproline in the polypeptide chains. Proline is frequently in the X position of the polypeptide and 4-hydroxyproline in the Y position. The presence of these two amino acids limits rotation of the polypeptide chains. 4-hydroxyproline is also required for the formation of hydrogen bonds and water bridges that further stabilise the triple helix. (See Kivirikko 1993, Prockop & Kivirikko 1995).

2.1.1 Collagen types

Collagens can be divided into subgroups according to their particular structural features. These groups are: fibrillar collagens, collagens that form network-like structures, fibril-associated collagens with interrupted triple helices (FACITs), collagens that form beaded filaments, collagens that form anchoring fibrils for basement membranes, and collagens with a transmembrane domain. Collagens II, V and XI belong to the fibrillar collagens and collagen IX to the group of FACITs. Collagen X, also mentioned in this review, belongs to network-like structures forming collagens. (See Kielty *et al.* 1993, Prockop & Kivirikko 1995).

2.1.2 Collagen biosynthesis

Collagens are synthesised as large precursor molecules called procollagens. They undergo several posttranslational modifications both inside the cell and after being secreted outside the cell. It is a multi-step process that involves a number of enzymes. The intracellular steps include the cleavage of signal peptides, hydroxylation of some of the proline and lysine residues into 4-hydroxyproline (Y-position), hydroxylysine (Y-position) and 3-hydroxyproline (X-position), glycosylation of some hydroxylysine residues, addition of a mannose-rich oligosaccharide to one or both of the propeptides, association and disulfide bonding of the C-terminal propeptides, and zipper-like folding of the triple helix starting from the C-terminus to the N-terminus. The molecule is then secreted from the cell. The propeptides are cleaved off the molecule in the extracellular space. This is followed by self-assembly of the collagen molecules into fibrils. Finally, covalent cross-links are formed. In the case of nonfibrillar collagens, there are some exceptions to this pattern. (See Prockop & Kivirikko 1995, Myllyharju & Kivirikko 2001).

2.1.3 Cartilage collagens

Cartilage provides permanent flexible components for the skeleton and also acts as a temporary template for developing skeletal structures. It is connective tissue that is rich in extracellular matrix and has only a few cells called chondrocytes. These cells produce and secrete the proteins of the extracellular matrix. In the extracellular matrix, collagens form a framework that gives tensile strength and shape and withstands the swelling pressure of the proteoglycans. Proteoglycans are extremely large aggregate molecules that have the ability to bind water to a great extent. This feature is most important for the elasticity of cartilage.

Collagen II is the major structural component of cartilage. Thirty percent of the dry weight of developing cartilage and sixty percent of adult articular cartilage is collagen II. It exists in copolymeric assembly with collagens IX and XI (Fig. 1). Collagen II represents approximately 85-90 percent of the total tissue collagen. Collagens II, IX and

XI are found only in hyaline cartilage, the inner ear, the vitreous body of the eye and intervertebral discs. Hence they are called cartilage collagens. (See Burgeson & Hollister 1979, Fassbender 1987, Eyre & Wu 1995, Muir 1995).

Collagen X is also found only in cartilage. Its expression is, however, limited only to the hypertrophic zone in cartilage (Reichenberger *et al.* 1991, see also Muir 1995).

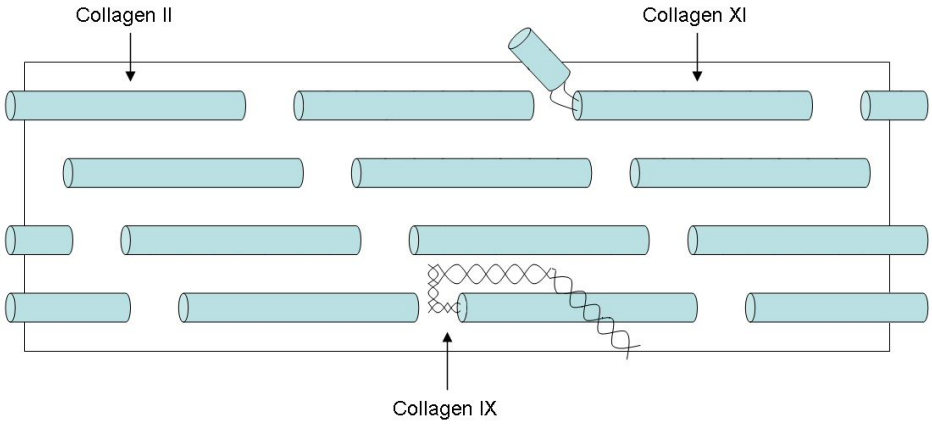


Fig. 1. The collagen II:IX:XI heterofibril. A simplified molecular model of collagen II, IX and XI interactions.

2.1.3.1 Collagen II

Collagen II is composed of three identical α chains called α1(II) chains that are encoded by the COL2A1 gene. This gene is about 30 kb in size and has 54 exons (Ala-Kokko & Prockop 1990, Ala-Kokko *et al.* 1995). It is located on chromosome 12q13.11-q13.12 (Takahashi *et al.* 1990). The first intron contains an alternatively spliced exon 2A (Ryan & Sandell 1990), and the gene product containing this exon is named collagen IIA. Collagens IIA and IIB have different tissue distributions during chondrogenesis, with IIB being expressed by chondrocytes and IIA by prechondrocytes surrounding the cartilage (Sandell *et al.* 1991). After birth, collagen IIA is only expressed in the vitreous body of the eye, whereas collagen IIB is predominantly expressed in cartilage (Parma *et al*. 2002). Collagen II forms fibres that interact with other matrix macromolecules. Fibrils are formed in a controlled manner so that approximately one quarter of a molecule overlaps with the preceding one. This results in the banded appearance of the fibre. (Eyre 1991, Eyre & Wu 1995).

2.1.3.2 Collagen XI

Collagen XI is a heterotrimeric molecule composed of three α chains, α1(XI), α2(XI), α3(XI), that are products of three distinct genes; COL11A1, COL11A2 and COL2A1. These genes are located on chromosomes 1p2 (Henry *et al.* 1988), 6p21 (Kimura *et al.* 1989) and 12q13.11-q13.12 (Takahashi *et al.* 1990) respectively. The α3(XI) chain is a more posttranslationally modified product of the COL2A1 gene. The α1(II) and α3(XI) chains differ in their degree of lysine hydroxylation and hydroxylysine glycosylation (Eyre & Wu 1987). The α3(XI) chain is a product of the alternative splicing variant IIB of the COL2A1 gene (Wu & Eyre 1995).

All three α(XI) chains have one long triple helical part (COL1), one short N-terminal triple helical part (COL2), and three non-collagenous domains (NC1-3). All domains, except the N-terminal NC1 domain, are very similar in size to the α chains. (Zhidkova *et al.* 1995).

The COL11A1 gene has 68 exons and is over 150 kb in size (Annunen *et al.* 1999a). Processing of the COL11A1 product involves complex alternative splicing. Six different variants have been identified in the chick pro-α1(XI) chain. At least two different splicing variants have been shown to exist in humans; exons numbered 6A and 6B are alternative to each other and never present in the same mRNA. (Zhidkova *et al.* 1995, Annunen *et al.* 1999). Alternative processing takes place in the N terminal part of the molecule called the variable region.

The COL11A2 gene is over 28 kb in size and is composed of 66 exons (Vuoristo *et al.* 1995). Alternative splicing occurs in the N-terminal part where exons 6 and 7 undergo alternative splicing in humans and exons 6-8 in mice. (Zhidkova *et al.* 1995, Lui *et al.* 1996).

During extracellular processing, a peptide called proline/arginine rich protein (PARP) is released from the N-terminus of the pro-α2(XI) chain. PARP is retained in the cartilage matrix where it can be isolated in significant amounts. Its function in unclear. Pro-α1(XI) and pro-α1(V) chains also have an N-terminal domain homologous to PARP, however it is not known if there is specific cleavage and release of these PARP-like domains in the matrix. (Zhidkova *et al.* 1995).

All α(XI) chains have been shown to retain at least to some extent their N terminal propeptides even after association into fibrils, in contrast to other fibrillar collagens (Wu & Eyre 1995). The same is true with the α1(V) chain that sequence analysis has shown to be closely related to the α1(XI) and α2(XI) chains. Collagens V and XI are closely related in their biological and structural features, although their tissue locations are different. Collagen V is a heterotrimer composed of three of four α(V) chains that are coded by four genes; COL5A1, COL5A2, COL5A3 and COL5A4. The α4(V) chain is only expressed in the nervous system. The molecular formula for the most common combination is $[\alpha1(V)_2\alpha2(V)]$. Collagen V is found associated with collagens I and III in non-cartilagenous tissues, whereas collagen XI is associated with collagen II fibrils in cartilage. Collagen V and XI are found buried within the major collagen types, but their N-terminal propeptides are projected onto the surface of the fibril. This feature may explain how collagens V and XI regulate fibril diameter. It is also possible that the N-propeptide projecting out from the fibril plays some role in the interactions between

fibrils and other matrix components. (Andrikopoulos *et al.* 1995, see Fichard *et al.* 1995, Li *et al.* 1995, Zhidkova *et al.* 1995, see also Myllyharju & Kivirikko 2001).

The α chains of collagens V and XI have been found to form heterotypic fibrils with each other. For example, in the vitreous body of the eye α2(XI) is completely replaced by the α2(V) chain, and during fetal development the α1(XI) and α1(V) chains have been found to replace each other in developing bone and cartilage. These findings of heterotypic fibrils composed of α(V) and α(XI) chains, together with knowledge of their functional and biological similarities, have lead to the suggestion that these collagens form a single collagen type, V/XI collagen (Mayne *et al.* 1993, see also Fichard *et al.* 1995).

2.1.3.3 Collagens IX and X

Collagen IX is a heterotrimer of the α1(IX), α2(IX) and α3(IX) chains. The genes COL9A1, COL9A2 and COL9A3 that code for these chains are located on chromosomes 6q12-q13, 1p32 and on 20q13.3 respectively (Warman et al. 1993, Warman *et al*. 1994, Tiller *et al*. 1998). The α1(IX) chain has two different forms due to the usage of an alternative promoter. One form has a large globular domain (NC4) in the N-terminus and the other one lacks it (Muragaki *et al.* 1990). Collagen IX is a member of the FACIT collagens. It consists of three triple-helical domains (COL1-3) and four non-collagenous domains (NC1-4). Unlike collagens II and XI, it does not form fibrils but is attached to the surface of collagen II fibrils in an antiparallel orientation. The collagen IX molecule is both a collagen and a proteoglycan since it has a single site of attachment for a chondroitin sulfate chain on the α2(IX) chain. (See Eyre 1991, Prockop & Kivirikko 1995, Eyre *et al*. 2002).

Collagen X is a homotrimer of α1(X) chain coded by COL10A1 on chromosome 6q21-22. It consists of a single triple helical domain, a globular C terminal domain and a short non-collagenous N-terminal domain. It is expressed only by the hypertrophic chondrocytes. (Eyre 1991, Eerola *et al*. 1998, see also Myllyharju & Kivirikko 2001). The suggested function of collagen X is to transiently reinforce the extracellular matrix in the hypertrophic zone of growth plates (Schmid *et al*. 1990).

