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Genetic Deafness, a clinical audiological approach.
Thesis University Antwerp.

**Universiteit Antwerpen**
**Faculteit Geneeskunde**

# GENETIC DEAFNESS / ERFELIJKE DOOFHEID

## A clinical audiological approach
## Een klinisch audiologische benadering

**Proefschrift voorgelegd tot het behalen van de graad van doctor in de Medische Wetenschappen aan de Universiteit van Antwerpen te verdedigen door**

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## Emerging fields in multidisciplinary research on genetic deafness

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## 1.1 Historical Note

It has been known for a long time that deafness could be inherited. The first written references date to the beginning of the 17th century when Paulus Zacchias, physician to the pope, wrote:

*"The deaf and dumb ought to abstain from marriage not only because they do not understand the end of marriage, but also for the good of the commonwealth, because there is evidence they beget children like themselves" (1)*

In 1865 Gregor Johann Mendel, a monk and biologist who studies pea pod plants, published his original work on what we call today Mendelian inheritance which is the cornerstone of current genetics.(2) However, about a decade earlier in 1853 Sir William Wilde, an Irish otologist, already described autosomal dominant inheritance of hearing loss in several families because he succeeded to add a questionnaire to an Irish census.(3) He also suggested the importance of parental consanguinity in autosomal recessive hearing impairment. In about that period Toynbee (1861) also described a familial pattern of a conductive type of hearing loss previously described by himself in 1837 as thickening of the anterior 2/3 of the stapes footplate resembling ivory.(4) In 1876 Magnus documented a family in which the father and 7 of his 13 children had also conductive hearing impairment and in one of them he verified ankylosis of the stapes.(5) It was Adam Politzer who coined the term otosclerosis for this conductive type of hearing impairment in 1894.(6) Already in 1887 Politzer endorsed Arthur Hartmann's evidence for autosomal dominant and recessive inheritance of hearing impairment (1880) in the second edition of his manual of otology called "Lehrbuch der Ohrenheilkunde". (7)

In the second part of the 19th century, science focussed on hearing loss as part of a syndromal disorder. The earliest reference on syndromal hearing loss is probably on maxillofacial dysostosis described by Thomson in 1846.(8) Another syndrome was described by Albrecht von Graefe, a famed ophthalmologist who published 3 cases of deafness combined with retinitis pigmentosa that causes impairment of vision, although it was his nephew Alfred Graefe that was the first ophthalmologist to notice these deaf-blind patients in 1858.(9) In the end the name of a Scottish ophthalmologist, Charles Howard Usher became eponym for hereditary deaf-blindness known as Usher syndrome after he gave a Bowman lecture in 1935.(10) A similar explorative journey in time is seen in the discovery of the Waardenburg Syndrome. The Dutchman Waardenburg was the first to thoroughly describe in 1948 a syndrome that combines hearing impairment with dystopia canthorum and hypo-pigmentation leading to variably colored irides and the typical white forelock and other features.(11) However, certain aspects of the Waardenburg Syndrome were previously described by Hammerslag in 1905, Van der Hoeve in 1916 and Mende in 1926. This Syndrome may have been first noted by Rizolli in 1877 or Urbantschitsch in 1910. Another syndrome combines congenital hearing loss with euthyroid goiter as more recently described by Pendred in 1896.(12)

With the invention of the audiometry and development of audiometric techniques since the 1930s it became possible to characterize genetic hearing loss not only by its presentation (autosomal dominant or recessive and syndromal or solitary) but also by its progress. Audiometry can characterize hearing loss (affected frequency range, symmetry between both ears, degree of seriousness) and also distinguish age of onset. Repeated measurements can reveal progression of hearing impairment. Progression rate is often unjustly forgotten when a certain type of hearing impairment is described clinically. Further on, clinical and audiological approaches of hereditary hearing impairment are discussed more thoroughly.

## 1.2 Anatomy and physiology of hearing

The ear is composed of three parts: the external ear, the middle ear, and the internal ear. (Figure 1) Functionally another subdivision can be made between a conductive part (external and middle ear) and a perceptive part (inner ear). External ear captures sound that is amplified in the inner ear and transmitted to inner ear for processing.

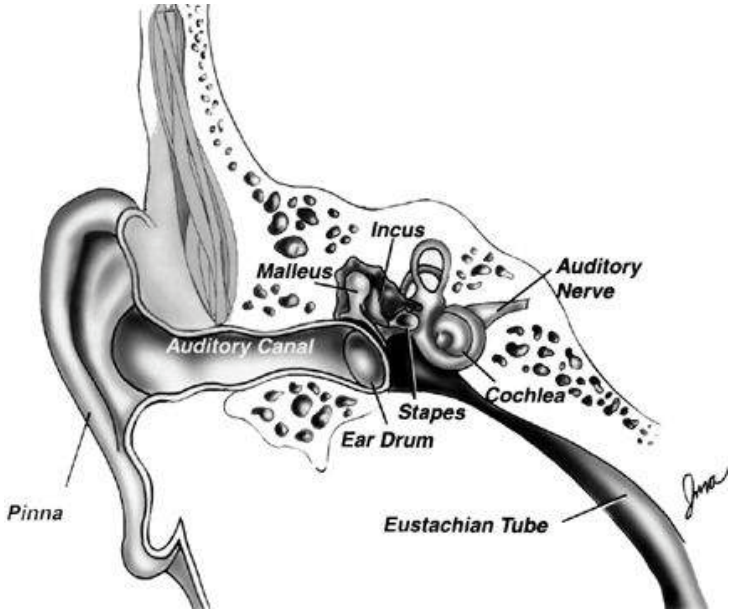



Figure1: The external ear is composed of the auricle (pinna) and the external auditory canal. The middle ear consists of the tympanic membrane (ear drum), the tympanic cavity, and the three auditory ossicles (malleus, incus and stapes). The inner ear is composed of bony labyrinth and membranous labyrinth. www.nih.gov/news/WordonHealth/ apr2001/story04.htm

External Ear

The auricle (or pinna) is composed of a single plate of elastic cartilage covered by thin skin containing sweat and sebaceous glands and hair follicles. The convex surface of the ear has more hair follicles than does the thinner concave surface. The external auditory canal extends from the auricle to the tympanic membrane. It is supported by elastic cartilage in the outer portion and by bone near the tympanic membrane. The skin that lines the canal contains small hair follicles, sebaceous glands, and ceruminous glands. Ceruminous glands are simple coiled tubular apocrine sweat glands. The secretions of the ceruminous and sebaceous glands combine with desquamated squamous cells to form cerumen (ear wax). These structures protect the ear canal from desiccation and the earwax has also a bactericide effect.

Middle Ear

The thin tympanic membrane (or ear drum) delimits the external auditory canal from the tympanic cavity. It is covered externally by stratified squamous epithelium and internally by a layer of simple squamous epithelium continuous with that of the tympanic cavity. Between these two epithelial sheaths is a connective tissue layer composed of collagen fibers. The tympanic cavity is an irregular, air filled space that lies within the temporal bone. In most regions, the tympanic cavity is lined by simple squamous epithelium. The lateral wall of the cavity is formed primary by the tympanic membrane. The medial wall of the cavity is a common wall shared with the inner ear with two openings; the vestibular (oval) window and the cochlear (round) window.

Three small bones, the malleus, incus, and stapes, traverse the middle ear connecting the tympanic membrane to the membrane of the vestibular (oval) window of the internal ear. The malleus is attached to the inner aspect of the tympanic membrane. The stapes is oriented at a right angle to the incus and is secured by a fibrous ligament into the oval window of the inner ear. The malleus and the incus are suspended from the roof of the cavity by suspensory ligament. The three ossicles are joined to one another by synovial joints.
Sound waves that impinge upon the tympanic membrane are transmitted to the malleus as mechanical vibrations. The mechanical vibrations are conveyed from the malleus to the incus and then to the stapes which leading to fluid displacement within the inner ear. The auditory tube (Eustachian tube) connects the tympanic cavity to the nasopharynx. The tube is surrounded by bone near the tympanic cavity and by an incomplete cartilaginous tube toward the pharynx. The primary function of the auditory tube is equalizing the air pressure between the tympanic cavity and external environment. Equilibration of the air pressure occurs during swallowing and yawning when the walls of the tube are separated, allowing air to the tympanic cavity from the nasopharynx.

Internal Ear
The internal ear is a system of canals and cavities in the petreous part of temporal bone containing the bony labyrinth and the membranous labyrinth.

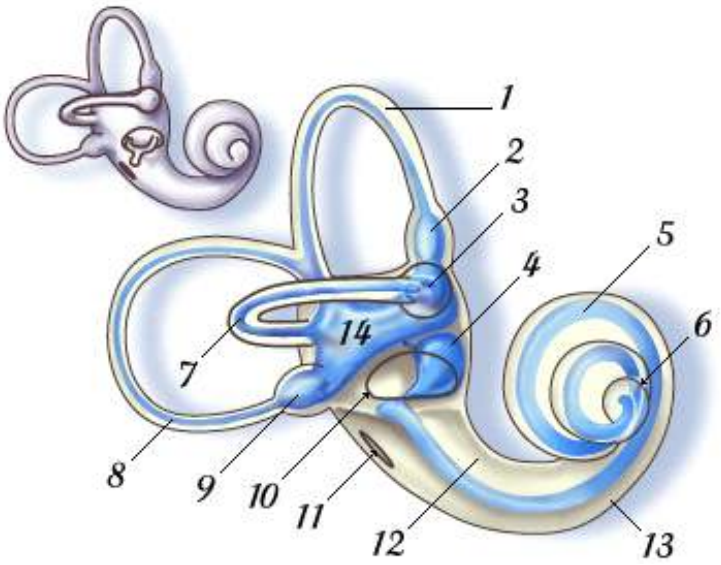



Figure2: The inner ear.

1. Anterior semicircular canal.
2. Ampulla superior canal.
3. Ampulla lateral canal.
4. Saccule.
5. Cochlear duct.
6. Helicotrema.
7. Lateral (horizontal canal) canal.
8. Posterior canal.
9. Ampulla (posterior canal)
10. Oval window.
11. Round window.
12. Vestibular duct.
13. Tympanic duct.

The bony labyrinth contains cavities filled with perilymph and consists of the vestibule, the semicircular canals, and the cochlea. The walls of the bony labyrinth are composed of an outer periosteal layer, a middle endochondral layer, and an inner endosteal layer. The central portion of the bony labyrinth is a bony cavity, called the vestibule. It has an elliptical recess and a spherical recess for two membranous sacs, the utricle and the saccule, respectively. The lateral wall of the vestibule has the vestibular (oval) window, in which the footplate of the stapes is inserted. The membranous endolymphatic duct lies in an opening of a small canal (vestibular aqueduct) in the medial wall of the vestibule. Three semicircular canals extend posterior from the vestibule. These semicircular canals, derive their names (superior, lateral, posterior) from their orientation to one

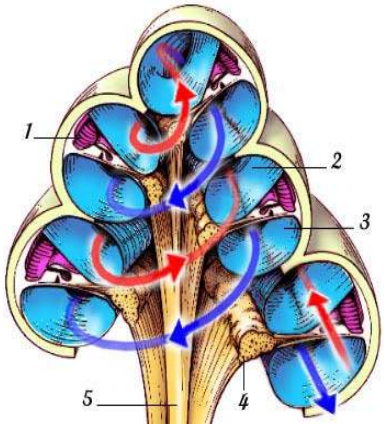



Figure 3.
1 Cochlear duct
2 Scala vestibuli
3 Scala tympani
4 Spiral ganglion
5 Auditory nerve fibres

another. Each canal forms about two-thirds of a circle and is located at approximately a right angle to the other two canals. The ampulla is a dilation at the end of each semicircular canal. The cochlea is a complex bony canal that coils like a snail shell housing for the membranous cochlea containing the organ of hearing, the spiral organ. The cochlea makes 2 3/4 turn in humans around an axis of spongy bone, the modiolus.

The membranous labyrinth contains cavities filled with endolymph and is composed of utricle, saccule, semicircular duct, endolymphatic duct, endolymphatic sac, and cochlear duct.

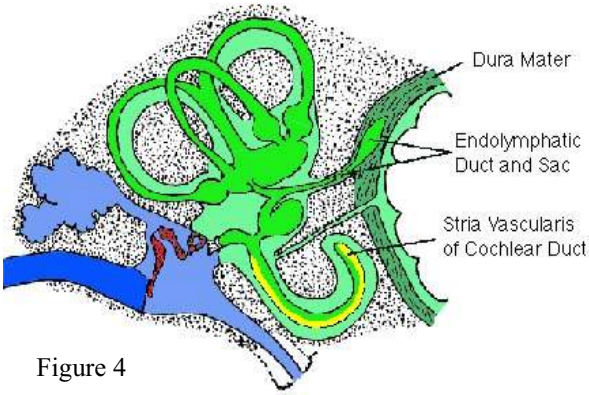
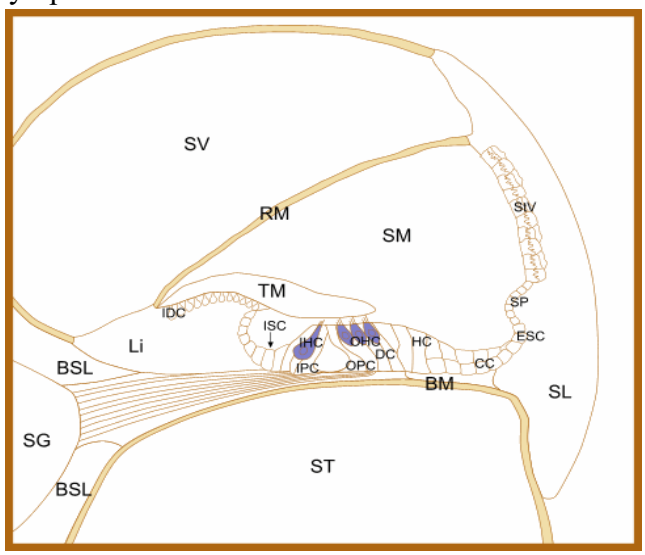



Figure 4

All of the components of the membranous labyrinth communicate with each other. The utricle is an irregular membranous sac located on the medial wall of the vestibule. Saccule is another membranous sac located on the medial wall of the bony vestibule. It is flattened and irregular in shape and is oriented perpendicular to the utricle. There are two openings of the saccule. One opening is a small duct that unites with the ductus utriculosaccularis of the utricles to form the endolymphatic duct. (Fig.4) The other opening communicates with the duct of the cochlea through the ductus reuniens. The anterior, posterior, and lateral semicircular ducts are located within their respective bony semicircular canals. Endolymphatic duct is extending both from the utricle and saccule. This duct opens into the endolymphatic sac. The endolymphatic sac is thought to be actively involved in absorption of the endolymph. The membranous cochlea extends into the bony cochlea from the saccule by a small duct (ductus reuniens) and ends as a blind sac at the apex of the cochlea. The triangular shaped (cross section) scala media (cochlear duct) splits the osseous cochlea into two compartments above (scala vestibuli) and below (scala tympani). (Fig.5) At the apex of the cochlea, the scala vestibuli and scala tympani are in communication through a narrow canal termed the helicotrema. The scala vestibuli and the scala tympani are filled with perilymph, whereas the scala media (cochlear duct) contains endolymph. The scala media is separated from the scala vestibuli by vestibular membrane (Reissner) and from the scala tympani by the basilar membrane. The basilar membrane is attached to the cochlea by the spiral ligament and extends to the other side, where it attaches to the spiral lamina. The basilar membrane varies in width from the base coil (where it is narrowest) to the helicotrema (where it is widest). The stria vascularis is as vascularized epithelium located in the lateral wall of the cochlear duct. It contains marginal cells that are generally believed to produce the endolymph of the scala media

Figure 5 Cross sectional scheme of cochlea




BM Basilar membrane
BSL Bony spiral lamina
CC Claudius cells
DC Deiters' cells
ESC External sulcus cells
HC Hensen cells
IDC Interdental cells
IHC Inner hair cell
IPC Inner pillar cell
ISC Inner sulcus cells
Li Limbus
OHC Outer hair cells
OPC Outer pillar cell
RM Reissners membrane
SG Spiral ganglion
SL Spiral ligament
SM Scala media
SP Spiral prominence
ST Scala tympani
StV Stria vascularis
SV Scala vestibuli
TM Tectorial membrane

Organ of Corti (Spiral Organ)

The organ of Corti is a collection of sensory and supporting epithelial cells that rests on the scala media side of the basilar membrane. The sensory cells of the organ of Corti are classified as inner hair cells and outer hair cells. The inner hair cells lie in a single row along the length of the basilar membrane and are surrounded completely by supporting cells. The outer hair cells lie in three to five rows along the basilar membrane. Only the apical and basal

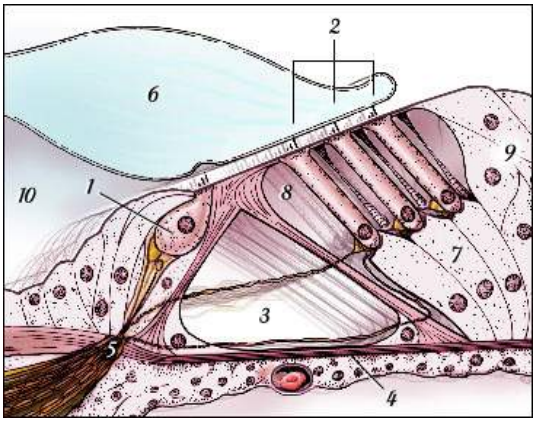



Figure 6

1-Inner hair cell
2-Outer hair cells
3-Tunnel of Corti
4-Basilar membrane
5-Habenula perforata
6-Tectorial membrane
7-Deiters' cells
8-Space of Nuel
9-Hensen's cells
10-Inner spiral sulcus

surfaces of the outer hair cells are surrounded by supporting cells. Each sensory hair cell has stereocilia on its apical surface. There are 100 to 300 stereocilia on the apical surface of each outer hair cell. Each inner hair cell has 50 to 70 stereocilia on its apical surface. The stereocilia of the outer row of hair cells are embedded in a gelatinous membrane, the tectorial membrane. The supporting cells of the organ of Corti are classified as inner and outer pillar cells, inner and outer phalangeal (Deiters') cells, border cells, Hensen cells. The inner and outer pillar cells line a triangular space (tunnel of Corti). These supporting cells rest on the basilar membrane extend upward to cradle the base of the hair cell, than send long cytoplasmic processes toward the surface. The columnar border cells lie between the inner row of hair cells. The Hensen cells, lateral to the outer phalangeal cells, constitute the outer border of the organ of Corti. Lateral to the Hensen cells are cuboidal cells known as the cells of Claudius. Boettcher's cells are found in clusters beneath the cells of Claudius.

Histophysiology of the Auditory Mechanism

Sound waves reaching the tympanic membrane cause it to vibrate at the same frequency.

The movement consequently imparted to the auditory ossicles moves the base of the stapes in and out of the oval window at the same frequency. Since the fluid perilymph on the other side of the oval window lies in a chamber with rigid bony walls and is itself incompressible, the inward movement of the stapes produces a pressure wave within the perilymph which can be relieved only by a compensating outward movement of the secondary tympanic membrane covering the round window. It could travel the length of the scala vestibuli and pass by way of the slender helicotrema to the perilymph of the scala tympani, thence to the round window, or it could be transmitted across the vestibular membrane to the endolymph of the cochlear duct (scala media). This would cause displacement of the basilar membrane toward the scala tympani; consequently, the pressure wave would be transmitted to the perilymph of the scala tympani and released at the round window. Thus a sound of a given frequency would cause movement of basilar membrane of equal frequency.

The hair cells are firmly supported within a framework mounted on the basilar membrane, their hairs contact the overlying tectorial membrane, and these membranes are "hinged" to maintain a parallel relationship when the basilar membrane is distorted. Such distortion thereby impacts a shear force between the tectorial and basilar membranes that results in bending of the hairs to a proportional degree. The hair cells transduce the bending into

generator potentials which stimulate appropriate signals in the afferent nerve endings. It is now known, however, that large region of the basilar membrane vibrate for all frequencies, but waves that travel up to the cochlear spiral produce maximum displacement of the membrane at different sites depending on the tone of the incident sound. The lower frequency of the sound waves, the farther from the oval window the maximum displacement of the basilar membrane occurs. Central nervous system mechanisms sort out the input signals so that the site of maximum basilar membrane displacement and thus the pitch and quality of a sound are discerned. The loudness of a tone is thought to be determined by the amount of basilar membrane set into maximum motion. It has been suggested that the outer hair cells are particularly concerned with determining the intensity of sound and the inner hair cells with pitch discrimination. Moreover, the nerve endings on hair cells are arranged not only for the reception of excitation but also for inhibition.

Primary pathway

The final neuron of the primary auditory pathway links the thalamus to the auditory cortex, where the message, already largely decoded during its passage through the previous neurons in the pathway, is recognised, memorised and perhaps integrated into a voluntary response. The first relay of the primary auditory pathway occurs in the cochlear nuclei in the brain stem, which receive Type I spiral ganglion axons (auditory nerve); at this level an important decoding of the basic signal occurs: duration, intensity and frequency. The second major relay in the brain stem is in the superior olivary complex: the majority of the auditory fibres synapse there having already crossed the midline. Leaving this relay, a third neuron carries the message up to the level of the mesencephalus (superior colliculus). These two relays play an essential role in the localisation of sound. A final relay, before the cortex, is in the thalamus (median geniculate body). Here an important integration occurs: preparation of a motor response (e.g. vocal response).

Non-primary pathway

From the cochlear nuclei, small fibers connect with the reticular formation where the auditory message joins all other sensory messages. The next relay is in the non-specific thalamus nuclei before the pathway ends in the polysensory (associative) cortex. The main function of these pathways also connected to wake and motivation centres as well as to vegetative and hormonal systems, is to select the type of sensory message to be treated first. For instance, when reading a book while listening to a record, this system allows the person to pay attention alternately to the most important task.

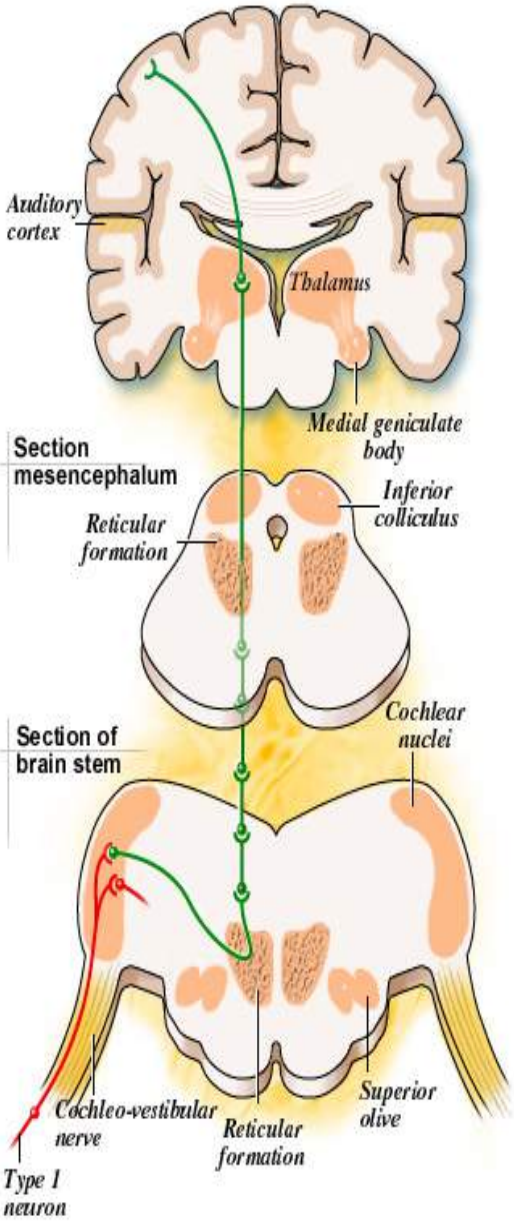



Figure 7 Primary pathway.

## 1.3 Defining the research field of this study

Hearing impairment is the most important sensory deficit, causing a significant handicap. Approximately 35 percent of people between 60 and 70 years old have 25 decibels or more hearing loss, whereas 1 in 1000 children are born deaf in our society.(13;14) Hearing loss is the third most prevalent handicap in persons over 65 years of age. The disability from hearing impairment in private and professional life is substantial, especially in a society as ours that is focussed on verbal communication.

Aetiology of hearing impairment comprises various items. Acquired types of deafness can be caused by excessive noise exposure, trauma to the ear, infections and ototoxic drugs for instance. Genetic deafness is an innate type of hearing impairment. Genetic deafness can be monogenic when one disease causing gene is responsible for the deafness. Sometimes genetic deafness is accompanied by other pathological manifestations so that it is referred to as a genetic syndrome. For instance, in the Usher syndrome deafness occurs together with blindness. The inheritance pattern is used for a further subdivision in autosomal dominant, autosomal recessive or X-linked type of hereditary deafness as shown in Table 1.
Complex genetic deafness is a term to define a genetic susceptibility that leads to hearing impairment when triggered by certain environmental factors. Complex genetic also implies that more than one gene can be involved. Presbyacusis, otosclerosis and noise induced hearing loss are examples that probably have a complex genetic origin.

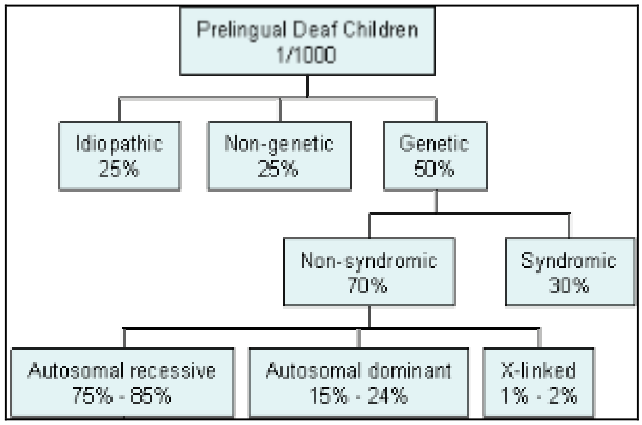



Table 1 Classification of hereditary hearing impairment.
Modified from: http://www.geneclinics.org/profiles/deafness-overview/details.html. R. Smith and G. Van Camp

A fundamental and primary step in research on genetic deafness is the clinical and audiological determination of the phenotype at the level of an individual person. This includes a clinical examination of the possibly affected patient with hearing impairment. Clinical signs that hint towards a syndromal type of deafness and audiological characterization of the hearing impairment are crucial for further genetic analyses. A clinician has to make a distinction between genetic and environmental factors for a specific person with hearing problems. Then, the otological and clinical observations have to be documented and reported in a useful manner to support genetic research.

This thesis aims at reviewing, elaborating, evaluating and applying several clinical and audiological investigation methods in view of the genetic molecular biological phase of genetic research.

## 1.4 Objectives of the study and structure of dissertation

The specific objectives of this study are:

1. To clinically report and audiologically characterize different types of hereditary hearing impairment in order to facilitate genetic linkage analysis.
2. To study and optimize the methods of assessing audiological and clinical data to classify and identify different types of hereditary hearing impairment (HHI).

These objectives will be pursued by;

1. Study of currently known types of HHI and their use in clinical diagnosis.
2. Audiological and clinical evaluation and study of non syndromic monogenic HHI.
3. Audiological and clinical evaluation of syndromal monogenic HHI associated with deafness-blindness in Usher Syndrome.
4. Audiological and clinical evaluation of otosclerosis: a complex genetic trait with a mixed type of hearing loss.
5. Audiological and clinical evaluation of more than 400 volunteers aged between 55 and 65 years and 15 small families of 5 or more siblings aged between 55 and 70 years in order to contribute to a European study of the genetic origin of Age-Related Hearing Impairment. At the same time this study population will be analysed for environmental factors that have a deleterious affect on hearing.

**Structure of dissertation**

Chapter 2 is a review of how audiological and clinical analyses can sometimes lead to direct genetic testing to make diagnosis of some well known types of hereditary hearing loss.

Chapter 3 describes audiological and clinical analyses in a family with sensorineural hearing loss that has led to successful linkage analyses with determination of the genetic locus.

Chapter 4 describes audiological and clinical analyses in a family with sensorineural hearing loss that has directly led to discovery of a mutation in a known deafness gene, without linkage analyses.

Chapter 5 describes audiological and clinical analyses in families with mutations in a known deafness gene but with different clinical presentations.

Chapter 6 describes audiological and statistical analyses for operated patients with otosclerosis: a complex genetic type of hearing impairment.

Chapter 7 describes audiological and clinical analyses in volunteers for the European Age-Related Hearing Impairment (ARHI) study reporting the preliminary risk factors.

Chapter 8 describes the preliminary results of genetic analyses of the European Age-Related Hearing Impairment (ARHI) study based on audiological and clinical analyses.

## 1.5 Current clinical and audiological approaches of genetic hearing impairment

For a successful genetic analysis a careful selection of genetically affected and non-affected study cases is essential. The basic condition for investigating genetic origin of hearing impairment is the correct identification of individuals with a similar phenotype. This is based on careful history taking (anamnesis) and clinical examination, often completed with clinical tests such as audiometry: a subjective test for hearing acuity. Non-genetic causes of hearing impairment, due to environmental factors or aging have to be excluded and cases must be compared to normative values for hearing in order to characterize a phenotype of a hearing impairment. This chapter covers current issues on the clinical audiological approach of phenotype determination of genetic deafness in phenotype-genotype correlation studies.

**Pure-tone audiometry**

The usual clinical purpose of pure-tone tests is to determine the type, degree, and configuration of hearing loss. Pure-tone audiometry is a behavioural test measure used to determine hearing sensitivity. This measure involves the peripheral and central auditory systems. Pure-tone thresholds indicate the softest sound audible to an individual at least 50% of the time. Hearing sensitivity is usually plotted on an audiogram for the clinician. The audiogram is a chart of hearing sensitivity with frequency plotted on the abscissa and intensity on the ordinate. Intensity is the level of sound power measured in decibels; loudness is the perceptual correlate of intensity. Frequency, perceptually correlating with pitch, is measured in hertz. Usually frequencies from 250 to 8000 Hz are used in testing because this range represents most of the speech spectrum, although the human ear can detect frequencies from 20-20,000 Hz. Some children can detect even higher frequencies.

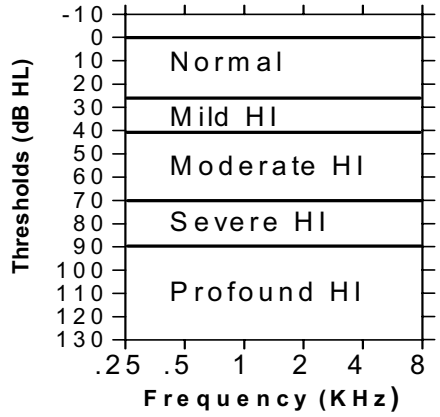



Figure 8 Audiogram with degrees of hearing loss indicated. Normal hearing (0-25 dB): At this level, hearing is within normal limits. Mild hearing impairment (HI) (26-40 dB) may cause inattention, difficulty suppressing background noise, and increased listening efforts. Patients with this degree of loss may not hear soft speech. Moderate HI (41-70 dB) may affect language development, syntax and articulation, interaction with peers, and self-esteem. Patients with this degree of loss have trouble hearing some conversational speech. Moderate-severe HI (56-70 dB) may cause difficulty with speech and decreased speech intelligibility. Severe HI (71-90 dB) may affect voice quality. With profound HI or deafness (>90 dB), speech and language deteriorate.

Audiometry is best performed with calibrated equipment according to the ISO389 standard in a sound booth with a permissible ambient noise level according to ISO8253 standard. (15;16) The ISO 8253 standard also describes a modified Hughson-Westlake method for threshold determination. Crossover occurs when sound presented to the test ear travels across the head to the non-test ear. This occurs at approximately 40 dB for circumaural earphones across all frequencies. When hearing sensitivity is much poorer in the test ear than the non-test ear, the signal may cross over and be perceived in the ear with better hearing, thus yielding a false impression of the intended test ear's sensitivity. Insert earphones reduce the crossover by reducing surface contact area. Masking presents a constant noise to the non-test ear also to prevent crossover from the test ear. The purpose of masking is to prevent the non-test ear from detecting the signal (line busy), so only the test ear can respond. Masked thresholds represent the true threshold of the test ear. The ISO8253 describes standardized instructions for masking as well.

Determination of air conduction (AC) thresholds assesses subjective response (whether pressing a knob or lifting the arm) to an acoustic signal, transmitted through the outer, middle, and inner ear. Testing may be performed using headphones, insert earphones, or sound fields. Sound-field (free-field) tests are used with infants and when earphone use may be problematic. During sound-field testing, an individual sits in the centre of the room, facing forward, halfway between 2 speakers. Typically, visual-reinforcement audiometry (toys light and animate when the child responds to sound); conditioned-orientation response audiometry (toys on both sides test localization); or play audiometry (various games, e.g. dropping a block in response to sound) are used. These conditioned responses to auditory stimulus provide reinforcement that allows for measurable responses and longer interest in the test situation. In a sound field, the auditory signals are warble tones or bursts of narrow-band noise. Pure tones cannot be used because they can create standing waves in a sound field, which can alter signal intensity. Sound-field testing also may assess hearing aid benefit. Placing the person in the centre of the room (facing the speakers) yields aided thresholds. The difference between aided and unaided thresholds is called functional gain.

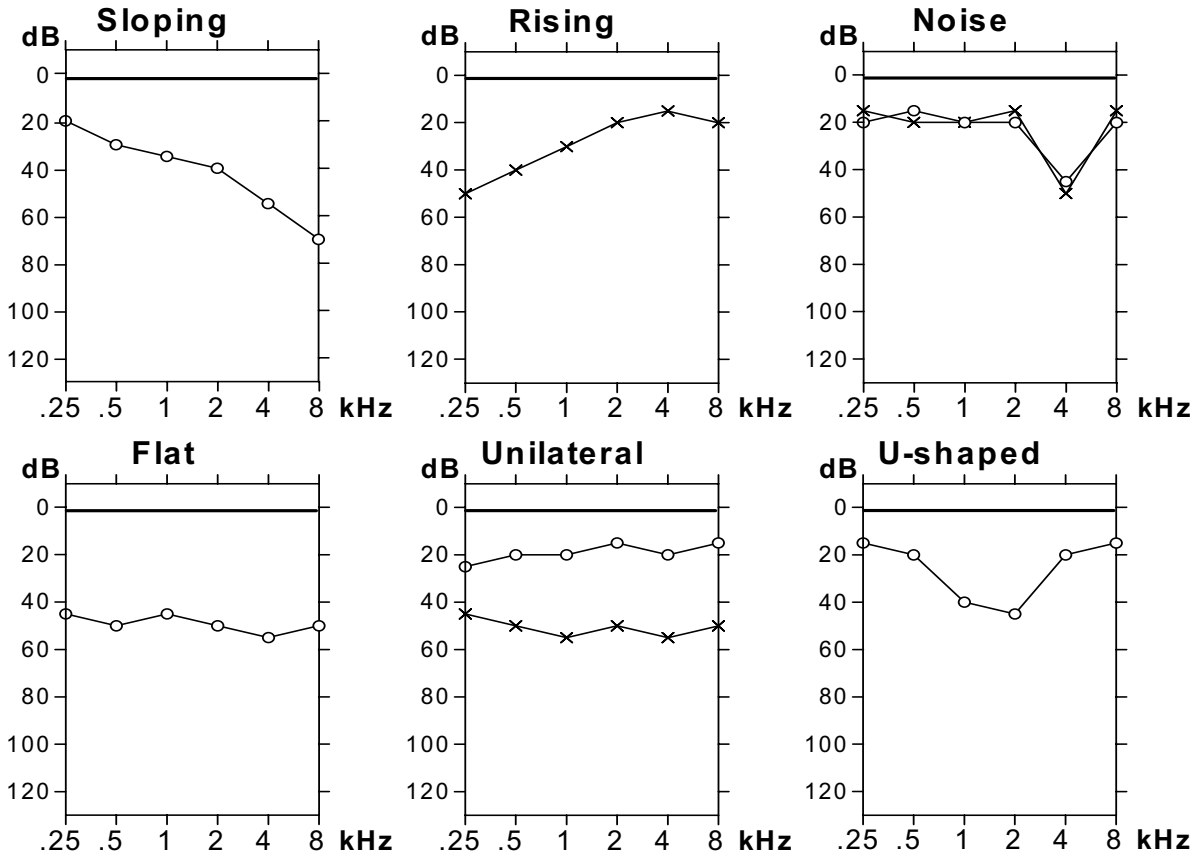



Figure 9 Air conduction thresholds indicated with O for right ear and X for left ear. Configuration of the hearing loss can be recognized as indicated. U-shapes are suggestive for genetic origin.

