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ABSTRACT

Alport syndrome is a clinically heterogeneous, progressive nephropathy caused by mutations in collagen IV genes, namely *COL4A3* and *COL4A4* on chromosome 2 and *COL4A5* on chromosome X. The wide phenotypic variability and the presence of incomplete penetrance suggest that a simple Mendelian model cannot completely explain the genetic control of this disease. Therefore, we explored the possibility that Alport syndrome is under digenic control. Using massively parallel sequencing, we identified 11 patients who had pathogenic mutations in two collagen IV genes. For each proband, we ascertained the presence of the same mutations in up to 12 members of the extended family, for a total of 56 persons studied. Overall, 23 mutations were found. Individuals with two pathogenic mutations in different genes had a mean age of renal function deterioration intermediate with respect to the autosomal dominant form and the autosomal recessive one, in line with molecule stoichiometry of the disruption of the type IV collagen triple helix. Segregation analysis indicated three possible digenic segregation models: i) autosomal inheritance with linked mutations *in trans* mimicking recessive inheritance (5 families); ii) autosomal inheritance with linked mutations *in cis* mimicking dominant inheritance (2 families); and iii) unlinked autosomal and X-linked inheritance having a peculiar segregation (4 families). This pedigree analysis provides evidence for digenic inheritance of Alport syndrome. Clinical geneticists and nephrologists should be aware of this possibility in order to more accurately assess recurrence risk, predict prognosis and identify other family members at risk.

Introduction

Alport syndrome is an inherited nephropathy characterised by haematuria, proteinuria, progressive renal failure and ultrastructural lesions of the glomerular basement membrane, often associated with sensorineural deafness and ocular lesions.[1] The primary defect resides in one of the alpha chains of type IV collagen produced by podocytes; the alpha chains assemble into a heterotrimeric triple helix (α3, α4 and α5) to create the three-dimensional network of the basement membrane.[2] The alpha 3 and alpha 4 chains are encoded by *COL4A3* and *COL4A4* genes, located head-to-head on chromosome 2, while the alpha 5 chain is encoded by *COL4A5* on chromosome X. All three main models of Mendelian inheritance have been demonstrated in Alport syndrome: X-linked semidominant, autosomal recessive, and autosomal dominant.[3,4,5] X-linked semidominant inheritance is associated with mutations in *COL4A5*, while autosomal recessive and dominant inheritance patterns are associated with one or two mutations in either *COL4A3* or *COL4A4.*[4]

Mutant alleles of all three primary loci demonstrate variable expressivity. In particular, heterozygous *COL4A5* females may be asymptomatic or have symptoms that range from microhaematuria alone to severe nephropathy leading to end-stage renal disease (ESRD).[6] Similarly, heterozygous carriers of *COL4A3* or *COL4A4* mutations, irrespective of gender, may be asymptomatic, have microhaematuria (carriers of recessive disease) or may progress to ESRD, albeit at a later age (apparently dominant form)[7,8]. A correlation between *COL4A5* mutations and both the rate of progression to ESRD and the course of hearing loss and ocular lesions has been established in X-linked Alport syndrome but, even within a single family, there is variability in disease severity.[6,9] Part of this variability may be due to a modifier effect of functional polymorphisms in the three collagen chains (of which *COL4A3* and *COL4A4* are particularly rich) or in other structural

proteins of the glomerular basement membrane, such as podocin.[10] Clarification of this relevant clinical variability warrants further investigation.

The wide phenotypic variability of patients with Alport syndrome and the presence of incomplete penetrance suggest that a simple Mendelian model is inadequate to explain the genetic control of this disease. An alternative genetic model that may apply to Alport syndrome is digenic inheritance. As explained by Shaffer in 2013, “inheritance is digenic when the variant genotypes at two loci explain the phenotypes of some patients and their unaffected (or more mildly affected) relatives more clearly than the genotypes at one locus alone”.[11] Digenic inheritance has previously been demonstrated in diseases such as retinitis pigmentosa[12] and left ventricular noncompaction cardiomyopathy.[13] During the past few years, the extensive use of comparative genomic hybridisation and microarray technology (array CGH) has improved knowledge of two-loci diseases. In particular, it has recently been shown, in patients with various neuropsychiatric diseases, that an enrichment in copy number variants (CNVs) correlates with a more severe phenotype, i.e. a specific microdeletion both predisposes to neuropsychiatric phenotypes as a single event and exacerbates neurodevelopmental phenotypes in association with other deletions or duplications.[14,15]

Research into the genetic causes of disease has been facilitated by the recent development of massively parallel sequencing techniques. This technology permit the sequencing of many genes simultaneously as a routine procedure, [5,7,16] and should accelerate the discovery and characterisation of disorders governed by digenic inheritance.[11] In the past, when Sanger sequencing was the dominant method, it was common to stop analysis when a single pathogenic mutation was identified; this approach prevented the ascertainment of individuals with mutations in more than one collagen gene. Since 2011, we have been using massively parallel sequencing to evaluate the three

collagen IV genes in patients with Alport syndrome, and this approach permitted us to identify 11 people who have mutations in two genes. In this study, we examined the mutation pattern and clinical characteristics of these 11 patients and members of their extended families, to explore the possibility that Alport syndrome is under digenic control.

Materials and methods

Patients and families

Genetic counselling was performed in six European institutes (University of Siena, Italy; Hôpital Necker – Enfants Malades, Paris, France; Guy's Hospital, London, England; Newcastle upon Tyne Hospitals NHS Foundation Trust, United Kingdom; Maastricht University Medical Centre, The Netherlands; and Université Catholique de Louvain, Belgium) where patients with Alport syndrome were selected for mutation screening in the *COL4A3*, *COL4A4* and *COL4A5* genes according to clinical criteria.[17] Eleven unrelated persons were found to have pathogenic mutations in more than one collagen gene. These patients (4 males and 7 females, 3–55 years old) and members of their extended families were recruited for the present study, allowing us to construct 11 pedigrees. Informed consent for clinical data sharing and DNA testing was obtained from each proband and family member; in case of minors, parental consent was obtained.

From all persons recruited, we collected clinical data regarding: family status (proband or family relation), gender and age at inclusion (or death), kidney function (haematuria, proteinuria, chronic renal failure (CRF) or ESRD), hearing loss and ocular lesions; for all comorbidities we recorded age at diagnosis and eventual treatment. Moreover, we obtained a sample of peripheral blood in EDTA tubes when possible

Genomic DNA, amplification and sequencing strategy

Blood was stored frozen prior to the extraction of genomic DNA using QIAamp DNA Blood Kits (Qiagen, Hilden, Germany); this work was done at each institute for the families

recruited there. In Siena, genomic DNA was supplied directly from the institutional biobank.

Genomic DNA from probands was assessed for mutations in *COL4A3*, *COL4A4* and *COL4A5* by locus-specific amplification followed by massively parallel sequencing. Each participating institute amplified and sequenced the DNA for the probands it recruited. Briefly, the ALPORT MASTR kit (Multiplicom, Niel, Belgium) was used to amplify 149 amplicons (representing 150 coding exons) of the three genes in a four-tube multiplex PCR reaction starting with about 4 X 50 ng genomic DNA. Amplification products were sequenced using either a GS Junior System (454 Life Sciences, Roche) or an Ion Personal Genome Machine (PGM; Life Technologies), as described below. Mutations of Probands 3, 4, 5, 7 and 9 have already been reported in Morinière et al.[16] Genomic DNA from family members was analysed by Sanger sequencing to determine if these persons had the same mutations as the probands.

GS Junior 454 sequencing

Our strategy for sequencing the *COL4A3*, *COL4A4* and *COL4A5* genes on a GS Junior system has been reported.[5] Briefly, amplification products were diluted and then reamplified with primers containing, at the 5' end, a multiplex identifier sequence which barcodes the samples. The second PCR products for each proband were pooled in predefined proportions according to the ALPORT MASTR protocol (Multiplicom). These libraries were purified using Agencourt AMPure XP system (Beckman Coulter) and quantified using Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies), following the protocol of 454 Life Sciences (http://454.com/downloads/my454/documentation/gs-junior-plus/454SeqSys_Amplicon-Library-Prep-MM_Apr2014.pdf).

For GS Junior sequencing, libraries were diluted to a concentration of 1 X 10^7 PCR fragment molecules/µl, annealed to carrier beads (SeqCap EZ Pure Capture Bead Kit,

Roche NimbleGen) and clonally amplified by emulsion PCR (emPCR) according to the manufacturer's protocol (http://454.com/downloads/my454/documentation/gs-junior/method-manuals/GSJunioremPCRAmplificationMethodManualLib-A_March2012.pdf). After amplification, the beads carrying single-stranded DNA templates were enriched, counted, and deposited into the PicoTiterPlate for sequencing (http://454.com/downloads/my454/documentation/gs-junior/method-manuals/GSJuniorSequencingManual_Jan2013.pdf). Sequence reads were analysed using GS Amplicon Variant Analyzer, version 2.9.

