Clinical and Genetic Studies of Hearing Impairment

CARINA FRYKHOLM
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Abstract

Monogenic disorders offer a possibility for studies of genetic disturbances in hearing impairment—a knowledge which could be essential for development of future treatment options. In this thesis, the underlying genetic disturbances in neurofibromatosis 2 (NF2) and familial Meniere’s disease (FMD) were evaluated, and familial X-linked hearing impairment was described from a clinical point of view.

In paper I, constitutional DNA from 116 individuals with NF2 of variable severity was studied using the array-CGH method focusing on a 7.6-Mb area surrounding the NF2 gene on chromosome 22q. Deletions were found in 20.7% of samples. In mild NF2, the deletions were small, but variable sizes of deletions were found in cases that were moderately or severely affected. Disease phenotype could not be predicted from the size of the deletions.

In papers II and III, a single five-generation family with autosomal dominant FMD was described. Anticipation concerning age of onset was observed. Genome scan revealed five candidate gene regions with a LOD score of > 1. Two additional families with autosomal dominant MD were analyzed for linkage to these five regions. A cumulative Zmax of 3.46 was obtained for a single 463-kb region on chromosome 12p12.3, containing only one known gene: PIK3C2G. This encodes a protein with a proposed role in hair cell regeneration in mammalian ears. No mutations were found in protein-coding sequences or exon-intron borders. In two of the three families, a shared haplotype, suggested common ancestry, was found to extend over 1.7 Mb, which could be a genomic region of importance for FMD.

In paper IV, a family in which five males displayed progressive low- and mid-frequency hearing impairment from the first or second decade was described. Female carriers were affected by a high-frequency hearing impairment from the fourth decade. The family could represent a novel X-linked dominant audiophenotype.

Keywords: NF2, array-CGH, Meniere’s disease, PIK3C2G, X-linked, progressive, hearing impairment

Carina Frykholm, Department of Surgical Sciences, Akademiska sjukhuset, Uppsala University, SE-75185 Uppsala, Sweden

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To Peter, Moa and Selma
This thesis is based on the following papers, which are referred to in the text by their Roman numerals:


IV **Carina Frykholm**, Sara Ekvall, Hans-Christian Larsen, Marie-Louise Bondeson, Ulla Friberg. A clinical description of a novel X-linked dominant hearing impairment in a five-generation family. *In manuscript.*
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Abbreviations and glossary

ABR      Auditory brainstem response
AN/AD    Auditory neuropathy/dyssynchrony
AUN-     Prefix for hearing impairment with test profile of auditory neuropathy/dyssynchrony
BAC      Bacterial artificial chromosome
BVS      Bilateral vestibular schwannomas
CGH      Comparative genomic hybridisation
cM       Centimorgan
CT       Computed tomography
DFN      X-linked inherited hearing impairment (recessive/dominant)
dB       Decibel
ddNTP    Dideoxy nucleotide triphosphate
DFNA     Prefix for hearing impairment with autosomal dominant inheritance
DFNB     Prefix for hearing impairment with autosomal recessive inheritance
DPOAE    Distortion product otoacoustic emissions
DNA      Deoxyribonucleic acid
EVA      Enlarged vestibular aqueduct
Exon     The expressed sequence of a gene
FISH     Fluorescent in situ hybridisation
FMD      Familial Meniere’s disease
Intron   Noncoding DNA that separate neighbouring exons
kb       1000 base pairs
LD       Linkage disequilibrium
LOD      Logarithm of odds
LOH      Loss of heterozygosity (loss of second allele)
Mb       Million base pairs
MD       Meniere’s disease
NF2       Neurofibromatosis 2
OAE      Otoacoustic emissions
OMIM   On Mendelian Inheritance of Man (online database); see references
PAC      Bacteriophage P1 artificial chromosome
PCR      Polymerase chain reaction, a method of amplifying DNA sequences
PTA      Pure tone average (average of 0.5, 1, 2 and 3 (or 4) kHz)
RNA      Ribonucleic acid
SNP      Single nucleotide polymorphism
TEOAE    Transient evoked otoacoustic emissions
UVS      Unilateral vestibular schwannoma
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>VOR</td>
<td>Vestibulo-ocular reflex</td>
</tr>
<tr>
<td>VS</td>
<td>Vestibular schwannoma</td>
</tr>
</tbody>
</table>
Genetic hearing impairment

Permanent hearing impairment (pure tone average (PTA) > 25 dB at 0.5–4 kHz in the better ear) affects 0.21% of newborn children (1). In the adult population, the prevalence is approximately 15% (2), but increases to 50% in people over 80 years of age (3).

The exogenous causes of early childhood hearing loss have decreased in the developed countries during the last decades, mainly due to immunisation programs and progress in neonatal care (4). For several years, the aetiology was unknown in 30% of children with hearing impairment. Recent advances in audiological, microbiological, molecular and imaging techniques may improve these figures. However, studies in which all the different diagnostic possibilities are combined are still lacking.

In a review of aetiological factors in childhood hearing impairment, genetic factors were estimated to account for more than 60% of cases (5) (Fig. 1).

Figure 1. The contribution of different aetiologies in hearing impairment at birth and at the age of 4, adopted from Morton CC. and Nance WE., 2006 (5).
In hearing impairment of adults, the proportion of underlying genetic factors is difficult to estimate (6). The most common diagnoses in adults are caused by a combination of genetic and environmental factors.

The high proportion of cases of hearing impairment with genetic causes implies that knowledge from this research field should be incorporated into audiological practice. People with hearing loss will require information about aetiology, prognosis, possible hereditary factors, and eventually individualised therapy based on findings in molecular genetics.

Hereditary hearing loss is usually classified either according to the mode of inheritance (autosomal dominant or recessive, X-linked or mitochondrial) or based on whether the hearing impairment is isolated (nonsyndromic) or is associated with other disorders (syndromic) (Fig. 2).

![Figure 2. Overview of aetiologies and naming of syndromic/nonsyndromic hearing impairment. DFNA (autosomal dominant), DFNB (autosomal recessive), DFN (X-linked) all followed by an accession number, and mitochondrial (mt) followed by mutation type and position.]

The risk of two individuals with hearing impairment having affected offspring is only 8%. Due to this, the heterogeneity of genetic hearing impairment has long been suspected. In the last decade, there has been remarkable progress in mapping of loci and identification of genes involved in hearing impairment. By July 2006, over 100 loci had been mapped and more than 40 genes causing nonsyndromic hearing loss had been identified (Fig. 3) (Hereditary hearing loss homepage).
Apart from the nonsyndromic hearing loss genes, at least 110 other genes are known to be associated with syndromic hearing loss (7). Over 400 clinically defined syndromes, where hearing impairment is included, are registered in the OMIM database. The high number of genes that are important for adequate hearing mirrors the complicated organisation of the inner ear.

The genes that have been identified belong to specific categories of genes. These encode transcription factors, extracellular matrix proteins, unconventional myosins and ion/transporter proteins, and some are involved in embryonic development (8). The genes known to be involved in hearing impairment and the locations of their expression in the inner ear are shown in Fig. 4 and 5.

**Figure 4.** Overview of the genes found in hearing impairment and their expression in the inner ear, the cochlear nerve and the spiral ganglion. Adopted from (5).
The identification of the genes involved in hearing impairment has revolutionized our understanding of inner ear physiology and pathophysiology.

Genetic studies of hearing impairment have shown that: 1) the same gene can cause either isolated hearing loss or a syndrome, 2) some syndromes can be caused by several genes, and 3) the classification into syndromic and nonsyndromic hearing impairment is not clear-cut; rather, there is rather a phenotypic continuum depending on the functionality of the protein involved.

Knowledge of how audiology and genetics are linked is beginning to emerge. There have been some important studies correlating pure tone audiograms and inner ear histology with genetic abnormalities. These involve only a few conditions. Traditionally, hearing impairment has been classified as conductive or sensorineural, and the latter as cochlear or retrocochlear. Functionally, sensorineural hearing impairment could be classified into cochlear afferent and efferent disorders, and even further subclassification would be feasible. The ways in which additional audiological test methods might be used in considerations of genetic aetiology warrant further investigations.

The contribution of genetic factors in hearing loss varies. In the monogenic disorders, the contribution of the genetic factor is high, but these conditions are uncommon. This thesis is based on studies of two uncommon familial disorders, neurofibromatosis 2 and familial Meniere’s disease, which are related to the relatively common sporadic vestibular schwannoma and sporadic Meniere’s disease, respectively. The work also includes a study of a family with an uncommon X-linked postlingual progressive hearing impairment affecting the low- and mid-frequencies.

