

# Alport syndrome

## Molecular genetic aspects

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This review has been accepted as a thesis together with nine previously published papers by the University of Aarhus, February 5, 2009, and defended on May 15, 2009.

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Dan Med Bull 2009;56:105-52

## 1. INTRODUCTION

### 1.1 HISTORICAL ASPECTS

In 1902, Leonhard G. Guthrie [10] presented a family in which several members had hematuria. He noticed that hematuria had been inherited through the maternal side only. The disease was named "*Idiopathic*", or "*Congenital, hereditary and family hematuria*". A follow-up on the same family was made ten years later in 1912 by George Kendal and Arthur F. Hertz [11], and again in 1923 by Arthur F. Hurst [12]. They named the disease "*Hereditary familial congenital haemorrhagic nephritis*". According to the pedigree presented by Hurst, [12] three of the affected patients were deaf, but it was not mentioned in the text as a part of the disease. The same family was re-evaluated in 1927 by A. Cecil Alport [13], who found that deafness is a marked feature in nearly all cases. He described the disease as a dominantly inherited hereditary nephritis characterized by hematuria and nerve deafness. He noticed a different clinical course of the disease in males and females. Males tend to develop nephritis and deafness and do not as a rule survive whereas females have deafness and hematuria and live to old age. The eponym (AS) was introduced in 1961 by Williamson [14], and refers to the author of this original description of the disease back in 1927. Ophthalmological changes in association with hereditary hematuria and nerve deafness was first recognized in 1954 by Reyersbach and Butler [15], but without specifying its nature. A few years later Sohar [16] presented a family in which spherophakia and congenital posterior cortical cataract were associated with chronic renal disease and progressive deafness. Castleman and Kibbee [17] described in 1957 ocular changes in form of a mottled white ring around the macula in association with familial hereditary nephropathy and deafness.

### 1.2 PREVALENCE AND INCIDENCE OF AS

No valid figures exist for the prevalence and incidence of AS. It has been difficult to obtain such figures due to the lack of generally accepted diagnostic criteria, and the fact that no simple diagnostic test for the disease has been available. AS has been reported in different ethnic groups, and does not seem to be restricted to certain geographic areas [18]. The most widely used estimate of the prevalence of AS is 1:5,000 based on the finding in United States of about 300 cases of AS in Utah and southern Idaho in a population of 1,500,000 people [19]. Another study on Rhode Island provides a figure of 77 cases in a state with a population of about 1,000,000 inhabitants, giving a prevalence of 1:13,000 [20, 21]. The incidence of AS was found to be 1:53,000 in live births in a nationwide survey in Finland [22], and 1:17,000 in southern Sweden [23].

Patients with AS constitute 2.3% (11/476) of the renal transplant population at the Mayo Clinic [24], and 1.3% of 1,000 consecutive kidney transplant patients from Sweden [25]. Approximately 0.56% of the European dialysis population suffers from AS [26], and similar figures have been found in other series. AS accounts for 18% of the patients undergoing dialysis or having received a kidney graft in 2003 in French Polynesia [27]. A common founder mutation was in this area. In Denmark, the percentage of patients with AS among all patients starting treatment for ESRD ranges from 0 to 1.21% (mean: 0.42%) in a twelve year period from 1990 to 2001 (Danish National Registry. Report on Dialysis and Transplantation in Denmark 2001). This is probably an underestimate due to the difficulties of establishing the diagnosis.

## 1.3 CLINICAL FEATURES OF X-LINKED AS

### 1.3.1 Renal features

AS in its classic form is a hereditary nephropathy associated with sensorineural hearing loss and ocular manifestations. The characteristic renal features in AS are persistent microscopic hematuria appearing in early childhood with or without episodes of gross hematuria. Sixty-seven per cent of males present with macroscopic hematuria during an intercurrent infection at an average age of 3½ years [28]. Male relatives without hematuria during the first 5-10 years of life are very unlikely to be affected. Proteinuria is often absent or minimal in the early stages of the disease, but increases progressively with age and a nephrotic syndrome may develop. Renal failure occurs in virtually all affected males, and in most cases during the third or fourth decade. Schneider [29] recognised two groups of patients with different progression of the disease. One group with onset before puberty, often within the first years of life, and death before the age of thirty. In the second group, onset is generally after puberty. Affected males in this group often survive to about the age of forty years. Juvenile AS is defined as mean age at onset of ESRD ≤30 years, and adult AS as mean age at onset of ESRD >30 years. There is, however, considerable inter- and intrafamilial variation in the age at onset of ESRD.

### *Renal light microscopic characteristics*

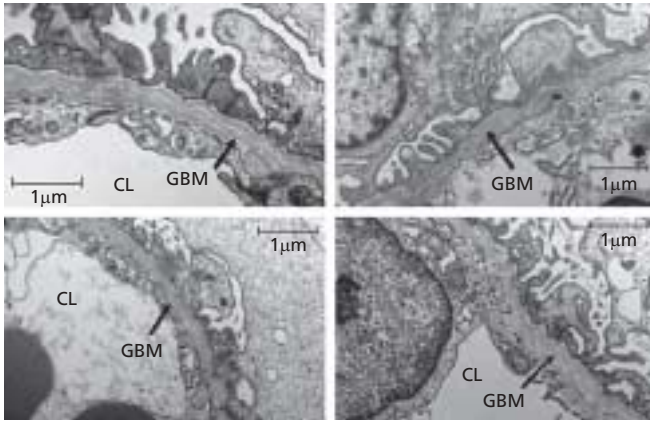
The light microscopic findings in renal biopsy specimens from patients with AS are not characteristic, and appear normal during the first years of life [30-33]. In older patients with renal impairment interstitial fibrosis, tubular atrophy, foam cells, and segmental proliferative glomerular changes are seen. The presence of interstitial foam cells has been considered as suggestive of AS, but they are frequently seen in other types of glomerulonephritis as well [31].

### *Renal ultrastructural characteristics*

Characteristic ultrastructural changes in the GBM in patients with AS were demonstrated independently by three groups in 1972 and 1973 [34-36]. The GBM is an approximately 150 nm thick amorphous layer, located between the capillary endothelial cells and the epithelial podocytes. Typical electron microscopic changes in AS are irregular thickening of the GBM of up to 4 or 5 times the normal thickness, multilamination of the lamina densa forming a "basket weave" pattern by inclusion of electron lucent areas, often containing round, dense granules, measuring 20-60 nm in diameter (Figure 1).

These changes are often widespread especially in adults, but they may be patchy and alternating with segments of normal thickness [32], and areas with a focally or diffusely thinned lamina densa to one third or one half of the normal thickness [37].

Widespread "basket weave" changes of the GBM are indicative of a tendency towards a progressive disease course [38]. Focal thickening and "basket weave" pattern, often confined to a few capillary loops, have been observed in other renal disorders [39], whereas widespread changes appears to be confined to AS, and seen in the majority of cases [32, 40, 41]. There is good correlation between the



**Figure 1.** Electron micrographs showing irregular thickening and splitting of the glomerular basement membrane (GBM) in a renal biopsy specimen from a 20-year-old male patient (IV:4) from Alport family DK008. GBM=glomerular basement membrane. CL=capillary lumen. EP=endothelial cell. EP=epithelial podocytes.

severity of the GBM splitting and the clinical course [42, 43]. It seems that progression of splitting means progression of the nephropathy. A positive correlation of the GBM splitting and the grade of proteinuria has been demonstrated.

The earliest manifestation of the GBM is irregular thinning, and the extent and severity of thickening and multilamellation increases with age [42, 44]. Typical changes from thin to thickened GBM with splitting of lamina densa have been demonstrated in repeated renal biopsies obtained from a patient with AS at the age of 9 years and again at the age of 21 years [45].

### 1.3.2 Audiological manifestations

A high-tone sensorineural deafness occurs in 55-83% of males with AS [28, 46]. Deafness is never congenital, but is usually detectable by late childhood or early adolescence in boys, and always accompanied by evidence of renal impairment. The hearing defect is in the beginning only detectable by audiometry, but becomes clinically apparent at an average age of 11 years [28]. A hearing-aid may become necessary. Deafness is slowly progressive in childhood, but usually static in adult life. Audiometry reveals a bilateral reduction in sensitivity to tones in the 2,000-8,000 Hz range (high-tone), but may extend to other frequencies. Uremia and treatment with nephrotoxic pharmaceuticals (e.g. aminoglycosides) may contribute to the hearing defect in the same range of frequencies. Improvement of hearing in patients with AS after renal transplantation has occasionally been described [47]. This improvement may, however, be attributable to a general improvement of hearing after renal transplantation due to absence uremia caused hearing loss [48]. Deafness may be late occurring and slowly progressive in some families. A relatively common *COL4A5* mutation in the western United States (p.Leu1649 Arg), present in 9 out of 121 independently ascertained families (7.4%), results in a relatively mild phenotype with renal failure occurring in the 4th or 5th decade in affected males, and with significant hearing loss occurring about 10 years after the onset of the renal failure [49].

The otopathology in AS is most probably due to a defect in the cochlear basement membranes. The  $\alpha 3$ -6(IV)-chains in the cochlea in guinea pigs are located exclusively to the tectorial and basilar membranes, suggesting a possible role of type IV collagen chains in the active tuning of the basilar and tectorial membrane which is an essential step in frequency discrimination and amplification of auditory signals [50]. Auditory-evoked responses implicate the cochlea as the site of lesion. Impaired vestibular function has been detected but without clinical significance. AS represents the first syndromic hearing loss condition in which the gene has been identified.

### 1.3.3 Ophthalmological manifestations

The typical ophthalmological manifestations of the X-linked form of AS are a dot-and-fleck retinopathy which occur in about 85% of affected adult males, and anterior lenticonus which occur in about 25% [51]. Many additional ocular abnormalities have been described, including, recurrent corneal epithelial erosions [52], posterior polymorphous corneal dystrophy, posterior lenticonus, macular holes [53], and retinal detachment [54, 55]. The ocular manifestations seen in AS are usually not present in childhood, but presents after renal abnormalities manifest [56].

The dot-and-fleck retinopathy is normally detectable at the onset of renal failure, and comprises numerous bilateral white and yellow dots and flecks with streaming. The lesions are located in the perimacular area, sparing the fovea. There is no associated visual impairment or night blindness. If careful ophthalmological investigation is performed, these dots and flecks are reported to be present in almost all patients. Gelisken et al. [57] found dot-and-fleck-retinopathy in 12 out of 13 patients (92%) with biopsy-proven AS.

Anterior lenticonus is associated with thinning of the anterior lens capsule and caused by a defect of the lens epithelial basement membrane [58, 59]. The anterior lens capsule is more fragile than normal, and spontaneous rupture of the lens has been reported [30, 60]. There is usually a history of gradual deterioration of vision over several years, with the development of axial myopia. Anterior lenticonus might exist exclusively as a part of AS [61].

### 1.3.4 X-linked AS in females

Females heterozygous for the X-linked form of AS have a more variable phenotype than males, ranging from intermittent microscopic hematuria to ESRD. It has previously been suggested that as many as 10-15% of female carriers never manifest hematuria [62]. However, Flinter [28] found that all obligate carriers have at least microscopic hematuria by the age of 20 years. Dagher et al. [63] found hematuria by phase-contrast microscopy in all 40 examined carrier females of the X-linked form of AS. Microscopic hematuria was found in the European Collaborative Study (ECASCA) in 96% of 323 obligate carriers of X-linked AS, belonging to 195 families with known *COL4A5* mutations, and as the presenting symptom in the majority of cases [64]. Proteinuria is present in 75% of the carriers. Gross hematuria in childhood, progressive increase in proteinuria or nephrotic syndrome, hearing loss, anterior lenticonus, and diffuse GBM thickening on electron microscopy are features suggestive of progression to renal failure [64, 65]. Renal ultrastructural changes were seen in 26 of 28 cases (93%), and as irregular GBM changes in 57%, diffuse thinning in 25%, and a thick GBM in 11%. Immunohistochemical staining for the  $\alpha 5$ (IV)-chain of a renal biopsy specimen and demonstration of an interrupted, discontinuous linear pattern in the GBM, can identify females as being heterozygous for the X-linked form of AS rather than having benign familial hematuria [66]. A significant association between the severity of the disease and the expression of the  $\alpha 5$ (IV)-chain in the epidermal basement membrane by immunohistochemical analysis was found by Kakani-shi et al. [67].

The risk of progression to ESRD before the age of 40 years in carrier females is 12%, and 30-40% after the age of 60 years [64]. The high-tone sensorineural hearing defect in female carriers occurs less frequently and later in life than in affected males [28], but can be seen even when renal function is normal. In the European Collaborative Study hearing loss was observed in 28% of the female carriers, and usually occurs after 30-40 years of age. Ocular manifestations were seen in 15% [64].

There does not seem to be a correlation between the genotype, i.e. the type of mutation, and the clinical manifestations in carrier females [64]. The development of clinical symptoms in carriers is not correlated to the severity of the disease in affected male relatives or to the age at which the males develop ESRD [63]. The variable phenotype in heterozygous females could be related to random X-chro-

mosome inactivation. Vetrie et al. [68] studied the X-chromosome inactivation pattern in DNA from peripheral blood lymphocytes from 43 females from 22 families with X-linked AS, and did not find a correlation between the X-inactivation pattern and the severity of the disease. Gou et al. [69] studied the X-chromosome inactivation pattern in a female patient with a severe phenotype and two different missense mutations on the same *COL4A5* allele. An extremely skewed X-chromosome inactivation pattern was found in DNA isolated from the kidney with approximately 90% of the X-chromosomes carrying the normal *COL4A5* allele being inactivated.

#### 1.4 DEFINITION OF AS AND DIAGNOSTIC CRITERIA

AS is a progressive hereditary nephritis, frequently associated with cochlear and ocular abnormalities, which result from mutation in type IV collagen genes [70]. The disease is phenotypically and genetically heterogeneous. The inheritance is X-linked in most of the cases, but autosomal recessive and dominant forms also occur.

Many different hereditary renal diseases have been grouped together under the eponym AS. AS is in its classic form an X-linked disease. In the original description by Alport [13] a different clinical course of the disease in males and females was noticed. Males tend to develop nephritis and deafness and do not, as a rule, survive whereas females have deafness and hematuria and live to old age.

Hasstedt et al. [71] classified the disease into six phenotypes based on inheritance, age at ESRD, and the occurrence of extrarenal manifestations: Type I is the classic juvenile form of AS with deafness and dominant inheritance; type II is an X-linked juvenile form with deafness; type III is an X-linked adult form without deafness; type IV is an X-linked dominant adult form without extrarenal manifestations; type V is an autosomal form with deafness and macrothrombocytopenia; and, type VI is an autosomal juvenile form with deafness. Ocular manifestations are restricted to the juvenile types I, II, and VI, and only present in a subset of the families. As information regarding the molecular genetic background of the disease appeared, this classification became inadequate.

Flinter et al. [72] suggested in 1988 a set of four diagnostic criteria for classical X-linked AS. In a patient with unexplained hematuria at least three of the following four criteria must be fulfilled for a diagnosis of AS to be made clinically:

1. A positive family history of hematuria with or without chronic renal failure.
2. Typical ultrastructural GBM changes in a renal biopsy specimen.
3. High-tone sensorineural deafness.
4. Characteristic ophthalmological signs (lenticonus and/or macular flecks)

A patient with a negative family history should therefore fulfil all three characteristic features of the disease to be diagnosed as having AS.

Gregory et al. [73] suggested in 1996 a set of 10 criteria for the diagnosis of AS.

1. Family history of nephritis or unexplained hematuria in a first-degree relative of the index case or in a male relative linked through any number of females.
2. Persistent hematuria without evidence of another possibly inherited nephropathy such as thin GBM disease, polycystic kidney disease, or IgA nephropathy.
3. Bilateral sensorineural hearing loss in the 2,000-8,000 Hz range: the hearing loss develops gradually, is not present in early infancy, and commonly presents before the age of 30 years.
4. A *COL4A3*, *COL4A4*, or *COL4A5* mutation.
5. Immunohistochemical evidence of complete or partial lack of the  $\alpha3(IV)$ ,  $\alpha4(IV)$ , and  $\alpha5(IV)$ -chain in glomerular, or epidermal basement membranes, or both.

6. Widespread GBM ultrastructural abnormalities, in particular thickening, thinning, and splitting.
7. Ocular lesions including anterior lenticonus, posterior subcapsular cataract, posterior polymorphous dystrophy, and retinal flecks.
8. Gradual progression to ESRD in the index case or at least two family members.
9. Macrothrombocytopenia or granulocytic inclusions.
10. Diffuse leiomyomatosis of oesophagus, female genitalia, or both.

To establish a diagnosis of AS in a family, at least four of these criteria must be fulfilled in close related family members. To diagnose AS in an individual, AS must be present in the family according to the previous mentioned criteria. The individual must be on the line of descent for the postulated mode of transmission and must satisfy one of the criteria no. 2-10 for the diagnosis of probable AS, and two criteria for a diagnosis of definite AS. To diagnose AS in an individual without a family history, at least four of the criteria must be present. However, one of these criteria – no. 9 – actually excludes AS. It has been demonstrated, that macrothrombocytopenia and granulocytic inclusions are part of Epstein and Fechtner syndrome, caused by a *MYH9* mutation, and not AS, caused by a type IV collagen gene mutation.

#### 1.5 MAPPING OF THE LOCUS FOR THE X-LINKED FORM OF AS

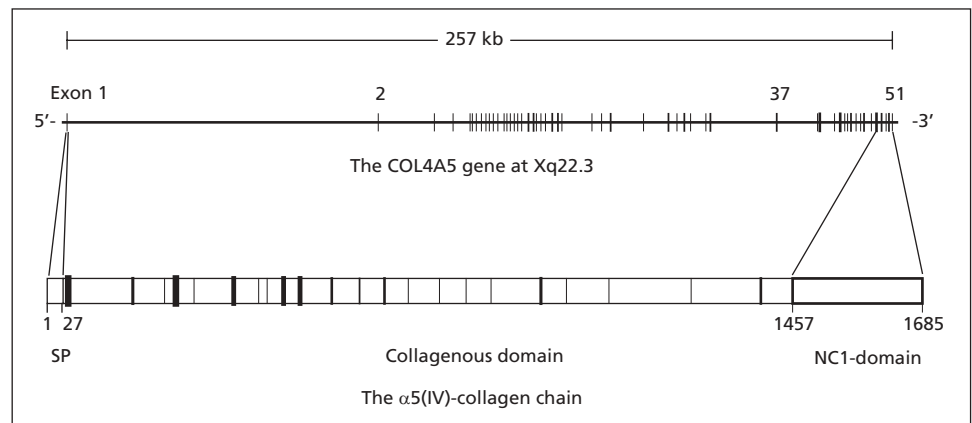
The inheritance of AS had long been a matter of much controversy. In the original description of the disease by Alport [13] a different clinical course for males and females was noted. Only affected males showed progressive nephritis and died of terminal renal failure at a mean age of 18 years without having produced offspring. Absence of father to son transmission of the disease could not be demonstrated, and discrimination between autosomal and X-linked inheritance was therefore difficult. Two large segregation studies comprising forty-one and twenty-three families with AS respectively, has demonstrated that AS is a genetic heterogeneous disease [71, 74]. The inheritance was found to be X-linked in about 85% of the families [72].

A locus for the X-linked form of AS was mapped to the middle of the long arm of the X-chromosome (Xq21.3-q22) in 1988 by demonstrating linkage to several anonymous X-chromosomal RFLP-markers [75-79]. However, the order of these markers, and the position of the locus of the X-linked form of AS in relation to these markers, was known only partially. The position of the gene for the X-linked form of AS in relation to different X-chromosomal RFLP-markers was determined by multipoint linkage analysis in twelve Danish families [1], in two very large Utah families [80], and in 31 French families [81] with AS.

#### 1.6 CLONING OF THE GENE FOR X-LINKED AS (*COL4A5*)

The gene for the X-linked form of AS was found in 1990 by a combined candidate gene approach and positional cloning strategy. Renal ultrastructural changes and immunohistochemical findings in the GBM suggested a type IV collagen defect in patients with AS [34, 82-84]. The gene for a new type IV collagen chain, the  $\alpha5(IV)$ -chain, was cloned independently by two different groups in 1990 [85, 86]. Hostikka et al. [85] used synthetic oligonucleotides coding for conserved type IV collagen sequences to screen a cDNA library from human placenta, resulting in the identification of six clones coding for a new type IV collagen  $\alpha$ -chain. Immunohistochemical staining for this new type IV collagen  $\alpha$ -chain named  $\alpha5(IV)$  showed it to be located in the kidney and almost exclusively in the GBM. The gene (*COL4A5*) was assigned to Xq22 by in situ hybridisation and somatic cell hybrids, the same region to which the locus for the X-linked form of AS previously had been assigned by linkage analysis. Mutations in the *COL4A5* gene were subsequently identified in patients with AS [87], thereby confirming the *COL4A5* gene

**Figure 2.** Structure of the *COL4A5* gene (upper part) encoding the  $\alpha 5$ -chain of type IV-collagen (lower part). Vertical bars indicate the 51 exons and two alternatively transcribed exons 41A and 41B. The  $\alpha 5$ (IV)-chain has a 26-residue signal peptide (SP) at the amino-terminal end, a 1,430-residue collagenous domain with a *Gly-Xaa-Yaa* repeat sequence with 22 interruptions (indicated by vertical bars), and a 229-residue non-collagenous domain (NC1) at the carboxyl-terminal end.



to be the gene for X-linked AS. The *COL4A5* gene spans approximately 257 kb with a transcript of 6.5 kb and 51 exons (Figure 2) [88].

Alternative splicing generates three different forms of *COL4A5* mRNA in human tissue. In RNA isolated from kidney biopsies from patients with AS and from normal kidney tissue, Guo et al. [89] detected an additional 18 bp fragment inserted between exons 41 and 42, preserving the reading frame and coding for two additional *Gly-Xaa-Yaa* repeats in the collagenous domain of the  $\alpha 5$ (IV)-chain. The 18 bp sequence was not present in mRNA isolated from white blood cells. This alternatively used 18 bp mRNA sequence is encoded by two 9 bp exons, 41A and 41B, located in intron 41, 1,989 bp and 6,516 bp downstream from exon 41, respectively [90]. Two different transcript variants are present in kidney tissue: A predominant transcript variant using both alternatively transcribed exons, and another transcript variant using neither exon 41A nor 41B. A third transcript variant using exon 41B only was detected in epithelial mRNA.

### 1.7 OTHER TYPE IV COLLAGEN GENES

The genes encoding the six individual collagen type IV  $\alpha$ -chains are localized in pairs on three different chromosomes, and transcribed from opposite strands. This quite unique organization of the genes allows coordinated regulation of a specific gene pair. It is likely that the genomic arrangement of the type IV collagen genes arose by a series of duplications from an ancestral gene [91]. The genes for each pair can be divided, by their homology, into two classes, the *COL4A1*- and the *COL4A2*-like genes, and thereby indicating their evolutionary origin. *COL4A5* differs from *COL4A1* and *COL4A3* in that it contains only 51 exons, as compared to 52 in the two other genes (Table 1).

Exon 19 of *COL4A5* results from a fusion of the corresponding exons 19 and 20 of the *COL4A1* and *COL4A3* [92].

#### *COL4A1* and *COL4A2*

The gene for the  $\alpha 1$ (IV)-chain (*COL4A1*) and the  $\alpha 2$ (IV)-chain (*COL4A2*) are located on chromosome no. 13 (13q34) [93-97]. The two genes are arranged head-to-head with overlapping 5'-flanking regions and sharing a common promoter between them [98-100].

The *COL4A1* gene is 158 kb in size and contains 52 exons. [101] *COL4A2* is approximately the same size but contains only 47 exons. The transcription start sites of the two genes are only 126 bp apart [98, 99].

#### *COL4A3* and *COL4A4*

The genes for the  $\alpha 3$ (IV)- and  $\alpha 4$ (IV)-chains, *COL4A3* and *COL4A4*, respectively, are located on chromosome no. 2 (2q35-q37), and arranged head to head [102, 103]. Heidet et al. [104] has characterized the entire intron-exon structure of *COL4A3*. The gene spans approximately 150 kb, and contains 52 exons. Boye et al. [105] has characterized the genomic structure of the *COL4A4* gene and determined the intron-exon boundaries of all 48 exons. Alternatively spliced *COL4A3* mRNAs leading to frameshift and putative truncated proteins missing the C-terminal end of the NC1-domain in the kidney and other tissues [106, 107], as well as an alternatively spliced *COL4A3* transcript introducing a stop codon within the collagenous domain in lymphocytes and kidneys, have been described [104]. If translated, these variant  $\alpha 3$ (IV)-chains are likely to be defective in triple helix formation. The significance of these alternatively transcribed  $\alpha 3$ (IV)-chains remains to be determined.

There are two alternative *COL4A4* transcripts presumably derived from two different promoters [103]. The transcription start of exon 1' is only 5 bp away from the transcription start of *COL4A3*, whereas the transcription start from exon 1 starts 373 bp downstream from the first one, generating two kinds of transcripts that differ in the 5'-UTR regions. The exon 1 transcript is expressed predominantly in epithelial cells, whereas the exon 1' transcript showed rather more ubiquitous and low level expression.

#### *COL4A6*

The *COL4A6* gene was identified in 1996 [91, 108]. The *COL4A6* intron-exon boundaries have been characterized, and the number of exons is 46 [109]. *COL4A6* is about 283 kb and is the largest type IV collagen gene known to date [110].

The *COL4A5* and *COL4A6* genes encoding the  $\alpha 5$ (IV)- and  $\alpha 6$ (IV)-chains, respectively, are arranged head-to-head on the X-chromosome [85, 111], with two promoters for *COL4A6* functionally separable from the *COL4A5* promoter [112].

**Table 1.** The six type IV collagen  $\alpha$ -chains and their genes. For references, see text.

Type IV collagen $\alpha$ -chain	Length of mature protein (no. residues)	Length of NC1-domain (no. residues)	Genesymbol	Chromosomal localization	Length of the gene (bp)	No. of exons
$\alpha 1$ (IV) . . . . .	1,642	229	<i>COL4A1</i>	13q34	158,106	52
$\alpha 2$ (IV) . . . . .	1,676	227	<i>COL4A2</i>	13q34	205,742	47
$\alpha 3$ (IV) . . . . .	1,642	232	<i>COL4A3</i>	2q35-q37	150,228	52
$\alpha 4$ (IV) . . . . .	1,652	231	<i>COL4A4</i>	2q35-q37	159,344	48
$\alpha 5$ (IV) . . . . .	1,659	229	<i>COL4A5</i>	Xq22	257,623	51
$\alpha 6$ (IV) . . . . .	1,670	228	<i>COL4A6</i>	Xq22	282,816	45

The *COL4A6* gene contains two alternative promoters that control the generation of two different transcripts [113]. One transcription start site for exon 1 is 754 bp away from the transcription start site of *COL4A5*. An alternative transcription start site for exon 1 is located 1,175 bp from exon 1, encoding a  $\alpha 6(\text{IV})$ -chain with a different signal peptide. The transcript from exon 1' is abundant in placenta, whereas the transcript from exon 1 is abundant in kidney and lung. The existence of different promoters for the two *COL4A6* transcripts explains their differential expression in different tissue [113].

## 1.8 THE TYPE IV COLLAGEN MOLECULE

### 1.8.1 The six type IV collagen $\alpha$ -chains

Type IV collagens belong to the group of collagens that form network-like structures and is a major structural component of basement membranes. The basement membrane separates epithelial cells from the underlying stroma and plays important roles in various normal biological functions such as cell adhesion, growth and differentiation, tissue repair, and molecular ultrafiltration.

Type IV collagen is a triple-helical, heterotrimer molecule composed of three  $\alpha$ -chains, of which six are known to date:  $\alpha 1(\text{IV})$  through  $\alpha 6(\text{IV})$ . Each type IV collagen chain is characterized by an approximately 10-20 residue noncollagenous N-terminal end (7S), containing a signal peptide; a long collagenous domain of approximately 1,400 residues of *Gly-Xaa-Yaa* repeats which are interrupted at 21-26 sites by short noncollagenous sequences; and an approximately 230 residue non-collagenous (NC1) domain at the C-terminal ends. In the collagenous domain every third amino acid is glycine, which is the only residue small enough to fit into the centre of the triple helix molecule [114]. The  $\alpha$ -chains undergo several enzymatic posttranslational modifications like hydroxylation of prolyl residues. Proline and hydroxyproline are frequently located at positions *Xaa* and *Yaa*. Hydroxyproline is essential for the collagen structure because it causes the formation of hydrogen bonds between the  $\alpha$ -chains and stabilizes the molecule. The 21-26 interruptions in the collagenous domain are assumed to provide flexibility to the molecule. The positions of these interruptions are highly conserved during evolution, and the generation of an additional interruption by exchanging a glycine residue may interfere with correct folding of the triple-helical molecule. The NC1 domains of type IV collagen chains exhibit a direct tandem repeat structure, with two approximately one hundred amino acid segments, each containing three cysteine residues which are involved in intra- and interchain disulfide bonds [115, 116]. Other amino acids in both tandem repeats are also highly conserved during evolution [117].

The  $\alpha 5(\text{IV})$ -collagen chain has 1,685 amino acid residues (Figure 2). There is a 26-residue signal peptide, a 1,430-residue collagenous domain starting with a 14-residue noncollagenous sequence, and a *Gly-Xaa-Yaa*-repeat sequence interrupted at 22 locations; and a 229-residue C-terminal non-collagenous (NC1) domain [2]. The complete primary structure for the other type IV collagen  $\alpha$ -chains has also been determined [91, 118-121].

### 1.8.2 Supramolecular organization and normal tissue distribution of the six type IV collagen $\alpha$ -chains

Three  $\alpha(\text{IV})$ -chains assemble to form triple helical molecules (protomers) that further associate to form supramolecular networks. Protomers form a network structure through tetramerization by disulfide cross-links at their N-terminal ends and dimerization by disulfide cross links at their NC1-domains [122]. Random recombination of the six type IV collagen  $\alpha$ -chains allows for 56 different triple-helical isoforms (protomers). However, only three of these isoforms have so far been identified in basement membranes: the  $[\alpha 1(\text{IV})]_2\alpha 2(\text{IV})$ ,  $\alpha 3(\text{IV}) \alpha 4(\text{IV}) \alpha 5(\text{IV})$ , and  $[\alpha 5(\text{IV})]_2\alpha 6(\text{IV})$  protomers (Figure 3).

The NC1 domain contains as yet unidentified recognition sequences that direct the selection of chain assembly in the triple heli-

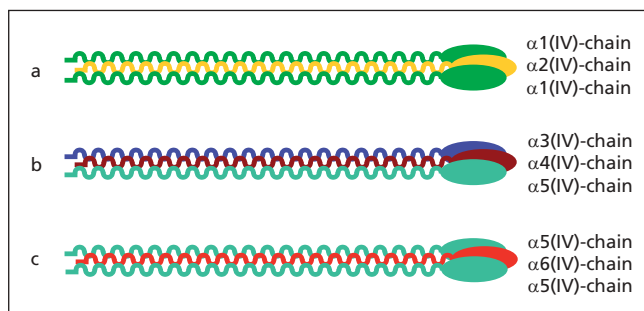


Figure 3. Schematic presentation of the three different type IV-collagen triple helix molecules (protomers) (a-c).

cal type IV collagen molecule, and the formation of the network through dimerization of type IV collagen molecules [123]. Another factor limiting the number of different protomers seen is the tissue-specific expression of the various chains.

The  $\alpha 1(\text{IV})$ - and  $\alpha 2(\text{IV})$ -chains are present ubiquitously in basement membranes as  $[\alpha 1(\text{IV})]_2\alpha 2(\text{IV})$  protomers [124], whereas the  $\alpha 3(\text{IV})$ -,  $\alpha 4(\text{IV})$ -,  $\alpha 5(\text{IV})$ -, and  $\alpha 6(\text{IV})$ -chains have a restricted tissue distribution. In the kidney, the  $\alpha 1(\text{IV})$ - and  $\alpha 2(\text{IV})$ -chains occur in the GBM, Bowman's capsule, and mesangial matrix. The  $\alpha 3(\text{IV})$ - and  $\alpha 4(\text{IV})$ -chains occur in the kidneys exclusively in the GBM, but also in basement membranes in the lungs, in the choroid plexus, and in neuromuscular junctions [125-127]. The  $\alpha 5(\text{IV})$ -chain is present in the kidney in the GBM, in distal tubular basement membranes, and in Bowman's capsule. It is also present in the basement membranes of the skin, trachea, eye, and neuromuscular junctions [127-130]. The  $\alpha 6(\text{IV})$ -chain is localized to the same structures except the GBM [129, 130]. Although the  $\alpha 1(\text{IV})$ -,  $\alpha 2(\text{IV})$ -,  $\alpha 3(\text{IV})$ -,  $\alpha 4(\text{IV})$ -, and  $\alpha 5(\text{IV})$ -chains are co-expressed in the GBM, they separate as  $[\alpha 1(\text{IV})]_2\alpha 2(\text{IV})$  protomers and  $\alpha 3(\text{IV}) \alpha 4(\text{IV}) \alpha 5(\text{IV})$  protomers [131]. Two distinct networks have been identified in the GBM, an embryonic network consisting of the  $[\alpha 1(\text{IV})]_2\alpha 2(\text{IV})$  protomers and a postnatal predominant  $\alpha 3(\text{IV}) \alpha 4(\text{IV}) \alpha 5(\text{IV})$  network [131]. Cross linking disulfide bonds in the cysteine-enriched  $\alpha 3(\text{IV}) \alpha 4(\text{IV}) \alpha 5(\text{IV})$  network confer increased tensile strength and greater resistance to proteolysis as compared to the  $[\alpha 1(\text{IV})]_2\alpha 2(\text{IV})$  network [132]. The NC1 domains of the  $\alpha 3(\text{IV})$ -,  $\alpha 4(\text{IV})$ -, and  $\alpha 5(\text{IV})$ -chains in the GBM dimerize through NC1-NC1 domain interactions, such that the  $\alpha 3(\text{IV})$ -,  $\alpha 4(\text{IV})$ -, and  $\alpha 5(\text{IV})$ -chains of one protomer connect with  $\alpha 5(\text{IV})$ -,  $\alpha 4(\text{IV})$ -, and  $\alpha 3(\text{IV})$ -chains of the opposite protomer [133].