2.2 Type II and XI collagenopathies and multiple epiphyseal dysplasia (MED)

2.2.1 Achondrogenesis II, hypochondrogenesis, spondyloepiphyseal dysplasia congenita and Kniest dysplasia

Achondrogenesis II (MIM #200610), also known as Langer-Saldino type achondrogenesis, is characterised by a short trunk and very short extremities. It is

frequently associated with prematurity and hydrops fetalis; thus the affected babies are stillborn or die soon after birth. Radiographic findings include a severely underossified axial skeleton and very short tubular bones. In hypochondrogenesis, ossification of the vertebral bodies is less severely retarded and the tubular bones are longer. Also the ilia are larger and the ends of the long bones are less severely splaying. Patients with hypochondrogenesis are short with an oval, flat face, widely spaced eyes and often have cleft palate. In severe cases, respiratory difficulties are present early and these patients die within hours or weeks. In less severely affected patients, the respiratory distress is transient and patients may survive. These survivors are, however, often reclassified as having spondyloepiphyseal dysplasia congenita. (Spranger *et al.* 1994, International Working Group on Constitutional Disease of Bone 1998, Horton & Hecht 2002).

Clinically and radiographically, achondrogenesis II, hypochondrogenesis and spondyloepiphyseal dysplasia (SED) congenita (MIM #183900) form a continuous spectrum, with SED congenita representing the mildest end of this spectrum. These patients are characterised by a short trunk and, to a lesser extent, short extremities. The head and the facies are usually considered normal. The neck is short, and the chest is barrel-shaped with kyphosis and prominent lumbar lordosis. The limb shortening is prominent proximally and the hands are characteristically normal. Clubfoot and cleft palate are sometimes present. Also, myopia with or without retinal detachment occurs. Infants with SED congenita may be hypotonic. The skeletal features become more apparent with time, and radiographic changes involve the spine and proximal extremities. The vertebral bodies of a newborn are ovoid and there is defective ossification at several sites. A wide range of clinical variability is seen in the long bone changes. Severely affected patients show marked metaphyseal abnormalities that are not seen in mild cases. Patients with severe metaphyseal alterations have been considered to have a distinct disorder called Strudwick-type spondylometaepiphyseal dysplasia (SEMD). Recent observations suggest, however, that SED congenita and SEMD are related disorders and a distinction between them is no longer useful. (Spranger *et al.* 1994, International Working Group on Constitutional Disease of Bone 1998, Horton & Hecht 2002).

Patients with Kniest dysplasia (MIM #156550) are more severely affected than patients with SED congenita. Kniest dysplasia patients have a short trunk and extremities and in contrast to SED congenita, the hands are also affected. Many of these patients are shorter than SED congenita patients. Joints are often large at birth and continue to enlarge during the childhood. Severe joint contractures and kyphoscoliosis are present. The face is round with midface hypoplasia and a flat nose. Associated findings include cleft palate and retinal detachment. Radiographically there is severe platyspondyly and tubular bones are shorter and wider at their ends than in SED congenita. (Spanger *et al.* 1994, International Working Group on Constitutional Disease of Bone 1998, Horton & Hecht 2002).

Inheritance of all the syndromes described above is autosomal dominant and all have been shown to result from mutations in the gene coding for collagen II. Most mutations are single base mutations that lead to the substitution of an obligatory glycine (Kuivaniemi *et al*. 1997). In 1989 Vissing *et al.* described a patient with achondrogenesis II who had a serine instead of a glycine at position 943. Bonaventure *et al.* (1995) described a substitution of aspartic acid-310 for glycine in achondrogenesis II. Several glycine substitutions have been reported in hypochondrogenesis (Bogaert *et al.* 1992,

Horton *et al.* 1992, Freisinger *et al.* 1994, Bonaventure *et al.* 1995). Körkkö *et al*. (2000) analysed 12 patients with achondrogenesis type II/hypochondrogenesis for mutations in the COL2A1 gene and found ten single base substitutions, one splicing defect and one 18 bp deletion of the coding sequence. They demonstrated that most, if not all, patients with this syndrome have a COL2A1 gene mutation. In SED congenita, several glycine substitutions, a cysteine for arginine substitution, two in-frame duplications and one in-frame deletion of the COL2A1 gene have been reported (Lee *et al.* 1989, Tiller *et al.* 1990, Chan & Cole 1991, Chan *et al.* 1993, Vikkula *et al.* 1993, Winterpacht *et al.* 1993, Chan *et al.* 1995). Several small deletions and single base substitutions resulting in splicing defect and exon skipping have been reported in patients with Kniest dysplasia (Anderson *et al.* 1990, Winterpacht *et al.* 1993, Spranger *et al.* 1994, Spranger *et al*. 1997, Weis *et al*. 1998, Wilkin *et al*. 1999, Yokoyama *et al*. 2003).

Three mutations in the COL2A1 gene that replace arginine codons at positions 75 (three patients), 519 (five patients) and 789 (two patients) with cysteine in the triple-helical domain have been reported (Kuivaniemi *et al*. 1997). Three of the five families with cysteine-519 for arginine apparently have an early Icelandic founder (Bleasel *et al.* 1996a). Cysteine is not normally present in the triple-helical domain of collagen II. This is also the only time reported when an amino acid substitution in the Y position results in a disease phenotype. The phenotype of the patients with the substitutions at positions 75 and 789 was SED, and the patients with the 519 substitution had early onset osteoarthrosis (OA) with mild SED (Ala-Kokko *et al*. 1990, Williams *et al*. 1995, Bleasel *et al*. 1996b, Kuivaniemi *et al.* 1997).

2.2.2 Stickler and Marshall syndromes

Stickler syndrome (MIM #108300, #604846, #184840) is an autosomal dominant disorder that affects the eyes, ears and skeleton. It is also known as hereditary progressive arthro-opthalmopathy. Ocular abnormalities include high myopia, retinal detachment, glaucoma, premature cataracts, optically empty vitreous cavities and retinal pigmentary changes. Non-ocular manifestations show a great variation in expression. Typical facial features include a flat mid-face with depressed nasal bridge, a short nose, anteverted nares and micrognathia. Cleft palate is often present in Stickler syndrome. The severity of the oral cleft can vary from a cleft of the soft palate to Pierre Robin sequence. Mild SED can often be defined radiologically, and there is joint hypermobility which declines with age. OA develops typically in the third or fourth decade of life. Mild sensorinoural deafness to high tones may be present, however, stature and intelligence are normal. Stickler syndrome diagnosis may be delayed due to variable expressivity. In some cases, systemic problems may be mild or non-existent (Richards *et al.* 2000, Parma *et al.* 2002, Donoso *et al.* 2003). There is also a form of Stickler syndrome in which the eyes are unaffected. (Stickler *et al.* 1965, Stickler & Pugh 1967, Hermann *et al.* 1975, Temple 1989, Snead & Yates 1999, Bowling *et al.* 2000).

Marshall syndrome (MIM #154780) holds most of the same characteristics as Stickler syndrome. It has been long debated whether these syndromes are different entities or represent the same clinical entity. There have been some suggestions regarding the

differencies between Marshall and Stickler sydromes. It has been observed that Marshall patients more often have a short stature, deafness, and abnormalities in cranial ossification with more-pronounced dysmorphic features including a retracted midface with flat nasal bridge, short nose, anteverted nostrils and a long philtrum. It has also been suggested that retinal detachment occurs less frequently in patients with Marhsall syndrome than in Stickler syndrome. (O'Donnell *et al.* 1976, Ayme & Preus 1984, Stratton *et al.* 1991, Shanske *et al.* 1997, Annunen *et al.* 1999a).

Wagner syndrome (MIM #143200) is a dominantly inherited ocular syndrome characterised by myopia and vitreoretinal degeneration, and non-ocular features are virtually non-existent. Some Wagner syndrome patients have been reported to have some of the non-ocular features of Stickler syndrome such as cleft palate and micrognathia. This has lead to some problems in distinguishing between these two syndromes. (Liberfarb *et al.* 1979, Temple 1989, see Horton & Hecht 2002). Recently, however, Wagner syndrome has been linked to 5q13-14 (Brown *et al*. 1995, Perveen *et al*. 1999). Parma *et al.* (2002) compared eye findings in these two syndromes and concluded that they probably really are separate entities and not an expression of Stickler syndrome variability.

Mutations in Stickler syndrome have been characterised in the COL2A1, COL11A1 and COL11A2 genes (see Kuivaniemi *et al.* 1997, Annunen *et al.* 1999a, Richards *et al.* 2000). Most of the Stickler mutations described so far in the COL2A1 gene have been substitutions, deletions or splicing mutations that cause premature termination of translation and lead to haploinsufficiency (Ahmad *et al.* 1991, Brown *et al.* 1992, Ahmad *et al.* 1993, Ritvaniemi *et al.* 1993, Ahmad *et al*. 1995, Williams *et al.* 1995, Donoso *et al*. 2002). In addition, two Stickler causing mutations in the COL2A1 gene have been described to alter an amino acid in the X position of the Gly-X-Y triplet in the triple helical region. These were a cysteine-365 for arginine substitution and a phenylalanine-467 for leucine substitution. (Richards *et al.* 2000). A substitution of glycine-97 by valine, exon skipping, and a multiexon deletion in the COL11A1 gene have been shown to result in Stickler syndrome (Richards *et al.* 1996, Martin *et al.* 1999). In addition, five patients with phenotypes overlapping those of both Stickler and Marshall syndrome were reported to have mutations in the COL11A1 gene. These mutations included a splicing mutation at the 5' half of the gene, deletions and amino acid substitutions. (Annunen *et al.* 1999a).

It has been suggested that Stickler patients with either the COL2A1 or COL11A1 gene mutations differ in their vitreous phenotype. The COL2A1 gene mutations produce a congenital "membranous" anomaly of the vitreous of all affected individuals, while the COL11A1 gene mutations produce a different "beaded" vitreous phenotype. These can be distinguished by vitreous slit-lamp biomicroscopy. (Martin *et al.* 1999, see Snead & Yates 1999, Richards *et al.* 2000).

In 1998 Griffith *et al.* published the first mutation in a large family with Marshall syndrome. It was a splicing defect in the COL11A1 gene that caused the in–frame deletion of a 54 bp exon. In 1999 Annunen *et al.* reported ten new mutations in the COL11A1 gene in patients with Marshall syndrome. All of those mutations altered splicing consensus sequences and resulted in 54 bp exon deletions in the 3' half of the gene. Eight of them were in exons 48-54, and four of these eight were located in exon 50. In this report, Annunen and her co-workers also reported eight new COL2A1 gene

mutations in Stickler patients and five new COL11A1 gene mutations in patients with a phenotype overlapping both, Stickler and Marshall syndromes. They compared the clinical features of the Stickler and Marshall patients and concluded that although these two syndromes are quite similar, there are some clear differences between the phenotypes. Almost all patients with the COL11A1 gene mutations had moderate to severe hearing defects that were congenital or were detected in early childhood. Patients with the COL2A1 gene mutations had normal hearing or minor hearing loss that usually developed later in life. But these patients had more severe ocular findings than patients with the COL11A1 gene mutations. Almost all of the patients with the COL2A1 mutations had vitreoretinal degeneration and retinal detachment. Also cataracts were more common in these patients. In addition, there were slight differences in the facial features and stature between these two groups. Midfacial hypoplasia, a short nose and flat nasal bridge were more pronounced in Marshall patients and did not disappear as the child aged. Cranial abnormalities were only present in Marshall patients. These abnormalities included abnormal frontal sinuses, intracranial calcifications and thickened calvaria.