Ion PGM sequencing

The strategy for sequencing the three collagen genes on an Ion PGM has recently been reported.[16] Briefly, amplification products were diluted, reamplified using the universal primers included in ALPORT MASTR kit, and pooled in predefined proportions according to the ALPORT MASTR protocol. This pool was used to prepare a barcoded library compatible with the Ion PGM according to the protocol of Life Technologies (http://ioncommunity.lifetechnologies.com/community/login.jspa?referer=http://ioncommunity.lifetechnologies.com/community/protocols-home).[16] Libraries were purified using Agencourt AMPure XP system and quantified using Qubit dsDNA HS Assay Kit (Life Technologies).

For Ion PGM sequencing, PCR fragments were diluted to 100 pM, annealed to carrier spheres (Ion Sphere Particles) and clonally amplified by emPCR using the Ion PGM Template OT2 200 kit (Life Technologies). Spheres carrying single-stranded DNA templates were transferred to Ion 314 chips for sequencing using the Ion PGM Sequencing 200 Kit v2. Data were processed using Torrent Suite software v4.0, while post-run analysis was conducted using Torrent Variant Caller plugin (v4.0-r72895).

In silico analyses

Pathogenicity was ascertained if following criteria were met: non-polymorphic missense mutations or in-frame deletions involving key amino acids, such as glycine in the collagen Gly-X-Y triple helical domain, splice-site mutations, and truncating mutations. Pathogenicity of non-synonymous variations other than Gly substitutions was predicted using Alamut software v2.3 (Interactive Biosoftware, Rouen, France). To determine if the identified sequence variants were novel or had been previously reported, we searched in the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/), the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php)[18], the Leiden Open Variation Database v2.0 Build 35 (https://grenada.lumc.nl/LOVD2/COL4A/variants.php?select_db=COL4A3&action=search_all&search_MutCol=%3E) and the ALPORT (*COL4A5*) database (http://www.arup.utah.edu/database/ALPORT/ALPORT_display.php?sort=2#alport; Last update: October 2013)[19].

Variants were described according to the *COL4A3* reference sequence LRG_230 (NM_000091.4), *COL4A4* reference sequence LRG_231(NM_000092.4), and *COL4A5* reference sequence LRG_232(NM_000495), where nucleotide number 1 corresponds to the first base of the translation initiation codon, and using the nomenclature recommended by the Human Genome Variation Society.[20]

Sanger sequencing and segregation analysis of pedigrees

Pathogenic variants identified in probands were confirmed by direct Sanger sequencing. Sanger sequencing was also used to determine if the pathogenic variants were present in family members for whom genomic DNA was available. Briefly, genomic DNA was amplified using the primers and PCR conditions described for amplicon library preparation in Artuso et al.[7] Sequencing was performed on an ABI Prism 310 genetic analyser (PE Applied Biosystems) and data were analysed with Sequencher software v4.9

(Gene Codes, Ann Arbor, USA). Genotypes of pedigrees were examined to determine if the *COL4A3* and *COL4A4* mutations on chromosome 2 were linked in *cis* or *trans* and to assess genotype-phenotype correlation.

Results

The study began with 11 Alport syndrome patients in whom massively parallel sequencing identified pathogenic mutations in two of the three collagen IV genes examined. In seven patients (Probands 1-7) there was a combination of mutations in *COL4A3* and *COL4A4*, whereas in four (Probands 8-11) one or two mutations in *COL4A4* associated with a mutation in *COL4A5*. In no case were there simultaneous *COL4A3* and *COL4A5* mutations. Altogether, 23 unique mutations were found, including seven in *COL4A3*, 12 in *COL4A4* and four in *COL4A5* (**Table 1**). The mutations involved all domains of the collagen molecules, although the majority of missense mutations (11 of 13) affected the triple-helical collagenous domain and 11 missense mutations substituted a critical glycine residue in this domain. Overall, 13 mutations have been previously reported (among missense mutations six are already listed in dbSNP and four[16] are pending assignment of a dbSNP reference ID) and 10 are novel.

Between 1 and 12 family members were recruited for each proband, for an average of 4 members per family (**Table 2**). Therefore, the study considered a total of 56 persons (27 males and 28 females; gender missing for one person) from 5 to 80 years of age (exact age missing for 11 persons). Seven individuals were dead at the time of study. A wide range of kidney functionality was observed in the study population, ranging from normal (in 7 person), to micro- and macrohaematuria, proteinuria, CRF (in 6 persons) and ESRD (in 12 subjects leading to death in **4** cases). Hearing loss was recorded in 8 of 44 persons for whom hearing test results were available, and ocular lesions were noted in 2 of 6 persons for whom ophthalmological data were available.

Genomic DNA was available for 34 family members (all alive at the time of study). Sanger sequencing of this DNA revealed which family members had the same mutations as the probands (including which were heterozygous or hemizygous for only one mutation or compound heterozygous in only one gene), permitting us to explore the relationship between mutations and disease severity as well as to investigate the form of genetic transmission. Individuals with two mutations tended to be more severely affected than those with one mutation. The few cases of hearing loss and ocular lesions were observed only in persons with mutations in two genes, and, among the 5 persons with ESRD and sequencing data, the age at onset was lower in the 2 cases with two genes affected (25 and 44 years) than in those with one gene affected (Family 4).

Family 1 was identified first (**Figure 1**). In the proband (II:2) (**Figure 1A**), massively parallel sequencing (**Figure 1B**) revealed the presence of a *COL4A4* glycine substitution inherited from the ascertained asymptomatic father and a splice site mutation in the *COL4A3* gene inherited from the microhaematuric mother. The disruption of the terminal part of the triple helix in the alpha3 chain and the kink formed by the presence of the bigger glutamic acid instead of the flexible, small glycine can be assumed to prevent the correct formation of the triple helix, which assembles from the C-terminal tail (**Figure 1C**). Although the proband was very young (7 years), the presence of recurrent episodes of macrohaematuria may indicate a poor prognosis.

Digenic autosomal inheritance with linked mutation *in trans*

In five of the seven families with *COL4A3/COL4A4* mutation combinations, the two mutations were definitely linked *in trans* (Families 1–5; **Figures 1, 2**). In these families, individuals with two heterozygous mutations had more severe phenotypes than those with a single heterozygous mutation. Family members having only one mutation in a collagen gene were asymptomatic (father in Family 1) or had haematuria (mothers in Families 1, 2

and 4; father in Family 2; niece in Family 3) or intermittent hematuria (daughters in Family 3).

In the digenic model with mutations linked *in trans,* the mode of inheritance mimics a recessive model, in the sense that the probability of having another child with the same genotype is 25%. The main difference is that the classic recessive model is determined by the combination of two alleles mutated at the same locus, while the digenic model is attributable to two different alleles mutated at two different loci. However, individuals with digenic disease have an intermediate phenotype between autosomal dominant and autosomal recessive form.

Digenic autosomal inheritance with linked mutation *in cis*

In the other two families with *COL4A3/COL4A4* mutation combinations, the two mutations were linked *in cis* (**Figure 3**). In Families 6 and 7, the inheritance pattern mimics an autosomal dominant mode: the probability of having another child with the same genotype is 50%, but the phenotype is more severe than expected for the classic autosomal dominant form. In fact, subject I:2 of Family 6 and subjects II:1 and I:2 of Family 7 had CRF at an early age, with two of them progressing towards ESRD at 40 years of age, which is unexpected for the autosomal dominant form.[21]

Digenic unlinked autosomal/X-linked inheritance

In Families 8-11, there was a combination of a mutation in the autosomal *COL4A4* gene and in the X-linked gene *COL4A5* (**Figures 4, 5**). In these families, double heterozygotes also have a more severe phenotypes than expected in individuals with a *COL4A4* heterozygous mutation or in *COL4A5* carrier females.[6,21] In fact, the female I:1 in Family 8 had ESRD at the age of 44 years, earlier than expected had she had just one mutation in either *COL4A4* or *COL4A5*.

In Family 11, in addition to the mutation in *COL4A5*, two mutations in *COL4A4* were found, resembling a triallelic inheritance (**Figure 5**). The proband (II:4) presented with intermittent haematuria and proteinuria, and a first genetic testing by Sanger sequencing revealed an autosomal recessive form of Alport syndrome due to compound heterozygosity at the *COL4A4* gene. The first male sib (II:1) had both *COL4A4* mutations, while three other sibs (II:2; II:3 and II:5) with a similar degree of the disease were carriers, as was the last sib who had isolated haematuria. At clinical re-evaluation, the father was found to have ESRD, so the proband was re-tested using massively parallel sequencing techniques. An additional pathogenic mutation resulting in a Gly substitution at codon 684 in *COL4A5* was identified. Not surprisingly, all sisters were carriers. We suggest that the concomitant mutations in two different genes may be associated with a more severe clinical picture, even if in this family a follow-up is needed.

Discussion

The present study provides evidence that digenic inheritance can occur in Alport syndrome as well as classic Mendelian inheritance. Using massively parallel sequencing, we investigated 11 families with variable degrees of clinical severity among their members who also varied in the genotype of two (or three) loci of two collagen type IV genes. In the reported pedigrees, the “two-locus model” explains the variable expressivity of the disease within the same family better than simple Mendelian inheritance: the different genotypes at two loci, roughly equal in importance, can explain the differences in age at onset of renal failure and in the severity of the symptoms. This discovery has implications for genetic counselling especially for risk assessment of patients’ relatives, because an erroneous definition of the inheritance model may result in incomplete cascade of testing relatives with consequent erroneous risk estimations.