Another network has been found in the basement membranes in smooth muscle tissue comprising the  $\alpha 1(\text{IV})$ -,  $\alpha 2(\text{IV})$ -,  $\alpha 5(\text{IV})$ -, and  $\alpha 6(\text{IV})$ -chains [133]. The  $\alpha 6(\text{IV})$ -chain assemble with the  $\alpha 5(\text{IV})$ -chain at the NC1-domains forming a  $[\alpha 5(\text{IV})]_2\alpha 6(\text{IV})$  protomer, that interacts with a  $[\alpha 1(\text{IV})]_2\alpha 2(\text{IV})$  protomer in a network. This network has been found to co-exist in smooth muscle cells with the  $[\alpha 1(\text{IV})]_2\alpha 2(\text{IV})$  network, and probably also in the skin and in Bowman's capsule.

Thus, the three to date known type IV collagen networks are assembled from three basic protomers with the main composition of  $[\alpha 1(\text{IV})]_2\alpha 2(\text{IV})$ ,  $\alpha 3(\text{IV}) \alpha 4(\text{IV}) \alpha 5(\text{IV})$ , and  $[\alpha 5(\text{IV})]_2\alpha 6(\text{IV})$ . The  $\alpha 5(\text{IV})$ -chain is involved in two of these networks; the  $\alpha 3(\text{IV}) \alpha 4(\text{IV}) \alpha 5(\text{IV})$  network in the GBM, and the  $\alpha 1(\text{IV}) \alpha 2(\text{IV}) \alpha 5(\text{IV}) \alpha 6(\text{IV})$  network in the basement membrane of smooth muscle tissue, skin and Bowman's capsule.

### 1.8.3 The type IV collagen $\alpha$ -chains in AS

In general, when one of the  $\alpha 3$ - $\alpha 5(\text{IV})$  chains is changed or absent due to a mutation in the underlying gene, all three fail to accumulate in the GBM. Variable presence of the  $\alpha 3(\text{IV})$ -,  $\alpha 4(\text{IV})$ - and  $\alpha 5(\text{IV})$ -chains has been reported in females and occasionally in male patients [134-136]. Negative immunohistochemically staining was

found in 88% (14/16) of patients with X-linked AS in the European Collaborative Study [137]. A normal result of the analysis does therefore not exclude a diagnosis of AS. The  $\alpha 6(\text{IV})$ -chain is present in basement membranes together with the  $\alpha 5(\text{IV})$ -chain in the  $\alpha 1(\text{IV})$ - $\alpha 2(\text{IV})$ - $\alpha 5(\text{IV})$ - $\alpha 6(\text{IV})$  network [133], which explains the concomitant loss of both the  $\alpha 5(\text{IV})$ - and  $\alpha 6(\text{IV})$ -chains from basement membranes of patients with X-linked AS.

Heidet et al. [138] studied the glomerular expression of the different type IV collagen chains at the transcriptional and protein level in renal specimens from male patients with X-linked AS. By *in situ* hybridisation they found in two patients affected with a frameshift mutation and a large deletion, respectively, no detectable *COL4A5* transcript, suggesting a nonsense mediated mRNA decay [139], whereas a *COL4A5* transcript was clearly expressed in a patient with a 3'-splice site mutation. In all three patients *COL4A3* and *COL4A4* transcripts were readily detected in podocytes indicating that there is no co-regulation of all three genes. Immunohistochemical staining demonstrated the  $\alpha 3(\text{IV})$ -chain in podocytes, which are not seen in normal kidneys. These results indicate that the absence of the  $\alpha 3(\text{IV})$ -chain from the GBM in X-linked AS is a post-translation event. The absence of the  $\alpha 5(\text{IV})$ -chain, or the synthesis of an abnormal  $\alpha 5(\text{IV})$ -chain, can prevent the integration of the  $\alpha 3(\text{IV})$ - and  $\alpha 4(\text{IV})$ -chains, which continue to be normally synthesized by podocytes.

The  $\alpha 1(\text{IV})$  through  $\alpha 6(\text{IV})$  collagen chains is present in the normal anterior lens capsule [140]. The anterior lens capsule obtained from a male patient with AS and anterior lenticonus lacked the  $\alpha 3(\text{IV})$  through  $\alpha 6(\text{IV})$  collagen chains [140]. However, normal reactivity to the  $\alpha 3(\text{IV})$ -,  $\alpha 4(\text{IV})$ - and  $\alpha 5(\text{IV})$ -chains in a male patient [141], and reduced reactivity in a female carrier with anterior lenticonus, has also been found [141].

The  $\alpha 5(\text{IV})$ -chain is normally present in both the GBM and the epidermal basement membrane. The absence of the  $\alpha 5(\text{IV})$ -chain in the epidermal basement membrane in skin biopsies from patients with X-linked AS can be used in the diagnosis for confirmation. However, a normal result does not exclude X-linked AS, as only about 82-88% of the patients have a normal  $\alpha 5(\text{IV})$  expression pattern [137, 142]. A skin biopsy is a less invasive procedure than a kidney biopsy, and can be used even in patients with renal fibrosis and atrophy.

The  $\alpha 3(\text{IV})$ -,  $\alpha 4(\text{IV})$ - and  $\alpha 5(\text{IV})$ -chains are absent from the GBM in the autosomal forms of AS, whereas the  $\alpha 5(\text{IV})$ - and  $\alpha 6(\text{IV})$ -chains remain in Bowman's capsule and epidermal basement membranes [135].

The basis for the progressive renal ultrastructural changes in the form of thinning, thickening, and lamellation in the GBM in AS is thought to be related to the normal developmental switch from the embryonic  $[\alpha 1(\text{IV})]_2\alpha 2(\text{IV})$  network to the mature  $\alpha 3(\text{IV})\alpha 4(\text{IV})\alpha 5(\text{IV})$  network [132]. The type IV collagen network formed by  $\alpha 1(\text{IV})$ - and  $\alpha 2(\text{IV})$ -chains exhibits a reduced stability and is more susceptible to proteolysis, probably due to a significantly lesser cysteine content in the  $\alpha 1(\text{IV})$ - and  $\alpha 2(\text{IV})$ -chains than in the  $\alpha 3(\text{IV})$ - and  $\alpha 4(\text{IV})$ -chains, and thereby a less extensive disulphide cross-linking in and between the  $\alpha$ -chains. One of the molecular mechanisms underlying the pathogenesis of AS is the defective assembly of the  $\alpha 3(\text{IV})$ -,  $\alpha 4(\text{IV})$ - and  $\alpha 5(\text{IV})$ -chains [143]. In addition, replacement of the normal mature  $\alpha 3(\text{IV})\alpha 4(\text{IV})\alpha 5(\text{IV})$  network with the  $[\alpha 1(\text{IV})]_2\alpha 2(\text{IV})$  network results in accumulation of various noncollagenous extracellular matrix molecules in the basement membrane. Ectopic accumulation of different laminins in the GBM occurs in patients with both the X-linked form and the autosomal recessive form of AS, and in different animal models of AS, which could play a role in the pathogenesis of the renal disease [144, 145].

#### 1.8.4 AS and renal transplantation

The only treatment available so far for the renal disease in AS is dialysis and transplantation. The introduction, with a renal allograft, of

a normal GBM into a patient with AS and a defect GBM, may elicit the formation of antibodies directed against the normal type IV collagen  $\alpha$ -chains. Since the first descriptions by McCoy et al. [146] and Milliner et al. [24] of anti-GBM mediated nephritis in the allograft following renal transplantation, several such cases have been reported [147-152]. Post-transplant anti-GBM mediated nephritis is characterized by presence of anti-GBM antibodies in the serum, proteinuria, crescentic glomerulonephritis, and potential graft loss. However, loss of an allograft caused by anti-GBM antibody mediated nephritis is a rare complication, occurring in only 3-4% of transplanted male Alport patients [153]. In other materials, the overall outcome of renal transplantation in patients with AS does not seem to differ from controls with other renal diseases [154, 155]. AS patients who develop post-transplant anti-GBM nephritis are usually males with hearing loss and a juvenile form of the disease with end-stage renal disease before the age of 30 [153]. In cases where graft loss occurs due to anti-GBM antibodies, re-transplantation usually fails [156, 157]. Browne et al. [158] summarized the results of 16 retransplantations, of which 14 received a second transplant, and 2 received a third transplant. Post-transplant anti-GBM nephritis developed in 15 out of 16 retransplants (93%), and 12 of the 15 grafts were lost from two weeks to 30 months following transplantation. Appearance of linear IgG staining of the GBM and/or circulating anti-GBM antibodies after renal transplantation in AS patients is frequently, but not necessarily, associated with poor graft outcome [24].

The  $\alpha 5(\text{IV})$  NC1-domain is the primary target for alloantibodies in most patients with X-linked AS, who develop post-transplant anti-GBM nephritis [159, 160]. Alloantibodies directed against the  $\alpha 3(\text{IV})$  NC1 domain have been described in patients with X-linked AS harbouring a *COL4A5* mutation [159, 161, 161], as well as in patients with autosomal recessive AS caused by a *COL4A3* mutation [160]. The  $\alpha 3(\text{IV})$ -alloantibodies may therefore also mediate the post-transplant anti-GBM nephritis, resulting in loss of graft function in some patients. The incidence of *COL4A5* deletions, as opposed to other *COL4A5* mutations, is much higher in AS patients who develop post-transplant anti-GBM nephritis than in the general AS-population [162]. In the material collected in the "European Community Alport syndrome Concerted Action", only in three out of 80 (4%) of transplanted male patients with an identified *COL4A5* mutation developed post-transplant anti-GBM glomerulonephritis. All three had large *COL4A5* deletions [137]. Post-transplant anti-GBM nephritis is, however, not restricted to patients with *COL4A5* deletions, as a *COL4A5* splice-site mutation [163] and a *COL4A5* missense mutation [164], also have been associated with post-transplant anti-GBM nephritis.

Females heterozygous for a *COL4A5* mutation are not expected to develop post-transplant anti-GBM nephritis, due to the production of a normal  $\alpha 5(\text{IV})$ -chain by the normal *COL4A5* allele. Among 13 carrier females who underwent renal transplantation, none developed anti-GBM nephritis on the graft [137]. Post-transplant anti-GBM nephritis has, however, also been described in females with an autosomal recessive form of AS due to *COL4A3* mutations [165, 166].

#### 1.9 THE CLINICAL SPECTRUM OF TYPE IV COLLAGEN DISEASES

A broad spectrum of phenotypes is associated with mutations in the *COL4A1*, *COL4A3*, *COL4A4*, *COL4A5*, and *COL4A6* genes (Table 2), whereas no diseases have so far been associated with *COL4A2* encoding the  $\alpha 2(\text{IV})$ -chain.

##### 1.9.1 Autosomal recessive AS

Parental consanguinity, absence of severe symptoms in parents, and full expression of the AS phenotype in affected females, are suggestive of *autosomal recessive AS* (OMIM: #203780).

Hematuria is persistent in both males and females and the disease

**Table 2.** Spectrum of phenotypes associated with mutations in the COL4A1, COL4A3, COL4A4, COL4A5, and COL4A6 genes.

Disease	OMIM#	COL4A1	COL4A3	COL4A4	COL4A5	COL4A6
AS, X-linked . . . . .	301050				+	
AS, autosomal recessive . . . . .	203780		+	+		
AS, autosomal dominant . . . . .	104200		+	+		
Benign familial hematuria . . . . .	141200		+	+		
AS with diffuse leiomyomatosis . . . . .	308940				+	+
The AMME complex . . . . .	300194				+	
The HANAC syndrome . . . . .	611773	+				

progresses to ESRD during the second or third decade of life. The spectrum of ophthalmological manifestations appears to be the same as seen in X-linked AS [167].

In 1993, linkage to the COL4A3/COL4A4 locus was demonstrated in three consanguineous families with an autosomal recessive form of AS [168]. Shortly after that, in 1994, Mochizuki et al. [165] found homozygous mutations in COL4A3 and in COL4A4 in consanguineous families with AS.

Forty-seven different pathogenic mutations in the COL4A3 gene in patients with autosomal recessive AS have so far been described [104, 165, 166, 169-175]. Twenty-two are frameshifts or nonsense mutations, six are splicing mutations, and eighteen are missense mutations. Furthermore, a number of variants of unknown significance (non-glycine substitutions in the collagenous domain), and coding region polymorphisms have been identified [104, 171]. Sixty percent (28/47) of the COL4A3 mutations seen in autosomal recessive AS are truncating.

Twenty-seven different mutations in the COL4A4 gene have been identified in patients with an autosomal recessive form of AS, of which twelve were nonsense mutations or frameshifts, two *in frame* deletions of 18 bp, four splice site mutations, and nine missense mutations [105, 165, 172-174, 176, 177]. One mutation, an *in frame* deletion of 18 bp, was found in homozygous form in a patient with AS. This mutation segregated in the patients family with the presence of clinical and histological features of benign familial hematuria [105]. In addition to these disease causing mutations, a number of polymorphisms and sequence variants of unknown significance have been identified. A patient compound heterozygous for mutations in the COL4A3 and COL4A4 genes presented clinical features consistent with benign familial hematuria rather than autosomal recessive AS [178]. Such cases are, however, not frequent [104].

Longo et al. [179] studied 36 cases presenting with either typical features of autosomal recessive AS (a rapidly progressive nephritis with males and females equally affected or parental consanguinity), or sporadic cases who had tested negative for COL4A5 mutations. They found 8 different mutations; seven in COL4A3, and one in COL4A4, in six patients (6/36 or 17%). Two patients had two different mutations in compound heterozygous form, and four patients had only one (heterozygous) mutation.

The distribution of the type IV collagen  $\alpha$ -chains in four patients with autosomal recessive AS have been studied by Gubler et al. [135] In three of the cases the  $\alpha 3(IV)$ -,  $\alpha 4(IV)$ -, and  $\alpha 5(IV)$ -chains were found to be absent from the GBM, whereas the  $\alpha 5(IV)$ -chain was found to be present in extraglomerular basement membranes including capsular, collecting ducts and epidermal basement membranes, which is never observed in X-linked AS. In one patient, a girl with a severe phenotype due to a COL4A4 mutation, normal renal and skin distribution of the  $\alpha 3(IV)$ - and  $\alpha 4(IV)$ -chains was found. The distribution of the  $\alpha 3(IV)$ -,  $\alpha 4(IV)$ -, and  $\alpha 5(IV)$ -chains can be used to differentiate the autosomal form of AS from the X-linked form. Absence of the  $\alpha 3(IV)$ - $\alpha 5(IV)$ -chains in the GBM and presence of the  $\alpha 5(IV)$ -chain in extraglomerular basement membranes, capsular, collecting ducts, and epidermal basement membranes indicate that it is an autosomal form of the disease.

### 1.9.2 Autosomal dominant AS

Only a limited number of families with an autosomal dominant

form of AS and a COL4A3 or a COL4A4 mutation have been described: Three families with a COL4A3 mutation, and three families with a COL4A4 mutation.

A large family from Northern Ireland with an *autosomal dominant form of AS* (OMIM #104200) has been described by Jefferson et al. [180]. Linkage to polymorphic markers flanking the COL4A3/COL4A4 loci at 2q35-q37 was found in this family. A COL4A3 splice site mutation resulting in skipping of exon 21, but without changing the reading frame, was found to segregate with the disease in this family [181]. The proband in this family reached ESRD at an age of 35 years, and audiometry revealed a mild high-tone sensorineural deafness. No ophthalmological features were seen. The proband's father also suffered from renal failure. The other affected family members were only mildly affected with moderate impairment of renal function and no hearing loss. Ultrastructural changes in the GBM characteristic for AS were found on a renal biopsy specimen from four affected family members. A non-pathogenic missense mutation (c.4582T>C) in exon 48, resulting in a p.Leu1472Pro substitution was found in a healthy person in the family, and in compound heterozygous form, together with the splice site mutation in exon 21, in five persons from the family including the proband with the most severe phenotype [169]. Those family members who do not carry the p.Leu1472Pro mutation seem to have a less severe phenotype.

Two different COL4A4 mutations have so far been identified in patients with presumed autosomal dominant AS. A Sardinian family with an autosomal dominant form of AS associated with hypercholesterolemia was described by Ciccarese et al. [182] Linkage to polymorphic markers at the COL4A3/COL4A4 locus was found, and sequence analysis revealed a G to T substitution at nucleotide position 1183 in COL4A4, which changes the codon for lysine at amino acid position 325 to asparagine (p.Lys325Asn), but might also cause aberrant splicing since it involves the last nucleotide in exon 16. The most severely affected family member with ESRD at age 29, inherited a mutated allele from both her affected and consanguineous parents. The age at ESRD was 60 and 66 years in two affected males in the family, all having moderate to severe deafness and no ocular signs. Another missense mutation, p.Gly448Ser, was found in heterozygous form in a 35-year-old man with hematuria, proteinuria, slowly progressing renal disease, and ultrastructural findings suggestive of AS [179]. However, no other family members were affected, so this throws doubt upon the autosomal dominant nature of this missense mutation.

Pescucci et al. [183] reported four families with an autosomal dominant form of AS of which two had a COL4A3 mutation, and two a COL4A4 mutation. The phenotypic spectrum seen in these four families range from ESRD in the fifth decade to a nonprogressive isolated hematuria. A reduced penetrance of about 90% was suggested, as some family members, heterozygous for the mutation, were completely asymptomatic.

### 1.9.3 Benign familial hematuria

*Benign familial hematuria* (OMIM: #141200), also referred to as *thin basement membrane disease*, is inherited as an autosomal dominant trait and characterised by persistent or recurrent microscopic hematuria, a uniformly thinned GBM, and normal renal function. Proteinuria and episodes of gross hematuria are uncom-

mon. The disease is not associated with extrarenal manifestations such as deafness or ocular symptoms, characteristic of AS [184]. Familial occurrence of benign microscopic hematuria was first described by McConville et al. [185], and Rogers et al. [186] first demonstrated ultrastructural changes in form of thinning of the GBM in a family with benign hematuria. For review, see reference [187]. AS and benign familial hematuria has been observed in two brothers with consanguineous parents, indicating that autosomal recessive AS and benign familial hematuria are allelic [188].

Benign familial hematuria is a much more frequent disease than AS, and accounts for 31% of patients presenting with persistent microscopic hematuria [189]. Schröder et al. [190] investigated 65 children with isolated hematuria persisting for at least one year. Eight cases (12%) of AS was diagnosed by the combination of characteristic ultrastructural lesions and a positive family history, and 36 children (55%) were classified as having benign hematuria of which 23 (70%) could demonstrate familial occurrence. Piqueras et al. [191] reviewed the renal biopsy findings in 322 children presenting with recurrent macro-, or continuous microscopic hematuria persisting for  $\geq 6$  months, and in whom non-glomerular causes were excluded. A nephropathy characteristic of AS was found in eighty-six patients (27%), IgA nephropathy in 78 patients (24%), and thin GBM in 50 patients (16%). AS may, however, be overrepresented in this material as relatives to index cases were also included.

A thin GBM is not restricted to patients with benign familial hematuria. An early ultrastructural change in boys with X-linked AS is thinning of the GBM. On the other hand, not all cases of thin basement membrane disease are familial, and the terms *benign familial hematuria* and *familial thin basement membrane disease* are therefore not exactly interchangeable [192]. Benign familial hematuria can only be differentiated clinically from early stages of X-linked AS in boys by the inheritance (e.g. father-to-son transmission), as diffuse thinning of the GBM is seen as the only abnormality in about 10% of boys with X-linked AS [137, 193]. Some lamellation of the GBM has also been seen in patients with benign familial hematuria [194, 195]. Recent immunohistochemical studies using confocal laser scanning microscopy have demonstrated significantly weaker labelling of the  $\alpha 5(\text{IV})$ -chain along the GBM of patients with benign familial hematuria [196]. In the "European Community Alport Syndrome Concerted Action" it was found, that 10.3% (12/117) of male patients with X-linked AS had a diffusely thin GBM as the only ultrastructural GBM change [137]. A thin GBM has also been demonstrated in females with AS [37]. Thin GBM is, therefore, in some patients just a stage in the development of a progressive glomerular disease, rather than a disease entity. Buzza et al. [197] found, that 10% of families with a biopsy proven diagnosis of benign familial hematuria showed linkage to *COL4A5* suggesting that they were really AS families. Distinction between AS and benign familial hematuria is essential for establishing prognosis and for genetic counselling.

Lemmink et al. [198] found linkage to the *COL4A3/COL4A4* locus at 2q35-q37 in a large Dutch family with benign familial hematuria. Benign familial hematuria can be caused by a mutation in heterozygous form in either *COL4A4* [105, 171, 198-202], or *COL4A3* [104, 171, 178, 179, 201]. The disease is therefore allelic to autosomal recessive AS. Not all carriers of *COL4A3* or *COL4A4* mutations, however, present with microscopic hematuria [105, 106], and not all families with benign familial hematuria could demonstrate linkage to the *COL4A3/COL4A4* locus or the *COL4A5* locus [197, 203]. Buzza et al. [197] has shown that hematuria segregates with the *COL4A3/COL4A4* locus in up to 36% of families with benign familial hematuria. Benign familial hematuria is therefore a genetic heterogeneous disease. No other loci have so far been identified.

#### 1.9.4 AS with diffuse leiomyomatosis

The association of AS with *diffuse leiomyomatosis* (DL) was first described by García-Torres and Guarner in 1983 [204], and has later

been established as an X-linked dominant disorder: *Diffuse leiomyomatosis with Alport syndrome* (DL-AS) (OMIM: #308940), caused by partial deletion of both the *COL4A5* and the *COL4A6* gene. More than 35 sporadic and familial cases have been described so far [110, 111, 205-223].

DL is a rare condition with benign proliferation of smooth muscle cells most frequently affecting the oesophagus, tracheobronchial tree, and female genital tract [224]. Sporadic as well as familial cases have been described [225-227]. The clinical presentation of oesophageal leiomyomatosis is diffuse thickening of the oesophageal wall resulting in dysphagia, retrosternal pain, vomiting, achalasia, regurgitation with respiratory symptoms, and bleeding. The proliferation of smooth muscle cells is predominantly in the lower third of the oesophagus but may extend variably through the rest of the oesophagus and into the upper stomach. Oesophageal leiomyomatosis appear more often in females than in males [225]. Tracheobronchial leiomyomatosis may cause paroxysmal dyspnoea, and genital leiomyomatosis presents as clitoral and vulva hypertrophy that may extend into the vagina and perineum. DL is inherited as an X-linked dominant trait, but, contrary to AS, DL is completely penetrant and fully expressed in both males and females [213]. For females with DL and hematuria it is important to notice that they might transmit fully penetrant AS their sons [220].

A patient with DL-AS has progressive renal disease, sensorineural deafness, and in some cases ocular symptoms characteristic of AS, in addition to DL. The renal disease associated with DL is generally severe with progression to ESRD early in life. Milder forms with adult onset ESRD ( $>31$  years of age) have also been described [221]. Another feature seen in patients with DL-AS is the development of severe, congenital and bilateral cataract, not usually seen as a part of the AS phenotype [210].

In 1992, Antignac et al. [209] found a deletion in the 5'-end of the *COL4A5* gene, and extending beyond its 5'-end, in three out of three patients with DL-AS. Zhou et al. [111] found, that patients with DL-AS from four unrelated families had deletions that disrupt both the *COL4A5* and *COL4A6* genes. The sizes of the deletions range from  $<10$  to 320 kb. The transcription start of exon 1 of *COL4A5* is separated by only 442 bp from the transcription start site of exon 1' of *COL4A6* [113]. The deletion in patients with DL-AS encompasses only the two first exons of *COL4A6*, with a breakpoint located in the very large intron 2 of *COL4A6*, whereas the *COL4A5* breakpoint can vary [212, 214]. *COL4A5-COL4A6* deletions extending beyond exon 3 of *COL4A6* are associated with AS but not with DL [110, 214]. This indicate that DL is due to either loss of promotor sequences located between the *COL4A5* and *COL4A6* genes [113], or a gain-of-function mutation involving either *COL4A6* or as yet unidentified regulatory sequences located in intron 2 of *COL4A6*, and involved in the regulation of smooth-muscle proliferation, and disrupted by the deletion [223]. A restricted deletion may allow the production of a truncated  $\alpha 6(\text{IV})$ -chain that might be capable of aberrant signalling and abnormal proliferation of smooth muscle cells [214]. Both the  $\alpha 5(\text{IV})$ - and  $\alpha 6(\text{IV})$ -chains of type IV collagen are normally present in smooth muscle cell basement membranes, and are absent in tumor basement membranes. Negative immunohistochemical staining for the  $\alpha 6(\text{IV})$ -chain in tumor basement membranes rules out the hypothesis that DL could be due to a truncated  $\alpha 6(\text{IV})$ -chain exercising a dominant effect through stable integration into the collagen network [228]. Negative staining for both the  $\alpha 5(\text{IV})$ - and the  $\alpha 6(\text{IV})$ -chain was observed not only in tumors from male patients, but also from female patients, suggesting that the tumors developed from cells in which the normal allele is inactivated. In most of these female patients, positive staining was observed in a few smooth muscle cells embedded in large areas of negatively stained tumor cells. By studying a Samoyed dog model of X-linked AS it has been demonstrated that the lack of the  $\alpha 6(\text{IV})$ -chain in AS is related to failure at the protein assembly level, and does not obviously result in oesophageal leiomyomatosis [229]. Another hypothesis is that the



deletions that cause DL-AS also cause disruption of a yet unidentified gene in intron 2 in *COL4A6*, or loss of a regulatory sequence, resulting in smooth cell proliferation [217]. Heidet et al. [230] found somatic deletion of the 5' ends of both the *COL4A5* and *COL4A6* genes in sporadic oesophageal leiomyomas, suggesting that the mechanism leading to sporadic oesophageal leiomyoma is the same as the mechanism leading to inherited DL.

A high percentage of *de novo* mutations in patients with DL-AS have been found (four out of six cases) [213]. Segal et al. [219] isolated and characterized two deletion junctions in two different patients with DL-AS. In a patient previously reported by Renieri et al. [212], a non-homologous recombination was found, fusing a LINE-1 (L1) repetitive element in intron 1 of *COL4A5* to intron 2 of *COL4A6*, resulting in a 13.4 kb deletion. In a second, and previously undescribed patient, an unequal homologous recombination was detected between neighbouring L1 elements in intron 2 of *COL4A6*, resulting in a more than 40 kb deletion.

By studying a canine model of AS it has been shown that smooth muscle cell proliferation or diffuse leiomyomatosis is not a characteristic of AS, even though both the  $\alpha 5(\text{IV})$ - and  $\alpha 6(\text{IV})$ -chains are absent [229]. In patients with AS and a *COL4A5* mutation both the  $\alpha 5(\text{IV})$ - and  $\alpha 6(\text{IV})$ -chains are usually absent in the  $\alpha 1(\text{IV})\alpha 2(\text{IV})\alpha 5(\text{IV})\alpha 6(\text{IV})$  network in smooth muscle basement membranes. The absence of this network is therefore not sufficient to cause diffuse leiomyomatosis. Mutation in a yet unidentified gene close to the *COL4A6* gene could be the cause of diffuse leiomyomatosis [214].

### 1.9.5 The AMME Complex

The AMME Complex (OMIM: #300194) is a contiguous gene deletion syndrome involving the *COL4A5* gene and at least four other genes at Xq22. In 1998, Jonsson et al. [231] described a family in which the mother, her two sons and a daughter had clinical features consistent with X-linked AS. Additional clinical features in the two affected males in the family included mental retardation, elliptocytosis, midface hypoplasia, and different minor dysmorphic features. A submicroscopic deletion encompassing the entire *COL4A5* gene was found in the affected males. The additional features in this family with X-linked AS might be caused by deletion or disruption of X-linked recessive genes adjacent to the *COL4A5* gene thus representing a new X-linked contiguous gene deletion syndrome. Piccini et al. [232] presented evidence that this was a true contiguous gene deletion syndrome by demonstrating deletion of another gene: *FACLA*, and suggested the name AMME due to the distinctive features of AS (A), mental retardation (M), midface hypoplasia (M), and elliptocytosis (E). In addition to *COL4A5* and *FACLA*, two other genes were found to be deleted: *KCNE5* [233] and *AMMECR1* [234] in the family originally described by Jonsson et al. [231] Another family has been described by Meloni et al. [235] with a smaller deletion of about 1 Mb compared to the approximately 2 Mb deletion in the first described family. Comparison with two other deletions extending beyond *COL4A5* in the telomeric direction which cause only AS and not mental retardation, made it possible to define a minimal critical region for mental retardation, which contain four candidate genes: *GUCY2F*, *NXT2*, *KCNE5*, and *FACLA* [235]. Of these, *FACLA*, encoding fatty acid CoA ligase 4, has been demonstrated to be mutated in patients with non-specific X-linked mental retardation [236, 237], and is therefore a good candidate as the cause of mental retardation in the AMME Complex.

### 1.9.6 The HANAC Syndrome

The HANAC syndrome (OMIM: #611773) is an autosomal dominant basement membrane disorder characterised by hematuria, cystic kidney disease, intracranial aneurysms, and muscle cramps. Plasier et al. [238] identified three different *COL4A1* mutations in three families, resulting in different glycine substitutions in the triple helix domain of the  $\alpha 1(\text{IV})$ -chain (p.Gly498Val, p.Gly519Arg, and

p.Gly528Glu). Histological analysis revealed a complex basement membrane defect in kidney, skin, and in both large and small vessels.

### 1.9.7 Other type IV collagen-related disorders

In addition to disorders caused by a type IV collagen gene mutation, two different autoimmune disorders involving the type IV collagen molecule have been described. *Goodpasture's disease* is a rare autoimmune disorder caused by circulating IgG autoantibodies that bind to the GBM and alveolar basement membrane and results in a rapidly progressive glomerulonephritis and pulmonary haemorrhage [239]. The NC1 domain of the  $\alpha 3$ -chain of type IV collagen, present in glomerular and alveolar basement membranes, is the target antigen in Goodpasture's disease [240, 241]. Two different epitopes have been identified in the  $\alpha 3(\text{IV})$  NC1 domain, close to the collagenous domain [242, 243].

Another, and probably very rare, type IV collagen autoimmune disorder with circulating IgG autoantibodies directed against the NC1 domain of the  $\alpha 5(\text{IV})$  and  $\alpha 6(\text{IV})$  collagen chains has been described [244, 245]. The disease is characterised by severe subepidermal bullous eruptions of the skin, mucosal lesions, and renal failure due to crescentic glomerulonephritis.

### 1.10 AS WITH ADDITIONAL FEATURES AND AS-LIKE PHENOTYPES

In rare instances, patients with AS have additional features or other inherited disorders not generally associated with the condition. Some might be incidental, and other as a part of a common genetic background.

#### 1.10.1 AS and mental retardation

Schafer et al. [246] described a family with a hereditary nephropathy in association with deafness, hyperprolinemia, and cerebral dysfunction. The index case in this family was mentally retarded. Gubler et al. [31] reported on a boy and his affected siblings with AS and mental retardation. Robson et al. [247] describes a family in which the affected males (two brothers and their maternal uncle) have a progressive renal disease with ultrastructural changes typical of AS, a mild bilateral sensorineural hearing defect, macrocephaly and mental retardation. The mothers of the affected males in this family had hematuria. No information concerning the *COL4A5* gene in this family was presented. A boy presented by Kawakami et al. [248] was found to have a nephrotic syndrome without hematuria, severe sensorineural deafness detected at the age of 4 weeks, psychomotoric retardation, hyperkinesia, cleft soft palate, and negative family history for renal disease and mental retardation. A renal biopsy revealed widespread irregular thickening of the glomerular basement membrane with splitting of the lamina densa on electron microscopy, characteristic of AS. Immunohistochemical staining of the GBM using Goodpasture antibodies was normal.

#### 1.10.2 AS associated with other disorders

Cases in which an AS-like phenotype have been combined with other disorders such as polyneuropathy, hyperprolinemia, and ichthyosis, have been reported [249]. A combination of AS and a polyneuropathy (Charcot-Marie-Tooth disease) have been reported in at least 12 cases [250-254]. The evidence that the nephropathy is AS is not convincing in all cases, however, and the polyneuropathy in some of the cases is probably related to the uremia.

#### 1.10.3 An AS-like phenotype associated with macrothrombocytopenia (Epstein and Fechtner syndrome)

The association of an AS-like phenotype (hereditary nephritis and deafness) with macrothrombocytopenia was first described by Epstein et al. [255] in 1972. Two large and unrelated families were described in which two and three members, respectively, were affected with an autosomal dominant disorder characterized by nephritis,

deafness, and macrothrombocytopenia. This disorder has later been referred to as *Epstein syndrome* (OMIM: #153650). More than 50 families with similar clinical features and autosomal dominant inheritance have so far been described [256-266]. *Fechtner syndrome* (OMIM: #153640), named after the surname of the proband in the family reported by Peterson et al. [267] in 1985, is another autosomal dominant variant of an AS-like phenotype associated with macrothrombocytopenia. In addition to the symptoms seen in Epstein syndrome, patients with Fechtner syndrome exhibit characteristic pale blue inclusions in polymorphonuclear leukocytes (Döhle-like bodies) and congenital cataracts.

The renal disease in Epstein and Fechtner syndrome is characterized by hematuria and proteinuria, and progression to ESRD in the fourth to fifth decade, earlier in some patients [268]. There is a wide intrafamilial variability in the clinical features [269]. Ultrastructural GBM changes resembling those seen in AS have been seen in patients with Epstein syndrome, whereas the distribution of the  $\alpha 3$ -,  $\alpha 4$ -, and  $\alpha 5$ -chains of type IV collagen is normal in renal specimens from patients with Epstein syndrome [270]. The deafness in Epstein and Fechtner syndrome is a high-tone sensorineural deafness, also characteristic of AS. The ocular manifestations characteristic of AS (dot-and fleck retinopathy and anterior lenticonus) is, however, absent [271].