Figure 5. Genes expressed on the apical surface of the outer hair cells. Adapted from Morton CC, and Nance WA. (5).
Neurofibromatosis 2

Neurofibromatosis 2 (NF2) is an autosomal dominant disorder in which the hallmark is bilateral vestibular schwannomas (BVS). Schwannomas are benign tumours that arise from specialised glial cells, the Schwann cells, which surround the axons and provide electrical insulation and protection. The tumours emanate from the vestibular branch of the eight cranial nerve and have secondary effects on the acoustic branch. The birth incidence of NF2 is estimated to be 1 in 25,000–33,000 (11).

Diagnostic criteria

The first diagnostic criteria for NF2 were published in 1987 by the National Institutes of Health (12), and were aimed at high specificity, since the causative gene(s) had not yet been identified. In clinical practice, the first criteria were too narrow and individuals with the first manifestations of NF2 were missed, which is why several other criteria have been suggested (13–15). In 2002, Baser (16) formally assessed the different criteria that prevailed in the literature. The criteria suggested by the Manchester group were found to have the highest sensitivity, and these are presented below (Table 1).

Table 1. The criteria for neurofibromatosis 2 suggested by the Manchester Group (15)

<table>
<thead>
<tr>
<th>The Manchester criteria for NF2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Bilateral vestibular schwannomas.</td>
</tr>
<tr>
<td>B. First-degree relative with NF2 and unilateral vestibular schwannoma or any two of the following: meningioma, schwannoma, glioma, neurofibroma, juvenile posterior subcapsular lens opacity.</td>
</tr>
<tr>
<td>C. Unilateral vestibular schwannoma and any two of the following: meningioma, schwannoma, glioma, neurofibroma, posterior lens opacity.</td>
</tr>
<tr>
<td>D. Multiple meningiomas (two or more) and unilateral vestibular schwannoma or any two of the following: schwannoma, glioma, neurofibroma, cataract.</td>
</tr>
</tbody>
</table>

Clinical picture of neurofibromatosis 2

The clinical picture of NF2 is highly variable, ranging from a mild (Gardner) to a severe (Wishart) phenotype. In mild NF2, the onset is usually after the
age of 25. Usually only bilateral vestibular schwannomas (BVS), with slow growth rates, are seen. In severe NF2, the onset is before 25 years of age. Apart from BVS, several other tumours such as meningiomas, schwannomas on other nerves, and ependymomas develop. The growth rate of the tumours is usually high and many surgical procedures are needed (17).

For all individuals with NF2, the average age of onset is 22 years (range 2–52). Usually, the diagnosis of this uncommon disease is delayed and the average age at diagnosis is 27 years (range 5–66) (15). In 10% of individuals with NF2, the onset is before the age of 10. The first symptom of NF2 differs between children and adults. While vestibular tumours often cause the initial symptoms in adults, the presentation in children is associated with spinal tumours, visual problems or skin manifestations (18, 19).

In cross-sectional studies of NF2, 92–94% of the individuals have been found to exhibit BVS (15–21). Meningiomas are seen in 58% of cases (21) and ependymomas in 6% (15). Spinal tumours occur in 90% (21), but only 30% of such individuals require surgical intervention (20, 21). The most common spinal tumours are either intramedullary ependymomas or intraspinal extramedullary schwannomas (22). Trigeminal nerve schwannomas are seen in 29% of cases, but schwannomas on other cranial nerves are uncommon (21). Schwannomas or neurofibromas of the peripheral nerves are also seen (15, 17, 20, 21). Juvenile posterior cataract is seen in 63–80% of cases (21-23), sometimes even from birth (15). Epiretinal membranes and retinal hamartomas are common (24). Many individuals with NF2 also have signs of mononeuropathy—often many years before the first tumour is noted (25, 26).

**Inheritance**

The inheritance pattern in NF2 is autosomal dominant and the penetrance is > 95% (17, 27). In 49% of the cases, NF2 is inherited from an affected parent (15). The other cases represent new mutations, which occur either in the germ line or in the somatic tissue (after conception). In NF2 cases with new mutations presenting with BVS, the frequency of mosaicism is 33% whereas those with unilateral vestibular schwannomas are mosaics at a frequency of 60% (28).

**The neurofibromatosis 2 gene and protein**

In 1987, *NF2* was found to be linked to chromosome 22 (29). The disease-causing gene, which codes for merlin/schwannomin, was cloned in 1993 (30, 31). This gene has 17 exons and spans 100 kb on chromosome 22q12.1-12.2. Two main isoforms of transcripts are produced because of alternative splic-
Merlin/schwannomin is located in the cell membrane, especially at ruffling membrane edges, and links the actin cytoskeleton to cell-surface CD44 (35). The protein encoded by NF2 acts as a tumour suppressor (36). The form of merlin/schwannomin that result from phosphorylation, promotes cell proliferation and motility. The dephosphorylated form of the protein induces a growth-inhibitory state (37). A recent report has suggested that merlin/schwannomin independently uncouples both Ras and Rac from growth factor signals (38) (Fig.6).

Figure 6. Overview of how Ras-GDP and Rac-GDP and merlin/schwannomin interact in intracellular signal transduction (38). Courtesy of Helen Morrison.
Mutations in the \textit{NF2} gene

Today, more than 300 different mutations have been identified in the \textit{NF2} gene (http://www.hgmd.cf.ac.uk). The majority of the mutations result in non-functional merlin/schwannomin, but some less severe mutations show some retention of function. No dominant negative effect is described in NF2 (39). Mutations have been described in all exons, except for exon 16 and 17, and only a few in exon 9.

Tumorigenesis

In NF2, tumorigenesis is a two-step process. Individuals with NF2 carry a predisposed cell, with the first hit, from birth. A somatic mutation, the second hit, is needed to initiate tumour formation. This 2-hit theory (Fig. 7), based on epidemiological data, was first described for retinoblastoma in 1971 (40) but also holds for NF2. This is the explanation for the average age of onset of 22 years in NF2, while sporadic VS occurs in the fourth decade since two somatic hits must occur during one’s lifetime.

\begin{figure}[h]
\centering
\includegraphics[width=0.7\textwidth]{tumorigenesis.png}
\caption{Knudson’s two-hit theory for tumorigenesis in tumour suppressor gene disorders. Courtesy of Carl Bruder.}
\end{figure}

Genotype-phenotype correlation

In general, constitutional mutations with no functional protein cause a severe phenotype, and those in which some function is retained cause a mild phenotype (15, 41-43). Individuals with splice site mutations have a variable phenotype, depending on in which intron the mutation resides (44). These genotype-phenotype correlations are, however, not always true. There are familial NF2 cases with mild disease despite the fact that they carry deletions that
cause a truncated non-functional protein. Furthermore, individuals with missense mutations can have either have mild or severe disease (45). In these cases, the molecular events that initiate tumour formation seem to be different from those that promote tumour progression (46). In NF2, the age at onset or diagnosis can usually be predicted from the mutation (47). Within families with NF2 the age at onset of symptoms, of hearing loss and of the number of meningiomas can usually be predicted (48). In monozygotic twins with NF2, there is discordance concerning the type intracranial tumour and progression. These variations are thought to be attributable to the stochastic process of the second hit (49), and indicate that the effect of the second hit is variable and cannot be predicted from the first hit. Volumetric MRI studies of VS growth rate in NF2 have shown no genotype-phenotype correlation, only a tendency towards lower growth rate with increasing age of onset or at diagnosis (46, 50, 51).

Clinical challenges

In severe NF2, repeated surgical procedures are required. There is a high risk of side effects, both due to surgery and to the aggressive tumour growth pattern (52). Middle fossa approach surgery with intent to preserve hearing in NF2 is successful in less than 50% of cases (53), in contrast to sporadic VS where the corresponding rate is 85% (54). To improve communication, auditory brain stem implant (ABI) and in some cases cochlear implant (CI) (55) can be offered. Gamma knife treatment of the vestibular tumours has been used to preserve hearing (56), but the risk of inducing additional chromosomal aberrations and malignant progression with this treatment cannot be neglected, which is why it should be offered only to selected NF2 cases (57). NF2 has a severe effect on quality of life, mainly due to hearing impairment, but sometimes also due to facial nerve palsy, visual problems and difficulties in movement (58–60). Life expectancy is also reduced (52, 61). A broader knowledge of the underlying molecular disturbances in NF2 is probably essential for the development of the therapeutic options in NF2.