All missense mutations except two affected glycine residues in the collagenous domain. Glycine is a small amino acid essential for making the protein flexible and allowing the coiling of the triple helix, building the final shape of the collagenous domain. The other two missense mutations were found in the main non-collagenous domain (NC1) relevant for the protein's self-assembly and formation of the irregular polygonal network. One such mutation, c.4760C>G in *COL4A4,* affects an evolutionarily conserved codon.[22] A different missense mutation having the same effect on the protein's sequence, p.(Pro1587Arg), was recently reported in another Alport syndrome patient.[23] The last missense mutation, c.4994G>A (p.Cys1665Tyr) in *COL4A3,* eliminates a cysteine. Cysteine residues are key amino acids in the non-collagenous domains because their disulphide bridges are important for the globular structure. These observations strengthen the pathogenic classification of these changes.

In our cohort, we identified seven families with associated mutations in *COL4A3* and *COL4A4* genes and four families with associated mutations in *COL4A4* and *COL4A5. W*e did not find kindreds with digenic inheritance attributable to mutations in *COL4A3* and *COL4A5*. This is likely due to the small size of our cohort; however we cannot exclude a possible biological mechanism. Present knowledge of basement membranes is based on the 1:1:1 model. Each alpha chain (α3, α4 and α5) interacts equally with the other two, concurring to form a triple helix. Therefore there is no molecular explanation for missing a *COL4A3*/*COL4A5* combination. It is likewise unlikely that this combination gives rise to an unrecognisable phenotype.

In our cohort, double heterozygotes reach ESRD at the age of 40 years (subject I:2 of Family 6 and I:2 of Family 7) and 44 years (subject I:1 of Family 8). It is interesting to note that this is older than the mean age expected in the autosomal recessive form (31 years) but earlier than expected in the autosomal dominant form (56 years).[21,5] This fits

well with the stoichiometry of the molecules of the triple helix (**Figure 6**). In double heterozygotes about 75% of triple helix molecules are expected to be defective, which is more than 50% in heterozygotes and less than 100% in homozygotes or hemizygotes.

Pedigree 11 may represent an example of the triallelic form of digenic inheritance. This kind of inheritance has been previously proved in Bardet-Biedl syndrome and other diseases.[24] Triallelic inheritance is defined when any combination of three deleterious alleles at two loci, but not three heterozygous mutations at three loci, is sufficient to cause the disease. In the case of Family 11, subject II:4 has two mutated alleles at the *COL4A4* locus (M1, M2) in addition to one mutated allele at the *COL4A5* locus (M3). In this family an accurate follow-up of clinical progression may enhance our understanding of how the combination of different mutated alleles contributes to the developing phenotype. While the pathogenicity of the splice site mutation in *COL4A4* (M1) and of the Gly substitution in the collagen domain of *COL4A5* (M3) is certain, one could question the pathogenicity of the *COL4A4* Pro to Arg substitution in the NC1 domain (M2; rs190148408). Two of three prediction tools scored this variant as pathogenic, and its reported frequency in the general population is 0.3%. Segregation analysis of Family 11 is suggestive of a role of this allele in worsening the phenotype. Undoubtedly more data are necessary to exactly define the role of this mutation before we can conclude that the pedigree is an example of triallelic inheritance.

In summary, in this paper we provide evidence for a digenic inheritance model for Alport syndrome and illustrate three possible segregation models: i) autosomal inheritance with linked mutations *in trans* mimicking the recurrence risk of a recessive disease; ii) autosomal inheritance with linked mutations *in cis* mimicking the recurrence risk of a dominant disease; and iii) unlinked autosomal and X-linked inheritance having its own

distinctive segregation. While the first case (linked *in trans*) represents a novelty with purely scientific interest, the other two have important implications in genetic counseling.

In cases of digenic inheritance linked *in trans*, the prognosis of affected individuals and the risk of recurrence for the couple overlap with those of the recessive form (**Figure 7A**). For digenic inheritance with linked mutations *in cis*, the risk of recurrence is the same as that of the autosomal dominant disorder, but the prognosis is worse and intermediate between the autosomal dominant and autosomal recessive forms (**Figure 7B**). The clinician may need to discuss this with their patients. In digenic unlinked autosomal and X-linked inheritance, neither recurrence risk nor prognosis fit with any previously known Alport model and need to be determined on a case by case basis. **Figure 7C** illustrates the case of segregation through an affected male, hemizygous for an X chromosome mutation (*COL4A5* gene) and heterozygous for a mutation on chromosome 2 (mutation at either the *COL4A3* or *COL4A4* locus). If only one mutation is detected, for example that on chromosome X, the risk of recurrence of the disease is about zero. This risk may increase because of the second event reaching up to 50%. In a similar situation with mating with a heterozygote, triallelic segregation will appear in the offspring and half of them will have an even worse prognosis (**Figure 7D**). Therefore, the present results are of interest both from a scientific point of view and for genetic counselling. Clinical geneticists should be familiar with more complex models of inheritance, which could alter the prognosis and recurrence risk.

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Legends to Figures

Figure 1 Molecular and segregation analysis of Family 1

A) Pedigree: the mutated alleles are linked *in trans* and the mode of inheritance is digenic autosomal. *wt*, wild type allele. B)Screenshots from the GS Amplicon Variant Analyzer software showing the position of the *COL4A4* missense mutation c.1553G>A (p.Gly518Glu; M1) and the *COL4A3* splice site variant c.2746+1G>T (M2) and. The upper histograms indicate the percentage of variation. In the lower panels, reads from different directions are displayed and the mutated base is highlighted. C) Locations of these mutations on the a3-a4-a5 triple helix of collagen.

Figure 2 Pedigrees of three other families having digenic autosomal inheritance with mutations in *COL4A3* and *COL4A4* linked *in trans.* A) Family 2. B) Family 3. C) Family 4. D) Family 5. *CRF*, chronic renal failure; *SNHL*, sensorineural hearing loss; *ESRD*, end-stage renal disease.

Figure 3 Families with digenic autosomal inheritance with mutations in *COL4A3* and *COL4A4* linked *in cis.* A) Family 6. B) Family 7. *CRF*, chronic renal failure; *SNHL*, sensorineural hearing loss; *ESRD*, end-stage renal disease

Figure 4 Families with mutations in *COL4A4* and *COL4A5,* with digenic unlinked autosomal and X-linked inheritance. A) Family 8. B) Family 9. C) Family 10. *SNHL*, sensorineural hearing loss; *ESRD*, end-stage renal disease.

Figure 5 Pedigree of Family 11 with a triallelic form of digenic inheritance, with mutations in *COL4A4* and *COL4A5*. *ESRD*, end-stage renal disease.

Figure 6 Triple helix combinations of defective alpha chains.

A) In heterozygotes, about 50% of triple helix molecules are expected to be defective. B) In homozygotes or hemizygotes, 100% of triple helix molecules are expected to be defective. C) In double heterozygotes, about 75% of triple helix molecules are expected to be defective.

Figure 7 Three possible segregation models of digenic inheritance in Alport syndrome.

A) Digenic autosomal inheritance with linked mutations *in trans* mimicking a recessive inheritance disease. B) Digenic autosomal inheritance with linked mutations *in trans* mimicking a dominant inheritance. C) Unlinked autosomal and X-linked inheritance having its own particular segregation. D) Trialleic inheritance as observed in Family 11.

Table 1. Molecular features and predicted pathogenicity of 23 mutations in collagen IV genes, found in 11 patients with Alport syndrome

DNA variant	Type	Exon (intron) number	dbSNP reference ID	Predicted effect on the protein	Collagen domain affected (for missense mutation)	Pathogenicity*		
						SIFT (score)[a]	Mutation taster (p value)[b]	PolyPhen2 (score)[c]
COL4A3								
del ex 1	Deletion	1	-	Whole gene deletion	NA	NA	NA	NA
c.898G>A	Missense	16	-	p.Gly300Arg	Collagenous	Deleterious (0)	Disease-causing (1)	Probably damaging (1)
c.1504+1G>A	Misplicing Lost 5′ splice site	(23)	-[16]	p.?	NA	NA	NA	NA
c.1558G>C	Missense	24	Pending[16]	p.Gly520Arg	Collagenous	Deleterious (0)	Disease-causing (1)	Probably damaging (1)
c.2065G>A	Missense	28	Pending[16]	p.Gly689Arg	Collagenous	Deleterious (0)	Disease-causing (1)	Probably damaging (1)
c.2746+1G>T	Misplicing Lost 5′ splice site	(33)	-	p.?	NA	NA	NA	NA
c.4994G>A	Missense	52	rs376550779	p.Cys1665Tyr	NC1	Deleterious (0)	Disease-causing (1)	Probably damaging (1)
COL4A4								
c.[1-?_192+?del]	Deletion	1-4	-[16]	Whole gene deletion	NA	NA	NA	NA
c.1293_1310del	In-frame deletion	20	-	p.Lys434_Gly439del	Collagenous	NA	NA	NA
c.1459+1G>A	Misplicing Lost 5′ splice site	(21)	-	p.?	NA	NA	NA	NA
c.1553G>A	Missense	22	-	p.Gly518Glu	Collagenous	Deleterious (0)	Disease-causing (1)	Probably damaging (1)
c.1623+5G>T	Misplicing Lost 5′ splice site	(22)	-	p.?	NA	NA	NA	NA
c.2075G>T	Missense	27	Pending[16]	p.Gly692Val	Collagenous	Deleterious (0)	Disease-causing (1)	Probably damaging (1)
c.2164G>A	Missense	27	-	p.Gly722Ser	Collagenous	Deleterious (0)	Disease-causing (1)	Probably damaging (1)
c.2906C>G[22]	Nonsense	32	rs35138315	p.Ser969X	Collagenous	NA	NA	NA
c.3452G>C	Missense	37	rs371803356	p.Gly1151Ala	Collagenous	Deleterious (0)	Disease-causing (1)	Probably damaging (1)
c.3817+1G>T	Misplicing Lost 5′ splice site	(40)	-	p.?	NA	NA	NA	NA