Leukocyte inclusions have also been observed in two other autosomal dominantly inherited disorders characterized by macrothrombocytopenia, but without nephropathy, deafness, and ocular defects: Sebastian platelet syndrome and the May-Hegglin anomaly. *Sebastian platelet syndrome* (OMIM: #605249), first described by Greinacher et al. [272], is a variant of hereditary macrothrombocytopenia combined with inclusions in polymorphonuclear leukocytes similar to those seen in patients with Fechtner syndrome, but without nephritis and sensorineural deafness. Low penetrance of the renal manifestations in a four-generation family with Fechtner syndrome has been described by Rocca et al. [273], indicating that the two conditions are related. *The May-Hegglin anomaly* (OMIM: #155100) is another autosomal dominant form of macrothrombocytopenia associated with leukocyte inclusions. Sebastian platelet syndrome and the May-Hegglin anomaly can be differentiated by subtle ultrastructural leukocyte inclusions. The clinical symptoms in patients with these forms of megathrombocytopenia range from no bleeding to moderate mucocutaneous bleeding, most often without requirement for therapeutic intervention.

A locus for Fechtner syndrome was mapped to the long arm of chromosome 22 (22q11-q13) in 1999 in an extended Israeli family of Iraqi origin, originally described by Gershoni-Baruch et al. [274, 275] Cusano et al. [276] further refined the locus in a large five-generation Italian family to a region less than 600 kb containing six known genes including the nonmuscle heavy chain 9 gene (*MYH9*), encoding the non-muscle myosin heavy chain II A (MYHIIA). In a linkage study comprising one of the families originally described by Epstein et al. [255], one family with Fechtner syndrome, and two families with Sebastian platelet syndrome, Toren et al. [277] found significant lod-scores using markers from the 22q11-q13 region, suggesting Epstein syndrome, Fechtner syndrome, and Sebastian platelet syndrome to be allelic. The May-Hegglin anomaly maps to the same region on chromosome 22 [278-280].

In 2000, the *May-Hegglin/Fechtner Syndrome Consortium* [281] and Kelley et al. [282], found *MYH9* to be mutated in patients with

the May-Hegglin anomaly, Fechtner syndrome and Sebastian platelet syndrome. Mutations in *MYH9* were later found also in patients with Epstein syndrome [264, 283]. In the kidney, MYHIIA is a major component of the actin-myosin contractile apparatus in the podocyte foot process, and is thought to play a role in maintaining capillary wall integrity against hydraulic pressure [264]. *MYH9* is expressed in the mature kidney mainly in the glomerulus in the epithelial visceral cells and in peritubular vessels [284, 285]. Expression of *MYH9* within the rat cochlea has been localized to the organ of Corti, in the subcentral region of the spiral ligament, and in the Reissner membrane [285]. In addition to these macrothrombocytopenias an autosomal dominant form of post-lingual deafness, characterized by progressive hearing loss and cochleosaccular degeneration (Scheibe dysplasia): *DFNA17* (OMIM: #603622) is also caused by mutation in *MYH9* [285, 286].

Twenty-three different putative disease causing *MYH9* mutations have so far been found, 12 of which are associated with a phenotype of Epstein or Fechtner syndrome [264, 266, 281-283, 285, 287-289]. However, no clear relationships between genotype and phenotype seem to exist.

*MYH9* mutations are thus responsible for a wide variety of different clinical phenotypes ranging from Epstein and Fechtner syndrome to isolated macrothrombocytopenia and non-syndromic hearing impairment (Table 3).

One of the six different AS phenotypes proposed by Hasstedt et al. [71], type V, was an autosomal form of AS with deafness and thrombocytopenia. The renal disease in Epstein and Fechtner syndrome should not be named AS due to differences in phenotype and the different molecular genetic background. The eponym AS should be restricted to patients with a type IV collagen disease.

Due to the common genetic background, the name *MYHIIA syndrome* has been proposed to encompass these autosomal dominant macrothrombocytopenias [283].

## 2. OBJECTIVES OF THE STUDY

The objectives of this thesis have been to:

1. Refine the mapping of the locus for the X-linked form of AS.
2. Establish and evaluate methods for mutation analysis of the *COL4A5* gene.
3. Identify and characterise mutations in the *COL4A5* gene in 135 patients suspected of having AS.
4. Establish genotype-phenotype correlations for prognosis.
5. Implement the results of the molecular genetic analyses in clinical practice for carrier detection and prenatal diagnosis of AS, in order to be able to offer better genetic counselling to the families.

## 3. MATERIALS AND METHODS

### 3.1 FAMILIES

A total of 494 persons from 135 families were included in the study (Table 4). Among the 135 index cases suspected of AS, 107 are males and 28 are females. Their countries of origin are Denmark (68), Sweden (32), Belgium (12), Norway (5), Germany (3), Ireland (3), England (2), Estonia (2), Finland (1), Greece (1), Iran (1), Iraq (1), Mexico (1), Slovenia (1), Turkey (1), and Yugoslavia (1). Some atypical forms of the disease and sporadic cases have been included in order to determine the phenotypic spectrum of AS and possible *de novo* mutations. Blood samples have been obtained from all 494

Table 3. Phenotypic spectrum of disorders caused by *MYH9* mutations.

Disease	OMIM#	Nephritis	Deafness	Cataract	Megathrombocytopenia	Leucocyte inclusions
Epstein syndrome . . . . .	153650	+	+	-	+	-
Fechtner syndrome . . . . .	153640	+	+	+	+	+
Sebastian platelet syndrome . . . . .	605249	-	-	-	+	+
May-Hegglin anomaly . . . . .	155100	-	-	-	+	+
DFNA17 . . . . .	603622	-	+	-	-	-

**Table 4.** Summary of the clinical findings in all 135 families studied.

A. Families with a disease causing *COL4A5* mutation detected.

Family	Country of origin	Probands sex	Affected relatives	Age at ESRD in M/F (years)	Hearing loss	Ocular lesions	GBM changes
DK001	Denmark	M	+	M: 17-22	+	NI	NI
DK002	Denmark	F	+	M: 26/F: 58	+	NI	NI
DK003	Denmark	M	+	M: 13-24-25	+	+	NI
DK005	Denmark	M	+	M: 30-58-60-62	+	NI	+ <sup>a</sup>
DK006	Denmark	M	+	M: 22-28	+	+	NI
DK007	Denmark	M	+	M: 20-32	+	NI	NI
DK008	Denmark	M	+	M: 16-23-24/F: 39	+	+	+
DK009	Denmark	M	+	M: 16-20-39	+	-	NI
DK010	Denmark	M	+	M: 25	+	-	+
DK011	Denmark	M	+	M: 37-39	+	NI	+
DK012	Denmark	M	+	M: 36	+	-	+
DK013	Denmark	M	+	M: 27-29	+	+	NI
DK014	Denmark	M	+	M: 14-31	+	-	NI
DK019	Denmark	M	+	M: 19/F: 72	+	+	NI
DK020	Denmark	M	+	M: 19-45-50	+	+	NI
DK021	Denmark	M	+	M: 29-37/F: 41	+	NI	NI
DK024	Denmark	M	+	M: 14-17-30	+	+	+
DK025	Denmark	M	+	M: 10	+	NI	NI
DK028	Denmark	M	+	M: 19	+	NI	NI
DK035	Denmark	M	+	M: 38-60	+	NI	NI
DK036	Denmark	M	+	M: 26	+	+	NI
DK043	Denmark	M	+	M: 31-39	+	+	NI
DK045	Estonia	M	-	M: >16	+	NI	NI
DK046	Denmark	M	+	M: 30	+	NI	NI
DK048	Sweden	M	+	M: 24-33-44/F: 35-73	+	NI	NI
DK050	Sweden	M	+	M: 20	+	NI	NI
DK054	Sweden	M	+	M: 19	+	+	NI
DK055	Sweden	M	+	M: 34/F: 45	+	NI	NI
DK057	Sweden	M	+	M: 28	+	NI	NI
DK058	Sweden	M	+	M: >25	+	NI	NI
DK064	Sweden	F	+	M: 47-74	+	NI	NI
DK066	Sweden	M	+	M: 17-23	+	NI	NI
DK068	Sweden	M	+	M: 20	+	NI	NI
DK072	Sweden	M	+	M: 35-67	+	NI	NI
DK073	Sweden	M	+	M: 17-19/F: 61	+	NI	NI
DK074	Denmark	M	+	M: >16	+	NI	+
DK075	Germany	M	+	M: 16-25	+	-	NI
DK076	Germany	M	+	M: 14	+	NI	NI
DK079	Belgium	M	+	M: 20/F: 55	+	+	+
DK081	Belgium	M	+	M: 27-44	+	+	NI
DK083	Denmark	M	+	M: 18/F: 50	+	+	NI
DK084	Greece	M	+	M: 25-30/F: 50	+	+	NI
DK086	Denmark	M	+	M: 25	+	NI	NI
DK088	Sweden	M	+	M: 20	+	+	NI
DK089	Denmark	M	+	M: 20	+	+	NI
DK090	Denmark	M	+	M: 23/F: 24	+	+	+ <sup>c</sup>
DK092	Germany	M	+	M: 22	+	-	NI
DK093	Yugoslavia	M	+	M: 38-60/F: 37	+	-	NI
DK095	Ireland	M	NI	-	+	NI	NI
DK096	Norway	M	+	M: 15	+	NI	NI
DK098	Denmark	M	NI	M: >16	+	NI	NI
DK099	Norway	F	+	M: 28	+	NI	NI
DK100	Denmark	M	+	M: >26	+	NI	NI
DK104	Sweden	F	+	-	+	+	NI
DK106	Estonia	M	+	M: 27	+	NI	NI
DK108	Norway	M	+	-	+	NI	NI
DK110	England	F	+	-	+	NI	NI
DK115	Ireland	F	+	M: 24	+	+	NI
DK119	Sweden	F	+	-	+	NI	NI
DK120	Denmark	F	+	-	+	NI	NI
DK121	Belgium	M	NI	M: 29	+	-	-
DK125	Mexico	M	+	M: >28	+	+	NI
DK126	Denmark	M	+	-	+	NI	NI
DK131	Denmark	M	+	-	-	-	+ <sup>c</sup>
DK134	Denmark	M	+	-	+	-	NI
DK138	Belgium	M	NI	-	+	NI	NI
DK142	Denmark	M	+	M: 21/F: 60	+	NI	+
DK143	Belgium	M	-	-	+	NI	+ <sup>c</sup>
DK145	Norway	M	+	-	+	NI	NI
DK148	Denmark	M	+	M: 18	+	+ <sup>d</sup>	+ <sup>b,e</sup>
DK149	Denmark	M	+	-	+	+	+
DK150	Denmark	M	+	M: 24	+	+	NI <sup>b</sup>

M = Male. F = Female. ESRD: End-stage renal disease. GBM = Glomerular basement membrane. NI = No information. a) Normal immunohistochemical staining for the  $\alpha 5(\text{IV})$  collagen chain on kidney tissue. b) Negative immunohistochemical staining for the  $\alpha 5(\text{IV})$  collagen chain on sections from a skin biopsy. c) Negative immunohistochemical staining for the  $\alpha 5(\text{IV})$  collagen chain on kidney tissue. d) Bilateral congenital cataract. e) Additional clinical findings include imperforate anus, cleft palate, and diffuse leiomyomatosis of the esophagus and stomach.

To be continued next page.

Table 4. Continued.

B. Families without a disease causing COL4A5 mutation detected.

Family	Country of origin	Probands sex	Affected relatives	X-linked inheritance excluded	Age at ESRD in M/F (years)	Hearing loss	Ocular lesions	GBM changes
DK004	Denmark	F	+	-	M: 35	+	NI	NI
DK015	Denmark	F	+	-	M: 30	-	NI	NI
DK016	Denmark	F	+	-	M: 27	-	NI	NI
DK017	Denmark	M	+	-	M: 14-14	+	NI	NI
DK018	Denmark	M	-	-	M: 18	+	NI	NI
DK022	Denmark	M	+	-	M: 36-43	-	-	NI
DK023	Denmark	M	+	+	-	NI	NI	NI
DK026	Denmark	M	+	-	M: 19-20	+	+	NI
DK027	Denmark	M	+	+	M: 25/F: 31-49	NI	NI	NI
DK029	Denmark	F	-	-	-	NI	NI	NI
DK030	Denmark	F	+	-	-	+	NI	NI
DK031	Denmark	F	-	-	F: 32	+	NI	NI
DK032	Denmark	F	-	-	F: 47	+	NI	NI
DK033	Denmark	F	-	-	F: 43	+	NI	NI
DK034	Denmark	F	+	-	-	-	NI	NI
DK037	Denmark	M	+	-	-	-	NI	+
DK038	Denmark	M	+	-	-	+	+	+
DK039	Denmark	M	+	-	-	NI	NI	NI
DK040	Denmark	F	+	-	-	NI	NI	NI
DK041	Denmark	M	+	+	M: 32-36	+	NI	+
DK042	Denmark	F	-	-	-	+	NI	NI
DK044	Denmark	M	-	-	-	NI	-	NI
DK047	Sweden	M	+	+	-	+	NI	NI
DK049	Sweden	M	+	-	M: 18-24	+	NI	NI
DK051	Sweden	M	+	-	M: 19-27	+	NI	NI
DK052	Sweden	M	+	-	M: 55/F: 50	+	NI	NI
DK053	Sweden	M	+	-	-	+	NI	NI
DK056	Sweden	M	+	-	M: 22	-	NI	NI
DK059	Sweden	F	+	-	-	-	NI	NI
DK060	Sweden	M	+	+	-	-	NI	NI
DK061	Sweden	M	+	-	M: 14-45/F: 50	+	NI	NI
DK062	Sweden	F	+	-	M: 19	+	NI	NI
DK063	Sweden	M	-	-	M: 37	+	NI	NI
DK065	Sweden	M	+	+	-	-	NI	NI
DK067	Sweden	M	+	-	M: 17-23/F: 26-26	+	NI	NI
DK069	Sweden	M	+	-	M: 27/F: 37	-	NI	NI
DK070	Sweden	F	+	-	-	-	NI	NI
DK071	Sweden	M	+	-	M: 25-26/F: 69	+	NI	NI
DK077	Belgium	M	NI	NI	-	NI	NI	NI
DK078	Belgium	M	NI	NI	-	NI	NI	NI
DK080	Belgium	M	+	NI	-	NI	NI	NI
DK082	Belgium	F	+	-	-	NI	NI	NI
DK085	Denmark	M	-	-	M: 16	+	NI	NI <sup>a</sup>
DK087	Iraq	M	+	+	-	NI	NI	NI
DK091	Slovenia	M	+	-	M: 20	+	-	+
DK094	Ireland	M	NI	NI	-	NI	NI	NI
DK097	Iran	F	+	-	-	-	-	+ <sup>b</sup>
DK101	Norway	M	-	-	M: 14	+	NI	+
DK102	Belgium	M	+	+	M: 25-36 F: 29-32-29	-	NI	NI
DK103	Sweden	M	+	-	M: 18	+	NI	+
DK105	Finland	M	+	-	M: 29	+	+	+ <sup>b</sup>
DK107	Denmark	M	+	-	M: 19/F: 53	-	-	+ <sup>b</sup>
DK111	Denmark	F	-	-	-	+	-	NI
DK114	Denmark	F	-	-	-	+	+	NI
DK116	England	M	+	-	-	+	NI	NI
DK127	Denmark	F	+	-	M: 51	-	NI	NI
DK128	Belgium	M	+	-	-	+	NI	+
DK129	Denmark	F	-	-	-	-	NI	+
DK130	Belgium	M	+	-	-	-	NI	+
DK132	Sweden	M	+	-	-	-	+	+ <sup>b</sup>
DK133	Denmark	M	+	-	NI	+	NI	NI
DK136	Turkey	M	+	-	M: 19-19	+	+	NI
DK144	Denmark	M	+	-	-	+	-	+

M = Male. F = Female. ESRD: End-stage renal disease. GBM = Glomerular basement membrane. NI = No information. a) Macrothrombocytopenia. b) Thin GBM.

persons for linkage studies and/or mutation analysis. Among the 494 persons included in the study, 137 are affected male patients, and 122 are affected females or carriers. Clinical information has been obtained from the patient's case records and through personal interview by the author for most Danish patients, or by collaborators when the patients were from abroad. Patients have been referred to the Department of Clinical Genetics, Aarhus University

Hospital, due to contact with all nephrological units in Denmark, personal contacts in "The International AS Consortium", and by advertising in "EDDNAL" (European Directory of DNA Diagnostic Laboratories (<http://www.eddnal.com>)). A clinical description of two of the families has previously been published: DK003 [290], and DK022 [291].

ESRD is defined as first occurrence of kidney transplantation, ini-

tiation of chronic dialysis, death in uremia or from a kidney disease of unknown type, and excluding all instances of renal failure due to trauma, infection, diabetes, toxic agents, or neoplasm [18]. Hearing loss is defined as either clinical hypacusis or a bilateral hearing deficit in the 2,000-8,000 Hz range detected by audiometry. Ocular changes are defined as characteristic changes recognised by ophthalmoscopy (retinopathy) and/or slit lamp examination (lenticonus).

There are 113 familial cases, 15 sporadic cases (seven males and eight females), and 7 cases with no information regarding their families. In forty-three of the families X-linked inheritance can be clearly demonstrated due to absence of father to son transmission of the disease, at least two affected males, a milder phenotype in affected females than affected males, and the presence of affected individuals over at least three generations. The pedigrees in the other 84 families are compatible with X-linked inheritance including the 13 sporadic cases. X-linked inheritance can be excluded in 8 of the families: DK023, DK027, DK041, DK047, DK060, DK065, DK087, and DK102 due to father to son transmission of the disease. No obvious autosomal recessive pedigrees have been seen. The study has been approved by The Ethical Committee.

### 3.2 CHROMOSOME ANALYSIS

The detection of a structural chromosome rearrangement in patients with a Mendelian disorder has facilitated the mapping and identification of the actual gene in many cases [292]. Early in the project, and before the gene for the X-linked form of AS was identified, we decided to karyotype all probands entering the study. Selected family members were also included to see if a severe phenotype in a female relative could be caused by a structural or numeric X-chromosome abnormality.

The probands from 44 families (32 males and 12 females) suspected of AS, and the probands from two families with benign familial hematuria (one male and one female) were therefore karyotyped.

Chromosome analysis was performed by conventional techniques on cultured peripheral lymphocytes after phytohemagglutinin stimulation. Cells were grown in RPMI 1640 medium supplemented with fetal bovine serum, penicillin (10,000 U/ml), streptomycin (10 mg/ml), and L-glutamine (200 mM). At least 10 metaphases were analysed in Q-banding by fluorescence using quinacrine mustard (QFQ).

### 3.3 LINKAGE ANALYSIS IN X-LINKED AS

#### 3.3.1 Multipoint linkage analysis in 12 AS families

About the time that the first reports on the mapping of the locus for the X-linked form of AS appeared [75-78], we decided to initiate a multipoint linkage study comprising a number of Danish families with a probable X-linked form of AS. Information regarding the order of the polymorphic markers flanking the AS locus could be useful for carrier detection and prenatal diagnosis of the disease in the families, and form the basis for positional cloning of the actual gene for AS.

Twelve families with classical AS or hereditary nephritis without deafness were selected for study. Both families with and without deafness were included, as no difference in recombination fraction was seen between a large Utah family described as an adult form of AS with deafness, and two other families, also with an adult form of AS, but without deafness, when analysed using one of the closely linked polymorphic markers [75].

The probands in the twelve families included in the study had hematuria and progressive renal failure, and met at least two of the following three diagnostic criteria: 1) a positive family history of hematuria, with, or without, renal failure, 2) electron microscopic signs of the disease in a renal biopsy specimen, and 3) extra-renal manifestations including sensorineural deafness and ophthalmological signs (lenticonus or macular flecks). One hundred seven individuals including 20 affected males and 36 affected females

entered this study. All twelve pedigrees were compatible with X-linked inheritance. Materials and methods are described in the accompanying publication [1].

#### 3.3.2 Analysis of microsatellite polymorphisms closely linked to and flanking the COL4A5 gene

Haplotype analysis in patients with identical mutations, and determination of the parental origin of *de novo* mutations, has been performed by analysing microsatellite polymorphisms flanking the COL4A5 gene. Oligonucleotide primer sequences were obtained from the Human Genome Database (<http://www.gdb.org>), and the 5'-end of the forward primer was end-labelled with a fluorescent dye: 6-FAM, -TET or -HEX (DNA Technology). PCR was carried out in a final volume of 10 µl containing genomic DNA (25 ng), Tris-HCl (10 mM, pH 8.3), KCl (50mM), MgCl<sub>2</sub> (1.5 mM), dNTPs (120 µM each; Boehringer), primers (0.1 µM each), Taq DNA polymerase (0.018 U/µl; Boehringer) for 30 cycles (denaturation: 30 s at 94°C, annealing: 30 s at 55-58°C, and elongation: 30 s at 72°C). Samples were denatured in formamide and electrophoresed in the polymer POP4 on an ABI 310 Prism Genetic Analyser and the results were analysed using Genotyper version 2.5 (PE Biosystems). The following polymorphic markers were used with their GenBank Accession no. indicated in parentheses (<http://ncbi.nih.gov/entrez>): DXS1191 (#Z23464), DXS1120 (#L21174), DXS1105 (#Z17275), 2B3 1/3 [293], DXS1210 (#Z23952), DXS456 (#L40351), DXS1059 (#Z3536), and DXS1072 (#Z23963). The listed order of loci is as described by Srivastava et al. [294] and the markers are spanning about 5 cM according to the Geneton Map. The COL4A5 gene is located in the physical map between DXS1105 and DXS1210. The marker 2B3 1/3 is isolated from one of the COL4A5 genomic clones [293].

### 3.4 DETECTION OF MUTATIONS IN COL4A5

Five different techniques have been applied to identify mutations in the COL4A5 gene: Southern blotting analysis using COL4A5 cDNA probes and multiplex ligation-dependent probe amplification (MLPA) for larger rearrangements; and PCR-SSCP analysis, direct sequencing, and RT-PCR analysis of mRNA from cultured skin fibroblasts for smaller (point) mutations. A larger COL4A5 rearrangement is defined as a rearrangement (deletion, duplication or inversion) of one or more exon.

#### 3.4.1 Southern blotting analysis using COL4A5 cDNA probes

In order to detect larger deletions, duplications and other major structural COL4A5 rearrangements, genomic DNA samples from 76 of the probands were screened by Southern blotting analysis using cDNA probes covering the entire coding region of COL4A5. Samples of 8 µg genomic DNA were digested to completion using each of 7 different restriction enzymes (endonucleases) under conditions as indicated by the manufacturer. The following restriction enzymes have been used: *Bam*HI, *Eco*RI, *Hind*III, *Msp*I, *Pst*I, *Taq*I, and *Xmn*I (Boehringer Mannheim). Electrophoresis was carried out in a 0.7% agarose gel, and the DNA was transferred to a Hybond-N membrane (Amersham) by Southern blotting [295]. The filters were subsequently hybridised with seven contiguous or overlapping cDNA probes: JZ-4, HT-14-1, HT14-2, HT14-3, PL-31, MD-6, and PC-4, spanning the entire COL4A5 gene [2, 85]. The <sup>32</sup>P-labeling of the probes was performed by oligonucleotide priming [296].

#### 3.4.2 Multiplex ligation-dependent probe amplification

Screening for COL4A5 deletions and duplications was performed by multiplex ligation-dependent probe amplification (MLPA) using the SALSA P191/P192 Alport V.04 MLPA assay (MRC-Holland, Amsterdam, The Netherlands), as indicated by the manufacturer. The assay consists of two reaction mixes containing probes for 48 of the 51 COL4A5 exons. Probes for exon 8, 25 and 40, and the alternatively transcribed exons 41A and 41B, are not included. In addition,

probes for *COL4A6*, exon 1, 1', and 2 are included. Each probemix contain probes for seven control fragments, and four DNA Quantity control fragments. Details on probe sequences are available on the MRC-Holland website (<http://www.mrc-holland.com>).

Approximately 100 ng genomic DNA was used for the annealing of the probes and subsequent ligation. Electrophoresis and relative quantification of the PCR products were carried out using an ABI 3100 Avant Genetic Analyzer (Applied Biosystems). The peak height for each fragment was measured and analysed using the program GeneMarker v. 1.70 (SoftGenetics, PA, USA). Data are presented in a ratio plot as the ratio of normalised peak intensities between the reference and the sample trace. Data have been normalised by using all peaks in the sample to correct for preferential amplification effects. Median peak intensities are derived from the first nine data points, then sliding to data point 2-10, 3-11, etc. to ascertain the local median intensities. With this method, a ratio of ~1 is obtained in normal males and females. A reduction in the ratio to ~0 in males, and ~0.5 in females, indicates a deletion in hemizygous and heterozygous form, respectively. An increase in the ratio to ~2 in males, and ~1.5 in females should be considered positive for duplication. A normal MLPA ratio is >0.70, and <1.30. All analyses were made in duplicates, and only samples showing consistent results between the two experiments were considered positive for a deletion. Absence of peaks corresponding to two or more contiguous exons in at least two experiments, were taken to represent a genuine deletion and no further investigations were performed. Samples demonstrating absence of a single peak, corresponding to a single exon, were sequenced.

### 3.4.3 PCR-SSCP analysis

#### PCR amplification

Intronic primers for each of the 51 exons and two alternatively spliced exons 41A and 41B were synthesized according to published sequences [6, 90, 92], with exons 11-12 and 14-15 being amplified together (Table 5). Genomic DNA was amplified in a reaction volume of 25 µl containing 200 ng DNA, 200 µM dNTP, 20 pmol of each primer and 1 U *Taq*-DNA polymerase (Boehringer Mannheim). The buffer used for PCR contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 1.5 mM MgCl<sub>2</sub>. The PCR conditions were as follows: Initial denaturation at 94°C for 4 minutes followed by 30 cycles of denaturation at 94°C for 1 minute, annealing for 1 min at a temperature as indicated for each primer pair in Table 5 (page 137), and extension at 72°C for 2 min. The final extension was 4 min at 72°C. Hot start PCR and AmpliTaq Gold DNA Polymerase (Perkin Elmer) was used for exons 1, 8, 36, 37, 41, and 44.

#### SSCP analysis

Single-stranded conformational polymorphism (SSC) analysis [297] was used to screen exon 1-51 of the *COL4A5* gene for mutations. The PCR-SSCP-technique is based on the principle that single-stranded DNA exhibit specific sequence-based secondary structures under non-denaturing conditions. DNA fragments differing in sequence can be detected by altered electrophoretic migration on one, or both single strands in a non-denaturing polyacrylamide gel. The PCR-SSCP method was chosen because it is a simple and effective mutation detection technique. Pre-screening by PCR-SSCP analysis was preferred to direct sequencing at the time the study was designed because it was found to be less time consuming due to a less laboratory workload. The PCR-SSCP method is most sensitive when applied to DNA fragments of approximately 150 bp in size [298].

PCR products for SSCP-analysis were diluted (1:2) in 97.9% formamide containing 2% glycerol, 0.05% bromphenol blue, and 0.05% xylene cyanol, heated for 5 min at 95°C, and then cooled on ice. The single stranded DNA fragments are then separated in a nondenaturing polyacrylamide gel using a semi-automatic electrophoresis system; *PhastSystem*<sup>TM</sup> (Pharmacia Biotech). The single-

stranded DNA fragments will fold in sequence-specific secondary or tertiary structures when entering the non-denaturing conditions in the gel and therefore migrate differently in the gel. The PhastSystem provides carefully controlled electrophoretic conditions and small precast gels with different densities. Different polyacrylamide gels were tested (*PhastGel Homogenous 12.5%*, *20%* and *High Density*) (Pharmacia Biotech) and electrophoresis running conditions were optimised for each DNA fragment to give good separation of the two single strands (Figure 4).

Each exon was screened at two different temperatures (4°C and 15°C) and the running conditions (Vh) at 200 V, 10 mA and 2.0 W for both temperatures are indicated in Table 5 (page 137). Gels were silverstained using *PhastGel DNA Silver Staining Kit* (Pharmacia Biotech), and all samples with abnormal band patterns were subsequently sequenced in both directions.

### 3.4.4 Direct sequencing

All migration shifts detected by SSCP have subsequently been sequenced in both directions, and all abnormal results have been confirmed by analysing a second PCR product. Exons 41A and 41B have been analysed by direct sequencing without pre-screening by SSCP-analysis. Sequencing has been performed by either solid-phase sequencing [299] on single-stranded DNA, or by cycle sequencing of double stranded DNA, on an automated sequencer (ABI 310 Genetic Analyser).

Single-stranded DNA for solid-phase sequencing was obtained by PCR amplification of 200 ng genomic DNA under conditions as described for each primer set listed in Table 5, with the forward primer labelled with biotin in the 5'-end. The double-stranded PCR product was immobilized on streptavidin-coated magnetic beads in a permanent magnet (Dynal A/S, Oslo, Norway). Thirty-five µl PCR-product, 35 µl 6 M LiCl, and Dynabeads M-280 Streptavidin (Dynal), previously washed twice with 100 µl 250 mM TRIS pH 8.0/0.1% Tween 20, were incubated at 48°C for 15 min. Immobilized single-stranded DNA, suitable for sequencing, was obtained by removal of the unbound strand with 200 µl 0.1 M NaOH at 20°C for 4 min. The bound DNA strand was washed first with 100 µl 250 mM Tris pH 8.0/0.1% Tween 20, then with 100 µl TE-buffer, and finally with 60 µl distilled H<sub>2</sub>O. The DNA-beads-complex were resuspended in 12 µl distilled H<sub>2</sub>O.

Solid-phase sequencing was performed using the PRISM Sequenase Terminator Single-Stranded DNA Sequencing Kit (Applied Biosystems) with the same primers as for PCR, and subsequently electrophoresed on an ABI 373A Automated DNA Sequencer (Applied Biosystems).

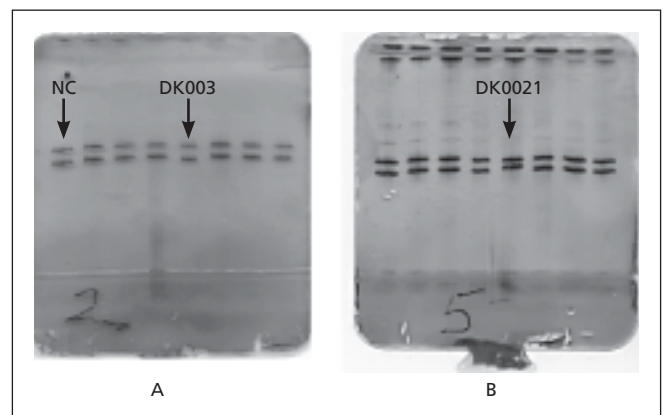


Figure 4. A. PCR-SSCP analysis of *COL4A5* exon 51 from a normal control (NC) and the probands from seven AS families. The gel is shown in full size. A migration shift is observed in DK003 (indicated by an arrow) using the running conditions: 250 Vh at 4°C in a 12.5% gel. B. PCR-SSCP analysis of exon 26 from the probands from eight AS families. A migration shift is observed in DK021 (indicated by an arrow) using the running conditions: 90 Vh at 15°C in a 12.5% gel.

**Table 5.** Primer sequences and conditions for PCR-amplification and SSCP-analysis of the *COL4A5* gene. Primers are synthesized according to published sequences [91, 6, 90]. Exonic sequences are underlined. The no. of intron bases between primer and exon are indicated in parentheses.