Determination of bone conduction (BC) thresholds assesses sensitivity when the acoustic signal is transmitted through the bones of the skull to the cochlea. This type of testing bypasses the outer and middle ear. A small oscillator is placed on the mastoid (or forehead). The device stimulates the bones of the skull, which in turn stimulates both cochleae. The oscillator may produce a vibration that the patient may feel at higher test intensities, thus eliciting a vibro-tactile response instead of a response to the auditory stimulus.

After having determined AC and BC thresholds, 3 types of hearing loss can be differentiated as shown in figure 3. A conductive type of hearing loss is secondary to outer ear or middle ear abnormality, which can include abnormalities of the tympanic membrane or ossicles. The abnormality reduces the effective intensity of the air-conducted signal reaching the cochlea, but it does not affect the bone-conducted signal that does not pass through the outer or middle ear. Examples of abnormalities include perforated tympanic membranes, fluid in the middle ear system, or otosclerosis of the stapes. Pure-tone air-conduction thresholds are poorer than bone-conduction thresholds by more than 10 dB over three alongside tested frequencies. A perceptive type of hearing loss is secondary to cochlear abnormality and/or abnormality of the auditory nerve or central auditory pathways and is also called sensorineural hearing loss. Because the outer ear and middle ear do not reduce the signal intensity of the air-conducted signal, both air- and bone-conducted signals are effective in stimulating the cochlea. Pure-tone air- and bone-conduction thresholds are within 10 dB. Examples include noise induced hearing loss, toxic hearing loss and age-related hearing impairment (ARHI). A mixed type of hearing loss has sensorineural and conductive components. Pure-tone AC thresholds are poorer than BC thresholds by more than 10 dB, and BC thresholds are less than 25 dB (HL). Examples are otosclerosis or combined diseases, as in ARHI with otitis media with effusion.

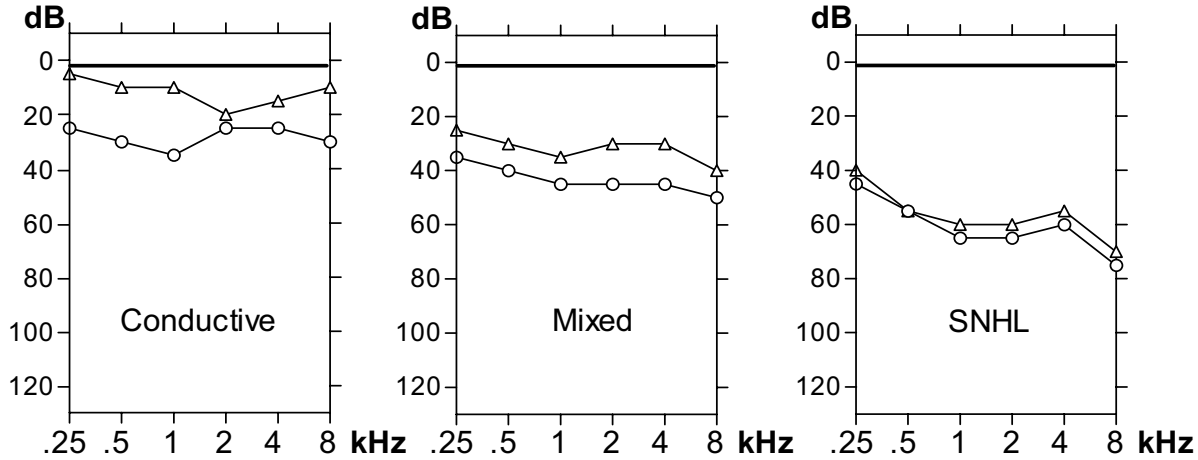



Figure 10. Three types of hearing loss. Conductive hearing loss has normal BC thresholds, but AC thresholds are poorer than normal by at least 10 dB in three alongside tested frequencies. Sensorineural hearing loss (SNHL) has AC and BC thresholds within 10 dB of each other, and thresholds are higher than 25 dB HL. Mixed hearing loss has conductive and sensorineural components. ○=Air conduction thresholds Δ=Bone conduction thresholds.

**Presbyacusis corrections**

Presbyacusis (literally elder hearing) is a general term for inevitable deterioration of hearing ability that occurs with age. The term encompasses all conditions that effect hearing acuity in elderly including central auditory processing that reduces with age. Age-related hearing impairment (ARHI) is a better term to reflect deterioration of sensorineural hearing acuity in function of age. The magnitude of this age effect varies considerably between individuals and between sexes. Therefore in 1984 standard thresholds for age and sex were published for the first time by the International Organisation of Standardization in Geneva, Switzerland (ISO7029).(17) These thresholds were republished in 2000 and are based on a synthesis of 8 highly screened populations. Whether these thresholds are a correct reflection of ARHI thresholds can be debated. Nevertheless, ISO7029 normative thresholds have been the reference in the last 15 years for audiogram interpretation in genetic research. When the concerning hearing impairment is considerably worse than the P95 ISO7029 normatives it is believed to be caused by other reasons than ARHI. A study case is labelled as affected by genetic deafness when three or more tested frequencies exceed the P95 values. A study case can be included in the non-affected group when hearing thresholds are better than the P50

value for all measured frequencies and the subject is older than the upper limit of the onset age of the hearing impairment in the concerning family pedigree.

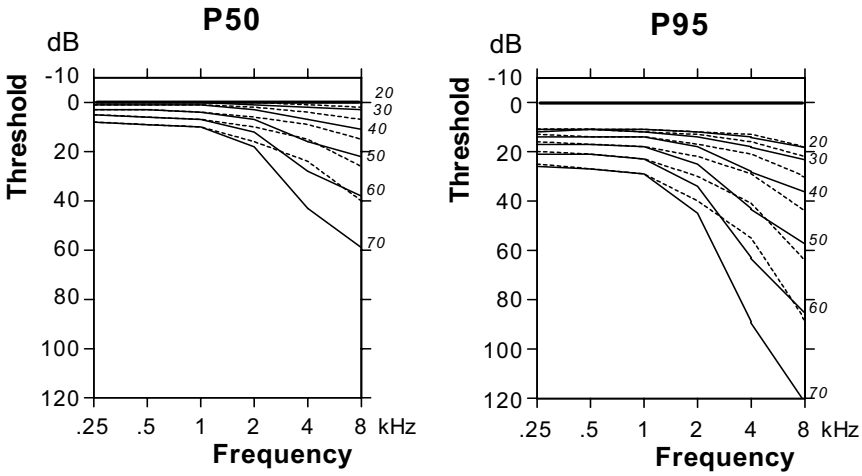



Figure 11 Precentile50 (P50) and percentile 95 (P95) of ISO7029 air conduction thresholds as a function of age and sex. Dotted lines for females and solid line for males. Age in italic.

Of course all other environmental factor that might lead to hearing impairment must have been excluded to begin with. This is not always possible to screen for especially in retrospective studies. In case of unscreened populations annex B of ISO1999 is probably a better comparison than ISO7029.(18) This issue is discussed in chapter 3.4.

Another problem arises when ARHI itself is studied as a complex genetic hearing impairment. About half of the variance of ARHI is attributed to genetic factors and the other half to environmental risk factors. No genes have been identified for ARHI in the human genome but three loci in mice are localized.(19) Genetic variation in humans probably plays a role in determining the range of individual susceptibility to ARHI, but no contributing loci have been identified because of the difficulties of dissecting complex traits in humans. A novel method to study ARHI as a quantitative trait consists of a conversion of audiometric data into a Z-score.(20) The conversion is based on ISO 7029 normative standards and comprises a sex independent and age independent value.

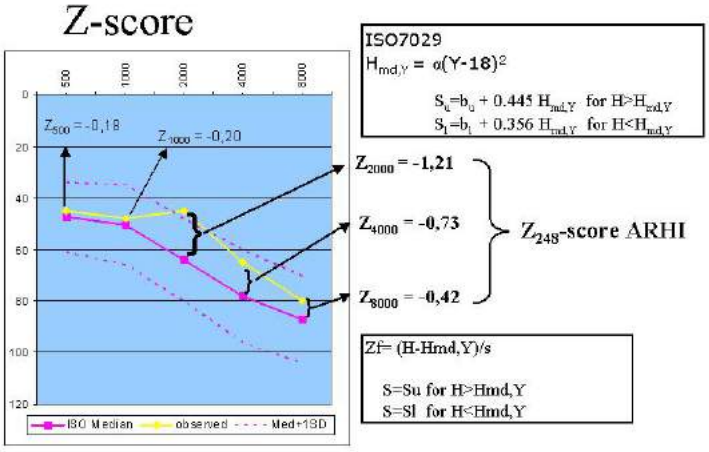



Figure12 Example of the Z-score conversion which is a calculation of how many standard deviations an observed threshold is away from the expected P50 ISO 7029 thresholds. The conversion averaged over 2, 4 and 8 kHz are referred to as the z-score that quantifies ARHI.

**Assessment of repeated or multiple audiometric threshold measurements.**
When several audiograms are available for one case at different times or for several cases from one family there are more possibilities to visualize these measurements in one plot.
It is even possible to perform statistical analysis on multiple measurements. Govaerts et al proposed box and whisker plots to graphically present pre- and post operative audiometric data for conductive hearing loss in otosclerosis. A single graph would thus present more than simply a measured threshold by demonstrating the mean threshold. (Fig. 13D)

This method has successfully been applied in genetic research for a Belgian family with hereditary otovestibular dysfunction that was proved to be caused by a mutation in the *COCH*-gene as shown in figures 13.

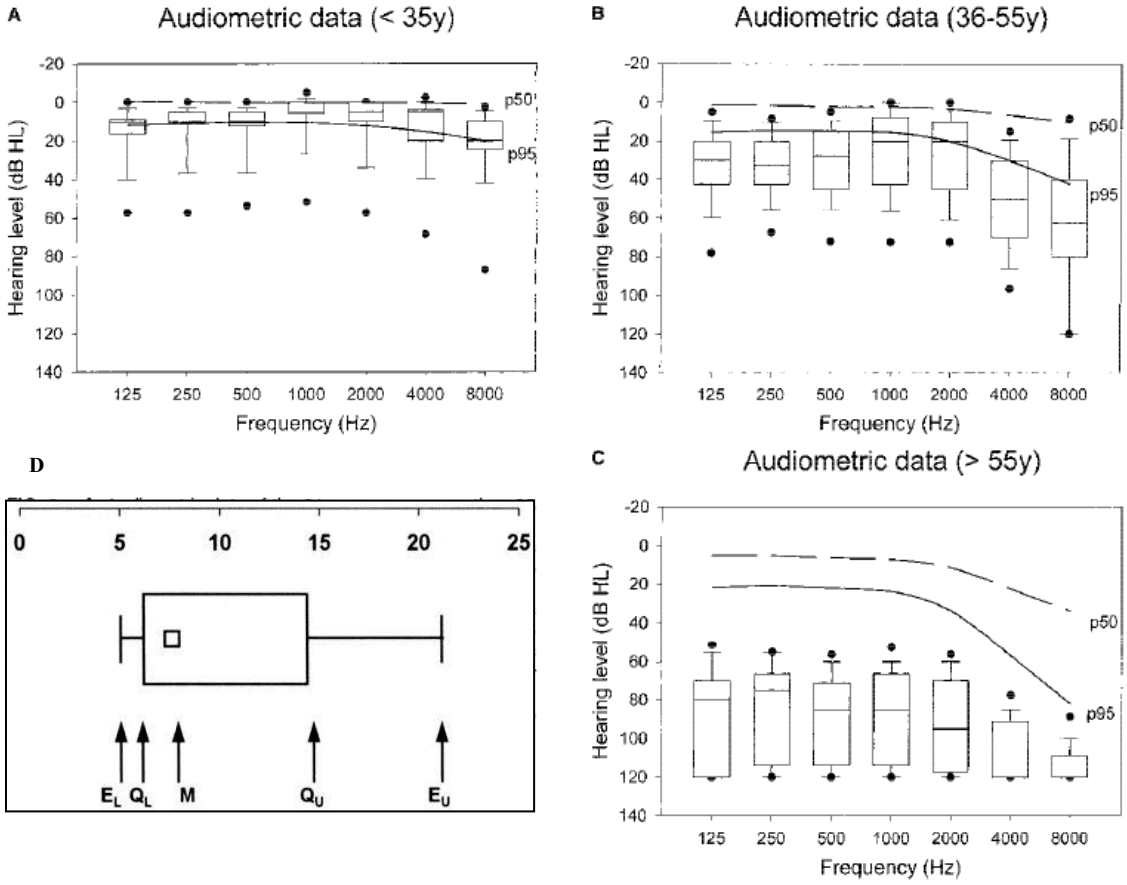



Figure 13 Audiometric data represented as box and whisker plots.
A) Data of family members younger than 35 years. The upper solid line is the p50 curve of a normal population and the lower dotted line is the p95 percentile. B) Thresholds of family members aged between 36 and 55 years C) Thresholds of family members of 55 years and older. D) Box and whisker plots of a hypothetical sample. $E_L$ lower extreme, $Q_L$ lower quartile (P25), M median, $Q_U$ upper quartile (P75), $E_U$ upper extreme. The upper scale represents thresholds in decibels.
Modified from Verstreken et al 2001 (21)

In figure 13 the family members have been divided into age-groups and the mean threshold of the group was plotted in box and whisker plots against ISO7029 P50 and P95 normative thresholds to recognize affected family members from unaffected.
Another graphical method of presenting multiple audiograms in one figure is by using three-dimensional audiograms. Although these plots seem impressive they have not found a useful application in characterizing phenotypes of hearing impairment yet. Nevertheless, some examples are demonstrated in figure15. The Glasgow benefit Plot has to be mentioned to be complete on the issue on graphical audiogram presentations.(22) This method is based on

graphical interpretation of audiograms to study benefit from middle ear surgery and is beyond the scope of clinical genetic research.

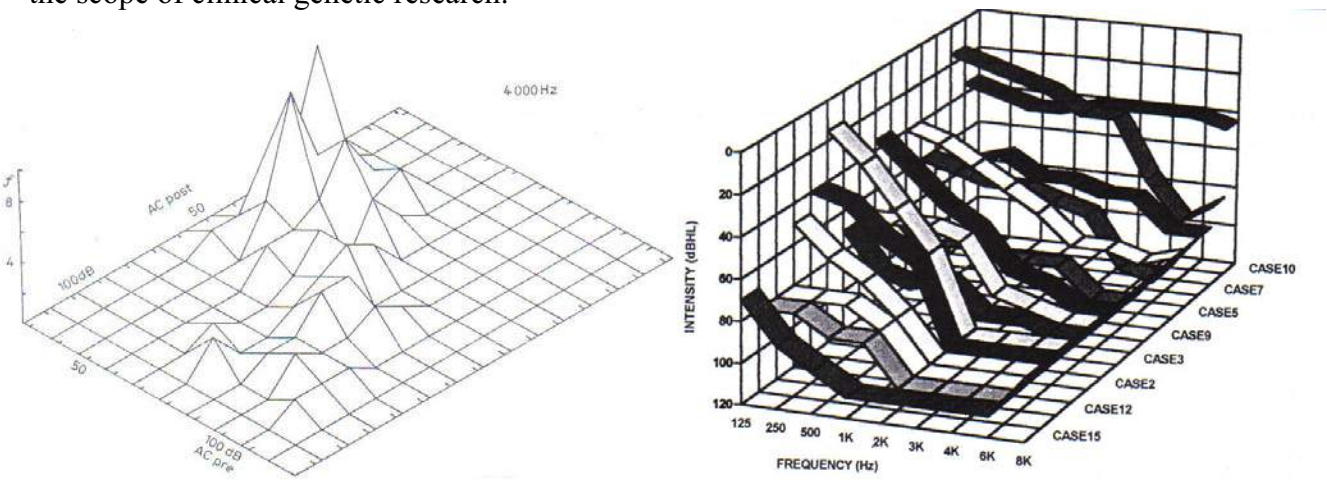



Figure 15 Left side from EHAM Mares, PLM Huygen, OWJM de Jong vd Brand, The classification of audiograms in Otosclerosis. ORL 35:205-209 1973.
Right side from thesis dissertation on progressive autosomal dominant hearing loss due to a genetic defect on chromosome 1 in families from west Java. BD Djelantik, 1996 University of Antwerp

**Progression of thresholds**

When at least three audiograms from different ages (preferably over decades) are available for an individual, longitudinal progression of hearing thresholds can be analysed with linear regression analysis. In case of symmetrical hearing loss a binaural mean of the hearing thresholds are plotted against time for each frequency and the best fitting line calculated. Thus deterioration of hearing thresholds in time related to the type of hearing loss in question can be tested. This concept is very valuable for counselling other patients with the same type of hearing impairment. The clinician can explain what is to be expected in time and possible precautions can be taken for threatening profound deafness. A comparison between types of hearing impairment is even more facilitated by these analysis because they also describe the progression rate (slope of best fitted line) and onset age (intercept with time axis). In fact, these analyses are currently applied in genetic research for family studies. A cross-sectional linear analysis is possible when audiograms of several affected members of different age are available. A powerful tool in comparing the regression analysis between several types of hereditary hearing impairment is supplied by Age Related Typical Audiograms (ARTA).(23) These are graphical presentations of expected thresholds per decade based on the results of linear regression analysis (slope and intercept) as show in figure 16.

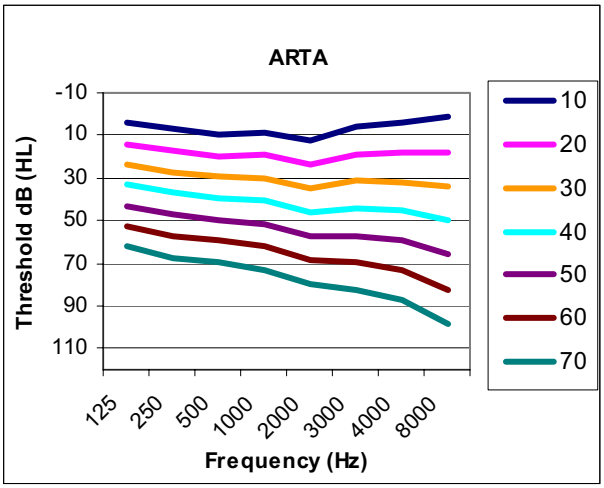



Figure 16 ARTA from own archive of the first Belgian Family with hereditary hearing Impairment that localizes to the DFNA22 locus. Mutation analyses for the MYO6 gene are being performed. Legend shows age in years. The ARTA indicates fairly flat thresholds with significant deterioration over the years in this family.

A more sophisticated method of comparing and charactering phenotypes is encompassed in the threshold features array (TFA) which is a one-dimensional array (N=9). With this array a conventional chi-square test can even be performed to compare TFA of different types of hearing impairments. Figure 17 describes how TFA and ARTA are derived from regression analyses.

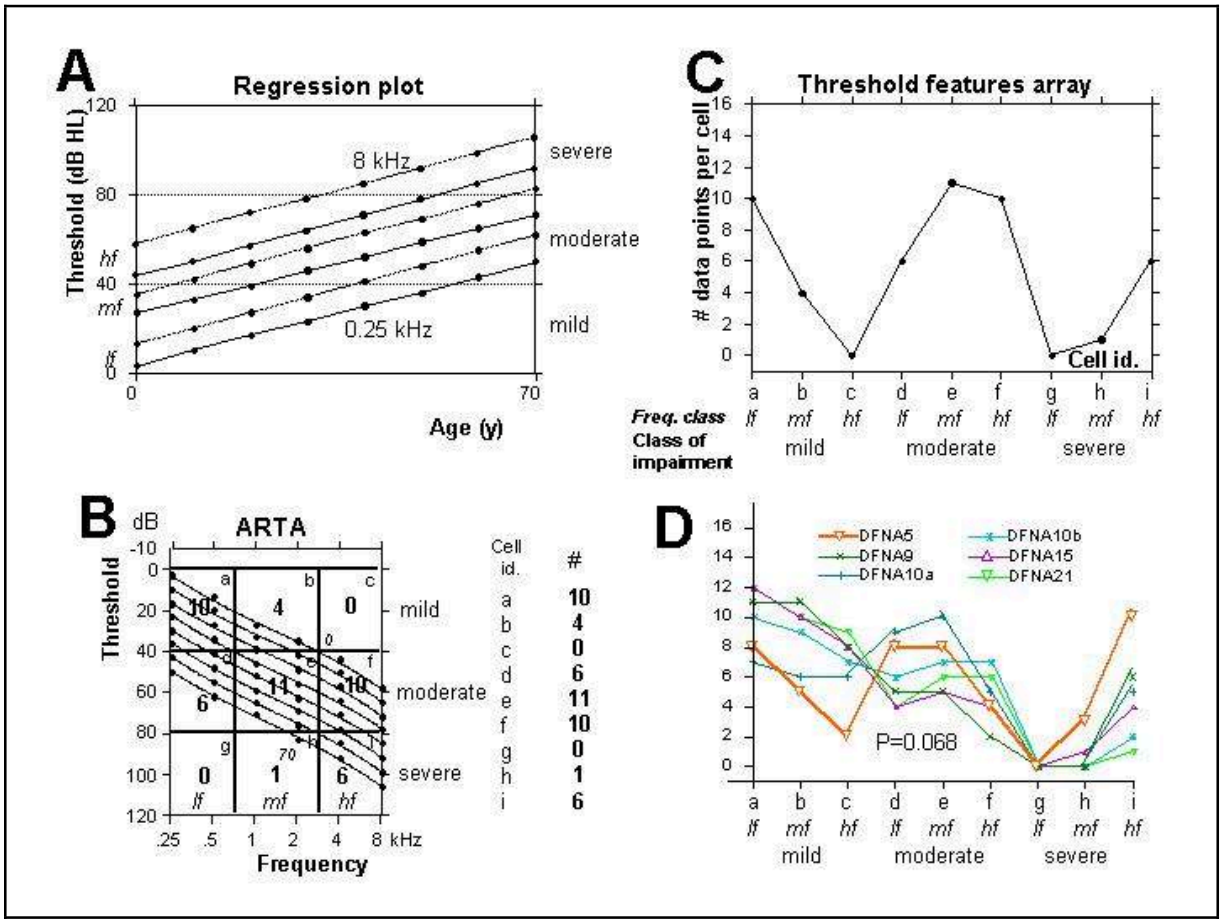



Figure 17 Illustrating the method used to transform age-related threshold findings obtained in regression plot (A) into ARTA (B) and from there into a threshold features array (C). The number of data points in (A) is counted for 3 frequency classes, i.e. the low frequencies (lf, 0.25-0.5 kHz), the mid frequencies (mf, 1-2 kHz) and the high frequencies (hf, 4-8 kHz) for each class of hearing impairment, mild, moderate and severe. The counts of data points per cell (bold) are included in the corresponding cells (identification, a-i) as indicated in each cell in the ARTA panel (B). The one-dimensional threshold features array is included in (B) and the count (#) per cell is plotted in (C). Italic figures 0 and 70 in (B) indicate age (year). Panel D gives an example how this method can be applied to compare several autosomal dominant types of deafness (DFNA5-21) with a Chi-square test (P-value indicated in panel D). From: Characterizing and distinguishing progressive phenotypes in nonsyndromic autosomal dominant hearing impairment PLM Huygen, RJE Pennings, CWRJ Cremers. Audiological Medicine 2003; 1; 37-46 with permission of R. Pennings and P. Huygen.

Various audiometric parameters were produced expeditiously short after the Second World War and since than many characteristics of hearing impairments have been recognized. Over the years many statistical and graphical tools have been created. However they all are based on clinical and audiometric tests. Table 1 enumerates these clinical and audiological items that are useful in characterizing hereditary hearing impairment (HHI).

Table 2 Essential Clinical and Audiological parameters for phenotyping hereditary hearing impairment

| | | |
|---|---|---|
| Audiological | Type | Conductive/Perceptive/Mixed See figure 3 |
| | Degree | Normal to profound deaf See figure 1 |
| | Configuration | See figure 2 |
| | Onset age | Estimated or calculated |
| | Progression | Estimated or calculated |
| Clinical | Mode of inheritance | Aut.Dom. / Aut.Rec. / X-linked / Mitochondrial |
| | Penetrance | Intrafamilial or interfamilial variability |
| | Consanguinity | Hints often at Aut.Rec. inheritance |
| | Origin | Ethnicity and geological origin |
| | Onset | Congenital, Prelingual or postlingual |
| | Tinnitus | |
| | Vestibular involvement | Assessed with electronystagmography |
| | Syndromal Signs | Most often extra clinical tests required |

Aut.Dom. = autosomal dominant and Aut.Rec. = autosomal recessive

Because the cochlea and vestibular system are embrologically and anatomically related it is important to ask for balance problems of patients with hearing impairment. Standard clinical electronystagmography (ENG) assesses the movements of each eye separately by placing electrodes to the left and right of each eye. The ENG records of spontaneous nystagmus, followed by tests for gaze-evoked nystagmus, saccades, optokinetic nystagmus and smooth pursuit. Subsequently, the horizontal rotational vestibulo-ocular reflex (VOR) is assessed using a chair which rotates sinusoidally with a maximum velocity of 50°/s and with a frequency of 0.05 Hz. This VOR test is performed in total darkness while the subject performs a mental task to ensure alertness. The head velocity is measured with an angular rate sensor that is placed to the subject's head by means of a velcro strap. The ratio of the eye velocity (response) to the head velocity (stimulus) defines the gain, whereas the time delay between the response and stimulus is called the phase. This test is very useful to assess the level of compensation and overall responsiveness of the subject's VOR. Last part of the investigation is the caloric test which is performed to provide information about the total responsiveness of the horizontal semicircular canals. For this test, the subject is placed such that the horizontal semicircular canal is positioned vertically. Both ear canals are consecutively irrigated with warm (44°) and cold (30°) water for 30 seconds with a volume of 180 cc. The subject is instructed to close the eyes and perform mental tasks. Labyrinth and nystagmus asymmetry are calculated using Jonkees'formula, based on the maximum slow component velocities. Additionally, total responsiveness of the horizontal semicircular canals (= the sum of the 4 irrigations) is assessed. All values are referenced to normative data, obtained in the same setting, using the same protocol. Calibration is performed repeatedly throughout the entire test and prior to caloric irrigation.

Electro-oculography and electro-retinography are also useful clinical test in assessing deaf-blindness in Usher syndrome. Sometimes embryological relations between various clinical manifestations are not that obvious. Therefore it is necessary to be informed about hearing impairment that occurs in the context of a genetic syndrome.

## 1.6 Strategies for genetic analysis of hearing impairment

Up to 1 percent of approximately 30.000 human genes are involved in hearing, which reflects the complexity of our hearing organ.(24) A gene is defined as a functional physical unit of a heredity that can be passed from parent to child, which is in fact a piece of DNA. As indicated before a distinction has to be made between monogenic diseases and complex genetic diseases. Monogenic diseases are caused by a defect (mutation) in a single gene. Genes responsible for monogenic deafness can be identified with positional cloning. With this strategy genetic linkage analysis aims to establish linkage between disease causing genes and genetic markers in large families with hearing impairment. The segregation of the investigated phenotype in the family pedigree is compared to the segregation of approximately 400 polymorphic marker alleles. The statistical estimate whether the phenotype and the marker are likely to lie near each other on a chromosome and therefore likely to be inherited as a package, is reflected with a Logarithm of Odds (LOD score).

$$\text{LOD score} = z = \log \left( \frac{\text{Probability pedigree data under hypothesis of linkage}}{\text{Probability pedigree data under hypothesis of no linkage}} \right)$$

A LOD score of 3.3 (or more) means that the odds are a thousand to one in favour of genetic linkage, which is generally accepted as statistically significant in a genome-wide linkage analysis. After linkage is found, a candidate region between 2 flanking markers can be determined that co-segregates in the family pedigree together with the deafness trait. In this region the genetic defect is located that causes the deafness. By systematically analyzing all genes located in this region by mutation analysis a mutation in the disease causing gene is eventually discovered.

Misinterpretation of one ore more individual's hearing can interfere with LOD score calculations and undermine the study. As a rule of thumb, a family with at least 10 volunteers with hearing impairment is suitable for linkage analyses. Participation of normal hearing family members is also required. A distinction between affected and unaffected family members has to be made by clinical and audiological examination, which is the main subject of this thesis. Many audiological and clinical pitfalls are described above. There are also some genetic pitfalls for clinical examinations such as phenocopies and genetic heterogenecity. A phenocopy is (an individual in which) an environmental factor mimics the genetic trait (hearing loss) that is at study. They have to be excluded from the linkage analyses. Genetic locus heterogenecity indicates that a certain disease can be caused by different genes. Monogenic hearing impairment is one of the genetic diseases with the highest locus heterogenecity. This generally means that linkage analysis needs to be carried out in each family separately. However, careful and thorough clinical examinations can make distinctions between some of the genes and this can help linkage analysis considerably. Moreover this type of phenotypic characterization can be very helpful for genetic testing. At the moment 21 genes causing autosomal dominant and 23 genes causing autosomal recessive non-syndromic deafness are identified.(25) The loci for non-syndromic hereditary hearing loss are indicated with abbreviations. DFN is the root for the locus symbol for deafness. A suffix A or B indicates that the mutant allele is segregating in an autosomal dominant or autosomal recessive pattern, respectively. Subsequently a numerical suffix is assigned to discern the loci (Table 3 and 4). Nearly all of these loci were discovered by linkage analyses on single large families.

| Table 3 Nonsyndromic autosomal dominant loci | | | | | | |
|---|---|---|---|---|---|---|
| **Locus name** | **Location** | **Gene** | **Onset** | **Freq.** | **Type** | **Reference** |
| DFNA1 | 5q31 | DIAPH1 | Po | L/A | P | (26;27) |
| DFNA2 | 1p34 | GJB3 / KCNQ4 | Po | H | P | (28-30) |
| DFNA3 | 13q12 | GJB2 / GJB6 | Pr | H (L) | S | (31-33) |
| DFNA4 | 19q13 | MYH14 | Po | M, A | P | (34;35) |
| DFNA5 | 7p15 | DFNA5 | Po | H | P | (36;37) |
| DFNA6/14/38 | 4p16 | WFS1 | Pr | L | S | (38-41) |
| DFNA7 | 1q21-q23 | Unknown | Po | H, A | P | (42) |
| DFNA8/12 | 11q22-24 | TECTA | Pr | M | S | (43;44) |
| DFNA9 | 14q12-q13 | COCH | Po | H | P | (45;46) |
| DFNA10 | 6q22-q23 | EYA4 | Po | A | P | (47;48) |
| DFNA11 | 11q12.3-q21 | MYO7A | Po | A | P | (49;50) |
| DFNA13 | 6p21 | COL11A2 | Pr | M, H | S | (51;52) |
| DFNA15 | 5q31 | POU4F3 | Po | A | P | (53) |
| DFNA16 | 2q24 | Unknown | Po | H | P | (54) |
| DFNA17 | 22q12-13 | MYH9 | Po | H, A | P | (55;56) |
| DFNA18 | 3q22 | Unknown | Po | | | (57) |
| DFNA19 | 10 pericentric | Unknown | Pr | M | S | (58) |
| DFNA20/26 | 17q25 | ACTG1 | Po | H | P | (59-61) |
| DFNA21 | 6p21-22 | Unknown | Po | M, H | P | (62) |
| DFNA22 | 6q13 | MYO6 | | | | (63) |
| DFNA23 | 14q21-q22 | Unknown | | | | (64) |
| DFNA24 | 4q | Unknown | Pr | M, H | S | (65;66) |
| DFNA25 | 12q21-24 | Unknown | | | | (67) |
| DFNA27 | 4q12 | Unknown | | L,M,H | P | (68) |
| DFNA28 | 8q22 | TFCP2L3 | Po | H | P | (69) |
| DFNA29 | | | | | | R |
| DFNA30 | 15q25-26 | Unknown | | | | (70) |
| DFNA31 | 6p21.3 | Unknown | Po | M,H | P | (71) |
| DFNA32 | 11p15 | Unknown | | | P | (72) |
| DFNA33 | | | | | | R |
| DFNA34 | 1q44 | | Po | | P | (73) |
| DFNA35 | | | | | | R |
| DFNA36 | 9q13-q21 | TMC1 | | | | (74) |
| DFNA37 | 1p21 | | Po | H | P | (75) |
| DFNA39 | 4q21.3 | DSPP | | | | (76) |
| DFNA40 | 16p12 | | | | | R |
| DFNA41 | 12q24-qter | Unknown | | | | (77) |
| DFNA42 | 4q28 | Unknown | Po | | P | (78) |
| DFNA43 | 2p12 | | Po | L,M,H | P | (79) |
| DFNA44 | 3q28-29 | | Po | | P | (80) |
| DFNA45 | | | | | | R |
| DFNA46 | | | | | | R |
| DFNA47 | 9p21-22 | Unknown | Po | | P | (81) |
| DFNA48 | 12q13-q14 | MYO1A | | | | (82;83) |
| DFNA49 | 1q21-q23 | Unknown | Po | L,M (H) | P | (84) |
| DFNA50 | 7q32 | Unknown | Po | L,M,H | P | (85) |
| DFNA51 | 9q21 | | | | | R |
| DFNA52 | | | | | | R |
| DFNA53 | 14q11-q12 | Unknown | | | | (86) |
| DFNA54 | 5q31 | Unknown | | | | (87) |

Table 3 Nonsyndromic autosomal dominant loci for hearing impairment. Age of onset is indicated with Prelingual ($P_r$) or Postlingual ($P_o$). Commonly affected frequencies are indicated with L for Low, M for Mid, H for High and A for All measured frequencies in standard audiometry. When data are present about the evolution of the trait in time it is indicated with P for Progressive and S for Stable. R=Reserved Locus name

| Table 4 Nonsyndromic autosomal recessive loci | | | | | | |
|---|---|---|---|---|---|---|
| **Locus name** | **Location** | **Gene** | **Onset** | **Freq.** | **Type** | **Reference** |
| DFNB1 | 13q12 | GJB2 | Pr | A | S | (88;89) |
| DFNB2 | 11q13.5 | MYO7A | Pr + Po | A | | (90-92) |
| DFNB3 | 17p11.2 | MYO15 | Pr | A | S | (93;94) |
| DFNB4 | 7q31 | SLC26A4 | Pr | A | S | (95;96) |
| DFNB5 | 14q12 | Unknown | Pr | A | S | (54) |
| DFNB6 | 3p14-p21 | TMIE | Pr | A | S | (97;98) |
| DFNB7 | 9q13-q21 | TMC1 | Pr | A | S | (74;99) |
| DFNB8 | 21q22 | TMPRSS3 | Po | A | P | (100;101) |
| DFNB9 | 2p22-p23 | OTOF | Pr | A | S | (102;103) |
| DFNB10 | 21q22.3 | TMPRSS3 | Pr | A | S | (101;104) |
| DFNB11 | 9q13-q21 | TMC1 | Pr | A | S | (74;105) |
| DFNB12 | 10q21-q22 | CDH23 | Pr | A | S | (106;107) |
| DFNB13 | 7q34-36 | Unknown | | | | (108) |
| DFNB14 | 7q 31 | Unknown | | | | (109) |
| DFNB15 | 3q21-q25 19p13 | Unknown | Pr | A | S | (110) |
| DFNB16 | 15q21-q22 | STRC | | | | (111) |
| DFNB17 | 7q31 | Unknown | Pr | A | S | (112) |
| DFNB18 | 11p14-15.1 | USH1C | Pr | | | (113;114) |
| DFNB19 | 18p11 | Unknown | | | | (115) |
| DFNB20 | 11q25-qter | Unknown | | | | (116) |
| DFNB21 | 11q | TECTA | Pr | | | (117) |
| DFNB22 | 16p12.2 | OTOA | | | | (118) |
| DFNB23 | 10p11.2-q21 | PCDH15 | | | | (119) |
| DFNB24 | 11q23 | Unknown | | | | R |
| DFNB25 | 4p15.3-q12 | Unknown | | | | R |
| DFNB26 | 4q31 | Unknown | | | | (120) |
| DFNB27 | 2q23-q31 | Unknown | | | | (121) |
| DFNB28 | 22q13 | TRIOBP | | | | (122;123) |
| DFNB29 | 21q22 | CLDN14 | | | | (124) |
| DFNB30 | 10p12.1 | MYO3A | | | | (125) |
| DFNB31 | 9q32-q34 | WHRN | | | | (126;127) |
| DFNB32 | 1p13.3-22.1 | Unknown | | | | (128) |
| DFNB33 | 9q34.3 | Unknown | | | | (129) |
| DFNB34 | | | | | | R |
| DFNB35 | 14q24.1-24.3 | Unknown | | | | (130) |
| DFNB36 | 1p36.3 | ESPN | Pr | A | | (131) |
| DFNB37 | 6q13 | MYO6 | Pr | A | | (132) |
| DFNB38 | 6q26-27 | Unknown | Pr | A | | (133) |
| DFNB39 | 7q11.22-q21.12 | Unknown | Pr | A | | (134) |
| DFNB40 | 22q11.21-12.1 | Unknown | Pr | A | | (135) |
| DFNB41 | | | | | | R |
| DFNB42 | 3q13.31-q22.3 | Unknown | | | | (136) |
| DFNB43 | | | | | | R |
| DFNB44 | 7p14.1-q11.22 | Unknown | | | | (137) |
| DFNB45 | | | | | | R |
| DFNB46 | 18p11.32-p11.31 | Unknown | | | | (138) |
| DFNB47 | 2p25.1-p24.3 | Unknown | | | | (139) |
| DFNB48 | 15q23-q25.1 | Unknown | | | | (140) |
| DFNB49 | 5q12.3-q14.1 | Unknown | Pr | A | S | (141) |
| DFNB50 | 12q23 | Unknown | | | | |
| DFNB51 | 11p13-p12 | Unknown | | | | (142) |
| DFNB52 | | | | | | R |
| DFNB53 | 6p21.3 | COL11A2 | | | | (143) |
| DFNB54 | | | | | | R |
| DFNB55 | 4q12-q13.2 | Unknown | | | | (144) |
| DFNB56 | | | | | | R |
| DFNB57 | | | | | | R |
| DFNB58 | 2q14.1-q21.2 | | | | | R |
| DFNB59 | 2q14.1-q21.2 | PJVK | Pr | | | (145) |
| DFNB60 | 5q22-q31 | Unknown | | | | R |
| DFNB61 | | | | | | R |
| DFNB62 | 12p13.2-p11.23 | | | | | (146) |
| DFNB63 | | | | | | R |
| DFNB64 | | | | | | R |
| DFNB65 | 20q13.2-q13.32 | | | | | (147) |
| DFNB66 | 6p21.1-p22.3 | LHFPL5 | | | | (148-150) |
| DFNB67 | 6p21.1-p22.3 | LHFPL5 | | | | (148-150) |

Table 4 Nonsyndromic autosomal recessive loci for hearing impairment. (Identical abbreviations as table 3).