c.4698delT	Frame shift	47	-[16]	p.Cys1566Trpfs*37	NC1	NA	NA	NA
c.4760C>G	Missense	47	rs190148408[d]	p.(Pro1587Arg)	NC1	Deleterious (0.01)	Polymorphism (1)	Probably damaging (0.913)
COL4A5								
c.1931G>A	Missense	25	Pending[16]	p.Gly644Asp	Collagenous	Deleterious (0)	Disease-causing (1)	Probably damaging (1)
c.2051G>T	Missense	27	rs104886160[e]	p.Gly684Val	Collagenous	Deleterious (0)	Disease-causing (1)	Probably damaging (1)
c.2858G>T	Missense	33	rs78972735[e, f]	p.Gly953Val	Collagenous	Deleterious (0)	Disease-causing (1)	Probably damaging (1)
c.4042G>A	Missense	46	-	p.Gly1348Arg	Collagenous	Deleterious (0)	Disease-causing (1)	Probably damaging (1)

*Pathogenicity predicted using Alamut software v.2.3 (Interactive Biosoftware, Rouen, France). [a] Substitutions with normalized probabilities <0.05 are predicted to be deleterious, those ≥0.05 are predicted to be tolerated. [b] P value indicates the security of the prediction as either 'disease-causing' or 'polymorphism', with 1 being most secure. [c] Benign, possibly damaging, and probably damaging correspond to posterior probability intervals [0, 0.2], (0.2, 0.85), and [0.85, 1], respectively. [d] Minor allele (C) frequency/count=0.003/6; [e] dbSNP clinical significance, pathogenic; [f] Minor allele (T) frequency/count =0.003/5.

NA, not applicable.

Table 2. Clinical characteristics and collagen gene mutations in 11 patients with Alport syndrome and their family members

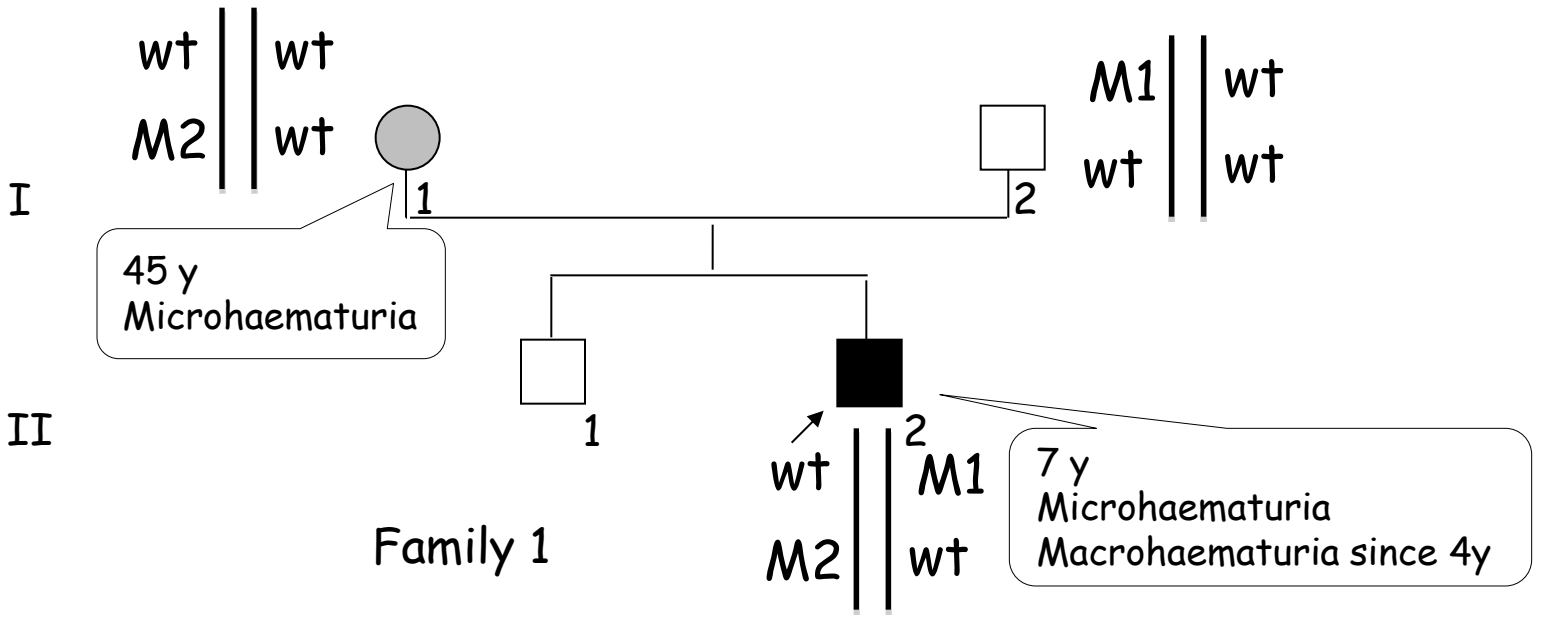
Family number	Family member (pedigree position)	Sex	Age, years[a]	Kidney disease (age, years[b])	Hearing loss (age, years[c])	Ocular lesions	Mutation (nucleotide change; effect on protein)		
							COL4A3	COL4A4	COL4A5
1	Proband (II:2)	M	7	Macrohaematuria (4)	No	No	c.2746+1G>T; p.?	c.1553G>A; p.Gly518Glu	None
1	Mother (I:1)	F	45	Microhaematuria	NA	No	c.2746+1G>T; p.?	Variant not present	NT
1	Father (I:2)	M	43	Ureteropelvic junction obstruction	NA	NA	Variant not present	c.1553G>A; p.Gly518Glu	NT
2	Proband (II:1)	F	36	Microhaematuria	No	No	c.898G>A; p.Gly300Arg	c.3452G>C; p.Gly1151Ala	None
2	Mother (I:1)	F	NA	Microhaematuria	NA	NA	c.898G>A; p.Gly300Arg	Variant not present	NT
2	Maternal grandfather	M	NA	ESRD (80)	NA	NA	NA	NA	NA
2	Father (I:2)	M	NA	Microhaematuria	NA	NA	Variant not present	c.3452G>C; p.Gly1151Ala	NT
3	Proband (I:2)	M	55	Proteinuria, CRF	Yes (39)	NA	c.1558G>C; p.Gly520Arg	c.4698delT; p.Cys1566Trpfs*37	None
3	Mother	F	Dead, 88, cancer	None	No	NA	NA	NA	NT
3	Maternal uncle	M	Dead, 45, ESRD	ESRD (<45)	NA	NA	NA	NA	NT
3	Sister (I:1)	F	60	Preemptive renal transplantation (50)	Yes	NA	c.1558G>C; p.Gly520Arg	c.4698delT; p.Cys1566Trpfs*37	NT
3	Father	M	Dead, cancer	None	No	NA	NA	NA	NA
3	Daughter (II:2)	F	23	Intermittent haematuria	No	NA	Variant not present	c.4698delT; p.Cys1566Trpfs*37	NT