Since the COL11A2 gene is not expressed in the vitreous body of the eye, mutations in this gene do not result in eye abnormalities. For this reason, Stickler syndrome patients who have mutations in the COL11A2 gene lack the eye involvement, but have other findings characteristic for Stickler syndrome. Vikkula *et al.* (1995) reported a non-ocular Stickler family with an in-frame exon skipping of COL11A2. Sirko-Osadsa and others (1997) reported another non-ocular Stickler patient, this time with a 27 bp deletion in the COL11A2 gene. The patients with non-ocular Stickler syndrome seem to have a higher prevalence of sensorineural hearing loss than the patients with classical Stickler syndrome (Admiraal *et al.* 2000, Vuoristo *et al***.** 2004).

In 1993 Körkkö *et al.* reported a Wagner syndrome family with a substitution of aspartic acid-67 for glycine in the α1(II) chain. The affected individuals had early-onset cataracts, lattice degeneration of the retina and retinal detachment. No findings suggesting non-ocular involvement were found, as is typical for Stickler syndrome. This finding suggested that Stickler and Wagner syndromes are related at molecular level but are distinct entities. In light of recent findings (Parma *et al*. 2002), it seems likely that the patients' phenotype was predominantly ocular Stickler syndrome and not Wagner syndrome.

2.2.3 Otospondylomegaepiphyseal dysplasia (OSMED)

In 1982 Giedion and his co-workers introduced the term oto-spondylo-megaepiphyseal dysplasia (OSMED, MIM #215150) to describe a subgroup of patients with spondyloarthropathy. They presented clinical data from four of their own patients and reviewed two previously reported OSMED cases (Insley & Astley 1974). One of these four patients had been originally described by Weissenbacher & Zweymüller in 1964 after a three-month follow-up from birth. He was reported to have Robin sequence, rhizomelic shortness of the limbs with dumbbell-shaped femora, coronal vertebral clefts and normal stature. The condition was named Weissenbacher-Zweymüller syndrome

(WZS, MIM #277610). A follow-up of this patient at age of 24 led to the incorporation of this case into the OSMED paper. The authors stated that the main clinical findings of OSMED were sensorineural deafness, enlarged 1st interphalangeal joints of the hands, relatively short extremities with abnormally large knees and elbows and normal total body length. Cleft palate was also a common finding. The diagnostic radiological findings were large epiphyses combined with a moderate platyspondyly, most marked in the thoracic region. The authors admitted that although OSMED is well defined, it is still possibly heterogeneous bone dysplasia. Later on Salinas *et al.* in 1986, Johnston *et al.* in 1987 and Kääriäinen *et al*. in 1993 reported additional patients with the OSMED phenotype.

In 1995 Vikkula *et al.* reported an OSMED family with a recessive mutation in the COL11A2 gene. This was a glycine-175 to arginine substitution in the main triple helical region of the α2(XI) chain. Clinical findings of this family were reported in detail by Steensel *et al.* in 1997. The findings included midface hypoplasia, sensorineural deafness and epiphyseal dysplasia. None of the family members had cleft palate. In 1998 Pihlajamaa and co-workers reported a mutation in the COL11A2 gene in the original case with Weissenbacher-Zweymüller syndrome that was also included after a follow-up to the original OSMED paper (Giedion *et al*. 1982, Weissenbacher & Zweymüller 1964). It was a heterozygous single base substitution that converted glycine-955 to glutamate. Based on clinical and molecular findings, the authors of this paper proposed that syndromes caused by mutations in the COL11A2 gene should be divided into two groups called heterozygous OSMED and homozygous OSMED (see also Spranger 1998). The former group would include the Weissenbacher-Zweymüller and non-ocular Stickler syndromes and the latter, OSMED cases. Pihlajamaa *et al.* (1998) summarised that the homozygous OSMED patients had a more pronounced midface hypoplasia and larger epiphyses compared to patients with heterozygous OSMED. Homozygous patients also had a shorter stature than the heterozygous patients.

2.2.4 Non-syndromic hearing loss (DFNA13)

Hearing impairment is an common feature in phenotypes caused by the COL11A2 gene mutations. The hearing deficit is usually severe and sensorineural. (Spranger 1998, McGuirt *et al.* 1999). McGuirt *et al.* (1999) reported two mutations in the COL11A2 gene that caused non-syndromic deafness previously mapped to the DFNA 13 locus on chromosome 6p (MIM #601868). They presented two families with mutations. One of them had an arginine-549 to cysteine substitution and the other had glycine-323 to glutamate substitution. Both substitutions are located in the major triple helical part of the α2(XI) molecule. Patients had non-progressive hearing loss (60-70dB) predominantly affecting middle frequencies and presented no other features usually present in the COL11A2 mutation phenotype.

2.2.5 Multiple epiphyseal dysplasia (MED)

Multiple epiphyseal dysplasia (MIM #132400) affects mainly the epiphyses. Patients have prominent, frequently painful joints with restricted mobility and normal or mildly short stature. MED can present itself in childhood as waddling gait, easy fatique, or joint pain after exercise. Mildly affected patients may not be detected until adulthood, when joint complaints are noted, with possibly a slightly short stature. At this time, OA diagnosis is usually made instead of MED. Hips and knees are usually affected, but problems with the ankles, shoulders, wrists and hands can occur too. The disease can be detected by radiographic evaluation before symptoms appear. In the initial stage epiphyseal ossification is delayed while later on the ossification centers are small and sometimes fragmented. In adulthood the articular surfaces resemble osteoarthrosis being irregular and flattened (see Horton & Hecht 2002, see Chapman *et al*. 2003).

Several mutations in patients and families with autosomal dominantly inherited MED have been reported. These mutations are located in the genes coding for collagen IX, COMP (cartilage oligomeric matrix protein) and matrilin-3. All collagen IX mutations are located in the COL3 domain of the protein and lead to the skipping of an exon or exons. The COL3 and NC4 domains have been suggested to mediate interactions between collagen IX and other components of the cartilage extracellular matrix. It is thus possible that mutations in COL3 may interfere with these interactions. (Czarny-Ratajczak *et al.* 2001, Unger & Hecht 2001, see Chapman *et al*. 2003).

A recessively inherited form of MED also exists. It has been found to result from mutations in the diastrophic dysplasia sulfate transporter (DTDST) gene. (see Superti-Furga & Rossi 2001).

2.3 Oral clefts and Robin sequence

2.3.1 Oral clefts (MIM #119530)

Cleft palate with or without cleft lip is one of the most common congenital malformations. One out of 1000 caucasian newborns have cleft palate. Clefts in humans are divided into those that encompass the lip with or without cleft of palate (CL/P) and to those that affect only the palate (CPO). Prevalence rates for CL/P are slightly higher and show more variation between populations than CPO. This division is supported on embryological grounds as well as by family studies that have shown that it is unusual to find CPO and CL/P in the same family. Though more than 300 syndromes with oral clefts have been reported, most of the oral clefts are considered non-sydromic. (See Murray 1995, Wyszynski *et al.* 1996).

Non-syndromic oral clefts have a substantial genetic component and show strong familial aggregation. They may be defined as complex traits since these clefts do not exhibit classic Mendelian inheritance attributable to any single locus. (See Wyszynski *et al.* 1996). Twin studies have provided concordance rates for monozygotic twins varying

from 36% to 42% for CL/P and from 22% to 50% for CP (See Fraser 1970, Gorlin *et al.* 1990, Christensen & Fogh-Andersen 1993, Wyszynski *et al*. 1996). Allelic association and linkage studies have been used in an attempt to identify the underlying genes for non-syndromic oral clefts. These studies have given contradictory results. This is not surprising since it seems evident that multiple genes are involved in the craniofacial development. (See Murray 1995, Hibbert & Field 1996, Wyszynski *et al.* 1996). Hibbert & Field (1996) wrote that since there are only a limited number of ways in which abnormal human development can manifest itself, it is therefore feasible that the same clinical condition may result from different genetic anomalies. What may increase the confusion is that the same mutation in two persons with different genetic background may produce two distinct phenotypes. Linkage and association studies have suggested some role in non-syndromic facial clefting for chromosomes 2q, 4q, 6p, 17p and 19q, the beta 3 subunit of the gamma-aminobutyric acid receptor (GABRB3), transforming growth factor α (TGFA), retinoic acid receptor α (RARA), a homeobox gene MSX1, epidermal growth factor, epidermal growth factor receptor, glucocorticoid receptor and estrogen receptor (see Murray 1995, Wyszynski *et al.* 1996, Scapoli *et al.* 1997, Sertie *et al.* 1999, Hecht *et al*. 2002, Scapoli *et al.* 2002, Zeiger *et al.* 2003). Murray (1995) and Hibbert & Field (1996) concluded that no research has conclusively determined the site of the major locus for CL/P, but the evidence suggests a potential major gene on 6p, and a modifying role for TGFA. Machida *et al*. (1999) published the genomic structure of TGFA and mutation analysis of 202 non-syndromic CL/P cases and 89 non-syndromic CP cases. While seven sequence variations were found in the 3' untranslated region, no direct evidence for dysfunction of the TGF-α protein were detected.

The only data for identified mutations in isolated cleft palate patients comes from Jezewski *et al*. (2003). They found five mutations in seven patients with isolated CPO in MSX1 gene or in its highly conserved non-coding sequencies. These seven cleft patients represented 2% of their panehtnic cleft study set.

Environmental influences to craniofacial development have also been studied. Prescribed drugs such as phenytoin, retinoic acid derivatives and methotrexate, among other drugs like alcohol and cigarette smoking have been associated with clefting (see Murray 1995, Granzow *et al*. 2003). Excessive administration of retinoic acids to pregnant women is known to cause micrognathia and cleft palate (Benke 1984, Lammer *et al.* 1985). Treatment of pregnant mice with retinoic acid in mid-gestation produces cleft palate and limbs defects. It has also been shown that administration of folic acid and methionine can reduce the frequency of retinoic acid-induced clefting in mice. (Reynolds *et al.* 2003).

2.3.2 Robin sequence

Robin sequence (RS, MIM #261800) is a neonatal triad of micrognathia, cleft palate and glossoptosis/upper airway obstruction. It bears the name of French stomatologist Pierre Robin who published his observations in 1923. (Robin 1923, Robin 1934). In the sequence, all or some of the associated anomalies are caused secondarily by one of the anomalies present. In Pierre Robin sequence, the primary anomaly has long been thought

to be micrognathia. (Shprintzen 1992, Olney *et al.* 1997, see Prows & Bender 1999). However, some evidence has recently been presented that suggest a primary defect role for cleft palate instead of micrognathia (Cohen *et al.* 1994, Marques *et al.* 1998). Then again the work of Ricks *et al*. (2002) with Disproportionate micromelia mice (Dmm) that present both micrognathia and cleft palate at birth, supported the original hypothesis of micrognathia as the underlying defect for RS.

RS occurs in at least 1 out of 8 850 live births. Neonates with RS often have respiratory obstruction varying from mild to severe. In most cases, conservative treatment with prone positioning and close observation is sufficient. More severe cases necessitate nasopharyngeal or endotracheal intubation, tracheostomy, lip-tongue adhesion or mandibular traction. RS neonates' mortality ranges between 2.2% to 26%. Death results mainly from obstructive apnea or failure to thrive. (Olney *et al*. 1997, see Prows & Bender 1999, van den Elzen *et al*. 2001).

RS is often present as part of a syndrome. Most frequently it is associated with Stickler syndrome, velocardiofacial syndrome, cerebrocostomandibular syndrome, fetal alcohol syndrome and trisomy 18. Therefore, RS diagnosis should be the first step of a diagnostic process instead of being the last. The percentage of isolated RS cases varies from one report to another, being between 11 to 75%. (van den Elzen *et al*. 2001).