Several mtDNA mutations have been demonstrated in families in which hearing loss shows a maternal inheritance pattern and affected males do not transmit the disease to their offspring. (25) In mitochondrial deafness, hearing impairment is often an additional symptom in a number of syndromic diseases caused by mitochondrial DNA defects. Most mitochondrial mutations have a pleiotropic phenotype leading to various clinical conditions comprising MELAS, Mitochondrial Encephalopathy, Lactic Acidosis and Stroke-like episodes, MERRF, Myoclonic Epilepsy and Ragged Red Fibers and MIDD, Maternally Inherited Diabetes and Deafness. Mitochondrial non syndromic hearing impairment does not occur often and is sometimes aminoglycoside induced or worsened.

Complex genetic deafness is defined as genetic susceptibility that leads to hearing impairment when triggered by certain environmental factors. Complex genetic also implies that more than one gene can be involved. Presbyacusis, otosclerosis and noise induced hearing loss are examples that probably have a complex genetic origin.

A prevalent strategy to analyse complex genetic traits is a genetic association study. It aims to associate variations in human DNA sequence with a disease. Genetic associations arise because human populations share common ancestry and in a sense association studies are a special form of linkage study in which the extended family is the wider population.(151) In most association studies single nucleotide polymorphisms (SNPs) are used as genetic markers. These SNPs are variants changing a single base pair of DNA and occur on average every 300 base pairs. Many SNPs have been genotyped by the HapMap project.(152) The HapMap project has set goal to develop an inventory in the human genome taking into acoount the haplotype structure. The most frequently used study design for genetic association studies is the case control study. Genetic association studies for hearing impairment also depend on careful clinical and audiological examination of a large population of unrelated cases. Patients and controls are matched for age, sex and ethnicity. When an association is found between a certain form (allele) of a genetic marker and a type of deafness the frequency of this allele will differ between the patient and control group. The detected allele can be the causative variant of a deafness trait (direct association) or it can be in linkage disequilibrium (LD) with the disease causing mutation that is inherited (indirect association). Linkage disequilibrium is defined as non random association between alleles on different but linked loci.

Today genome-wide association studies are possible thanks to progress in genetic research as well as technological progress. However they are accompanied by high financial costs. Therefore association studies are more frequently applied on selected candidate genes based on functional studies or candidate regions. As an example, chapter 8 describes an association study for age-related hearing impairment (ARHI) in the candidate gene *KCNQ4*. The *KCNQ4* gene is a known deafness gene associated with DFNA2. The type of sensorineural hearing loss in DFNA2 is fairly similar to age-related hearing loss and therefore this gene was considered a good candidate gene for ARHI.

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# Chapter 2

## Genetic testing for hearing impairment

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# Genetic testing for hearing impairment

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**Key-words.** Hereditary hearing impairment; deafness genes; genetic screening

**Abstract.** *Genetic testing for hearing impairment.* For some patients, genetic testing can reveal the etiology of their hearing impairment, and can provide evidence for a medical diagnosis. However, a gap between fundamental genetic research on hereditary deafness and clinical otology emerges because of the steadily increasing number of discovered genes for hereditary hearing impairment (HHI) and the comparably low clinical differentiation of the HHIs. In an attempt to keep up with the scientific progress, this article enumerates the indications of genetic testing for HHI from a clinical point of view and describes the most frequently encountered HHIs in Belgium. Domains of recent scientific interest, molecular biological aspects, and some pitfalls with HHIs are highlighted.
The overview comprises bilateral congenital hearing loss, late-onset progressive high frequency hearing loss, progressive bilateral cochleo-vestibular deficit, and progressive low frequency hearing loss. Also, several syndromal forms of HHI are summarized, and the availability of genetic tests mentioned. Finally, the requirements for successful linkage analysis, an important genetic research tool for localizing the potential genes of a trait on a chromosome, are briefly described.

## Introduction

The last 15 years, molecular genetics has contributed enormously to our understanding of the inner ear. Nowadays, more than 100 chromosome loci are known to carry genes necessary for the proper functioning of the inner ear. Moreover, 43 deafness genes have been successfully identified, and their DNA sequences are known.[1] A specific website on genetic deafness is regularly updated and can be found at http://www.uia.ac.be/dnalab/hhh.[2] Since 1995, the number of identified deafness genes has been rapidly increasing, but the clinical applications remain limited to counselling. Today, it is possible to test whether an individual is a carrier of certain sequenced deafness alleles. The main otologic phenotype characteristics relevant to identification of a HHI are abnormal ear morphology (pinna, middle ear, cochlea), onset age of the hearing loss, frequency profile of the hearing loss, progression rate of the hearing loss, and vestibular function.[3,4]

The aim of this article is to give an overview of available diagnostic tests for HHI and their indications in Belgium from a clinician's viewpoint. Indications for genetic testing of common deafness genes are described and genotype-phenotype correlations are highlighted.

Figure 1 shows a conceptual framework on the aetiological, clinical, and hereditary aspects of hearing impairment. The upper level shows a spectrum representing the relative contribution of environmental and inherited aetiological factors for hearing impairment; purely acquired deafness is indicated on the left , and purely genetic deafness on the right.[5] It is currently believed that about half of the congenital hearing impairments are caused by genetic factors.[6] Most of those inherited congenital hearing impairments are monogenic (caused by a mutation in a single gene) and can be subdivided according to their mode of inheritance. A distinction can also be made between non-syndromal (70%) and syndromal forms (30%), in which deafness is accompanied by other pathological manifestations. Monogenic traits can have phenotypic variability and a variable expression or reduced penetrance. These

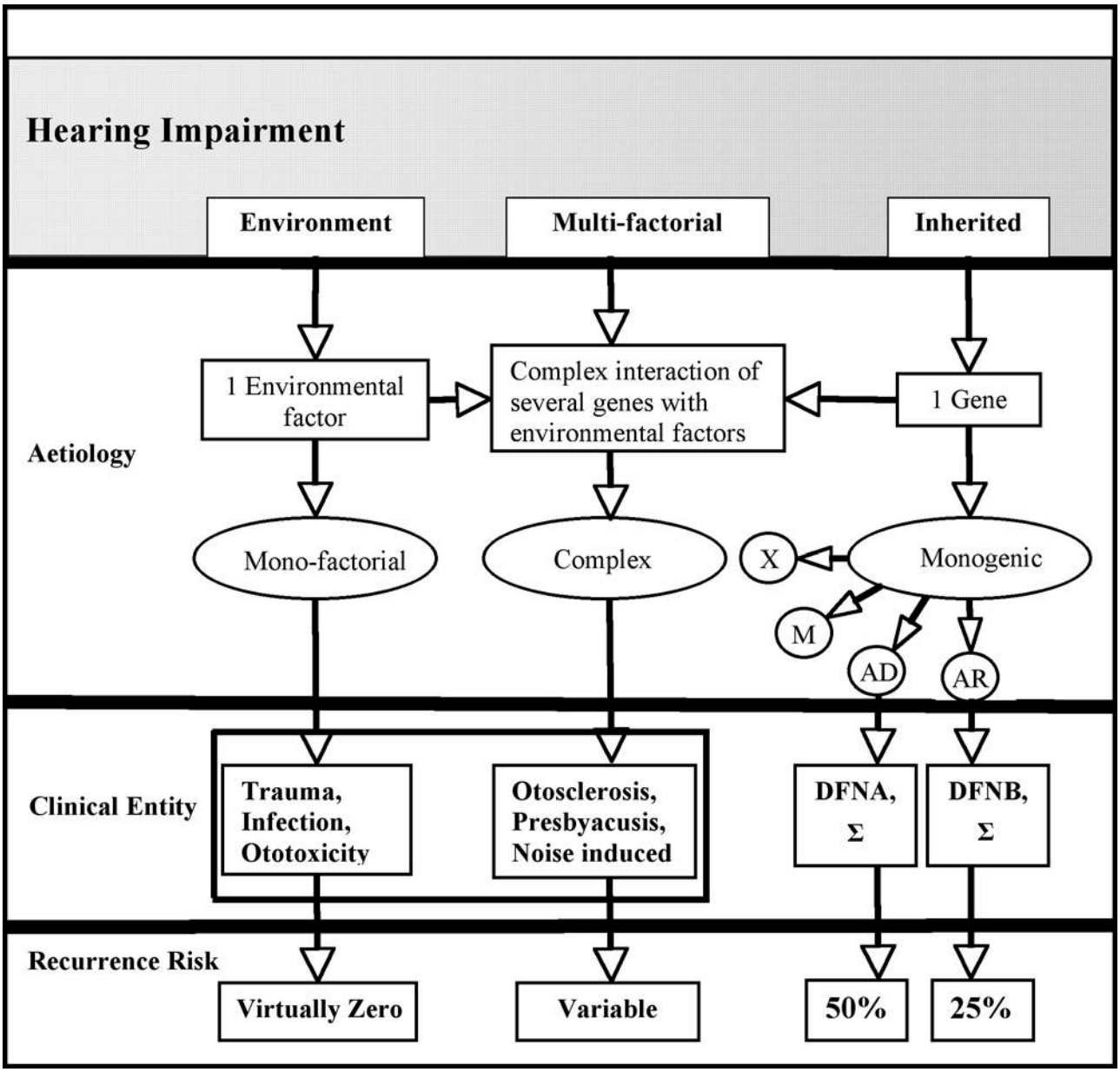


*Figure 1*

Aetiology, clinics, and hereditary aspects of hearing impairment.
The graded shading of the upper level represents the relative contribution of environmental and inherited factors for hearing impairment: purely environmental aetiology on the left, and purely inherited on the right.[5] Multi-factorial aetiology (or complex genetic) is shown in the middle. The dotted line symbolizes that acquired forms of hearing impairment can also depend on genetic susceptibility, and therefore be considered as complex genetic. Today only some monogenic forms of HHI are unravelled sufficiently to calculate recurrence risks (in percentage) as indicated on the lowest level. (AD= Autosomal dominant, AR = Autosomal Recessive, X= X-linked and M= Mitochondrial inheritance, Σ = Syndromal Hearing Impairment).

inconsistencies with Mendelian inheritance can be attributed to environmental factors and modifier genes. Therefore, some types of HHI that were initially considered as monogenic, might in fact be complex genetic traits.

Multi-factorial hearing impairment as indicated in the centre of Figure 1 has a complex genetic founding (involving several genes) but its occurrence is facilitated by numerous environmental factors. The term complex deafness also refers to an interaction

between several genes and different (unknown) environmental triggers. Otosclerosis, presbyacusis and noise induced hearing loss are members of this group. Some of them have circumstantial evidence for a genetic origin but the susceptibility genes are not yet found. For Age Related Hearing Impairment (ARHI), a complex genetic heredity has been reported.[7] Two large scale multicenter studies on ARHI and otosclerosis are currently running under coordination of the department of Medical Genetics of the University of Antwerp.

Acquired deafness in our conceptual framework is due to purely environmental factors such as infection or trauma. Absence of any inheritance factor, of course, can be disputed for any trait. All types of hearing impairment, except for trauma, are likely to have genetic susceptibility genes. For example, it is well know that otitis serosa or cholesteatomata cluster in families.

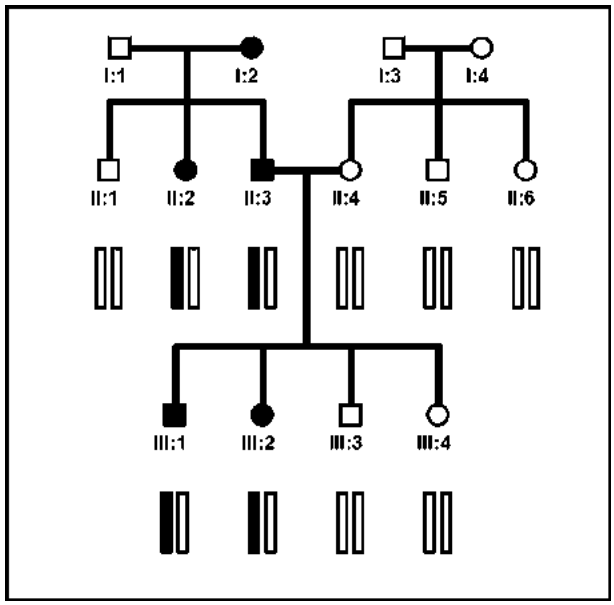


*Figure 2*
Pedigree chart of with autosomal dominant inheritance pattern of a DFNA2/KCNQ4 family with progressive high frequency hearing loss. Typical for autosomal dominant inheritance is that the trait does not skip a generation and that every offspring has a 50% risk for the trait. Unaffected family members do not pass on the trait to their children. Males and females are equally affected. Circles represent females and squares represent males. Filled symbols represent affected individuals. The bars underneath individuals represent pathogenic and wild-type alleles. Roman numbers indicate generations and Arabic numbers indicate individuals within a generation.

*Classical inheritance patterns*

Autosomal dominant inheritance describes a pattern of inheritance in which the phenotype is expressed in those who have inherited only one copy of a particular gene mutation (allele) and refers to a gene on one of the 22 pairs of autosomes (non-sex chromosomes). Today, 21 autosomal dominant non-syndromic deafness genes are known.[2] Their chromosomal localization is indicated with DFNA, where DFN stands for "deafness" and "A" for autosomal dominant. The abbreviation is followed by a number that represents the order of discovery of the chromosome locus. Currently, DFNA54 is the most recently reported chromosome locus.[8] A chromosome locus is a physical site on a chromosome. Prior to identifying and sequencing a putative disease causing gene, its chromosome locus has to be determined; this is achieved by linkage analysis. Linkage analysis tests for co-segregation of any chromosomal locus and a trait locus of interest.

Figure 2 demonstrates autosomal dominant inheritance in a pedigree chart of a DFNA2 family. DFNA2 is characterized by progressive, high-frequency sensorineural hearing impairment. At its locus on chromosome 1p34, two deafness genes have been identified: the GJB3 gene that encodes connexin 31, a gap junction subunit, and the KCNQ4 gene that encodes the subunits of a voltage-gated potassium channel.[2] Both genes presumably play a role in the recycling of potassium ions from the hair cells to the endolymph. Currently, at least 15 families with progressive high-frequency sensorineural hearing impairment linked to the DFNA2 locus have been studied. Ten of these DFNA2 families harbour a mutation in the KCNQ4 gene and five in the GJB3 gene. For three families linked to DFNA2, no mutation has been found yet, and thus, a third gene might be involved in this chromosome locus. Other late onset, progressive high frequency HHI loci are DFNA5 and DFNA7 reported in

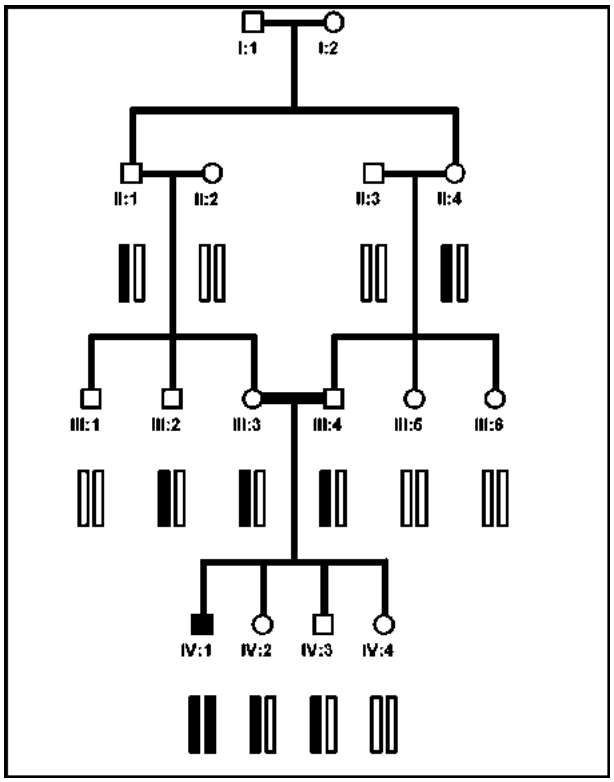


*Figure 3*
Pedigree chart representing autosomal recessive inheritance pattern in a DFNB1/GJB2 family. The pathogenic GJB2 allele is indicated with a filled bar, and the wild-type allele with a non-filled bar. When both parents are heterozygous carriers, each offspring has a 25% risk for the deafness, and a 66% risk for carrying the pathogenic allele. When one of the parents is homozygous for the wild-type allele, none of the offsprings will have the deafness. Males and females are equally affected. Consanguinity is indicated with a double marriage line (same symbols as Figure 2).

Dutch and Norwegian families respectively.[2]

Autosomal recessive inheritance refers to the need for two mutated alleles in order to cause a certain trait. About 75% of the congenital HHIs are autosomal recessive.[9] Recessive traits occur more often in consanguineous marriages. Autosomal recessive HHIs are usually severe and prelingual, and are indicated with DFNB, with "B" standing for autosomal recessive. Today 59 DFNB loci are known, and 21 genes are identified.[2] In 1994, the first non-syndromic autosomal recessive deafness, DFNB1, was mapped to chromosome 13q12-13.[10] In most cases, the phenotype is asymmetrical and non-progressive, severe to profound prelingual deafness. Figure 3 shows a pedigree chart of a DFNB1 family. In 1997, three years after the discovery of the locus, the corresponding gene, GJB2, was identified. GJB2 encodes for a transmembrane protein called Connexin 26 (Cx26). Connexins are the structural components of gap junctions which allow intercellular passage of ions, messenger molecules, and metabolites.[11] Cx26 is present in the supporting cells of the organ of Corti, in the spiral ligament, and in the limbus. Gap junctions presumably play a role in the recycling of potassium ions from the hair cells to the endolymph. The size of GJB2 is small and therefore, it is relatively easy to screen for mutations. Moreover, since pathogenic alleles of GJB2 are relatively prevalent, screening for GJB2 is the most frequently performed genetic test for HHIs. (See paragraph: "Connexin26").

Mitochondrial inheritance of hearing loss is less frequent than autosomal inheritance, but a number of mutations have been described.[2] In case of mitochondrial inheritance, the deafness is mostly one of the signs of a syndrome. Mitochondrial non syndromic hearing impairment, however, has been demonstrated by the finding of several mtDNA mutations in families in which hearing loss shows a maternal inheritance pattern. Affected males do not transmit the disease to their offsprings. The first mutation associated with non-syndromic HHI was described in an Arab-Israeli family where deafness occurred after aminoglycoside exposure. A specific mitochondrial mutation that has been associated to hearing loss is frequent in certain countries including China and Spain, but has not been reported in Belgium,[2] see Table 1.

X-linked inheritance refers to a sex specific inheritance pattern, and can be subdivided into a dominant and recessive form. A father can never pass an X-linked trait to his son. An X-linked recessive trait gives rise to an inheritance

*Table 1*

Common diagnostic DNA tests for non-syndromic deafness genes

| Genotype | | | | Phenotype | | | |
|---|---|---|---|---|---|---|---|
| **Gene** | **Mutation** | **Protein** | **Biological role** | **Locus** | **Characteristic features** | **Audiometry** | **Onset** |
| *GJB2* | 35delG | Connexin26 | Gap junction | **DFNB1** | Caucasians | Moderate to profound | Prelingual |
| | 167delT | | | | Ashkenazi Jews | | |
| | 235delC | | | | Japanese, Korean | | |
| *GJB2* | Several | | | **DFNA3** | Associated epidermal defects | Profound high frequency | 2nd decade |
| *GJB6* | Several | Connexin30 | | | | Mid-high frequency | Not stated |
| *GJB3* | Some | Connexin31 | | **DFNA2** | Tinnitus | High frequency | 3rd decade |
| *KCNQ4* | Several | Subunit of $K^+$ channel | Voltage gated $K^+$ channel | | Progressive | High frequency | Prelingual |
| | | | | | | | |
| *COCH* | P51S | Cochlin | Extra cellular matrix | **DFNA9** | Belgian and Dutch progressive HI with vestibular involvement | Initially high frequency loss eventually all frequencies | 3rd or 4th decade |
| | | | | | | | |
| *WFS1* | Several | Wolframin | Transmembrane protein in E.R. | **DFNA6/14** | Allelic with Wolfram Σ | Low frequency loss | 2nd decade |
| | | | | | | | |
| *SLC26A4* | H723R IVS7-2A > G | Pendrin | Anion ($Cl^-$, $I^-$) transporter | **DFNB4** | Allelic with pendred Σ, postponed goitre onset, associated dilated vestibular aqueduct | Sloping, severe to profound high frequency loss | Prelingual |
| | | | | | | | |
| *OTOF* | Q829X | Otoferlin | Vesicle trafficking | **DFNB9** | Spanish and Cuban auditory neuropathy | Profound ABR=abnormal OAE=present | Prelingual |
| | | | | | | | |
| *12S rRNA* | 1555A → G | 12S Ribosomal RNA | Mitochondria | **DFN** | Mitochondrial inherited, often syndromale appearances aminoglycoside enhanced | Variable from normal to profound | Prelingual |

HI = Hearing impairment. Σ = Syndromal Deafness. E.R. = Endoplasmic Reticulum. $K^+$ = Potassium, $Cl^-$ = Chloride, $I^-$ = Iodide. ABR = Auditory brainstem response. OAE = Otoacoustic emission.

pattern in which all males are affected, and females are unaffected but transmit the disease. Families with X-linked HHI are rare. For X-linked non-syndromic HHI, eight loci, indicated with DFN, have been mapped, but one locus was withdrawn and another one appeared to be syndromic after restudy.[2] Only for DFN3, the gene is identified as POU3F4 (POU domain, class 3, transcription factor 4).[12] DFN3 is characterized by a profound deafness with or without a pseudo-conductive component, and is associated with a unique developmental abnormality of the ear.[13]

*HHIs in Belgium*

Bilateral congenital sensorineural hearing loss

Connexin 26 (GJB2)

In several races, mutations in the GJB2 gene account for approximately 50% of the severe to profound congenital HHIs.[14] Although more than 85 disease causing mutations are reported, one mutation, 35delG, predominates in the Caucasoid race.[15] Today, in Belgium and surrounding west-European countries, GJB2 is routinely analyzed by diagnostic laboratories. In Asian populations, the 235delC mutation is more prevalent, and in Ashkenazi Jewish populations, the 167delT. Each of these three mutations have probably their common founder.[16] The phenotype of DFNB1 can be described as bilateral, non-progressive, prelingual and profound deafness. Recently, it has been shown that homozygotes with two inactivating mutations in GJB2 have more severe hearing loss than homozygotes with two non-inactivating mutations. Individuals with one inactivating and one non-inactivating mutation have a hearing loss between both extremes, making the phenotype predictable to a certain extent in genetic counselling.[15] Some, less frequent, mutations in GJB2 cause autosomal dominant hearing impairment (DFNA3). These dominant forms are often associated with epidermal defects, as indicated in Table 1. Currently, a large multicenter study is collecting data on the phenotype of the 35delG mutation in the GJB2 gene.

Otoferlin (OTOF)

Otoferlin, coded by the OTOF gene, is a protein that is predominantly expressed in the inner hair cells of the cochlea, and probably plays a role in synaptic vesicle trafficking.[17] Mutations in the OTOF gene cause prelingual, severe to profound, non-syndromic HHI linked to DFNB9. A characteristic of the phenotype is that Otoacoustic Emissions (OAE) are present (normal outer hair cell function) but Auditory Brainstem Responses (ABR) are absent. Therefore, this trait is sometimes – according to us, wrongly - referred to as Auditory Neuropathy. Although several mutations are known, the Q829X mutation is very frequent in Spain, probably due to a common founder.[18] No Belgian DFNB9-families are known, but genetic tests are available.

Progressive bilateral cochleovestibular deficit

Cochlin (COCH)

The COCH gene is expressed in the cochlea and semicircular canals.[19] Today, six different mutations have been found, and the P51S mutation predominates in Belgium and the Netherlands because of a common founder.[20] The exact pathogenic mechanism is not yet fully understood, but defects in the COCH gene cause autosomal dominant non-syndromic HHI associated with vestibular symptoms (DFNA9). A direct genetic tests for the COCH gene is indicated when progressive, initially high frequency but eventually pan-cochlear HHI is associated with vestibular dysfunction.

Progressive low frequency hearing loss

Wolframin (WFS1)

The WFS1 gene encodes a glycoprotein that is localized in the endoplasmic reticulum, but its function is not yet completely understood. Homozygous inactivating mutations in the WFS1 gene cause the autosomal recessive Wolfram syndrome, whereas heterozygous non-inactivating mutations[3] account for the non-syndromic autosomal dominant low frequency sensorineural HHI DFNA6/14. Although DFNA6 and DFNA14 were originally reported as non-overlapping, re-evaluation of the original DFNA6 locus indicated that they were in fact the same. WFS1 is the gene of interest in both DFNA6 and DFNA14, now designated as DFNA6/14.[2] The hearing impairment is characterized by slowly increasing hearing thresholds at low frequencies. Four Dutch families and one German DFNA6/14 family have been described in Europe. Although no Belgian families are reported yet, this gene has an important diagnostic value because it is allelic with the Wolfram Syndrome. This is a progressive neurodegenerative disease characterized by Diabetes Insipidus, Diabetes Mellitus,

Optic Atrophy and Deafness (DIDMOAD). Psychiatric disorders, renal tract anomalies, gonadal atrophy and gastrointestinal dysmotility are also features of this rare syndrome (Table 2). In contrast to DFNA6/14, hearing impairment with DIDMOAD starts during the second decade, and affects primarily the basal cochlea. Hearing thresholds deteriorate with 4 dB per year, and the median life expectancy is 30 years.[3]

*Syndromic HHI*

Pendrin (SLC26A4)

The SLC26A4 gene encodes the chloride-iodide transporter pendrin which is expressed in the cochlea and the thyroid.[21] Some mutations in this gene cause non-syndromic autosomal recessive HHI linked to DFNB4. These patients have severe to profound HHI and an enlarged vestibular aqueduct (EVA), but initially no accompanying goiter. Other mutations in SLC26A4 cause the autosomal recessive Pendred syndrome that associates congenital, profound HHI with goiter. This thyroid abnormality can be demonstrated by the perchlorate test. All Pendred syndrome patients have cochlear malformations such as EVA, and many have a Mondini dysplasia.[22]

Stickler Syndrome and Osteogenesis imperfecta (Collagen genes)

As indicated in Table 2, mutations in COL2A1, COL11A1 or COL11A2 genes cause the autosomal dominant Stickler syndrome (STL). The classic phenotype, STL1, is caused by mutations in COL2A1, which encodes a fibrillar collagen, and is characterized with progressive myopia, vitreoretinal degeneration, premature joint degeneration, midface hypoplasia (flat facial profile), irregularities of the vertebral bodies, cleft palate and a sensorineural hearing loss of variable severity. STL2 is caused by mutations in COL11A1, and is also characterized by ocular, auditory and orofacial features whereas mutations in COL11A2 (STL3) do not cause visual dysfunction because this protein is not present in the vitreous body.[2] The phenotype of STL is in general highly variable. Therefore, this syndrome is probably underdiagnosed, and it is difficult to differentiate the genetic subtypes. In STL2 and STL3 high frequency sensorineural HHI is reported to be more severe.[23] Conductive or mixed hearing losses sometimes occur due to a hypermobility of the tympano-ossicular chain (tympanograms type $A_D$ according to Jerger's classification). Oto-Spondylo-Mega-Epiphyseal-Dysplasia (OSMED) has similar clinical features as STL but the inheritance is autosomal recessive.

Osteogenesis Imperfecta (OI) is another disease that involves collagen genes: COL1A1 and COL1A2. Although fragile bones are the hallmark of this syndrome, non-osseous features such as blue sclerae, hearing loss, dentinogenesis imperfecta (DI), easy bruising, cardiopulmonary abnormalities and neurological complications occur. The inheritance pattern is usually autosomal dominant, and four subtypes exist. Type I is characterized by mild to moderate bone fragility, blue sclerae, DI, and almost 50% of these patients have hearing loss that can be conductive, sensorineural or mixed.[24] Type II is often lethal during the perinatal period. Type III is characterized by severe bone fragility and hearing loss is common. In type IV, moderate bone fragility is seen occasionally with hearing loss. In Belgium, genetic laboratories of the University of Ghent provide diagnostic service for Osteogenesis imperfecta.

Usher Syndrome

Three subtypes of the Usher syndrome, characterized by hereditary retinitis pigmentosa with deafness, are clinically discernable.[25] Type I is determined by congenital, profound deafness associated with vestibular areflexia and retinitis pigmentosa. Usher syndrome type II is characterized by moderate to severe sensorineural hearing impairment, intact vestibular responses and retinitis pigmentosa, whereas Usher syndrome type III is characterized by progressive hearing impairment, variable vestibular function and retinitis pigmentosa. Currently, 7 loci have been mapped for Usher syndrome type I (USH1A-USH1G), 3 loci for Usher syndrome type II (USH2A-USH2C) and 1 locus for Usher syndrome type III (USH3).[3] For these 11 loci, 8 genes have been identified and can be used for diagnostic screening as indicated in Table 2. In Belgium, routine genetic testing is available for the USH1B gene. However, mutation analysis is successful in only about 20 % of Usher 1B patients. Recently, in Nijmegen, a microarray that covers all known Usher mutations and reaches mutation diagnosis of 50 % for Usher 2A, the most common subtype of Usher syndrome, has become available.

Treacher Collins Syndrome

Treacher Collins syndrome, alter-

*Table 2*

Common diagnostic DNA tests for syndromic deafness genes

| Genotype | | | | | Phenotype | | |
|---|---|---|---|---|---|---|---|
| **Syndrome** | | **Location** | **Gene** | **Biological role** | **Characteristic features** | **Audiometry** | **Onset** |
| Wolfram | | 4p16.1 | *WFS1* | Transmembrane protein in E.R. but function unknown | Aut. rec.,<br>DIDMOAD = diabetes indsipidus, diabetes mellitus, optic atrophy and deafness, hypogonadism in males urological, neuropsychiatric and cardiac abnormalities | High frequency hearing loss | Most signs in 2nd decade |
| | | | | | | | |
| Stickler | *STL1* | 12q13.11-13.2 | *COL2A1* | Structural collagens | Aut. dom.<br>midface hypoplasia and cleft palate<br>premature joint degeneration, irregular vertebra<br>progressive myopia, vitreoretinal degeneration | Variable HI | Congenital |
| | *STL2* | 6p21.3 | *COL11A1* | | | | |
| | *STL3* | 1p21 | *COL11A2* | | *STL3* is WITHOUT ocular symptoms | | |
| | | | | | | | |
| Alport | | Xq22 | *COL4A5* | Structural collagens | X-linked or aut. rec. or aut. dom.<br>nephritis, anterior lenticonus and macular flecks | Progressive high frequency | 2nd decade |
| | | 2q36-37 | *COL4A3/A4* | | | | |
| | | | | | | | |
| Pendred | | 7q21-34 | *SLC26A4* | Anion ($Cl^-$, $I^-$) transporter | Aut. rec. congenital deafness and goitre later in life, thyroid dysfunction possible,<br>associated with EVA and mondini dysplasia | Profound | Prelingual |
| | | | | | | | |
| Usher | USH1B | 11q13.5 | *MYO7A* | Motor molecule | Aut. rec.<br>retinitis pigmentosa onset before puberty,<br>vestibular areflexia | Severe to profound | 1st decade |
| | USH1C | 11p15.1 | *USH1C* | PDZ domain protein | | | |
| | USH1D | 10q | *CDH23* | Cadherin | | | |
| | USH1F | 10q21-22 | *PCDH15* | Protocadherin | | | |
| | USH1G | 17q24-25 | *SANS* | Scaffold protein | | | |
| | USH2A | 1q41 | *USH2A* | Extra cellular matrix | Aut. rec., retinitis pigmentosa onset after puberty,<br>normal vestibular function | Moderate to severe sloping audiogram | 1st or 2nd decade |
| | USH2C | 5q14 | *VLGR1* | G-protein coupled receptor | | | |
| | USH3 | 3q21-25 | *USH3* | Transmembrane protein | Aut. rec., retinitis pigmentosa,<br>variable vestibular function | Progressive SNHI | Variable |
| | | | | | | | |
| Treacher Collins | | 5q32-33.1 | *TCOF1* | Nuclear cytoplastic transport protein | Aut. dom., coloboma of the lower eyelid, down-slanting palpebral fissures, malformation external and middle ears, micrognathia, hypoplasia os zygoma, macrostomia | Variable HI | Congenital |

Aut. rec. = Autosomal Recessive. Aut. dom. = Autosomal dominant. HI = Hearing impairment. EVA = Enlarged vestibular aquaduct.