Exon	Forward primer (5'→3') Reverse primer (5'→3')	PCR-amplification			SSCP-analysis	
		Annealing temp. (°C)	Product size (bp)	Gel	Running conditions	
					Vh at 4°C	Vh at 15°C
1	TCAATTGGTTAGAGCCA AGCGTGAAGAGCGGTGATG (28)	59 <sup>a</sup>	195	12.5%	150	110
2	GATGTGATTTTCAGTTGAGCTGT (63) CTAAGTACTGAGATAGAAGCT (77)	63	244	12.5%	60	50
3	TCTCAACCATGCCTGTGCTT (44) TGATGTGACACCTACTCCAC (54)	62	229	12.5%	250	175
4	AAAACTAAAAAATATTGCA (8) GTATTTGATATAGAGAAAATAC (7)	45	101	12.5%	70	50
5	GTTTAAGGATTTTATTCTT (7) TCAAAGTAAAAAGTGAATG (8)	45	100	12.5%	110	70
6	ATTATACATGTGTTATGTCG (10) ACTACCAAGATTAATGGA (10)	45	123	12.5%	120	80
7	TCCATGCTCTTATTTTTAA (10) TCATCATAATCCAAATTTT (9)	45	113	12.5%	120	80
8	CAGAAATGACGCTACAGCAG (97) ATTATCCTATTGAAGTTGCCAGC (50)	55 <sup>a</sup>	214	12.5%	70	50
9	AGAACTCCATTGATGGCTTCT (5) AAAAAAGAAAATCACTGGATAC (4)	55	129	12.5%	150	90
10	GGCGACACAAGTGAGACTTT (78) GAATGTTGAGAATGCATTATGTTTT (81)	58	268	12.5%	300	225
11/12	TATTTTCTCTTTGTCTTCTTCTC (4) GAATAACCAGCTCTCTTTTCTTAC (4)	55	224	12.5%	350	175
13	CCACTGTCTTATTTTATCTTGC (6) CATTGACTTCTCCCTACTTAC (4)	55	157	12.5%	150	90
14/15	GATTTCTTTCCCTACTACTG (5) TGCAAAGAATATTAGCAGTTACATC (6)	55	264	12.5%	350	275
16	GCCCTATCATTCTTTGTATCC (5) AGGGGGAAGAACCTTAGCTAC (4)	55	98	12.5%	70	50
17	CTGATGTACCCCTACTCTATG (8) TAGACTAAACCAGTCACTTCAAAG (15)	55	124	12.5%	100	80
18	CTAACCTATTTTACAATTGC (9) ATTTGATAAACGAAGACTAA (15)	55	106	H.D.	900	700
19	GCATTTCTTTATTTTTTTTTTCT (13) CAAGGCCATAAATGCAATC (8)	59	195	H.D.	1,000	800
20	AAGATGAAATCATTGATCAC (15) GAACTTAATAGGAGAAAATATAGC (4)	55	240	H.D.	900	700
21	GCTTGCTATCCTTTCTTATCTTAC (4) GAAGGAATGAATATGTTTGAGATC (4)	55	142	12.5%	130	70
22	TGTTATTATGATTTCACTAG (0) TTAGAAGGTTACCTGAGGC (9)	55	142	12.5%	130	70
23	AAGCTTACGTTATTGTGT (33) TGTAATAATGCCTTCTTCTC (36)	59	176	12.5%	160	300
24	CTTTTTTCTTACTCATTTC (2) AACCAAAAATATCAAACCAAC (4)	57	240	20%	600	300
25	ATATGTTTCTGTATTAAC (14) TAAGCACCAGTTTAAAAC (18)	44	239	12.5%	250	225
26	ACTTCTCATTACCATTGATTTAC (12) GTTACTTTGAAATAAATTCCTCAC (4)	55	157	12.5%	130	90
27	GTTTTCTTTCAATAACTGCTGTTTC (7) ACTCTGCCTGCTACCCATTCC (7)	63	166	12.5%	150	80
28	TCCTTTGGTGGTTAAAAAATGAC (14) GAGAAGGAATAAAGAAAATGTCCC (4)	58	164	12.5%	150	80
29	ATGGGAGTTTTGTTGTGTTTTGTC (15) CAAGTTGAGATGCAAGTGACAGCC (7)	60	221	12.5%	250	150
30	TTAAACTGTATTTATTCTTA (3) TACAAAATGCACATTTACTCTA (7)	44	168	20%	600	300
31	CTTATTAATATTGATATTGATT (6) AAATCAGAGAAAACCTTTAAAC (7)	45	225	12.5%	250	150
32	AATAGTTTTCTGGTTGACATC (68) TATTCTGACTGACATAAAGC (51)	55	251	12.5%	285	175
33	ATATTGTGTTTTACACACATTGA (6) AAATATTCATAATAAATTCATTAC (4)	55	210	12.5%	200	130
34	CTTGCTCTTCTACTCATTCTTG (4) CAATTGCTACAAATGGCCTATCAC (6)	55	156	20%	500	300
35	TTAATTTTACCAATTTGACCTTCC (3) CTAAATATTTGGAAGATTTTCATC (6)	53	147	20%	550	300
36	AATATTATATATCACATTTTTCAAC (2) TGCCTAAAATATATGCCAAAG (5)	54 <sup>a</sup>	195	12.5%	150	85
37	ATTTACATCAAGTACTTACTGGAG (99) AGTCTGCCAATAAAGAAGCTGC (65)	63 <sup>a</sup>	337	12.5%	100	80
38	AAAGCAATGCAGTTTTTCTTTC (27) AACAGCAAACCTGTTATTTTTCATG (5)	55	159	12.5%	85	60
39	GGTGTAACCTGCTGACTCAATT (6) AATAGGAAAAATGAAAACTACAG (3)	55	155	20%	550	300

To be continued next page.

Table 5. Continued.

Exon	PCR-amplification		SSCP-analysis				
	Forward primer (5'→3')	Reverse primer (5'→3')	Annealing temp. (°C)	Product size (bp)	Gel	Running conditions	
						Vh at 4°C	Vh at 15°C
40	TGTTTTGTTTTGTTTTGACTCTG (4)	TTGATTTAGCATGTTTTATTAAGG (5)	53	108	12.5%	85	300
41	TTATCTTCTAATTATACCTTTACTTTT (4)	AGACCATTCTCTACCACCT (10)	55a	246	12.5%	250	225
41A	CTTTTTGTTAATGATGACAT (31)	ACAGAAACACTGGGTTCTACA (151)	55	232	Direct sequencing		
41B	CTTCTGTATGGTTCTGTTTGC (164)	TTGCATTTCTCTATCACACAC (51)	57	267	Direct sequencing		
42	AATGTCGTCATTTGCTGTGGATTA (5)	CATCAGATATCTACTTCCATTTC (3)	55	191	12.5%	210	110
43	CAATCACCTTCTCCCTCG (40)	CAAATCAGAAAATGGCTATCTTG (50)	65	206	12.5%	250	150
44	AAAACCTGATGTACCTTCTGTG (3)	TATAACTATCTTCAGGAATAAGTC (4)	55 <sup>a</sup>	125	20%	600	300
45	CCCTTCAAATTTGTGTGTTTTGTC (6)	GATAATAAAGATGATCTGCATTGG (3)	55	186	12.5%	210	110
46	TATTTGAATGCCTCATTCTTTTC (5)	ACCAACAGCATGTTTACTTGTG (4)	55	155	12.5%	175	125
47	TCTTGATACTGATTATTTCTGTGG (6)	CAGTAGGAAATTAGATATTGATTA (6)	55	273	H.D.	900	600
48	CTTTACTGTTTTCTCCTCAAATCT (6)	TAAAAGTCACAGCTAAATCAATGCC (4)	55	237	12.5%	375	275
49	ATTATGTTCTTCTCCTTTTCTT (5)	ATGACAAATGCAAGGAAGAGTGTA (6)	58	175	12.5%	250	150
50	TTGCGGCACATTTTCTTGTCT (6)	GGACCTGAATTAAGCTATAAGCAC (5)	55	233	12.5%	300	175
51	GATCTGATTGTCTTATTCTTATT (6)	TAAAACACAAAAGGAATTCTCAA	55	139	20%	350	300

a) Hot start PCR. H.D. = High Density.

Cycle sequencing was performed using ABI PRISM Big Dye Terminator Labelling Cycle Sequencing Kit (PE Applied Biosystems) as recommended by the manufacturer, and run on an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems).

Obtained sequences were analysed by Sequence Navigator (Applied Biosystems) and compared to control sequences from normal individuals. Nucleotides and amino acids have been numbered as recommended by Human Genome Variation Society (<http://www.hgvs.org/mutnomen/>), with nucleotide no. 1 being the first nucleotide in the *COL4A5* initiation codon ATG [NM\_000495] (<http://www.ncbi.nlm.gov/entrez/>). The consequences of the mutations at the protein level have been predicted from the same sequence data set.

### 3.4.5 Long range PCR

The result of the Southern blotting analysis of genomic DNA from DK054 indicated a possible structural *COL4A5* rearrangement in intron 6, 7, 8, or 9, but the precise location and the nature of the rearrangement was not obvious (see section 4.3.1) [8]. Expand Long range PCR System (Roche) was used to determine more precisely the location of the breakpoints related to the structural rearrangement detected in DK054. To a 50 µl solution containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 1.75 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 0.1 mM EDTA, 0.5% Nonidet P40, 0.5% Tween 20, 50% glycerol, 0.35 mM dNTP's, 0.3 µM of each primer, and 3.75 U Expand Long Template enzyme mix, was added 1.5 µg genomic DNA. The PCR reaction was performed on an Eppendorf Mastercycler Gradient thermocycler under the following conditions: An initial cycle of denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C at 15 sec, annealing at 55-62°C for 30 s, and extension at 68°C for 4-8 min depending on the length of the fragment. The resulting PCR fragments were separated on a 1% agarose gel. A combination of the primers used for amplification of exon 6, 7, 8, and 9 were tested (see section 4.3.1, and Table 5).

### 3.4.6 Inverse PCR

The nature of the structural rearrangement in DK054 was determined using inverse PCR [8, 300-302]. Inverse PCR is a method by which genomic DNA from the patient is treated with a restriction enzyme and subsequently circularised by ligation. PCR is then performed around the circle using primers from the known sequence across the breakpoint and into the unknown sequence. Sequencing of the PCR product then allows determination of the breakpoints and the origin of the obtained and unknown sequence could be determined by searching the Human Genome Database. The inverse PCR reaction was performed as described by Williams et al. [303] Five µg genomic DNA from DK054 and a normal control were digested for 3 h at 65°C with 20 U *TaqI* restriction enzyme (Boehringer Mannheim) under conditions as recommended by the manufacturer. Digested DNA was then precipitated by phenol/chloroform and resuspended in 50 µl H<sub>2</sub>O. Ligation was carried out overnight at room temperature using 10 µl *TaqI* digested DNA, 50 µl T4 DNA ligase buffer, 440 µl H<sub>2</sub>O, and 0.2 U of T4 DNA ligase (New England Biolabs). The reaction mix was placed on ice before ligase was added. The ligated DNA was phenol/chloroform precipitated and resuspended in 30 µl H<sub>2</sub>O. Two rounds of semi-nested PCR were then performed using the following primers:

1. 5'-CAGTCTGCATTTAGTTATTGGG-3'
2. 5'-AAAGTGTGACGAAAGAACTCAAG-3'
3. 5'-CTTAAAGCCATGGGAACACAGG-3'.

The first round of PCR contained 10 µl of the ligated DNA in a solution with 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP's, 0.2 µM of primer 1 and 2, and 2.5 U AmpliTaq. The PCR reaction was performed on a Eppendorf Mastercycler Gradient thermocycler under the following conditions: 33 cycles of denaturation at 96°C at 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. For the second round of PCR, 1 µl first-round PCR product was used as template and the same set up as used for the first



round, except that primers 1 and 3 were used. A PCR product of approximately 1.35 kb was obtained in the reaction containing DNA from DK054, and 1.45 kb in the reaction containing DNA from the normal control. Both fragments were subsequently sequenced in both directions using primers 1 and 3 as sequencing primers.

### 3.4.7 RNA analysis

#### RT-PCR analysis of mRNA from EBV transformed lymphocytes

RNA was prepared from Epstein-Barr virus (EBV) transformed lymphocytes by acid guanidinium thiocyanate [304] by the RNAzol B Method (WAK-CHEMIE, Bad Homburg).

Reverse transcriptase- (RT-) PCR was performed using the Titan One Tube RT-PCR System (Boehringer Mannheim) as recommended by the manufacturer.

For the c.385-1G>C splice site mutation in intron 6 in family DK050, cDNA was amplified with an exon 6 forward primer, nucleotides 342-359:

5'-GGCCCCAGGACCTCAAGG-3',

and an exon 10 reverse primer, nucleotides 580-560:

5'-TTGGCCCTGGTATACCAGTGG-3'.

PCR conditions were as recommended with an annealing temperature of 62°C. cDNA from the patient in family DK007 with the c.466-2A>G splice site mutation in intron 8 was amplified with the same exonic primers as for the c.385-1G>C mutation.

cDNA from two heterozygous female sisters from family DK002 with the c.3454+1G>T splice site mutation in intron 38 was amplified with an exon 36 forward primer, nt. 3186-3205:

5'-AGGCGACAAAGGTGATCCTG-3', and an exon 39 reverse primer, nt. 3548-3529: 5'-TCACCCCTTGTCCAGCTGG-3', and a PCR program: 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min. All RT-PCR products were separated by electrophoresis in a 1% SeaKem gel (FMC BioProducts), and analysed by sequencing.

#### RT-PCR analysis of mRNA from cultured skin fibroblasts

The skin biopsies were collected in a transportation medium containing RPMI 1640 (Invitrogen), supplemented with fetal calf serum (10%), L-glutamine (1%), and Penicillin/Streptomycin/Phenol red (PSP) (1%), for cell culture [9]. Fibroblasts from explants of the skin biopsies were grown in 25 cm<sup>2</sup> flasks in Bioamf-1 Basal Medium (Invitrogen), supplemented with Bioamf-1 Supplement, L-glutamine and PSP. The skin biopsies were cut into pieces of 1 to 2 mm in size and placed in plastic flasks. Growth medium was supplemented after 1-2 hours and the flasks placed in an incubator at 37°C, and with an atmosphere of 95% air and 5% CO<sub>2</sub>. Cells were grown for 2-4 weeks, and used in 2nd or 3rd passage.

Fibroblasts were grown to confluence, and cytoplasmic RNA was prepared according to the method by Favalaro et al. [305] cDNA was prepared by reverse transcriptase- (RT-) PCR using Qiagen-Omniscript® RT Kit (Qiagen, Hilden, Germany). The entire coding sequence of COL4A5 was PCR amplified from cDNA in nine overlapping fragments using a set of primers modified from Wang et al. [306] (Table 6), and GoTaq® DNA polymerase (Promega, Madison, WI, USA). COL4A6 exon 1'+2 and 3 were PCR amplified as previously described by Mothes et al. [221].

### 3.5 ASSAYS FOR SPECIFIC MUTATIONS

An assay for most of the mutations identified has been set up in order to test for the presence or absence of the mutation in controls and in relatives available for study. The presence or absence of these mutations has been checked in a panel of 50 normal female controls (100 X-chromosomes). Different PCR based methods have used: PCR and gel-electrophoresis for deletions/duplications; PCR and restriction enzyme digestion if the mutation causes a change in a restriction site in the DNA sequence, either by introducing or by abolishing a site. A restriction site can be introduced using a mismatch

**Table 6.** Primers used for amplification of the entire coding sequence of COL4A5. The alternatively transcribed exons 41A and 41B were found not to be included in RT-PCR amplified COL4A5 mRNA from cultured skin fibroblasts. \*) Exon 41 is distributed on two adjacent fragments.

Fragment	Primer sequences	Annealing temp. (°C)	Size (bp)	COL4A5 exons included
A	F: 5'-TCTCTTCACCCAAGCCTCAC-3' R: 5'-CACCAGGGTAACCAGGATCA-3'	58	1101	1-16
B	F: 5'-ACCAGGCAAAGATGGAGAAA-3' R: 5'-CCAGTCCAATGCAGTTGAAG-3'	55	567	17-21
C	F: 5'-GGATCTCCAGGTGATAAAGG-3' R: 5'-CCGGCTGGGTTATAGTCTGA-3'	55	610	22-25
D	F: 5'-AAGGTGTGGCAGGAAATCCA-3' R: 5'-CTATAGGACCAGGTGCTCCGG-3'	58	714	26-30
E	F: 5'-GGCTGCCAGGAATAGTG-3' R: 5'-ACCTTTGGACCAGGAAGAC-3'	58	809	31-35
F	F: 5'-AAGGAACCATCGGTGATATG-3' R: 5'-GGTTGCCTTTAGGTCTTTC-3'	55	685	36-41*
G	F: 5'-GGAATCCTGGCCTCCAG-3' R: 5'-AGACCTGGGTCTCCTTTC-3'	55	645	*41-45
H	F: 5'-GCCTGGCTAAAGGGTCTAC-3' R: 5'-CATTGGCATGGGCTCTGG-3'	55	495	46-47
I	F: 5'-GAGCCCAAGGTCAGACTGG-3' R: 5'-CATTGACGGCAGCAGTAGTA-3'	55	673	48-51

primer if the mutation alone does not cause a change. PCR-SSCP analysis can be used if an easily recognized band pattern is seen. Finally, allele-specific oligonucleotide (ASO) hybridisation on dot-blots can be used. The method of choice for each of the mutations identified is indicated in Table 7, and examples are given below.

#### 3.5.1 PCR and gel-electrophoresis for deletion/duplication

PCR and gel-electrophoresis can visualize all three in-frame deletions identified in this study.

*Assay for the c.2404\_2421del18 mutation in exon 30 in family DK024*  
PCR-amplified exon 30 is electrophoresed in a 3% Metaphor gel and visualized by ethidium bromide staining. The normal allele is 168 bp long, and the deleted allele is 150 bp long.

#### *Assay for the c.3657\_3728del72 mutation identified in DK045*

PCR-amplified exon 41 is electrophoresed in a 3% Metaphor gel and visualized by ethidium bromide staining. The normal allele is 246 bp long, and the deleted allele is 174 bp long.

#### 3.5.2 PCR-based restriction enzyme assays (RFLA)

Many mutations will change the recognition sequence for a restriction enzyme. If the sequence variation caused by the mutation does not alone cause a change in a restriction site, a recognition site for a restriction enzyme can be introduced by using a PCR primer with a single nucleotide mismatch.

#### *Assay for the c.1856C>T missense mutation (p.Pro619Leu) in exon 25 in family DK074*

The c.1856C>T mutation identified in family DK074 changes the recognition sequence for the restriction enzyme *DraII*. Exon 25 is PCR-amplified, digested with *DraII*, and electrophoresed in a 3.5% NuSieve gel (Cambrex Bio Science, Rockland). Four different fragments of 124 bp, 61 bp, 45 bp, and 9 bp are normally seen due to three internal *DraII* restriction sites. The mutation abolishes one of the *DraII* restriction sites resulting in the appearance of an aberrant 54 bp fragment, and the disappearance of the 45 bp and 9 bp fragments.

#### *Assay for the c.4688G>A missense mutation (p.Arg1563Gln) in exon 48 in family DK013*

Genomic DNA has been amplified using a forward primer (nt. 4668-4687).

**Table 7.** Mutations in the COL4A5 gene detected in this study. M = Male. F = Female.

## A. Missense mutations.

Family	Exon	Nucleotide change	Predicted effect	Sex	Reference	Assay(s)
DK036	10	c.584G>A	p.Gly195Asp	M	7	-NlaIV
DK055	10	c.602G>T	p.Gly201Val	M	7	-SexAI
DK145	13	c.689G>A	p.Gly230Asp	M	-	-BtsCI
DK088	13	c.697G>A	p.Gly233Ser	M	-	SSCP
DK096	18	c.1001G>A	p.Gly334Val	M	-	-MaellI, +HincII
DK093	22	c.1507G>C	p.Gly503Arg	M	-	-DrallI, +MaellI
DK021	23	c.1561G>T	p.Gly521Cys <sup>a</sup>	M	2, 7	+HindIII
DK028	24	c.1718G>A	p.Gly573Asp	M	6, 7	+BspHI, -Bst1107I <sup>b</sup>
DK043	25	c.1780G>A	p.Gly594Ser	M	7	Dot blot
DK057	25	c.1780G>A	p.Gly594Ser	M	7	Dot blot
DK064	25	c.1780G>A	p.Gly594Ser	M	7	Dot blot
DK072	25	c.1780G>A	p.Gly594Ser	M	7	Dot blot
DK149	25	c.1780G>A	p.Gly594Ser	M	7, 9	Dot blot
DK046	25	c.1783G>A	p.Gly595Arg	M	7	Dot blot
DK100	25	c.1816G>A	p.Gly606Arg	M	-	-ApyI, +BlnI, +Bfal
DK098	25	c.1835G>A	p.Gly612Asp	M	-	-StuI
DK074	25	c.1856C>T	p.Pro619Leu	M	7, 327	-DrallI
DK131	25	c.1871G>A	p.Gly624Asp	M	6	-BstNI, +Bfal
DK035	25	c.1877G>C	p.Gly626Ala <sup>a</sup>	M	7	Dot blot
DK099	27	c.2042G>A	p.Gly681Asp	F	332	-BstEII
DK108	28	c.2244G>T	p.Lys748Asn	M	-	SSCP
DK115	29	c.2246G>T	p.Gly749Val <sup>a</sup>	F	-	-Hpy166II
DK012	29	c.2297G>A	p.Gly766Asp	M	7	+XbaI, -BsaI
DK119	30	c.2431G>A	p.Gly811Arg	F	-	-NlaIV
DK143	31	c.2344G>A	p.Gly849Glu	M	-	-AvalI
DK005	33	c.2821G>T	p.Gly941Cys	M	6, 7	SSCP
DK121	35	c.3106G>A	p.Gly1036Arg	M	-	-BstNI
DK104	37	c.3319G>A	p.Gly1107Arg	F	321	-MspI
DK010	38	c.3428G>A	p.Gly1143Asp	M	3, 6, 7	-MspI
DK142	39	c.3508G>A	p.Gly1170Ser	M	335, 9	-MspI, +Bsrl
DK075	41	c.3763G>A	p.Gly1255Arg	M	7	-BstNI, +BlnI
DK011	42	c.3808G>A	p.Gly1270Ser	M	7	-AvalI
DK014	44	c.4069G>A	p.Gly1357Ser	M	6, 7, 321	-ApyI
DK086	44	c.4069G>A	p.Gly1357Ser	M	6, 7, 321	-ApyI
DK013	48	c.4688G>A	p.Arg1563Gln	M	4, 7, 68, 335	+MspI
DK076	49	c.4756T>C	p.Cys1586Arg	M	7	SSCP

a) Another sequence variation detected in this patient. b) Using a mismatch primer.

## B. In-frame deletions.

Family	Exon	Nucleotide change	Predicted effect	Sex	Reference	Assay(s)
DK058	21	c.1371_1379del9	p.Pro458_Gly460del	M	7	PCR and gelelectrophoresis
DK024	30	c.2404_2421del18	p.Gly802_Pro807del	M	6, 7	PCR and gelelectrophoresis
DK045	41	c.3657_3728del72	p.Gly1220_Pro1243del	M	7	PCR and gelelectrophoresis

## C. Nonsense mutations.

Family	Exon	Nucleotide change	Predicted effect	Sex	Reference	Assay(s)
DK106	2	c.136G>T	p.Glu46X	M	-	SSCP
DK001	20	c.1219C>T	p.Gln407X	M	6, 7	+NdeIa, +MseIa
DK090	27	c.2098G>T	p.Glu700X	M	9	SSCP
DK126	29	c.2305G>T	p.Gly769X	M	-	SSCP
DK008	36	c.3212C>G	p.Ser1071X	M	6, 7	Dot blot
DK006	48	c.4563C>A	p.Cys1521X	M	7	Dot blot
DK003	51	c.5029C>T	p.Arg1677X	M	6, 7, 321, 335, 140	+AluI
DK083	51	c.5029C>T	p.Arg1677X	M	6, 7, 321, 335, 140	+AluI

a) Using a mismatch primer

## D. Frameshifts.

Family	Exon	Nucleotide change	Predicted effect	Sex	Reference	Assay(s)
DK092	1	c.41_42insTCTT	p.Leu14fsX40	M	-	SSCP
DK066	10	c.548insC	p.Leu184fsX207	M	7	+ApaI
DK095	21	c.1340delG	p.Ser447fsX473	M	-	-HphI
DK068	29	c.2318insA	p.Gly775fsX776	M	7	SSCP
DK084	30	c.2452_2454delATA/2454insT	p.Ile818fsX853	M	7	-BsiYI
DK134	32	c.2690_2694del5	p.Glu897fsX920	M	-	PCR
DK019	33	c.2802insT	p.Gly935fsX942	M	7	+StyI
DK138	33	c.2861_2862insC	p.Met955fsX1010	M	-	+NlaIV
DK073	39	c.3474delG	p.Gln1159fsX1298	M	6, 7	-BsaJI
DK089	42	c.3873_3874insG	p.Leu1292fsX1320	M	-	+BsiYI
DK079	46	c.4253_4262del/4263_4280dup	p.Arg1422fsX1482	M	7	+CviRI
DK009	47	c.4436-4437delGA	p.Gly1479fsX1484	M	5, 7	Dot blot

To be continued next page.

**Table 7.** Continued.

e. Splice site mutations.

Family	Intron	Nucleotide change	Predicted effect	Sex	Reference	Assay(s)
DK050	6	c.385-1G>C	Skipping of exon 7	M	7	- <i>MaeI</i>
DK007	8	c.466-2A>G	Skipping of exon 9	M	7	Dot blot
DK081	9	c.547-1G>A	N.D.	M	7	SSCP
DK048	10	c.609+1G>A	N.D.	M	7	SSCP
DK125	33	c.2918-2A>C	N.D.	M	-	- <i>HpyAV</i>
DK025	35	c.3107-2A>G	N.D.	M	6, 7	+ <i>NlaIV</i>
DK120	38	c.3454+1G>T	Skipping of exon 38	F	6, 7	+ <i>MseI</i>
DK002	38	c.3454+1G>T	Skipping of exon 38	F	6, 7	+ <i>MseI</i>
DK020	38	c.3454+1G>T	Skipping of exon 38	M	6, 7	+ <i>MseI</i>

f. Larger structural rearrangements.

Family	Exon	Nucleotide change	Predicted effect	Sex	Reference	Assay(s)
DK148	1-18 <sup>a</sup>	c.1-?-1032+?del	Absent protein	M	9	MLPA
DK110	20-22	c.1166-?-1516+?del	p.Ala390_Gly506del	F	9	MLPA
DK150	26-37	c.1949-?-3373+?del	p.Pro651_Pro1124del	M	9	MLPA
DK054	9-51	Inversion of exon 9 through 51, contained in a 21 Mb fragment with breakpoints in COL4A5 intron 8 at Xq22.3, and in RAB33A at Xq26.1	Truncated or absent protein	M	8	+ <i>EcoRI</i>

a) The deletion comprises COL4A6, exon 1, 1', and 2.

g. Non-pathogenic sequence variations, polymorphisms, and mutations of unknown significance.

Family	Exon	Nucleotide change	Predicted effect	Sex	Reference	Assay(s)
DK035	1	c.1-13G>A (5'-UTR)	None <sup>a</sup>	M	7	+ <i>NlaIV</i>
NC	10	c.609+21G>T	None	M	7, 320	Dot blot
DK115	20	c.1289C>T	p.Ala430Val <sup>a</sup>	F	-	- <i>BanII</i>
DK021	26	c.1992G>T	p.Lys664Asn <sup>a</sup>	M	332, 321, 7	+ <i>BstYIb</i>
DK032	26	c.1992G>T	p.Lys664Asn	M	332, 321, 7	+ <i>BstYIb</i>
DK082	41B	IVS41B+7C>T	Unknown	M	-	- <i>Eam1104I</i>

a) A COL4A5 missense mutation was detected in the same patient. b) Using a mismatch primer.

5'-CATCCAGCCATTCATTAGCC-3', and the reverse primer 5'-ACCTAGGAGGAATATCATCAG-3'.

The forward primer introduces a single mismatch (T>C) at nucleotide position 4687, and thereby creates a *MspI*-restriction site. The mutation abolishes this *MspI*-restriction site. *MspI*-digestion of PCR-amplified DNA results in fragments of 101 bp and 19 bp in persons without the mutation. A single 120 bp fragment is seen in hemizygous males and in undigested DNA from normal control DNA. Heterozygous females have both the 120 bp fragment and the 101 bp fragment. The PCR fragments are electrophoresed in a 3% NuSieve gel (Cambrex Bio Science, Rockland) along with a molecular weight marker.

*Two different assays for the c.2297G>A missense mutation (p.Gly766Asp) in exon 29 in family DK012*

Two different assays for the c.2297G>A mutation in DK012 were set up. Primers were designed for each assay to introduce one restriction site if the normal sequence is present and another if the mutated sequence is present, respectively. Genomic DNA was PCR-amplified using the forward primer for exon 29 (Table 5) and a reverse primer: 5'-TTTTGGACCAAGTGCTCCTTTGAGA-3' (nucleotides 2298-2322) [92] introducing a single mismatch (T>C) at nucleotide position 2299. The resulting PCR product is 118 bp long and digestion with the restriction enzyme *BsaI* results in fragments of 98 bp, and 20 bp (too small to be seen) when the normal sequence is present. For detection of the mutated sequence, a forward primer was used: 5'-ACCTGGGCCACCAGGACTTCTAG-3', corresponding

to nucleotides 2274-2296 and introducing a single mismatch (C>T) at nucleotide position 2294, and the reverse primer for exon 29 (Table 5). The resulting PCR-fragment is 152 bp and digestion with the restriction enzyme *XbaI* results in two fragments of 133 bp, and 19 bp (too small to be seen) if the 2297G>A mutation is present. The PCR-products are separated in a 3.5% NuSieve gel (Cambrex Bio Science, Rockland) and stained with ethidium bromide.

*Two different assays for the c.1219C>T nonsense mutation (p.Gln407X) in exon 20 in family DK001*

Two different assays for the c.1219C>T mutation in DK001 were set up. Primers were designed for each assay to introduce a restriction site if the mutated sequence is present, and another one if the normal sequence is present. For detection of the mutated sequence, genomic DNA was PCR-amplified using the forward primer for exon 20 (Table 5) and a reverse primer:

5'-TGGTCCTTCATCACCTTTTT-3' (nucleotides 1239-1220) [92] introducing a single mismatch (G>A) at nucleotide position 1220. The resulting PCR product is 111 bp long and digestion with the restriction enzyme *Tru9I* results in fragments of 90 bp and 21 bp (not seen) if the mutated sequence is present. For detection of the normal sequence, a forward primer was used:

5'-TGGATTTCCTGGAGAAAGGGAT-3', corresponding to nucleotides 1297-1218 and introducing a single mismatch (G>A) at nucleotide position 1217, and the reverse primer for exon 20 (Table 5). The resulting PCR-fragment is 172 bp and digestion with the restriction enzyme *NdeII* results in two fragments of 153 bp and 19 bp

(not seen) if the normal sequence is present. The PCR-products are separated in a 3.5% NuSieve gel (Cambrex Bio Science, Rockland) and stained with ethidium bromide.

### 3.5.3 Allele-specific oligonucleotide (ASO) hybridisation on dot-blot

Labelled oligonucleotides specific for the normal and the mutated sequence are hybridised to denatured genomic DNA on dot-blot. A single mismatch is sufficient to render the heteroduplex unstable at high hybridisation stringency.

#### Dot-blot analysis for the c.4436\_4437delGA frameshift mutation in exon 47 in DK009

The method is described in paper 5 [5].

#### Dot-blot analysis for the c.609+21T>C polymorphism in intron 10

Duplicate filters were made by loading 5 µl PCR-amplified exon 10 from each person on a dot-blot apparatus (Minifold). Each filter was hybridised overnight at 42°C in 5 X SSPE, 10 X Denhardt's solution and 0.1% SDS with either an oligonucleotide corresponding to the normal sequence: 5'-TCTTTAATTTAATTTCCCC-3' (filter-N), or the mutated sequence: 5'-CTTTAATTTCAATTTCCCC-3' (filter-M), end-labelled with  $\gamma^{32}\text{P}$ -dCTP by Terminal Transferase (Boehringer Mannheim). Washing was performed once with the hybridisation buffer at room temperature for 10 min. followed by 2.5 × SSPE and 0.1% SDS at 54°C (filter-N), or 51°C (filter-M) for 1 hour. The filters were then autoradiographed at room temperature overnight (filter-N) or for 5 hours (filter-M).

### 3.6 DETECTION OF MUTATIONS IN MYH9 BY DIRECT SEQUENCING

All 40 coding exons of *MYH9* were PCR-amplified in a reaction volume of 25 µl containing 20 µM of each primer, 200 µM dNTP, 10 mM Tris pH 8.8, 50 mM MgCl<sub>2</sub>, and 1 U AmpliTaq Gold DNA polymerase (Perkin Elmer), and sequenced. Intronic primer sequences for each of the 40 exons and PCR cycle condition were as previously published by Heath et al. [283] Sequencing was performed using ABI PRISM Big Dye Terminator Labelling Cycle Sequencing Kit (PE Applied Biosystems) as recommended by the manufacturer, and electrophoresed on an ABI PRISM 310 Genetic Analyser (PE Applied Biosystems). Sequencing was performed in both directions, using the PCR-amplification primers as sequencing primers. The sequences obtained were analysed by Sequence Navigator (Applied Biosystems) and compared to control sequences from a normal individual. Nucleotides and amino acids have been numbered according to GenBank Accession No.: NM\_002473 (<http://ncbi.nih.gov/entrez>).

## 4. RESULTS AND DISCUSSION

### 4.1 CHROMOSOME ANALYSIS

No structural or numeric chromosome abnormalities have been detected.

Only a few cases of cytogenetic abnormalities in patients with AS have been reported. Kapoor and Dasgupta [307] described a 20-year-old female with clinical features of Turner syndrome and microscopic hematuria, albuminuria, bilateral mild perceptive deafness, anterior lenticonus, and a family history of deafness and anterior lenticonus. Her karyotype was found to be 45,X, compatible with hemizygoty for a *COL4A5* mutation and full expression of the phenotype seen in X-linked AS. Renieri et al. [308] presented a patient with Klinefelter syndrome and a missense mutation (p.Gly406Asp) in *COL4A5* in heterozygous form. Father-to-son transmission of a disease normally exclude X-linked disorders. Ars et al. [309] presented a boy with Klinefelter syndrome and a 47,XXY karyotype, who inherited a *COL4A5* splice site mutation and AS from his father.

A boy with AS, mild mental retardation, minor dysmorphic fea-

tures, and a *de novo* 17p11.2 duplication confirmed by FISH analysis was described by Balarin et al. [310] The diagnosis of AS was based on hematuria and typical ultrastructural features on a renal biopsy specimen. No ophthalmological or audiological abnormalities were detected, and no familial history of renal disease. The most frequent mutation seen in Charcot-Marie-Tooth disease type 1A is a 1.5 Mb duplication at 17p11.2 involving the *PMP22* gene and D17S122 [311]. The duplication identified in the boy described by Balarin et al. [310] is proximal to D17S122 and no clinical features typical of Charcot-Marie-Tooth disease type 1 in the patient were recognized. The occurrence of both a 17p11.2 duplication and renal disease in this boy might be incidental.

### 4.2 LINKAGE STUDIES IN FAMILIES WITH X-LINKED AS

At an early point in this study a substantial amount of effort was put into mapping the gene for X-linked AS precisely. At that time the reason for performing these studies was to be able to perform carrier detection and prenatal diagnosis in the families, and to form the basis for positional cloning of the gene for X-linked AS. At present, marker analysis can still be of use in cases where the mutation analysis does not reveal any mutation. In such families one possible solution is to investigate polymorphic markers linked to the genes underlying the X-linked and autosomal forms of AS.

By taking into account the consensus map and the results from other studies at that time, the best supported order of loci was found to be: DXYS1-DXS3-DXS17-(AS,DXS101)-DXS94-DXS11-DXS42-DXS51 [1]. We were not able to place DXS101, DXS88, and DXS11 in relation to the AS-locus because of lack of recombination between the locus and these polymorphisms. The evidence that the AS-locus is proximal to DXS94 comes from only one of the families included in the study: DK004. Subsequently, we have not been able to identify a disease causing *COL4A5* mutation in DK004, suggesting that the disease in this family is not X-linked AS. However, the linkage analysis in this family could neither exclude nor verify X-linked inheritance due to insignificant lod-scores.