Cohen (1999, 2001) clarified that RS is causally heterogeneous and pathogenetically variable. Thus, it can be malformational, when based on intrinsic mandibular hypoplasia, or deformational when based on constraint. This distinction has clinical implications for catch-up growth. In addition to this, RS is also phenotypically variable. Cohen suggested that the classical Robin sequence term should be used when micro/retrognathia and cleft palate are present with glossoptosis. When breathing difficulty results from other types of pharyngeal respiratory compromise or from the central nervous system, the triad should be called the Robin complex. In 1999 Cohen defined RS with four different groups all presenting mandibular defect with variable combinations of upper airway obstruction and U- or V- shaped cleft palate. In 2001 he added a fifth group to this definition, one that straddles the interface between RS and ordinary cleft palate. In group five, cleft palate is present but the mandible is normal or slightly small and some respiratory compromise is evident with polysomnography.

In 2001 Houdayer and others reported association of RS with deletion 2q32.3-q33.2 due to an unbalanced chromosomal translocation t(2;21). This deletion maps to a previously determined chromosomal region known to associate with CP. An association between isolated Robin sequence and balanced chrosomal translocation t(2;17) was reported in 2004 by Jamshidi *et al*.

2.4 Osteoarthrosis

Softening, fibrillation, ulceration and eventually loss of articular cartilage with sclerosis of subchondral bone, osteophytes, and subchondral cysts are common findings in osteoarthrosis (MIM #165720). OA symptoms include joint pain, tenderness, limitation of movement, crepitus, occasional effusion and variable degrees of inflammation without systemic effects. While the mechanism of how OA develops is somewhat unclear, it has

been stated that OA results from both mechanical and biological events that destabilise the normal coupling of degradation and synthesis of articular cartilage chondrocytes, extracellular matrix and subchondral bone. (Creamer & Hochberg 1997).

OA is the most frequent cause of musculoskeletal disability in developed countries and generates huge costs for society. In 2001 Arokoski and others estimated that for Finland it meant costs of hundreds of millions (Fim) per year. The prevalence of OA increases with age from 4% (18-24 years) to 85% (75-79 years). It is classified as primary (idiopathic) and secondary OA. Trauma, obesity, joint surgery, congenital or developmetal diseases of bone, and metabolic diseases, such as hyperparathyreosis, are common causes of secondary OA. OA that develops without such factors is titled primary. (Moskowitz 1989, Cicuttini & Spector 1996, Creamer & Hochberg 1997).

2.4.1 OA genetics

Primary OA is considered to be, besides common and multifactorial, also an oligogenic, genetic disease. Twin and family studies have revealed OA to have a major genetic component. It is rarely transmitted as a Mendelian trait and environmental factors play a significant role in disease expression. Linkage and association analyses have identified several regions that may harbor OA suceptibility genes. The number of these locations together with the failure to repeat these results in different populations and patient groups support the ideas that several genes are involved in OA pathogenesis and the genetic background of OA varies between populations, sexes, and affected joints. (see Loughlin 2001, Spector *et al*. 1996).

The first evidence of genetic factors in OA was presented by Stecher in 1941. He showed that Heberden's nodes of the fingers were three times as common in the sisters of 64 affected subjects as in the general population. Subsequent studies provided similar results for the familiar occurrence of hand and generalised OA (Allison & Blumberg 1958, Crain 1961, Kellgren *et al*. 1963). More evidence of Heberden's nodes heritability was provided by studies which showed the association of hand OA and HLA haplotypes (Ercilla *et al*. 1977, Pattrick *et al*. 1989).

In 1996 Spector *et al*. published the first large-scale twin study. The subjects were examined with radiographs of the hands and knees. The study revealed a heritability of 39% to 65% with a concordance rate of 0.64 for monozygotic twins and 0.38 for dizygotic. A twin study focusing on the radiographic hip OA in female twins obtained a heritability value of 50 % (MacGrecor *et al*. 2000). Kaprio *et al*. (1996) did a twin study which included patients with OA in any joint group. Both men and women were included. The obtained heretability value was 44% for women, and no genetic component was detected for men.

A heritability of 27% for severe OA of the knee and hip was calculated by Chitvanis *et al.* (1997) for a set of UK siblings. Hirsch *et al*. (1998) determined a sibling correlation for OA among patients collected by the Baltimore Longitudinal Study of Ageing. They showed aggregation of OA, particularly in families with severe polyarticular disease. The study by Felson's *et al*. (1998) revealed a significant genetic component in hand and knee OA diagnosed by radiographs. They collected families with both parents and at least one

offspring. In addition, the results showed a consistent correlation for the OA genetic effect between family pairs involving women, but not for family pairs involving men. Bijkerk *et al*. (1999) were also able to show a high level correlation for hand OA (0.56 heritability) in a set of Dutch probands with radiographic OA, but no correlation for knee or hip OA.

Several groups have reported linkage analysis of candidate genes to OA pedigrees with dominant trait inheritance and incomplete penetrance. However, the affected probands were diagnosed later on to have mild osteochondrodysplasias. (Loughlin 2001). Recently Meulenbelt *et al*. (1997) reported a genome-wide scan of a large Dutch pedigree with knee, hand and spine OA but no chondrodysplasia. OA in this family was linked to markers in chromosome 2q. In another family of Dutch origin with hip OA, the disease was linked to chromosome 4q35 (Roby *et al*. 1999). In an Icelandic pedigree with hip OA, the strongest evidence for linkage was obtained with markers on chromosome 16p. (Loughlin 2001).

Affected sibling pairs have also been used as material for linkage analysis in OA. These analyses have been done using either the candidate gene approach or a genome-wide scan. Loughlin *et al*. (1994) tested linkage in a small cohort of UK sibling pairs with generalised OA to COL2A1, cartilage link protein gene (CRTL1) and the cartilage matrix protein (CRTM). None of them were positive. Mustafa *et al*. (2000) used a number of candidate genes with almost 500 affected sibling pairs with OA in either the hip or knee or both joints. Suggestive linkage was obtained to COL9A1 in female pairs with hip OA (LOD score 2.3). (Loughlin 2001). Genome-wide scans with affected sibling pairs have identified several suggestive loci for OA ascertained either radiographically or by joint replacement surgery. Wright *et al*. (1996) reported linkage to 2q in set of siblings with hand OA. A set of Finnish patients with hand OA showed linkage to 2q12-q21, 4q26-q27, 7p15-p21 and the X centromeric region in a genome-wide scan by Leppävuori *et al*. (1999). The families used by Mustafa *et al*. (2000) were subjected to genome-wide linkage analysis. A two-step analysis showed suggestive a linkage to chromosome 2q and 11q. Further analysis revealed that 2q linkage was principally founded in affected pairs with hip OA, and 11q linkage in female hip OA pairs. (Chapman *et al*. 1999, Loughlin 2001). Loughlin *et al*. (1999) stratified the data obtained in the first step of the Chapman *et al*. study and revealed additional regions of linkage. Female hip pairs showed linkage to 4q12-q21.2 and to 16p13.1-q12.1 and hip pairs to 6p21.1-q22.1.

Several OA association studies have been done using case-control cohorts. These studies have focused around twelve candidate genes that each either code for structural proteins of the extracellular matrix or bone or function in the regulation of bone mass or density. Patient groups used for these studies included both sexes and all types of primary OA. Of the collagen genes, COL1A1, COL2A1, COL9A1 and COL11A2 have shown a positive association with OA. Only two of the twelve candidate genes reside in a chromosomal region that has positive linkage in the genome-wide scans. Therefore, major genes for OA susceptibility are still to be identified (Loughlin 2001).

2.5 Animal models for cartilage collagen diseases

Genetically modified mice models have been used in several studies to clarify the role of cartilage collagen genes in normal and abnormal cartilage and bone development and function.

2.5.1 Collagen II

Vanderberg *et al.* (1991) presented transgenic mice with a large central deletion of human COL2A1. A large proportion of these mice expressing the deleted gene developed a chondrodysplasia with dwarfism, short and thick limbs, a short snout, a cranial bulge, a cleft palate, and delayed mineralisation of bone. A number of mice died shortly after birth. Microscopic evaluation of cartilage revealed decreased density and organisation of collagen fibrils. Abnormal collagen was disulfide-linked to normal mouse pro-α1(II) chains. The authors concluded that the phenotype was probably a result of the depletion of endogenous mouse type II collagen through procollagen suicide. Helminen *et al*. (1993) studied these trangenic mice further. They found that comparison of these mice at the age of 1 day and 15 months revealed that there was decreasing evidence of a chondrodysplasia as the mice grew older. They also noted that at the age of 15 months the most striking feature of these mice was degenerative changes of articular cartilage similar to osteoarthrosis.

Garofalo *et al*. (1991) generated transgenic mice with a glycine to cysteine mutation in residue 85 of the triple-helical domain of the α1(II) chain. These mice presented severe chondrodysplasia with short limbs and trunk, cranio-facial deformities and cleft palate. The mice died right after birth of acute respiratory distress. Closer analysis of the skeleton indicated severe retardation of growth for practically all bones. The authors saw a strong resemblance of these findings to those of patients with SED. They proposed that the consequence of the mutation is a reduction in the density of the typical thin cartilage collagen fibrils, and this causes severe disorganisation of the growth plate. Metsäranta *et al*. (1992) produced trangenic mice with a deletion of exon and intron 7 of Col2a1. They expected this mutation to disturb the assembly and processing of the collagen II molecule. These mice had virtually the same clinical findings as the transgenic mice with the glycine to cysteine mutation (Garofalo *et al*. 1991). In microscopic evaluation, they noted a defect in endochondral ossification. It was concluded that this deletion acts as a dominant negative mutation disrupting the assembly and secretion of collagen II molecules. Later on, a transgenic mouse line that over-expressed collagen II was generated. It was found that the overproduction disrupted the mechanisms that control fibril assembly (Garofalo *et al*. 1993).

Shi-Wu Li and co-workers (1995) generated Col2a1 knockout mice. Heterozygous mice were found to have a minimal phenotype whereas homozygous died just before or right after birth. These mice had no endochondral bone or epiphyseal growth plate in their long bones. However, many skeletal structures such as the cranium and ribs were normally developed and mineralised. Sahlman *et al*. (2001) also studied heterozygous Col2a1 knockout mice and found that at birth these mice had shorter limb bones, skulls,

and spines, as well as thicker and more irregular vertebral endplates. At the age of 15 months, some of these features were compensated, but long bones and skulls remained shorter than those of wild-type mice. Lapveteläinen *et al*. (2001) studied these mice further and noted that heterozygous inactivation of Col2a1 made the mice more susceptible to OA.

Homozygous disproportionate micromelia (Dmm) mice have a severe shortening of the long bones, a shortened vertebral column, a small rib cage, and a cleft palate (Seegmiller *et al*. 1988). Pace et al. (1997) defined Dmm mice to have a three-nucleotide deletion in the region coding for the globular C-propeptide domain of collagen II. The mutation leads to the substitution of one amino acid, asparagine, for two amino acids, lysine and threonine. They showed that the amount of collagen II was significantly reduced in Dmm mice and that the mutation dramatically affected interchain disulfide bond formation.

Saamanen *et al*. (2000) generated transgenic mice with a small deletion in Col2a1 and found that this truncated gene, together with a maturation related reduction in collagen II production significantly contributed to the early-onset degeneration of knee joints in mice heterozygous for the defective gene.