*Table 3*

Basic diagnostic indications for common genetic tests for HHI in Belgium

| Type of hearing impairment | Occurrence | Genetic test for |
|---|---|---|
| Congenital bilateral HHI | S or F | DFNB1 Connexin26 gene[1]<br>DFNB9 Otoferlin gene[2] |
| Progressive low frequency HHI | F | DFNA6/14 Wolframin gene |
| Progressive bilateral cochleo-vestibular deficit | F | DFNA9 Cochlin gene |
| HHI with other clinical feature | S or F | Syndromal HHI (see table 2) |

S = Solitary case in family, F = Familial occurrence with several family members affected. [1] = Specific mutations reported for specific ethnicities (See Table 1). [2] = Mainly Spanish origin.

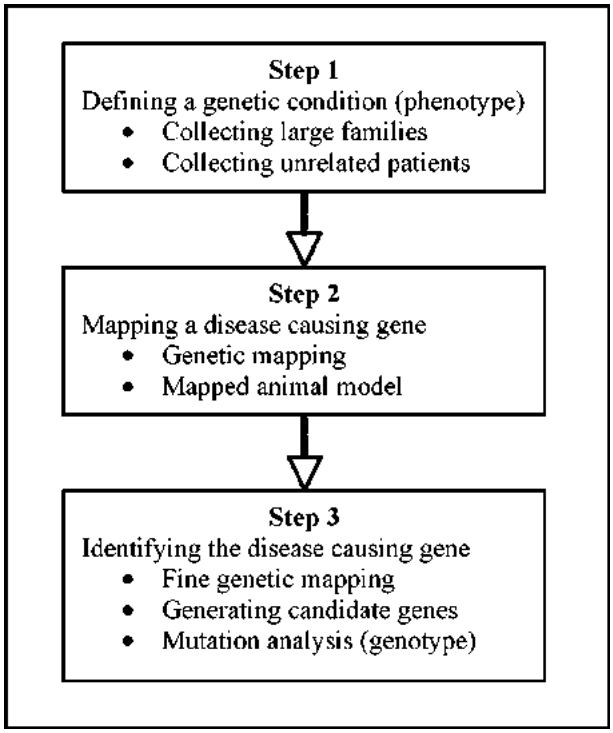


*Figure 4*

Steps to identify the disease-causing gene for monogenic HHI. Modified from Textbook of Audiological Medicine.[26]

natively called mandibulofacial dysostosis (MFD), is an autosomal dominant disorder caused by mutations in the *TCOF1* gene which encodes a trafficking protein involved in nucleolar-cytoplasmic transport.[2] It is characterized by coloboma of the lower eyelid, micrognathia, microtia, hypoplasia of the zygomatic arches, macrostomia, and inferior displacement of the lateral canthi with respect to the medial canthi leading to a recognizable "fish-like" facial appearance. A single gene is responsible for Treacher Collins syndrome, and if a clear clinical picture is available, a high chance of finding a mutation in TCOF1 might be expected. The auditory ossicles, cochlear and vestibular apparatus can be absent or severely malformed leading to conductive, mixed or perceptive HHI.

### *Investigating genetics of HHI*

Unravelling the putative genes of a hearing impairment follows at least three stages.[26] In the first stage, a genetic cause for the hearing impairment has to be confirmed on the basis of family data. The simplest genetic conditions yield Mendelian inheritance patterns that are easily recognizable from the pedigree chart. If an inheritance pattern for a hearing impairment could be confirmed, recurrence risk is available for counselling. In the second stage, the putative gene has to be mapped to a particular chromosome locus. This is achieved with the so-called linkage analysis which requires large families. Linkage analysis tests for co-segregation of a chromosomal locus and the deafness. In families, parents can pass on either the disease-causing gene or its normal version (allele) to each of several children. The strategy is to check which chromosome fragment co-segregates together with the disease in the pedigree chart. The chromosome fragments are identified with specific genetic markers, available on the market. Of course this is an oversimplification and in practice it is not that easy. But, the theory of linkage analysis postulates that if a chromosome fragment co-segregates together with a disease more than would be expected on the basis of chance, then that chromosome fragment must carry the disease-causing gene. The logarithm of odds (LOD) score is a statistical parameter that represents the odds or likelihood of linkage over non-

linkage. A LOD score of +3 is significant for linkage, whereas a LOD score of -2 is significant against linkage. How closely a disease-causing gene can be localized depends on the size of the family, i.e. how many informative meioses are available. Genetic mapping, thus, relies on large families with a high number of affected individuals. The last step, and often not the easiest step, is to identify the disease-causing gene. This will eventually allow precise genetic diagnosis for individuals who want to know their carrier status for a particular allele. When a gene is identified, studies on the function of the encoded protein can be started. On long-term, this might lead to new strategies for therapy.

There are several techniques that tackle the quest for the mutation in the candidate gene. These techniques are not in the objective of this review and can be found in genetic handbooks. However, it is important to realize that none of these methods are perfect or guarantee success. Much research is focussed on increasing the speed and reliability of screening for candidate genes. When allelic variability for a candidate gene is observed, it is not directly clear whether an allele is pathogenic or a harmless variant of the wild-type allele. In conclusion, investigating HHIs has provided new insights on the functioning of the inner ear, and further efforts will surely contribute to further understanding of normal hearing.

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# Chapter 3

## A Belgian family with non-syndromic, autosomal dominant, progressive, sensorineural hearing loss linked to DFNA22

**To be submitted.**

**STRUCTURED ABSTRACT**

**Objective:** To clinically and genetically study a family with autosomal dominant sensorineural hearing loss (ADSNHL).

**Study design:** Family study.

**Setting:** Tertiary referral center.

**Patients:** Sixty-eight family members from a 4 generation Belgian family with ADSNHL.

**Methods:** All participants completed a questionnaire, were clinically examined and underwent standard pure-tone audiometry. First, genetic linkage analysis in 6 chromosomal regions implicated in ADSNHL was carried out. Subsequently, a genome wide linkage scan was carried out using 400 microsatellite markers.

**Results:** Eighteen family members were diagnosed as affected, all having moderate to severe sensorineural hearing loss starting during the third decade. The hearing loss can be characterized as a flat hearing loss affecting all tested frequencies when age is taken into account. A genome-wide scan revealed linkage to DFNA22, with *MYO6* as the identified deafness gene. All family members that were clinically affected shared a common haplotype on chromosome 6q13-q14.1, which was not present in unaffected individuals. A maximum LOD score higher than 5 was reached, proving linkage to this region. Sequence analysis of *MYO6* did not reveal any mutation in the exons and intron-exon boundaries.

**Conclusions:** We have localized the gene responsible for ADSNHL in a large 4 generation family to a region of chromosome 6q13-6q14.1, containing the previously known deafness gene *MYO6.* However, we were unable to find a mutation by DNA sequencing of the coding region. Although a mutation outside this region could be responsible, it is also possible that another gene in this region is responsible for the hearing loss in this family.

**Introduction**

This study reports a Belgian family with early onset non syndromic progressive hearing loss that is linked to DFNA22. This locus for hearing impairment has been described before in an Italian kindred that has a mutation in the *MYO6* gene that encodes for myosin VI, a member of the myosin super-family.(1) Myosins are motor proteins that use hydrolysis of ATP to move on F-actine through which they convert chemical energy into mechanical energy. Nearly 40 myosin genes have been identified in humans. They are grouped into 12 different classes and play an important role in several cellular processes, including endocytosis, exocytosis and cell motility.(2) Unconventional myosins are a subfamily of myosins that has been associated with hearing impairment. In 1995, mutations in the *shaker1 (s1)* mice have been identified almost simultaneously with human Usher Syndrome type 1B mutations in the *MYO7A* gene.(3;4) Shortly afterwards, both dominant and recessive types of human hearing loss were identified at loci DFNA11 and DFNB2. (5) Another unconventional myosin is identified in *shaker 2 (s2)* mice with mutations in the *MYO15* gene in 1998 and was associated with human recessive hearing impairment in DFNB3, one year later.(6;7) A third unconventional myosin was also identified in 1995 in the *Snell's waltzer (sv)* mouse but the human homologue was identified many years later (2001) in an Italian family with autosomal dominant hearing impairment because of a missense mutation in the *MYO6* gene. (1;8) Myosin VI also has another unique feature of moving toward the negative end of actine filaments.(9) Table 1 summarizes the concordance between human and mouse mutations in unconventional myosins and shows the comparably late discovery of the human homologue for the *sv* locus. Mouse models are excellent model systems to study genetic deafness in humans because the anatomy of the ear is similar and many mutations causing deafness in mice are known.

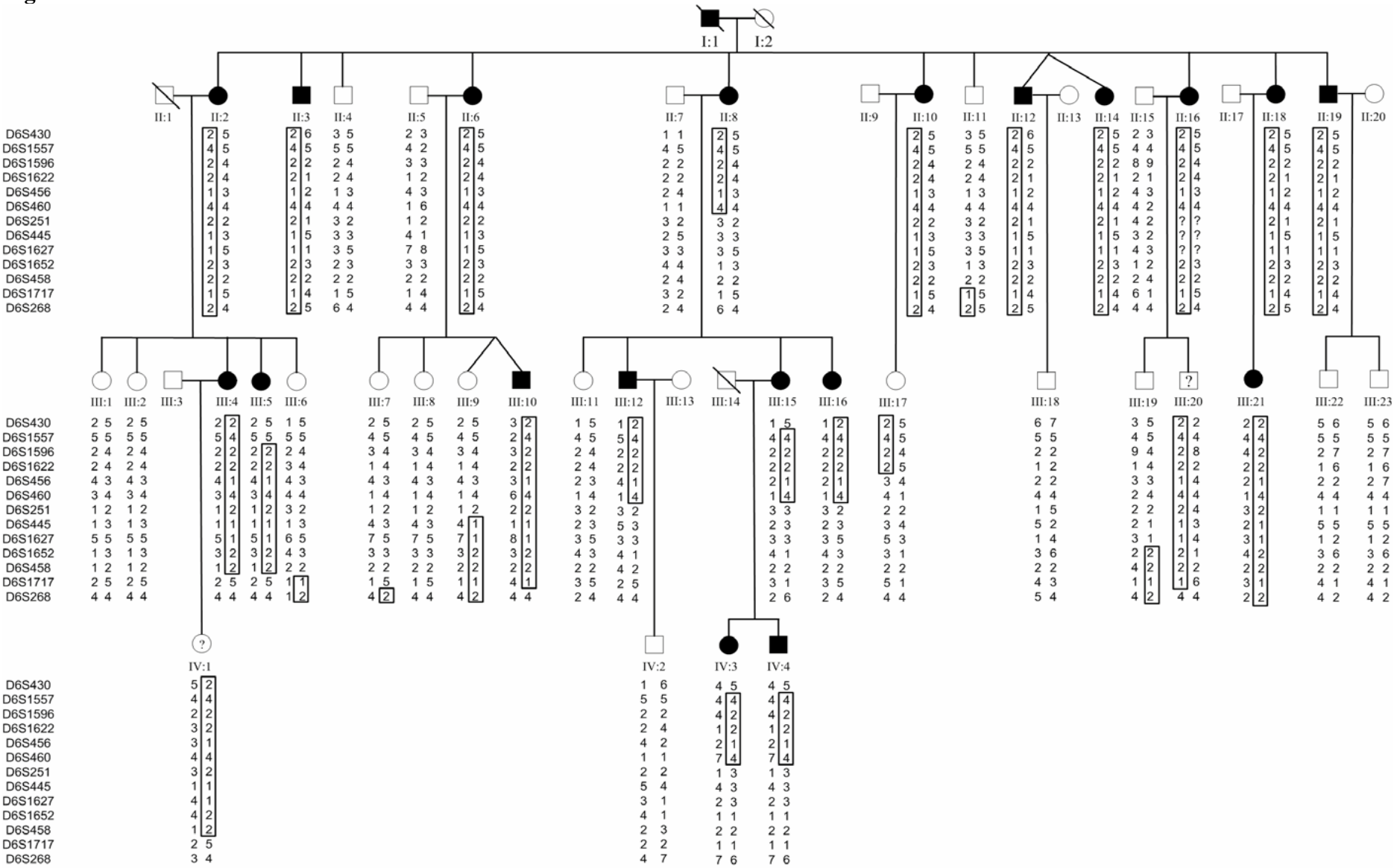
**Table 1** Concordance between animal models and human deafness in mutated Myosin genes

| Gene | Animal model | | | Human Homologue | | |
|---|---|---|---|---|---|---|
| | **Name** | **Year** | **Ref** | **Locus** | **Year** | **Ref** |
| ***MYO1A*** | *Mouse 22* | 1996 | Hasson et al | DFNA48 | 2003 | Donaudy et al |
| ***MYO3A*** | *Drosophila* | 2002 | Walsh et al | DFNB30 | 2002 | Walsh et al |
| ***MYO6*** | *sv* | 1995 | Avraham et al. | DFNA22<br>DFNB37 | 2001<br>2003 | Melchionda<br>Ahmed et al |
| ***MYO7A*** | *s1* | 1995 | Gibson et al | USH2A<br>DFNA11<br>DFNB2 | 1995<br>1997<br>1994 | Weil et al<br>Liu et al<br>Guilford et al |
| ***MYO15*** | *s2* | 1998 | Probst et al | DFNB3 | 1999 | Wang et al |

Although the *sv* mouse model has otovestibular dysfunction the human homologue DFNA22 only shows deafness. In the first reported DFNA22 family, a missense mutation has been identified in exon 12 of the *MYO6* gene on chromosome 6q13. This causes replacement of a cysteine with a tyrosine at residue 442 of the protein (C442Y), which results in non-syndromic progressive hearing impairment without vestibular dysfunction.(1) The second family with another missense mutation in the *MYO6* gene is reported to have progressive late onset autosomal dominant hearing impairment combined with cardiac hypertrophy. Because of this mutation a histidine residue is replaced with arginine (H246R) in the motor region of myosin VI.(10)

Here we report a third family that has been linked to DFNA22. This first Belgian DFNA22 family did not harbour the previously reported missense mutations in the MYO6 gene. This family is only characterized by autosomal dominant, late onset, progressive sensorineural hearing loss (SNHL).

**Figure 1**


I:1
I:2
II:1
II:2
II:3
II:4
II:5
II:6
II:7
II:8
II:9
II:10
II:11
II:12
II:13
II:14
II:15
II:16
II:17
II:18
II:19
II:20
III:1
III:2
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III:14
III:15
III:16
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III:18
III:19
III:20
III:21
III:22
III:23
IV:1
IV:2
IV:3
IV:4
D6S430
D6S1557
D6S1596
D6S1622
D6S456
D6S460
D6S251
D6S445
D6S1627
D6S1652
D6S458
D6S1717
D6S268

## Patients and Methods

*Patients*

Figure 1 shows the ascertained pedigree of a Belgian family spanning 4 generations. Initially, individual number III:12 consulted the University Hospital in Antwerp for hearing improving clinical intervention. He declared that 10 out of 12 siblings of his mother had documented hearing problems. After several contacts with family members individual II:14 acted as a proband. All family members were given the opportunity to participate in a family study on a voluntary basis. Sixty-eight participants (N=68) signed an informed consent, which also covered the retrieval of audiograms previously obtained elsewhere.

*Phenotyping the hearing loss*

Every participant completed a questionnaire and underwent otoscopic examination to exclude other causes of hearing impairment. Pure tone audiometry to current standards was performed on site to determine hearing thresholds at frequencies 0.25, 0.5, 1, 2, 3, 4, 6 and 8 kHz. Participants were considered to be affected when 3 or more measurements exceeded the expected thresholds given by percentile 90 of ISO 7029 normative values specific age and sex.(11) Unaffected members had to have lower thresholds than percentile 50 over all measured frequencies. Cross-sectional threshold analysis was performed, comprising 18 affected family members, to characterize the phenotype of the present trait. The binaural mean air conduction threshold was assessed after having confirmed that the hearing impairment was fairly symmetric. A commercial program (SPSS, version 12) was used to perform linear regression analysis to evaluate progression of hearing impairment in this family, with extrapolation to age zero (offset threshold). For each measured frequency it was tested whether progression was significant, which implies that the regression coefficient differs significantly from zero. The regression coefficient -i.e. the slope- is called Annual Threshold Deterioration (ATD, in decibels per year). Based upon the cross-sectional analysis, “Age Related Typical Audiograms” (ARTA) were constructed, which show the expected threshold for a number of decade steps in age.(12) “Thresholds features arrays” were derived from the ARTA (data not shown) for formal statistical testing against previously reported data from Huygen et al.(12) Electrocardiography was retrieved from general practitioners from family members that indicated heart problems.

*Genetic analyses*

DNA was isolated from peripheral blood lymphocytes obtained from blood samples of all participating family members. Based on an autosomal dominant mode of inheritance and on the clinical diagnoses of all participating family members, SLINK simulations were performed to estimate whether the family was informative enough for linkage analysis.(13) Six known dominant loci were screened beforehand to check linkage. All genotyping was done by polymerase chain reaction (PCR) and polyacrylamide gel electrophoresis, using standard procedures. After screening of the 6 selected loci, a genome-wide search was performed by the company deCODE Genetics (Reykjavik, Iceland), using a polymorphic set of 400 microsatellite markers, spread throughout the whole genome. Linkage analysis was performed by calculating two-point and multipoint LOD-scores with the program Easylinkage (version 4.01 Berlin, Germany). (14) Primers were designed for the amplification of the coding region (34 exons), the intron-exon boundaries and 1 none-coding exon of *MYO6* (MIM 600970), using primer3 input. (15) PCR products were sequenced by standard procedures using an ABI 3130 automated DNA sequencer (Applied Biosystems).

**Figure 2**

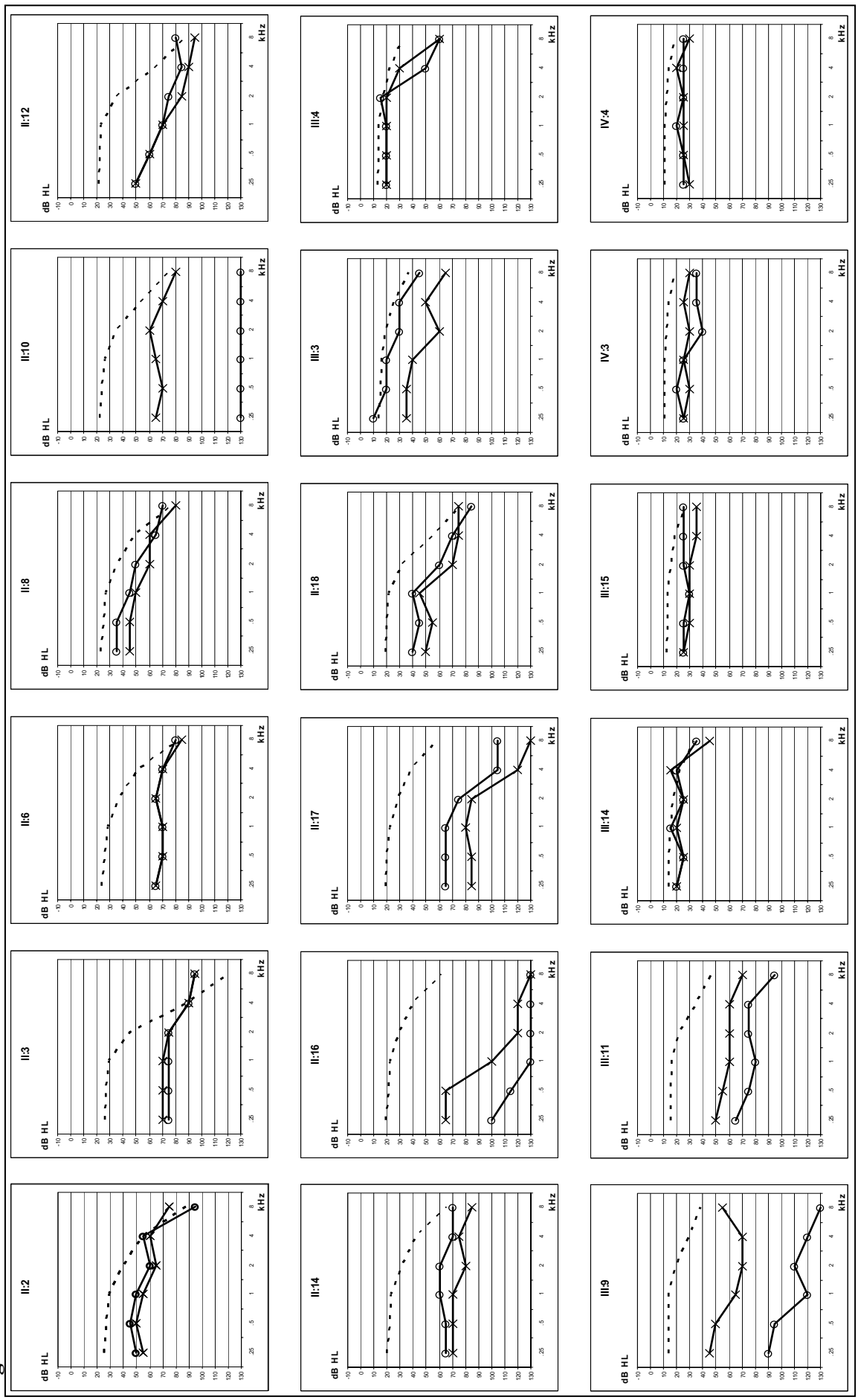
II:2
II:3
II:6
II:8
II:10
II:12
II:14
II:16
II:17
II:18
III:3
III:4
III:9
III:11
III:14
III:15
IV:3
IV:4
dB HL
kHz

**Table 2** Results of the questionnaire

| Pedigree Number | II:2 | II:3 | II:6 | II:8 | II:10 | II:12 | II:14 | II:16 | II:18 | II:19 | III:4 | III:5 | III:10 | III:12 | III:15 | III:16 | IV:3 | IV:4 | M:F | Mean | %Y |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AGE (years) | 74,8 | 73,5 | 67,9 | 66,4 | 65,0 | 61,0 | 61,0 | 59,4 | 58,0 | 56,6 | 44,6 | 41,3 | 42,4 | 45,5 | 41,7 | 35,7 | 22,0 | 20,2 | | 52,1 | |
| Gender | F | M | F | F | F | M | F | F | F | M | F | F | M | M | F | F | F | M | 1:2 | | |
| Length (cm) | 155 | 168 | 160 | 161 | 160 | 170 | 168 | 165 | 162 | 172 | 165 | 170 | 178 | 168 | 163 | 168 | 173 | 172 | | 167 | |
| Weight (kg) | 57 | 77 | 59 | 66 | 65 | 78 | 63 | 47 | 47 | 69 | 52 | 80 | 78 | 65 | 54 | 64 | 48 | 65 | | 63 | |
| Body Mass Index | 24 | 27 | 23 | 25 | 25 | 27 | 22 | 17 | 18 | 23 | 19 | 28 | 25 | | 20 | 23 | 16 | 22 | | 23 | |
| Hearing difficulty | Y | Y | Y | Y | Y | Y | Y | Y | Y | Y | N | N | Y | Y | N | N | Y | N | | | 72 |
| Which side | B | B | B | B | B | B | B | B | B | B | | | R | R | | | B | | | | |
| Onset of first notice | 60 | 60 | 25 | 40 | 26 | 28 | 28 | 15 | 28 | 28 | | | 23 | 15 | | | 8 | | | 29,4 | |
| Progressiveness | Y | Y | Y | Y | Y | Y | Y | Y | Y | Y | | | Y | Y | | | Y | | | | 100* |
| Cocktail party effect | Y | N | Y | Y | Y | Y | N | Y | Y | Y | N | N | Y | N | N | N | N | N | | | 50 |
| Hyperacusis | N | N | Y | Y | Y | Y | Y | Y | Y | Y | N | N | N | N | N | N | N | Y | | | 50 |
| Fullness or blockage in ears | N | N | N | N | Y/L | N | Y/R | Y/B | N | N | N | N | N | N | Y/B | N | N | N | | | 22 |
| Tinnitus | N | N | N | N | Y | N | Y | Y | N | N | N | N | N | N | Y | N | N | N | | | 22 |
| Ear problems affecting hearing | N | N | N | N | Y | N | N | Y | N | N | N | N | N | N | N | N | N | N | | | 11 |
| Ear infections | N | N | N | N | Y | N | N | Y/R | N | N | N | N | N | N | N | N | Y | N | | | 17 |
| Ear operations | N | N | N | N | N | N | N | Y/ch | N | N | N | N | N | N | N | N | N | N | | | 6 |
| Dizziness | N | N | N | N | Y/c | N | N | Y | N | N | N | N | N | N | N | N | N | N | | | 11 |
| Unsteady in the dark | N | N | N | N | Y | N | N | Y | N | N | N | N | N | N | N | N | N | Y | | | 17 |
| Migraine | N | N | N | N | Y | Y | N | Y | Y | Y | N | N | N | N | N | N | N | N | | | 28 |
| Meningitis or encephalitis | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | | | 0 |
| Whiplash | N | N | N | N | N | N | N | N | N | N | N | N | N | Y | N | N | N | N | | | 6 |
| Knocked unconscious | N | Y | N | N | Y | Y | N | Y | N | N | N | N | N | N | N | N | N | N | | | 22 |
| Heart attack | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | | | 0 |
| Heart surgery | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | | | 0 |
| Stroke | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | | | 0 |
| Coronary artery surgery | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | | | 0 |
| Intermittent claudication | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | | | 0 |
| Heart / circulation | N | N | N | N | Y | N | N | N | N | N | N | N | N | N | N | N | N | N | | | 6 |
| Diabetes Mellitus | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | | | 0 |
| IV or IM antibiotic | N | N | N | N | Y | N | N | N | N | N | N | N | N | N | N | N | N | Y | | | 11 |
| Aspirin for dilution | N | Y | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | | | 6 |
| Gunfire | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | N | Y | | | 6 |
| Occupational Noise | N | Y | N | N | N | Y | Y | N | N | Y | N | N | N | N | N | N | N | N | | | 22 |

**Results**

Remarkably five of eighteen affected family members (27.8 %) declared not to experience any hearing problems in their questionnaire although they were affected as shown in figure 2. The average age of onset according to the questionnaire was about 30 years. Table 2 summarizes some results of the questionnaire. The examined family members had an average age of 50 years and a male to female ratio of 1:3.75 indicating that more females are affected. Forty seven percent complained of a cocktail party effect whereas 52 % indicated hyperacusis. Tinnitus was present in 21% and in 5% accompanied with fullness or blockage of the ears. None of the participant had a typical history and clear symptoms of a possible Meniere disease. However, individuals II:10 and II:16 had atypical dizziness complaints whereas case IV:4 experienced insecure feeling in the dark rather than instability. Individual II:16 had had surgery for cholesteatoma because of uncontrolled recurrent otitis on the right ear during childhood. Therefore audiometric data of the ear without history of infections was used for statistical analysis instead of the binaural mean. None of all affected family members had a clear history of hart problems indicating cardiomyopathy. Individual II:10 has also heart palpitations since she had had surgery for breast cancer, however recent electrocardiography did not show abnormalities indicating cardiomyopathy nor aerhythmias.

*Audiograms:* Figure 2 shows the last-visit audiograms of 18 affected patients including ISO7029 P95 normative values for matching age and gender. In general, most affected cases show fairly symmetric air conduction thresholds except for II:10, II:16, III:4, III:10 and III:12. Ten affected family members were from the second generation, seven from the third generation and only two from the fourth generation, which again indicates a late onset. The hearing impairment in this family can be characterized by a flat audiogram affecting all frequencies, when age related hearing impairment is taken into account for elderly cases.

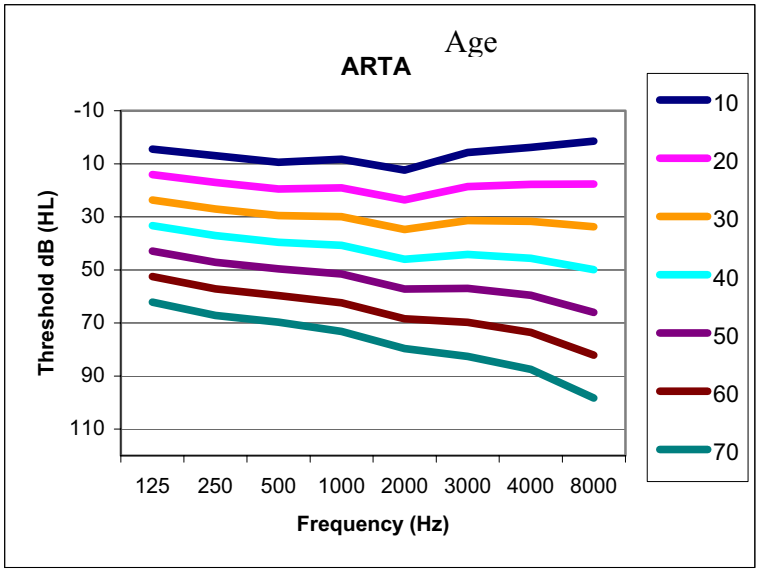


Figure 3 Age-Related Typical audiograms based on last-visit audiograms of 19 affected cases

*ARTA:* Figure 3 shows the ARTA based on the results of the cross-sectional analysis of 19 affected family members examined in this study. Significant progression was demonstrated for all frequencies measured. The average ATD value for all frequencies was 1.2 dB/year. The ATD was slowest at 125 Hz with 0.96 dB/year and fastest at 8000 Hz with 1.61 dB/year. The expected thresholds per decade in the ARTA indeed show a mild hearing loss starting at the age of 30 and the hearing loss evolves to moderate to severe at the age of 50 years.

*Genetic analyses:* Figure 1 clearly shows an autosomal dominant pattern of inheritance. The SLINK simulation showed a maximal LOD-score of 9.63. Initially, linkage analysis was performed for a known dominant locus based on phenotypic similarities between the Belgian family and the hearing loss associated with DFNA13/DFNA21. Five other loci that frequently cause dominant hearing loss were also selected for linkage analysis, namely DFNA2, DFNA3, DFNA5, DFNA8/12 and DFNA36. Linkage could be excluded for all selected loci by calculating two-point and multipoint LOD-scores (data not shown). Subsequently, a sample set as shown in figure 1 was used for a genome-wide search using 400 micro-satellite markers. Linkage was found to DFNA22 on chromosome 6q, with *MYO6* as the disease-causing gene for this known locus. Extra markers in the region were analysed to confirm linkage and to refine the candidate region. Table 3 summarizes the two-point LOD-scores for all markers analysed. After constructing the haplotypes as shown in figure 1, the minimal region was established to chromosome 6q13-q14.1 between markers D6S456 and D6S460, a region of 2.37cM. (Figure 4) Sequencing of the coding and non-coding exons, as well as the intron-exon borders of *MYO6* didn't reveal any mutations.

**Table 3** Two-point LOD scores.

| | **Recombination fraction** | | | | | | | **Position** |
|---|---|---|---|---|---|---|---|---|
| **Marker** | **0** | **0,01** | **0,05** | **0,1** | **0,2** | **0,3** | **0,4** | **(cM)** |
| D6S430 | *-inf* | *4.75* | *5.05* | *4.81* | *3.92* | 2.72 | 1.23 | 81.91 |
| D6S1557 | *-inf* | *5,87* | *6,06* | *5,68* | *4,53* | 3,08 | 1,36 | 84,33 |
| D6S1596 | *4,14* | *4,07* | *3,77* | *3,39* | 2,61 | 1,77 | 0,81 | 87,69 |
| D6S1622 | *-inf* | 3,2 | *3,58* | *3,47* | 2,87 | 2,03 | 0,99 | 88,21 |
| **D6S456** | *5,54* | *5,45* | *5,09* | *4,62* | *3,61* | 2,47 | 1,19 | 88,21 |
| **D6S460** | 0,9 | 0,89 | 0,84 | 0,77 | 0,61 | 0,44 | 0,24 | 90,58 |
| D6S251 | -inf | 2,57 | 2,96 | 2,86 | 2,3 | 1,52 | 0,58 | 91,2 |
| D6S445 | -inf | 2,18 | 3,79 | 4,07 | 3,6 | 2,58 | 1,19 | 91,56 |
| D6S1627 | -inf | 0,95 | 2,65 | 3,04 | 2,8 | 2,03 | 0,91 | 92,94 |
| D6S1652 | -inf | -1,19 | 1,83 | 2,7 | 2,84 | 2,17 | 1,03 | 93,4 |
| D6S458 | -inf | -1,7 | -1 | -0,7 | -0,4 | -0,22 | -0,1 | 99,71 |
| D6S1717 | -inf | -8,08 | -3,44 | -1,67 | -0,28 | 0,15 | 0,18 | 103,95 |
| D6S268 | -inf | -13,36 | -6,01 | -3,14 | -0,8 | 0,04 | 0,18 | 111,73 |

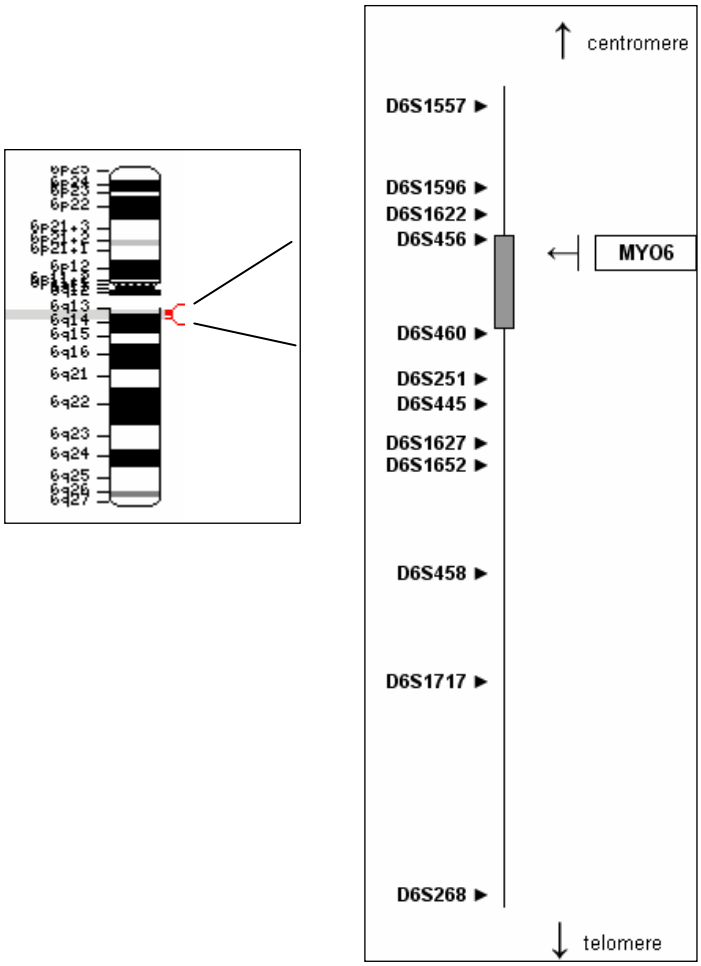


**Figure 4** After constructing the haplotypes as shown in figure 1, the minimal region was established to chromosome 6q13-q14.1 between markers D6S456 and D6S460, a region of 2.37cM.