Barker et al. [80] presented a high-density genetic and physical map of a large set of polymorphic markers flanking the AS locus. The localization of these polymorphisms and their position in relation to the *COL4A5* gene has been determined in a linkage study of 221 members of two large AS families from Utah, and in a set of somatic cell hybrids with different X-chromosome breakpoints. Their physical map data places DXS101 and DXS94 proximal to the AS-locus.

### 4.3 MUTATIONS IN COL4A5

#### 4.3.1 COL4A5 rearrangements detected by Southern blotting in this study

Three different, presumably structural, *COL4A5* rearrangements were detected by Southern blotting analysis, two of which were found to be caused by changes in restriction sites due to single base substitutions: DK010 [2] and DK021. [3] Only one larger structural *COL4A5* rearrangement was found by Southern blotting analysis (DK054) [8].

#### DK010

Analysis of *MspI* digested DNA from the proband (II:3) from family DK010 showed after hybridisation with the *COL4A5* cDNA probe PL-31 the absence of two fragments of 1.3 kb and 1.9 kb, and the presence of a variant 2.2 kb fragment [3]. This variant 2.2 kb fragment has not been detected in 50 other individuals tested, indicating that this is not a polymorphism. Analysis of DNA from the proband's mother (I:2) revealed that she was heterozygous, having the normal 1.3 kb and 1.9 kb fragments as well as the variant 2.2 kb fragment. The proband's half-sister (same mother) had only the normal 1.3 kb and 1.9 kb fragments. This change in restriction site was found to be caused by a single base substitution: c.3428G>A in exon 38 [3].

### DK021

Normally, HindIII digested genomic DNA reveals four constant bands of approximately 6.2 kb, 5.1 kb, 3.8 kb, and 1.35 kb after hybridisation with the *COL4A5* cDNA probe HT14-2. In the affected proband (IV:4) from family DK021 there was loss of the 1.35 kb fragment, and appearance of a variant 1.25 kb fragment [2]. Analysis of DNA from the other members of the DK021 family demonstrated that the proband's mildly affected female cousin (IV:1) was heterozygous for the mutated allele, having the normal 1.35 kb fragment as well as the variant 1.25 kb fragment. Her two sisters (IV:2 and IV:3), and the daughter of IV:1, had only the 1.35 kb fragment, and, therefore are homozygous for the normal allele.

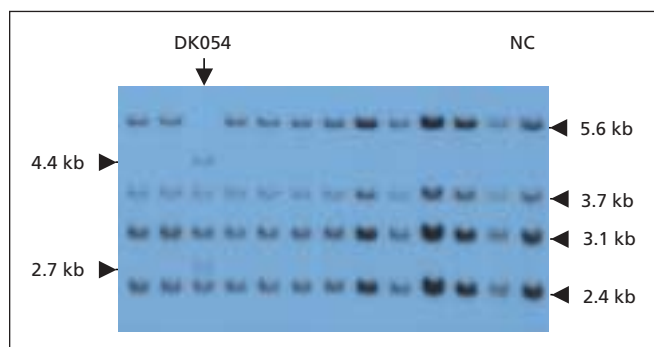
This change in restriction site was found to be caused by a single base substitution: c.1561G>T in exon 23 [2].

### DK054

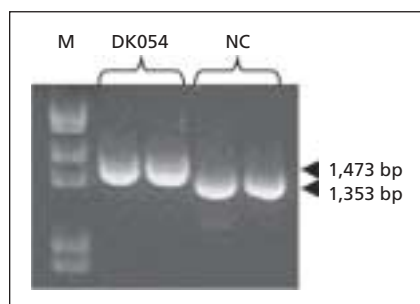
Southern blotting analysis revealed an abnormal band pattern in all restriction enzyme digestions using the *COL4A5* cDNA probe HT14-1, and in a few of the digests using the probe JZ4 [8]. HT14-1 is a 690 bp fragment from nucleotide position 443 in exon 4, to nucleotide position 1,132 in exon 16 [2, 85]. Southern blotting analysis using the restriction enzyme *EcoRI* and hybridisation with HT14-1 normally detects four different bands of 5.6 kb, 3.7 kb, 3.1 kb, and 2.4 kb, respectively. Analysis of genomic DNA from DK054 revealed a missing 5.6 kb band, and two aberrant bands of 4.4 kb, and 2.6 kb, respectively (Figure 5).

A structural aberration is therefore expected to be located within the 5.6 kb *EcoRI* fragment. A more precise location of the breakpoint within this 5.6 kb fragment was found by long range PCR and inverse PCR. Long range PCR was set up to amplify intron 6 through 9, in four different PCR reactions (Table 8).

Since no abnormalities were detected in DK054 by PCR-SSCP



**Figure 5.** Southern blotting analysis of *EcoRI* digested genomic DNA from the probands from family DK052 (left) through DK063 (right), and a normal control person (NC), hybridised to the *COL4A5* cDNA probe HT14-1. An arrow indicates DK054. Normal fragment sizes are indicated on the right, and aberrant fragment sizes from DK054 on the left.



**Figure 6.** Inverse PCR and semi-nested PCR amplification across the breakpoints in DK054 and normal control DNA (NC). Fragments were separated in a 1% agarose gel. M=DNA size marker. The PCR products were subsequently sequenced for identification of the structural rearrangement.

**Table 8.** Long range PCR of intron 6, 7, 8, and 9 of the *COL4A5* gene using a combination of primers described in table 5 (page 137). PCR amplification across the boundary of exon 8 and exon 9 was not possible, whereas amplification across any combination of exons 6-8 or exons 9-10 yielded the expected products. These findings suggested that a change in intron 8 is responsible.

Forward primer	Reverse primer	PCR fragment size (bp)	PCR-product obtained from	
			DK054	Normal control
Exon 6-F	Exon 7-R	2,816	+	+
Exon 7-F	Exon 8-R	528	+	+
Exon 8-F	Exon 9-R	2,045	-	+
Exon 9-F	Exon 10-R	2,426	+	+

**Table 9.** Long range PCR of different intron 8 fragments from the *COL4A5* gene. Each of the four reverse primers is combined with the forward primer: 5'-AAAGCTGGCAACTTCAATAG-3'. For details, see text.

Reverse primer no.	Primer sequence	PCR fragment size (bp)	PCR-product obtained from	
			DK054	Normal control
1	5'-GAATACACATAGCTAACTCAG-3'	480	+	+
2	5'-GGTAAGGGCGGAGTAGTG-3'	828	+	+
3	5'-GATAGGAGGAAGAATATTAGGC-3'	1,138	+	+
4	5'-GGGTAATCAGTAATGGAGAAG-3'	1,408	-	+

analysis of each of the exons 6, 7, 8, and 9 with flanking intronic sequences (see section 4.3.3), and since a long range PCR product was obtained for all except intron 8, the most likely position for the structural rearrangement was within the 1,736 bp intron 8.

To determine more precisely the position of the structural rearrangement within intron 8, an intron 8 forward primer positioned 47 bp downstream from exon 8 was designed:

5'-AAAGCTGGCAACTTCAATAG-3' and combined with several reverse primers located downstream in intron 8 (Table 9).

It appears from Table 9, that the most likely location for the breakpoint is in the distal part of intron 8 within a 270 bp fragment between the position of primer no. 3 and no. 4.

Inverse PCR was used to localize the breakpoint in intron 8 more precisely, and to characterize the nature of the structural rearrangement. A *TaqI* restriction site is positioned proximal in intron 8, and *TaqI* was therefore the restriction enzyme of choice to be used prior to the inverse PCR reaction. Genomic DNA from DK054 and a normal control were *TaqI* digested and subsequently ligated. Two PCR products were obtained; an approximately 1.35 kb fragment from the reaction containing normal control DNA, and an approximately 1.47 kb fragment in the reaction containing DNA from DK054 (Figure 6).

Sequencing of the PCR product obtained from the reaction containing DNA from DK054 revealed a 1,473 bp fragment with breakpoint in intron 8, 1,362 bp downstream from exon 8. The sequence distal for the breakpoint was identified in the Human Genome Database to be an inverted sequence with a breakpoint positioned 56,373 bp upstream from the first nucleotide of the initiation codon for methionine, in the first protein coding exon in the *RAB33A* gene at Xq26.1. *RAB33A* (NM\_004794) (<http://ncbi.nih.gov/entrez>) is a member of the RAS oncogene family, and encodes a small GTP-binding protein. Both breakpoints; the proximal in *COL4A5* intron 8 at Xq22.3, and the distal in *RAB33A* at Xq26.1, have subsequently been sequenced on PCR amplified genomic DNA from DK054 using flanking primers (Figure 7).

Sequencing of the distal breakpoint revealed deletion of the first *COL4A5* intron 8 nucleotide distal to the breakpoint, and insertion (duplication) of four *RAB33A* nucleotides downstream from the

distal breakpoint. These findings indicate, that the structural rearrangement detected in DK054 is a large paracentric inversion of the long arm of the X-chromosome comprising a fragment of 21,366,999 bp, and including *COL4A5* exon 9 through 51, with deletion of a single nucleotide and duplication of four nucleotides in the proximal and distal breakpoints, respectively (Figure 8).

The inversion was not seen by routine chromosome analysis on metaphase preparations in Q-banding. No recommendation exists regarding description of an inversion at this size at the genomic level [312, 313].

The mutation causes a disruption of the gene and will result in a truncated or absent  $\alpha 5(\text{IV})$ -chain, and is therefore expected to be disease causing. This is a type of mutation comparable to the intron 22 inversion seen in severe haemophilia A in about 50% of the cases [314]. This mutation will not be detected by the most commonly used strategies for mutation screening of the *COL4A5* gene: PCR-SSCP analysis or direct sequencing, but only by Southern blotting analysis, as demonstrated in this case, and perhaps by a RT-PCR based mutation-searching approach [315].

#### 4.3.2 Larger structural *COL4A5* rearrangements detected by MLPA in this study

Three larger deletions were detected by MLPA (Table 7f) [9]. The deletion of *COL4A5* exon 1 through 18 in DK148, who presented with AS and diffuse leiomyomatosis, was found to be extended into, and comprising *COL4A6* exon 1, 1', and 2. *COL4A6* exon 3 was

found not to be deleted. A deletion encompassing exon 20 through 22 in heterozygous form was detected in the female probands in DK110. She was previously tested negative for a *COL4A5* mutation by SSCP-analysis. The exon 26-37 deletion in DK150 was found in heterozygous form in his carrier mother, and absent in his healthy sister (Figure 9). No duplications were detected.

#### 4.3.3 Spectrum of larger structural *COL4A5* rearrangements in patients with X-linked AS

Only 6.3% (4/64) of the mutations in the present material can be classified as a large structural rearrangement: Inversion of exon 9 through 51, and three larger deletions of  $\geq 2$  exons.

The frequency of larger *COL4A5* rearrangements in other series vary from 1-2% (1/61) in Japanese patients [316, 317], to 5% (9/172) in Italian patients [318], 10% (2/20) in German patients [319], 10% (3/29) in American patients [320], 14% (11/77) in U k. patients [321], and 16% (14/88) in French patients [322]. More than 45 different larger structural *COL4A5* rearrangements have so far been published. These rearrangements include deletions ranging from one or more exons [87, 162, 316, 318, 319, 322-325] to deletion of the whole gene [319, 322], partial gene duplications [317, 326], and combined partial gene deletion and duplication [326]. No frequent deletion breakpoints have been identified.

#### 4.3.4 *COL4A5* point mutations detected in this study

A summary of the mutations identified in this study is presented in

Figure 7. Upper part: Sequencing of the proximal and distal breakpoints at Xq22.3 and Xq26.1, respectively. Lower part: Partial *COL4A5* and *RAB33A* sequences. Joined sequences are underlined. Arrowheads indicate breakpoints. Deleted and inserted sequences are shown in grey. The arrows indicate the reading direction.

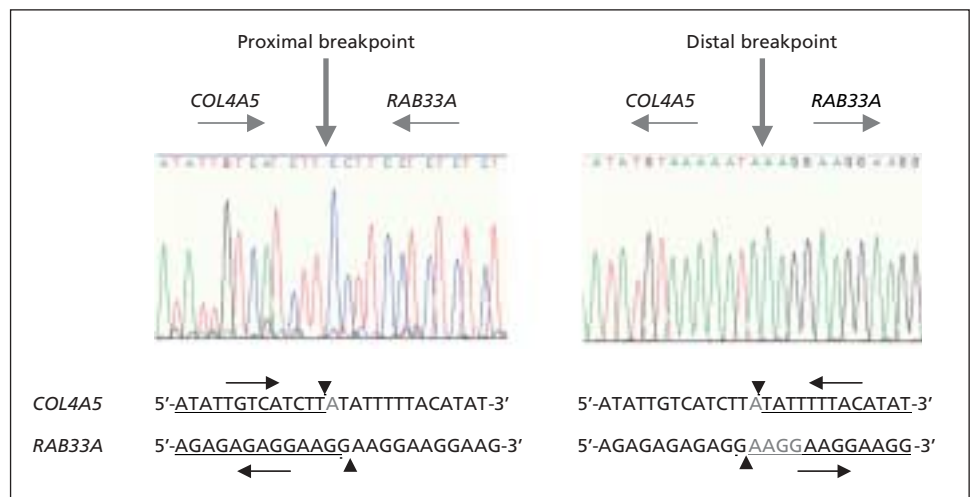
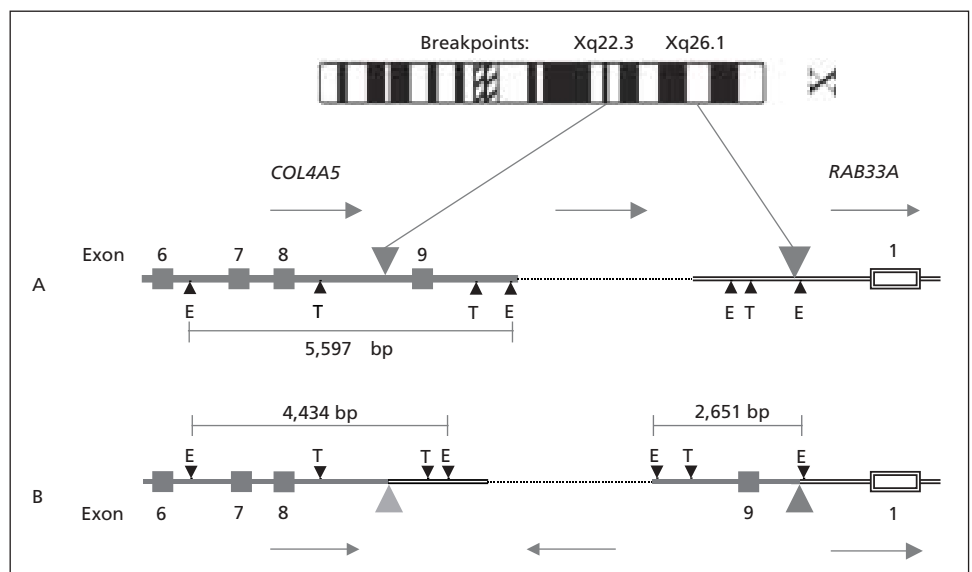
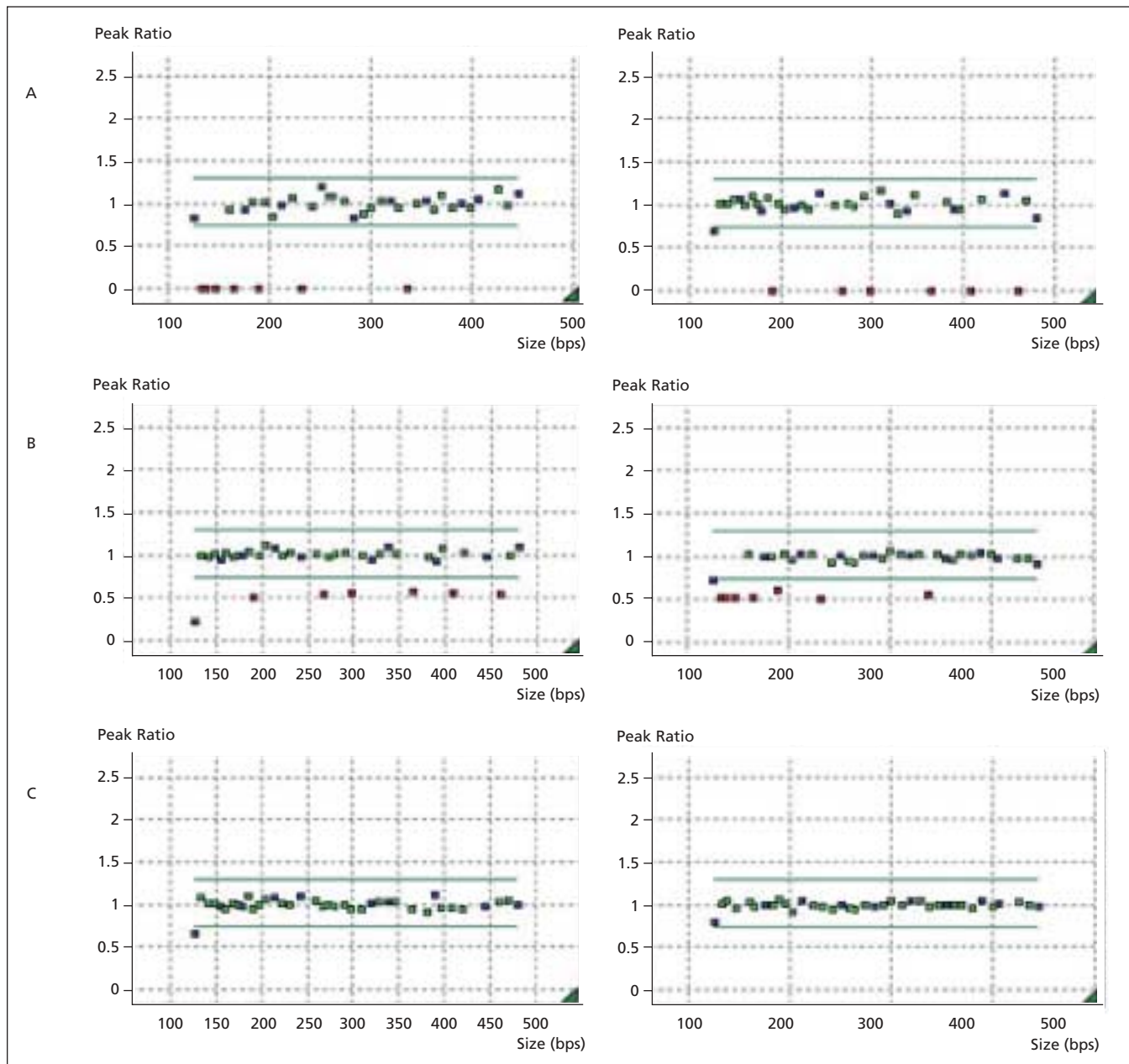


Figure 8. Schematic presentation of the structural *COL4A5* rearrangement found in DK054: Inversion of a 21 Mb fragment comprising exon 9 through 51 with breakpoints in intron 8 at Xq22.3, and in *RAB33A* at Xq26.1. A=Wild type. B=DK054. Large arrowheads indicate the breakpoints. Small arrowheads indicate recognition sites for restriction enzymes: T=*TaqI*, and E=*EcoRI*. Horizontal arrows indicate the normal reading direction. The *EcoRI* restriction fragment lengths refer to Figure 5.





**Figure 9.** MLPA analysis of DK150, and his family, using the SALSA MLPA P191/P192 V.04 Alport assay (MRC-Holland). Data are presented as ratio plots, where each plot point represents the normalised peak height ratio of the reference versus the sample. A: The proband, DK150, with deletion of exon 26-37 in hemizygous form. The deleted *COL4A5* exons (red dots) in P191 are, from left to right: 29, 32, 33, 35, 36, and 26; and in P192: 27, 28, 37, 29, 30, 31, and 34. The undeleted exons and control fragments are represented by green and blue dots, respectively. B: The probands carrier mother, with the same deletion in heterozygous form. C: The probands younger and healthy sister, without deletion.

Table 7, and in Table 10. Point mutations were detected by either PCR-SSCP-analysis [7], direct sequencing [6], or RT-PCR analysis of mRNA from cultured skin fibroblasts [9]. All putative disease causing mutations (except nonsense mutations, frameshifts and in-frame deletions) were confirmed to be causative by their absence in at least 100 X-chromosomes from 50 normal female controls. In those families in which a mutation was identified and with relatives available for study, an assay for the mutation was set up. All mutations were found to co-segregate with the disease in the family.

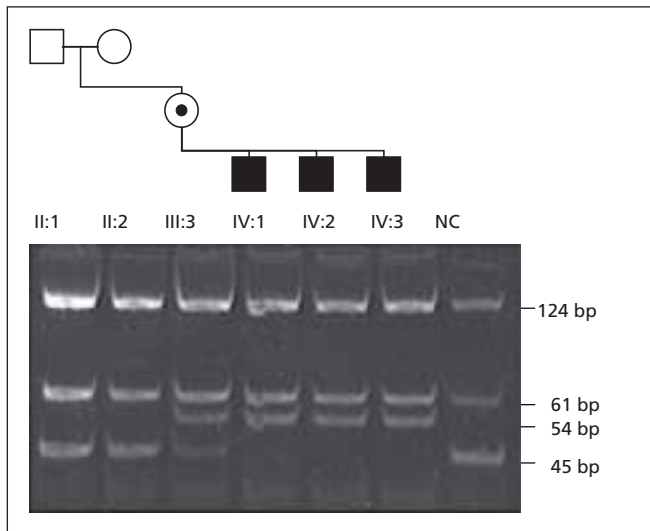
#### Missense mutations

Thirty-one different missense mutations were found in 36 families. Among the 29 different amino acid substitutions detected in the collagenous domain of the  $\alpha 5(\text{IV})$ -chain in this study, 27 were glycine substitutions, interrupting the normal *Gly-Xaa-Yaa* repeat structure. Only two mutation was a non-glycine substitution in the colla-

genous domain. The mutation detected in family DK074, a proline to leucine substitution at amino acid position 619 (p.Pro619Leu), is also assumed to be pathogenic, even though it is a non-conserved residue at position *Xaa* in the *Gly-Xaa-Yaa* repeat structure. The

**Table 10.** Summary of the different mutations detected among the 72 probands in this study.

Mutations	No.	(%)
Missense mutations	36	(50.0)
– Collagenous domain	34	(47.2)
– glycine substitutions	32	(44.4)
– non-glycine substitutions	2	(2.8)
– NC1-domain	2	(2.8)
In frame deletions	3	(4.1)
Nonsense mutations	8	(11.1)
Frame shifts	12	(16.7)
Splice site mutations	9	(12.5)
Larger structural rearrangements	4	(5.6)



**Figure 10.** Assay for a *de novo* c.1856C>T (p.Pro619Leu) missense mutation in exon 25 in the *COL4A5* gene in Alport family DK074. *DralI* digestion of PCR amplified exon 25 results normally in four fragments of 124 bp, 61 bp, 45 bp, and 9 bp (not seen). The mutation abolishes a *DralI* restriction site resulting in the appearance of a 54 bp fragment and loss of the 45 bp and 9 bp fragments as seen in hemizygous form in the three affected brothers IV:1-IV:3, and in heterozygous form in their mother III:3. Two other *DralI* restriction sites serve as internal controls for digestion. The mutation is not present in II:1 or II:2 indicating a *de novo* origin in III:3. NC=normal control.

mutation has arisen *de novo* in the proband's mother, and was found to co-segregate with the disease in the family (Figure 10).

The mutation could not be detected in 50 normal female controls (100 X-chromosomes examined). The same mutation has later been detected in several members of a Spanish family in which all affected male patients had juvenile onset of ESRD, hearing loss, and no ophthalmological changes [327]. The collagen  $\alpha$ -chain normally undergoes important post-translational modifications. Hydroxylation of prolines in the *Xaa*-position to hydroxyprolines results in the formation of hydrogen bonds between  $\alpha$ -chains, and thereby contributes to the stability of the triple helix [235].

Exchange of proline with another amino acid can therefore affect the stability of the  $\alpha$ -chain. The c.2244G>T (p.Lys748Asn) missense mutation detected in DK108 involves the last nucleotide in exon 28, and may affect the normal splicing.

Two different missense mutations have been identified in the NC1-domain. The c.4688G>A substitution in the last nucleotide in exon 48 detected in family DK013 is predicted to substitute arginine at amino acid position 1563 with glutamine (p.Arg1563Gln). Arginine at amino acid position 1563 in the first half of the NC1 domain is highly conserved through evolution from *Drosophila* (IV) and *Caenorhabditis elegans* to mouse, bovine, and human type IV collagen chains [117, 328]. Conservation through evolution indicates functional correlation and thereby importance for the normal

function of the type IV collagen molecule. Most of the conserved NC1 residues seem to be important for structural integrity, whereas the Arg1563 residue seems to modify surface properties of the NC1 domain, and thereby may affect interactions with other extracellular components [329]. The effect of an identical mutation detected in a Dutch patient has been studied by Lemmink et al. [330] By looking at RNA they found the mutation to cause aberrant splicing, resulting in skipping of exon 48. In the same way, substitution of the last nucleotide in exon 49, predicted to result in the missense mutation, p.Met1601Ile, led in fact to aberrant splicing [331]. The c.4069G>A substitution in exon 44 identified in family DK014 and DK086 involves the last nucleotide in the exon. The c.1780G>A substitution in exon 25 identified in family DK043, DK057, DK064, DK072, and DK149, and the c.2042G>A substitution in exon 27 in family DK099, involves the first nucleotide in the exon. In total, eight of the 31 different missense mutations detected (25%) involves either the first nucleotide (2), the second nucleotide (2), or the last nucleotide in the exon (4), and may potentially affect the normal splicing process. We have analysed cDNA prepared from cultured skin fibroblasts from DK149 harbouring the missense mutation c.1780G>A, but no aberrant splicing products were detected. Abnormal splicing in kidney tissue can not, however, be excluded. The importance of the last nucleotide in an exon for correct splicing has also been demonstrated for the *COL4A3* gene. Substitution of the last nucleotide in exon 21 in *COL4A3*, c.1315G>A, was found by van der Loop et al. [181] in a family with an autosomal dominant form of AS. This single nucleotide substitution is predicted to result in a glycine to serine substitution, p.Gly440Ser, but analysis of mRNA from peripheral blood lymphocytes from the patient however, demonstrated abnormal splicing resulting in skipping of exon 21.

Missense mutations affecting the NC1-domain may act by affecting the correct folding of the NC1-domain and thereby prevent its incorporation into the protomer. The other mutation detected in the NC1 domain was detected in the proband in family DK076. This missense mutation: c.4756T>C, change the codon for cysteine to arginine at amino acid position 1586, and thereby affecting one of the cysteine residues important for protein folding and chain assembly.

#### *In-frame deletions*

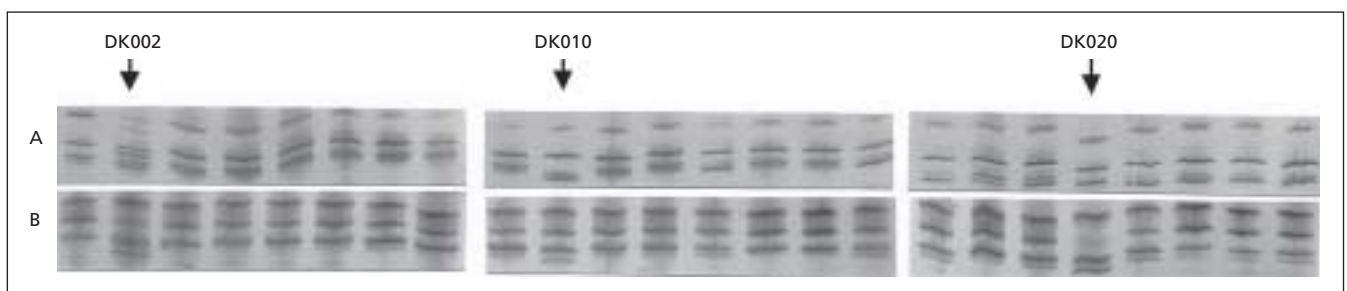
Three different in-frame deletions were found in exons 21, 30 and 41 in three families, resulting in the deletion of one, three, and twenty-four *Gly-Xaa-Yaa* repeats, respectively, in the collagenous domain of the  $\alpha5$ (IV)-chain.

#### *Nonsense mutations*

Seven different nonsense mutations were identified in 8 families. One mutation, p.Arg1677X, was identified in two apparently unrelated patients.

#### *Frameshifts*

Twelve different frameshifts in 12 patients have been detected: six insertions of 1-4 nucleotides, four small deletions of 1-2 nucleotides, and two combined deletions/insertions were found. All of



**Figure 11.** PCR-SSCP analysis of exon 38 with flanking intronic sequences with two different conditions: A 4°C and 85 mVh, and B 15°C, and 60 mVh, separated in 12.5% gel. Aberrant band patterns are seen in DK002, DK010, and DK020, at both temperatures.



them cause a change in the reading frame and introduce a premature stop codon. These mutations, as well as the seven different non-sense mutations, are all expected to result in truncated  $\alpha 5(\text{IV})$ -chains or degradation of the transcript by nonsense-mediated mRNA decay [139].

#### Splice site mutations

Seven different alterations of conserved splice sites were identified in 9 families; two at donor splice sites, and five at acceptor splice sites. In three of the families in which EBV-transformed peripheral lymphocytes were available for study, RT-PCR and sequencing were performed in order to determine the consequences of the mutation at cDNA level.

The same splice site mutation affecting the first nucleotide of the splice donor site in intron 38 (c.3454+1G>T) was identified in two families: DK002, and DK020 (Figure 11 and Figure 12).

RT-PCR and gel electrophoresis of EBV-transformed peripheral blood lymphocytes from the two heterozygous sisters, IV:6 ad IV:9, from family DK002 revealed two different cDNA fragments of 363 bp and 282 bp, respectively, and only the 363 bp fragment in normal control cDNA (Figure 13).

Sequencing of the two fragments revealed that the upstream exon 38 was skipped in the 282 bp fragment, preserving the reading frame (Figure 14).

The two mutations at splice acceptor sites in intron 6 in family DK050, and in intron 8 in family DK007, results in skipping of the downstream exon 7 and 9, respectively. The reading frame is also preserved in these two mutations.

#### Non-pathogenic sequence variations, polymorphisms, and mutations of unknown significance

Three different, presumably non-pathogenic, sequence variations were found. One of these, a G to A transition at nucleotide position 190 in the 5'-UTR in exon 1, was identified in the proband in family DK035. A second and putative disease causing missense mutation, c.1877G>C (p.Gly626Ala), was found on the same allele in this family. This variant was not identified in 80 other AS probands (95 X-chromosomes investigated), and was found to co-segregate with the c.1877G>C missense mutation and the disease in family DK035.

The other sequence variation, a polymorphic T to C transition in intron 10 at nucleotide position 21 downstream from exon 10, was not detected by the PCR-SSCP analysis, but identified fortuitously by direct sequencing of DNA from a normal control. Thirty-two AS probands (26 males and 6 females) (38 X-chromosomes) and 54 normal female controls (108 X-chromosomes) have been typed for this polymorphism by dot-blot analysis, and an allele frequency of 76% for the normal allele (T), and 24% for the variant allele (C) was found. The observed heterozygosity was 28% (17/60). This polymorphism was found to co-segregate with the disease in AS family DK008 and can be used for linkage studies in AS families.

The p.Lys664Asn substitution was identified in two unrelated patients; in DK032 (female) in whom no other mutation was found, and in DK021 (male) as a secondary mutation to the missense mutation p.Gly521Cys. Lys664 is located in the collagenous domain and is not a conserved amino acid. The p.Lys664Asn substitution has previously been identified in other AS patients [7, 321, 332] and also in combination with a second, and disease causing mutation: p.Pro1517Thr [68].

However, the p.Lys664Asn mutation was found not to segregate with p.Pro1517Thr and the disease in the family [68]. p.Lys664Asn is therefore not expected to be pathogenic.

In addition to these normal variants, one mutation of unknown significance was found. Screening of the two alternatively transcribed exons 41A and 41B by direct sequencing revealed a sequence variation in the proband in family DK082. A C>T substitution seven nucleotides downstream from exon 41B was found in heterozygous form in the female proband: IVS41B+7C>T. The mutation abolishes

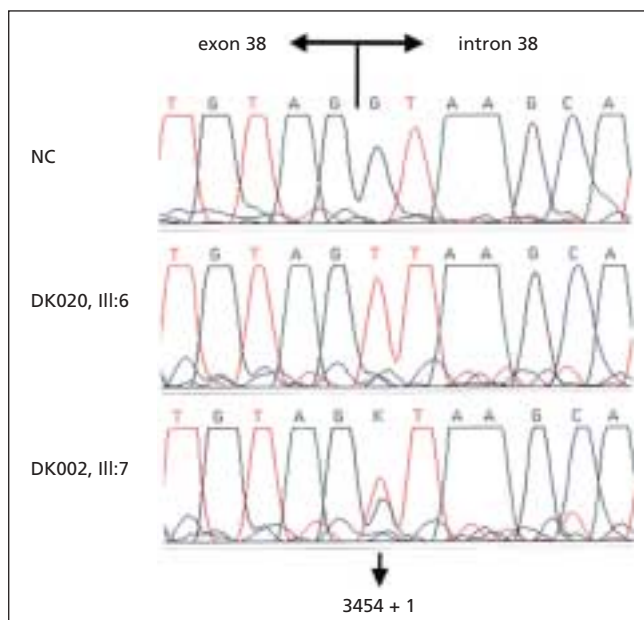


Figure 12. Sequencing of exon 38 with flanking intronic sequence demonstrating a splice site mutation at nucleotide position c.454+1G>T in hemizygous form in the proband in family DK020, in heterozygous form in the proband in family DK002, and the normal sequence in a normal control DNA (NC).