Matyas and his co-workers (2002) studied the expression of canine collagen II and aggrecan core protein in artificially induced OA of the knee joint. They found that as OA progressed the gene expression of both proteins elevated. The number of collagen II mRNA copies exceeded that of aggrecan in OA tissue, whereas the ratio was found to be the opposite in control cartilage tissue.

2.5.2 Collagen XI

Mice that are homozygous for the autosomal recessive chondrodysplasia (cho) mutation die at birth with abnormalities in the cartilage of limbs, ribs, mandible, and trachea. Limb bones are wider at the metaphysis and only half of the length of normal bones. The underlying mutation was found to be a deletion in the collagen α1(XI) gene that results in premature termination of translation. Therefore, the cho mice are functional knockouts. This data demonstrated that collagen XI is essential for the normal formation of cartilage collagen fibrils and that normal differentiation and spatial organisation of growth plate chondrocytes is critically dependent on the presence of collagen XI. (Li *et al*. 1996). Lavrin *et al*. (2001) studied cho mice in order to find out the mechanisms underlying cleft palate in these mice. They found that the major effect of the mutant collagen on the palate is likely to be via mandibular growth disruption and not the inability of palatal shelves to fuse. Xu *et al*. (2003) studied in detail the articular cartilage of mice heterozygous for the cho mutation. They hypothesised that the reduced amount of collagen XI in cartilage may be one of the initiation factors in the pathogenesis of OA. They found that the diameter of collagen fibrils in articular cartilage was increased and that OA-like degenerative changes appeared at the age 3 months and became more severe with aging.

Li *et al*. (2001) produced a Col11a2 knockout mice line. These mice had a milder phenotype than those of functional Col11a1 knockouts. They had a small body size, receding snouts, and deafness. Chondrocytes in growth plates of all long bones were

markedly disorganised. No obvious OA was observed in these mice up to one year of age, although some minor morphological abnormalities in the articular cartilages were noted.

2.5.3 Collagen IX

The COL9A1 is the only one of the three collagen XI genes whose function in cartilage disorders has been modelled using transgenic or knockout approaches. Nakata *et al*. (1993) showed that transgenic mice expressing the α1(IX) collagen chain with a central deletion had articular cartilage changes in the knee joints resembling OA. Homozygous mice also had mild chondrodysplasia with mild dwarfism, changes in vertebral bodies and ophthalmopathy. In 1996 Kimura *et al*. took another look at these mice and noted that in addition to OA, the mice had accelerated intervertebral disc degeneration. Therefore, they concluded that mutations of this gene may cause certain forms of degenerative disease in the spine as well as in joints. Fässler *et al*. (1994) studied the Col9a1 knockout mice and noted these mice develop severe degenerative joint disease resembling that of human OA. Haimes et al. (1995) also found changes similar to OA in their mice with over expression of the globular N terminal domain of the α1(IX) chain. These results taken together suggested that collagen IX plays an important role in maintaining cartilage integrity (Haimes *et al*. 1995). Using Col9a1 knockout mice (Fässler et al. 1994) Hägg *et al*. (1997) showed that the absence of the α1(IX) chain leads to the functional knockout of the entire collagen IX protein. They also found out that collagen IX is not essential for fibrillogenesis in cartilage, but is required for long-term tissue stability, presumably by mediating interactions between fibrillar and other macromolecules.

3 Outlines of the present research

When this study was initiated, only two mutations had been reported in the COL11A2 gene. Compared to the major cartilage collagen, collagen II, collagen XI had not been studied extensively and there was only a fairly limited amount of information available about collagen XI. One of the reported mutations was found in a family with OSMED and the other in a large family with non-ocular Stickler syndrome.

Syndromes reported to result from mutations in genes coding for cartilage collagens II and XI often include cleft palate in their phenotypes. Isolated cleft palates have also been shown to have a clear genetic component. Since mutations in collagen II seem mostly to result in more severe phenotypes, it seemed possible that mutations in the genes coding for a minor cartilage collagen, collagen XI, could result in milder phenotypes such as isolated cleft palate. We also wanted to find out what type of mutations in COL11A2 resulted in more severe phenotypes, like OSMED, and what sort of mutations would cause the milder end of the phenotypic spectrum.

Primary osteoarthrosis also has a strong genetic component in its etiology. A few mutations have been characterised in the COL2A1 gene in families with primary osteoarthrosis and mild chondrodysplasia. Although it has been shown that certain types of mutations in collagen II can cause osteoarthrosis, these mutations explain only a small number of cases in this common disease. We hypothesized that the minor cartilage collagens, collagens IX and XI, may play a more significant role in primary osteoarthrosis than collagen II.

The aims of this study, therefore, were:

1. to clarify the role of COL11A2 mutations in OSMED and to obtain more information about the function and importance of the α2(XI) chain,
2. to test our hypothesis that mutations in genes coding for collagen XI may cause isolated cleft palate and Robin sequence,
3. to reveal the role of cartilage collagens II, IX and XI in primary osteoarthrosis by screening the corresponding genes for mutations.

4 Materials and methods

The materials and methods are described in more detail in original articles I-III.

4.1 Patients and controls (I-III)

Patients with OSMED were referred to us by clinicians from Finland (Dr. H. Kääriäinen), The Netherlands (Drs. H.G. Brunner, R. Hennekam, T. van Essen), the USA (Drs. M.L. Warman, R.M. Pauli), France (Drs. J. Bonaventure, S. Manouvrier) and Switzerland (Drs. P. Miny, A. Superti-Furga). Clinical examination of the patients was performed as described in original article I.

Patients with isolated cleft palate or Robin sequence were selected from patient material of a center in Helsinki University Hospital focused on treating patients with clefts (HUSUKE) by Dr. Hannele Koillinen. She contacted the patients and selected the ones that met the selection criteria (see article II for details) and were willing to participate. Altogether 40 patient DNA samples were obtained for genetic analysis and referred to us. An additional group of 17 patients with non-syndromic micrognathia was collected from areas of New York and Cleveland and referred to us for genetic analysis by our collaborator Dr. Matthew L. Warman. Patients were evaluated and selected using mandibular radiographs and a health questionnaire. One North American patient with Robin sequence and one Finnish Marshall syndrome patient were also included. The patient with Robin sequence was referred to us by Drs. Deborah Krakow and Daniel Cohn, and the patient with Marshall syndrome was referred to us by Dr. Helena Kääriäinen. A random population sample of 150 unrelated Finnish adults with no personal or family history of oral clefts was used as the control in article II.

OA patients were selected from the patient population of the Department of Surgery in two Finnish hospitals; Kuopio University Hospital and the Central Hospital of Jyväskylä. Patients were referred to us by Drs. Heikki Kröger and Ilkka Kiviranta. Patients who were included in the study had (a) two or more affected joints or (b) one affected joint with a positive family history for OA or age of onset under 40 years. Other selection criteria included (c) an age of onset 50 years or under, (d) a body mass index (BMI) under 30, and (e) no history of trauma or joint infection. Seventy-two of the original 116

patients considered met these criteria and were included in the study. Of the 72 patients, 42 had only hip OA, 21 had only knee OA and 9 had both hip and knee OA. Thirty-three of them were females and 39 were males. One hundred three controls were selected from the same region. The controls consisted of 47 females and 56 males aged over 45 and with no history or symptoms of OA. A North American family with early-onset OA was also included in the OA study. They were referred to us for genetic study by Dr. Matthew L. Warman.

The studies were approved by the local ethical committee. Signed informed consent was obtained from all the subjects participating the studies. DNA used for analysis was extracted from venous blood by standard protocols.

4.2 CSGE (I-III)

Mutation analysis was done using the conformation-sensitive gel electrophoresis (CSGE) method (Ganguly *et al*. 1993, Körkkö *et al*. 1998). The sequences corresponding to the 66 exons of COL11A2, the 66 (original article II) or 68 (original article III) exons of COL11A1, and the 52 exons of COL2A1 and exon-flanking sequences were amplified by PCR to obtain products of 181bp to 441 bp (Ampli Taq Gold DNA polymerase, Applied Biosystems). To generate heteroduplexes for CSGE, the samples were denatured and re-annealed after PCR. The PCR products were analysed for quality and quantity on an agarose gel, followed by heteroduplex analysis by CSGE. Products that showed new or obscure patterns on CSGE analysis were sequenced. Since CSGE recognises only heterozygous sequence variations, samples with suspected homozygous variations (original article I) were mixed before the heteroduplexing phase with an equal amount from PCR product of a control sample.

4.3 Sequencing (I-III)

Sequencing was mostly carried out using an ABI Prism 377 or 3100 automatic sequenator and the dRhodamine or BigDye Terminator Cycle Sequencing Kit (Applied Biosystems).

PCR primers, or nested primers in some cases, were used for sequencing. If a possible disease causing sequence variation was found, extra samples and more detailed clinical data were collected from the patients' relatives when that was possible. In some instances a new blood sample was obtained from the patient or relatives to be used in RNA analysis.

4.4 RT-PCR (I-III)

Lymphoblasts from venous blood samples were immortalized by Epstein-Barr virus (EBV). Total RNA from EBV-transformed cells was extracted and used as a template for RT-PCR (Rapid RNA isolation Kit, Qiagen). The first strand was synthesised using

oligo(dT) or random hexameres (Superscript Preampliafication System, Gibco BRL). Obtained cDNA was amplified using PCR primers designed to flank the sequence of interest. Nested primers were used for the second PCR reaction. After each PCR, a few microliters of the reaction product were analysed on an agarose gel. After the second PCR, the products were sequenced using nested primers. Each RT-PCR was repeated at least once to make sure the results were accurate.

4.5 Genotyping (III)

Six to twelve single nucleotide polymorphisms (SNP) or small insertion/deletion polymorphisms initially detected in CSGE analysis were selected from each of the six genes. The selected polymorphisms were genotyped from the 72 unrelated Finnish probands with OA and from the 103 controls. The genotyping was done using either sequencing or restriction enzymes, if the polymorphisms altered a restriction enzyme recognition site (article III, Table 1).

4.6 Statistical analysis (III)

The computer program Polyphen, based on sequence alignment, was used to help us predict whether the observed amino acid subtitutions were likely to be pathogenic or not (Sunyaev *et al*. 2001, Ramensky *et al*. 2002).

The observed allele frequencies for all the genotyped polymorphisms were compared between cases and controls. Negative control analysis was also performed between the two groups of controls and cases (Jyväskylä vs. Kuopio) to make sure that the allele frequencies did not differ within controls or cases. Fisher's exact test was used for the analysis of individual diallelic polymorphisms, one polymorphism at a time. A joint analysis of all polymorphisms within a gene was also performed. This was done using the Dismult computer program, which implements a multiple two-point approach for linkage disequilibrium analysis (Terwilliger 1995).

All the statistical analysis was performed by Dr. Harald H. H. Göring at the Department of Genetics, Southwest Foundation for Biochemical Research, San Antonio, Texas.

5 Results

5.1 Analysis of OSMED patients (I)

Blood samples were obtained from seven unrelated OSMED families with 1-3 affected children (Fig. 1 in I). The families originated from Turkey, Morocco, The Netherlands, and Northern Europe. Three of the seven families had consanguineous parents. All 66 exons and the flanking sequences of the COL11A2 gene were amplified from the genomic DNA of one affected member of each family. The amplified products were analysed by CSGE together with the control samples. Also, equal amounts of the PCR products of the affected individuals were mixed with the control samples to generate heteroduplexes for CSGE analysis.