3	Daughter (II:3)	F	20	Intermittent haematuria	No	NA	c.1558G>C; p.Gly520Arg	Variant not present	NT
3	Niece (II:1)	F	28	Haematuria	NA	NA	c.1558G>C; p.Gly520Arg	Variant not present	NT
4	Proband (III:1)	F	36	Haematuria, proteinuria	No	NA	c.1504+1G>A; p.?	c.1293_1310del; p.Lys434_Gly439del	None
4	Mother (II:1)	F	64	Haematuria	No	NA	c.1504+1G>A; p.?	Variant not present	NT
4	Father (II:2)	M	80	ESRD (68)	No	NA	Variant not present	c.1293_1310del; p.Lys434_Gly439del	NT
4	Paternal aunt (II:3)	F	76	ESRD (64)	No	NA	Variant not present	c.1293_1310del; p.Lys434_Gly439del	NT
4	Paternal granduncle (I:3)	M	Dead, ~70	Haematuria	No	NA	NA	NA	NA
4	Paternally related	M	48	Haematuria	No	NA	Variant not present	c.1293_1310del; p.Lys434_Gly439del	NT
4	Paternal grandaunt (I:4)	F	Dead	Haematuria, proteinuria, ESRD	No	NA	NA	NA	NA
4	Paternally related	M	47	Haematuria, proteinuria, CRF (47)	No	NA	Variant not present	c.1293_1310del; p.Lys434_Gly439del	NT
4	Paternally related	F	80	Haematuria, proteinuria, CRF (70)	No	NA	Variant not present	c.1293_1310del; p.Lys434_Gly439del	NT
4	Paternally related	M	69	CRF (69)	No	NA	Variant not present	c.1293_1310del; p.Lys434_Gly439del	NT
4	Paternally related	F	NA	CRF (80)	No	NA	Variant not present	c.1293_1310del; p.Lys434_Gly439del	NT
4	Paternally related	F	51	ESRD (50)	No	NA	Variant not present	c.1293_1310del; p.Lys434_Gly439del	NT
4	Paternally related	NA	NA	ESRD (60)	No	NA	NA	NA	NA

5	Proband (II:1)	F	3	Macrohaematuria	No	No	c.2065G>A; p.Gly689Arg	c.1459+1G>A; p.?	None
5	Mother (I:1)	F	NA	Haematuria	No	NA	c.2065G>A; p.Gly689Arg	Variant not present	NT
5	Father (I:2)	M	NA	None	No	NA	Variant not present	c.1459+1G>A; p.?	NT
6	Proband (II:1)	F	37	Intermittent haematuria	NA	NA	c.4994G>A; p.Cys1665Tyr	c.2906C>G; p.Ser969X	None
6	Mother (I:1)	F	NA	None	NA	NA	Variant not present	Variant not present	NT
6	Father (I:2)	M	Dead	CRF (21); ESRD (40)	Yes	NA	NA	NA	NA
7	Proband (II:1)	M	45	Haematuria, proteinuria, CRF	Yes (32)[d]	NA	del exon 1 (same allele)	c.[1-?_192+?del];[=](del ex1-4)	None
7	Son (III:1)	M	19	None	No	NA	Variant not present	Variant not present	None
7	Father (I:2)	M	Dead, 40, ESRD	ESRD (<40)	NA	NA	NA	NA	NA
7	Paternal uncle (I:1)	M	Dead, 61, ESRD	ESRD (<61)	NA	NA	NA	NA	NA
8	Proband (I:1)	F	54	ESRD (44)	Yes	Yes	None	c.3817+1G>T; p.?	c.2858G>T; p.Gly953Val
8	Son (II:1)	M	17	Haematuria	NA	NA	NT	c.3817+1G>T; p.?	Variant not present
9	Proband (II:1)	F	45	Haematuria, proteinuria	Yes (34)	NA	None	c.2075G>T; p.Gly692Val	c.1931G>A; p.Gly644Asp
9	Mother (I:1)	F	69	Haematuria, proteinuria during pregnancies	No[e]	NA	NT	Variant not present	c.1931G>A; p.Gly644Asp
9	Son	M	11	Microalbuminuria (91 mg/l)	No	NA	NT	c.2075G>T; p.Gly692Val	Variant not present
9	Son	M	9	None	No	NA	NT	Variant not present	Variant not present

9	Paternal nephew	M	NA	Possible renal disease	Yes	NA	NA	NA	NA
10	Proband (II:1)	M	26	Haematuria, proteinuria	No	No	None	c.2164G>A; p.Gly722Ser	c.4042G>A; p.Gly1348Arg
10	Father (I:2)	M	65	NA	No	NA	NT	c.2164G>A; p.Gly722Ser	Variant not present
10	Mother (I:1)	F	55	NA	No	NA	NT	Variant not present	Variant not present
11	Proband (II:4)	F	13	Intermittent haematuria, proteinuria	No	NA	None	c.1623+5G>T; p.? c.4760C>G; p.(Pro1587Arg)	c.2051G>T; p.Gly684Val
11	Mother (I:1)	F	32	Haematuria, proteinuria	No	NA	NT	c.1623+5G>T; p.?	Variant not present
11	Father (I:2)	M	33	ESRD (25)	Yes	Yes	NT	c.4760C>G; p.(Pro1587Arg)	c.2051G>T; p.Gly684Val
11	Brother 1 (II:1)	M	17	Haematuria, proteinuria	No	NA	NT	c.1623+5G>T; p.? c.4760C>G; p.(Pro1587Arg)	Variant not present
11	Brother 2 (II:2)	M	17	Haematuria, proteinuria	No	NA	NT	c.4760C>G; p.(Pro1587Arg)	Variant not present
11	Sister 1 (II:3)	F	14	Haematuria, microalbuminuria	No	NA	NT	c.1623+5G>T; p. ?	c.2051G>T; p.Gly684Val
11	Sister 2 (II:5)	F	9	Haematuria, proteinuria	No	NA	NT	c.1623+5G>T; p.?	c.2051G>T; p.Gly684Val
11	Sister 3 (II:6)	F	5	Haematuria	No	NA	NT	c.1623+5G>T; p.?	c.2051G>T; p.Gly684Val

[a] Cause of death indicated when available; [b] Age at diagnosis or intervention; [c] Age at diagnosis; [d] -40 dB; [e] Tested at 65 years
NA, data missing or DNA not available for analysis; *NT*, gene not tested in relatives because not mutated in proband; *CRF*, chronic renal failure; *ESRD*, end-stage renal disease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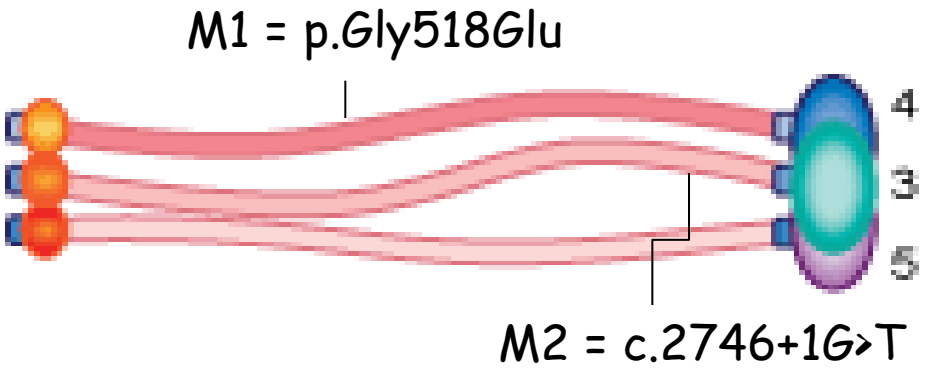
wt
M2
wt
I
1
2
M1
wt
wt
wt
45 y
Microhaematuria
II
1
2
wt
M1
M2
wt
7 y
Microhaematuria
Macrohaematuria since 4y
Family 1

a


M1 = p.Gly518Glu
4
3
5
M2 = c.2746+1G>T

b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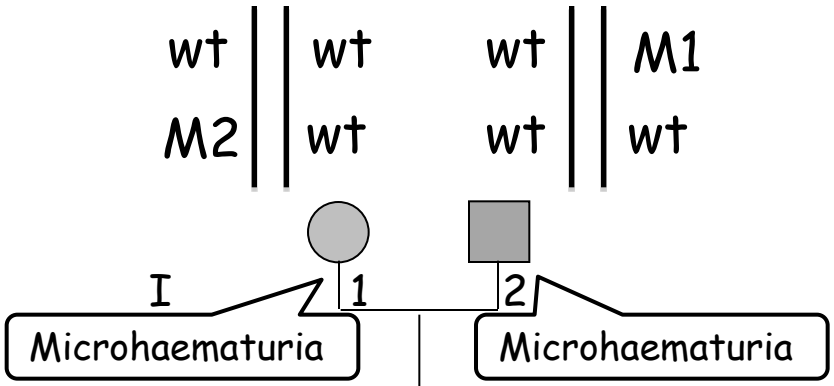
Number of Reads
Reference Sequence Position
Variation %
Number of Reads
Reference Sequence Position