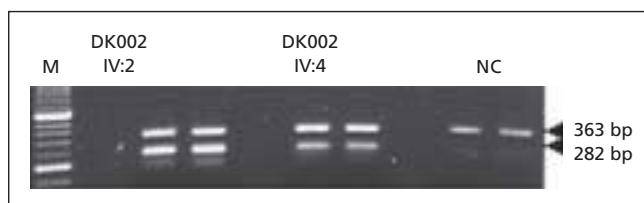


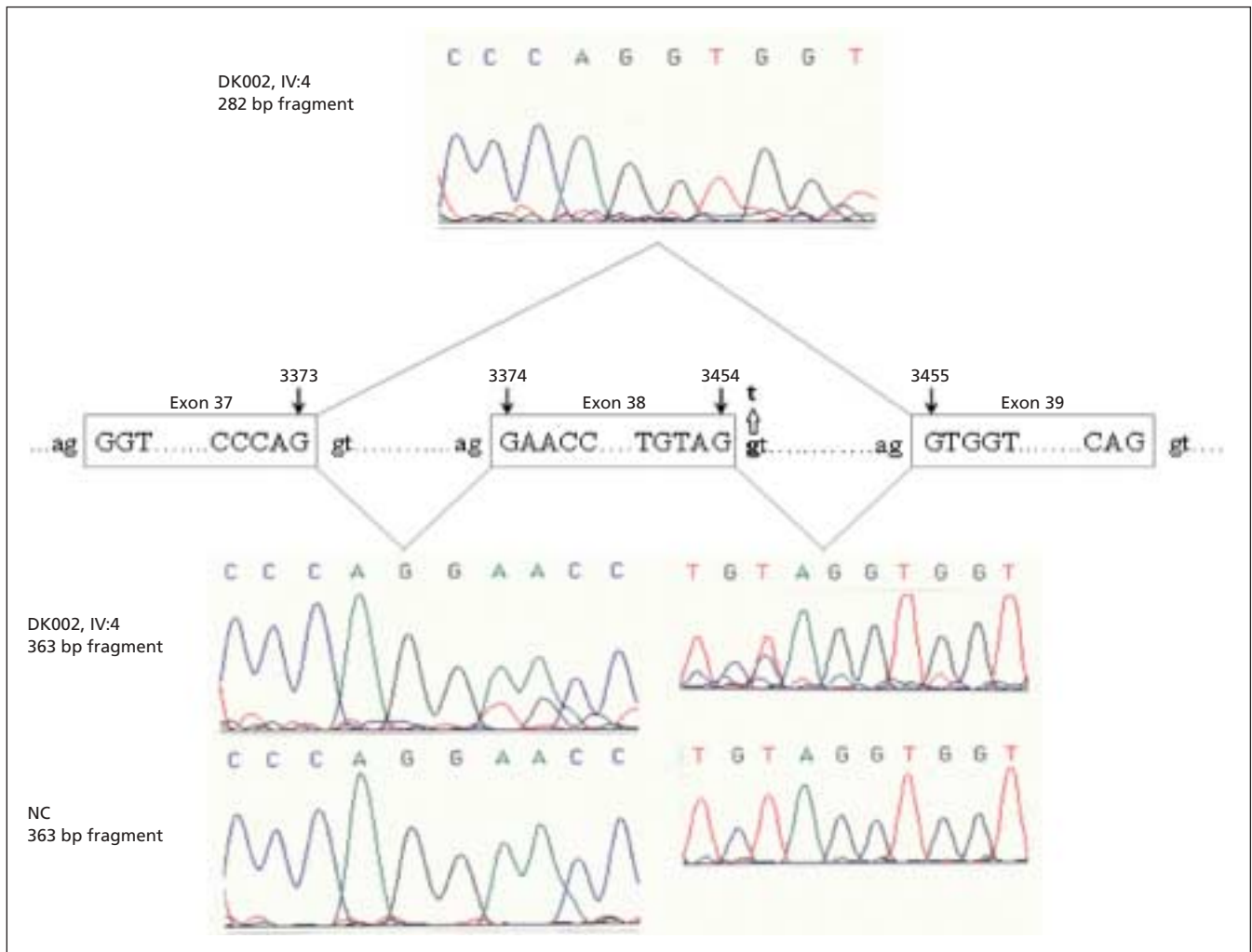
Figure 13. Reverse transcription and PCR amplification of mRNA from EBV-transformed peripheral blood lymphocytes from DK002 IV:2 and IV:4, both heterozygous for the c.3454+1G>T splice site mutation. M=DNA size marker. NC=normal control DNA.

a *Eam1104I* restriction site, and was found to co-segregate with the disease in the family. The sequence variation was not found by screening 53 normal controls (106 X-chromosomes). We have, however, not been able to demonstrate any effect of this mutation on the splicing process.

#### 4.3.5 Spectrum of COL4A5 point mutations in patients with X-linked AS

Worldwide, a total of 490 different point mutations have been identified in the *COL4A5* gene in patients with AS, including those identified in the present study. For review see Lemmink et al. [68], and later publications [6, 8, 172, 306, 309, 315, 320, 321, 327, 332-354]. The mutations are evenly distributed throughout the gene, with no hot spots and only a few recurrent changes. The *COL4A5* mutations can be divided into five different groups: missense mutations, accounting for 45% (219/490); frameshift mutations, accounting for 22% (110/490); splice site mutations, accounting for 21% (104/490); nonsense mutations, accounting for 7% (33/490); and *in frame* deletions or duplications, accounting for 3% (15/490) of the mutations. Glycine substitutions in the conserved *Gly-Xaa-Yaa* repeat sequence in the collagenous domain of the  $\alpha 5(\text{IV})$ -chain are the most frequent mutations accounting for 85% (186/219) of the missense mutations. Ten out of 219 missense mutations (5%) are non-glycine substitutions in the collagenous domain, and twenty-three (10%) are located in the NC1 domain. Most of the mutations in the NC1 domain affect conserved residues [328].

No putative disease causing mutations have so far been detected



**Figure 14.** Sequencing of the 363 bp and 282 bp fragments obtained by RT-PCR of mRNA from EBV-transformed peripheral blood lymphocytes from DK002, IV:4, who is heterozygous for the c.3454+1G>T splice site mutation, and a 363 bp fragment from a normal control (NC). Sequencing of the 282 bp fragment shows that the mutation results in skipping of exon 38.

in the promotor region or in the two small alternatively transcribed exons, 41A and 41B, or their flanking intronic sequences, in AS patients. The promotor region has been screened in 99 cases, and exons 41A and 41B in 199 cases (including the present cases) [90, 320, 338]. The only mutation detected so far in or around the alternatively transcribed exons 41A and 41B is the IVS41B+7C>T mutation detected in DK082 in the present material, the pathogenicity of which is dubious (for details, see section 4.3.3).

#### 4.4 RECURRENT *COL4A5* MUTATIONS

In general, only a few *COL4A5* mutations have been identified in more than one family. Two of the amino acid substitutions identified in the present series have been found in more than one family. The missense mutations p.Gly594Ser and p.Gly1357Ser was found in five (DK043, DK057, DK064, DK072, and DK149) and two (DK014 and DK086) apparently unrelated families, respectively. Using closely linked polymorphic markers flanking the *COL4A5* gene, we can demonstrate that four of the families with the p.Gly594Ser substitution share a common haplotype indicating a common ancestral origin of the mutation (Table 11a).

A core haplotype of markers flanking *COL4A5*, comprising the following markers: DXS1105, 2B3 1/3, DXS1210, DXS456, DXS1059, and DXS072, is identical in affected probands from each family, strongly supporting a common ancestral origin of the mutation. If we assume allele frequencies of 0.1-0.3 for each marker and little or no linkage disequilibrium between the markers, then the frequency of the common haplotype is  $<10^{-3}$ , and therefore very un-

likely to be identical by chance in all affected males. The presence of a DXS1120 allele of 128 bp in DK043 (originating from Denmark), as compared to the 126 bp allele in DK057, DK064, and DK072, all originating from Sweden, can be due to a crossing over event or to mutation at the polymorphic site in DK043, extending the dinucleotide repeat.

A common haplotype in the two families (DK014 and DK086) with the p.Gly1357Ser missense mutation also indicates a common ancestor for these two families (Table 11b).

A common origin of the normal variant p.Lys662Asn (c.1992G>T) in DK021 and DK32 is supported by a common haplotype of mark-

**Table 11a.** Haplotype analysis of the probands from four families with identical *COL4A5* mutations using eight different polymorphic markers flanking the *COL4A5* gene at Xq22.

Polymorphism	c.1780G>A (p.Gly594Ser)			
	DK043 (M)	DK057 (M)	DK064 (F) <sup>a</sup>	DK072 (M)
DXS1191	235 bp	235 bp	235-235 bp	235 bp
DXS1120	128 bp	126 bp	126-126 bp	126 bp
DXS1105	208 bp	208 bp	208-208 bp	208 bp
2B3 1/3	150 bp	150 bp	152-154 bp	150 bp
DXS1210	199 bp	199 bp	199-201 bp	199 bp
DXS456	150 bp	150 bp	150-156 bp	150 bp
DXS1059	189 bp	189 bp	189-189 bp	189 bp
DXS1072	271 bp	271 bp	271-269 bp	271 bp

M=male. F=female. a) phase unknown.

**Table 11b.** Haplotype analysis of the probands from families with identical *COL4A5* mutations using eight different polymorphic markers flanking the *COL4A5* gene at Xq22.

Polymorphism	c.4069G>A (p.Gly1357Ser)		c.5029C>T (p.Arg1677X)		c.3454+1G>T	
	DK014 (M)	DK086 (M)	DK003 (M)	DK083 (M)	DK002 (F) <sup>a</sup>	DK020 (M)
DXS1191	235 bp	235 bp	235 bp	239 bp	237-237 bp	237 bp
DXS1120	128 bp	128 bp	116 bp	116 bp	124-116 bp	124 bp
DXS1105	220 bp	220 bp	220 bp	208 bp	220-208 bp	220 bp
2B3 1/3	146 bp	146 bp	146 bp	152 bp	146-152 bp	146 bp
DXS1210	199 bp	199 bp	197 bp	201 bp	191-199 bp	191 bp
DXS456	150 bp	150 bp	150 bp	150 bp	156-156 bp	156 bp
DXS1059	193 bp	193 bp	193 bp	193 bp	193-189 bp	193 bp
DXS1072	271 bp	271 bp	269 bp	269 bp	269-269 bp	269 bp

M=male, F=female, a) phase unknown.

**Table 12.** Haplotype analysis using eight different polymorphic markers flanking the *COL4A5* gene at Xq22 in patients with identical *COL4A5* mutations (c.1992G>T).

Polymorphism	DK021 (M)	DK032 (F) <sup>a</sup>
DXS1191	239 bp	239
DXS1120	122 bp	122
DXS1105	220 bp	208 bp
(c.1992G>T)	+	-
2B3 1/3	146 bp	146
DXS1210	191 bp	191
DXS456	156 bp	150
DXS1059	193 bp	193
DXS1072	269 bp	269

M=male, F=female, a) phase unknown

ers: DXS1191, DXS1120, DXS1105, 2B3 1/3, and DXS1210 flanking *COL4A5* (Table 12).

Another, and disease causing mutation (p.Gly521Cys) was detected in DK021, but not in DK032. The same splice site mutation (c.3454+1G>T) was found in two independently ascertained families: DK002 and DK020. Haplotype analysis in these two families has indicated a common ancestral origin of the probands from these two families (Table 11b).

A search for a common ancestor to these two families was therefore initiated. By genealogical studies it was found that the probands maternal grandmother in family DK002 and the mother of the probands maternal grandfather in family DK020 are first cousins as their mothers are sisters (Figure 15). The two sisters are born in 1814 and 1832, respectively.

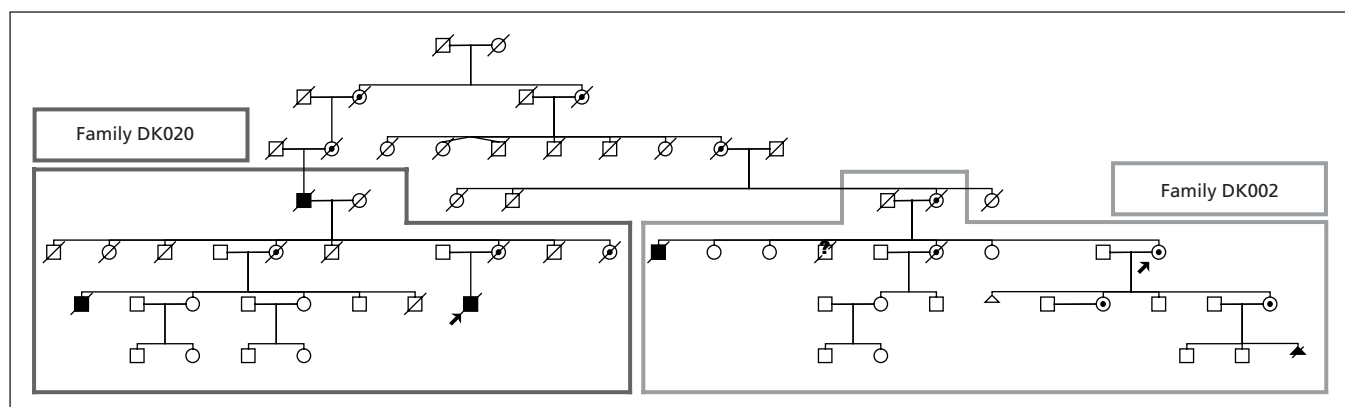
Transitions at hypermutable CpG sites are frequent mutations in a number of different disorders and a frequent cause of recurrent mutations. However, only three such mutations have been identified

in the present series: the c.4688G>A transition (p.Arg1563Gln) in DK013, and the c.5029C>T transition (p.Arg1677X) in DK003 and DK083. The mechanism behind a C>T transition at CpG dinucleotides is 5-methylation of cytosine and subsequent deamination of 5-methylcytosine to thymine. When the 5-methyldeamination occurs on the opposite strand it will result in a G>A transition. Mutations involving the codon for Arg1563 and Arg1677 in the NC1 domain have been identified in a number of cases. Both codons, 1563 and 1677, involves a CpG dinucleotide as the first and second nucleotide, and are highly conserved during evolution in each symmetrical halves of the NC1 domain of all known type IV collagen  $\alpha$ -chains from *Drosophila* to human [4]. The codon for arginine 1563 (CGA) can be changed to either CAA for glutamine (p.Arg1563Q) as described in DK013,4 and three other patients from the United States [4], the Netherlands [68], and Japan [335], respectively; or to a TGA stop codon (p.Arg1563X) as seen in two patients from the Netherlands [117] and Germany [343], respectively. The CpG site transitions (G>A or C>T) at codon 1677 for arginine (CGA) will result in either CAA for glutamine (p.Arg1677Gln), or a TGA stop codon (p.Arg1677X). The missense mutation p.Arg1677Gln has been detected in three independently ascertained American-Ashkenazi families [355] and causes a relatively mild phenotype with onset in the fourth or fifth decade. However, haplotype analysis strongly supports a common ancestral origin of these three families. The nonsense mutation p.Arg1677X has been found in two independently ascertained families from our series: DK003 and DK083 [7]. Haplotype analysis of the probands from these two families demonstrated that the mutation most probably has arisen independently in each family (Table 11b). The same mutation has been detected in three other patients, two from Japan [140, 335] and one from United Kingdom [321], respectively. The p.Arg1677X nonsense mutation is predicted to produce a truncated NC1 domain, lacking 9 amino acids, including two cysteine residues normally involved in intermolecular cross-linkages and thereby affecting the protomer formation. Transitions at CpG sites are therefore a cause of recurrent mutations also in AS, but not as frequent as seen in other collagen disorders such as osteogenesis imperfecta [356].

A relatively high prevalence of 17% (12/167) of the p.Leu1649Arg substitution has been found in United States families [320, 355].

#### 4.5 DE NOVO MUTATIONS

The frequency of *de novo* mutations among the probands in the present material is 2.7% (2/72), which is lower than reported in other studies. Lemmink et al. [68] reviewed the published *COL4A5* mutations and found that 12% (21/176), or 18% (7/38) of larger rearrangements, and 10% (14/138) of smaller mutations, have arisen *de novo*. Similar figures of 18% (4/22) in Japanese patients [335],



**Figure 15.** Genealogical studies in family DK002 and DK020. The probands from these two families can be traced back to common ancestors. The proband's maternal grandmother in DK002 and the mother of the probands maternal grandfather in DK020 are first cousins as their mothers are sisters. These two sisters were born in 1814 and 1832, respectively. By looking at eight different polymorphic markers closely linked to the *COL4A5* gene at Xq22, it was found that the probands from the two families share a common haplotype (Table 11b).

18% (5/28) in UK patients [321], and 11% (7/63) in French patients [332] have been found. The frequency of *de novo* mutations in the "European Community Alport Syndrome Concerted Action" (ECASCA) is 12% (22/195) [137]. These figures are comparable to what have been found in other X-linked disorders. A very high percentage of *de novo* deletions involving both the *COL4A5* and the *COL4A6* gene have been found in patients with DL-AS, although the number of cases identified to date is limited. Dahan et al. [213] found a *de novo* mutation in four out of six cases (67%).

The percentage of *de novo* mutations is related to the way the families have been selected for study. If a sporadic case should fulfil the diagnostic criteria as proposed by Flinter et al. [72], both hearing

loss and ophthalmological symptoms should be present in the absence of a positive kidney biopsy. Very few of the mutation positive probands in this study (15/72 or 21%) have had a kidney biopsy performed, and informations regarding ocular signs are only available for 46% (33/72) of the probands.

We have been able to trace the mutation back in the family in six of the present cases and to determine the parental origin of these six mutations. The male to female ratio of the origin of the mutation was found to be 4:2. The c.1219C>T nonsense mutation in III:1 in family DK001 (Figure 16), the c.3107-2A>G splice site mutation in V:1 in family DK025 (Figure 17), the c.1718G>A missense mutation in IV:3 in family DK028 (detected by direct sequencing), and the c.1856C>T missense mutation in IV:1 in family DK074 (Figure 10) were all found in heterozygous form in their mothers, but could not be detected in their maternal grandparents.

These four mutations were all found to be paternal of origin by haplotype analysis using closely linked and flanking polymorphic markers (Table 13). Two mutations were found to be of maternal origin: The mothers of the probands in family DK145 and family DK045, were found not to carry the mutations found in their affected sons (Figure 18).

The excess of male compared with female origin of mutations in this material is similar to what has been found in other X-linked disorders. The male to female ratio in *Haemophilia B* was found to be 3.5:1 for all point mutations, and 11:1 for C>T transitions at CpG sites [357]. The male to female ratio for deletions was found to be close to one. Most of the mutations in the X-linked *Rett syndrome* occur at CpG sites and are paternal of origin [358]. Male germ cells have a considerably higher level of CpG methylation than female germ cells [359]. A large proportion of germline mutations are caused by replication errors, which can explain a male excess in mutation rate, since the number of cell divisions in spermatogenesis exceeds that in oogenesis. A paternal age effect, with increasing mutation rates with increasing paternal age has been observed in some, but not all disorders [360].

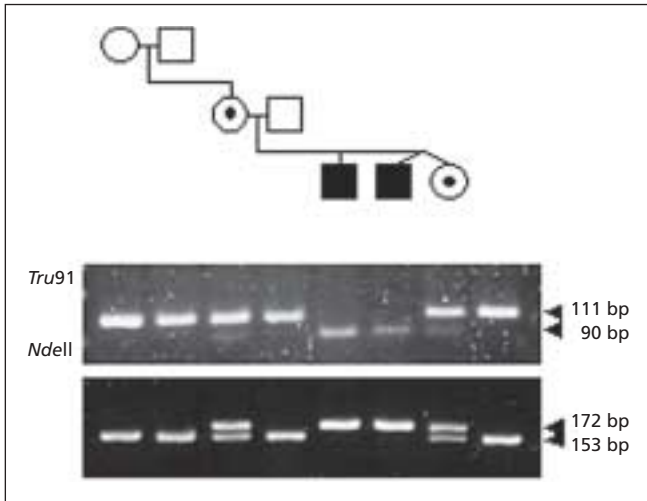


Figure 16. Two different PCR based restriction enzyme assays for the c.1219C>T nonsense mutation (p.Gln407X) in exon 20 in family DK001. The mutation has arisen *de novo* in III:1, and, according to haplotype analysis, on the paternal allele.

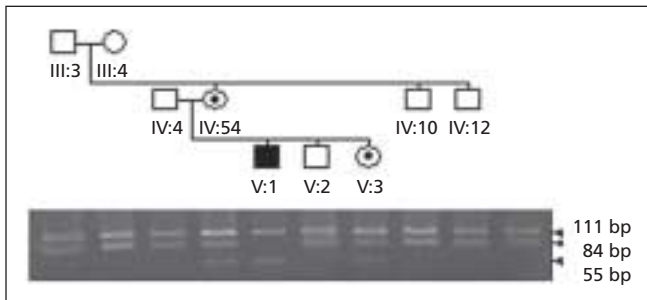
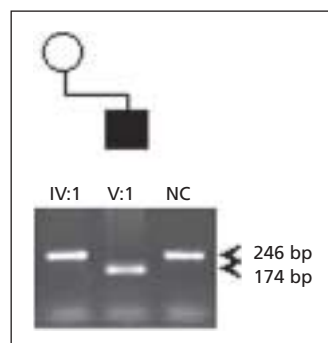


Figure 17. *NlaIV* digestion of a 195 bp PCR product from *COL4A5* exon 36. A *de novo* splice site mutation (c.3107-2A>G) in Alport family DK025, creates a new *NlaIV* restriction site. A 29 bp fragment in the heterozygous females (IV:5 and V:3), and in the hemizygous affected male (V:1), is not seen. Another *NlaIV* restriction site serves as an internal control for digestion.

Figure 18. A *de novo* deletion of 72 bp of *COL4A5* exon 41 in Alport family DK045. NC=normal control DNA.



#### 4.6 EVALUATION OF THE MUTATION DETECTION METHODS

##### 4.6.1 Mutation detection rates

The overall mutation detection rate was found to be 53% (72/135). A total of 64 different and putative disease causing mutations have been identified in 72 patients (Figure 19). Nineteen of the mutations have not previously been published, and 55 of the mutations have only been found in patients in this material (Table 7). No specific frequent mutations or hot spot area for mutations have been identified, although 5 different mutations in 5 families were found by screening exon 10, and 7 different mutations in 11 families were found by screening exon 25.

The mutation detection rate was 60% (64/107) in males, and 29% (8/28) in females. A mutation was identified in 82% (23/28) of families with clear X-linked inheritance. Among the sporadic cases only two mutation was identified in 7 males (29%) and none in 8 females (0%). The mutation detection rate in patients fulfilling  $\geq 3$  of the AS criteria proposed by Flinter et al. [72], is 72% (28/39), which is comparable to the 74% found in the large meta-analysis by Gross et al. [343], comprising data from 256 *COL4A5* mutations.

The *PCR-SSCP-technique* has been used to screen the *COL4A5* gene for mutations in six other studies, with mutation detection rates of 30-67%. Kawai et al. [317] screened 60 unrelated patients with characteristic ultrastructural GBM changes by *PCR-SSCP-technique* and found a mutation detection rate of 30% (18/60).

Renieri et al. [361] screened a heterogeneous population of 201 Italian patients also by *SSCP-technique* and found a mutation detection rate of 30%. In patients with certain or likely diagnosis of X-linked AS however, a mutation detection rate of 45% (43/96) was found. Knebelmann et al. [332] screened 131 patients using *SSCP-analysis* of 48 of the 51 exons and found a mutation detection rate of

**Table 13 .** The parental origin of six *de novo* mutations. The parental origin of the mutations have been determined by haplotype analysis using closely linked and flanking polymorphic microsatellite markers.

Family	COL4A5 mutation	Mutation type	Sex	Parent of origin	
				Mother	Father
DK001 ...	c.1219C>T	Nonsense	Female		+
DK028 ...	c.1718G>A	Missense	Female		+
DK074 ...	c.1856C>T	Missense	Female		+
DK143 ...	c.2344G>A	Missense	Male	+	
DK025 ...	c.3107-2A>G	Splice site	Female		+
DK045 ...	c.3657_3728del72	In-frame deletion	Male	+	

49% (64/131). In a series of 153 families, Plant et al. [321] found an overall mutation detection rate of 50% (62% in males and 17% in females) by SSCP-analysis. In 44 of these families in which X-linked inheritance was apparent, a mutation was detected in 62%. Cheong et al. [337] studied 25 unrelated Korean patients and found a mutation detection rate of 40%.

Barker et al. [320] developed a multiplex PCR-SSCP method with a single buffer condition and PCR profile, and identified 31 mutations in a series of 46 American AS patients (detection rate of 67% (31/46); 68% (21/31) in males, and 67% (10/15) in females). However, three of the mutations were larger deletions of two or more exons detected directly by PCR and gel-electrophoresis, and not by the SSCP analysis.

Our mutation detection rate of 52%, or 82% if we only include families with obvious X-linked inheritance, is comparable to the detection rates in these six previously reported studies. We have screened the intron-exon boundaries of exon 2, 10 and 37, which were not included in five of the other series using PCR-SSCP-technique. No mutations were found by screening exons 2 and 37, but at least three of the mutations found by screening exon 10 (c.602G>T in DK055, c.548insC in DK066, and c.609+1G>A in DK048) are close to the intron-exon boundaries, and can therefore not be expected to be identified in the five studies using primer pairs with only a few nucleotides apart from the intron-exon boundaries. This became less of a problem later when the whole sequence became available.

Inoue et al. [335] analysed RNA extracted from peripheral blood lymphocytes from 22 unrelated Japanese AS patients by *RT-PCR and sequencing*, and found a mutation detection rate of 77%

(17/22); 92% (12/13) in males and 56% (5/9) in females. However, only patients showing abnormal expression of the  $\alpha 5$ (IV)-chain in the GBM or in the epidermal basement membrane were included, and a high frequency of patients with a mutation in the *COL4A5* gene could therefore be expected. Detection of *COL4A5* mutations by RT-PCR and sequencing has some limitations in its use. Do to a very low amount of *COL4A5* mRNA in peripheral blood lymphocytes it may, however, fail to amplify. Another limitation in its use is that females may not express equimolar amount of the mutant transcript do to nonsense-mediated mRNA decay, resulting in a lower detection rate in heterozygous females.

A higher mutation detection rate has been obtained by *direct sequencing* of each of the 51 exons including 35-190 bp flanking the intronic sequence as demonstrated by Martin et al. [6] Analysis of 50 randomly chosen patients suspected of AS from a group of 250 patients gave a mutation detection rate of 82% (41/50).

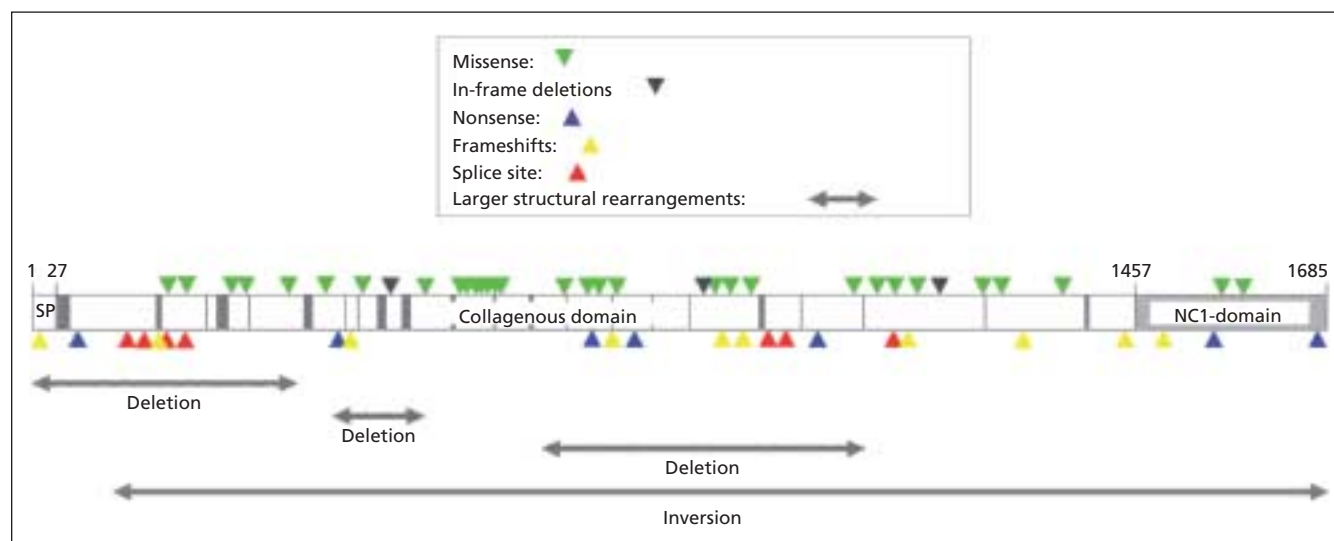
*Denaturing gradient gel electrophoresis* (DGGE) [362] and *chemical cleavage* [363] have only been used sporadically, and not in large series. *The protein truncation test* (PTT) [364, 365] detects mutations that interrupt reading frames and thereby changing the length of protein products translated from amplified DNA molecules. Due to the high frequency of missense mutations in *COL4A5* in AS, PTT will only detect a fraction of mutations.

Detection of mutations in the *COL4A5* gene is laborious and time consuming due to the large size of the gene (51+2 exons and a transcript size of approximately 6.5 kb). Screening of each of the 51 exons as described in the present study is practicable with an acceptable mutation detection rate, as compared to other studies, on an unselected group of patients clinically suspected of X-linked AS. A maximum detection rate of 85% in sporadic cases should be expected, since the X-linked form of AS constitute about 85% of cases. The frequency of sporadic cases was found to be 12% in a large European study [137].

A very low mutation detection rate has also been detected in the *COL4A3* and *COL4A4* genes in patients with benign familial hematuria. Wang et al. [178] found a mutation detection rate of 35% in 46 patients with benign familial hematuria. The mutation detection rate increased to 67% if only families where benign familial hematuria segregates with the *COL4A3/COL4A4* locus were included.

#### 4.6.2 Validation of the PCR-SSCP method

The PCR-SSCP method has been validated by additional direct sequencing of the entire coding sequence of the *COL4A5* gene with



**Figure 19.** Distribution of the 64 different putative disease causing *COL4A5* mutations identified in this study. The  $\alpha 5$ -chain of type-IV collagen contains a collagenous *Gly-Xaa-Yaa* repeat sequence with 22 interruptions (indicated by vertical bars), and a non-collagenous domain (NC1) at the carboxyl-terminal end. SP = signal peptide.

flanking intronic sequences in 15 of the 135 patients studied [6]. In family DK001, DK002, DK003, DK005, DK008, DK014, DK024, DK025, and DK028, both methods detected the disease causing mutation. In DK004, DK022, DK023, DK026, DK027, and DK030, no mutation could be detected by any of the methods used.

In total, 69 sequence variations have been identified in this study comprising both disease causing mutations and normal variants. Three mutations were missed by the PCR-SSCP analysis: A large inversion of a 21 Mb fragment with a breakpoint in intron 8 in DK054 was only detected by the Southern blotting analysis [8]. The p.Glu700X nonsense mutation in DK090 was only detected by analysing cDNA prepared from cultured skin fibroblasts, and the deletion of exon 20-22 in heterozygous form in DK110 was only detected by MLPA analysis [9]. A sequence variation in intron 10: c.609+21T>C, was detected incidentally by direct sequencing of exon 10 in a normal male control. Analysing DNA containing this sequence variant could demonstrate no abnormal PCR-SSCP band pattern. Sixty-five of the mutations were detected by the PCR-SSCP method. The sensitivity of the PCR-SSCP technique can therefore be estimated to be 94.2% (65/69). It is, however, not possible to determine the exact sensitivity of the PCR-SSCP method in all 135 patients, because the true status of the 63 patients without a *COL4A5* mutation is unknown.

The sensitivity of the PCR-SSCP method varies with the size of the DNA fragment being analysed, and the type of mutation. The optimal size fragment for sensitive single base substitution detection has been determined to about 150 bp in other studies [298]. Higher sensitivity can be expected for detection of deletions and insertions. To see if the size of the DNA fragment analysed influence the sensitivity of the present PCR-SSCP method, the observed number of mutations have been compared to the expected number of mutations in each of three classes of fragment sizes: Fragments <150 bp, fragments of 150-200 bp, and fragments >200 bp.

A total of 42 single-base substitutions were included in this study, of which 26 are missense mutations, 8 are splice site mutations, 5 are nonsense mutations, and 3 are normal variants. Screening 49 different *COL4A5* fragments in each of the 100 patients investigated has identified these mutations. Only single-base substitutions have been included because the other types of mutations (frameshifts, in-frame deletions) are more easily detected by the PCR-SSCP-analysis. Fifteen fragments are below 150 bp, comprising 1,764 bp, sixteen fragments are between 150 and 200 bp, comprising 2,749 bp, and eighteen fragments are above 200 bp, comprising 4,426 bp. In total, 8,939 bp have been screened. The null hypothesis is that the probability of identifying a mutation is independent of the size of the fragment analysed. It is assumed in the calculations that the probability of mutation is equal for all nucleotide positions. The expected number of mutations per nucleotide can be calculated to  $42/100 \times 8,939$ , or  $4.7 \times 10^{-5}$  mutation per nucleotide. The expected number of mutations is therefore  $4.7 \times 10^{-5} \times 100 \times 1,764$  or 8.3 in the class of fragments below 150 bp,  $4.7 \times 10^{-5} \times 100 \times 2,749$  or 12.9 in the class of fragments between 150 bp and 200 bp, and  $4.7 \times 10^{-5} \times 100 \times 4,426$  or 20.8 in the class of fragments over 200 bp. The observed number of mutations in the same three classes of fragments is 8, 13, and 21, respectively (Table 14).

The number of mutations identified in fragments of less than 150 bp is not significantly different from the number of mutations identified in larger fragments ( $\chi^2=0.0135$ ,  $p>50\%$ ). The assumption that the probability of identifying a mutation by PCR-SSCP-analysis is greater in fragments below 150 bp can therefore not be confirmed. In conclusion, the probability of detecting a single base substitution is not dependent of the size of the fragment being analysed in the present material.