Meniere’s disease

Prosper Ménière described Meniere’s disease (MD) in 1848. In those days, vertigo was thought to be caused by cerebral congestion. However, in a paper from 1861 (62), he emphasised that vertigo could originate from damage to the inner ear.
Definitions – diagnostic criteria

Meniere’s disease (MD) was defined by the American Academy of Otolaryngology-Head and Neck Surgery, Committee on Hearing and Equilibrium (AAO-HNS), 1995, as the idiopathic syndrome of endolymphatic hydrops (63). Endolymphatic hydrops is a variable degree of distention of the endolymphatic system of the inner ear. The aetiology of hydrops has been the subject of many theories. Increased production of endolymph (64, 65), reduced resorption in the endolymphatic duct and sac (65–69), disturbed ion regulation (70), genetic (71–74) or immunological factors (75, 76), viral (77–79) or bacterial (80, 81) infections, vascular disturbances (79, 82, 83), altered glycoprotein metabolism (84, 85), dietary factors (86) and allergy (66) have all been suggested.

The most recent diagnostic criteria (from 1995) are listed Table 2 (63).

Table 2.

**Diagnostic criteria for Meniere's disease**

<table>
<thead>
<tr>
<th>Certain Meniere's disease</th>
<th>Definite Meniere's disease, plus histopathological confirmation.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Definite Meniere's disease</td>
<td>Two or more definitive spontaneous episodes of vertigo of 20 minutes or longer. Audiometrically documented hearing loss on at least one occasion. Tinnitus or aural fullness in the affected ear. Other causes excluded.</td>
</tr>
<tr>
<td>Probable Meniere's disease</td>
<td>One definitive episode of vertigo. Audiometrically documented hearing loss on at least one occasion. Tinnitus or aural fullness in the affected ear. Other causes excluded.</td>
</tr>
<tr>
<td>Possible Meniere's disease</td>
<td>Episodic vertigo of the Meniere type without documented hearing loss, or sensorineural hearing loss, fluctuating or fixed, with dysequilibrium but without definitive episodes of vertigo. Other causes excluded.</td>
</tr>
</tbody>
</table>

**Epidemiology**

Epidemiological data indicate that MD has an annual incidence of 8.2–157 in 100,000 (87, 88). The prevalence in different studies ranges from 17 in 100,000 in Japan to 218.2 in 100,000 in the United States (89, 90). The disease is found all over the world, but may be more prevalent among Caucasians. The onset of MD is usually in middle age, but onset during the sixth and seventh decades is not uncommon (91). The disease is rare in children; only 1.5% of those affected have been found to be less than 15 years of age (92).
Audiological tests in Meniere’s disease

In MD, there is initially a peak type of curve in 30% of cases, a rising type in 20%, a falling type in 10%, and flat loss type in 20% of cases (93). Fluctuation of the threshold occurs mainly in the first years after onset of the disease. After 10 years, 57.9% of patients have been found to display a flat loss type of curve, with a PTA ranging from 41 to 70 dB (94). Bilateral involvement can be seen in up to 47% of cases who have had the disease for 30 years (93). Endolymphatic hydrops can be revealed by two diagnostic tests, the glycerol test (95) or the urea test (96, 97). The tests are considered positive if two and a half hours after ingestion, the PTA improves 10 dB in 3 adjacent frequencies and/or speech recognition scores are improved by 12%. Electrocochleography has been recommended as a method of verifying endocochlear hydrops, but in long-term follow-up of individuals with MD, the association with hydrops was unsure (98). Other audiological tests, such as Fowler test, ABR, and acoustic reflexes can be performed to verify recruitment — a finding that could also indicate MD.

Pathology of the temporal bone and inner ear in MD

Early radiological studies of the temporal bone in MD patients have shown a lack of periaqueductal pneumatisation and a short and narrow vestibular aqueduct (99, 100). Light microscopic investigations of the inner ear in MD have shown endolymphatic hydrops (64, 101). Signs of focal inflammation, fibrosis of subepithelial tissue and altered vascular anatomy in the endolymphatic sac may explain reduced endolymph resorption (68, 69). A partial degeneration of hair cells and vascular stria, especially in the apical part of the cochlea, has been described. However, the latter findings are not pronounced and cannot explain the degree of hearing loss (102).

Treatment

Treatment modalities used in MD, include hydrochloro-thiazide diuretics (103) and beta-histidine (104). In severe MD, sac surgery according to Portmann 1927 (105) or intratympanic gentamycin treatment (106) may be considered. Most treatment options are effective in reducing vertigo, but have not proven effective in preventing hearing loss or in reducing tinnitus.

Familial Meniere’s disease

Brown first described familial Meniere’s disease (FMD) in 1949. Of individuals affected by MD, it appears that 5-14% have relatives with the disease (72, 73). Several families with MD have been described in the literature.
FMD can often be seen in two to three generations. Morrison described 46 families with FMD in two or more generations. The most common pattern of inheritance was autosomal dominant, but recessive inheritance was also seen, e.g. in one of the families reported by Birgersson et al.. FMD sometimes presents with an earlier onset in successive generations, which could indicate anticipation. Penetrance is estimated to be 60% (73, 112). Morrison and co-workers performed a genetic study of 17 families focusing on chromosome 14. Linkage to an 11-Mb region on 14q was found in 50% of the families studied (Morrison, personal communication, 2005).

### Genetics in vertigo and fluctuating hearing impairment

**Genetically defined diseases with symptoms that resemble MD**

MD-like symptoms are sometimes associated with conditions in which the genetic background is known. Some of these are listed below (Table 3).

Table 3 Diseases with known genetic background and in which symptoms that resembles MD have been described

<table>
<thead>
<tr>
<th>Disease</th>
<th>Gene</th>
<th>Function</th>
<th>Inner ear histology</th>
<th>Reference</th>
<th>“MD”-frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsyndromic hearing loss</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DFNA9</td>
<td>COCH</td>
<td>Not known. (In eyes, involved in glaucoma)</td>
<td>(113)</td>
<td>25–30%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Acellular eosinophilic deposits in cochlear and vestibular labyrinths. Highly expressed, especially in spiral limbus and ligament.</td>
<td>(114)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DFNB4</td>
<td>SLC26A4</td>
<td>Anion transport</td>
<td>(115)</td>
<td>unusual</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Enlarged vestibular aqueduct. Expressed in vascular stria and the endolymphatic sac</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Syndromes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X-linked hypophosphatemic rickets</td>
<td>PHEX</td>
<td>Phosphate regulation</td>
<td>Thickening of temporal bone, precipitate in scala media, degeneration of organ of Corti and spiral ganglion, endolymphatic hydrops</td>
<td>(116)</td>
<td>2/19</td>
</tr>
<tr>
<td>Fabry disease</td>
<td>GLA</td>
<td>Lysosomal storage</td>
<td>Hydrops of the apical cochlea. Atrophy of vascular stria and the spiral ligament. Glycosphingolipid accumulation</td>
<td>(117)</td>
<td>3/4 males</td>
</tr>
<tr>
<td>Von Hippel Lindau</td>
<td>VHL</td>
<td>Tumour suppressor</td>
<td>Occasionally: benign tumours in endolymphatic sac. Not known if it interferes with its function.</td>
<td>(118)</td>
<td>8/13 (only 11% have ELS-tumours)</td>
</tr>
<tr>
<td>Kanzaki disease</td>
<td>NAGA</td>
<td>Lysosomal storage</td>
<td>Not known</td>
<td>(119)</td>
<td>Few cases</td>
</tr>
<tr>
<td>Congenital nephrogenic diabetes incipidus</td>
<td>AQP2</td>
<td>Water transport</td>
<td>Expressed intra-epithelially in the endolymphatic sac</td>
<td>(120-122)</td>
<td>2 cases (twins)</td>
</tr>
</tbody>
</table>
In summary, several of these genes appear to have a function in the vascular stria, the spiral ligament or in the endolymphatic sac, and could be involved in inner ear homeostasis. Some of the conditions are associated with accumulation of a metabolic product in the inner ear that could also interfere with the normal function. Of these, only the \textit{COCH} and the \textit{AQP2} mutations have been studied in sporadic MD patients, and they do not seem to be associated with the disease (123, 124).

\textit{Genetic causes of low-frequency or fluctuating hearing loss}

Low-frequency hearing loss is seen only with DFNA1, DFNA6/14/38, and DFNA58, but none of these have fluctuations in hearing or vertigo. Fluctuating hearing loss is seen in DFNA16, in which individuals have rapidly progressing or fluctuating hearing loss. Already in 1999, DFNA16 was linked to 2q23-24.3 (125). In 2001, the interval was further refined and two sodium channel genes, \textit{SCN2A} and \textit{SCN3A}, were found in the defined interval. No causative mutation was found, however (126).

\textit{Genetic causes of spells of vertigo}

The vertiginous spells of MD are characteristic. Around 20\% of the population suffer from chronic imbalance, but only 2\% suffer from recurrent spells of vertigo (127). The genetic cause of familial benign recurrent vertigo is not known. In studies of families where this is inherited in an autosomal dominant fashion, a linkage to 22q12 in some families and 5p15 in others has been shown (128). A gene causing familial bilateral progressive vestibulopathy could be predicted to cause spells of vertigo if the reduction in vestibular function is stepwise. In genetic studies of families in which bilateral progressive vestibulopathy is inherited in an autosomal dominant fashion linkage to 6q has been found (129), but not in all (129, 130), which indicates genetic heterogeneity.