c



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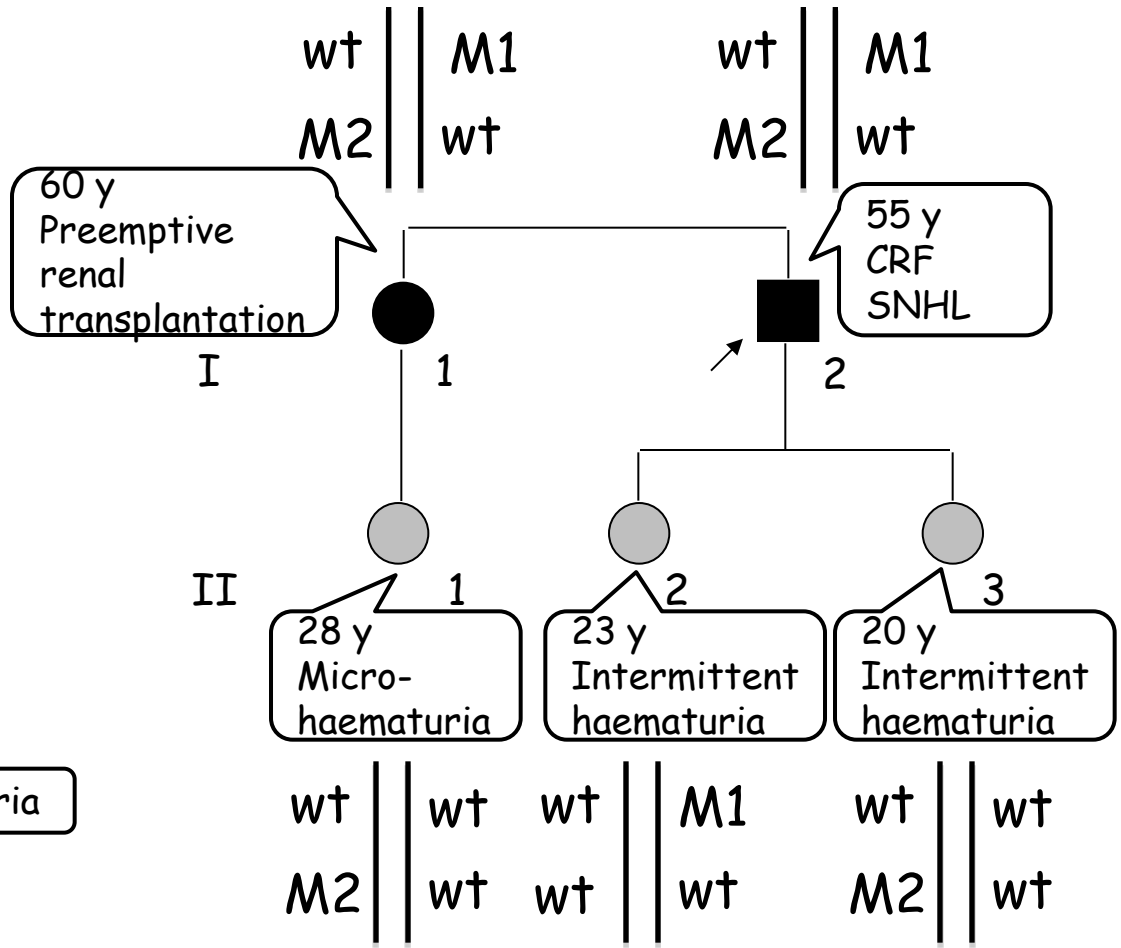
M1 = *COL4A4* p.Gly1151Ala