#### 4.6.3 Validation of the MLPA assay

The sensitivity of the MLPA assay was validated on samples from 57 patients clinically suspected of AS (35 males and 22 females), all pre-

viously tested negative for a *COL4A5* mutation by PCR and SSCP-analysis. A deletion of exon 20 through 22 in heterozygous form was detected in one of the female probands [9]. In addition, samples from 10 AS patients (all males) with a previously identified *COL4A5* point mutation involving the annealing site for the corresponding MLPA probe, were tested. Some point mutations involving the annealing site for the corresponding MLPA probe will be detected, as demonstrated in five of the ten samples with a known mutation included (Table 15).

All five point mutations detected were small insertions or deletions close to the MLPA annealing site. Another five mutations, all single base substitutions, remain undetected. The sequence variations in these five cases were positioned  $\geq 5$  bp from the MLPA ligation site. Generally, only very few of the more than 400 *COL4A5* point mutations identified to date can be expected to be detected by MLPA. A copy number variation detected by MLPA involving only a single exon should always be verified by another quantitative technique, or by sequencing.

The specificity of the MLPA assay was tested on samples from 20 normal controls. No deletions or duplications were detected among the 20 normal controls.

#### 4.6.4 An algorithm for molecular genetic diagnostics in patients with X-linked AS

Based on the results obtained in this study, and the literature, an algorithm for a comprehensive molecular genetic diagnostics in patients suspected of X-linked AS, is presented. The algorithm is shown in details in Figure 20.

First step is MLPA-analysis of the *COL4A5* gene for deletions and duplications on genomic DNA from a blood sample [9]. Deletion of two, or more, exons confirm the diagnosis. Deletion of a single exon should be confirmed by another quantitative technique, or by sequencing of the actual exon. Deletion of two or more exons were seen in 4.2% (3/72) in this study. In addition, a number of sequence variations involving the annealing site for the MLPA-probes will be detected, as demonstrated for 5 of the 10 point mutations studied. If no deletions or duplications are detected by MLPA, next step should

Table 14.  $\Sigma\chi^2=0.0135$ ,  $f=3-1-1=1$ ,  $p>0.5$

Fragment size	Observed no. of mutations (O)	Expected no. of mutations (E)	$\frac{(O-E)^2}{E}$
<150 bp . . . . .	8	8.3	$\frac{(8-8.3)^2}{8.3} = 0.0108$
150-200 bp . . . . .	13	12.9	$\frac{(13-12.9)^2}{12.9} = 0.0008$
>200 bp . . . . .	21	20.8	$\frac{(21-20.8)^2}{20.8} = 0.0019$

Table 15. *COL4A5* mutations analysis by MLPA. All mutations have previously been identified using PCR and SSCP-analysis of the *COL4A5* gene, exon-by-exon. Five of the 10 point mutations were detected by MLPA, whereas the other five remain undetected.

Sex	Exon(s)	MLPA ligation site <sup>a</sup>	Nucleotide change	RPH <sup>b</sup> Ratio	Mutation detected by MLPA
Male . . . . .	1	41-42	c.41_42insTCTT	0	+
Male . . . . .	21	1368-1369	c.1371_1379del9	0	+
Male . . . . .	30	2421-2422	c.2404_2421del18	0	+
Male . . . . .	33	2798-2799	c.2802insT	0	+
Male . . . . .	39	3475-3476	c.3474delG	0.543	+
Male . . . . .	18	1008-1009	c.1001G>T	0.857	-
Male . . . . .	24	1725-1726	c.1718G>A	1.010	-
Male . . . . .	38	3437-3438	c.3428G>A	1.000	-
Male . . . . .	42	3813-3814	c.3808G>A	1.052	-
Male . . . . .	47	4426-4427	c.4436_4437delGA	1.088	-

a) For detail, see the MRC-Holland website (<http://www.mrc-holland.com>).

b) RPH=relative peak height

be RT-PCR analysis of mRNA extracted from cultured fibroblasts from a skin biopsy. Negative immunohistochemical staining for the  $\alpha 5(\text{IV})$  collagen chain on skin sections from the biopsy support a diagnosis of X-linked AS. Four out of five mutations were detected by cDNA analysis in this study [9].

If a skin biopsy is not available, then an exon-by-exon screening strategy on genomic DNA from the blood sample should be initiated. A mutation detection rate of about 80% could be obtained by using direct sequencing [6]. An alternative to sequencing is PCR-SSCP analysis [7]. In the present study, PCR-SSCP analysis detect a mutation in 52% of all cases (70/135). The mutation detection rate is, however, 72% in patients fulfilling  $\geq 3$  of the clinical criteria for AS, and 82% in families clearly demonstrating X-linked inheritance. Therefore, a linkage analysis could be performed, depending on the family constellation, to support or exclude a diagnosis of AS. Identification of a mutation confirm a diagnosis of AS. A negative result of the cDNA-analysis or screening method should led to Southern blotting analysis. This method will detect some point mutations also detected by the exon-by-exon screening strategy [2, 3], but also larger structural rearrangements such as the inversion of exon 9 through 51 as demonstrated in this study, and exclusively detected by Southern blotting analysis [8].

The presented molecular genetic approach allows a definite diagnosis of X-linked AS in case a *COL4A5* mutation is detected. If no mutations were found, the diagnosis should be reconsidered, and a kidney biopsy should be considered, if not already performed.

#### 4.6.5 Explanations for a low mutation detection rate

In 47% (63/135) of the patients included in the study no *COL4A5* mutation could be detected. Some of the patients might not have AS, as demonstrated for DK085 diagnosed with Epstein syndrome (described in section 4.8.1), but a low mutation detection rate can also be due to failure of the mutation detection techniques to detect the mutations, the disease could be caused by mutations in intronic or unexamined regulatory sequences, or can be due to locus heterogeneity with involvement of other genes.

#### Technical limitations of the mutation detection methods

Some mutations, such as certain larger structural rearrangements and deep intronic mutations will not be detected by direct sequencing or PCR-SSCP-analysis. Some mutations will not cause SSCP shifts of the PCR product screened under the gel conditions used. One sequence variation, a polymorphic variant in intron 10: c.609+21T>C, found by direct sequencing of exon 10 in normal control DNA, do not result in a mobility shift, and was therefore not detected by the PCR-SSCP analysis. The nonsense mutation p.Glu700X detected in DK090 was not detected by PCR-SSCP analysis, but only by cDNA analysis [9]. On the other hand, some mutations will be overlooked by cDNA analysis. Females heterozygous for a nonsense or frame shift mutation may not express equimolar amounts of the mutant transcript due to nonsense-mediated mRNA decay, which may result in a lower mutation detection rate by cDNA analysis in heterozygous females.

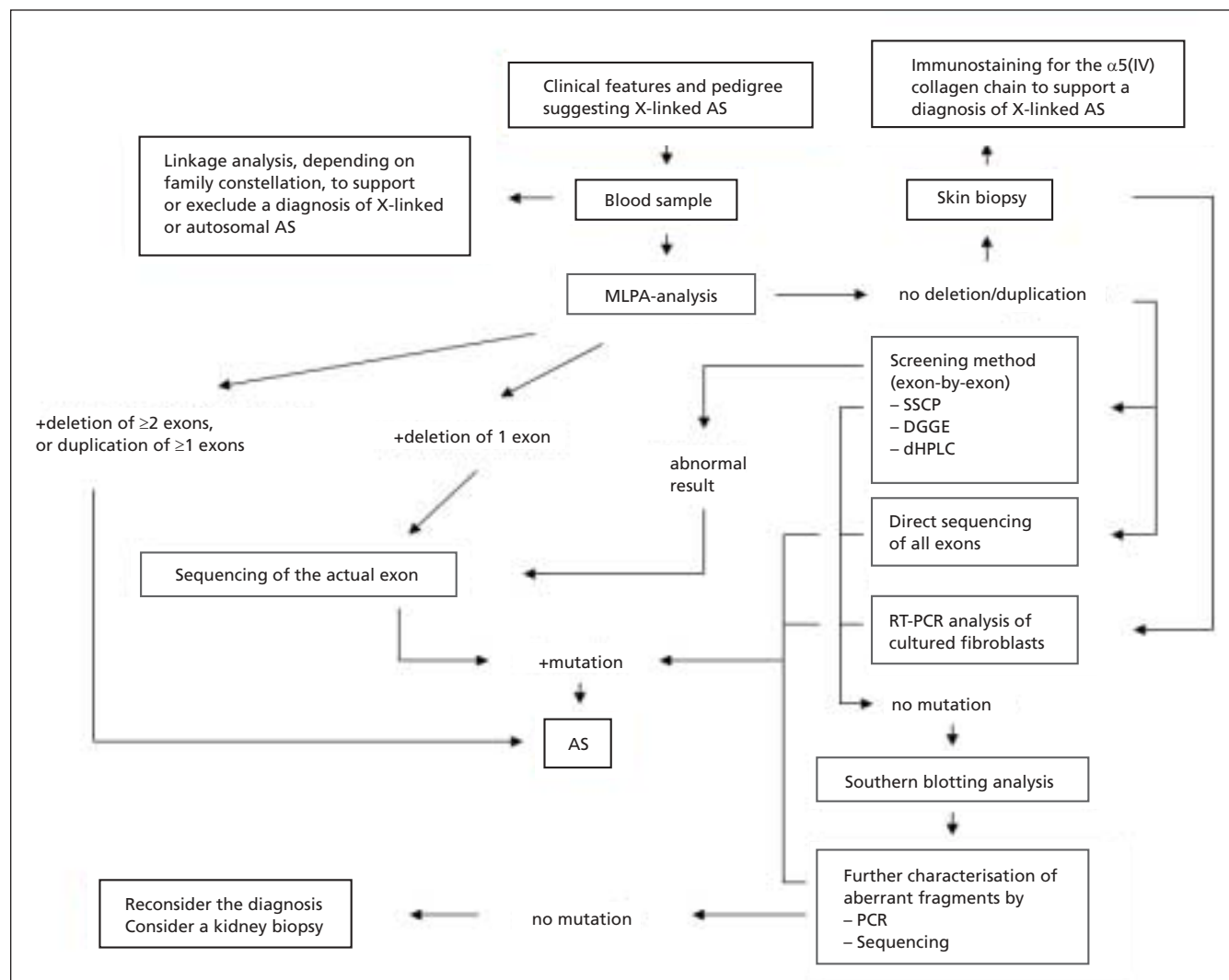


Figure 20. An algorithm for molecular genetic testing of the *COL4A5* gene in patients clinically suspected of X-linked AS. For details, see text.

#### Mutations in intronic sequences not tested

The *COL4A5* gene contains more than 250 kb intronic sequence, and rearrangements within introns can interfere with structure and production of normal mRNA. King et al. [315] analysed the entire coding region of the *COL4A5* gene in four overlapping nested RT-PCR reactions using RNA isolated from hair roots. By this approach they found three different intronic mutations of which at least two would not have been seen in an exon by exon search on genomic DNA. One of the mutations, a single base substitution 2,730 bp downstream from intron 29 (c) IVS29+2730A>G) was found to create a novel acceptor splice site resulting in the inclusion of a cryptic 30 bp exon between exon 29 and 30. The second mutation, c) IVS6+1873G >A, results in the inclusion of a cryptic 147 bp exon between exon 6 and 7. The third mutation, c) IVS32-10T>G, results in skipping of exon 32. These three mutations were found in a study that identified 24 disease-causing mutations and therefore represents 12.5%.

An intronic mutation causing activation of a cryptic splice site has also been demonstrated in *COL4A3* in a family with autosomal recessive AS. Knebelmann et al. [170] found a transcript disrupted by a 74 bp insertion by screening lymphocyte *COL4A3* mRNA. The insertion was derived from an antisense *Alu* element in *COL4A3* intron 48 and spliced into the mRNA due to a point mutation activating a cryptic acceptor splice site in this *Alu* sequence. Because this mutation is located in the middle of the intron it would not have been detected by analysis of exons and intron-exon boundaries on genomic DNA.

These examples, and the present case DK054 described in section 4.3.1, emphasize the importance of using other methods such as Southern blotting analysis, RT-PCR analysis, or MLPA as a supplement to an exon-by-exon screening approach.

#### Mutations in the *COL4A5* promotor region and other regulatory sequences not tested

The promotor region (820 bp upstream from exon 1) has been analysed in 96 patients, including 15 patients from the present study [6, 113, 320]. So far, no mutations have been identified in the *COL4A5* promotor region.

Transcription factors regulating the  $\alpha3$ (IV)- and  $\alpha4$ (IV)-chains have been described. The Nail-Patella syndrome (OMIM: #161200) is an autosomal dominant disorder caused by mutation in the gene for the LMX1B transcription factor located at 9q34 [366, 367]. LMX1B regulates the coordinated expression of the  $\alpha3$ (IV)- and  $\alpha4$ (IV)-chains required for normal GBM morphogenesis [368]. Mutations in the LMX1B gene therefore contribute to the renal symptoms and the ultrastructural GBM changes seen in patients with Nail-Patella syndrome. No such transcription factors have been detected for the  $\alpha5$ (IV)-chain gene.

#### Locus heterogeneity

Patients with a pedigree excluding X-linked inheritance, and some of the isolated cases, may have an autosomal recessive or dominant form of AS. The reduced detection rate in females may well also be due to genetic heterogeneity with autosomal forms of AS or benign familial hematuria resembling a carrier state of the X-linked form of AS. Mutation analysis of the *COL4A3* and *COL4A4* genes could clarify this. Another explanation for a low mutation detection rate in *COL4A5* could also be the existence of other X-linked genes. *COL4A6* encoding the  $\alpha6$ (IV)-chain is another type IV-collagen molecule and positioned within 452 bp from *COL4A5* at Xq22 [111]. However, *COL4A6* is probably not a candidate for another X-linked AS gene, since the  $\alpha6$ (IV)-chain is not expressed in the GBM [130]. Deletion of the 5'-end of *COL4A6* is involved in DL-AS, but only in combination with a *COL4A5* deletion. Heiskari et al. [369] sequenced four *COL4A6* exons in 250 patients suspected of AS without finding any mutations.

Genes for processing enzymes for collagen type IV molecules, as known for Ehlers-Danlos syndrome [370] might be another pos-

sibility. Ehlers-Danlos syndrome is a genetically heterogeneous disorder characterized by joint hypermobility, skin hyperextensibility, skin fragility, and other signs of connective tissue involvement. Most of the different types of Ehlers-Danlos syndrome have been found to be caused by mutations in different collagen genes. Ehlers-Danlos syndrome type VI has some special characteristics such as kyphoscoliosis and ocular abnormalities. This form is caused by mutation in the gene for *lysyl hydroxylase*, which is an enzyme catalysing the formation of hydroxylysine in fibrillar collagen. Hydroxylysine residues are used to build intermolecular collagen crosslinks, and thereby important for the stability of the tissue. If similar collagen processing enzymes specific for type IV collagen exist, they might be candidates for AS.

#### 4.7 GENOTYPE-PHENOTYPE CORRELATION

The clinical findings in each of the 135 families are presented in Table 4).

The families have been divided into two groups, depending on, whether or not, a putative disease causing *COL4A5* mutation has been identified. A summary of the findings is presented in Table 16.

Sixty-five per cent of the families with a disease causing mutation detected have a juvenile form of the disease with a mean age at ESRD in males  $\leq 30$  years. Hearing loss is present in at least one affected person in 99% of the families, and ocular lesions in 69%. GBM changes are present in 100%, but such data are only available for 14 of the families.

The progression rate of the renal disease, the age at ESRD, and the presence or absence of extrarenal manifestations, is to some extent influenced by the underlying mutation. Deletions in the 5'-end of both the *COL4A5* and the *COL4A6* gene are associated with a combination of AS and leiomyomatosis, whereas deletion of the *COL4A5* gene and other neighbouring genes are associated with the contiguous gene deletion syndrome: *The AMME Complex* [232]. Only one patient with the combination of AS and leiomyomatosis, and no cases with a contiguous gene deletion syndrome were found in the present material.

Genotype-phenotype correlations in X-linked AS are, however, difficult to establish. First of all because nearly all *COL4A5* muta-

**Table 16.** Summary of clinical findings in 135 families with, or without, a putative disease causing *COL4A5* mutation detected.

	+ Mutation	- Mutation
No. families (probands) . . . . .	72	63
No. male probands . . . . .	64	43
No. female probands . . . . .	8	20
Sporadic cases (males) . . . . .	2	5
Sporadic cases (females) . . . . .	0	8
Percentage of families with mean age at ESRD $\leq 30$ years . . . . .	65% (41/63)	60% (23/38)
Percentage of families with mean age at ESRD $> 30$ years . . . . .	35% (22/63)	40% (15/38)
Percentage of families with hearing loss in at least one affected person . . . . .	99% (71/72)	67% (34/51)
Percentage of families with ocular lesions in at least one affected person . . . . .	70% (23/33)	46% (6/13)
Percentage of families with GBM changes in at least one affected person . . . . .	100% (15/15)	100% (14/14)

**Table 17.** The number of AS criteria fulfilled in families with, or without, a putative disease causing *COL4A5* mutation detected.

No. AS criteria fulfilled	+ Mutation	- Mutation	Total no.
0 . . . . .	0	5	5
1 . . . . .	5	26	31
2 . . . . .	38	21	59
3 . . . . .	23	10	33
4 . . . . .	5	1	6
	72	63	135



**Table 18.** The mean age at ESRD in male patients depending on the type of the underlying *COL4A5* mutation with SEM indicated in parentheses.

Mutation type	Mean age at ESRD in years in males (SEM)	No. patients
Missense mutations (all)	33.8 (2.3)	42
– Glycine substitutions in the collagenous domain	35.8 (2.7)	36
– Exon 1-20	23.8 (3.5)	5
– Exon 21-47	37.3 (2.7)	32
– Missense mutations in the NC1 domain	23.3 (4.7)	3
In frame deletions	20.3 (4.9)	3
Splice site mutations	30.3 (3.4)	13
– Splice donor site	34.4 (4.5)	7
– Splice acceptor site	25.5 (4.8)	6
Nonsense mutations	21.7 (1.2)	13
Frameshifts	21.9 (1.6)	14
Large structural rearrangements	20.3 (1.9)	3

**Table 19.** Comparison of the clinical findings between 50 patients with a non-truncating *COL4A5* mutation and 31 patients with a truncating mutation.

	Missense mutations In frame deletions Splice site mutations <sup>a</sup>	Nonsense mutations Frameshift mutations Large structural rearrangements
No. of families	50	22
No. of male patients with ESRD	50	29
No. of female patients with ESRD	5	7
Mean age at ESRD in years in males (SEM)	33.1 (2.1)	21.6 (1.0)
Mean age at ESRD in years in females (SEM)	48.2 (4.6)	50.1 (5.8)
Percentage of male patients with hearing loss	97.3% (37/38)	100% (22/22)
Percentage of male patients with ocular lesions	61.1% (11/18)	76.9% (10/13)

a) Only patients harbouring a splice site mutation with exon skipping (non-truncating), as demonstrated by RT-PCR analysis, have been included.

tions detected to date are restricted to one, or at least very few families, and because the number of affected persons in each family is limited. Secondly, all relevant clinical information is not always available. Three of the four diagnostic clinical criteria for AS [72]: a positive family history of hematuria with or without chronic renal failure, typical ultrastructural GBM changes in a renal biopsy specimen, high-tone sensorineural deafness, and characteristic ophthalmological signs (lenticonus and/or macular flecks), are fulfilled in only 39% (28/72) of the patients with a *COL4A5* mutation detected (Table 17).

One explanation for this low percentage is the lack of clinical information regarding ocular signs and GBM changes in 57% (41/72) and 79% (57/72) of the families, respectively.

A *COL4A5* mutation has been detected in 72% (28/39) of the probands fulfilling  $\geq 3$  of the clinical criteria for AS, and no mutations were detected in 28% (11/39). It also appears from Table 17, that 7% (5/72) of the mutations have been detected in patients fulfilling  $\leq 1$  of the criteria.

Another important aspect that makes it difficult to correlate genotype and phenotype is the late, and age related occurrence of some of the symptoms in AS. The proband in family DK010 was initially, at the age of 24 years, described as having a juvenile form of AS without hearing loss [3]. Later, from the age of 33 years, he developed a progressive hearing loss, and started to use a hearing aid from the age of 39 years. The mean age at ESRD in males with a missense mutations was found to be 33.8 years (Table 18).

Missense mutations in the NC1-domain and in-frame deletions result in a mean age at ESRD of 23.3 and 20.3 years, respectively, but the number of patients in each group is limited.

The mean age at ESRD in patients with splice site mutations is

just above 30 years, and higher in the 7 patients with donor splice site mutations than in the 6 patients with acceptor splice site mutations. A distinction in the phenotypic effect of splice acceptor and splice donor site mutations was suggested by Gross et al. [343]. Acceptor splice site mutations were found to result in a significantly lower number of juvenile type cases with hearing loss than donor splice site mutations. We found a mean age at ESRD of 34.4 years in the seven patients with donor splice site mutations as compared to 25.5 years in the six patients with acceptor splice site mutations (Table 18). Three of the present splice site mutations (two acceptor and one donor splice site mutation) were investigated by RT-PCR. All three mutations were found to cause skipping of a single exon and to be non-truncating. The effect of splice site mutations should be evaluated by RT-PCR analysis rather than just by the position of the mutation in the splice acceptor, or the splice donor site.

*COL4A5* mutations can be classified into two groups: truncating and non-truncating mutations, depending on the predicted effect on the protein. Truncating mutations comprise nonsense mutations, frame shifts, and larger rearrangements. Non-truncating mutations comprise missense mutations and in-frame deletions. To look for genotype-phenotype correlations in the present material we have pooled patients from each group with either a truncating or a non-truncating mutation in order to obtain a sufficient number of cases for comparison. The effect of splice site mutations were predicted by RT-PCR analysis, if cells were available for study. Three different splice site mutations have been analysed by RT-PCR and all were found result in exon skipping and preservation of the reading frame. All seven patients from these families have therefore been grouped together with patients with non-truncating mutations.

The mean age at ESRD in males with a non-truncating mutation is higher than seen in patients with a truncating mutation (33.1 vs. 21.6 years) (Table 19). This difference is highly significant (Mann-Whitney test,  $p < 0.0001$ ). The mean age at ESRD in females is higher in females with a truncating mutation than in females with a non-truncating mutation (50.1 vs. 48.2 years), but the difference is not statistically significant (Mann-Whitney test,  $p = 0.45$ ). There does not seem to be a difference in the presence of hearing loss between patients with a truncating and patients with a non-truncating mutation (Fisher's exact test,  $p > 0.5$ ) (Table 19).

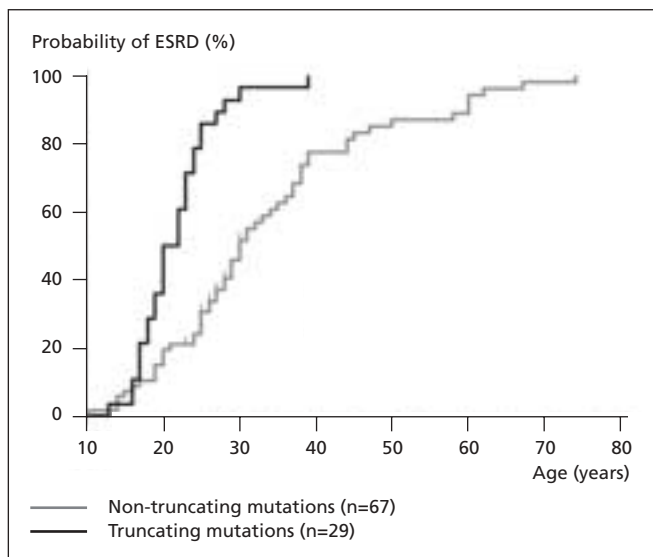
In some families with missense mutations, both ESRD and hearing loss develop after the age of 50 years [49, 137]. Ocular lesions were observed in the present material in 76.9% of the patients with a truncating mutation, and in only 61.1% of patients with a non-truncating mutation. The difference is, however, not statistically significant (Fisher's exact test;  $p = 0.098$ ). There does not seem to be a correlation between the type of the ocular abnormalities and the type of mutation in *COL4A5* [371].

Renal survival, or the age at ESRD as the parameter for disease severity, was analysed in 96 males with AS in which the *COL4A5* mutation were known. Graphs describing the probability of ESRD at a certain age in relation to the type of mutation were computed according to the Kaplan-Meier method.

Figure 21 compares the probability of ESRD in relation to age in patients harbouring a truncating versus patients harbouring a non-truncating mutation. Statistical analyses were done using the SPSS program package version 10.0. The age at ESRD was documented in 85 cases. Another 11 cases without ESRD were censored at the age obtained at the time the data was collected. Sixty-seven of the mutations were predicted to be non-truncating, and 29 to be truncating. All patients except one with a truncating mutation have ESRD at the age of  $\leq 30$  years, as compared to less than half of patients with a non-truncating mutation (Figure 21).

Median renal survival, or the age at which half of the patients have ESRD, is 22 years in the group with truncating mutations, and 30 years in the group with non-truncating mutations.

The two curves differ significantly (Logrank test,  $\chi^2 = 30.04$ ,  $df = 1$ ,  $p < 0.0001$ ), indicating that the underlying mutation, truncating or



**Figure 21.** Kaplan-Meier plot comparing the probability of ESRD in 96 males according to the type of *COL4A5* mutation: truncating or non-truncating. Non-truncating mutations comprise missense mutations, in-frame deletions, and splice site mutations. Truncating mutations comprise nonsense mutations, frame shifts, and major rearrangements. The age at ESRD was documented in 85 cases, and another 11 cases were censored. Tick marks on the curves represents the time a patient was censored. The difference between the two groups is highly significant (Logrank test,  $\chi^2=30.04$ ,  $df=1$ ,  $p<0.0001$ ).

non-truncating, has a significant influence on the age at ESRD in male patients.

AS with both juvenile and adult onset of ESRD have been reported for members of the same family [372, 373]. Our findings in the present material confirm this intrafamilial variability of phenotype. Twenty-four families were identified in which two or more males were affected by the disease (Table 4). In 17 of these families there was consistency of phenotype, i.e. either juvenile or adult onset of ESRD in all affected persons.

The phenotype was found to vary in 7 other families with both juvenile and adult onset of ESRD in different members of the same family. Interfamilial variability, or variability of the phenotype in association with recurrent mutations, has also been detected. Juvenile onset of ESRD was detected in one patient in family DK057, and adult onset in other 5 patients from family DK043, DK064, and DK072, all harbouring the missense mutation p.Gly594Ser. Variation in the phenotype was also seen in association with the missense mutation p.Gly594Ser in the two families DK014 and DK086, whereas all patients carrying the nonsense mutation p.Arg1677X have juvenile onset of ESRD.

Genotype-phenotype correlations in X-linked AS have been the subject in two larger studies. One of these, the "European Community Alport Syndrome Concerted Action (ECASCA)", was initiated in order to delineate accurately the AS phenotype, and to determine genotype-phenotype correlations in a large number of families [137]. In this study, genotype-phenotype correlations were determined in males from 195 families, in which the disease causing mutation was known. The other study is a meta-analysis of genotype-phenotype correlation in X-linked AS comprising clinical information related to 267 published *COL4A5* mutations [343].

According to the ECASCA study [137], larger structural *COL4A5* rearrangements, nonsense mutations, and frame shift mutations confer to affected male patients a probability of 90% of developing ESRD before the age of 30 years; the risk is 70% for male patients with splice site mutations, and 50% for male patients with missense mutations. The figures from the present material are in agreement with these results. In the same study it was found that the risk of de-

veloping hearing loss in male patients with larger *COL4A5* structural rearrangements, nonsense mutations, frame shift mutations or splice site mutations was 50% at the age of 10 years, and 90% at the age of 30 years. The risk for patients with missense mutations was found to be 50% at the age of 20 years, and 60% at the age of 30 years. Larger structural *COL4A5* rearrangements lead to a juvenile type of AS in more than 95% of the cases, and are associated with deafness in 77% [343].

Gross et al. [343] found, that the effect of glycine substitutions on the phenotype depends on the distance of the mutation from the NC1-domain. A glycine substitution in the collagenous domain of the  $\alpha 5(IV)$ -chain alters the folding of the type IV-collagen triple-helix. The triple-helix formation starts at the NC1 domain at the C-terminal end, and proceeds in a zipper-like way to the N-terminal end [374]. Glycine substitutions in the  $\alpha 5(IV)$ -chain were divided into two groups depending on the position of the mutation [374]. Mutations located in exon 1-20 were found to have a significantly less severe effect on the phenotype than mutations located in exons 21-47.

We found a mean age at ESRD of 23.8 years in the 5 patients with a glycine substitution in exon 1-20, compared to 37.3 years in the 32 patients with a glycine substitution caused by a mutation in exon 21-47 (Table 18). A more severe effect of the glycine substitutions in the N-terminal end can therefore not be confirmed in the present material. However, the number of mutations in the former group of mutations (exon 1-20), is limited.

The nature of the mutation, truncating or non-truncating, is not the only factor of importance for the phenotype. Either different environmental influences, variation in genetic background, or both, can cause the phenotypic variation in patients with the same mutation. Andrews et al. [375] demonstrated evidence, that factors other than the nature of the mutation, influence the disease progression. Using a knockout mouse model of autosomal recessive AS with mutated *Col4a3*, two lines of mice with different genetic background were compared. Genome scans provided significant evidence of the existence of two quantitative trait loci on mouse chromosome 9 and 16, respectively. This indicates the existence of modifier genes that act by modifying the GBM and thereby the disease progression.

#### 4.7.1 Double mutant alleles

No double mutant alleles with two disease-causing mutations have been identified in this study. The p.Lys664Asn substitution was identified in two unrelated patients; in DK032 (female) in whom no other mutation was found, and in DK021 (male) in whom a missense mutation (p.Gly521Cys) also was found. The sequence variation: c.1-13G>A in the 5'-UTR of exon 1, was identified in the proband in family DK035. A second and putative disease causing mutation, c.1877G>C (p.Gly626Ala), was found at the same *COL4A5* allele in this family. Both this 5'-UTR variant and the p.Gly626Ala missense mutation were found to co-segregate with the disease in family DK035, and were not identified in the 80 other AS probands (95 X-chromosomes) investigated. Two different missense mutations were also detected in DK115: p.Gly749Val, and p.Ala430Val. The pathogenic effect of p.Ala430Val is, however, dubious.

The presence of two different and putative disease causing mutations carried by the same *COL4A5* allele has previously been demonstrated in two cases. Guo et al. [69] detected two different missense mutations (p.Gly289Val and p.Arg1421Cys) in a female patient severely affected with AS. Chromosome analysis revealed a normal female karyotype, 46,XX. Both mutations were present in >90% of the mRNA in peripheral white blood cells and kidney tissue, while at the genomic level she was heterozygous for both mutations, suggesting the two mutations to be present on the same *COL4A5* allele. Skewed X-chromosome inactivation with >90% of the normal *COL4A5* allele inactivated in DNA from kidney tissue and white blood cells could explain the severe phenotype in this

woman. Knebelmann et al. [332] found two different glycine substitutions (p.Gly953Val and p.Gly1211Glu) on the same allele in a male patient, both segregating with the disease in the family. The polymorphic variant p.Lys664Asn found in DK021 together with the missense mutation p.Gly521Cys has also been detected together with another missense mutation (p.Pro1517Thr) in a previous study [68, 117].

The presence of more than one mutation in a gene, and interactions between them, may have implications for the phenotype. The functional consequences of the normal sequence variant in the three double mutant alleles identified in this study (p.Gly521Cys together with p.Lys664Asn, p.Gly626Ala together with c.1-13G>A in 5'UTR), and p.Gly749Val together with p.Ala430Val, are difficult to assess. The phenotypes in DK021 (juvenile onset of ESRD, hearing loss and lenticonus), and DK115 (juvenile onset, hearing loss, and lenticonus), are more severe than expected from the presence of an isolated missense mutation (p.Gly521Ser) in the collagenous domain of the  $\alpha 5(\text{IV})$ -chain. The phenotype in DK035 (adult form of AS with hearing loss) is, however, no more severe than expected in a patient with a glycine substitution in the collagenous domain of the  $\alpha 5(\text{IV})$ -chain.

There are, however diagnostic implications of double mutant alleles, especially in families too small for linkage studies. Failure to identify the presence of a second and disease causing mutation on the other *COL4A5* allele in an affected female may result in errors in the carrier diagnosis and in prenatal diagnosis. Failure to identify a second and disease causing mutation on the same *COL4A5* allele in both male and female patients may result in diagnostic errors if recombination between the two mutations occurs in a heterozygous female relative, although the frequency of such cases will be low. It is therefore important to analyse the entire coding sequence of the gene, and not stop the screening of the gene after identifying a single mutation.

#### 4.8 EVALUATION OF FAMILIES WITHOUT A *COL4A5* MUTATION

Forty-eight of the probands studied showed no disease causing *COL4A5* mutation. A number of these cases can, however, be excluded as possible X-linked AS due to additional clinical findings and/or pedigree information. The proband in family DK085 presented with macrothrombocytopenia in addition to progressive renal failure and hearing loss. A diagnosis of Epstein syndrome was established in this patient by sequencing the *MYH9* gene (for details, see section 4.9.1). X-linked inheritance can be excluded in seven other families due to father to son transmission of the disease. Another eight cases were isolated cases, so that X-linked inheritance was not evident based on pedigree information. Some of these cases may have an autosomal form of AS. A comparison of the clinical findings between the remaining 25 families without a *COL4A5* mutation detected, and 49 families with a *COL4A5* mutation detected,

**Table 20.** Comparison of clinical findings in 74 families with a pedigree compatible with X-linked inheritance with, and without a *COL4A5* mutation detected. Sporadic cases, families with a pedigree excluding X-linked inheritance, families without relevant information, and DK082 with Epstein syndrome were excluded.

	+ Mutation	- Mutation
No. families	49 (66%)	25 (34%)
No. male patients	78	26
Mean age at ESRD in years in males (SEM)	29.3 (1.6)	27.5 (2.7)
No. female patients	12	7
Mean age at ESRD in years in females (SEM)	52.5 (3.6)	40.3 (6.4)
Percentage of probands with hearing loss	98% (48/49)	60% (15/25)
Percentage of probands with ocular changes	68% (15/22)	29% (2/7)
Percentage of probands with GBM changes	100% (7/7)	100% (4/4)

is presented in **Table 20**. Only families from which relevant clinical information was available, were included.