Several unique patterns were detected by CSGE, and all available family members were then analysed for the exons of interest. Sequencing revealed that all the patients had mutations in the COL11A2 gene (Table 1). All the families with consanguineous parents (A-C) and one non-consanguineous family (D) were homozygous for mutations. In the remaining three families (E-G), the affected individuals were compound heterozygous for the mutations. Nine of the ten mutations resulted or were predicted to result in premature termination of translation. One mutation altered the splicing consensus sequence. Analysis of all available parents (12/14) confirmed that all mutations were inherited rather than sporadic.

RNA analyses were conducted for two samples (E:I2 and G:I1) and one control to reveal the consequences of two intronic mutations (IVS22-2A→G and IVS53+5G→A). Total RNA extracted from immortalised lymphoblasts was used for RT-PCR. The PCR products were generated from exon 21 to exon 26, and from exon 50 to exon 56, respectively. The second PCR was done using nested primers. Analysis of the PCR product of E:I2 on an agarose gel revealed two bands of about 150 bp and 200 bp with equal intensities, but only one band of about 200 bp in the control sample. Sequencing of the shorter product indicated skipping of exon 23, and thus an in-frame deletion of 54 nucleotides. Analysis of the PCR products on an agarose gel showed no difference between the control and G:I2. Sequencing revealed that the G:I2's mutant allele utilised a

cryptic splice site in exon 53, eliminating 17 bp at the end of the exon. This frameshift results in a premature translation termination codon in exon 56.

The clinical findings of the parents, who were all heterozygous for one of the COL11A2 mutations, were mild or non-existent (Table II in paper I). The clinical findings of all OSMED patients were quite similar, affecting hearing, facial features, joints, spine and body proportions. Their mental development was normal. Other features are listed in Table 2.

Table 1. COL11A2 Mutations in the OSMED families.

Subject	Mutation[a]		Location	Expected
	Homozygous	Heterozygous		Consequence
A:II3,4	732delC		E5	Frameshift stop in E6
B:II3,4	2492C>A		E33	S345X
C:II1	2406-2409del; 2405-2410ins9bp		E31	Frameshift stop in e32
D:II1	4821-4843del		E64	Frameshift stop in E64
E:II2		1636C>T, IVS22-2A>G	IVS22, E17	Inframe deletion of E23 (54 bp), R60X
F:II1		3032-3033insC, 4750G>T	E41, E63	Frameshift Stop in E43, G1098X
G:II1,2		3991C>T, IVS53+5G>A	E55, IVS53	R845X, Frameshift stop in 56

[a]A from ATG of the initiator methionine codon is denoted as nucleotide +1

Table 2. Main Clinical Features of the OSMED Patients in Seven Families.

Feature	Patients									
	A:II3	A:II4	B:II3	B:II4	C:II1	D:II2	E:II2	F:II1	G:II1	G:II2
Age (years)	3	1,5	7	3	6	3	25	20	10	10
Height (cm)	86	78	115	89	106	83	150	147	122	124
Disproportionate shortness of the limbs	+	+	+	+	+	+	+	+	+	+
Facial features										
cleft palate	+[a]	+[a]	+	+	+	+	+	+[b]	+	+
micrognathia	-	-	+	+	+	+	-	+	+	+
mid-face hypoplasia	+	+	+	+	+	+	+	+	+	+
flat mala	+	+	+	+	+	+	+	+	+	+
flat nasal bridge	+	+	+	+	+	+	+	+	+	+
short nose	+	+	+	+	+	+	+	+	+	+
anteverted nares	+	+	+	+	+	+	-	+	+	+
long philtrum	+	+	+	+	+	+	+	+	+	+
hypertelorism	+	+	+	+	-	+	-	-	-	-
midfacial haemangioma	+	+	-	-	-	-	-	-	+	+
Enlarged joints	+	+	+	+	+	+	+[c,d]	+	+[c]	+[c]
Limited flexion of metacarpophalangeal joints	+	+	+	+			+	-	+	+
Pectus excavatum	nd[f]	nd	+	+	+	-	-	nd	+	+
Vertebral body anomalies	+	+	+	+	+	+	+[e]	+[e]	+	+
Kyphosis	+	+	+	+	+	-	+	+	+	+
Lumbar lordosis	-	+	+	+	+	+	+	+	+	+
Sensorineural hearing loss (dB)	80	50-70	80-90	100	70	60-110	90	50-90	90-95	90-95
High myopia	-	-	-	-	-	-	-	+	-	-

[a] cleft of soft palate [b] bifid uvula and submucosal palatal cleft [c] joint pain/stiffness [d] osteochondrosis in knee joint [e] osteochondrosis-like changes [f]nd = not determined

5.2 Analysis of patients with isolated Robin sequence, cleft palate and micrognathia (II)

Altogether 62 patients with non-syndromic Robin sequence, cleft palate or micrognathia were analysed for sequence variations in the COL11A2 gene. The 23 patients with Robin sequence were also analysed for the two other genes coding for collagen XI, COL11A1 and COL2A1. All the exons and flanking sequences of the COL11A1, COL11A2 and COL2A1 genes were amplified by PCR and analysed using CSGE. Samples that had heteroduplexes on CSGE analysis were sequenced to identify the underlying sequence

variation. Sequence variations were also scanned by CSGE for 47 control samples. If the detected variation seemed rare or was not found among the controls, an additional control set of 103 samples was scanned by CSGE.
Several heteroduplexes were detected. Sequencing revealed that most of the variations were located in the intronic sequences and less than 20% in the exons. Altogether nine nucleotide substitutions causing missense or nonsense mutations were found in these three genes. Six of them were unique in that they were not found in any of the 150 controls (Table 3).

Table 3. Unique sequence variations.

Gene	Nucleotide change	Predicted consequence	Diagnosis
COL11A2	E4+86C>T	R-310X	Robin sequence
	E13+4C>T	R-32W	Micrognathia
COL11A1	IVS31+92T>A	nd[1]	Robin sequence
	IVS45+3G>A	nd	Robin sequence, Marshall syndrome
	IVS50+3insT	Splicing defect	Robin sequence, Marshall syndrome[2]
COL2A1	E17+99C>T	nd	Robin sequence

[1]nd = not determined [2]Annunen *et al*. 1999; patient 6.

5.2.1 COL11A2 sequence variations

A single nucleotide mutation, C to T, was found in exon 4 of the COL11A2 gene in a patient with Robin sequence. This changed a codon CGA for arginine to TGA for translation termination. Since the termination codon was in exon 4 of the 66-exon gene, it is likely that the mutant allele cannot produce any functional protein. DNA analysis of the parents indicated that the patient had inherited the mutation from her father. Even though the father did not have Robin sequence, he had a partial phenotype involving a high-arched palate and a small, upturned nose.

A C to T change converting the codon CGG for Arg32 to TGG for Trp was found in exon 13 in a patient with micrognathia. This affected amino acid -32 in the short N-terminal non-triple helical region between the minor and major triple helical domains. This substitution was interesting because tryptophan is not found in this region in any of the fibrillar collagens. In addition, tryptophan substitutions in the α2 and α3 chains of collagen IX have recently been found to associate with lumbar disc disease (Ala-Kokko 2002). The patient also had early onset OA in one knee, mild disc degeneration at L4, normal palate, uvula and hearing. She wears reading glasses nowadays (in her fifties), but previously had normal vision. Her brother and son have normal jaws. Her niece has a small jaw, but refused DNA analysis.

5.2.2 COL11A1 sequence variations

Analysis of exon 50 revealed the insertion of a T at the donor splice site of intron 50, in nucleotide position +3 in a patient with Robin sequence. This variation was not present in any of the 150 control samples, but it has been reported earlier in a patient with sporadic Marshall syndrome (Annunen *et al*. 1999a; patient 6). The present male patient was of normal stature (186 cm) and had mild myopia (-1.5/-1.5D) but no hearing defect. All the other family members were unaffected. Neither of the parents had the mutation, and therefore, this patient also had a sporadic mutation. Even though RNA was not available to enable the consequence in terms of splicing to be examined, the mutation is predicted to result in a splicing defect (see Cartegini *et al*. 2002).

The second unique sequence variation, IVS45+3G>A, was in the COL11A1 gene in another patient with Robin sequence. The mother was found to have the same sequence change. She had died of diabetic complications at the age of 28. Neither parent of patient 22 had cleft palate or any other craniofacial malformations, but the son of the proband's paternal aunt had cleft lip and palate. A blood sample was not available from him. The same sequence variation was identified in a patient with Marshall syndrome who had Robin sequence, severe myopia (-20/-20D), midfacial hypoplasia, hyperflexible finger joints and a mild conductive hearing defect. The father of this latter patient was found to have the same mutation. He had no cleft palate but had a small mandible at birth. He also had mild myopia (-2.75/-2.5D). The paternal grandmother had myopia (-6/-6D) and hyperflexible joints, but she did not have a cleft palate or hearing impairment (her blood sample was unavailable). Both G and A nucleotides are commonly observed in position 3 of a donor splice site, but there are several examples of A to G mutations in this position causing aberrant splicing (Zeniou *et al*. 2002). RT-PCR was done to reveal possible splicing defects, but the analysis failed to indicate any. To exclude the possibility of nonsense mediated mRNA decay by means of a cryptic splice site, the exon 5+9T>C and exon 63+162C>T polymorphisms were analyzed from patient RNA. Sequencing showed that both polymorphisms were present in the mRNA, and thus both alleles were expressed. The analysis could not rule out the possibility of a quantitative splicing defect, however.

One additional nucleotide substitution (IVS31+92T→A) was found in two Robin sequence patients and in none of the 150 controls. Analysis of the parents indicated that both patients had inherited the substitution from one of their parents. Since the parents or other family members did not have any cleft defects, the substitution is not likely to be pathological, but it may still predispose carriers to the phenotype.

5.2.3 COL2A1 sequence variations

A unique nucleotide change was found in exon 17 of the COL2A1 gene in two unrelated patients with Robin sequence, the last nucleotide C of this exon being replaced with T. This did not change the amino acid encoded. The father of patient 35 was heterozygous for the same change, but no one else in this family had cleft palate except the patient. The mother of patient 39 was found to be homozygous for the nucleotide change and had non-

syndromic cleft palate. She had three unaffected sisters and a brother. All four of her grandparents originated from the same region. Since RT-PCR analysis did not reveal any splicing defects and showed that both alleles were expressed, this mutation is not likely to be pathological, but it may be a predisposing, as three of the four individuals with this nucleotide variation had a clefting defect.

5.3 Analysis of OA patients (III)

5.3.1 Family study

A small three-generation family (Fig. 2) with early onset OA was referred to us for genetic study. The family had two affected individuals, a mother and her son. The sibling daughter was unaffected, as were the son's two children. The mother had hip and knee problems, and the first joint replacement was done at the age of 49 years on her right hip. The other hip was replaced two years later. Both knees were replaced with prostheses at 55 years of age. Her right thumb needed surgical correction two years later. She had near-normal vision but developed hearing loss with advancing age.

Her 44-year-old son had experienced knee pain after exercise since the age of 17. At 33 years of age, his knee cartilage was repaired in an arthroscopic surgery. He is of normal height, 183 cm, with normal body proportions. He has mild nasal anteversion, mild myopia since age 13 and mild sensorineural hearing defect bilaterally. No other signs of Stickler syndrome were found. Radiographs from knees and cervical spine revealed OA, without evidence of chondrodysplasia.

CSGE analysis showed a unique pattern for these two affected individuals. Sequencing revealed a deletion of one nucleotide at IVS42-2. The analysis of the son's RNA showed, in addition to the wild-type allele, an allele that lacked exon 43 completely. Thus, the mutation resulted in an in-frame deletion of 108 bp or 36 amino acids.