\textbf{X-linked hearing impairment}

The X chromosome is a relatively large chromosome that is estimated to contain around 1,000 genes, 4\% of the human genome and 10\% of all known Mendelian inherited traits (131). X-linked hearing impairment accounts for 1–5\% of genetic hearing impairment (7, 132) and is thought to be responsible for the excess of males experienced in schools for children with severe to profound hearing impairment (134, 135). In male congenital impairment,
6.5% of cases are estimated to be X-linked (134). Hearing impairment is a part of the clinical features of several X-linked syndromes. However, there have been several reports on pedigrees with X-linked nonsyndromic hearing impairment as well. Based on age of onset and the audiological test result, four different X-linked hearing impairment phenotypes have been delineated: congenital profound (type 1), progressive (type 2), high-frequency (type 3) and mixed hearing impairment (type 4) (136). More recent studies have reclassified X-linked nonsyndromic hearing loss according to the cytogenetical location.

*Congenital profound X-linked hearing impairment*

The first type of X-linked congenital profound hearing impairment has been described in several pedigrees. The phenotype has been linked to both the Xp21.2 and Xq22 regions.

In 1996, Tyson described a family with hearing impairment linked to Xq22 in a region that includes the *COL4A5*, but no mutation in the gene was found (137). Cui reported another family in which the defect was mapped to an overlapping region (138). Both *COL4A5* and *TIMM8A* in the region were sequenced, but no mutation was found.

Two families with congenital X-linked hearing impairment, DFN4, have been linked to the Xp21.2 region (139, 140). The gene causing Duchenne muscular dystrophy, *DMD*, resides in the defined region, and by combining data from the two known families, a region inside the *DMD* gene was defined. A selection of the exons, in the large *DMD* gene, was studied for mutations, but none were found. It is not known whether the *DMD* gene is involved in DFN4 or if an unknown gene inside the gene is the cause. A mouse model—mdx mouse of Duchenne muscular dystrophy—does, however, exhibit sensorineural hearing loss (141). Other structural genes such as myosin and diaphaneous are involved in hearing impairment, which suggests that *DMD* could be a possible candidate gene.

*Progressive X-linked hearing impairment*

The second type, progressive hearing impairment, is described in DFN6, and is reported in two additional families described by Tyson et al. (137) and by Manolis et al. (142).

In DFN6, progressive hearing impairment starts at the age of 5–7 years, initially in the high frequencies and later progressing to involve all frequencies. DFN6 was mapped to Xp22 in 1996 (143).

In one family described by Tyson et al. (137), the progressive hearing impairment started at the age of 3–9 years and females were mildly affected. No linkage could be found to the DFN4 and Xq21-22 region.
In the family described by Manolis et al., affected males had a progressive hearing impairment, starting in the low- and mid-frequency range at 7–20 years of age. The hearing impairment was assigned the name DFN2, but significant linkage was obtained at Xq21.

**X-linked high-frequency hearing impairment**

In 1992, a family with a nonsyndromic stable high-frequency hearing impairment affecting males was reported (144). This involved frequencies in the 1,500–8,000 Hz range, and female carriers were not affected; hence, the pattern of inheritance was X-linked recessive. No genetic study was reported.

**X-linked mixed hearing impairment**

The most common X-linked hearing impairment, DFN3, is that of the mixed type. DFN3 is connected to congenital fixation of the stapedial footplate and a risk of perilymphatic gusher at stapes surgery. The sensorineural component of the hearing impairment is progressive and prelingual, starting at the age of 1–2 years. Radiological malformations including a wide cochlear aqueduct are seen (145, 133). In 1991, Reardon significantly linked this audiological phenotype, DFN3, to Xq13-Xq21 in two out of three families (146), and in 1995 the gene—POU3F4—causing this audiological phenotype was identified (147). This was aided by a deletion found in a family in which DFN3 segregated with choroidermia and mental retardation caused by a contiguous gene syndrome (148). However, there are also families with the DFN3 phenotype but with no mutation in POU3F4. Mutations in the regulatory region, found 400 kb from the coding region on the centromeric side, may be an explanation (149), but the possibility of other genes causing the DFN3 phenotype cannot be excluded.

**Phenotypes of females in X-linked hearing impairment**

In the family with stable high-frequency hearing impairment, type 3 X-linked nonsyndromic hearing impairment, the female carriers were not found to be affected (144). In all other recently described families with X-linked nonsyndromic hearing impairment, females are usually affected, but to a milder degree and at a later stage. In DFN3, females usually have a mild low-frequency conductive hearing impairment, not enough to justify medical attention, but still not normal (150). There are also minor signs of malformations on CT scans of their temporal bones (151). In DFN2 (137), DFN4 (139) and DFN6 (143), females are affected at a later stage. This indicates
that almost all genes and loci identified in X-linked nonsyndromic hearing impairment are X-linked dominant, with a reduced penetrance in females.

Females have two X chromosomes, but one of them is to a large extent inactivated in the early embryonic period. This is a random process, and this inactivation pattern is stable throughout life. This means that females are mosaics in their cells concerning their X chromosome, i.e. in half of their cells the expressed X chromosome is derived from the father and in the other cells the expressed X chromosome is derived from the mother (152). In 10% of the population, there is, however, a skewed form of inactivation (153), which can modify the expression in a female.

X-linked recessively inherited disorders are generally more common than X-linked dominant disorders (152). This does not seem to be the case, though, in X-linked hearing impairment. This indicates that the redundancy of the proteins involved in hearing impairment is not sufficient and in females, with 50% of the functional protein, the disease will be expressed later in life.

**X-linked syndromes with hearing impairment**

Many X-linked syndromes with hearing impairment as part of the phenotype have been described, but to date no allelic heterogeneity, for which hearing impairment is the only expressed feature, has been described. This is suspected in DFN4, but no mutation in the \textit{DMD} gene has been reported in the two families (139, 140). In DFN2, the connection with X-linked Alport syndrome, caused by a mutation in \textit{COL4A5} has also been discussed (137).

Sometimes, hearing impairment is the presenting symptom of a syndrome. This has been reported in two diseases in which there is a phenotype of auditory neuropathy, showing preserved otoacoustic emissions and distorted or no ABR responses. Mohr-Tranebjaerg syndrome has this audiological phenotype (154) and hearing impairment is the presenting symptom, which is why this hearing impairment was initially assigned the name DFN1. Later, however, cortical blindness, dystonia, fractures, and mental deficiency develop, which is why it was renamed. The gene causing Mohr-Tranebjaerg syndrome, \textit{DDP1/TIMM8A} was identified in 1996 (155) —aided by a deletion encompassing both the \textit{DDP1} gene and the adjacent \textit{BTK} gene (156).

Today, several families with different mutations in \textit{TIMM8A} have been described, but no family in which isolated hearing impairment prevails has been reported. A family with the auditory neuropathy linked to a separate region, Xq23-27.3, was reported in 2006 (157). In this disorder also, hearing impairment is the presenting symptom, while peripheral neuropathy develops later.
Genetic investigations

Genetic linkage

Genetic segments, which have a physical proximity on a chromosome, have a tendency to be inherited together. A recombination between the two homologous chromosomes occurs during meiosis, in which there is a reciprocal exchange of genetic material between the maternal and paternal chromosome (Fig. 8).

![Diagram of single and double recombinations between two homologous chromosomes](image)

*Figure 8. Single (a) and double recombinations (b) between two homologous chromosomes and the resulting DNA exchange are illustrated.*

The likelihood that two genetic segments will be separated depends on how far from each other they are situated on the chromosome. The recombination fraction ($\theta$) indicates how often two chromosomal loci are separated by a recombination event. If two chromosomal segments are far apart on a chromosome, the recombination fraction is high. The recombination fraction can be used for calculation of genetic distance, and is measured in Morgans (M). A Morgan is defined as the average distance for which one recombination occur per meiosis. A distance of 1 cM corresponds to approximately 1 Mb in physical distance. This is, however, not always true since some chromosomal regions have a higher recombination rate.

Genetic markers

To trace inheritance of specific DNA segments in genetic mapping, genetic markers are used. These markers must be variable—polymorphic—in the studied population to be useful for a genetic study. How informative the markers are may be measured by the heterozygocity value, which is the frequency of individuals that are heterozygous for the marker.
Variable sequences in the genome are used as markers. The most commonly used markers are microsatellites and single nucleotide polymorphisms (SNPs). In general, microsatellite markers are more informative than SNPs. On the other hand, the number/density of SNPs in the genome exceeds that of the microsatellites.