**Discussion**

Many families from a rather small geographic region covering Belgium and the Netherlands have lead to the localization of several deafness loci. Their phenotypes have been reported extensively.(16;17) Not surprisingly some studies reported co-founder effects for hearing impaired families that were initially reported separately.(18) Correlations between phenotypes of known deafness traits have been very helpful in genotype phenotype correlation studies.(19) However, in this study phenotype characterization was not sufficient to directly discover a mutation in 6 chromosomal regions involved with similar types of ADSNHL. A genome wide scan revealed linkage to DFNA22 that contains *MY6A* as a known deafness gene.

The first reported Italian family linked to DFNA22 showed moderate to profound hearing loss.(1) The present family doesn't only seem to have less severe hearing impairment; the age of onset is also around the third decade, which is about 20 years later than the first family linked to DFNA22. The fact that the hearing impairment in the Belgian family is mild is also reflected in the questionnaire where nearly a quarter of affected individuals did not have any complaints. This Belgian family can also be discerned from the second family that was previously linked to DFNA22 because in this family hyperthropic cardiomyopathy is not a hereditary trait.(10) We are aware that vestibular function in affected members of this family has not been thoroughly evaluated. At the moment we have ascertained another Belgian

family with autosomal dominant sensorineural hearing loss that has also been linked to the DFNA22 region. Also in this second Belgian DFNA22 family affected members do not have vertigo complaints. At the moment co-founder effect is being checked and sequence analyses are examining possible mutation sites. We plan to investigate vestibular involvement in these Belgian families with electronystagmography. Although the phenotypes of these families are similar we have to be careful with comparing these. If genetic analyses in the Belgian families identify possibly new mutations more refined phenotypes can be determined.

Already in 1994 Hasson and Mooseker characterized porcine myosin VI.(20) One year later Avraham et al identified the gene encoding myosin VI in the mouse recessive deafness mutation: Snell's waltzer (*sv*).(8) In 1997 human MYO6 cDNA was cloned and characterized.(21) In 1996 the human homologue was predicted to be located on 6p12-q16.(22) However it took till 2001 before the first family could be linked to this region. In contrast to the mouse model this family showed autosomal dominant hearing impairment without vestibular involvement.(1) It took even longer to identify families segregating autosomal recessive congenital sensorineural deafness linked to this region: DFNB37.(23) Vestibular involvement is today still not associated with human MYO6 gene.

Today we rapport another DFNA22 family but the mutation has not yet been found. Because of the size of the pedigree at issue, lots of recombination was found, which allowed refining the minimal region to 2.7cM. A combination of the two-point LOD-scores (table 3) and the haplotypes for all markers genotyped (figure 1) revealed linkage to chromosome 6q13-q14.1. The deafness gene identified for DFNA22, is still located in this region, so the chance of finding a mutation in this gene was rather high. Unfortunately, no mutations were found in the coding region of *MYO6*. These results do not rule out the possibility that MYO6 is indeed the disease-causing gene in the family reported. Although the possibility that a mutation outside the coding region is present remains this type of mutation is much more difficult to find. Nevertheless genetic analyses to screen for this are ongoing.

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# Chapter 4

## Phenotype determination guides swift genotyping of a DFNA2/KCNQ4 family with a hot spot mutation (W276S)

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**Otol Neurotol. 2005 Jan; 26 (1):52-58.**

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# Phenotype Determination Guides Swift Genotyping of a DFNA2/*KCNQ4* Family With a Hot Spot Mutation (W276S)

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**Objective:** Genotype a family trait with autosomal dominant nonsyndromic sensorineural hearing impairment guided only by the phenotype.
**Study design:** Family study.
**Setting:** Tertiary referral center.
**Patients:** Fifteen family members.
**Methods:** In the first phase, sequence analysis was performed on DNA isolated from buccal swabs of the proband and her daughter, guided by the phenotype based on audiometric data that were already available. After detection of the W276S missense mutation in the *KCNQ4* gene in both patients, this finding was confirmed in the other affected family members. All participants completed a questionnaire, were clinically examined, and underwent standard pure-tone audiometry. The results were analyzed to refine the phenotypic features of the family trait.
**Results:** All clinically affected participants were carriers of the W276S hotspot mutation in exon 5 of the *KCNQ4* gene on chromosome 1p34. Refined phenotypic features confirmed previously described phenotypes of DFNA2 families.
**Conclusions:** Phenotype determination can be cost saving and very effective in detecting the genotype of autosomal dominant nonsyndromic hearing impairment, especially when phenotype analyses can be performed on data that are already available or easily collected. **Key Words:** DFNA2/*KCNQ4*–Genetic hearing impairment–Phenotypic characterization.
*Otol Neurotol* **26:**52–58, 2005.

Autosomal dominant nonsyndromic types of sensorineural hearing impairment are classified by numbers in order of discovery of their corresponding chromosomal loci. At present, 51 autosomal dominant loci are known (1). One of the more frequently encountered loci is DFNA2, discovered in 1994 as the second locus for DFNA (DFN for deafness, A for autosomal dominant) (2). DFNA2 is clinically characterized by progressive, high-frequency sensorineural hearing impairment. At this locus on chromosome 1p34, two deafness genes have been identified: the *GJB3* gene that encodes connexin 31, a gap junction protein, and the *KCNQ4* gene that encodes the subunits of a voltage-gated potassium channel (3,4). Both genes presumably play a role in recycling potassium ions from the hair cells to the endolymph (5). A third gene at this locus has been postulated to underlie the hearing impairment in an Indonesian family (6). Also, an American family has been linked to this region, but again, no mutation could be found in the two genes that already had been identified (7). Currently, at least 14 families with progressive high-frequency sensorineural hearing impairment linked to the DFNA2 locus have been studied. Ten of these DFNA2 families harbor a mutation in the *KCNQ4* gene (Table 1) (2,8–16).

The corresponding DFNA2 phenotype has been described in eight families from the United States, Belgium, Japan, and The Netherlands (17,18). Their phenotypes were compared and age-related typical audiograms (ARTA) were derived as described previously (19). These phenotype descriptions and the method to analyze and compare them paved the way for rapid genotyping of the present hearing impairment trait. The W276S missense mutation has been found repeatedly in three apparently unrelated families from The Netherlands and Japan, and the analysis of closely linked polymorphic markers and intragenic single nucleotide repeats indicates that the mutation is likely to be a hot spot for mutation (20).

In this study, we tested the value of our current knowledge about genotype-phenotype correlations in practice. A fifth Dutch family (Dutch V) was identified. The analysis of already available audiograms of seven family members led to the assumption of a *KCNQ4* mutation. Subsequently, mutation analysis revealed a W276S mis-

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**TABLE 1.** *DFNA2/KCNQ4 families and pathologic mutations*

| Family origin | Mutation | Exon | Region in protein | Reference |
|---|---|---|---|---|
| Dutch I | W276S | 5 | Pore region | 8,9 |
| Dutch II | G321S | 7 | Transmembrane domain | 8,10 |
| Dutch III | L274H | 5 | Pore region | 11,12 |
| Dutch IV | W276S | 5 | Pore region | 13 |
| Dutch V | W276S | 5 | Pore region | Present study |
| Belgian | Q71fs | 1 | N-terminal, cytoplasmatic | 8 |
| Japanese | W276S | 5 | Pore region | 14 |
| USA I | G285C | 6 | Pore region | 2,15 |
| USA II | L281S | 6 | Pore region | 16 |
| French | G285S | 6 | Pore region | 4 |

sense mutation in DNA isolated from buccal swabs of the proband and her daughter (IV:10 and V:5). It took only 2 weeks to complete this first part of our study. In the second part, the mutation was verified in all affected family members willing to participate, and thus refined phenotypic features were established.

## PATIENTS AND METHODS

### Patients

Figure 1 shows the pedigree of the Dutch V family spanning five generations. Genetic counseling for hereditary hearing im-

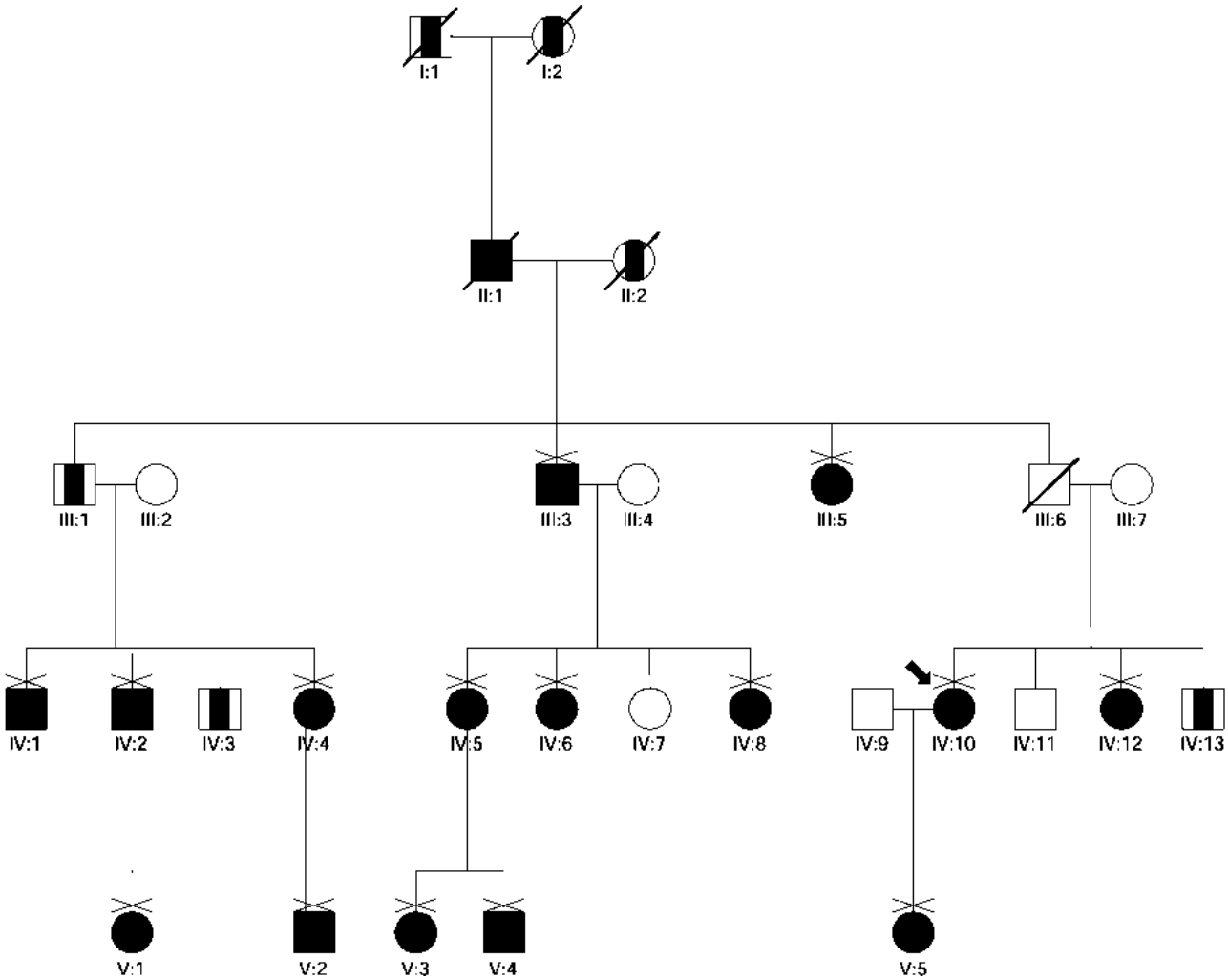


**FIG. 1.** Pedigree of family, adjusted for recognizability reasons. *Circle* for female and *square* for male. ■ = affected; ◧= affected by hearsay but preferred not to participate; □ = not affected by hearsay, not examined; ⍁ = deceased; × = participated in this study.

pairment was initially attempted in 1974, based on audiograms of the individuals II:1, III:3, III:5, IV:1, IV:5, IV:6, and IV:8. At that time, the conclusion was limited to the identification of an autosomal dominantly inherited, probably progressive, high-frequency hearing impairment. The audiograms obtained at that time were now used for initial cross-sectional analysis to see whether it was possible, with our present knowledge, to recognize a specific phenotype. Because we did not intend to perform linkage analysis, only family members affected by history ($n = 15$) were examined. All participants signed an informed consent, which also covered the retrieval of audiograms previously obtained elsewhere.

### Phenotyping the hearing loss

The participants were asked to fill out a questionnaire and underwent otoscopic examination to exclude other causes of hearing impairment. Pure-tone audiometry was performed in a sound-treated room according to current standards. Thresholds that were out of scale or related to vibro-tactile sensation were arbitrarily fixed at 130 dB HL (4–8 kHz). Cross-sectional threshold analysis was performed, comprising 15 family members, to characterize the phenotype of the present trait. The binaural mean air-conduction threshold was used for analysis after having confirmed that the hearing impairment was fairly symmetric. A commercial program (Prism, version 3; GraphPad, San Diego, CS) was used to perform linear regression analysis to evaluate progression of hearing impairment in this family, with extrapolation to age 0 (offset threshold). It was tested whether progression was significant, which implies that the regression coefficient differs significantly from 0. The regression coefficient (i.e., the slope) is called annual threshold deterioration (ATD, in decibels per year). Based upon the cross-sectional analysis, ARTA were constructed, which show the expected threshold for a number of decade steps in age. From the ARTA, a "thresholds features array" was derived (data not shown) for formal statistical testing against similar data documented for DFNA2/*KCNQ4* traits as previously described (19).

### Genetic analysis

DNA was isolated from buccal swabs taken in patients IV:10 and V:5 according to a protocol adapted from Richards et al. (21). Exons 5, 6, and 7 of the *KCNQ4* gene were amplified using standard conditions with the after primers exon 5 (forward) 5′-GAGATGGGGGACCTTTATCC-3′, exon 5 (reverse) 5′-AGCCCTACAAAGACCCTCAC-3′, exon 6 (forward) 5′-GACCAGTCCTGCCTGTAACC-3′, exon 6 (reverse) 5′-AACTGAGCAGGAGGCAACTC-3′, exon 7 (forward) 5′-ACCCTTGCAGCCTCTTACTG-3′, and exon 7 (reverse) 5′-CTGCTCCTAGGGCTTCTTCC-3′. Exons were polymerase chain reaction-amplified using the DYAD PTC200 thermo cycler (MJ Research, Inc., Waltham, MA, U.S.A.), and sequencing was performed with the ABI PRISM Big Dye Thermal Cycle Sequencing V2.0 Kit (Applied Biosystems). Reactions were analyzed with the ABI PRISM 3700 DNA analyzer. DNA from additional family members was isolated from blood samples as described by Miller et al. (22).

## RESULTS

The initial ARTA for the seven already known patients of the present Dutch V family are shown in Figure 2 (center panel), next to the ARTA of DFNA2/*KCNQ4* traits with previously identified genotypes (Table 1).

Only the Belgian family showed different ARTA, which might be because this family had a truncating mutation N-terminal of the pore encoding region (18). All other ARTA demonstrate a fairly similar type, degree, and rate of deterioration of thresholds with advancing age. The apparent similarity with ARTA from families with known defects in the *KCNQ4* protein within or close to the channel pore region instigated us to search for *KCNQ4* mutations. The already known pathologic W276S missense mutation in exon 5 of the *KCNQ4* gene was detected in the proband and her daughter. At the nucleotide level, guanine is replaced by cytosine at position 827. This causes the tryptophan (W) to change into a serine (S) amino acid at position 276, affecting the pore region of the potassium voltage-gated channel. Subsequently, the W276S mutation was confirmed in all additional affected family members by mutation analysis.

All participants were clinically hearing impaired and had fairly symmetric, down-sloping audiograms. None of them had a history of noise exposure or had any clear vestibular symptoms. Six patients mentioned having tinnitus occasionally. Patient IV:8 had undergone bilateral mastoidectomy and was the only one that revealed an abnormal otoscopic finding; myringosclerotic spots were seen in both ears. This patient was excluded from the analysis.

In Figure 3, the last-visit audiograms of 16 patients are shown, including a retrieved audiogram of subject II:1 who had already died. The presbyacusis ISO7029 P95 values for matching age and gender (23) are also drawn in the audiograms. Participants were considered clinically impaired when the thresholds at three or more frequencies were worse than the corresponding presbyacusis P95 thresholds.

Figure 4A and B shows the results of the cross-sectional analysis of 15 affected family members examined in the second part of this study. Significant progression was demonstrated for all frequencies measured. The average ATD value for all frequencies was 0.65 dB per year. The offset threshold increased from 19 dB at 0.25 kHz to 66 dB at 8 kHz, suggesting congenital onset of hearing impairment. The ARTA derived from this analysis are shown in Figure 4C.

## DISCUSSION

The present family is the first one in which we immediately applied mutation analysis only based on regression analysis of previously obtained audiometric data (threshold on age) to characterize the phenotype. This study was deliberately scheduled as a pilot study with initial phenotype determination and attempted initial mutation analysis of only a small number of affected family members. Later mutation analysis of all the other affected relatives participating in the remainder of the study confirmed the presence of the disease-causing mutation.

In the past, we had already experienced in another DFNA2/*KCNQ4* family (Dutch IV) that the phenotype

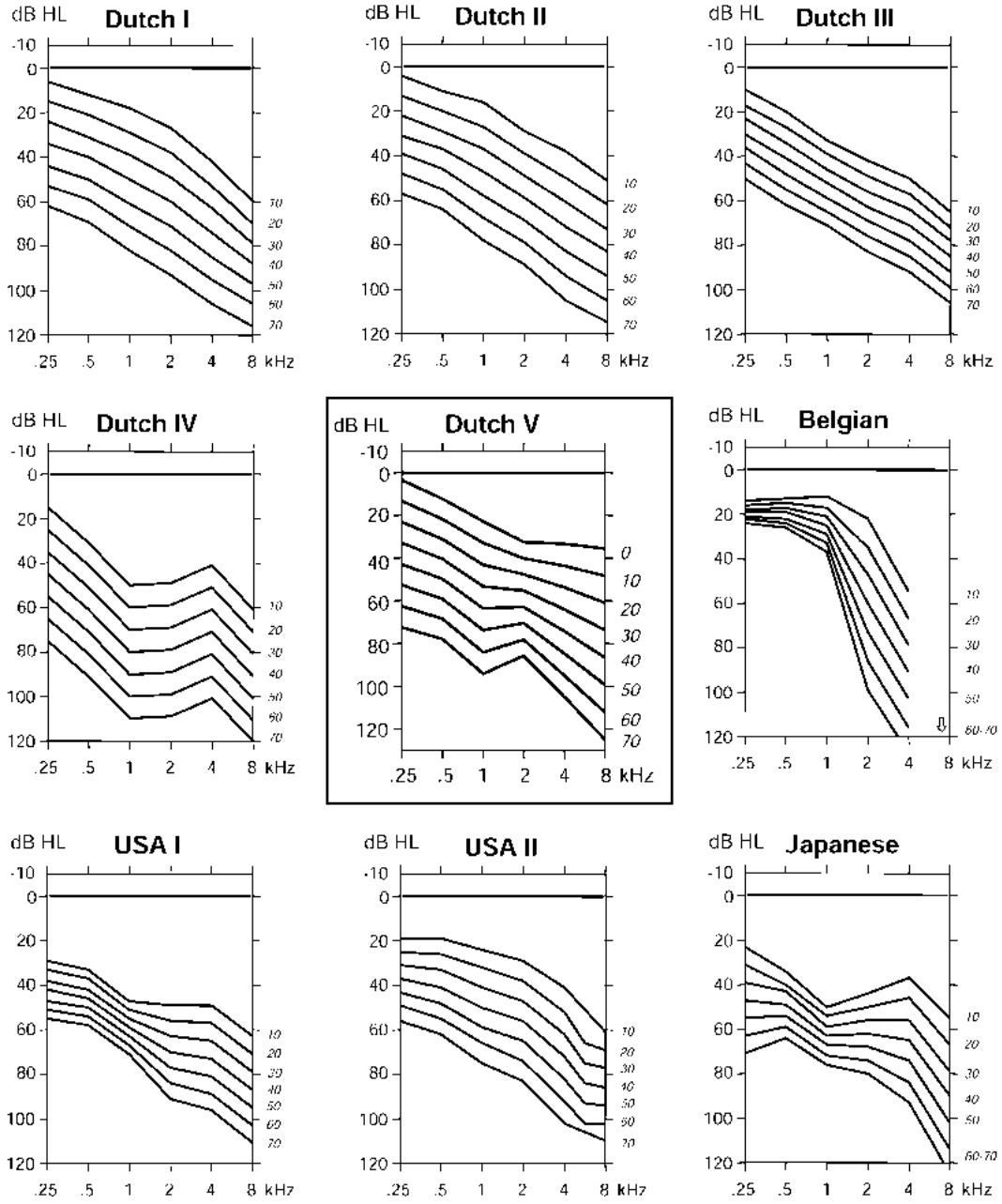


**FIG. 2.** Initial ARTA of family Dutch V (*center panel*), compared with ARTA of eight known DFNA2 families reported by De Leenheer et al. (17). Age in italics.

was so characteristic that we decided to skip linkage analysis and successfully endeavored mutation analysis of *KCNQ4* on affected family members (13). An important difference between the study of the Dutch IV family and the present one (Dutch V) is that the clinical study of family Dutch IV had already been completed, including collection of audiometric data and the extraction of DNA from the blood samples, before the start of the genetic analysis. The results of the study on the Dutch IV DFNA2/*KCNQ4* family helped us to focus on ARTA, including the possibility of deriving a threshold features array and formally testing this against available normative or standard data (19), especially within the context of analyzing progressive autosomal-dominant hearing impairment traits (18).

The Dutch V family is the fourth DFNA2 family in which the W276S mutation was identified. Two of the previously identified families were also Dutch (I and IV), and the other family originates from Japan. Van Camp et al.(20) examined whether these three families were related by analyzing flanking microsatellite markers and intragenic single nucleotide polymorphisms. Because

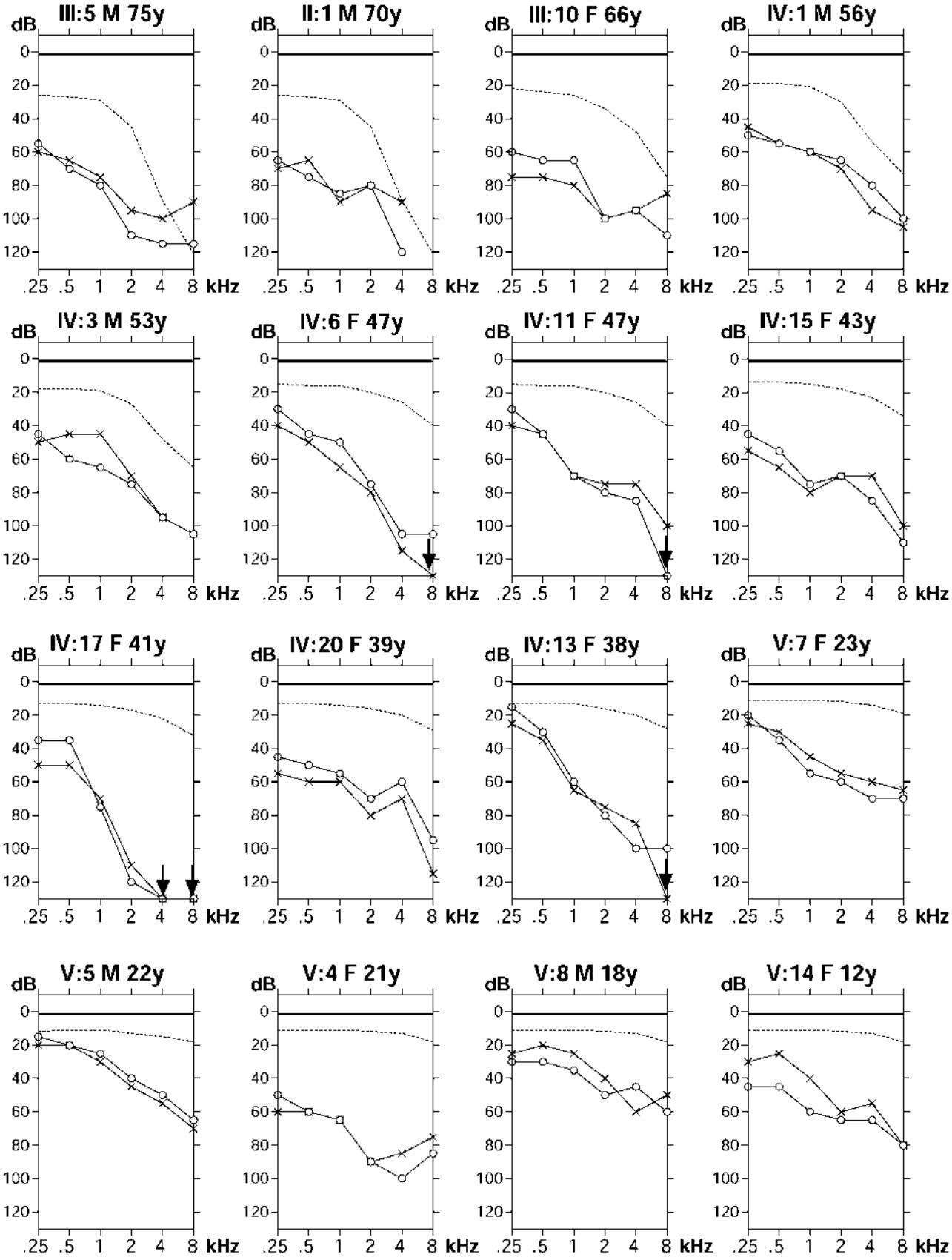


**FIG. 3.** Individual last-visit audiograms in 16 members of the family, ordered by age (y, year). Audiogram of case II:1 was retrieved after his death. *Circles*, right ear (air conduction level); *crosses*, left ear; *dotted line*, threshold according to P95 presbyacusis; *Downward arrow*, out-of-scale measurement; *M*, male; *F*, female.

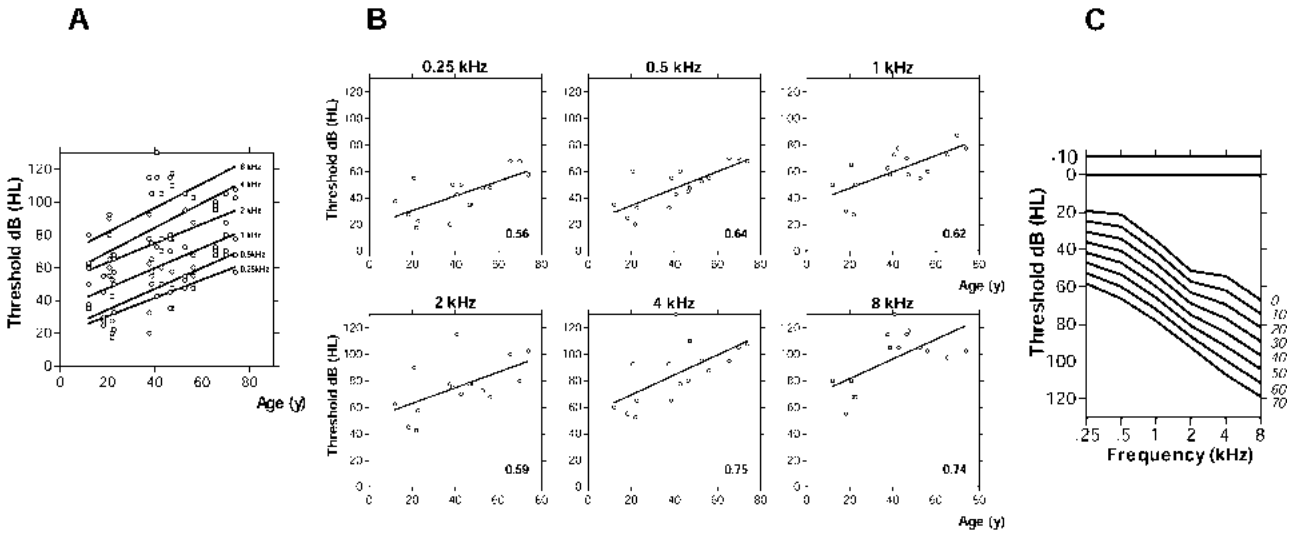


**FIG. 4.** Cross-sectional linear regression analysis of binaural mean air-conduction threshold on age (years) in 15 family members for all frequencies shown together (*A*) or separately (*B*), as well as final ARTA derived from the threshold values predicted by regression at given ages in decade steps (*C*, age in italics). ATD value is shown in separate frequency panels (*B*).

they identified differences between the families in both the flanking markers and the intragenic polymorphisms, they concluded that tryptophan at position 276 is a hot spot for mutation. Although the present family might be related to one of the previously described families, Dutch I and IV, the results of Van Camp et al.(20) show that the similarity of the phenotype in the different W276S mutation is not the result of the genetic background of the families.

DFNA2 families with a different mutation in the pore region of KCNQ4 show a phenotype that is comparable with that of W276S families. Hearing impairment in DFNA2 families in general affects all but is most severe in the high frequencies and has an early onset. It shows progressive hearing impairment, which is comparable in all frequencies, although the progression can be somewhat larger in the high frequencies. In the low frequencies, the hearing loss does not proceed to profound. Using these characteristics, the ARTA of DFNA2 families can be distinguished from the ARTA of other DFNA phenotypes (18). So far, only one mutation (FS71) has been identified outside the pore region of KCNQ4 in a Belgian family, and the related ARTA show a distinct phenotype with purely high-frequency hearing loss. Minor differences in the ARTA of the families with mutations affecting the channel pore region might be due to the genetic background. Also, intrafamiliar differences might contribute to apparent differences in the ARTA. For example, in the present family, patient V:4 has similar hearing impairment at age 21 years as patient IV:3 at age 53 years. Of course, individual variations in hearing impairment have a greater bearing on familial ARTA in families with a relatively small number of affected individuals.

It has already been shown that phenotype analysis is most helpful to guide the diagnostic efforts more directly to the gene involved for early childhood, low-frequency, autosomal-dominant, sensorineural hearing impairment (DFNA6/14) (24) and for midlife-onset progressive cochleovestibular impairment (DFNA9) (25). On the other hand, phenotypic differences among different mutations in one and the same gene, related to different effects of the mutations or genetic background, are also seen. For example, autosomal dominantly inherited mid-frequency hearing impairment (DFNA8/12) caused by mutations in the *TECTA* gene may be progressive or nonprogressive. Autosomal recessively inherited mutations in this gene may even cause prelingual severe to profound deafness (DFNB21) (26,27).

Most of the 51 DFNA types show a high-frequency progressive sensorineural hearing loss manifesting in the first or second decade of life. For these DFNA types, the current study demonstrates that audiometric analysis can be helpful in genotyping. It is, therefore, very useful to be aware that in such a situation an audiometric analysis may guide the investigation directly to the gene involved. If it succeeds, such an approach can be very cost- and time-effective.

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# Chapter 5

## Variable clinical features in patients with CDH23 mutations (USH1D-DFNB12)

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# Variable Clinical Features in Patients with *CDH23* Mutations (USH1D-DFNB12)

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**Objective:** To describe the findings of audiovestibular and ophthalmologic examinations in four families with mutations in the *CDH23* gene.
**Study Design:** Family study.
**Setting:** Tertiary referral center.
**Patients:** Four DFNB12 patients from a large consanguineous Dutch family and six patients from three different Usher syndrome Type ID families were examined. All were identified by at least one pathogenic mutation in the *CDH23* gene.
**Methods:** Audiovestibular examinations consisted of standard pure-tone audiometry, vestibulo-ocular reflex, optokinetic nystagmus, and in some cases the cervico-ocular reflex. Linear regression analysis was used to evaluate progression of hearing impairment, and the degree of hearing impairment of DFNB12 was compared with that found for USH1D. Ophthalmologic examinations consisted of best-corrected visual acuity, Goldmann perimetry, slit-lamp examinations, color vision testing, dark adaptation, electroretinography, electro-oculography, funduscopy and photography of the retina, and sometimes fluorescein angiography.
**Results:** The USH1D patients had significantly worse hearing impairment than the DFNB12 patients. The DFNB12 patients, identified by missense mutations in *CDH23*, had normal retinal and vestibular function. All USH1D patients had splice-site mutations in *CDH23* and a typical Usher syndrome Type I phenotype. One DFNB12 patient had slightly abnormal yellowish flecks in the posterior poles of both eyes.
**Conclusion:** Recessive missense mutations in *CDH23* lead to a milder phenotype (DFNB12) than splice-site mutations (USH1D); however, abnormal bilateral flecks, suggestive for lipofuscin accumulation, can be observed in DFNB12 patients. **Key Words:** *CDH23* gene—DFNB12—Hearing impairment—Genotype-phenotype correlation—Retinitis pigmentosa—Usher syndrome—USH1D.

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"Usher syndrome" covers a group of autosomal recessive inherited disorders characterized by sensorineural hearing impairment and visual impairment mainly caused by retinitis pigmentosa. In some of the patients, vestibular dysfunction also occurs. This syndrome is named after Charles Usher, a Scottish ophthalmologist who described familial retinal pigment disorders and noted that some of his retinitis pigmentosa patients also had hearing impairment (1). Three different clinical types of Usher syndrome are known. Usher syndrome Type I is characterized by congenital, profound deafness associated with vestibular areflexia and retinitis pigmentosa. Usher syndrome Type II is characterized by moderate to severe sensorineural hearing impairment, intact vestibular responses, and retinitis pigmentosa. Usher syndrome Type III is characterized by progressive hearing impairment, variable vestibular function, and retinitis pigmentosa (2).

Several genotypes were found to be causing these three clinical types of Usher syndrome (Table 1). Currently, seven loci have been mapped for Usher syndrome Type I (USH1A-USH1G), three loci for Usher syndrome Type II (USH2A-USH2C), and one locus for Usher syndrome Type III (USH3) (3). Five of the seven genes involved in Usher syndrome Type I have been cloned. Cadherin 23 (*CDH23)* is the gene involved in Usher syndrome Type ID (USH1D) and has been mapped to chromosome 10q21-22. This gene is also known to be involved in DFNB12, an autosomal recessive type of nonsyndromic hearing impairment. Therefore, USH1D

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**TABLE 1.** *Genetic subtypes of Usher syndrome*

| | Genotype | Locus | Gene |
|---|---|---|---|
| Usher Type I | USH1A | 14q32 | — |
| | USH1B | 11q13.5 | *MYO7A* |
| | USH1C | 11p15.1 | *USH1C* |
| | USH1D | 10q21-22 | *CDH23* |
| | USH1E | 21q | — |
| | USH1F | 10q21-22 | *PCDH15* |
| | USH1G | 17q24-25 | *SANS* |
| Usher Type II | USH2A | 1q41 | *USH2A* |
| | USH2B | 3p23-24.2 | — |
| | USH2C | 5q14.3-q21.3 | — |
| Usher Type III | USH3 | 3q21-q25 | *USH3* |

and DFNB12 are allelic disorders (4–6). The *CDH23* gene is not the only gene involved in syndromic as well as nonsyndromic hearing impairment; other genes are: *USH1C* (USH1C/DFNB18), *SLC26A4* (Pendred syndrome/DFNB4), *WFS1* (Wolfram syndrome/DFNA6/14), *COL11A2* (DFNA13/Stickler syndrome), and *MYO7A* (USH1B/DFNA11/DFNB2) (7–12). This report describes the findings of audiovestibular and ophthalmologic examinations in 10 patients from four different families (three USH1D, one DFNB12) carrying *CDH23* mutations and relates the encountered features to the underlying genotype.