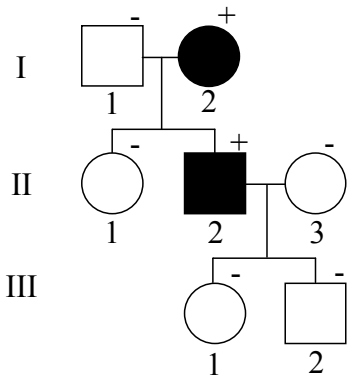


Fig. 2. Pedigree of the North American family with OA.

5.3.2 Analysis of unrelated patients

Seventy-two Finnish probands with primary early onset hip and/or knee OA were screened by CSGE for mutations in the six genes coding for collagens II, IX and XI. All different heteroduplex patterns observed by CSGE analysis were sequenced at least from one sample, and the control samples were analyzed for the presence of the observed sequence variations. Altogether 239 sequence variations were identified in the patients, sixteen of which were not present in the controls. Two of these unique variants were in the COL2A1 gene, five in the COL11A1 gene, three in the COL11A2 gene, two in the COL9A1 gene, one in the COL9A2 gene, and three in the COL9A3 gene (Table 4). Seven of the variations, one in the COL2A1 gene, four in the COL11A1 gene and two in the COL11A2 gene, were studied in more detail because they were predicted to result in amino acid substitutions or cause splicing abnormalities.

Table 4. Sequence variations unique to the OA patients.

Gene	Nucleotide variation	Predicted consequence	Proband
COL11A1	E2+33T>A[1]	F-482I	K123
	E8+31G>C[1]	E-188Q	J14
	IVS8+87T>C	nd	J61
	E39+5C>A[1]	P446E	J64
	E59+60C>A[1]	D944E	J14
COL11A2	E17+4C>T[1]	R53W	J38
	IVS42-2delA[1]	deletion of e43 (36 amino acids)	Family EC (Fig. 2)
	e63+118C>T	T1044T (nd[2])	K136
COL2A1	E37+57C>T	G658G (nd)	K136
	E38+54G>A[1]	decreased mRNA level	J35
COL9A1	IVS14+24T>C	nd	J14
	IVS17+60-64delcttta	nd	J3
COL9A2	IVS30-112- -114deltct	nd	J51
COL9A3	IVS13+69a>g	nd	K137
	IVS14-18c>g	nd	K113
	E30+170C>T	D591D (nd)	K115

[1]Studied in more detail [2]nd = not determined

5.3.2.1 COL11A1 sequence variations

A unique one base substitution, E39+5C→A that converted Pro446 to Gln was found in a man who had undergone hip replament surgery at age 40. His sister, who was diagnosed with bilateral hip and knee OA, had also inherited the mutant allele. The sister's daughter, also with the mutant allele, was diagnosed with bilateral knee OA (Fig. 3). Four additional family members were found to have this allele, two of whom had OA symptoms, but their OA status could not be determined radiologically. Thus, this mutation may associate with the phenotype but is not fully penetrant. This substitution affects a highly conserved amino acid and is likely to be damaging (Fig. 6C in III), although the Polyphen program gave a result of status unknown due to the unavailability of data.

A proband (Fig. 4) with bilateral hip OA was found to have two nucleotide changes in the COL11A1 gene, which converted Glu188 to Gln and Asp944 to Glu. His mother and maternal grandmother had a history of OA but were deceased and no DNA was available for them or the father. The only blood sample from the family was from an unaffected sister. She had not inherited the mutant alleles. No one in the control set had these alleles, but one person in the random population set of 100 persons was found to possess both of these variants. This was a 52-year-old woman who had suffered from hip pain. Radiological examination showed OA in the left hip and mild OA changes in both knees. Polyphen analysis predicted these changes to be benign even though both of them substituted a conserved amino acid (Fig. 6B,D in III).

A proband with bilateral hip OA was found to have a single nucleotide variation in the COL11A1 gene that converted Phe482 to Ile. Co-segregation analysis could not be performed since family members refused to participate. Again, none of the controls had the variation, but one individual in the random population set was found to have it. This individual was a 37-year-old female with evidence of Osgood Schlatter disease in the left knee and traumatic meniscus lesion in the right. She had no radiological findings of OA in her knee, but she had a history of lumbar disc herniation. Hip radiographs were unobtainable. Polyphen analysis predicted this substitution to be possibly damaging (Fig. 6A in III).

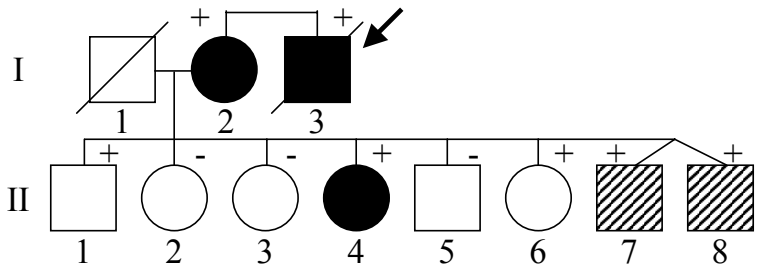


Fig. 3. Pedigree of the proband with COL11A1 Pro446 to Gln.

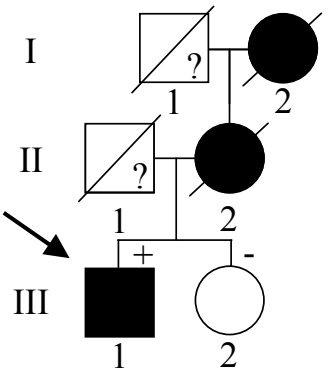


Fig. 4. Pedigree of the proband with COL11A1 Glu188 to Gln and Asp944 to Glu.

5.3.2.2 COL11A2 sequence variations

A man with OA symptoms since age 38 was found to have an exonic variation, E17C→T in the COL11A2 gene that converted a codon for Arg53 to a codon for Trp. He had undergone hip replacement surgeries, the right one at 54 years of age and the left nine years later. Tryptophan is not normally present in the collagen triple helix and neither was this W53 found in any of the 103 controls or in the 100 random population samples. Family members of the original proband were included in the study (Fig. 5). Four additional family members had inherited the tryptophan allele. Individual II.7 had hip, knee, and hand pain and Heberden's nodes. Her hip, knee and hand OA have been confirmed radiologically. Individual II.8 had suffered from hip and hand pain but there is no radiological evidence of hip or knee OA. He had Heberden's nodes and spinal OA. Individual II.9 had hip, knee and shoulder pain and radiologically confirmed knee OA. The son of proband (III.1), aged 45, had pain in the left knee. Radiological and MRI examinations revealed no signs of hip or knee OA, but showed signs of previous Osgood Schlatter disease in the right knee. One family member without the tryptophan allele was diagnosed to have bilateral hip OA at the age of 65 years. She had no other joint complaints and no evidence of knee OA was detected radiologically. The Polyphen program predicted this substitution to be probably damaging as can be expected for a change of a highly conserved amino acid (Fig. 6E in III).

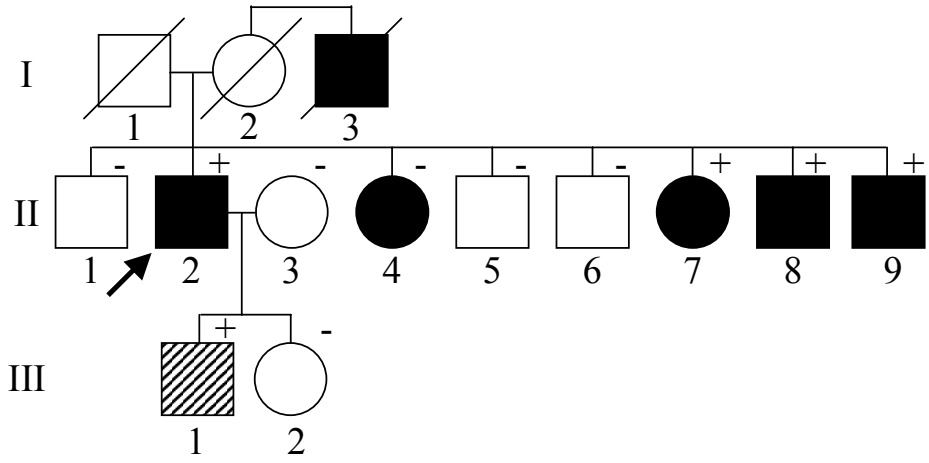


Fig. 5. Pedigree of the proband with 11A2 Arg53 to Trp.

5.3.2.3 COL2A1 sequence variations

A unique nucleotide substitution was found in the last nucleotide of exon 38 of the COL2A1 gene. It resulted in a silent mutation (Table 4). The proband had had OA

symptoms since her thirties and had undergone right hip replacement surgery. She also had Heberden's nodes and mild sensorineural hearing defect in the left ear, but no cleft palate or myopia. Her sister also had the mutation (Fig. 6). She was diagnosed with OA at age fifty and now thirty years later she was found to have OA in the knees, hands and left hip. The sister had no findings typical for Stickler syndrome.

Since the mutation could potentially cause a splicing defect, the proband's RNA was analysed. Three distinct RT-PCRs were performed, but all of them gave only one fragment of the right size corresponding to the wild-type allele. Sequencing of the RT-PCR products, however, revealed that only in one case out of three were both alleles present. Therefore, the mutation probably causes a quantitative splicing defect that leads to aberrant splicing and nonsense-mediated RNA decay in most but not all mutant allele mRNAs.

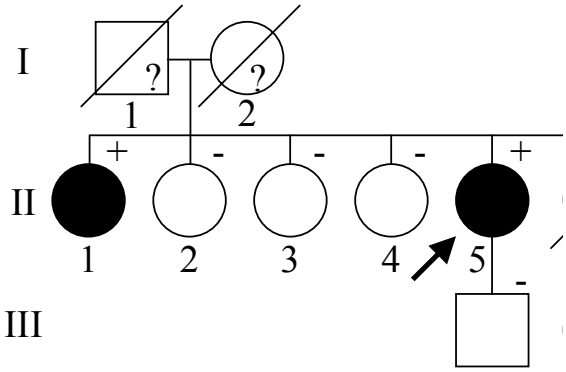


Fig. 6. Pedigree of the proband with splicing defect of COL2A1.

5.3.3 Allelic association analysis

Six to twelve polymorphisms were selected within each of the analysed six genes. These polymorphisms were genotyped from all of the 72 unrelated Finnish probands and 103 controls. The observed allele frequencies were compared between the probands and controls. Since the probands and controls were enrolled from two different hospitals, although located quite close to each other, allele frequencies were also compared between probands from both hospitals and between controls from both hospitals as a negative control. Fisher's exact test was used to analyse each of the diallelic polymorphisms, one at a time. No evidence of genetic difference between the proband and control groups was observed.

Neither were there statistically significant differences between the allele frequencies of the probands and the controls at any site in any of the genes. This was true when considering all the subjects and when subdividing them into cases of knee and hip OA. When multiple testing was taken into account (Terwilliger 1995), no significant differences were observed either after stratification of the data by sex.

6 Discussion

Mutations in the COL2A1 gene coding for the major cartilage protein, collagen II, are known to cause a spectrum of phenotypes, ranging from mild to lethal. A number of patients with the COL2A1 gene mutations have been reported with well-defined phenotypes (Kuivaniemi *et al*. 1997, Horton & Hecht 2002). This information, together with results obtained from animal studies, have clarified the role of collagen II and the consequence of different types of mutations at different locations in the COL2A1 gene on extracellular matrix function and disease phenotypes. A lot less is known about the two minor cartilage collagens that are present in cartilagenous tissues, and some other tissues as well, in close relation with collagen II. These are collagens IX and XI.