**Linkage analysis**

In linkage analysis, genetic markers are used to identify genomic regions that are associated with a disease. If a marker is close to a disease-causing mutation they will be inherited together more often than what could be expected with random segregation. A recombination seldom occurs between two thus linked genomic regions.

**LOD score**

To evaluate genetic linkage statistically a logarithm of odds (LOD) score is used \((Z)\). The LOD score is a \(\log_{10}\) ratio between the probability that a given locus is linked to a marker and the probability that the locus is not linked. A LOD score \(Z > 3.0\) is considered evidence of linkage, but inconclusive for \(Z\) values between -2 and 3. For complex disorders, a LOD score cut off value of \(Z = 3.3\) is recommended to avoid false positive results.

Linkage analysis can be performed using one or several markers simultaneously. In two-point analysis, a calculation is done separately for each marker. In multipoint analysis, several markers close to each other are analysed simultaneously against the disease locus and this can be an advantage in studies when the heterozygosity values of the markers are low.

**Parametric linkage analysis**

Parametric linkage analysis is the method used for LOD score calculation, when information on inheritance pattern, penetrance and allele frequency is available.

**Fluorescent in situ hybridisation (FISH)**

Fluorescent *in situ* hybridisation (FISH) is a technique that can be used to study chromosomal rearrangements. A labelled probe is hybridised to metaphase, prophase, or interphase chromosomes, where it binds to the DNA sequence that corresponds to the probe. The probe is labelled with a fluorescent dye and can thus be visualised under a fluorescence microscope. In the
normal individual the probe will bind to two locations—one on each homologous chromosome. If the probe hybridises only to one locus this indicates that a deletion is present on one chromosome. The resolution of FISH is relatively high—deletions as small as 1 Mb can be detected.

Sequencing

DNA to be sequenced forms a template in a PCR reaction, in which a DNA polymerase is used to create a new single stranded DNA chain, which is complementary to the DNA that is studied. To start this polymerisation, the DNA is heated and a primer of a known sequence initiates the reaction. To terminate the reaction dideoxy nucleotides (ddNTP) labelled in four different colours, corresponding to the four different bases, are used. The ddNTP contain a hydrogen group instead of a usual hydroxyl group at the 3’ carbon position of the deoxyribose. The hydroxyl group is essential for elongation of the DNA chain, and that is why ddNTP causes a termination of the reaction. These ddNTP molecules are incorporated randomly and cause a termination of the synthesis at different positions. By a combined analysis of the different lengths of the obtained DNA chains and the four different fluorescent types of the ddNTP, the DNA sequence is defined (Fig.9).

![Figure 9. An illustration of the principles of sequencing of DNA.](image-url)
Microarray -Comparative Genomic Hybridisation (array- CGH)

The array-CGH is a way of detecting variations in DNA copy number, such as deletions or amplifications (158). In array-CGH, test and reference DNA are differentially labelled by fluorescent dyes and cohybridized to the array together with Cot DNA to block repetitive sequences. On the array, DNA (e.g. BAC-clones, PCR products or oligonucleotides) is spotted onto a glass slide. If the test DNA has a complementary sequence to the pieces of DNA on the slide it will hybridise. However, the hybridisation occurs in competition with the reference DNA with another colour. If both the colours bind equally to a spot, the fluorescence from both colours will be equal. If test DNA fluorescence is higher or lower than the reference DNA, amplification or deletion of DNA has been found, respectively. A special array reader is used to register the differences in the fluorescent signals (Figure 10).

Figure 10. Principles of the array-CHG-method (158).

The resolution of the array-CGH depends on the size and distribution of the clones spotted on the array. For full coverage, no gaps and overlapping clones are desirable.
Present Investigations

Aims

Paper I
- To perform high-resolution mapping of constitutional deletions in a comprehensive series of 116 NF2 patients with clinically well-defined phenotypes.
- To investigate the size of NF2 gene deletions in order to see whether other genes on 22q are involved in creating the clinical type of NF2.

Papers II and III
- To describe the clinical and audiological findings in three generations of a five-generation family segregating for familial Meniere’s disease.
- To study candidate genes for FMD.
- To identify the subchromosomal region for the gene underlying FMD in one large family, by linkage analysis.
- To analyse FMD for genetic homogeneity or genetic heterogeneity by including two additional FMD families.

Paper IV
- To assess the phenotypes of a 5-generation family with X-linked hearing impairment both clinically and audiomically.
- To compare the phenotype of the family to those of previously described families with X-linked hearing loss.
Materials

In paper I, 116 individuals with NF2 of variable severity were included in this study of constitutional DNA. Patients from 10 European countries and from North America were included. All severe NF2 cases were included, irrespective of their mutational status, but mild or intermediate NF2 cases were included only if no point mutation was found in the NF2 gene. In total, 83 individuals with severe NF2 (Wishart type) and 16 with mild NF2 (Gardner type) were included. The 16 individuals who did not fit clearly into any of these groups were classified as having intermediate-phenotype NF2. In addition, one individual who was an obligate carrier with no symptoms of NF2 at age 54 was included and classified as mild. All the individuals who were included fulfilled the 1988 NIH criteria for confirmed NF2, except for the obligate carrier mentioned above. Three subjects had additional clinical features, which included mental retardation.

Results

Of the 116 NF2 individuals included, 24 (20.7%) had deletions of variable sizes. Eight of 83 severely affected individuals, 8 of 16 moderately affected individuals, and 6 of 17 mildly affected individuals had deletions. A family history of NF2 was found in 50% of subjects with deletions. In the mildly affected group, 2 out of 5 individuals had a family history of NF2. Of the moderately affected individuals, 5 out of 10 had a family history of NF2, and in the severely affected group, 5 out of 8 had a family history of NF2.

The deletions found in paper I are shown in Figure 11. In the severely affected individuals, the sizes of the deletions varied from 40 kb to 2.5 Mb. The NF2 gene was always included in these deletions.

In the moderately affected group, the size of the deletions could be determined in 9 out of 10 individuals. In one case, the deleted region was larger than the studied section of chromosome 22 and the centromeric border could not be defined. The size, of around 10 Mb, was the largest deletion found in the study. The size of deletions in the intermediate group varied between 120 kb and 6.6 Mb. The NF2 gene was always included in the deletions.

In the mildly affected individuals, the deletions were intragenic or did not stretch outside the NF2 gene.
Figure 11. Overview of the sizes of deletions in each disease phenotype and one-third of the clones that were included in the array-CGH.

All the deletions detected were predicted to result in unstable non-functional merlin/schwannomin proteins, which is why this did not explain the variable phenotype. The largest deletions spanned adjacent genes on chromosome 22.

The majority of individuals who were included were severely affected, and in these individuals, the mutational status was unknown. The moderately and mildly affected individuals had all been tested for mutations in the NF2 gene and no point mutations could be found. The frequency of NF2 deletions cannot be estimated with any accuracy from a population point, and the resolution does not allow small intragenic deletions to be detected.

In mildly affected individuals, the deletions were intragenic or encompassed only the NF2 gene, which is why some genotype-phenotype correlation could be found in these cases. In the moderately or severely affected individuals, there was no correlation between the size of the deletion and the phenotype. There were NF2 cases with similar sizes and locations of deletions in both the mildly affected and the severely affected groups.

Somatic mosaicism could be an explanation for any milder phenotype than would be predicted from the size of the deletion. In non-familial cases, the possibility of a somatic mosaicism causing a milder phenotype cannot be excluded. The fluorescence intensity ratios in the non-familial cases studied did not deviate in any clear way from values found in other clones, which further supports the idea that there was no mosaicism of any significance.
If individuals with new mutations are excluded, the risk of mosaicism appears highly unlikely. Even if these are excluded from the genotype-phenotype study, and we concentrate only on familial cases, no correlation between the size of the deletions and the phenotype is seen. There are familial cases with similar deletion sizes and location, but with diverse phenotypes. Even when specific variables such as age at onset or age at diagnosis in familial cases were studied, no correlation was found.

Discussion

In paper I, an array-CGH covering a 7.4-Mb area defined by previous linkage studies of NF2 families was used. The results indicate that deletions are common in the pathogenesis of NF2.

The genotype-phenotype correlation in NF2 is not clear-cut. A 3-hit model, rather than a 2-hit model, fits better with known epidemiological data (159). Such data have been analysed with the assumption that the first hit does not have any effect on the second hit, which is debatable. The relative lack of merlin/schwannomin, both in the Schwann cell itself and its surroundings, could have some impact on the second hit.