The mean age at ESRD in males is not significantly different between the two groups (Mann-Whitney test,  $p > 0.4$ ). Hearing loss was present more frequent in patients with a mutation detected than in patients without (Table 20). The difference between the two groups was highly significant (Fisher's exact test,  $p < 0.0001$ ). Ocular lesions are also presented more frequent in the group with a mutation detected than in the group without, but the difference is not statistically significant (Fisher's exact test,  $p = 0.092$ ). From Table 17 it appears, that 39% (28/72) of the probands with a *COL4A5* mutation detected have  $\geq 3$  of the clinical criteria for AS fulfilled, compared to only 17% (11/63) of the probands in which no mutation was detected.

Forty-nine per cent (31/63) of the probands without a known mutation have  $\leq 1$  of the criteria fulfilled. There are, however, no significant differences with respect to the age at ESRD and the presence of extra-renal manifestations between patients with a mutation detected and patients without a mutation detected, within the group of patients fulfilling  $\geq 3$  of the clinical criteria for AS (data not shown). In conclusion, the evidence for a clinical diagnosis of AS in the group of patients without a mutation detected is less convincing than in the group in which a mutation has been detected.

Fifteen of the probands were studied by both PCR-SSCP analysis and direct sequencing of all coding exons [6]. Even if the PCR-SSCP method is not adequately sensitive to ascertain all mutations in the *COL4A5* gene, the consistent result in these 15 patients studied by both techniques suggest that the six patients in which no *COL4A5* mutation could be detected, could actually have another disorder.

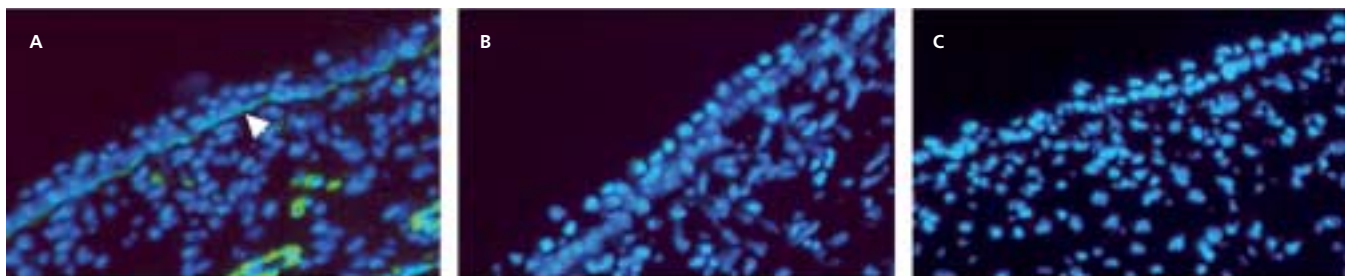
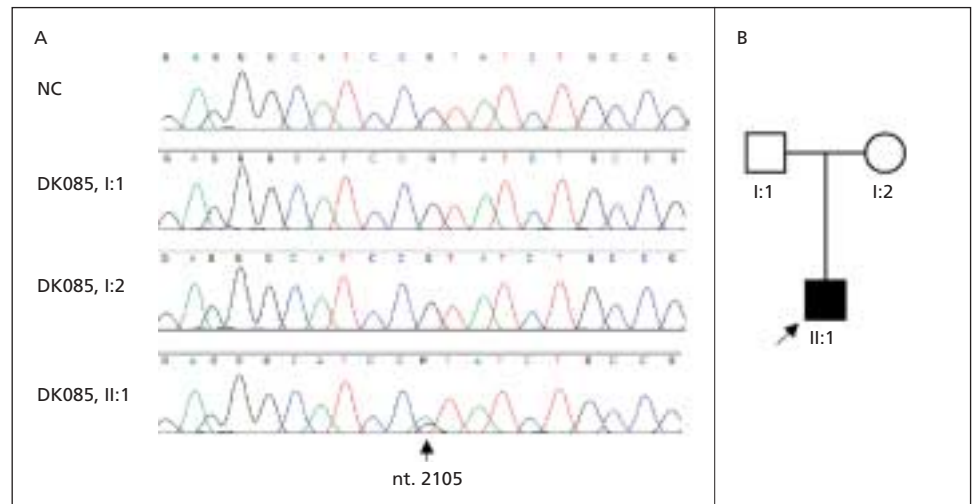
The pedigrees in five of the families (DK022, DK052, DK071, DK080, and DK082) in which no disease causing *COL4A5* mutation have been identified, strongly indicate X-linked inheritance. The proband in family DK022 has been evaluated by both PCR-SSCP analysis and direct sequencing. No mutation was detected by any of the methods used. Cells were not available for RT-PCR analysis. A *COL4A5* sequence variation: IVS41B+7C>T, was detected in DK082, co-segregating with the disease in the family. However, the number of family members from which blood samples were available was too small to obtain significant lod-scores in a linkage study. We have not been able to demonstrate any effect on the splicing process with respect to this mutation. Neither the age at ESRD nor the presence of extra-renal manifestations differs significantly between patients with a mutation detected and patients without a mutation detected within the group of patients with pedigrees strongly indicating X-linked inheritance (data not shown). These findings indicate, that the disease in these five families could be X-linked AS, even though no *COL4A5* mutation could be demonstrated.

#### 4.8.1 Detection of a *MYH9* mutation in family DK085

The proband in family DK085 was initially suspected of AS due to his clinical features in form of progressive renal failure accompanied by sensorineural hearing loss. However, no mutation was found by screening the entire coding sequence of the *COL4A5* gene by PCR-SSCP-analysis. Re-evaluation of his clinical phenotype disclosed macrothrombocytopenia, but neither cataract nor leukocyte inclusions. The correct clinical diagnosis in DK085 is therefore not AS, but more likely *Epstein syndrome*, which is an autosomal dominant disease caused by mutation in the *MYH9* gene, encoding the non-muscle myosin heavy chain IIA (MYHIIA).

Direct sequencing of *MYH9* exon 16 in the proband in DK085 revealed a single nucleotide substitution: c.2105G>A [NM\_002473] in heterozygous form, and thus confirming a diagnosis of Epstein syndrome. This single base substitution is expected to change the codon for arginine at amino acid position 702 to histidine in MYHIIA (p.Arg702His). Direct sequencing of *MYH9* exon 16 in both parents was normal, indicating that the mutation in the proband has arisen *de novo* (Figure 22).

**Figure 22.** A *de novo* missense mutation, c.2105G>A (p.Arg702His) in *MYH9* in the proband in family DK085 with Epstein syndrome. Direct sequencing of *MYH9* exon 16 revealed a single nucleotide substitution, c.2105G>A, in heterozygous form in the proband in family DK085 (DK085, II:1). This missense mutation changes codon 702 from CGT for arginine to CAT for histidine. B. Pedigree of the family.



**Figure 23.** Immunohistochemical staining of skin sections from the aborted fetus in Alport family DK002 (V:5) using the antibodies MAB1, MAB3, and MAB5 (Wieslab, Lund, Sweden) directed against the  $\alpha 1(\text{IV})$ -,  $\alpha 3(\text{IV})$ -, and  $\alpha 5(\text{IV})$ -collagen chains, respectively. Nuclei are counterstained (blue) using Hoescht 33258 (Aldrich) and double exposure. MAB1 stains the epidermal basement membrane (green) indicated by the arrowhead in (A), whereas no staining was seen using MAB3 (negative control) (B) and MAB5 (C). The epidermal basement membrane is normally negative for MAB3.

The p.Arg702His missense mutation has previously been identified in a patient with Epstein/Fechtner syndrome from USA, but originating from Europe [283], and in five patients with Epstein syndrome; one of Finnish, and four of Italian origin [266, 376]. p.Arg702His has not been observed in 60 normal controls, and alignment of the human MYH9 amino acid sequence from the globular head domain with the amino acid sequence of non-muscle and smooth muscle myosin from various species reveals complete conservation [376].

#### 4.9 IMPLICATIONS OF THE MOLECULAR GENETIC FINDINGS FOR GENETIC COUNSELLING

##### 4.9.1 Carrier detection and prenatal diagnosis

The identification of a disease causing mutation in a patient with AS makes carrier detection and prenatal diagnosis possible in relatives. Determination of the carrier status in a female at risk is important for accurate genetic counselling. Identification of the disease causing mutation in a patient with AS is important for accurate genetic counselling in future pregnancies, especially since both X-linked and autosomal forms of the disease exist. Seventeen prenatal tests for X-linked AS have been performed in 7 of the families in which the disease causing *COL4A5* mutations have been identified. The results are presented in Table 21. Five carrier females requested prenatal diagnosis in two or three subsequent pregnancies. Five couples decided on termination of the pregnancy due to result of the prenatal test, and twelve children have been born. In nine of the cases in which the result of the test was normal, no symptoms of AS have evolved in the children yet. However, most of these children are still too young to be out of risk. None of the children have been tested for the mutation after birth. Five affected male foetuses were detected, and the parents decided on termination of the pregnancy in all five cases. Two female foetuses were found to be heterozygous for

the mutation and the parents in both these cases decided to continue the pregnancy.

To assess the accuracy of the analysis in three of the cases we studied the expression of the  $\alpha 5(\text{IV})$ -chain by immunohistochemical staining of sections from fetal skin tissue after termination of the pregnancy. No expression of the  $\alpha 5(\text{IV})$ -chain could be demonstrated in skin sections from a male foetus predicted to be affected by the mutation analysis (Figure 23).

Normally, the  $\alpha 1(\text{IV})$ - and  $\alpha 2(\text{IV})$ -chains are present in early stages of human nephron development but are replaced in mature glomeruli on day 75 by  $\alpha 3(\text{IV})$ -,  $\alpha 4(\text{IV})$ -, and  $\alpha 5(\text{IV})$ -chains in the GBM [132]. However, the timing of the appearance of the  $\alpha 5(\text{IV})$ -chain in skin basement membranes is not investigated, and the significance of the absence of the  $\alpha 5(\text{IV})$ -chain from fetal skin sections from affected males is, therefore, so far unknown.

Attitudes towards genetic testing for AS have been evaluated in two studies. Levy et al. [377] interviewed a group of 27 females and 24 males with X-linked AS about their attitudes towards prenatal testing for the disease. They found that a majority of them, 78% of the females and 63% of the males, would make use of prenatal testing. About two thirds of the females in favour of prenatal testing would terminate the pregnancy in case of an affected male foetus, and 39% in case of an affected female foetus. Among the affected males in favour of prenatal testing, 53% would terminate a pregnancy in case of an affected foetus. Pajari et al. [378] evaluated attitudes towards genetic testing in 53 individuals (patients with AS and their healthy relatives) from 37 families, and found abortion acceptable in cases of an affected male and female foetus with AS in 28% and 19%, respectively. No significant association was found between the severity of the individual's own disease and the acceptability of selective abortion. The less severe clinical features in females as compared to males, and the fact that it is not possible to determine

the severity of the disease in affected females by the prenatal test, can explain a low acceptability of selective abortion of an affected female foetus.

Prenatal diagnosis of AS by molecular genetic analysis can be performed using two different approaches: direct investigation for the disease causing mutation [5], or indirect diagnosis by linkage analysis using closely linked and flanking polymorphic DNA markers [379].

Prenatal diagnosis by direct mutation analysis on DNA obtained from chorionic villus tissue or amniotic fluid cells is only a possibility in families in which the disease causing mutation has been identified prior to pregnancy. *COL4A5* mutations can rarely be identified in a short time due to the large size of the gene and the fact that there are no known hot spot areas for mutation and no significantly common mutations. Most of the *COL4A5* mutations identified so far seem to be restricted to single families. It is therefore important in the genetic counselling process of a family with AS to initiate mutation detection prior to pregnancy. This approach will allow specific prenatal diagnosis also in sporadic cases in which prenatal diagnosis by linkage analysis is impossible.

Carrier detection and prenatal diagnosis by linkage analysis is, however, still helpful in situations where the mutation in a family is unknown. No causative *COL4A5* mutation can be detected in 10-50% of patients in most series.

Prenatal testing for AS should only be performed in a family after careful genetic counselling.

#### 4.9.2 Gonadal mosaicism

Prenatal diagnosis should not be restricted to those in the family who have been identified as carriers because of the possibility of gonadal mosaicism. When the mother of an isolated case of AS with a known mutation can be clearly demonstrated as being non-carrier by direct mutation analysis, the recurrence risk is probably low. However, gonadal mosaicism in the mother remains possible, although the frequency remain to be determined. Nakazato et al. [380] identified a nonsense mutation, c.5035C>T (p.Gln1679X) in a boy with AS. The mutation was also present in heterozygous form in his sister, but could not be detected in peripheral blood leucocytes, hair roots or skin fibroblasts in their mother, suggesting the mutation only to be present in primordial germ cells. Plant et al. [381] screened the mothers of 25 affected male patients with a known *COL4A5* mutation, and both parents of three affected female patients, and identified three instances of somatic mosaicism (10.7%). One other case of somatic mosaicism in an Italian patient with AS has been reported by Bruttini et al. [340].

These findings indicate, that prenatal diagnosis based on mutation analysis still should be offered, even if the mother of a sporadic case was found not to be carrier of the mutation detected in her affected child.

## 5. CONCLUSIONS

The wide phenotypic spectrum, the genetic heterogeneity with several underlying loci, the large size of these genes, and the allelic heterogeneity with individual mutations make molecular genetic diagnosis of AS difficult, especially in sporadic cases and in families too small for linkage analysis. We have demonstrated a highly efficient and sensitive approach for molecular genetic testing of the *COL4A5* gene in putative AS cases. The combination of MLPA-analysis and either RT-PCR analysis of mRNA from cultured fibroblasts from a skin biopsy, if available, or an exon-by-exon screening strategy using direct sequencing or PCR-SSCP analysis, results in the detection of a disease causing mutation in a substantial number of cases. One advantage of using a skin biopsy is the possibility of also doing immunohistochemical staining for the  $\alpha 5(IV)$ -chain on sections from the skin biopsy to support a diagnosis of X-linked AS. Some larger structural rearrangements such as the inversion detected in this series was exclusively ascertained by Southern blotting analysis, which should be included in the diagnostic set-up for AS. MLPA-analysis is a fast and reliable method for detection of *COL4A5* deletions and certain point mutations, and should be the first choice method for screening patients clinically suspected of AS, and for carrier detection in female relatives of AS patients with a known deletion. In particular, MLPA should be used in patients presenting with diffuse leiomyomatosis and AS, because these patients are highly suspected of having a larger *COL4A5* deletion extending into *COL4A6*.

In total, 64 different and putative disease causing mutations were detected in 72 families by screening 135 probands. The mutation detection rate was 72% in patients fulfilling  $\geq 3$  of the clinical criteria for AS, and 82% in families clearly demonstrating X-linked inheritance.

A clinical diagnosis of AS in a patient with hematuria or progressive renal failure is based on the presence of a positive family history of hematuria with or without progressive renal failure, high-tone sensorineural deafness, characteristic ophthalmological signs, and typical ultrastructural or immunohistochemical GBM changes in a renal biopsy specimen. The availability of direct mutation testing for AS is a significant advance in the diagnosis of the disease, and therefore also an improvement in relation to genetic counselling of the family. It will allow carrier detection even in families with only a sin-

Table 21. Results of the prenatal tests.

Family	COL4A5 mutation detected			Week of pregnancy	Fetal sexing	Result of mutation analysis	Pregnancy outcome
	Exon	Nucleotide change	Predicted effect				
DK002 . . . . .	38	c.3454+1G>T	Exon 38 skipping	12th	46,XY	-M	Live birth
DK002 . . . . .	38	c.3454+1G>T	Exon 38 skipping	12th	46,XY	+M	Termination <sup>a</sup>
DK009 . . . . .	47	c.4436_4437delGA	p.Gly1479fsX1484	10th	46,XY	+M	Termination
DK009 . . . . .	47	c.4436_4437delGA	p.Gly1479fsX1484	13th	46,XY	-M	Live birth
DK009 . . . . .	47	c.4436_4437delGA	p.Gly1479fsX1484	11th	46,XY	-M	Live birth
DK009 . . . . .	47	c.4436_4437delGA	p.Gly1479fsX1484	11th	46,XX	Het.	Live birth (carrier)
DK009 . . . . .	47	c.4436_4437delGA	p.Gly1479fsX1484	12th	46,XX	-M	Live birth
DK012 . . . . .	29	c.2297G>A	p.Gly766Asp	15th	SRY+	-M	Live birth
DK012 . . . . .	29	c.2297G>A	p.Gly766Asp	12th	SRY+	+M	Termination <sup>a</sup>
DK012 . . . . .	29	c.2297G>A	p.Gly766Asp	12th	SRY+	-M	Live birth
DK014 . . . . .	44	c.4069G>A	p.Gly1357Ser	11th	46,XY	-M	Live birth
DK024 . . . . .	30	c.2404_2421del18	p.Gly802_Pro807del	10th	46,XX	-M	Live birth
DK024 . . . . .	30	c.2404_2421del18	p.Gly802_Pro807del <sup>b</sup>	10th	46,XY	-M	Live birth
DK024 . . . . .	30	c.2404_2421del18	p.Gly802_Pro807del	10th	46,XX	-M	Live birth
DK035 . . . . .	25	c.1877G>C	p.Gly626Ala <sup>b</sup>	9th	46,XY	+M	Termination
DK035 . . . . .	25	c.1877G>C	p.Gly626Ala <sup>b</sup>	10th	46,XX	Het.	Live birth (carrier)
DK036 . . . . .	10	c.594G>A	p.Gly195Asp	10th	SRY+	+M	Termination <sup>a</sup>

a) Immunohistochemically verified in skin sections from the aborted foetus. b) Prenatal diagnosis was initially performed by linkage analysis and later, when the mutation was identified, verified by mutation analysis. +M = The actual mutation detected. -M = The actual mutation not detected. Het. = heterozygous for the actual mutation.

gle affected male patient and will help differentiate the X-linked form from the autosomal forms of the disease. It could furthermore be helpful in distinguishing carrier females of the X-linked form of AS, presenting only with microscopic hematuria, from females with benign familial hematuria. We have shown that this method is applicable to mutational screening prior to carrier detection and prenatal diagnosis.

We found that the underlying *COL4A5* mutation, truncating or non-truncating, can significantly predict the age at ESRD in male patients. Truncating mutations, comprising nonsense mutations, frame-shifts, and larger structural rearrangements, were found to cause a juvenile form of the disease with a mean age at ESRD of 21.6 years, compared to 33.1 years in patients with a non-truncating mutation. The effect of non-truncating mutations is, however, less clear-cut. Glycine substitutions in the collagenous domain of the  $\alpha 5(\text{IV})$ -chain will result in an adult form of AS with a mean age at ESRD of 35.8 years. Missense mutations in the NC1-domain and in-frame deletions result in a juvenile form of the disease with a mean age at ESRD of 23.3 and 20.3 years, respectively, but the number of patients in each group is limited. We found no significant differences in the presence of hearing defects or ocular manifestations between patients with the different types of mutations. The lack of distinct genotype-phenotype correlations implies that the usefulness of the result of the *COL4A5* mutation analysis to predict the prognosis is limited.

## 6. PERSPECTIVES

The molecular genetic basis of AS is now established, and the spectrum of mutations in the *COL4A3*, *COL4A4*, and *COL4A5* genes is well characterized. Mutation detection rates are still low due to the large size of the gene, but future technological improvements e.g. automated sequencing strategies and implementation of *microarray technology*, may increase the mutation detection rates, and lower the time and costs of the analyses. Functional studies are another possibility, but are hampered by the restricted expression of the type IV collagen chains. The many different animal models for AS are an obvious and promising target for functional studies.

### 6.1 ANIMAL MODELS FOR AS

Animal models can be used for the study of the pathogenesis of the disease and the relationship between the underlying mutation and the biochemical defect in the basement membranes and the clinical phenotype. In addition, new therapeutic approaches can be evaluated. Several dog and mouse models for X-linked, autosomal recessive, and autosomal dominant AS are available for such studies.

Two different, naturally occurring, canine models for X-linked AS are known. One model for X-linked AS is described in Samoyed dogs [44, 382-385] in which the renal disease mimics the human X-linked form. Affected male dogs suffer from early-onset of renal disease at about 2-3 months of age, with rapid progression to renal failure and death at 8-10 months of age. Expression of the  $\alpha 3(\text{IV})$ -,  $\alpha 4(\text{IV})$ - and  $\alpha 5(\text{IV})$ -chains is totally absent. A nonsense *COL4A5* mutation in exon 35 has been identified in these dogs [386]. Another model for X-linked AS with almost the same features seen in the Samoyed dogs, occurs in a family of mixed-breed dogs from Novasota, Texas [387]. The underlying mutation in these dogs is a 10 bp deletion in exon 9 of *COL4A5*, creating a frame shift and a stop codon in exon 10 [388]. The mutation results in the production of a truncated protein lacking most of the collagenous region and NC1 domain essential for the assembly of the three collagen type IV  $\alpha$ -chains.

A naturally occurring autosomal recessive form of AS has been described in English cocker spaniels [389, 390]. Expression of the  $\alpha 3(\text{IV})$ - and  $\alpha 4(\text{IV})$ -chains are totally absent from the GBM, whereas the expression of the  $\alpha 5(\text{IV})$ -chain is markedly reduced but not absent [391]. No *COL4A3* or *COL4A4* mutations have so far been identified in these dogs. No hearing impairments or ophthal-

mological features are seen in either the X-linked or the autosomal recessive AS dog models.

Three different models for autosomal recessive AS have been generated in transgenic mice, with either knockout of the *Col4a3* gene [392, 393], or both the *Col4a3* and *Col4a4* gene [394]. The *Col4a3* knockouts were produced by either deletion of the last 3 exons encoding parts of the NC1 domain, or by a larger inverted insertion in *Col4a3*. In the *Col4a3/Col4a4* knockout mouse a region comprising exon 1-12 in *Col4a3*, exon 1-2 in *Col4a4*, and the intergenic promoter region, is deleted. The phenotypic features in these three transgenic mice are almost the same with onset within the first weeks to months, and ESRD after 2-4 months. No obvious extrarenal manifestations have been determined, and heterozygotes are phenotypically normal. One of the *Col4a3* knockout mouse models for autosomal recessive AS has been used for the study of ultrastructural changes in the inner ear [395], not previously studied in humans. Another application of the *Col4a3* knockout mouse model is the study of modifying genes affecting the AS phenotype [375].

Bull terrier hereditary nephritis represents a model for autosomal dominant AS with characteristic GBM changes, although all type IV collagen chains are present [396-398]. No significant hearing loss can be demonstrated, but anterior lenticonus is present.

Another autosomal dominant form of AS has been described in Dalmatian dogs [399]. The clinical features are very much the same as seen in the Bull terrier form, except for a less severely affected GBM and absence of in *uteri* ultrastructural GBM-changes.

The different phenotypic expression in patients with the same type IV collagen mutation is another aspect, which can be studied, in animal models. Quantitative trait loci influence renal disease progression in a mouse model.

A better understanding of the pathogenesis that leads to ESRD in AS and the factors that influence this pathway, environmental or genetic, may provide evidence and targets for new therapies for the disease.

### 6.2 GENE THERAPY OF AS

The only treatment available so far for the renal disease in AS is dialysis and transplantation. There are, however, several factors making AS a promising candidate for somatic gene therapy [400]. First of all the organ most seriously affected in AS is the kidney, which is accessible for both *ex vivo* and *in vivo* studies. The symptoms from the other organs involved in AS, the inner ear and the eye, are not life-threatening. Secondly, expression of the mutated gene in X-linked AS does not seem to be a problem, as heterozygous females usually only have mild features in the form of microscopic hematuria, and rarely develop renal failure.

Heikkilä et al. [401] studied the efficiency of adenovirus-mediated transfer of a  $\beta$ -galactosidase reporter gene into cultured human endothelial cells, glomerular mesangial cells, intact human glomeruli *in vitro*, and *ex vivo* and *in vivo* intra-arterial infusion into porcine kidneys in an organ perfusion model system [402]. A transfer efficiency in the organ perfusion method of up to 85% of renal glomeruli was obtained when infected during *ex vivo* renal perfusion for up to 12 hours, and up to 75% during *in vivo* renal perfusion for up to 2 hours. Only glomerular cells showed expression, while other kidney structures were negative for expression.

In an *in vitro* experiment using adenovirus mediated transfer into cultured human cells, Heikkilä et al. [403] could obtain expression of full-length human  $\alpha 5(\text{IV})$  cDNA. The recombinant  $\alpha 5(\text{IV})$ -chain was incorporated into type IV collagen protomers together with endogenous  $\alpha 3(\text{IV})$ - and  $\alpha 4(\text{IV})$ -chains and secreted from the cells. *In vivo* perfusion of pig kidneys with the recombinant adenovirus using the organ perfusion model system resulted in expression of the  $\alpha 5(\text{IV})$  chain into the GBM as shown by *in situ* hybridisation and its deposition into the GBM as shown by immunohistochemistry. It was demonstrated, that expressed recombinant human  $\alpha 5(\text{IV})$  chains were deposited extracellularly into the GBM.

Using an adenovirus vector and full-length canine  $\alpha 5(\text{IV})$  cDNA injected directly into the bladder smooth muscle in the Samoyed dog model of AS, Harvey et al. [404] could demonstrate expression of the  $\alpha 5(\text{IV})$ -chain in the smooth muscle basement membrane. Furthermore, this recombinant  $\alpha 5(\text{IV})$  was able to restore the expression of the  $\alpha 6(\text{IV})$ -chain in the basement membrane that normally is required for incorporation into the  $[\alpha 5(\text{IV})]_2\alpha 6(\text{IV})$  protomer. The  $[\alpha 5(\text{IV})]_2\alpha 6(\text{IV})$  protomer is normally seen in the basement membranes in smooth muscle cells, and lacks in X-linked AS.

A very promising approach was published by Sugimoto et al. in 2006 [405]. They demonstrated, that transplantation of wild-type bone marrow cells into irradiated *Col4A3<sup>-/-</sup>* mice results in a possible recruitment of bone marrow-derived progenitor cells as podocytes and mesangial cells within the damaged glomerulus, leading to a partial restoration of expression of the  $\alpha 3(\text{IV})$ -chain with concomitant emergence of the  $\alpha 4(\text{IV})$ - and  $\alpha 5(\text{IV})$ -chain expression, significant reduction of proteinuria and improvement of the overall kidney histology.

The several available animal models for AS are an important resource for future gene therapy studies, and for making AS a reliable candidate for gene therapy in humans also.

## 7. SUMMARY

Alport syndrome (AS) is a progressive renal disease that is characterised by hematuria and progressive renal failure, and often accompanied by progressive high-tone sensorineural hearing loss and ocular changes in form of macular flecks and lenticonus. AS is a genetic heterogenous disease, and X-linked dominant in about 85% of the families. The autosomal recessive and dominant forms constitute about 15% of the cases.

In the first part of the study is a multipoint linkage analysis of 12 families suspected of X-linked AS. The aim of that part of the study was to map a number of X-chromosomal polymorphic markers in relation to the locus for AS, in order to be able to perform carrier detection and prenatal diagnosis in the families. In addition, a more precise map of the region could form the basis for positional cloning of the gene for X-linked AS. In 1990 it was found that the X-linked form of AS is caused by mutation in the *COL4A5* gene located at Xq22, and encoding the  $\alpha 5$ -chain of type IV-collagen. The *COL4A5* gene is a very large gene spanning 257 kb with a transcript of 6.5 kb distributed on 51 exons. In addition, two alternatively transcribed exons have been identified.

In the second part of the study methods were set up for detection and characterisation of mutations in the *COL4A5* gene in 135 patients suspected of AS. The aims of that part of the study were to develop an efficient and reliable approach for mutation detection, and to implement the results of the mutation analysis in clinical practice for carrier detection and prenatal diagnosis, in order to be able to offer a better genetic counselling to the families. Knowledge of a possible correlation between genotype and phenotype can be of help in predicting the prognosis.

Samples from 135 probands suspected of AS and 359 of their relatives were collected, together with available clinical information. Southern blotting analysis and multiplex ligation-dependent probe amplification (MLPA) were used to screen for larger structural rearrangements (deletions and duplications). cDNA probes covering the entire coding region of the *COL4A5* gene were hybridised on restriction enzyme digested genomic DNA on Southern blots. Three different rearrangements were found by Southern blotting, two of which were caused by single base substitutions, and also detected by the PCR-SSCP analysis. One larger rearrangement was found, an inversion of 21 Mb with a proximal breakpoint in *COL4A5* intron 8 at Xq22.3, and a distal breakpoint in the *RAB33A* gene at Xq26.1. This rearrangement was exclusively ascertained by the Southern blotting analysis. Three deletions of  $\geq 2$  exons were detected by MLPA. One of these was detected in a female proband. A deletion in heterozygous form will not be detected by PCR-SSCP or direct sequencing.

A method based on the PCR-SSCP technique was set up for screening of the *COL4A5* gene exon-by-exon for mutation. All 51 *COL4A5* exons with flanking intronic sequences were screened by this technique. The two alternatively transcribed exons 41A and 41B were directly sequenced. The PCR-SSCP method was compared to direct sequencing in 15 of the cases. No difference in mutation detection rates were found. Finally, a method based on RT-PCR analysis of mRNA extracted from cultured skin fibroblasts was established. A mutation in a patient previously screened by PCR-SSCP analysis with normal result, was detected. Another advantage of analysing a skin biopsy is that it is also possible to perform immunostaining for the  $\alpha 5(\text{IV})$ -chain in the epidermal basement membrane on sections from the biopsy. Absence of the  $\alpha 5(\text{IV})$ -chain support a diagnosis of X-linked AS.

A total of 64 different and putative disease causing mutations were found in 72 of the families. Half of the mutations identified were missense mutations. The most frequent mutations in AS were glycine substitutions in the *Gly-Xaa-Yaa* repeat sequence in the collagenous domain of the  $\alpha 5(\text{IV})$ -chain, accounting for 47% of all mutations and 89% if the missense mutations. Frame-shift mutations accounted for 17% of the mutations, splice site mutations for 13%, nonsense mutations for 11%, in-frame deletions for 4%, and larger structural rearrangements for 6%. In addition, 5 different non-pathogenic sequence variations, polymorphisms and mutations of unknown effect on the phenotype, were found. Nineteen of the mutations are new and have not previously been published, and 55 of the mutations have exclusively been detected in this material. Two of the mutations (3%) are *de novo* mutations, and it has been possible to trace the mutation back in six of the families, and to determine the parental origin of the mutation in these six families. The origin of the mutation was found to be paternal in 4 of the families (67%), and maternal in 2 of the families (33%).

We have demonstrated a highly efficient and sensitive molecular diagnostic approach for analysing the *COL4A5* gene in putative AS cases. Based on the present results and the literature, an algorithm for molecular genetic analysis of the *COL4A5* gene is suggested. The overall mutation detection rate was found to be 53%. The mutation detection rate was 72% in patients fulfilling  $\geq 3$  of the clinical criteria for AS, and 82% in families clearly demonstrating X-linked inheritance.

No *COL4A5* mutation could be detected in 63 (47%) of the families. X-linked inheritance could be excluded in seven of these families solely based on a pedigree analysis, and a diagnosis of Epstein syndrome was established in one of the patients by *MYH9* mutation analysis.

We found that the underlying *COL4A5* mutation, truncating or non-truncating, can significantly predict the age at ESRD in male patients. Truncating mutations, comprising nonsense mutations, frame-shifts, and larger structural rearrangements, were found to cause a juvenile form of the disease with a mean age at ESRD of 21.6 years, compared to 33.1 years in patients with a non-truncating mutation. The effect of non-truncating mutations is, however, less clear-cut. Glycine substitutions in the collagenous domain of the  $\alpha 5(\text{IV})$ -chain will result in an adult form of AS with a mean age at ESRD of 35.8 years. Missense mutations in the NC1-domain and in-frame deletions result in a juvenile form of the disease with a mean age at ESRD of 23.3 and 20.3 years, respectively, but the number of patients in each group is limited. We found no significant differences in the presence of hearing defects or ocular manifestations between patients with the different types of mutations. The lack of distinct genotype-phenotype correlations implies that the usefulness of the result of the *COL4A5* mutation analysis to predict the prognosis is limited.

Future technological improvements e.g. automated sequencing strategies and implementation of *microarray technology*, may increase the mutation detection rates, and lower the time and costs of the analyses. Functional studies are hampered by the restricted ex-

pression of the type IV collagen chains. The many different animal models for AS are obvious and promising targets for functional studies, and an important resource for gene therapy studies. This makes AS a reliable candidate for future gene therapy in humans.

#### LIST OF ABBREVIATIONS:

AS:	Alport syndrome
ASO:	Allele-specific oligonucleotide
AS-DL:	Alport syndrome with diffuse leiomyomatosis
Bp:	Base-pairs
CEPH:	The Centre d'Etude du Polymorphisme Humain
CM:	Centi-Morgan
DGGE:	Denaturing gradient gel electrophoresis
dHPLC:	Denaturing high performance liquid chromatography
EBV:	Epstein-Barr virus
EDDNAL:	European Directory of DNA Diagnostic Laboratories: <a href="http://www.eddnal.com">http://www.eddnal.com</a>
ESRD:	End-stage renal disease
GBM:	Glomerular basement membrane
Kb:	Kilobases
Mb:	Megabases
MLPA:	Multiplex ligation-dependent probe amplification
NC1:	Non-collagenous domain
OMIM:	Online Mendelian Inheritance in Man. McKusick-Nathans Institute for Genetic Medicine, Johns Hopkins University (Baltimore, MD) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, MD), 2000. World Wide Web URL: <a href="http://www.ncbi.nlm.nih.gov/omim/">http://www.ncbi.nlm.nih.gov/omim/</a>
PCR:	Polymerase chain reaction
PTT:	Protein truncation test
RFLA:	Restriction fragment length analysis
RT-PCR:	Reverse transcriptase PCR
SDS:	Sodium dodecyl sulphate
SEM:	Standard error of the mean
SSCP:	Single-stranded conformational polymorphism
UTR:	Untranslated region.

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