6.1 Otospondylomegaepiphyseal dysplasia

Vikkula & others (1995) reported the first collagen XI gene mutations. Both of the reported mutations were in the COL11A2 gene. One was found in a large family with non-ocular Stickler syndrome (deletion of exon 60) and the other in a family with OSMED (homozygous Gly175→Arg). Encouraged by these findings, we decided to perform a large study to elucidate the role and importance of COL11A2 gene mutations in OSMED. We collected blood samples and clinical data from seven unrelated OSMED families with ten affected individuals. The clinical analysis revealed that their phenotypes were very similar, with the typical features being Robin sequence, disproportionate shortness of the limbs, enlarged joints, vertebral body anomalies, sensorineural hearing loss and normal vision. CSGE analysis was performed using DNA samples from both the probands and their parents. All the affected family members were found to be homozygous or compound heterozygous for mutations in the COL11A2 gene. Ten new mutations in the COL11A2 gene were identified; nine of them lead to the generation of a premature translation termination codon and one altered the consensus sequence for splicing. The data suggests that affected members of six of seven families completely lacked the α2(XI) chains or produced truncated α2(XI) chains that are unlikely to be incorporated into type XI collagen molecules.

These OSMED patients, therefore, present the human phenotype of a lack of functional COL11A2 alleles. Li & others (2001) generated Col11a2 knockout mice. The phenotype of these mice resembled that of OSMED patients. It was interesting that the lack of the α2(XI) chains produced a rather mild phenotype both in humans and mice, since the complete lack of either of the α1(XI) or α1(II) chains is known to result in a lethal phenotype in mice (Li *et al*. 1995, Li *et al*. 1996). A complete lack of α1(XI) or α1(II) chains has not been described in humans, presumably because of early embryonic lethality. Collagen V and XI are known to form hybrid molecules, and collagen V α chains can replace collagen XI α chains, and *vice versa*. For example, this can be seen in bovine vitreous where α2(XI) is completely absent and substituted by the α2(V) chain in the collagen XI molecules (Mayne *et al*. 1993). Thus, partial compensation of the α2(XI) chain by the α2(V) chain in cartilage could explain why the absence of the α2(XI) chain is non-lethal. Further support for the replacement idea comes from the finding that α1(XI) and α1(V) form triple-helical molecules in the absence of the α2(XI) chain in the rib cartilage of Col11a2 knockout mice (Li *et al*. 2001).

All but one of the parents of the OSMED patients were heterozygous for a COL11A2 mutation leading to premature termination of translation. They had no detectable phenotype. One parent with the in-frame deletion of exon 23 of the COL11A2 gene had a mild phenotype resembling those of the patients with non-ocular Stickler syndrome (Vikkula *et al*. 1995, Sirko-Osadsa *et al*. 1998). The in-frame deletion mutation is likely to lead to a dominant negative effect rather than a non-functioning allele and thus, possibly explain the phenotype of this individual.

The previously reported OSMED family with a recessive arginine for glycine mutation had a slightly different phenotype than the OSMED patients we reported or the ones reported previously in the literature (Insley & Astley 1974, Giedion *et al*. 1982, Salinas *et al*. 1986, Johnston *et al*. 1987, van Steensel *et al*. 1997). These patients had no signs of Robin sequence but had severe OA. The phenotypic variation may reflect the fact that haploinsufficiency and mutations with a dominant negative effect usually result in different phenotypes or degrees of severity (Kuivaniemi *et al*. 1997, Horton & Hecht 2002).

6.2 Robin sequence and isolated clefts

The syndromes that are known to result from mutations in genes coding for collagen XI often include Robin sequence, cleft palate or micrognathia in their phenotype. Also, is sensorineural hearing defect often present. McGuirt & others (1999) reported two mutations in the COL11A2 gene that lead to non-syndromic hearing defect with no syndromic findings, and thus established that the COL11A2 gene mutations can cause both syndromic and non-syndromic phenotypes. Non-syndromic cleft palate has been shown to have a strong genetic component as discussed in the review of the literature chapter. A major clefting locus has been proposed to be located on chromosome 6p where the COL11A2 gene is located (Murray 1995). We analysed 24 Robin sequence patients, 17 non-syndromic cleft palate patients and 17 patients with non-syndromic micrognathia for mutations in the COL11A2 gene. Twenty-three Robin sequence patients were also

analysed for mutations in the two other genes coding for collagen XI, COL11A1 and COL2A1.

The results showed that sequence variations in the genes coding for collagen XI can play a role in the etiology of non-syndromic Robin sequence and micrognathia, but are not common causes of these phenotypes. Interestingly no disease predisposing sequence variations were defined for isolated cleft palate patients. CSGE analysis revealed two possibly disease-associated sequence variations in the COL11A1 gene in two Robin sequence patients, one in the COL11A2 gene in a patient with micrognathia and one in the COL2A1 gene in two patients with Robin sequence (Table 3). Two disease-associated mutations were identified in two patients with Robin sequence. One was a stop codon mutation in the COL11A2 gene. The mutation caused non-syndromic Robin sequence in the patient but was not fully penetrant in the father, who also had the mutation. He had no cleft but had a high-arched palate and a small upturned nose. Although the parents of OSMED patients, who also were heterozygous for this type of mutation causing premature termination of translation, had no obvious phenotype, it seems possible that this stop codon mutation predisposes to cleft palate or Robin sequence. Because the consequences of this mutation were not examined at the mRNA level, it is not certain if this mutation resulted in nonsense-mediated mRNA decay. Alternatively, it could have resulted in nonsense-related altered splicing and exon skipping, and thus resulted in a dominant negative effect. It has become evident that some, but a minority, of the nonsense mutations induce exon skipping (Cartegini *et al*. 2002, Vuoristo *et al*. 2004).

One of the other Robin sequence patients had a mutation in the COL11A1 gene that was predicted to alter normal splicing. The patient had not inherited the mutation from her healthy parents. The same IVS50+3insT mutation has been reported earlier in a Marshall syndrome patient (Annunen *et al*. 1999a; patient 6). Our Robin sequence patient was of normal height and had only mild myopia. The Marshall patient had high myopia, mixed type hearing defect, short stature and cleft palate. This finding of the same mutation causing different but overlapping phenotypes is not unforeseen; there are several examples of identical mutations in the COL1A1 gene causing osteogenesis imperfecta that can vary from mild to lethal (Körkkö *et al*. 1997).

It is likely that a single genetic factor is not in itself enough to result in clefting, and that other, as yet unknown genetic or environmental factors modify the effects of mutations that predispose to clefting. It is thus possible that the unique sequence variations found here may be associated with the phenotypes, but cannot alone induce clefting. This hypothesis is supported by the findings that non-syndromic oral clefting is multi-factorial and involves both genetic and environmental factors, and that the concordance for non-syndromic cleft palate in monozygotic twins is only 23.5% (Fraser 1970, Murray 1995, Hibbert & Field 1996, Wyszynski *et al*. 1996).

6.3 Osteoarthrosis

Association analysis of OA patients revealed no common predisposing alleles in any of the cartilage collagen genes. Still, eighteen percent of the OA probands were found to have a unique sequence variation (Table 4). Although functional significance and co-

inheritance could not be studied in all cases, this finding itself may be significant. There is a lot of evidence supporting a strong genetic factor to primary OA, but little direct evidence of gene defects behind the disease. Seven of the 16 unique sequence variations were studied in more detail because they were predicted to result in amino acid substitutions or cause splicing defects. Co-segregation of the sequence variation with the phenotype was observed in four of the six families. Family members refused to participate in one family, and the other was uninformative because the only obtainable close relative was an unaffected sister. Though in both of these cases, the sequence variations found from the proband were observed once in the random population set. Clinical and radiological examinations revealed that these individuals in the random population set also had OA suggesting some significance for these variations in OA.

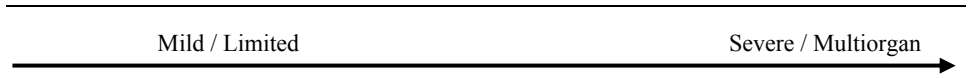
Some mutations in the COL2A1 gene are known to result in OA associated with very mild chondrodysplasia (see Reginato & Olsen 2002). It is possible that some OA cases present the mild end of CD phenotypic spectrum. Dysplastic changes may be evident in childhood but go unnoticed if no clinical attention is required. Later in life, these changes may be expressed as OA. Haploinsufficiency of the COL2A1 gene typically causes Stickler syndrome. We found a COL2A1 mutation that probably results in a partial reduction of mRNA from the mutant allele, but not haploinsufficiency, therefore resulting in OA without any syndrome characteristics. This is supported by the finding that reduced expression of one of the COL2A1 alleles can contribute to the development of OA (Loughlin *et al*. 1995).

Xu & others (2003) noted that a reduced amount of collagen XI lead to an increased diameter of collagen II fibrils in mouse cartilage collagen. These mice developed OA progressing with age. A splicing mutation in the COL11A2 gene was found to lead to the skipping of exon 42, and thus an in-frame deletion of 108 bp or 36 residues. The α1(II) chains with a smaller, 18-residue deletion have been shown to be able to incorporate into collagen II molecules, creating a loop in normal chains in the site of deletion (Weis *et al*. 1998). It seems possible that significantly larger deletions might lead to only partially folded collagen XI molecules. This might lead to a reduced amount or the impaired function of collagen XI in cartilage collagen fibrils. We also identified another COL11A2 mutation that introduced tryptophan into the collagen triple helix. This amino acid is not normally present in the triple-helical parts of collagens, and tryptophan mutations in collagen IX have been reported to associate with lumbar disc disease (Annunen *et al*. 1999b, Paassilta *et al*. 2001). Tryptophan, being the most hydrophobic amino acid, may have an affect on triple helix conformation or interactions with the other matrix molecules.

Surprisingly we found no sequence variations in collagen IX genes that would obviously alter the protein structure. Collagen IX mutations lead to MED that is typically associated with knee OA, sometimes being hard to distinguish from primary OA in adulthood. We did identify six unique sequence alterations. It remains possible, however, that some of them may alter regulatory sequences or exonic splicing elements (Faustino & Cooper 2003) and associate with the disease.

6.4 Collagen XI

Mutations in the genes coding for collagen XI cause a spectrum of phenotypes. The variation extends from virtually non-existing to early lethality, from a single-sense defect to syndromes with multiple dysfunctions. Figure 7 summarises the phenotypes associated with collagen XI. The COL2A1 gene mutations usually lead to more severe phenotypes than mutations in the COL11A1 and COL11A2 genes. This is likely due to the fact that collagen II is the most abundant protein in cartilage and it has an important function in development (Eyre & Wu 1995). COL11A1 gene defects can also lead to relatively severe phenotypes, but not as often as COL2A1 mutations. COL11A2 mutations lead to relatively mild phenotypes. The reason for this is not clear. However, it is possible that the replacement of the α2(XI) chain by α2(V) chain may partially explain this phenomenon (Mayne *et al.* 1993).



*A severe human phenotype for the COL11A1 mutation has not been described, probably due to early lethality during pregnancy. This is supported by the finding that Col11a1 functional knockout mice die at birth (Li *et al.* 1995).

Fig. 7. The phenotypic spectrum of collagen XI gene defects in humans.

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