The constitutional alteration in the NF2 gene is only one factor of many in creating the NF2 phenotype. The second hit is usually a larger mutation than the first hit, but not always (160). This stochastic event is responsible for the fate of the remaining 50% activity of merlin/schwannomin. If the second hit involves larger parts of 22q than the constitutional mutation, this might affect other genes close to the NF2 gene and may have a larger effect on the phenotype than the first hit. If that is the case, no genotype-phenotype correlation can be expected. In studies of schwannomas, different cell populations can be found within the tumours (161), indicating that the second hit is variable and that factors other than the constitutional mutations dictate the second hit.

The possibility of a third hit is supported by studies on conditional mouse models that harbour a second hit in schwann cell hyperplasia, before schwannomas are seen (162). There are human schwannomas in which no alteration of the NF2 gene is seen (160). A tumour suppressor gene near the NF2 gene might explain both the variability in phenotype and why the NF2 gene is in fact intact in some tumours. A thorough study of the NF2 gene and adjacent regions might reveal other genes that are of importance for the phenotype in NF2.

Another issue to consider is the high rate of mosaicism in NF2 individuals affected by new germ line mutations. In de novo cases of NF2, this is estimated to occur in 45% of cases. There are studies that indicate that LINE repetitive elements are important in mosaicism (163). In the present study,
the repetitive elements were blocked by Cot-1 DNA and this might obscure the importance of repetitive sequences in NF2.

Revertant mutations causing somatic mutations to be corrected have been shown to operate in several disorders (164). If a reparative DNA process is valid in NF2, variation in this function between individuals could also explain a variable phenotype. One hypothesis on age-related decay in DNA reparative function operating in NF2 mutations has already been suggested as an explanation of the increase in mutations seen with increasing age (165).

The phenotype in NF2 is highly variable, and the results of this study of deletions in the disease support the idea that no simple genotype-phenotype correlation exists. Further mechanisms must be considered in future studies in order for us to fully understand the phenotypic variations in NF2.

Meniere’s disease

Materials

Individuals with familial Meniere’s disease have been investigated for this study since more than 30 years (73).

A family in which Meniere’s disease was present in five generations was studied in paper II (Figure 12, family 1). Thirteen individuals were included in the clinical assessment, which was completed by retrospective evaluation of patient charts of deceased family members. In paper III, two more families with autosomal dominant Meniere’s disease (Figure 12, families 2 and 3) were added. In total, 13 affected and 8 unaffected individuals were assessed both clinically and genetically. All the families originated from the middle part of Sweden, and there was no relationship between the families.
Families 1 and 3 were described in a study of familial Meniere’s disease by Birgerson et al. (73), and were described as family G and family C. One generation in each of the two families included in that study was deceased, and no DNA could be obtained. The clinical data were, however, saved.

All individuals in the three FMD families were assessed clinically using a questionnaire concerning the MD phenotype, including data on age of onset and the symptoms hearing loss, vertigo, tinnitus and aural pressure, and whether one or both ears were affected. Information on the clinical picture of the deceased individuals was obtained through relatives and through clinical data from the study published in 1987 (73).

Results

In family 1, the disease could be followed for five generations, in family 2 for four, and in family 3 for three generations. The pattern of inheritance was autosomal dominant. The clinical evaluation of all the families is shown in Table 4. The phenotypes in the three families showed some variability. Even within families, a variable clinical picture was observed.

In families 1 and 2, the clinical course was more severe than in family 3. The suspected anticipation seen in family 1 was not seen in family 2 or family 3. In all three families, some female members did not develop a progressive MD, but described disappearance of symptoms—both vertigo and hearing impairment (family 1: IV:7, family 2: III:6 and in family 3 II:3 and III:3).

Audiograms in family 2 showed that the progression did not stop at a flat loss at PTA 60dB, but continued to a severe-to-profound hearing impairment in the affected ear. This was observed in 4 of 5 affected individuals.
Table 4. An overview of clinical data in the three families included in study III. PTA = pure tone average, BPPV = benign paroxysmal positional vertigo, pc = patient chart, q = questionnaire

<table>
<thead>
<tr>
<th>Identity</th>
<th>MD ear</th>
<th>Age at onset</th>
<th>PTA right/left</th>
<th>fluctuations</th>
<th>vertigo</th>
<th>tinnitus</th>
<th>Aural pressure</th>
<th>Data collection</th>
</tr>
</thead>
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<tr>
<td><strong>Family 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III:3</td>
<td>left</td>
<td>65</td>
<td>55/61</td>
<td>yes</td>
<td>yes</td>
<td>?</td>
<td></td>
<td>pc</td>
</tr>
<tr>
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<td>left</td>
<td>64</td>
<td>75/110</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>?</td>
<td>pc</td>
</tr>
<tr>
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<td>right</td>
<td>42</td>
<td>83/8</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>q</td>
</tr>
<tr>
<td>IV:3</td>
<td>Bilat/partial</td>
<td>42</td>
<td>80/34</td>
<td>no</td>
<td>BPPV yes/no</td>
<td>yes/no</td>
<td>no</td>
<td>q</td>
</tr>
<tr>
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<td>45</td>
<td>10/14</td>
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<td>yes</td>
<td>yes</td>
<td>no</td>
<td>q</td>
</tr>
<tr>
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<td>right</td>
<td>20</td>
<td>66/0</td>
<td>no</td>
<td>yes</td>
<td>yes/no</td>
<td>yes</td>
<td>q</td>
</tr>
<tr>
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<td>30</td>
<td>3/23</td>
<td>yes</td>
<td>yes</td>
<td>no/yes</td>
<td>no</td>
<td>q</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II:5</td>
<td>right</td>
<td>27</td>
<td>&gt;105/41</td>
<td>?</td>
<td>yes</td>
<td>yes</td>
<td>?</td>
<td>pc</td>
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<td>yes</td>
<td>yes</td>
<td>no</td>
<td>q</td>
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<td>left</td>
<td>44</td>
<td>2/15</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>q</td>
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<td>27</td>
<td>4/&gt;105</td>
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<td>yes</td>
<td>yes</td>
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<td>q</td>
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<td>bilateral</td>
<td>26</td>
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<td>yes</td>
<td>yes</td>
<td>q</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
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<td>pc</td>
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<td>q</td>
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<td>29</td>
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<td>q</td>
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<td>right</td>
<td>35</td>
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<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>q</td>
</tr>
</tbody>
</table>

In FMD family 1, a linkage analysis to regions of several candidate genes was performed. These included: DFNA1, DFNA6/14, DFNA15, DFNA9, DFNB4, antiquitin 1, aquaporins 1-12, antisecretory factor/PSMD4 and HLA-region genes. This study did not yield a positive result, which is why a total genome scan was performed.

The total genome screening of FMD family 1 yielded five regions in the genome with a LOD of > 1. These loci were found on chromosome 2, chromosome 18, and in three separate regions on chromosome 12. When further markers were added to these regions, chromosome 18 could be excluded, while the region on chromosome 2 and the three regions on chromosome 12 segregated with MD.

Two families were added, assuming a common cause for FMD in all three families. The aim was to be able to refine the regions of interest and to test for genetic homogeneity. Several steps of genotyping aided by recombination defined a 463-kb region at 12p12.3. In this region, the highest cumulative LOD score of 3.46 was obtained for marker TA171. The result of the linkage analysis is shown in Table 5.
Table 5. LOD scores. Those verified by segregation analysis are marked in bold.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Family 1</th>
<th>Family 2</th>
<th>Family 3</th>
<th>Total</th>
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<td>-∞</td>
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<td>-1.74</td>
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<tr>
<td>D12S363</td>
<td>2.20</td>
<td>0.40</td>
<td>-2.70</td>
<td>-0.09</td>
</tr>
<tr>
<td>D12S1595</td>
<td>1.66</td>
<td>0.72</td>
<td>0.38</td>
<td>2.76</td>
</tr>
<tr>
<td>D12S301</td>
<td>1.11</td>
<td>-0.12</td>
<td>-2.70</td>
<td>-1.71</td>
</tr>
<tr>
<td>TA35</td>
<td>0.16</td>
<td>0.86</td>
<td>0.12</td>
<td>1.15</td>
</tr>
<tr>
<td>TA171</td>
<td>2.10</td>
<td>0.98</td>
<td>0.38</td>
<td>3.46</td>
</tr>
<tr>
<td>GT11</td>
<td>0.21</td>
<td>0.59</td>
<td>0.05</td>
<td>0.84</td>
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<tr>
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<td>0.06</td>
<td>0.18</td>
<td>0.27</td>
<td>0.50</td>
</tr>
<tr>
<td>TA23GA22</td>
<td>1.72</td>
<td>-∞</td>
<td>0.15</td>
<td>-∞</td>
</tr>
<tr>
<td>GATA20</td>
<td>-1.85</td>
<td>-2.70</td>
<td>-2.70</td>
<td>-4.64</td>
</tr>
<tr>
<td>D12S310</td>
<td>0.69</td>
<td>-2.70</td>
<td>-1.53</td>
<td></td>
</tr>
<tr>
<td>D12S1591</td>
<td>0.73</td>
<td>-∞</td>
<td>0.12</td>
<td>-∞</td>
</tr>
<tr>
<td>D12S1057</td>
<td>-1.35</td>
<td>0.38</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>D12S1042</td>
<td>-2.78</td>
<td>-∞</td>
<td>0.10</td>
<td>-∞</td>
</tr>
</tbody>
</table>

This defined region contained only one gene, PIK3C2G. The coding exons and the exon-intron borders were sequenced, but no mutations could be found. Families 1 and 2 shared the same haplotype in a 7-Mb region, but a recombination restricted this common region to 1.7 Mb (Fig. 9).