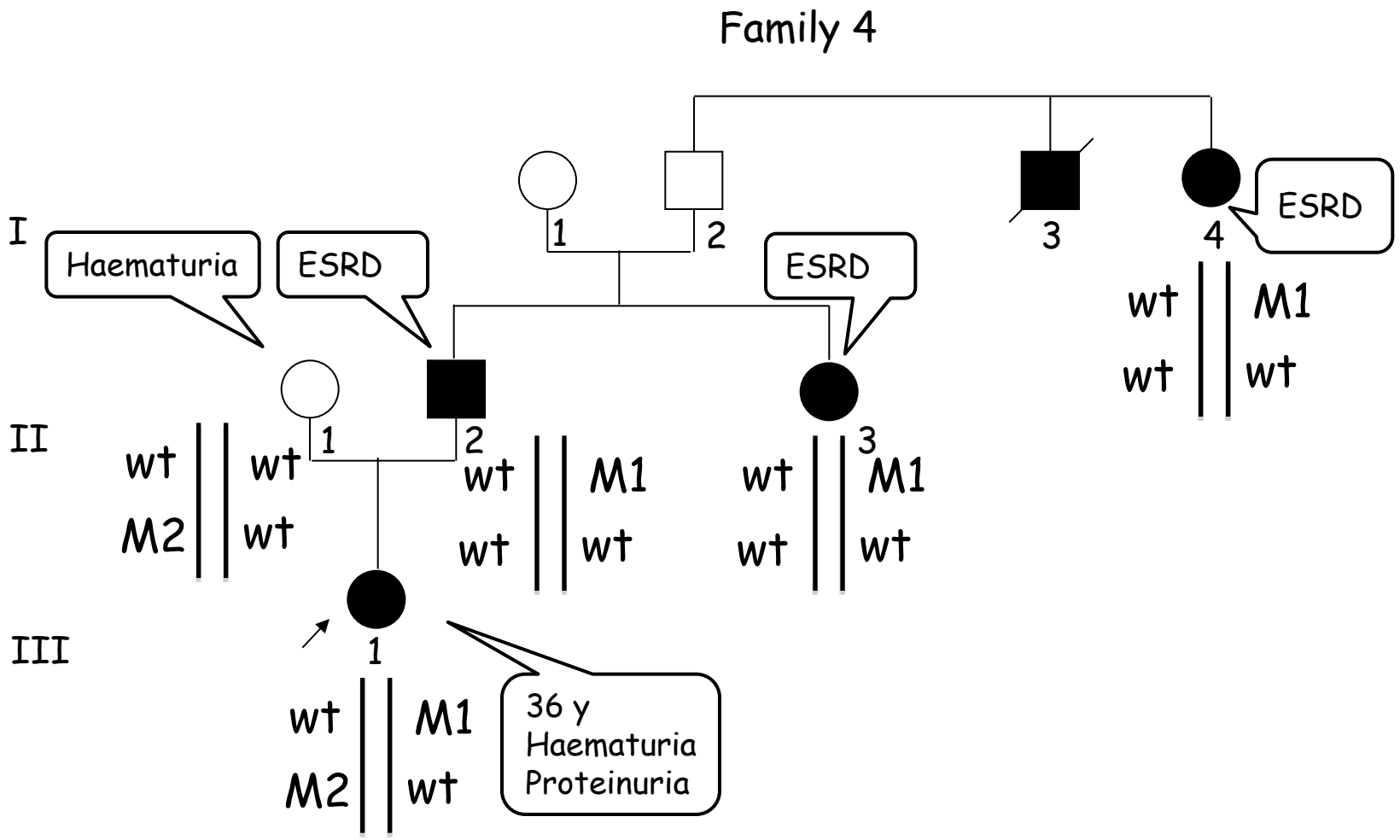
M2 = *COL4A3* p.Gly300Arg



M1 = *COL4A4* p.Cys1566Trpfs*37

M2 = *COL4A3* p.Gly520Arg

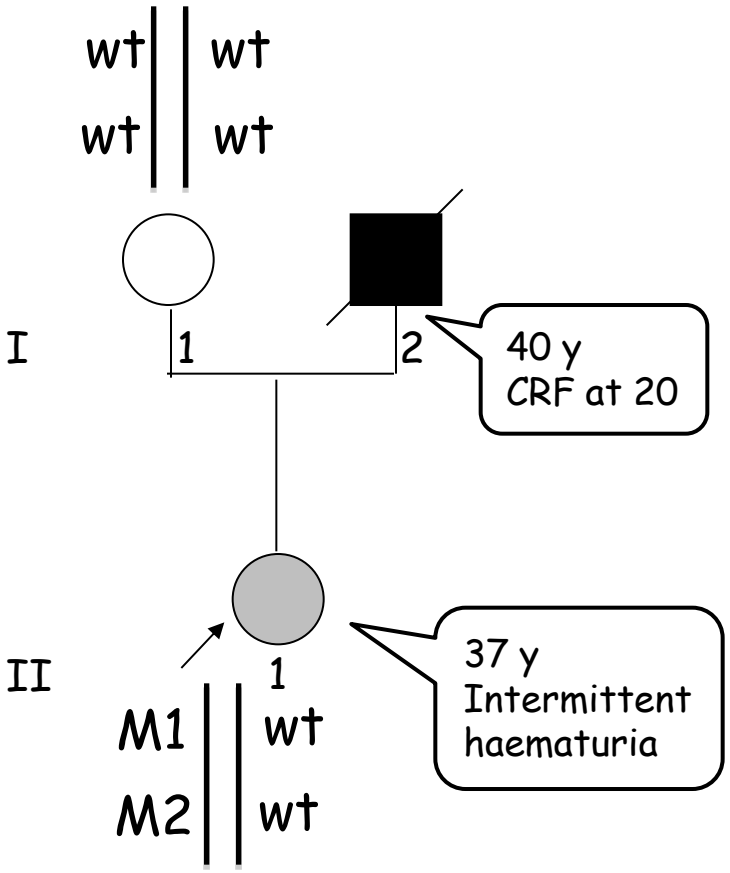
Family 3



M1 = *COL4A4* p.Lys434_Gly439del

M2 = *COL4A3* 1504+1G>A

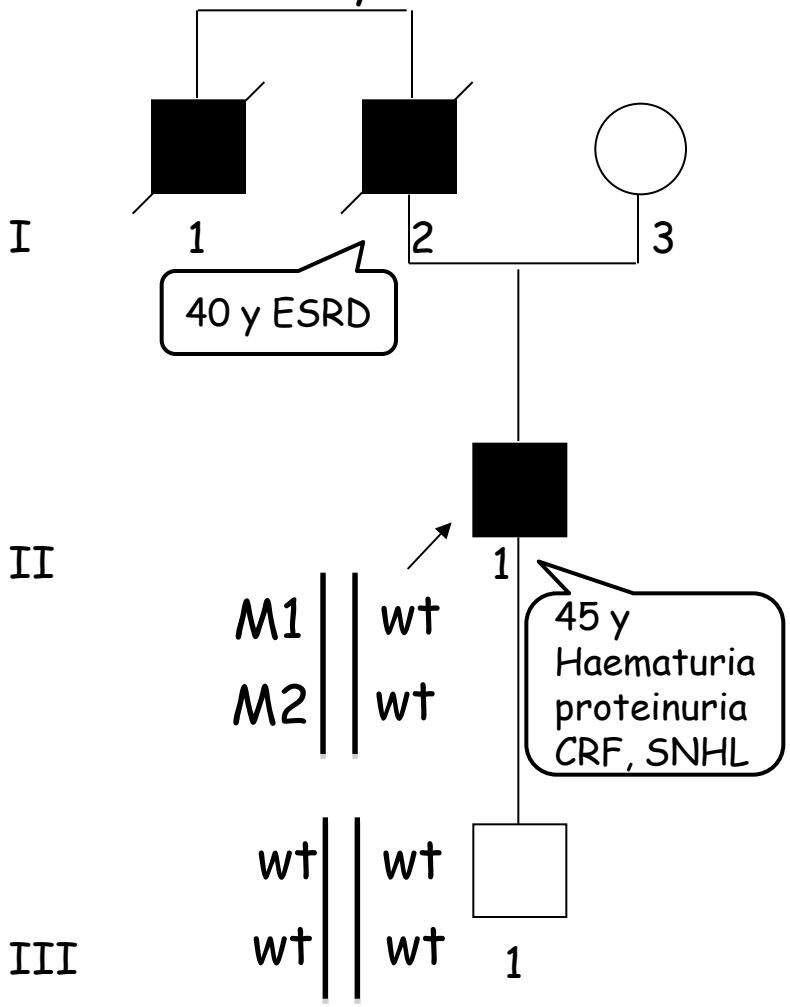
Family 5



M1 = COL4A4 p.Ser969X

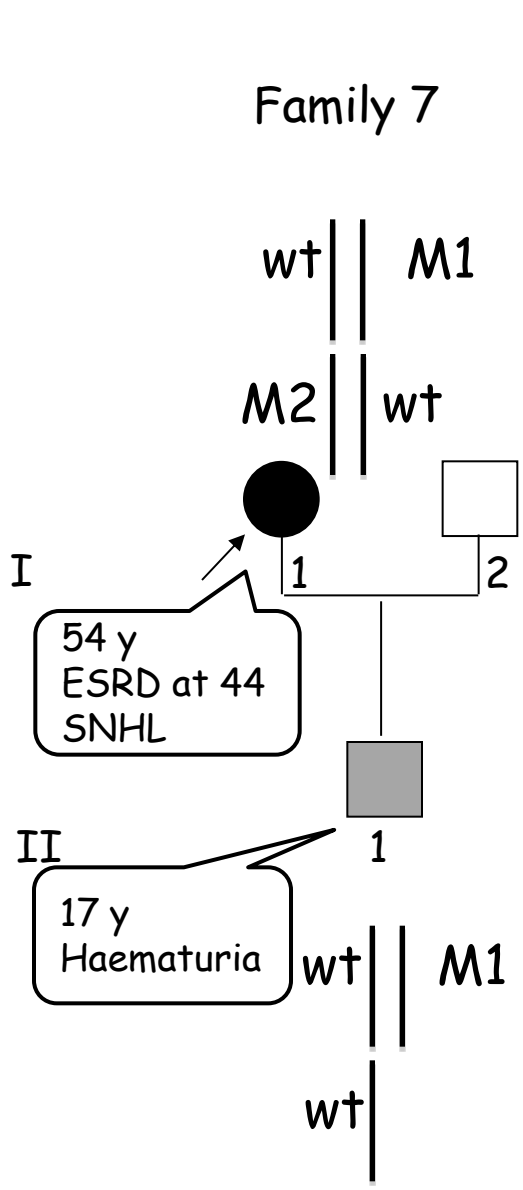
M2 = COL4A3 p.Cys1665Tyr

Family 6



M1 = COL4A4 del ex1-4

M2 = COL4A3 del ex1



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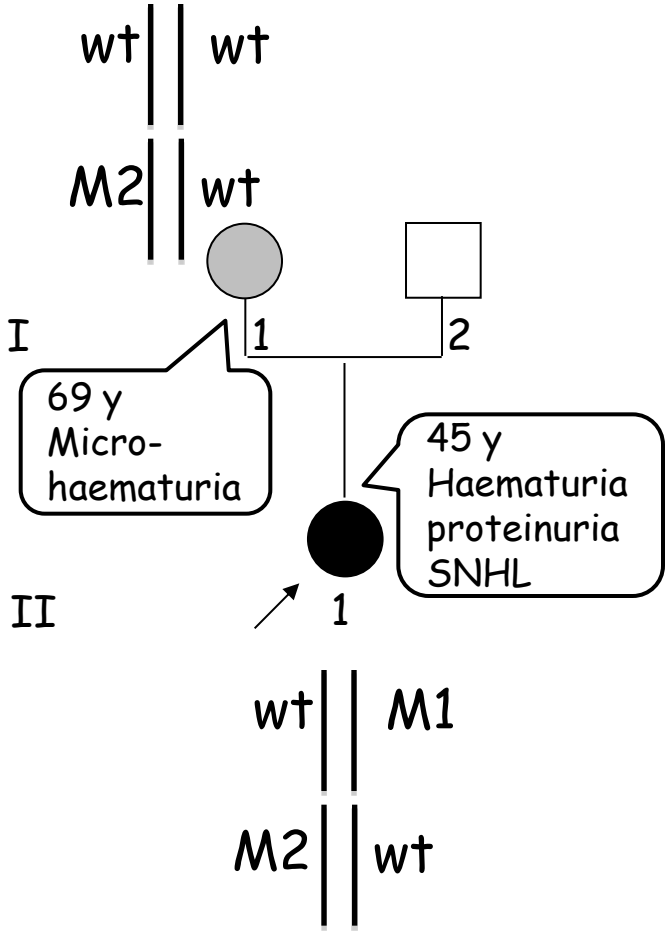
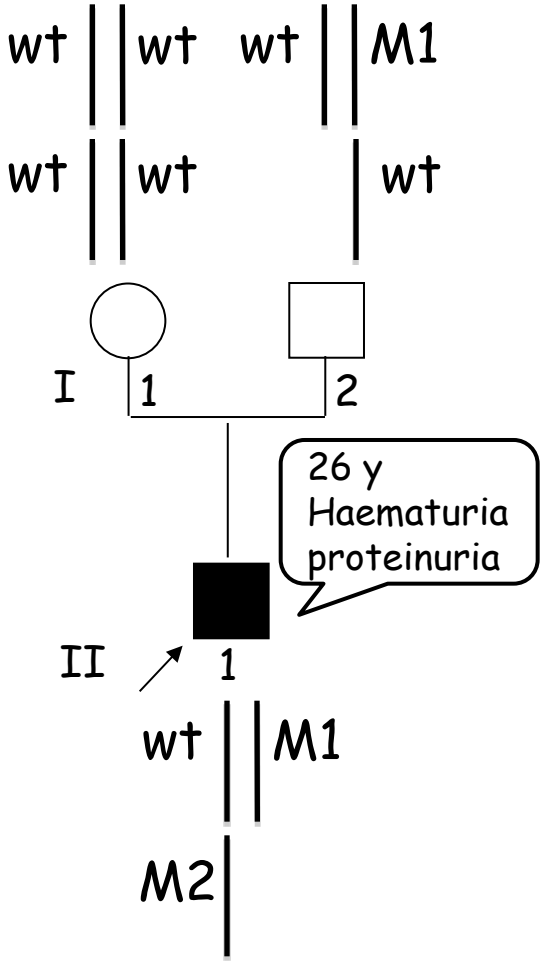
M2 = COL4A5 p.Gly953Val