## PATIENTS AND METHODS

### Patients, families, and the associated genotype

In this study, four families were examined, and their pedigrees are shown in Figure 1. After the identification of pathogenic mutations in the *CDH23* gene (Table 2), these families were contacted again for additional audiovestibular and ophthalmologic examinations. A written informed consent was ob-

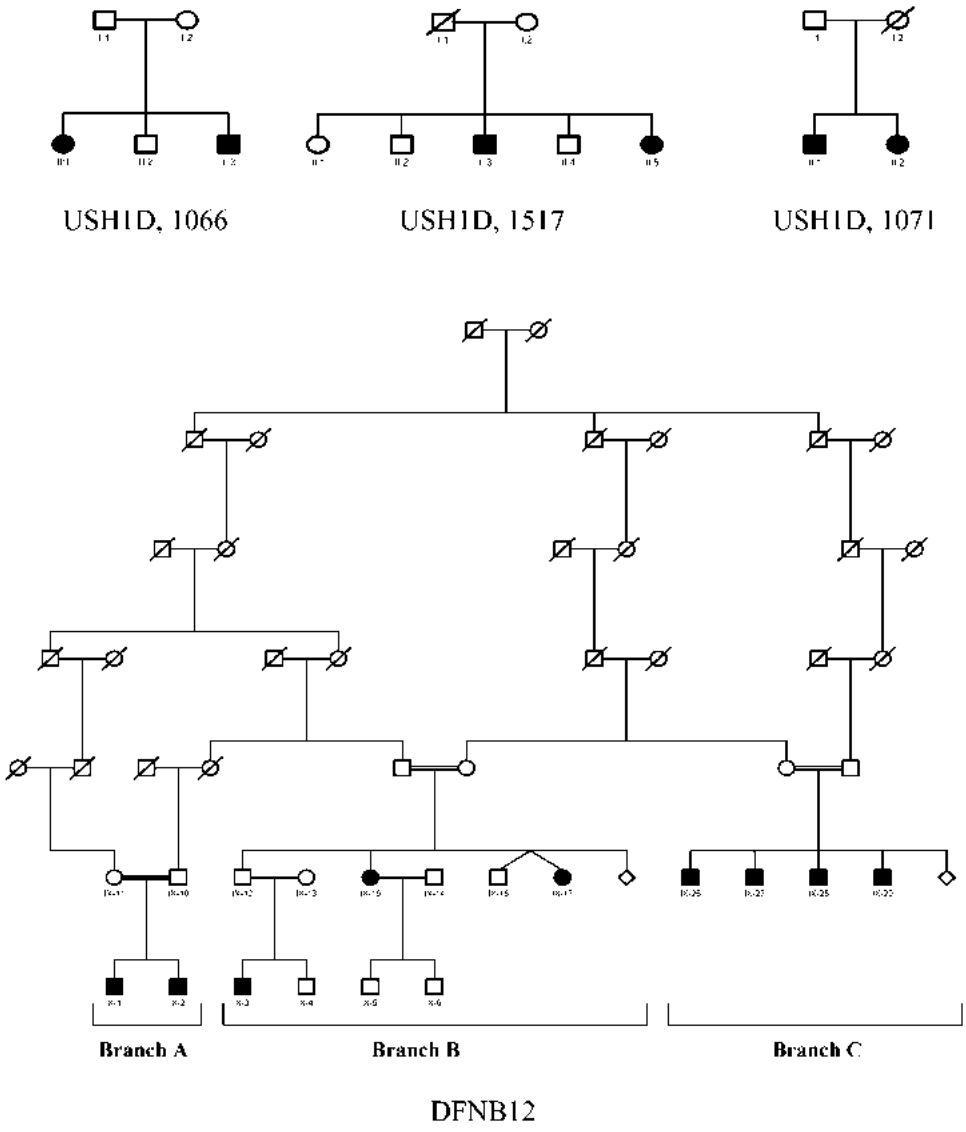


**FIG. 1.** Pedigrees of the families.

**TABLE 2.** *Genotype of three USH1D families and one DFNB12 family*

| | Family | Mutation 1 | Mutation 2 | Exon | Domain |
|---|---|---|---|---|---|
| USH1D | 1517 | 1450G>C (A484P) | 1450G>C (A484P) | 14 | EC5 |
| | 1071 | IVS45-9G>A | Unknown | 46 | EC19 |
| | 1066 | IVS20+1G>A | Unknown | 20 | EC7 |
| DFNB12 | Branch A | D2148N | D2148N | 47 | EC20 |
| | Branch B | D2148N | D1341N | 47, 31 | EC20, EC13 |

tained from all patients and nonaffected family members. In addition, a medical history was taken for all patients, focusing on audiovestibular and visual impairment.

### *USH1D families*

Two siblings each were affected by Usher syndrome Type I in two Dutch families (1066 and 1071). In a third family (1517), originating from Flanders, Belgium, again two siblings were affected by Usher syndrome Type I. Individual II-5 from this family did not participate in the study; however, her previous clinical data were retrieved with her permission. Mutation analysis of *CDH23* in the USH1D families was performed by heteroduplex and confirmation of the identified mutations was performed by sequencing. Some exons were studied directly by sequencing. In this way, more than 95% of the coding region of *CDH23* was screened (13). A homozygous 1450 G>C mutation was identified in both affected individuals of the Belgian Family 1517. This mutation not only leads to an amino acid substitution of proline for alanine at position 484 but is also predicted to cause a splicing defect of exon 14 (14). In Family 1071, one heterozygous mutation in both affected individuals was identified in the *CDH23* gene: IVS45-9 G>A; this mutation affects the splice-acceptor region preceding exon 46 (14). So far, the second mutation has not been found. In family 1066, a IVS20 + 1 G>A mutation was identified in *CDH23*, whereas the second mutation in this family remains to be identified as well. The IVS20 + 1 G>A mutation also disrupts the donor splicing sequence (14). All identified mutations are located within the extracellular cadherin (EC) domains of cadherin 23 and lead to truncation of the protein.

### *DFNB12 family*

Four patients of the fourth family (W90-004), a large consanguineous family with nonsyndromic autosomal recessive sensorineural hearing impairment, were shown to be affected by DFNB12, and three of them could be contacted and decided again to participate in this study. For Patient IX-17, the data retrieved from her previous clinical examinations dating from the 1970s and 1980s were used. Mutation analysis recently showed that mutations in two different genes were responsible for the hearing impairment in this family (15). The hearing impairment in Branch C of this family (Fig. 1) was caused by a homozygous 35delG mutation in the *GJB2* gene (DFNB1). In Branch A, Patients X-1 and X-2 were found to have a homozygous D2148N mutation in *CDH23*, whereas both Patients IX-15 and IX-17 of Branch B are compound heterozygous for this mutation and the D1341N mutation. These two amino acid substitutions are located in the highly conserved calcium-binding sites of the EC domains of cadherin 23.

## Audiometric examinations

Audiometric examination consisted of standard clinical pure-tone audiometry in a sound-treated room. Previously performed audiometric examinations were retrieved to evaluate possible individual progression of hearing impairment. Some of the previously recorded audiometric data of Family W90-004 were described by Marres and Cremers (16). Individual, longitudinal pure-tone thresholds were analyzed for progression of hearing impairment using linear regression analysis (binaural mean air-conduction threshold on age). It was checked whether progression could be called significant (i.e., 0 outside 95% confidence interval for slope at two or more of six or seven frequencies). At each frequency, the last-visit binaural mean pure-tone threshold was compared between USH1D and DFNB12 patients using a $2 \times 2$ contingency table and Fisher's exact probability test. The level of significance used in all tests was $p = 0.05$.

## Vestibular examinations

Vestibulo-ocular examinations were performed with the patient sitting in the upright position in a rotatory chair. Visually guided eye movements were evaluated (saccades, optokinetic nystagmus, and smooth pursuit eye movements), including monitoring of the presence of any spontaneous or gaze-evoked nystagmus. Vestibular tests were performed in the dark with eyes open. The vestibulo-ocular reflex was evaluated using velocity steps of 90 deg/s in either direction with electronystagmography and computer analysis as previously described (17). The cervico-ocular reflex was elicited only in patients showing a lack of the vestibulo-ocular reflex (18).

## Ophthalmologic examinations

Ophthalmologic examinations consisted of corrected visual acuity measurements, slit-lamp examinations, and funduscopy. Visual fields were evaluated by Goldmann perimetry using Test Targets V-4, III-4, and I-4. Test Target III-4 was evaluated according to American Medical Association guidelines (19). Additional ophthalmologic tests included electroretinography and electro-oculography, both of which were performed and evaluated according to International Society for Clinical Electrophysiology of Visions standards (20,21). Color vision was tested using the Tokyo Medical College color vision test, the Standard Pseudoisochromatic plates, the Ishihara test, the New Color test by Lanthony, the light discrimination test, and the Farnswoth-Munsell 100 Hue test, as previously described (22). Dark adaptation was performed with the Goldmann-Weekers adaptometer. Finally, the fundus was photographed and fluorescein angiography was performed to record the retinal picture.

# RESULTS

## Audiometric findings

Longitudinal analysis of pure-tone thresholds could be performed in three USH1D patients (n = 5–12; age range, 2–39 yr) and in two DFNB12 patients (n = 4; age range, 10–40 yr). None of these patients showed significant progression of hearing impairment (data not shown). Figure 2 shows the individual last-visit audiograms of both patient groups. The USH1D patients generally only

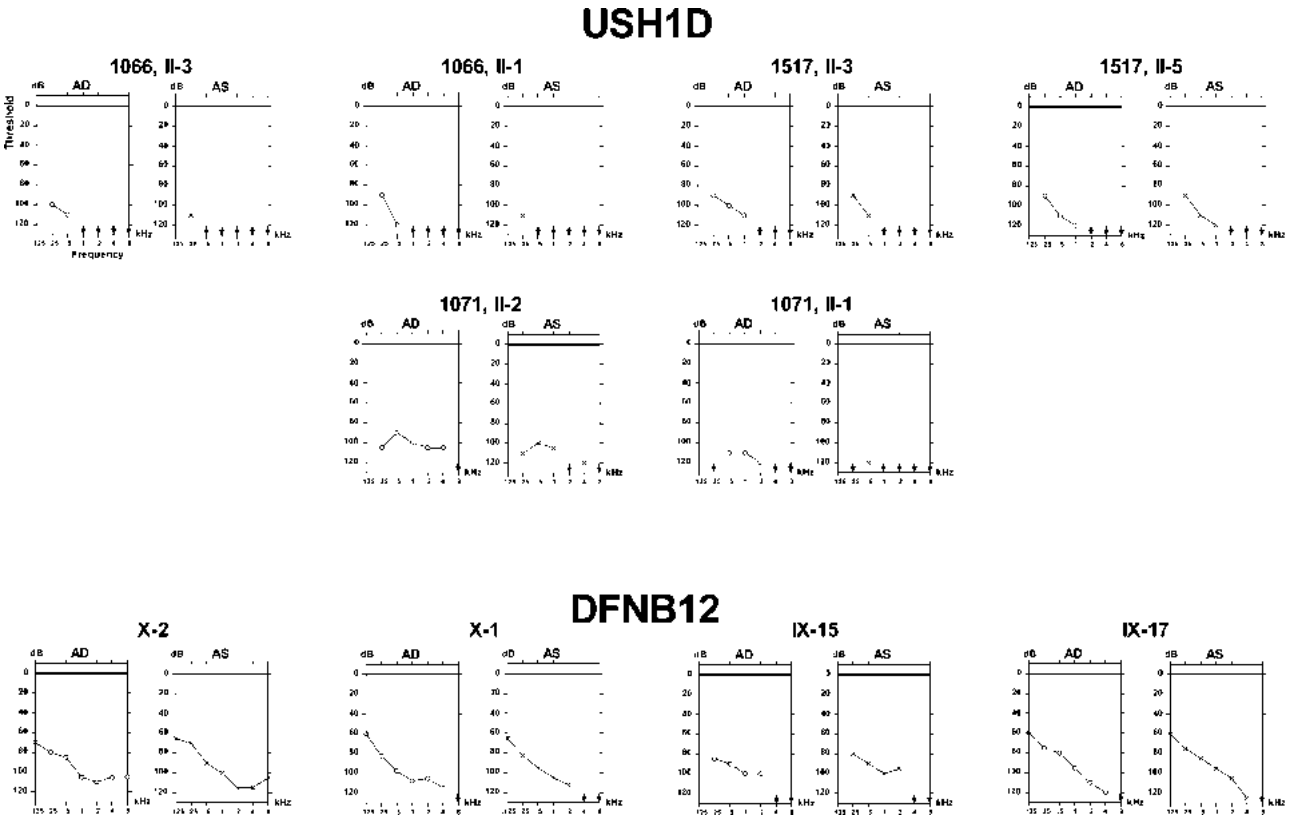


**FIG. 2.** Individual last-visit audiograms. (*Circles*) right ear (air conduction level); (*crosses*) left ear; (*downward arrows*) out-of-scale measurement.

had residual hearing at the low frequencies (125–500 Hz), whereas the DFNB12 patients showed severe to profound sensorineural hearing impairment with thresholds that could be measured up to and including 4 kHz in most cases.

Comparison between USH1D and DFNB12 patients revealed that the USH1D patients had significantly worse hearing than the DFNB12 patients at all frequencies except for the highest ones ($p < 0.05$). Figure 3 shows the "mean audiogram" for the DFNB12 patients ($n = 4$) and the USH1D patients ($n = 6$).

### Vestibular findings

All examined USH1D patients started to walk independently at age older than 18 months, except for Patient III-1 of Family 1066 (at age 12 mo). Smooth pursuit was not tested in the USH1D patients because they had difficulty with catching up to the target and was found to be normal in the DFNB12 patients. USH1D Patient II:3 of Family 1517 (aged 40 yr) tended to show spontaneous nystagmus in the dark. Saccades were normal in all patients. Optokinetic nystagmus could be elicited in all USH1D patients, except for the oldest one (II-1, Family 1071), who had very poor vision. Optokinetic nystagmus showed sufficiently high slow-phase velocity except in Patient II-3 of Family 1066 (aged 18 yr). Vestibular examinations disclosed vestibular areflexia in all patients, and in three patients the cervico-ocular reflex was found to be enhanced, which is in line with the findings in labyrinthine-defective subjects previously described by Huygen et al. (18).

### Ophthalmologic findings

All of the examined Usher syndrome Type I patients suffered from nyctalopia in childhood; most of them had been diagnosed to have retinitis pigmentosa in the first decade of life. As expected, none of the patients with DFNB12 complained about their vision.

Table 3 shows the results of the ophthalmologic examinations. None of the three DFNB12 patients had evidence of malfunction of the retina. Funduscopy revealed remarkable findings in two of the three DFNB12 patients. In Patient X-1, almost symmetric, small, yellowish flecks with central clustering of pigment at the level of the retinal pigment epithelium (RPE) were seen in the posterior pole of both eyes. These flecks were most clearly seen in the right fovea (Fig. 4A). Patient X-2 showed decreased filter action of the RPE on fluorescein angiography and had increased reflexes and minifolds of the internal limiting membrane in the macular area. Patient IX-15 had no retinal abnormalities on funduscopy.

Five USH1D patients had ophthalmologic findings characteristic for retinitis pigmentosa with attenuated vessels, bony spicules, thinning of the RPE, and a waxy

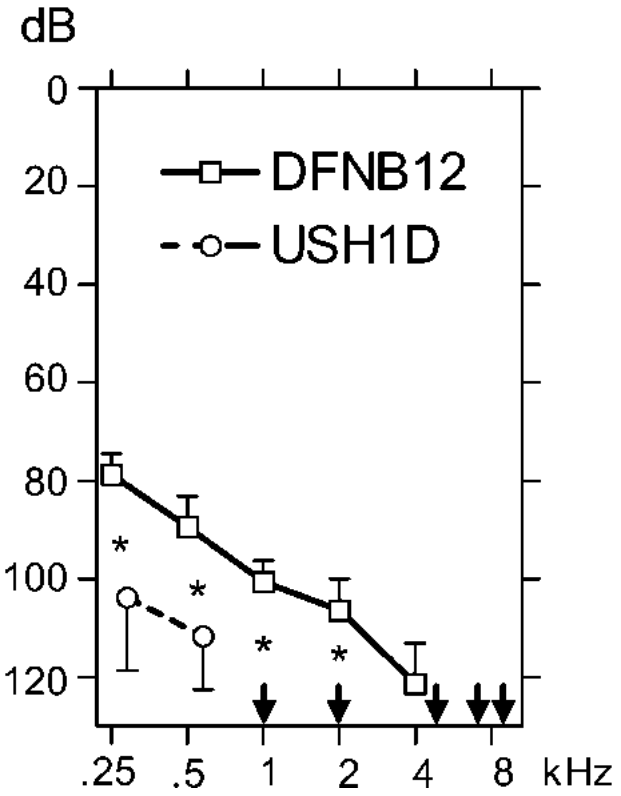


**FIG. 3.** "Mean audiograms" in both patient groups. Bar represents 1 SD. *Significant result of Fisher's exact probability test, which included out-of-scale measurements (*downward arrow*).

disk appearance. In Patient II-2 of Family 1071, a white elevated structure above the disk was seen, as was a common choroidal nevus (Fig. 4B). This whitish lesion was not unlike the retinal changes seen in Bourneville's tuberous sclerosis (23). Patient II-3 of Family 1517 had remarkable asymmetry of eye findings. His right eye only had light perception and showed severe bony spicules, whereas both were less severe in his left eye. In all USH1D patients, the electroretinogram and electrooculogram were extinguished and poor best-corrected visual acuity scores were found. All patients had tunnel vision confirmed by Goldmann perimetry and all had functional vision scores below 45% (<50% indicates severe loss of vision) (19). One patient (II:1, Family 1071) had total loss of vision. In four of the five Usher syndrome patients, subcapsular posterior cataracts were seen; two of them have had cataract extraction for this condition, whereas the other patients may need surgery in the future.

## DISCUSSION

Comparison of the audiometric features of six USH1D and four DFNB12 patients, all identified by at least one mutation in the *CDH23* gene revealed that the USH1D patients had significantly more hearing impairment than the DFNB12 patients. In addition, only the USH1D patients had vestibular areflexia and progressive retinitis pigmentosa. Two DFNB12 patients showed slightly abnormal funduscopic findings. In Patient X-1, bilateral abnormal flecks of the RPE were seen, and in Patient X-2, slight wrinkling of the internal limiting membrane and a decreased filter action of the RPE were noticed, although neither of these features caused any functional problems. The present findings are in line with previous observations (6,13), which noted that amino acid substitutions in *CDH23* cause severe to profound hearing impairment with normal vestibular responses and retinal function (DFNB12) and that mutations that lead to *CDH23* protein truncation cause congenital profound deafness, vestibular areflexia, and the development of retinitis pigmentosa (USH1D).

Astuto et al. (13) reported on mutation analysis of *CDH23* in a large group of patients. In a selected panel of probands with Usher syndrome Type I, they identified *CDH23* mutations in 35 of 69 probands with Usher syndrome. USH1D was found to be caused by nonsense, frameshift, splice-site, and missense mutations. In only three families, two missense mutations were identified, and these families all had an atypical form, with variation in the retinal phenotype, of Usher syndrome Type I. All other missense mutations were compound heterozygous, with a truncating mutation of the other allele. Nonsyndromic autosomal recessive hearing impairment families were also examined, and only amino acid substitutions were observed. Results of ophthalmologic examinations in these patients with nonsyndromic autosomal recessive deafness in some cases showed asymptomatic retinitis pigmentosa-like manifestations. Missense mutations in the *CDH23* gene, therefore, may have a subtle effect on the retina, which was also noted in two of the present DFNB12 patients. In addition, Astuto et al. (13) also describe that USH1D patients display a wide range of hearing loss and retinitis pigmentosa phenotypes, differing in severity, age of onset, type, and the presence or absence of vestibular areflexia. The findings in the present USH1D patients are typical for Usher syndrome Type I. In line with these results, the findings in the USH1D patients presented in this article suggest a second mutation (missense or truncating) in *CDH23*. However, in Families 1066 and 1071, the second mutation in *CDH23* was not identified. This can have several explanations. First, heteroduplex analysis can detect insertions, deletions, and most but not all single-base substitutions (24). Furthermore, branch site mutations, splice-site mutations in an intron, or mutations in regulatory elements cannot be detected by heteroduplex analysis.

Cadherin 23 is a transmembrane protein with 27 extracellular cadherin repeats, a transmembrane domain, and a cytoplasmic domain. It is encoded by *CDH23*, which consists of 69 exons (5,6). Cadherins are important for cell-to-cell contact and the organization of the extracellular matrix. Binding of calcium ions to these proteins is essential for linearization, rigidification, and dimerization of the cadherin molecules (25,26). Mutations in *Cdh23* lead to disorganization of the stereocilia

**TABLE 3.** *Visual characteristics in three DFNB12 patients and five USH1D patients*

| | DFNB12 (patient) | | | USH1D (patient, family) | | | | |
|---|---|---|---|---|---|---|---|---|
| | X-2 | X-1 | IX-15 | II-3, 1066 | II-1, 1066 | II-3, 1517 | II-2, 1071 | II-1, 1071 |
| Gender | Male | Male | Female | Male | Female | Male | Female | Male |
| Age (yr) | 29 | 33 | 52 | 26 | 33 | 40 | 47 | 51 |
| VAS (%) | | | | | | | | |
| RE | 105 | 105 | 100 | 90 | 90 | lp | 60 | No lp |
| LE | 90 | 105 | 100 | 80 | 95 | 80 | 60 | No lp |
| VFS (%) | | | | | | | | |
| RE | 100 | 97 | 91 | 51 | 44 | 0 | 31 | NA |
| LE | 100 | 98 | 93 | 50 | 36 | 43 | 32 | NA |
| FAS (%) | 102 | 105 | 100 | 88 | 94 | 64 | 60 | 0 |
| FFS (%) | 108 | 104 | 93 | 51 | 44 | 43 | 32 | 0 |
| FVS (%) | 100 | 100 | 93 | 45 | 41 | 28 | 19 | 0 |
| Media | | | | | | | | |
| RE | Normal | Normal | Normal | Minor SPC | Vitreous body strings | SPC ++, divergent strabism | SPC ++, surgery | Minor SPC |
| LE | Normal | Normal | Normal | SPC + | Normal | Minor SPC, some cells in vitreous body | SPC ++, surgery | SPC ++, surgery |
| EOG | Normal | Normal | Normal | Extinguished | Extinguished | Extinguished | NA | NA |
| ERG | Normal | Normal | Normal | Extinguished | NA | Extinguished | Extinguished | Extinguished |
| Dark adaptation | Normal | Normal | Normal | NA | NA | No lp | NA | NA |
| Color vision | Normal | Normal | Normal | Red-green defect | Normal | Normal | NA | NA |

VFS, Visual Field Score; VAS, Visual Acuity Score; FAS, Functional Acuity Score; FFS, Functional Field Score; FVS, Functional Vision Score; EOG, electro-oculogram; ERG, electroretinogram; NA, not available; SPC, subcapsular posterior cataract; lp, light perception; RE, right eye; LE, left eye.

of the hair cells in the inner ear of *waltzer*, the mouse model for USH1D (26). The missense mutations found in the present DFNB12 family and in other DFNB12 families reported in the literature are located in the highly conserved extracellular calcium-binding motifs. Modeling has shown that these mutations are likely to induce a decrease in the capacity for calcium binding (15). As calcium provides rigidity to the elongated structure of cadherin molecules, it is likely that mutations in these calcium-binding motifs lead to a disturbance of the elongated shape of cadherin. Cadherin 23 has been suggested to be a candidate molecule for forming the lateral links or tip links between the stereocilia (26,27). A disturbed elongated shape of cadherin 23 may therefore lead to disorganization of the stereocilia because two mutated cadherin 23 molecules are not able to interact directly or with an additional molecule to correctly establish these links (15). This report shows that the hearing impairment caused by mutations in *CDH23* is significantly more severe in USH1D than in DFNB12. It therefore seems

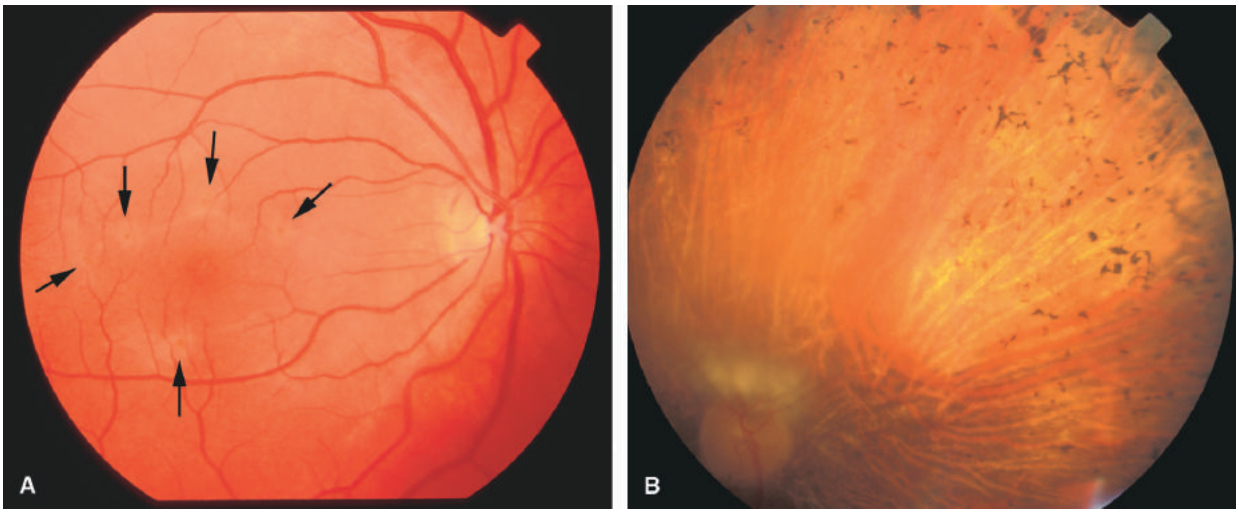


**FIG. 4.** (*A*) Fundus photograph of the right eye of DFNB12 Patient X-1; around the macula, depigmentations with central clustering of pigment can be seen (*arrows*). (*B*) Fundus photograph of the right eye of USH1D Patient II-2 of Family 1071; a white elevated spot can be seen above the waxy disk. Bone spicules and attenuated vessels are also seen. The retinal pigment epithelium has vanished almost completely.

likely that the truncated protein in USH1D totally disturbs the interaction and elongated shape of the stereocilia and thus negatively influences the mechanoelectrical transduction in cochlear hair cells, whereas the disturbance in function of cochlear hair cells is less severe in the DFNB12 patients with reported amino acid substitutions in the conserved calcium binding motifs. Another pathogenic mechanism is suggested by Wilson et al. (28), who claimed that cadherin 23 may in addition be involved in ion homeostasis of the endolymph in the inner ear, because of expression of *Cdh23* in the utriculosaccular foramen, the ductus reuniens, and Reissner's membrane.

The abnormal funduscopic findings in Patient X-1 of the DFNB12 family are atypical for retinitis pigmentosa; however, they are clearly abnormal. The small flecks may have been caused by previous inflammation of the RPE as described by Krill and Deutman (29), but this could not be substantiated in this patient. Forgacs and Bozin (30) in 1966 described similar flecks in two sisters and therefore suggested a genetic cause. An alternative explanation of the bilateral flecks and the decreased filter action of the RPE in the present two brothers could be that these two findings relate to accumulation of lipofuscin. Lipofuscin accumulation is known to occur in Stargardt's disease, age-related macular dystrophy, and in some forms of retinitis pigmentosa (31). The protein of another Usher syndrome Type I gene, *MYO7A*, has a crucial function in the normal processing of ingested disk membranes in the RPE, primarily in the basal transport of phagosomes into the cell body, where they then fuse with lysosomes (31). When myosin VIIa is absent, the half-life of the phagosomes is extended, and it is suggested that impaired phagosome degradation may be responsible for the accumulation of lipofuscin in the RPE in some forms of retinitis pigmentosa (31,32). In the cochlea, several Usher syndrome Type I proteins (USH1B, USH1C, USH1D, and USH1G) form a functional complex that shapes the sensory hair cell bundle (33–35). Hypothetically, these genes may also interact in the process of ingestion of outer segment disk membranes and phagosome degradation in the RPE. Possibly, the lipofuscin accumulation seen in the present two DFNB12 patients is caused by a disturbance of the phagosome degradation in the RPE.

Both patients with abnormal retinal findings have a homozygous missense mutation (D2148N) in *CDH23*, whereas a normal retina was seen in the older patient (IX-15), who was compound heterozygous for two missense mutations (D2148N/D1341N). Possibly, the homozygous combination of the D2148N mutation affects the retina more than a combination of this mutation with D1341N. Another possibility is that this difference is caused by the different position of these two mutations and subsequent relationship with the EC domains. Also, some previously studied USH1D families, characterized as atypical, showed absent, delayed, or atypical ophthalmologic features suggestive of retinitis pigmentosa (36). It may therefore be useful to examine the retina of patients with nonsyndromic autosomal recessive hearing impairment for abnormalities, indicating the possible involvement of *CDH23*.

## CONCLUSIONS

From this study, we conclude that the two missense mutations in *CDH23*, found in four patients of the DFNB12 family, cause nonsyndromic sensorineural hearing impairment, which is accompanied by clinically silent funduscopic abnormalities in two of the present patients. More elaborate, ophthalmologic studies of DFNB12 patients need to be performed to answer the question of whether there is a true relationship between the mutations and the encountered funduscopic abnormalities. Inactivating splice-site mutations in *CDH23* were found in six patients from three USH1D families that lead to significantly more severe hearing impairment than in the DFNB12 patients and, in addition, to retinitis pigmentosa and vestibular areflexia.

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# Chapter 6

## Audiological analyses confirm a cochlear component, disproportional to age, in stapedial otosclerosis

**V. Topsakal, E. Fransen, S. Schmerber, F. Declau, M. Yung, F. Gordts, G. Van Camp, P. Van de Heyning. Otol Neurotol. 2006 Sep; 27 (6):781-787**

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# Audiometric Analyses Confirm a Cochlear Component, Disproportional to Age, in Stapedial Otosclerosis

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**Objective:** To report the preoperative audiometric profile of surgically confirmed otosclerosis.
**Study Design:** Retrospective, multicenter study.
**Setting:** Four tertiary referral centers.
**Patients:** One thousand sixty-four surgically confirmed patients with otosclerosis.
**Interventions:** Therapeutic ear surgery for hearing improvement.
**Main Outcome Measures:** Preoperative audiometric air conduction (AC) and bone conduction (BC) hearing thresholds were obtained retrospectively for 1064 patients with otosclerosis. A cross-sectional multiple linear regression analysis was performed on audiometric data of affected ears. Influences of age and sex were analyzed and age-related typical audiograms were created. Bone conduction thresholds were corrected for Carhart effect and presbyacusis; in addition, we tested to see if separate cochlear otosclerosis component existed. Corrected thresholds were than analyzed separately for progression of cochlear otosclerosis.
**Results:** The study population consisted of 35% men and 65% women (mean age, 44 yr). The mean pure-tone average at 0.5, 1, and 2 kHz was 57 dB hearing level. Multiple linear regression analysis showed significant progression for all measured AC and BC thresholds. The average annual threshold deterioration for AC was 0.45 dB/yr and the annual threshold deterioration for BC was 0.37 dB/yr. The average annual gap expansion was 0.08 dB/year. The corrected BC thresholds for Carhart effect and presbyacusis remained significantly different from zero, but only showed progression at 2 kHz.
**Conclusion:** The preoperative audiological profile of otosclerosis is described. There is a significant sensorineural component in patients with otosclerosis planned for stapedotomy, which is worse than age-related hearing loss by itself. Deterioration rates of AC and BC thresholds have been reported, which can be helpful in clinical practice and might also guide the characterization of allegedly different phenotypes for familial and sporadic otosclerosis. **Key Words:** Otosclerosis—Age-related typical audiograms—Sensorineural hearing loss.
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Otosclerosis is an isolated disorder of bone homeostasis of the otic capsule in the middle ear that can cause a conductive and a sensorineural hearing loss (SNHL). The disease is characterized by resorption of healthy bone tissue and subsequent formation of abnormal bone tissue, a process referred to as *otospongiosis*. During this process, a fixation of the stapes occurs by focal bone formation around the foramen ovale that generates a conductive hearing loss (1,2). The etiology is not fully understood, but both genetic and environmental factors are assumed to be involved (3). Autosomal dominant heritability with incomplete penetrance has been described for otosclerosis, but large families are rare and, for most of the patients, the cause remains unknown. Since 1998, five genes have been localized for autosomal dominant forms, but none of them have been identified (4). The *OTSC1* gene is located on chromosome 15q25–q26, the *OTSC2* gene on chromosome 7q34–q36, and the *OTSC3* gene on chromosome 6p21.3–22.3 (5–7). In addition, the *OTSC5* gene was recently localized to chromosome 3q22–q24 (8). The names *OTSC4*, *OTSC6*, and *OTSC7* were reserved with the Human Genome Nomenclature Committee for possible new localizations, but are not yet published.

The pathological pathway of otosclerosis is thought to be enzymatic. Proteolytic enzymes are released from otosclerosis foci, damage the inner ear, and concurrently initiate bone remodeling that leads to stapes fixation. The initial trigger for enzyme release may be an autoimmune process, and lysosomal activity is increased by

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estrogens (9). Pregnancy is known to cause exacerbation of otosclerosis because of hormonal changes. However, there has been some controversy regarding the autoimmune nature of otosclerosis. The role of the measles virus as a possible viral cause has been proven more significantly with elevated level of immunoglobulin G specific for paramyxovirus in perilymph of patients with otosclerosis and with reverse transcriptase polymerase chain reaction amplification of the measles virus from otosclerotic foci (10,11).

It has been argued that bone lesions affect intracochlear structures and also cause perceptive hearing loss (9,12). Many studies have assessed the correlation between the hydrolytic enzyme activity of perilymph and the deterioration of bone conduction (BC) thresholds. True cochlear otosclerosis is an issue that has been widely debated (13). Nevertheless, recent studies question the relationship between cochlear otosclerosis and SNHL (14). Audiological studies have shown mostly stable BC thresholds in otosclerosis (15,16), whereas some studies have also reported the progression of BC thresholds (17,18). Bone conduction thresholds in otosclerosis are not a true indicator of the inner ear function because of an audiological artifact due to stapes fixation. Carhart was the first to describe an improvement of BC thresholds in patients with otosclerosis of the stapes after successful surgery (19). Other studies confirm this preoperative overestimation of BC thresholds because of the artifact known as the *Carhart effect* (20–22). This effect should not be overlooked in any audiological assessment of cochlear otosclerosis.

The present study assesses the phenotype of otosclerosis from an audiological perspective to characterize hearing deterioration originating from either cochlea or ossicles involvement. In doing so, we contribute to phenotype-genotype correlation studies that may help to unravel the etiology of otosclerosis. The preoperative audiometric profile of surgically confirmed patients with otosclerosis is reported. The influence of age, sex, and deterioration rate of the hearing level was statistically analyzed to describe the preoperative profile of this complex genetic trait. Then, BC thresholds were corrected for Carhart effect and normative presbyacusis values in an attempt to distinguish otosclerosis-related SNHL from age-related hearing loss (ARHL) in otosclerosis. These corrected thresholds, indicating a separate cochlear component due to otosclerosis itself, were also tested for progression.