Figure 13. Overview of location of the linked markers in 12p12 and the genes in the defined regions of 463 kb (for all families) and 1.7 Mb (shared by family 1 and 2).
Discussion

Papers II and III describe three families with FMD, which segregated for five, four and three generations respectively. There were some variations concerning the clinical expression. In most individuals, MD affected only one ear—despite the fact that many years had passed since onset. Only one individual had bilateral MD. Sporadic MD cases are known to develop bilateral disease—in 30% of cases after 15 years and 47% of cases after 30 years from onset (93).

In sporadic MD, genetic factors could constitute an individual susceptibility for the disease that can be modified by environmental factors. No population-based association studies of sporadic MD have yet been conducted. The HLA antigens are known to be of importance in the individual response to infections. The frequencies of HLA A2 and HLA B44 are 28.9% and 5%, respectively, in the general population (166). In sporadic MD, these HLA types are found in 75% and 37% and in FMD in 90% and 60%, respectively (166). This might suggest that a host factor is of importance for the susceptibility to exogenous factors in MD. Recently, the importance of the HLA region in MD was evaluated in a southern European population. A polymorphism in the HLA-DRB1 was associated with development of bilateral MD (167). In the present study, no linkage to the HLA region was found in family 1—neither in the candidate gene search nor in the genome screening.

Interestingly, a study of different K+-ion channel proteins in sporadic MD patients compared to non-MD individuals was recently published (168). Polymorphisms in $KCNE1$ and $KCNE3$ were found to be associated with a tendency to develop MD. The genes encoding these proteins were located on chromosomes 11 and 21. No linkage to these chromosomes was found in FMD family 1.

The linkage analysis performed in paper III yielded a single common gene, $PIK3C2G$. $PIK3C2G$ has a proposed role in hair cell regeneration and Ca-dependent intracellular protein transport (169). No mutation was found in the coding regions or exon-intron borders of $PIK3C2G$. Some new polymorphisms were found, but they did not segregate with MD. Other non-coding alterations in the gene could still be possible.

Family 3 is small, and the co-segregation of marker alleles with the disease may be coincidental. Families 1 and 2, on the other hand, contributed more to the positive accumulated LOD score and they shared the same haplotype in a 7-Mb region. Hypothetically, this might suggest a common ancestry. A single recombination event restricted this candidate region to 1.7 Mb. This region may contain a gene of potential importance for FMD. Still, in the event of there being different aetiologies in all three FMD families, five areas of interest for further genetic studies of FMD have been defined. These are three regions on chromosome 12, and one each on chromosomes 2 and 18.
Future studies could, however, focus on the defined region on 12p12 by inclusion of more individuals—not only from FMD families, but also with sporadic MD and with unaffected individuals as controls.

X-linked hearing impairment

Materials

In paper IV, a family (Fig. 14) in which only males were affected by childhood/adolescent hearing impairment was studied. The family originated from an area in the middle part of Sweden with a stable population.

*Figure 14.* The pedigree of a family with X-linked hearing loss, where males were affected by hearing impairment with childhood/adolescent onset (black symbols). Women were affected from the age of 40 (half-filled symbols) and two other males had partly noise induced hearing impairment (grey symbols).

All individuals from generations III and IV, and the affected male in generation V were clinically assessed. Individual II:3 and the other individuals in generation V were assessed through audiograms from patient charts.
Results

According to several sources, a deceased male member, I:1, of the family in generation I (Fig. 10) had a severe hearing handicap, which is why the hearing disorder affecting only males could extend over five generations in the studied family. According to this, the pattern of inheritance was considered to be X-linked dominant with reduced penetrance in female carriers.

Phenotype in males

Five male members were considered to have a similar phenotype. They were all affected with postlingual progressive hearing impairment with age of onset ranging from 4 to 17 years. In the affected males, the deterioration of the hearing threshold started at different points in the audiogram. The later the onset of hearing impairment was, the more the starting point of deterioration shifted to the higher frequencies. The sensorineural hearing loss started at 0.25–0.5 kHz in one individual (V:4, age 4), at 0.25–1 kHz in one individual (IV:2, age < 7), at 1 kHz in one individual (IV:1, age 8), at 1–3 kHz, left ear, and at 0.25–2 kHz in right ear, in one family member (IV:5, age 14) and could not be assessed in individual IV:4 (Fig. 15).

The clinical audiological assessment revealed significant TEOAE response from one ear in individual IV:2, where the hearing impairment was severe. None of the other early affected males had any significant TEOAE responses. The ABR resulted in reproducible responses if the PTA was below 70 dB. Acoustic reflexes could only be registered in individuals IV:4 and IV:5 at the time of the study. The responses at 50–70 dB above the PTA level could indicate recruitment. The vestibulo-ocular reflex (VOR) was preserved, even in the worst affected individuals. Individual IV:4 had been exposed to occupational noise, which could affect the audiogram in the high frequency range. The predicted median audiogram adjusted for age, gender and 10 years of noise exposure is inserted in his audiogram.

Two men (III:1 and III:3) with a mid- and high-frequency hearing loss, had been exposed to occupational noise. Individual III:1 had an asymmetric hearing loss with an onset of left-sided hearing loss after acoustic-impulse trauma during military service (Fig. 11). After adjustment for age, gender and 30 years of noise exposure (170, 171) a high-frequency hearing impairment remains in individual III:1 and a mid- and high-frequency hearing impairment in individual III:3.
Figure 15. Pure-tone audiograms of males from generations III, IV and V. Y = years, ● = right ear, x = left ear. Dotted line = predicted audiogram (median) after adjustment for age, gender and noise exposure.

Phenotype in females

In female carriers, high-frequency, and sometimes mid-frequency, hearing impairment could be detected. This probably starts from the fourth decade, but the female carriers had undergone few audiograms and this assumption is based on one audiogram only. The mid- and high-frequency hearing loss was in generations III and IV, however, more pronounced than would be expected from age-related hearing loss alone (Fig. 12). TEOAE were undetectable in female carrier III:2 and present in the normal ear of female IV:3. The females V:1 and V:3 had normal hearing (not shown), at the age of seven.

Figure 16.. Pure-tone audiograms of females in generations II, III and IV. Y = years, ● = right ear, x = left ear. Dotted line = predicted audiogram (median) after correction for age and gender.
Additional clinical evaluation

Individual V:4 had reduced fine and gross motor skills. He also had some retinal pigmentation, which was considered to be within the normal range, in the absence of clinical symptoms of retinitis. None of the affected individuals in generation IV had any symptoms of neuromuscular or ophthalmological disease, and the ophthalmological investigations in IV:2 did not reveal any retinal abnormalities.

To screen for X-linked Alport syndrome, urine tests were performed in individual IV:2 and V:4; the results were normal. Magnetic resonance imaging in individual V:4 showed normal temporal bones, acoustic nerves and brain, and CT showed normal temporal bone configuration in individual IV:2.

Discussion

Few families with X-linked hearing impairment that is not congenital have been described. A family with DFN6 (143), a family described by Manolis et al. (146) and a family described by Tyson et al. (137) had an age at onset that was similar to that of the family described here. In DFN6, the high frequencies are affected. Low- or mid-frequency hearing impairment, progressing to involve all frequencies in affected males, has only been described as a phenotype linked to the X-chromosome by Manolis et al. In the family reported by Tyson et al., no information on the audiophenotype was included.

The genetic study performed on the family described by Manolis et al. yielded a positive linkage to Xq21-22, and the significant linkage was partly in the DFN3 region. There are individuals with a POU3F4 mutation without the characteristic mixed hearing impairment and individuals with the typical DFN3 phenotype but with no mutations in POU3F4. More evaluation of possible genes involved in impairment in this region is needed.