M1 = COL4A4 Gly692Val

M2 = COL4A5 Gly644Asp

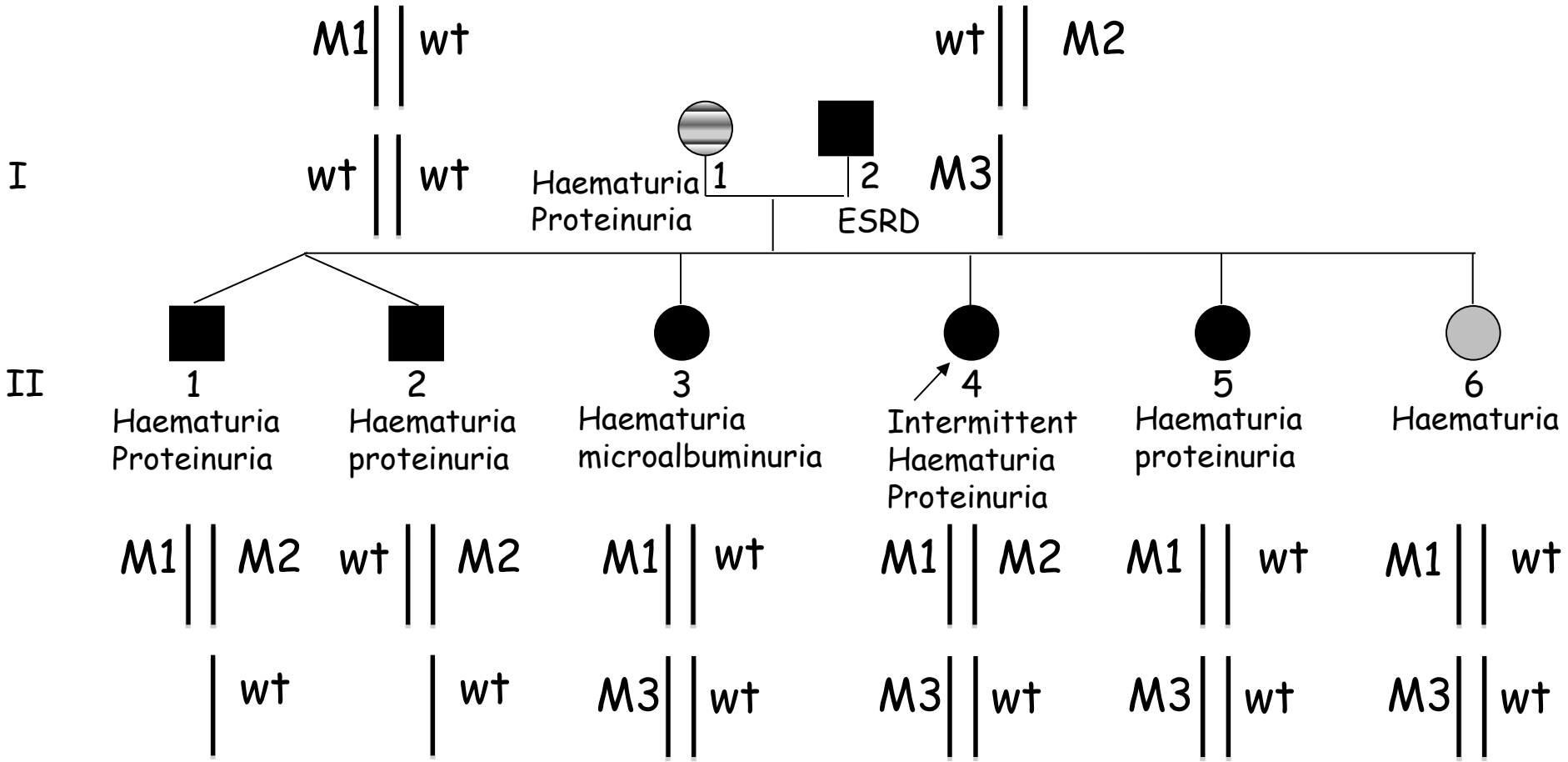
Family 8



M1 = COL4A4 p.Gly722Ser

M2 = COL4A5 p.Gly1348Arg

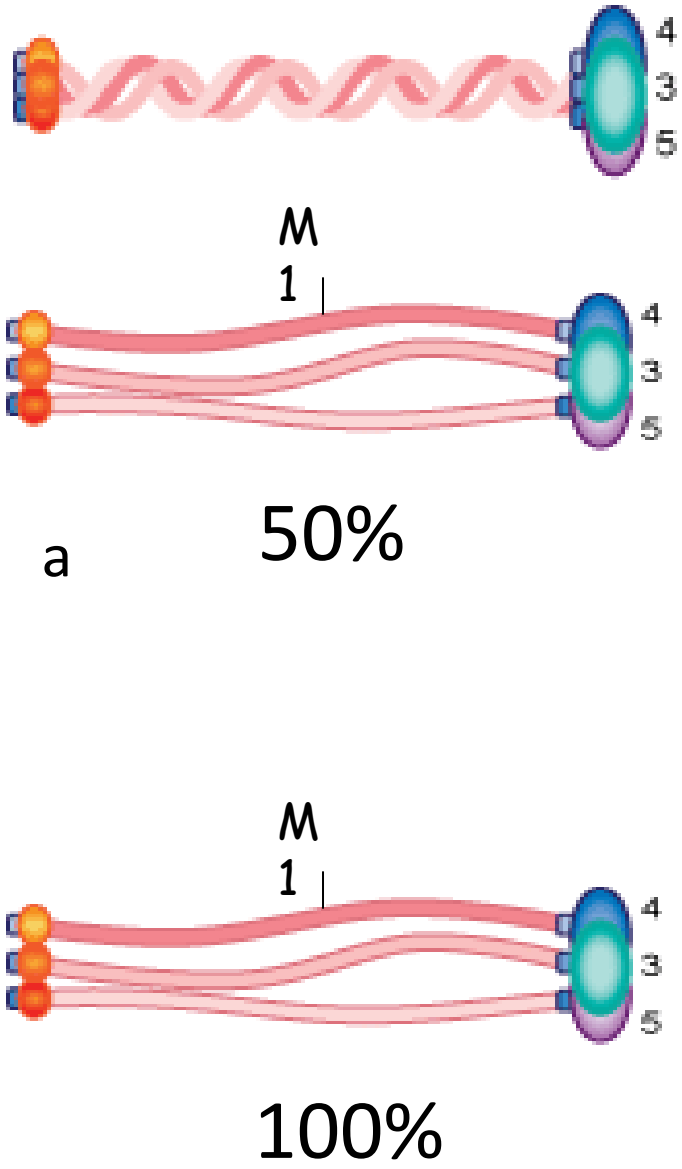
Family 10



M1 = COL4A4 c.1623+5G>T

M2 = COL4A4 p.Pro1587Arg

M3 = COL4A5 p.Gly684Val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100%
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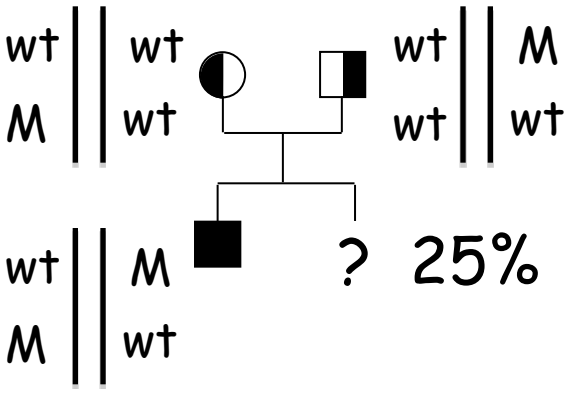
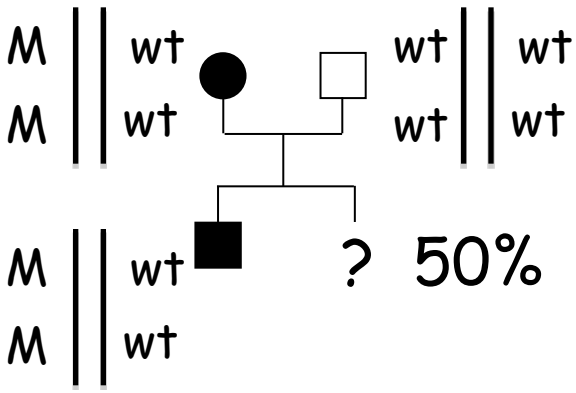
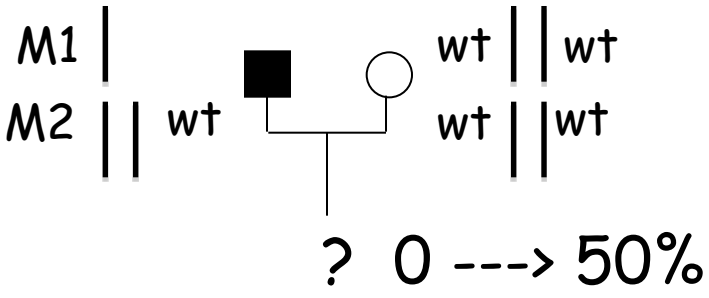


a

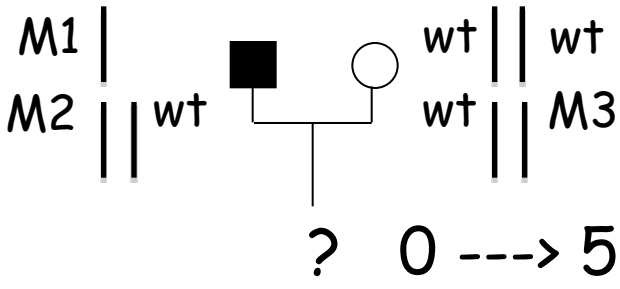


b



M1 = Ch X
M2 = Ch 2

c



M1 = Ch X
M2, M3 = Ch 2

half of them with very severe disease

d