## PATIENTS AND METHODS

### Patients

Using otosclerosis surgery registers, patient records and last preoperative pure-tone audiograms were obtained retrospectively from four tertiary referral centers in Belgium, France, and the United Kingdom. The onset age of otosclerosis usually occurs in the third decade; therefore, patients younger than 20 years were excluded. Analyses were performed on preoperative audiometric data from affected ears from patients who had undergone stapedotomy. In those cases when both ears were affected (and operated on), only the data from the worse-functioning ear was used. In total, 1064 audiograms were included in the analyses. Thus, we have studied the worst, most advanced state of otosclerosis before surgery.

### Statistical Analysis

#### *Regression Models*

A multiple linear regression (MLR; all calculations were performed using SPSS 12.0 software [SPSS, Inc., Chicago, IL, U.S.A.]) model was built for each frequency, for BC, air conduction (AC), and for the air-bone gap (GAP). Hearing thresholds were taken as dependent variable, with age and sex as predictors. The linear regression model was fitted in a backward-stepwise manner, starting with a saturated model that includes the interaction term *age-sex*. If this interaction term is significant, it means that the age-related change in decibels hearing level (dB HL) per year (i.e., the slope of the regression curve) differs between men and women. In cases where the age-sex term was not significant, it was left out and a new model that included only the main effects of sex or only of age was fitted. In this model, the slope of the dB HL versus age curve is the same for men and women. Therefore, the model with only main effects allows for differences between men and women that are constant across the entire studied age range. The $p$ value of the coefficient for sex indicates whether this difference is significant. The significance of the coefficient for age indicates whether the slope of the hearing threshold versus age curve was significantly different from zero (i.e., whether the hearing loss is progressive at that frequency).

#### *Age-Related Typical Audiograms Construction*

Age-related typical audiograms (ARTAs) are graphical representations of progressive audiological thresholds, allowing for easy visual comparison between different age groups or even between different types of progressive hearing impairment (23). The method of deriving ARTA is reported in detail by Huygen et al. (23). In the past, ARTAs have proven to be an easy way of comparing phenotypes of genetic deafness. The regression equations obtained by MLR analysis give, at each frequency for AC and BC, the expected threshold as a function of age. We calculated the expected AC and BC thresholds and the GAP for age 30, 40, 50, 60, and 70 for men and women. On the basis of these regression equations, ARTAs were constructed for AC and BC thresholds.

#### *Sign Test*

Bone conduction thresholds are overestimated with preoperative audiometry. Therefore, they were corrected according to the most recent figures for Carhart effect provided by Gatehouse and Browning (20). Then, we subtracted the expected ARHL thresholds according to the 50th percentile ($P_{50}$) of the ISO7029 normative values specified for age and sex (24). In addition, the 90th percentile ($P_{90}$) was used for correction. In an attempt to quantitatively distinguish otosclerosis-related SNHL in otosclerosis from ARHL, we combined deduction of figures for Carhart effect with subtracted $P_{50}$ presbyacusis ISO7029 normative values from the BC thresholds in each individual subject. Under the null hypothesis that cochlear otosclerosis is only due to ARHL, the medians of the age-corrected thresholds should be zero. This hypothesis was tested for each frequency using the sign test. Inasmuch as we

consider otosclerosis as a complex genetic trait, it seems valid to us to use ISO7029 because most environmental factors can be ruled out. We could not actively screen our own study population for environmental factors, but the surgeons probably have; it also seemed necessary to correct the thresholds with $P_{50}$ and $P_{90}$ values from annex B of ISO1999 (25), which describes the hearing thresholds associated with age for the better ear of a typical unscreened population in an industrialized country.

Again, additional linear regression analyses were performed on the residual thresholds acquired after Carhart correction and ISO7029 and ISO1999 annex B for both $P_{50}$ and $P_{90}$ percentiles, respectively, to test whether a separate cochlear component due to otosclerosis is also progressive.

## RESULTS

### Patients

The study population consisted of 370 men (35%) and 694 women (65%) leading to a men-women ratio of 1:1.8. The average age was 44 years, with no difference between sexes ($t$ test, $p > 0.05$). Figure 1A shows the distribution of age, ranging from 21 to 89 years, for each sex. Most patients with otosclerosis who have been surgically treated are in the age group of 40 to 50 years, comprising patients between 35 and 54 years. Figure 1B shows that the average ages are comparably equal for each participating hospital.

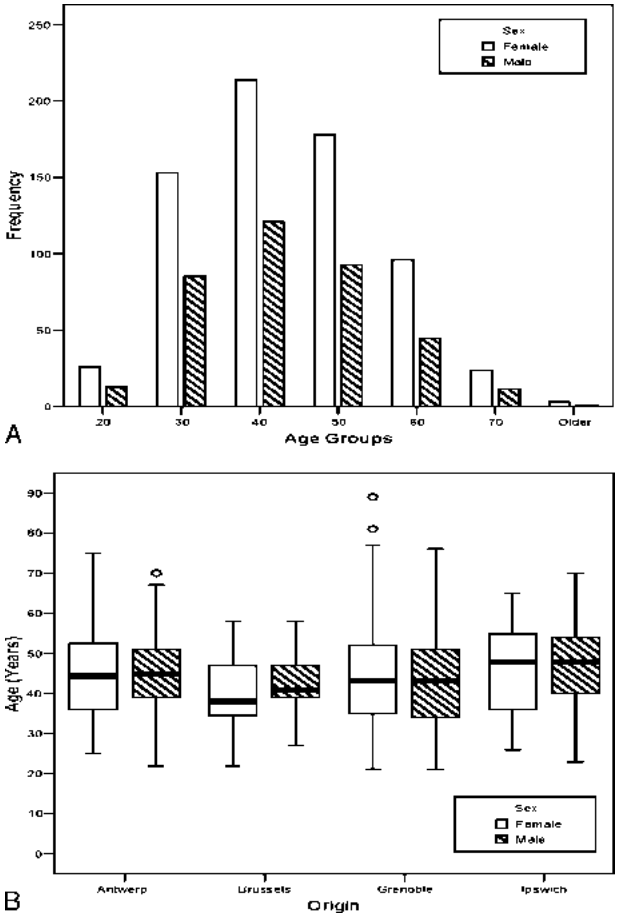


**FIG. 1.** Graphs showing (*A*) age distribution of the study population separated by sex, and (*B*) box plots of the average age per participating hospital with standard deviations. Median value is indicated within box plots with a horizontal line. Circles indicate outliers.

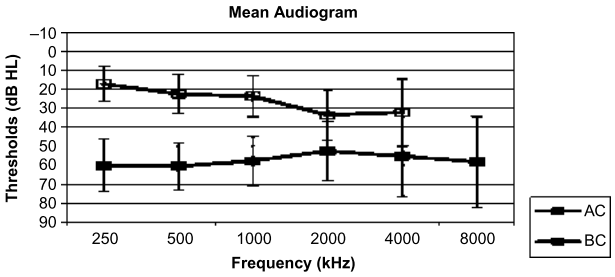


**FIG. 2.** Graph showing the mean preoperative audiometric thresholds of 1064 patients with otosclerosis (with 95% confidence interval).

In Figure 2A, the mean audiometric AC and BC thresholds are shown in a classical audiogram with the 95% confidence intervals. There is an air-bone gap typical for otosclerosis, and Carhart effect is clearly visible as a notch at 2 kHz. The mean pure-tone average at 0.5, 1, and 2 kHz is 57 dB HL.

### Statistical Analysis

#### *Regression Model*

To demonstrate how AC, BC thresholds, and GAP increase with age, and to check for the differences between men and women, we fitted the regression models of dB HL versus age at each of the frequencies studied. First, we generated scatter plots of thresholds versus age (data not shown). These plots showed that it was reasonable to fit a linear relationship at each of the tested frequencies within the age range studied. Next, the best-fitting linear model was fitted by MLR analysis. One separate MLR analysis for each frequency (0.25, 0.5, 1, 2, 4, and 8 kHz for AC, and 0.25, 0.5, 1, 2, and 4 kHz for BC) was performed. In addition, we performed an MLR for each measured frequency for the deducted GAP.

**TABLE 1.** *Results of multiple linear regression analyses*

| | Frequency (kHz) | | | | |
|---|---|---|---|---|---|
| | 0.5 | 1 | 2 | 4 | Mean |
| AC ($p$ value) | 0.000 | 0.000 | 0.000 | 0.000 | 0.45 |
| ATD (dB HL/year) | 0.28 | 0.29 | 0.47 | 0.75 | |
| BC ($p$ value) | 0.000 | 0.000 | 0.000 | 0.000 | 0.37 |
| ATD (dB HL/year) | 0.18 | 0.23 | 0.45 | 0.61 | |
| GAP ($p$ value) | 0.002 | 0.050 | 0.465 | 0.000 | 0.08 |
| AGE (dB HL) | 0.10 | 0.06 | 0.02 | 0.14 | |

All measured frequencies show significant deterioration for AC and BC thresholds. The 2 kHz is the only frequency with no significant expansion of the GAP.

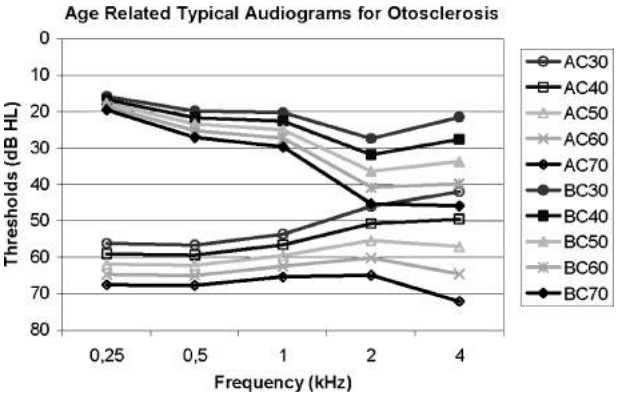


**FIG. 3.** Graph showing cross-sectional ARTA based on 1064 preoperative audiograms of surgically confirmed patients with otosclerosis.

None of the analyzed frequencies previously mentioned showed a significant interaction between age and sex. This means that regression lines of dB HL versus age have similar slopes in men and women. In other words, the progression rate of the hearing loss is not significantly different between men and women. However, we observed higher thresholds in men than in women in the high frequencies (4 and 8 kHz for AC and 4 kHz for BC thresholds, and, as a consequence, GAP at 4 kHz). Although the progression rate (annual threshold deterioration) is the same, there is a difference in dB HL, which is constant across the entire age range.

Next, we studied the regression coefficient for age, which indicates whether the hearing loss is progressive. Progression was significant at all frequencies for AC and BC thresholds. Table 1 shows the results of MLR analyses for AC, BC, and GAP. Progression rate differs between frequencies as shown. It is remarkable that GAP does not show significant expansion at 2 kHz.

The AC thresholds demonstrate highly significant deterioration at all measured frequencies, with average annual threshold deterioration (ATD) of 0.45 dB/yr. The BC thresholds are also progressive at all measured frequencies and have an average ATD of 0.37 dB/yr. The GAP is also significantly progressive except for the 2-kHz frequency where the Carhart Notch is situated. There is an average annual GAP expansion (AGE) of 0.08 dB/yr.

### *Age-Related Typical Audiogram Construction*

With the regression coefficients obtained from MLR, we constructed expected audiograms per decade for otosclerosis. These cross-sectional ARTAs for otosclerosis are shown in Figure 3. Again, a Carhart notch can be recognized at 2 kHz. The lower frequencies (range, 0.5–1 kHz) in Figure 3 show less progression compared with the higher frequencies (range, 2–8 kHz), especially for BC thresholds.

### *Sign Test*

The sign test indicated that the residual BC thresholds at frequencies 0.5, 1, 2, and 4 kHz remained significantly different from zero ($p < 0.001$) for presbyacusis corrections

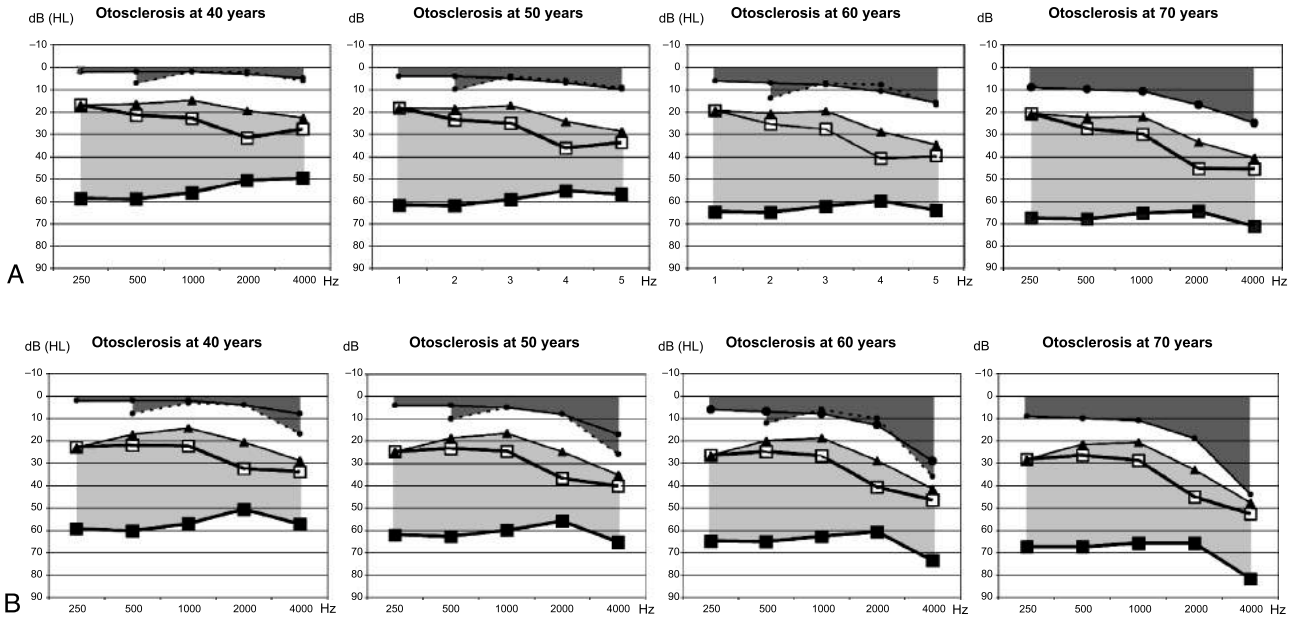


**FIG. 4.** Graphs showing the expected AC (■), BC (□), and Carhart-corrected BC thresholds (▲) thresholds plotted, together with the age-corresponding $P_{50}$ normative values of the ISO7029 (● with straight line) and $P_{50}$ normative values of annex B from ISO1999 (● with dotted line), separately for women (Fig. 4A) and men (Fig. 4B). The frequencies 250 and 8000 Hz are not defined in annex B of ISO1999, and only the thresholds from 30 to 60 years are defined; ISO7029 is more complete on these issues and covers the thresholds from 18 to 70 years.

with $P_{50}$ values of both ISO7029 and ISO1999 annex B (data not shown). This indicates that SNHL in patients with stapedial otosclerosis scheduled for stapedotomy is worse than can be expected by merely ARHL. When the more rigorous corrections with $P_{90}$ values are applied, only ISO7029 corrected data show residual threshold, significantly higher than zero. To visualize the $P_{50}$ thresholds of ISO7029 and annex B of ISO1999, they are plotted in Figure 4 against expected AC, BC, and Carhart-corrected BC thresholds from our data set, separated for sex. Figure 4 does not represent statistical analysis; it shows where expected hearing thresholds in otosclerosis are situated, with stapedial and cochlear component of otosclerosis, against normative thresholds for age and sex from ISO1999 annex B and ISO7029. However, there seems to be a slight overlap in gray-colored areas at 4 kHz between men aged 60 for ISO1999 annex B and men aged 70 for ISO7029, indicating that the hearing loss in the patients with otosclerosis may well be caused by ARHL. Age 30 is not plotted in Figure 4 because ARHL is not manifest at this age.

The final linear regression analyses were performed on corrected residual thresholds that represent SNHL in otosclerosis due to this disease and without involvement of ARHL.

When $P_{50}$ correction values from ISO7029 were applied, significant progression of thresholds was only found at 2 kHz. Four other tested frequencies (0.25, 0.5, 1, and 4 kHz) showed no progression. Noteworthy is that Carhart correction is not possible for 0.25 kHz because it is undefined. When $P_{50}$ correction values from annex B from ISO1999 were applied, significant progression was found at 0.5 and 2 kHz from tested frequencies (0.5, 1, 2, and 4 kHz). Annex B of ISO1999 does not define values for 0.25 kHz. Performing the same analyses with $P_{90}$-corrected data is not that meaningful because the thresholds start from negative values for most frequencies with ISO1999 annex B, especially those for men. Nevertheless, no significant progression of residual hearing thresholds could be found at every tested frequency when $P_{90}$-corrected data were tested from ISO7029 and from ISO1999 annex B.

## DISCUSSION

At present, the exact cause of otosclerosis is unknown and bewilders researchers of several fields of interest. In the seventies, there was a flourishing discussion regarding the true nature of cochlear otosclerosis. Currently, SNHL disproportional to the patient's age is an accepted feature of otosclerosis. Several authors of histological studies considered that cochlear otosclerosis is invariably associated with stapedial otosclerosis (13). Radiological studies report evidence of cochlear otosclerosis as double ring on axial computed tomography images (26). However, audiological studies are often in the scope of analyzing preoperative and postoperative results of stapedotomy. Some audiological studies have assessed the progression in otosclerosis, but the present study is the first to quantify the audiological deterioration and to distinguish SNHL in otosclerosis from ARHL.

Most of the patients operated on are aged between 35 and 54 years (Fig. 1). As can be expected, few patients older than 65 years have undergone stapedotomy because of the copathology, which can complicate anesthesia during surgery. With 370 men and 694 women, our study population has a men-women ratio of 1:1.8. Histological otosclerosis has a prevalence of 3.4%, with no difference between sexes (27). In contrast, clinical otosclerosis has a lower prevalence of 0.30 to 0.38% with a men-women ratio of 1:2, which is fairly consistent with our results (3,27). This sex ratio is often attributed to hormonal changes during pregnancy that provoke an abrupt exacerbation of otosclerosis. Unfortunately, we could not correct our analyses for pregnancy because these data were not always available. However, considering that our population was selected by surgery registers and that the surgical procedure is equally complex for both sexes, a preference for surgeons to operate mainly on women is not likely. An interesting matter in this case is the trigger that leads a patient with otosclerosis to seek the physician's advice and even undergo surgery. Women may have a lower tolerance for hearing loss and agree more readily to surgery to improve their hearing.

We are aware that this approach of data collection is susceptible to bias because we collect a clinical sample. All clinical populations are distorted toward greater severity of the condition in question because people with severe hearing problems, as in our case, are more likely to seek treatment than do people with mild hearing problems. However, people complain more easily of hearing problems nowadays because our society is more and more dependant on fast communication, and hearing aids are not as stigmatized as they once were. Moreover, many surgeons master stapedotomy with good results on hearing acuity. Therefore, our study population should not be as biased as could be expected. In theory, our population is also screened by the surgeon's anamnesis for other perceptive types of hearing loss, such as ototoxicity and noise-induced hearing loss, because these patients cannot profit from stapedotomy with hearing improvement as an objective.

Spreading the age of patient at surgery enables us to study whether there is a "typical" audiometric pattern at the age of patients at surgery and whether there is an audiological difference between young and old surgically treated patients. By means of MLR, we have shown the audiological characteristics for stapedotomy at different ages. The conduction component in otosclerosis is deteriorating more slowly than does the perceptive component. Our data demonstrate that in preoperative patients with otosclerosis, the ATD, on average, for BC and AC thresholds, is 0.37 and 0.45 dB/yr, respectively (Table 1). To our knowledge, this is the first study that quantifies audiological progression in otosclerosis. However, cautiousness is necessary in drawing conclusions from longitudinal trends in cross-sectional studies such as ours. Our

study may well include selection biases arising from cohort effect. To illustrate this hypothesis, imagine a population of 1000 people who are destined to develop clinically significant otosclerosis but with differing rates of progression and differing severity of overall hearing loss caused not only by otosclerosis but also by ARHL. At age 30, only those with the worst hearing will probably see the physician and be accepted for surgery. By age 60, many in the original group of 1000 will have undergone surgery, and only those with less severe (and less rapidly progressive) forms of otosclerosis (and ARHL) will be left to see the physician and undergo surgery. A cross-sectional sample of 60-year-old patients must be assumed to lead to an underestimation of the severity of hearing loss that would have been observed if everyone (including those who wanted surgeries at ages 30, 40, and 50 years) had been forced to wait until the age of 60 years. Nevertheless, cross-sectional studies can illuminate the progression rate of otosclerosis, which can help in planning surgery and in counseling patients. The patient will subjectively benefit more when operated on at a maximum air-bone gap, which might justify delaying stapedotomy until the GAP has expanded. However, the mean AGE in our data is 0.10 dB/yr, which corresponds with 1-dB air-bone gap expansion in 10 years. A hesitant attitude toward surgery should carefully be considered against the deteriorating sensorineural component in otosclerosis. Moreover, it is known that patients with otosclerosis generally need amplification to regain socially adequate hearing, even after successful stapedotomy (13).

Our data also show that SNHL in patients with stapedial otosclerosis is more severe than the average SNHL because of presbyacusis. Cochlear otosclerosis is attributed to severe involvement of the cochlear bony labyrinth in association with degenerative changes in the spiral ligament, stria vascularis, organ of Corti, and cochlear neurons (13). Presbyacusis can also involve stria vascularis, organ of Corti, and cochlear neurons because of metabolic, sensory, and neural presbyacusis, respectively; moreover, stiffening of the basilar membrane is reported in association with mechanical presbyacusis (28). Therefore, it is considered that SNHL in otosclerosis deteriorates faster than in pure ARHL, although the two effects are not necessarily additive.

To asses an age effect of hearing thresholds, two different references were used. The ISO7029 is generally used in studies on hereditary hearing impairment. Annex B of ISO1999 also describes normative hearing thresholds specified for age and sex, and it has proven use in medical-legal diagnosis and allocation of hearing loss in noise exposure (29). Because annex B of ISO1999 is based on a survey conducted in the seventies in industrialized populations, men have probably been exposed to more industrial noise than did women. In a sense, this argument is also applicable for ISO7029 values. Our analyses indicate a significant sex effect at higher frequencies, which is congruent with the ISO7029 normative data because they also indicate that ARHL on men is more pronounced at the higher frequency range, as shown in Figure 4B. Nevertheless, the $P_{90}$ values from annex B from ISO1999 seem too rigorous for correcting our thresholds. Besides, why should patients with otosclerosis be compared with the worst 10% (the $P_{90}$) of a population? Although otosclerosis might even protect against ARHL in theory, it seems more likely that patients with otosclerosis have ARHL like everybody else. Therefore, the $P_{50}$-corrected analyses seem more consistent, and we may conclude that there is a separate SNHL component in otosclerosis that is worse than can be expected from ARHL on itself. Whether this SNHL component in otosclerosis is progressive on its own is a difficult matter. Our analyses show that thresholds at 2 kHz significantly increase with the age of patient at surgery in both ISO7029 and ISO1999 annex B corrections, whereas thresholds do not increase with age in at least 2 other frequencies. Although this does not prove in our study that this separate SNHL component in otosclerosis is progressive, the mean age of our study population is 44 years, which is not an age when ARHL is expected to be an important cause of hearing loss. Nevertheless, all our cases deteriorated to the point requiring surgery because of otosclerosis. Given that the average age of onset of otosclerosis is somewhere in the third decade, our data suggest that the disease progresses up to a stage where surgery is required within approximately 20 years. It is very likely that this separate SNHL in otosclerosis is progressive, but we would need a longitudinal study on a population of patients with otosclerosis who have never been surgically treated to prove this, together with audiological studies. Such a population is hard to find in clinical settings, and animal models cannot help with this issue because otosclerosis is exclusively a disease of the human otic capsule.

The discussion whether cochlear otosclerosis exists is very relevant in the enigma between sporadic and familial otosclerosis. Where families with clear Mendelian inheritance patterns are said to be rare, isolated cases of otosclerosis have been accounted for as phenocopies of the disease, new mutations, incomplete penetrance, or multifactorial genetic-environmental etiology (30). Recently, Mazzoli et al. (31) asked if both forms of otosclerosis were distinct disorders because their study did not show clinical differences between familial and sporadic forms. Today, in large otosclerosis families amenable to genetic linkage analysis, both audiological and radiological investigations are performed to distinguish the affected from the unaffected family members. Radiology most often confirms stapedial involvement. Recent radiological techniques have detected stapedial otosclerosis on high-resolution computer tomography scans of patients with only a perceptive hearing loss. In addition, it is well known in practice that families with otosclerosis often also have family members with perceptive hearing loss disproportional to their age. Therefore, patients with sporadic otosclerosis may not be that sporadic after all. Maybe, otosclerosis should be regarded as a pleiotropic disease causing otosclerotic foci at random, which can either involve or spare the stapes. It might even be considered to regard all hearing-impaired family members,

whether of perceptive or conductive nature, as affected individuals in linkage analysis. Perhaps, this approach can lead to the discovery of more otosclerosis loci.

Further audiological characterization and anatomical typology (either histological or radiological) will refine the phenotype of otosclerosis, which will eventually benefit genetic research. In the recent past, genotype-phenotype association studies have improved our knowledge of hearing and hearing impairment enormously. Genetic research will probably also provide illumination on the pathological pathway of otosclerosis. Contributing to this issue, this audiological study confirms the existence of a cochlear component in stapedial otosclerosis.

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# Chapter 7

## Environmental factors that have a deleterious effect on hearing.

**To be submitted**

## INTRODUCTION

In any individual, the acuity of hearing declines with age. Speech understanding in noisy environment and the localization of sound sources are reduced, while hearing thresholds steadily elevate. Between age 60 and 70, about one-third of the population has an average hearing loss of 25 dB or more for 0.5, 1, 2, and 4 kHz. Between age 70 and 80, the fraction of individuals with an average PTA over 25 dB has increased to 50%, which makes age-related hearing impairment (ARHI) the most common sensory impairment in the elderly. Typically, ARHI is sensorineural, symmetric and more pronounced in the high frequencies, with males more severely affected than females. There is a large variation between individuals, which is higher in males compared to females. Variability increases with age, and is larger at the high frequencies.

### Z-scores

When comparing age-related hearing impairment in males and females of different ages, one needs to correct for the gender and age differences between the subjects, and this correction is different depending on the frequencies studied. The expected median hearing threshold as a function of age, sex and frequency, plus the standard deviation around this median, is given by the ISO 7029 standard (1) In a previous paper, we have developed a system to quantify how severely a person is affected by age-related hearing loss, given his/her age and sex.(2) In this method, a Z-score is defined as the standardized difference between an individual's observed hearing threshold at a given frequency, and the age- and sex-specific median for that frequency. This allows comparing individuals of different age and sex. Ideally, in a randomly collected and highly screened population, Z-scores should have a standard normal distribution, with no differences between males and females, and no relation to age. However, several studies indicate that typical populations have a slightly worse hearing than predicted by the ISO7029 standard, whereby the typical populations seem to have an excess ageing of 10-15 years compared to the population described by the ISO7029 standard. (3;4).

### Pathology of age-related hearing impairment

Based upon temporal bone studies, Schuknecht and Gacek found that the main structures in the cochlea affected by ageing are the hair cells, the cochlear neurons and the stria vascularis.(5) They subdivided ARHI into four categories (neural, sensory, strial, mixed) based upon the affected structure, and correlated the type of pathology to the structure of the audiogram. While the role of the three structures mentioned above is now widely recognized, it is now acknowledged that in most patients ARHI is a mixture of pathologies, and it is very difficult to correlate an audiogram to the type of pathology.(6)

### Importance of genetics

Predictably, a complex pathway like the ageing of the cochlea can be influenced by a complex interplay between genetic, medical and environmental factors. The relative importance of these factors is age-dependent. A heritability estimate by Karlsson indicated that in the age stratum 56-65, 58% of the variance was attributable to the influence of genes, declining to 47% in the stratum over 65.(7) In the Framingham cohort, Gates et al. found a clear familial aggregation of the hearing thresholds was found. The heritability also seems to be frequency-dependent, with a higher heritability in the low frequencies. In the Framingham cohort identified several loci with suggestive evidence for linkage. (8;9)

Environmental risk factors

While the first genetic variant associated with ARHI is still to be identified, there is substantial literature about environmental and medical risk factors leading to ARHI. The influence of occupational noise is well-documented. The permanent threshold shift caused by exposure to occupational noise with a given intensity during a given amount of time, is predicted by the ISO1999 standard. This effect is most pronounced at 2, 3 and 4 kHz.(10) However, individual noise susceptibility shows great variability. This may be due to complex interactions with non-occupational noise, other environmental factors or to genetic predisposition.(11) The most deleterious source of non-occupational noise is gunfire noise, primarily due to hunting and shooting.(12;13) People exposed to both occupational noise and gunfire noise have poorer hearing in both ears, compared to people exposed to occupational noise only.(14)

The main damage from long-term noise exposure is disappearance of the outer hair cells, followed by inner hair cell damage.(15) The same pathological changes are observed in histologic analyses of presbyacusis patients, which make it difficult to distinguish between NIHL and ARHI. It is unclear whether noise exposure and subsequent NIHL at an early age has any influence on the severity of ARHI at a later age. A longitudinal study in retired subjects between age 57 and 65 showed no difference in hearing deterioration rate between subjects with no history of noise exposure and retired subjects with NIHL, although these latter subjects had significantly elevated thresholds.(16)

Several aromatic solvents, including styrene, toluene and trichloroethane lead to irreversible hearing loss in the rat.(17) The effects were complex and nonlinear, including a synergistic effects between solvent and noise exposure.(18-20) It is not clear whether the ototoxic effects observed in rats can be extrapolated to human, as ototoxicity seems to be species-dependent.(21) In human, a more-than additive effect between noise and organic solvent exposure was found in a study of plastic factory workers co-exposed to a noise and styrene (22), whereas an additive effect was observed in dockyard labourers co-exposed to noise and a mixture of solvents.(23) For toluene, a synergistic effect was demonstrated, but this effect may not be present for other solvents.(24)

There is controversy on the effect of smoking. Rosenhall found as association between hearing levels and smoking(25); Cruickshanks and Ushida found indications for a dosage effect.(26;27) On the other hand, no association was found in the Framingham cohort.(9). Recent studies suggest an interaction between smoking and occupational noise, whereby the deleterious effect of noise exposure is exacerbated by smoking. (28-30)

An association with clear alcohol abuse has been observed, but with moderate alcohol intake, the results are less clear.(11;25)

Medical risk factors

In addition to these environmental factors, several medical risk factors are suspected to have an influence on hearing. Many studies have focused on cardiovascular diseases, as they are very prevalent in the elderly population. In the Framingham cohort, an association between cardiovascular events (stroke, coronary heart disease, or intermittent claudication) and low frequency hearing loss was reported.(9) They also reported an inverse relation between high-density lipoprotein levels and hearing thresholds. Torre et al. found a significant association between myocardial infarction and hearing loss was in women, but not in man.(31) Brant et al. reported an association between hearing thresholds and hypertension and systolic blood pressure.(18) A causal relationship between high-frequency sensorineural hearing impairment and diabetes mellitus has been found by several investigators.(32;33)

Here we test these previously identified environmental factors in a collection of 852 randomly collected subjects from a residential suburb. The present study is part of the contribution of the organising partner of an international multi-centre study over 7 different European countries on identification of genetic causes leading to ARHI.

## METHODS

*Sample collection*

Unrelated Caucasian subjects from a residential suburb of Antwerp, Belgium, were collected through population registries. Letters of invitation were written to a total of 3171 inhabitants aged between 55 and 65 years. All responding subjects underwent clinical examination, otoscopy and completed a detailed questionnaire on medical history and exposure to environmental risk factors. Subjects with ear diseases, possible monogenic forms of hearing impairment or other major pathologies with a possible influence on hearing, were excluded from the study. Relatively common pathologies such as diabetes mellitus were regarded as "environmental factors" and thus were included. The main goal was to include healthy cases and therefore patients with multiple hospitalisations were excluded. A complete list of exclusion criteria is available upon request. In the subjects passing the medical exclusion criteria, audiometric thresholds were determined for air conduction (0.25, 0.5, 1, 2, 3, 4, 6, 8 kHz) and bone conduction (0.5, 1, 2, 4 kHz) according to current clinical standards.(34) We excluded subjects with an asymmetric hearing loss (difference in air conduction threshold larger then 20 dB for at least 2 frequencies out of 0.5, 1 and 2 kHz) and subjects having a conductive hearing loss (air-bone gap of 15 dB or more in one or both ears at 0.5, 1 and 2 kHz.

*Z scores*

Z scores were calculated as described by Fransen et al.(2) In brief, for each individual we calculated the age- and sex-specific median hearing loss at each frequency, based upon the ISO7029 standards. This value was subtracted from the observed hearing loss at each frequency. The difference, which may be negative (=better hearing than median) or positive (= worse hearing than median), was normalized by dividing by the age-, sex- and frequency-specific standard deviation given by the ISO7029 standards. This calculation gives us frequency-specific Z scores. The high-frequency Z score ($Z_{high}$) is the average of the Z scores at 2, 4 and 8 kHz. In all analyses presented below, we use the high-frequency Z score of the best hearing ear ($Z_{high}$) as dependent variable.

*Statistical analysis*

Association between the $Z_{high}$ and binary factors was tested using ANOVA. Ordinal or numeric risk factors were tested via linear regression. To avoid confounding by gender effects, gender was always entered as a covariate into the model. All models were built in a stepwise backward way. First, a full model was fitted including the risk factor of interest, along with gender and the interaction term between these. In such model, the interaction term tests whether the effect of the risk factor is significantly different between the two sexes. If the interaction term was not significant, it was omitted from the model and a new model with only the two main effects is fitted. In this latter, simplified model, we test for the significance of the risk factor. To check the appropriateness of the fitted model and to find outlying observations, residual plots were visually inspected (normality of the raw residuals, predicted value vs. raw residuals, studentized residuals vs. independent variable).

## RESULTS

*Collection of subjects*

From the invited 3171 persons, 1420 (44.8%) volunteered to participate in our study. Exclusion criteria, as described in the methods section, were applied. A total of 852 subjects were included into this study, including 446 females and 406 males. Age range was 55 to 65, with a mean age overall of 61,4. Females were on average slightly younger than males (61,1 versus 61,8).

*Z score distributions*

In our population, the mean of the $Z_{high}$ was 0,294 with a standard deviation of 0,684. This means that our population has on average a slightly worse hearing that the ISO7029 reference population. The difference between the ISO7029 population and a 'typical' population has been noticed before.(35) The average $Z_{high}$ in females was slightly, but significantly higher compared to the males (difference = 0,219 with 95%CI: <0,128; 0,309>, p<0,001, two-tailed t-test). This does not mean that females have a worse hearing than males, but rather indicates that that the age and sex-correction using the Z score method is less optimal in females. This was reflected in the distribution of the $Z_{high}$. In males, this fits the normal distribution very well, whereas $Z_{high}$ in females shows a slight left skew. Also the overall distribution of $Z_{high}$ showed this slight right skew. Taking the square root of $Z_{high}$ improved the fit, but in our statistical tests the effect on the p-values was marginal and never affected the conclusions (not shown). Therefore, we performed our analyses on untransformed $Z_{high}$.