A mid-frequency hearing impairment is, however, seen in DFNA8/12, DFNA13 and DFNA49. In DFNA8/12, mutations in the part of TECTA that encodes the zona pellucida domain of α-tectorin cause stable or progressive cookie bite: mid-frequency hearing impairment of variable onset (172). In DFNA13, COL11A mutation causes progressive hearing impairment, not always congenital and most prominent at 1–2 kHz (173). In DFNA49, where no gene has been identified yet, the onset is from age 8 and a progressive hearing impairment affecting frequencies 0.5–2 kHz is seen (174). An autosomal dominant hearing impairment with reduced penetrance is a possible but unlikely cause in the family presented. Only males are affected by this specific audioprofile, and the offspring of individual IV:1 had no symptoms of hearing impairment at age 7 and 9 respectively.
Low-frequency hearing impairment is seen in DFNA1 (175) and DFNA6/14/38 (176), with first-decade and prelingual onset, respectively. DFNA54 affects both low and mid frequencies starting at a variable age ranging from 5 to 40 (177). All of these cases have autosomal dominant inheritance, and could be a possible, but unlikely, cause for the hearing impairment in the family described here.

Low-frequency hearing impairment is also seen in one-third of individuals with auditory neuropathy (178), but this study could not verify a pattern of auditory neuropathy—although this could not be excluded in some of the individuals. OAE testing was not included in the early assessment at onset age. Acoustic reflexes were present in individuals IV:4 and IV:5, a finding that does not support auditory neuropathy.

There were few audiograms available from female carriers. The only woman studied before retirement had a gently sloping high frequency hearing impairment in one ear. This might indicate an X-linked dominant pattern of inheritance with reduced penetrance in females. The high- and sometimes mid-frequency hearing impairment, which was more pronounced than expected from age alone, in female carriers from generations II and III also supports the idea of an X-linked dominant trait.

The audiograms of individuals in generation III are similar. All of them seem to have more hearing impairment than predicted after adjustment for age, gender and in the two males for noise exposure. It is not possible to evaluate if these findings in males are due to genetic background factors, but none of the males have children affected by hearing impairment.

Imaging with CT in one affected male and MRI in another did not disclose any malformations of the temporal bones. This and the age of onset and audiogram phenotype excluded *POU3F4* and DFN3. In two individuals, urine tests excluded X-linked Alport syndrome. The slightly pigmented retina and the fine and gross motor difficulties in individual V:4 were not found in generation IV, despite the fact that a long period of time had passed since the onset of hearing impairment. This might indicate that the findings in individual V:4 were not connected to the hearing impairment. An overview of clinical findings is given in Table 6.

This family is suitable for future genetic studies. More individuals—from an extended family—could be included, provided that they have a similar phenotype.
Table 6. Clinical data for a 5-generation family with nonsyndromic progressive hearing impairment affecting male individuals before adult age. M = male, F = female.

<table>
<thead>
<tr>
<th>ID</th>
<th>Sex/ status</th>
<th>Age at onset</th>
<th>Type PTA progression</th>
<th>Audiometric configuration right/left</th>
<th>Unilateral/ bilateral</th>
<th>Age/speech discrimination (%) right/left</th>
<th>ABR retro-cochlear-ity Y/N</th>
<th>OAE right/left age</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>M/ affected</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>age 83, 78/80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II:2</td>
<td>F/ carrier</td>
<td>sen- son- nor- neural</td>
<td>51/46</td>
<td>steeply sloping</td>
<td>bilateral symmetrical</td>
<td>age 74, 98/84</td>
<td>no/no, age 73</td>
<td></td>
</tr>
<tr>
<td>III:1</td>
<td>M/Un- af- fected</td>
<td></td>
<td>30/61</td>
<td>steeply sloping</td>
<td>bilateral asymmetrical</td>
<td>age 74, 98/84</td>
<td>no/no, age 73</td>
<td></td>
</tr>
<tr>
<td>III:2</td>
<td>F/ carrier</td>
<td>sen- son- nor- neural</td>
<td>37/36</td>
<td>steeply sloping</td>
<td>bilateral asymmetrical</td>
<td>no/no, age 69</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III:3</td>
<td>M/un- af- fected</td>
<td>sen- son- nor- neural</td>
<td>49/48</td>
<td>steeply sloping</td>
<td>bilateral symmetrical</td>
<td>N</td>
<td>not done</td>
<td></td>
</tr>
<tr>
<td>IV: M/ 8</td>
<td>af- fected</td>
<td>sen- son- nor- neural</td>
<td>79/70</td>
<td>U-shaped/ ascending</td>
<td>bilateral symmetrical</td>
<td>age 43, 34/46</td>
<td>not done</td>
<td>no/no, age 42</td>
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<td>af- fected</td>
<td>sen- son- nor- neural</td>
<td>82/83</td>
<td>ascending/ ascending</td>
<td>bilateral symmetrical</td>
<td>age 4, 8/4</td>
<td>No response</td>
<td>yes/no, age 42</td>
</tr>
<tr>
<td>IV: F/ 40</td>
<td>carrier</td>
<td>sen- son- nor- neural</td>
<td>11/13</td>
<td>normal/gently sloping</td>
<td>unilateral</td>
<td>not done</td>
<td>yes/no, age 39</td>
<td></td>
</tr>
<tr>
<td>IV: M/ 17</td>
<td>af- fected</td>
<td>sen- son- nor- neural</td>
<td>51/54</td>
<td>flat/ flat</td>
<td>bilateral symmetrical</td>
<td>age 38, 96/92</td>
<td>No</td>
<td>no/no, age 37</td>
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<tr>
<td>IV: M/ 14</td>
<td>af- fected</td>
<td>sen- son- nor- neural</td>
<td>56/80</td>
<td>flat/ U-shaped</td>
<td>bilateral asymmetrical</td>
<td>age 32, 42/12</td>
<td>No</td>
<td>no/no, age 31</td>
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<tr>
<td>V:1 F/ un- known</td>
<td></td>
<td></td>
<td>20/20</td>
<td></td>
<td></td>
<td></td>
<td>yes/yes</td>
<td></td>
</tr>
<tr>
<td>V:2 M/ Un- af- fected</td>
<td></td>
<td></td>
<td>20/20</td>
<td></td>
<td></td>
<td></td>
<td>yes/yes</td>
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<tr>
<td>V:3 F/ un- known</td>
<td></td>
<td></td>
<td>20/20</td>
<td></td>
<td></td>
<td></td>
<td>not done</td>
<td></td>
</tr>
<tr>
<td>V:4 M/ af- fected</td>
<td>sen- son- nor- neural</td>
<td>48/50</td>
<td>ascending/ ascending</td>
<td>bilateral asymmetrical</td>
<td>age 9, 70/76</td>
<td>No</td>
<td>no/no age 8</td>
<td></td>
</tr>
</tbody>
</table>
Conclusions

Genetic factors play an important but variable role in hearing impairment. This thesis is concerned with the genetic factors in neurofibromatosis 2, familial Meniere’s disease and a clinical characterisation of a family with X-linked hearing impairment.

Constitutional deletions were found to be common in NF2. The deletions found in the groups of NF2 patients studied are predicted to cause an unstable NF2 gene product. Despite this, the phenotype was highly variable. The result indicates that future studies cannot rely on a simple genotype-phenotype correlation, but must incorporate the insight that other factors are of importance in the phenotypic variation of NF2. The method used for the assessment, microarray-CGH, was shown to be a comprehensive, accurate and fast method to assess variations in DNA copy number in constitutional DNA. It is also possible to develop the method for studies of larger parts of DNA, and improved resolution is possible if smaller clones are used.

A five-generation family with Meniere’s disease (MD) revealed an autosomal dominant pattern of inheritance, and in one family, there were signs of anticipation. In three familial MD families, a linkage to a 463-kb region on 12p12.3 containing only one gene, PIK3F2G, was found. No mutation was found in the exons or exon-intron border, but a mutation in the non-coding regions could not be excluded. In two of the families studied, the region delineated was 1.7 MB, and included PIK3F2G and several other candidate genes. This region could harbour a gene of importance for familial MD, and will be studied in more detail in the future.

A clinical/audiological study of a five-generation family suggested a novel type of X-linked dominant low- or mid-frequency hearing impairment starting in the first or second decade in males, and a mid- and high-frequency hearing impairment in females from the fourth decade.
Acknowledgements

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Online resources

Ensembl http://www.ensembl.org/

Genome Bioinformatics (UCSC) http://genome.ucsc.edu/


Hereditary Hearing Loss Homepage (HHH) http://dnalab.uia.ac.be/dnalab/hhh

Hereditary Hearing Loss in Mice (HHIM) http://www.jax.org/


The Children’s Tumor Foundation https://www.ctf.org/

The Human Gene Mutation Database http://www.hgmd.cf.ac.uk

Others


167. Lopez-Escamez JA, Vilchez JR, Soto-Varela A, Santos-Perez S, Perez-Garrigues H, Aran I, Lopez-Nevo MA. HLA-DRB1*1101 Allele May Be As-


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)