*General health and medical risk factors*

The prevalence of some common diseases is shown in table 1. If the numbers were sufficiently large (at least 10), association with $Z_{high}$ was tested.

**Table 1.** Prevalence in % (number) of common diseases in our study population

| Disease | Present | Not present/don't know | Total N |
|---|---|---|---|
| Heart attack | 3.17 (27) | 96.82 (823) | 850 |
| Heart surgery | 2.46 (21) | 97.53 (831) | 852 |
| Heart Catheterization | 4.94 (42 | 95.05 (808 | 850 |
| Whiplash Injury | 10.5 (89) | 89.48 (757) | 846 |
| Carotid Artery Surgery | 0.24 (2) | 99.76 (848) | 850 |
| Intermittent Claudication | 0.96 (8) | 1.01 (841) | 835 |
| Heart Problems | 17.91 (151) | 82.08 (692) | 843 |
| Diabetes | 1.65 (14) | 98.35 (835) | 849 |
| Osteoporosis | 6.64 (56) | 93.36 (788) | 844 |
| OsteArthritis | 34.37 (288) | 65.63 (550) | 838 |
| Multiple Sclerosis | 0.24 (2) | 99.765 (840) | 842 |
| Epilepsy | 0.47 (4) | 99.5 (842) | 846 |
| Lung problems | 16.31 (138) | 83.69 (708) | 846 |
| Allergy | 7.32 (62) | 92.68 (785) | 847 |

We found no association between hearing loss and length, weight, body-mass index, left/right handedness, susceptibility to sunburn and eye colour (data not shown). No association was found between hearing level and whiplash injuries, or between hearing levels and heart catheterisation. There was no association either with the use of painkillers or aspirin, nor with osteoporosis, osteoarthritis, allergy or pulmonary problems.

The number of persons with heart attack, heart operation and intermittent claudication, was too small to test these conditions for association with $Z_{high}$. To test the association of any cardiovascular events on $Z_{high}$, we created an indicator variable denoting the occurrence of coronary heart disease, stroke, and heart attack or stroke. No association with this indicator variable was found. There was no association either with cardiac problems in general.

*Gunfire noise*

To score the exposure to gunfire noise, we used the methodology previously used by Lutman and Spencer.(13) Subjects were asked how many rounds of ammunition they had fired with either rifles or machine guns (hereafter referred to as light weapons) or large infantry weapons and artillery on the other hand (hereafter referred to as heavy weapons). Only rounds fired without ear protection were taken into account. Three levels of noise exposure were used: less than 100 rounds, 100-1000 rounds, and more then 1000 rounds. We combined the exposure from the light and heavy weapons, by multiplying the number of rounds with heavy weapons by ten. Hence, gunfire exposure was coded into an ordinal variable with three exposure levels. To test the influence of gunfire exposure on hearing, we regressed the $Z_{high}$ from the best ear on the gunfire exposure level. Since only 8 females had ever fired a gun, this analysis was performed on males only. Fitting a linear model did not give a significant association, but analysis of the residual plot revealed a quadratic trend. Indeed, when adding the square of the gunfire exposure to the model, a significant relationship was found (p=0,008) (Figure 1)

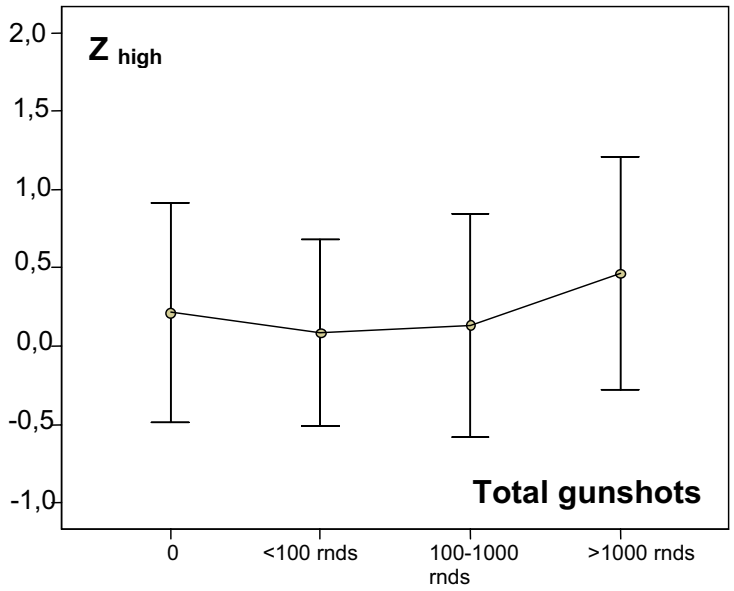


**Figure 1.** Influence of gunfire noise on $Z_{high}$. People who never fired a gun are given a zero. Only unprotected rounds are taken into account, weighted for heavy and light artillery as described in the text. A quadratic trend is observed, which suggests that the influence of gunfire on $Z_{high}$ is most pronounced among people who fired more then 1000 rounds (rnds).

*Leisure noise and Occupational noise*

Only 22 subjects reported repeated exposure to noise during their leisure time, and the time they had been exposed varied considerably. Therefore, the effect of leisure noise was not further analyzed. Work histories of the subjects were collected. Since this population was collected at random from population registries, there was a large variation in the number of jobs held, the length of time since employment, and the tenure of the job. In order to classify the subjects according to occupational noise exposure, we asked every subject whether (s)he

had ever worked for more than a year in an noisy environment, where a raised voice was necessary to be able to communicate. In addition, we asked for the duration of the exposure. People who always or almost always used hearing protection, were considered unexposed.
No significant association was found between occupational noise exposure and hearing levels, when exposure was scored as a binary trait. Neither did we find a significant effect when exposure time (in years) or daily exposure time (hrs/day) was taken into account.

*Solvents and toxic chemicals*
Subjects were asked for occupational exposure to organic solvents and other toxic substances. Organic solvents include aromatic carbohydrates (Toluene, Xylene, Styrene), trichloroethylene, and hexane. Since only 13 females were exposed to solvents, we restricted this analysis to males. No significant association was found between solvent-exposure and hearing levels, when exposure was scored as a binary trait. Neither did we find a significant effect when exposure time (in years) or daily exposure time (hrs/day) was taken into account. The number of subjects exposed to other toxic substances was too small to analyze.

*Noise – solvent interaction*
We tested for non-additive effects of noise and solvent exposure, since synergistic effect between these two risk factors have been reported.(19;23) Fifteen of our subjects had been exposed to both occupational noise and solvents, but this group did not show a significantly increased hearing loss compared to unexposed subjects, or subjects exposed to only one of the two risk factors alone.

*Smoking*
Subjects were asked for smoking habits by first asking whether they had ever smoked regularly. Dichotomizing the population into smokers and never-smokers showed a trend towards association with $Z_{high}$ (p=0,08). Then we subdivided the smokers into ex-smokers and current smokers. As shown in Figure 2, there is a linear trend (p=0,01) between $Z_{high}$ and smoking status (0=never smoker, 1=ex-smoker and 2= current smoker).

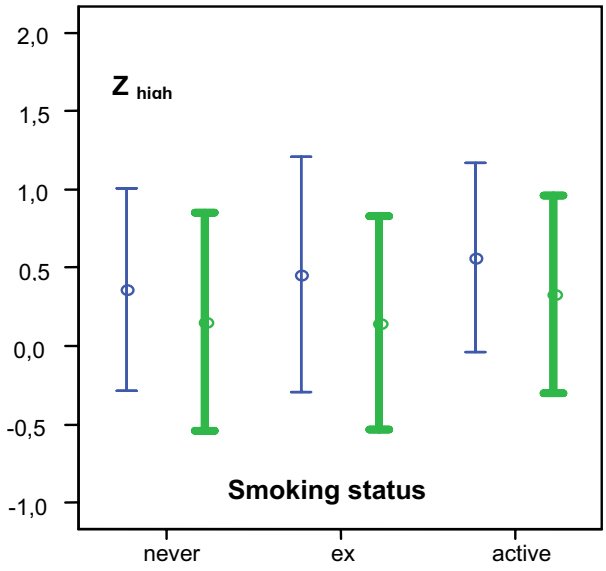


**Figure 2**. $Z_{high}$ in people who never smoked, former smokers and active smokers. A linear trend is observed in both males (thick line) and females (thin line). (never = never smoker, ex = ex-smoker and active = current smoker)

To further elucidate this possible association, we estimated the number of pack years, by multiplying the time (in years) an individual had been smoking by a weight factor for daily consumption of tobacco (<10 cigarettes/day = 0,5 ; 10-20 cigarettes/day = 1 ; >20 cigarettes/day = 1,5). For non-smokers, the number of pack years was set to zero. Linear regression revealed a significant association between pack years and $Z_{high}$ (P=0,018) as shown in Figure 3. The estimated effect on the $Z_{high}$ is small, though, with the 95%confidence interval ranging from 0,0006 to 0,0060 Z score units per pack year.

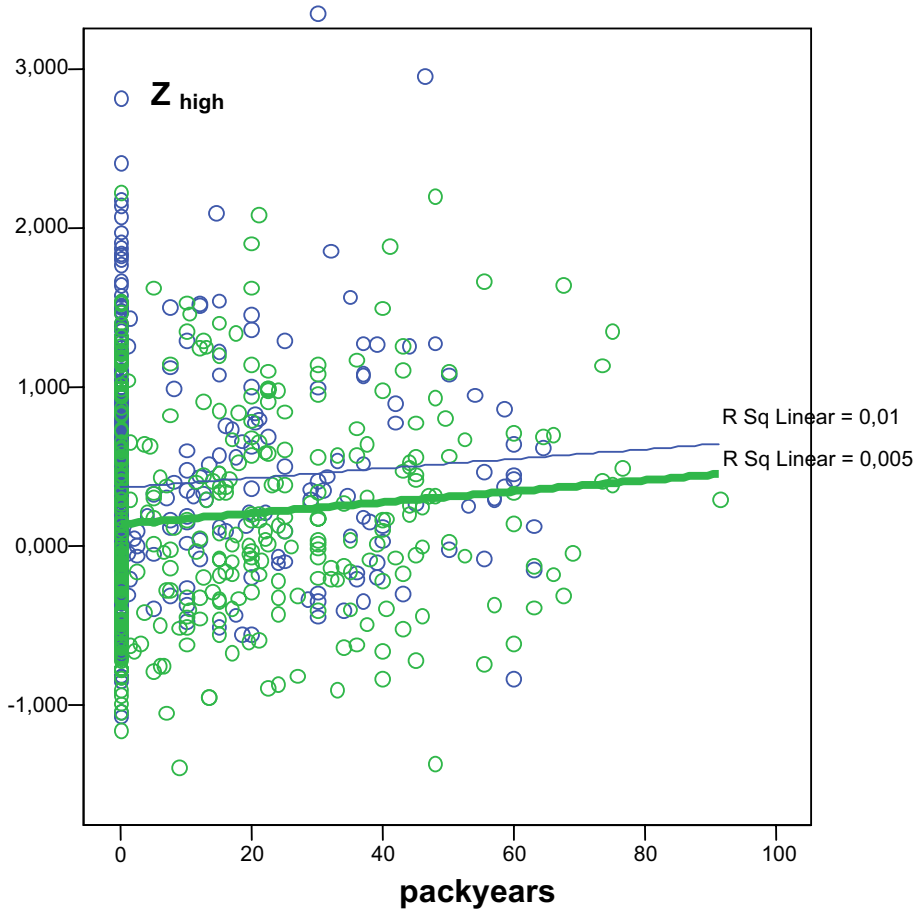


**Figure 3.** Plot of $Z_{high}$ versus number of pack years smoking. Males (thick line) and females (thin line), both show a significant linear relationship. People who never smoked were assigned a zero.

*Noise-smoking interaction*

In the male subjects, we tested for non-additive effects of occupational noise and smoking on hearing. $Z_{high}$ was regressed on the binary variables for smoking and occupational noise and the interaction between them. This showed a trend towards significance for the interaction term (p=0,07), indicating the effect of noise on hearing may be different between smokers and non-smokers. Subdividing the population into four groups on exposure to occupational noise, smoke or both, showed that subjects exposed to both cigarette smoke and occupational noise had worse hearing than the non-exposed, or the people exposed to only one of these risk factors (Figure 4) (p=0.02, contrast ANOVA).

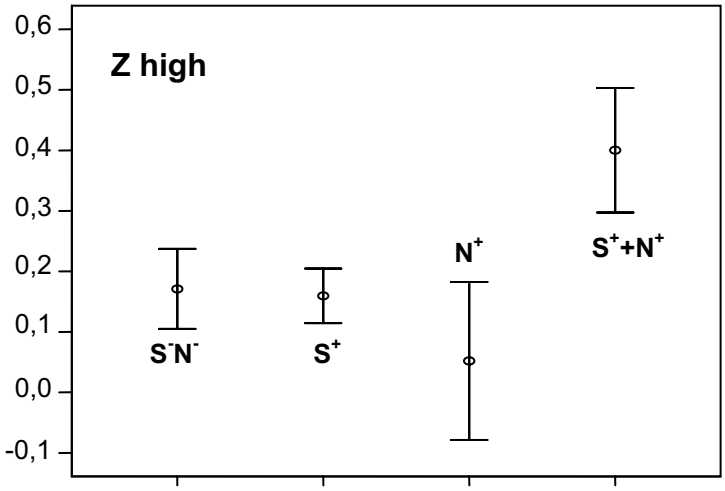


**Figure 4.** Noise-smoking interaction. Although the overall ANOVA on 4 groups is not significant, contrasting the people exposed to both smoking and occupational noise against the others show a significantly increased hearing loss in the former group.($S^-N^-$ = no smoking and no noise, $S^+$ = Smoking only, $N^+$ = Noise only and $S^+N^+$ = smoking and occupational noise)

*Alcohol consumption*

Subjects were asked if they regularly (at least once a week) drank alcohol. One glass of wine, spirit or beer counted as one consumption. Analyzing alcohol consumption as a binary variable using two-way ANOVA showed a significant (p=0,008) interaction between alcohol consumption and gender. We then subdivided the subjects into 4 categories according to their alcohol consumption (0= less then 1 consumption/week; 1 = less then 1 consumption/day but at least 1/week, 2= 1-3/day, 3= > 3/day; ). Linear regression of the Zhigh on this quantitative variable for alcohol consumption again showed a significant interaction between alcohol consumption and gender. As shown in Figure 5, alcohol consumption seemed to improve hearing in males, whereas in females it seems to decline.

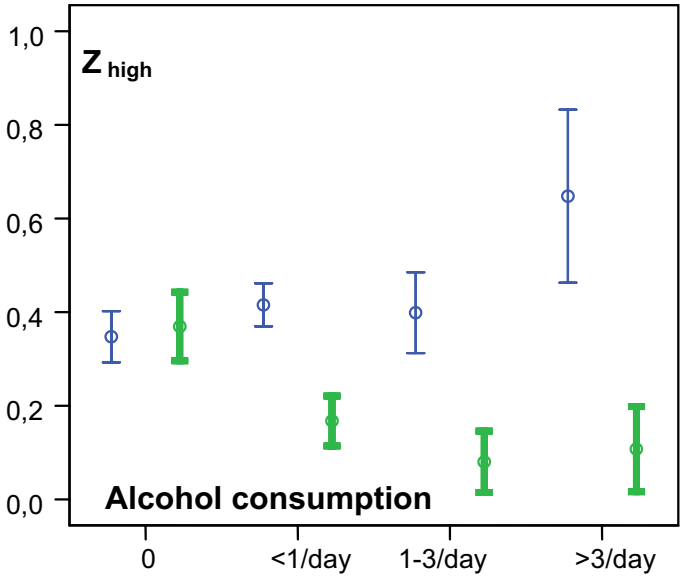


**Figure 5.** Effect of moderate alcohol consumption. Alcohol seems to have a beneficial effect on hearing in males (thick line), and a harmful effect in females (thin line).

## DISCUSSION

The study presented here is part of an ongoing study into the genetic and environmental causes of age-related hearing impairment across 7 European countries. This study has been primarily designed to find genetic variants leading to ARHI, and to provide guidelines for hearing protection via the study of 'avoidable' risk factors. Many medical conditions with a possible or proven role in hearing impairment, or subjects with ear diseases other than presbyacusis, were excluded beforehand as they represent nuisance factors for our study. Moreover, people with multiple hospitalisations were excluded from the study, and we specifically called for persons in good health. This caused a very low response rate among subjects with common diseases like cardiovascular disease (CVD) or diabetes.

The fact that we are unable to replicate previous associations between CVD events and hearing loss is probably due to the low prevalence of CVD in our study population. Only 42 subjects had suffered a CVD event in their life (5%), which is much lower than could be expected. As result, our study population has very weak power to detect a possible association between CVD and hearing loss.

A similar problem applies to the study of noise and solvent effects. The subjects in this sample set were not selectively sampled for this type of analysis. They were collected in a residential suburb with relatively few working-class people, so there were not many noise exposed or solvent exposed people present in our sample set. Again, this is an advantage when studying the genetics causes of hearing loss, but it makes environmental influences like noise-solvent interactions very difficult to study due to the small number of people in the double exposed group. Even when the marginal effects of noise and solvents were studied, there was still considerable variation in the type of noise and solvents our subjects had been exposed to. The intensity of the noise and the type of noise (impulse noise or steady noise) varied considerable, and the data did not allow taking all this into account. Large variation was also observed regarding the different solvents subjects had been exposed to. Some subjects had been exposed to a mixture of solvents, and the exposure time varied considerably. Studying the effects of solvents and the synergistic effect between noise and solvent exposure requires a different, more homogeneously exposed study population. Therefore, this sample set is not very powerful to detect effects of occupational noise and solvents on age-related hearing loss.

A weak but significant association between smoking and hearing loss in the elderly has been reported before, in a cohort study of persons between age 70 and 85.(25) The effect was only found in males, not in females. In our study, we found a significant effect of smoking, but no significant difference between the two sexes. This difference may be attributable to several reasons. First of all, the two studies have a different age range: 70-85 in the Rosenhall study versus 55-65 in the study presented here. The two studies also use a different statistical analysis technique. Rosenhall et al. analysed the two genders separately, and the number of smoking females in their cohort may have been too low to detect a significant effect. Also in our analysis, there were fewer smoking females than males, but there were still enough individuals to jointly analyze all individuals in one analysis. Moreover, our way of analysing the data using Z-scores and including gender as a covariate should eliminate possible confounding effects of gender. In the two-way ANOVA ($Z_{high}$ vs. smoking) as well as in the regression analysis ($Z_{high}$ vs. number of pack years) the interaction term between smoking and gender was not significant. This means the effect of smoking on $Z_{high}$ is not significantly different between males and females. Fitting a model without interaction term showed a

significant main effect of smoking on $Z_{high}$. In the ANOVA with smoking as a binary variable, this effect was borderline significant, but it became more pronounced when the number of pack years was taken into account, and a significant trend was observed when the group of the smokers was subdivided into ex-smokers and active smokers.

The study of cardiovascular disease, cardiovascular risk factors and hearing loss in the Framingham cohort did not show a significant association between smoking (in pack years) and hearing status. In general, they found stronger associations between cardiovascular disease events (coronary heart disease, stroke, intermittent claudication) and hearing loss than between risk factors for cardiovascular events and hearing loss. They conclude that the cardiovascular disease events, rather than their triggers like smoking, lead to hearing loss.(9)
If this were true, the association we found between smoking and hearing loss would be attributable to a higher occurrence of CVD in smokers, with the CVD leading to the hearing loss. To test this possibility, we created an indicator variable denoting the occurrence of any cardiovascular disease event, and we added this variable to the regression model, which already contained gender and pack years. In the new model, the regression coefficient for pack years was still significant ($p=0.012$), which indicates that an increased occurrence of CVD events in smokers does not explain the association between smoking and hearing loss. It supports the hypothesis that smoking in itself is, at least in part, responsible for a decrease in hearing ability, regardless of whether a CVD event took place. It does not, however, rule out an additional effect of CVD events on hearing loss. As stated above, our dataset is not well-suited to study this latter effect.

The prevalence of CVD in our study population (5%) is much lower than could be expected. In the Framingham study reported by Gates, 36% of the males and 22% of the females suffered from CVD, while only 11,7% of the males and 14,6% of the females were smokers. In our subject collection, 48% of the study subjects report they have ever smoked regularly. Therefore, we have a relatively large number of smoking subjects without CVD, we have more power to detect effects of smoking alone, in the absence of a CVD event. This enables us to disentangle the effects of smoking and CVD events on hearing.

We found a significant effect of gunfire noise on hearing loss, but could not prove a significant effect of occupational noise. However, we found a significant interaction between noise exposure and smoking, with noise exposure and cigarette smoking having a more-then additive effect. These results are in line with previous reports suggesting that noise-induced hearing loss may be exacerbated by long-term smoking.(28;30)

In contrast to reports that alcohol abuse can lead to increased hearing loss, a protective effect of moderate alcohol consumption on hearing has also been noted before in some but not all studies.(11;36) In this last publication, the effect was gender- and race-specific, being only present in black women. Possibly, the cardioprotective effect of moderate alcohol intake extends to a decreased risk of hearing loss regarding our results.

Despite the limitations of our study population, we were able to analyze several potential ARHI risk factors. Several findings were in line with previous epidemiological studies on environmental risk factors and hearing loss, including the effect of smoking, alcohol and gunfire. Still, these main effects only explain a minute fraction of the variance found in age-related hearing impairment. More is to be expected from the study of interactions between risk factors. We and others have found an interaction between smoking and occupational noise, and previous papers have described interactions between noise and solvents, between

solvents. Complex interactions are difficult to study due to the large number of degrees of freedom in the statistical tests, and they often require a dedicated study design.

Heritability studies indicate a roughly equal importance of environmental and genetic factors. Therefore, the analysis of genetic risk factors will also be very important. The completion of the human genome project has lead to the discovery of millions of genetic variants (single-nucleotide polymorphisms or SNPs) in the human genome. These SNPs are held responsible for phenotypic variation between individuals. Several SNPs have been found responsible for an increased susceptibility to several common diseases. It is tempting to speculate that in the upcoming years, SNPs in different genes will be identified that underlie an increased susceptibility to age-related or noise-induced hearing impairment. Here too, the effects will probably involve complex interactions between genes, or between genes and environmental factors.

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# Chapter 8

## KCNQ4, a Gene for Age Related Hearing Impairment

**E. Van Eyken, L. Van Laer, E. Fransen, V. Topsakal, N. Lemkens, W. Laureys, N. Nelissen, A. Vandevelde, T. Wienker, P. Van De Heyning, G. Van Camp.**
**Hum. Mutat. 2006 Oct;27(10):1007-1016**

# *KCNQ4*: A Gene for Age-Related Hearing Impairment?

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*Communicated by Michael Dean*

**Age-related hearing impairment (ARHI) is the most common sensory impairment among the elderly. It is a complex disorder influenced by genetic as well as environmental factors. SNPs in a candidate susceptibility gene, *KCNQ4*, were examined in two independent Caucasian populations. Two quantitative trait locus (QTL) values were investigated: $Z_{high}$ and $Z_{low}$, a measure of high and respectively low frequency hearing loss. In the first population, the statistical analysis of 23 genotyped SNPs spread across *KCNQ4* resulted in significant p-values for two SNPs for $Z_{high}$—SNP9 (NT_004511:g.11244177A>T) and SNP15 (NT_004511: g.11257005C>T; NP_004691:p.Ala259Ala), and one SNP for $Z_{low}$—SNP12 (NT_004511: g.11249550A>T). The linkage disequilibrium (LD) structure of *KCNQ4* was subsequently determined in a 34-kb region surrounding the significant SNPs, resulting in three LD-blocks. LD-block 1 contains SNP9 and covers an area of 5 kb, LD-block 2 measures 5 kb and surrounds SNP13 (NT_004511:g.11253513A>G) to SNP18 (NT_004511:g.11257509G>A; NP_004691:p.Thr293Thr), and LD-block 3 spans 7 kb. Five tag-SNPs of block 1 and 2, and 2 extra SNPs were subsequently genotyped in the second population. Again, several SNPs were positively associated with ARHI: one SNP (SNP18) for the high frequencies and three SNPs (SNP9, SNP12, and SNP18) for the low frequencies, although only a single SNP (SNP12) resulted in significant p-values in both populations. Nevertheless, the associated SNPs of both populations were all located in the same 13-kb region in the middle of the *KCNQ4* gene. Hum Mutat 27(10), 1007–1016, 2006.** 

KEY WORDS: KCNQ4; age-related hearing impairment; ARHI; presbyacusis; quantitative trait; association study; complex disease

## INTRODUCTION

Age-related hearing impairment (ARHI), alias presbyacusis, is the most common sensory impairment among the elderly. The prevalence of clinically significant hearing loss (25 dB and over) is 37% for people aged 61 to 70 years and increasing to 60% for people aged 71 to 80 years [Davis, 1994]. In general, men are more severely affected than women [Davis, 1994; Gates et al., 1999]. As the overall population in developed countries is aging, an increasing proportion will develop ARHI in the near future. In its most typical presentation, ARHI is symmetrical, sensorineural, and more pronounced in the high frequencies. Even though every individual shows a steady decline in hearing ability with aging, there is a great variation in the age of onset, the severity of hearing loss, and the progression of the disease. Pathologically, ARHI can be caused by several combinations of deficits in hair cells, cochlear neurons, and stria vascularis, leading to the classical definition of four types of ARHI: sensory, strial, neural, and cochlear, in addition to mixed presbyacusis [Schuknecht and Gacek, 1993].

ARHI is a complex disorder. Environmental and genetic factors contribute to the etiology of the disease. The best studied environmental factor is noise exposure [Flock et al., 1999; Mulroy et al., 1998; Pujol and Puel, 1999; Yamasoba et al., 1998]. Other non-genetic risk factors include: ototoxic medication [Aran et al., 1992; Boettcher et al., 1992; Stypulkowski, 1990], exposure to chemicals [Johnson and Nylen, 1995; Rybak, 1992], medical conditions such as diabetes [Kurien, 1989], cardiovascular disease [Gates et al., 1993], and renal failure [Antonelli, 1990]. A lot of controversy still exists on the relationship between tobacco smoking and ARHI [Brant, 1996; Fuortes et al., 1995; Gates et al., 1993; Mellstrom et al., 1982; Rosenhall, 1993].

The relative importance of the genetic component of a disease is expressed as heritability. Karlsson et al. [1997] performed a first twin study that estimated heritability values for ARHI, by

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combining a questionnaire with audiometric data. Twin similarity decreased with age between monozygotic twins and increased between dizygotic twins. This indicates that environmental factors become more important with age. The heritability for the age group above 64 years was 0.47, indicating that about half of the population variance for this age category is due to genetic factors. Another study comparing audiometric data from genetically related subjects (sibling pairs, parent–child pairs) and genetically unrelated subjects (spouse pairs), revealed a familial aggregation for ARHI. This study resulted in heritability estimates between 0.35 and 0.55 depending on the frequencies that were analyzed. Interestingly, the highest heritability was found for the low frequencies [Gates et al., 1999].

Little is known about genetic involvement in ARHI. The perception of sound requires complex molecular pathways and age-related changes in any component of these pathways might contribute to hearing loss. Therefore, it is expected that many genes will participate in the etiology of ARHI. Up to now, not much research effort has been put into the identification of ARHI susceptibility genes in humans. This is partly due to the fact that ARHI is still considered by many to be an inevitable part of aging, rather than a potentially preventable or even curable disease.

A first genome-wide linkage study for ARHI on the basis of families from the Framingham Heart study, resulted in six candidate regions on four chromosomes [DeStefano et al., 2003]. In mice, three different loci have been found linked to ARHI, *Ahl1* [Johnson et al., 1997], *Ahl2* [Johnson and Zheng, 2002], and *Ahl3* [Nemoto et al., 2004], and one of the responsible genes, *CDH23*, was identified as the responsible gene for *Ahl1* in inbred mouse strains [Noben-Trauth et al., 2003].

One of the ways to identify ARHI susceptibility genes, is to perform association studies on functional candidate genes. These genes are selected based on biological and physiological information and the biochemical pathways in which they are involved. In addition, genes causing monogenetic disease are excellent candidate susceptibility genes for the complex form of the disease [Tabor et al., 2002]. *KCNQ4* is such a candidate gene for ARHI. *KCNQ4* (MIM♯600101) encodes a voltage-gated potassium channel [Kubisch et al., 1999] and is expressed in hair cells of the cochlea and the vestibular apparatus, and in the auditive nuclei of the brainstem [Kharkovets et al., 2000]. Because of its expression in the basal membrane of hair cells, KCNQ4 is thought to play a role in the release of potassium out of the hair cells and the recycling of potassium in the inner ear. Mice with altered KCNQ4 channels display progressive hearing loss paralleled by a selective degeneration of outer hair cells [Kharkovets et al., 2006].

Mutations in *KCNQ4* cause an autosomal dominant type of nonsyndromic hearing loss, DFNA2. These mutations are mainly missense mutations [Coucke et al., 1999]. One of these families linked to DFNA2 displayed a very unique pattern of hearing loss.

One of the families linked to DFNA2 displayed a very unique pattern of hearing loss. Only the high frequencies were progressively affected while the lower frequencies remained intact until an older age. This resulted in an age-related typical audiogram (ARTA) that resembled the most typical presentation of ARHI (Fig. 1). In all known families with progressive nonsyndromic hearing loss starting at the high frequencies (either linked to the DFNA2 locus or to other autosomal dominant loci), the lower frequencies are progressively affected as well (Fig. 1). The hearing loss in this unique family is caused by a small 13-bp deletion early in the open reading frame of *KCNQ4* (FS71) resulting in gene inactivation or early truncation [Coucke et al., 1999]. Due to the striking phenotypic resemblance of this family, *KCNQ4* was given the highest priority among the monogenic hearing loss genes, and association studies were initiated. To investigate the association of *KCNQ4* with ARHI, we genotyped *KCNQ4* SNPs in two Caucasian populations, consisting of random independent samples. Subsequently, we analyzed the SNPs for association with ARHI by

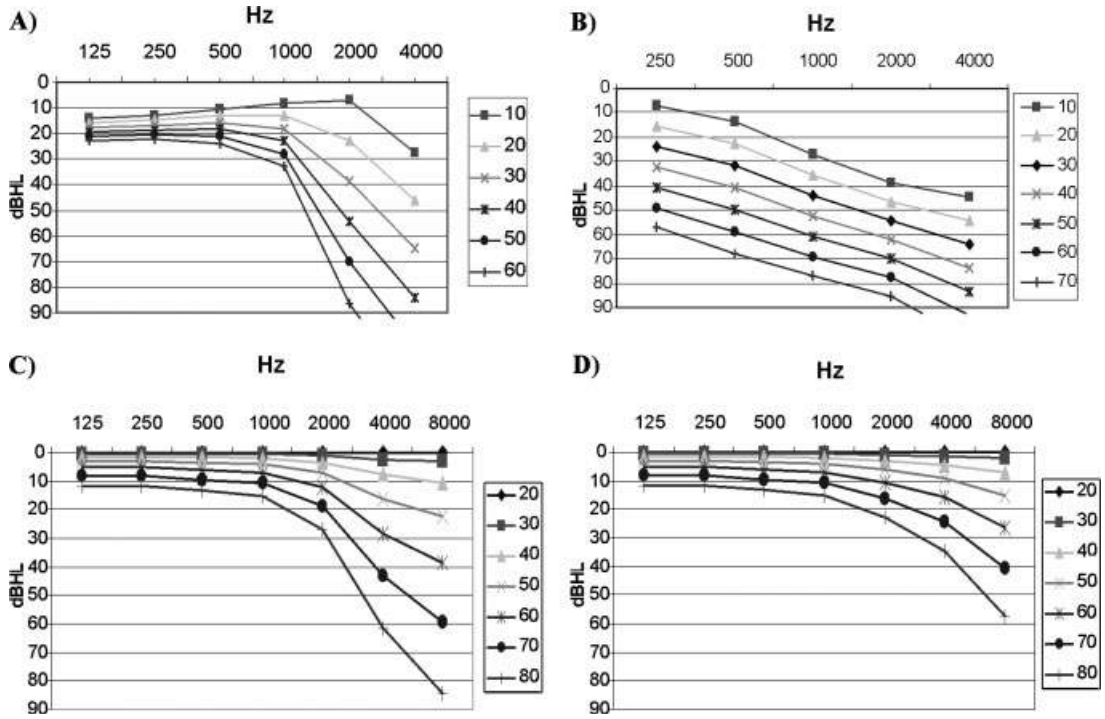


FIGURE 1. **Comparison of hearing loss between A: the family with the FS71 mutation (an age-related typical audiogram (ARTA) is presented) [Coucke et al., 1999], B: a family with the L274H mutation in *KCNQ4* [Talebizadeh et al., 1999] (ARTA), and the ISO 7029 standard for males (C) and females (D).**

treating ARHI as a quantitative trait [Fransen et al., 2003]. An average measure was calculated, both for the high frequencies and the low frequencies. Linkage disequilibrium (LD) pattern, tag-SNPs, and individual haplotypes were determined to enable haplotype-based association studies. For both populations several SNPs in a region spanning 13 kb in the middle of the *KCNQ4* gene were significantly associated with ARHI.

## MATERIALS AND METHODS

### Calculating Z-Scores

Frequency-specific thresholds were converted to sex- and age-independent Z-scores based on the ISO 7029 standards [International Organisation for Standardisation, 2000; Fransen et al., 2004]. The Z-score is defined as the number of standard deviations the hearing threshold differs from the median value at a specific frequency. Cases that hear better than the age- and sex-specific median at a certain frequency have a negative Z-score. For each subject, the better hearing ear was selected by averaging the Z-scores at 250, 500, and 1,000 Hz ($Z_{low}$), because these frequencies show the highest heritability [Gates et al., 1999], and at 2,000, 4,000, and 8,000 Hz ($Z_{high}$), because these frequencies are most affected in the elderly. Further calculations were performed on the better hearing ear.

### Power Calculations

Power calculations were performed using "Genetic power calculator" (http://pngu.mgh.harvard.edu/) [Purcell et al., 2003]. A case-control model for a threshold-selected quantitative trait was used. We calculated the power to detect association with a p-value of 0.05, when a putative KCNQ4 variant is responsible for 2% of the genetic variance of ARHI, under an additive model. The dominant genetic variance was put to zero. This showed that the sample size required to reach a power of 80% was 319 cases and an equal number of controls. To obtain a more stringent p-value of 0.001, we need to double the number of cases and controls ($N = 2 \times 694$).

### Population 1

Pure-tone audiometry was performed on Caucasian volunteers from Flanders and the Netherlands, ages 40 to 80 years old. Air conduction was measured at 125, 250, 500, 1,000, 2,000, 4,000, and 8,000 Hz, and bone conduction at 250, 500, 1,000, 2,000, and 4,000 Hz. Conductive hearing loss was expressed as the mean air-bone differences at 500, 1,000, and 2,000 Hz. Subjects with a conductive loss more than 10 dB were excluded from the study. Noise dips were calculated as the difference between air thresholds at 4,000 and 8,000 Hz and were excluded if they exceeded 20 dB. Subjects that reported an age-at-onset below age 30, subjects with a strong asymmetric hearing loss or middle ear pathology, and subjects with other pathological findings affecting hearing sensitivity were excluded from the study. After considering these exclusions, average Z-scores at 250, 500, and 1,000 Hz ($Z_{low}$) and at 2,000, 4,000, and 8,000 Hz ($Z_{high}$) were calculated for 645 subjects.

### Population 2

Inhabitants from a residential village of Antwerp, between 55 and 65 years old, were all invited through population registries. Air conduction was measured at 125, 250, 500, 1,000, 2,000, 3,000, 4,000, 6,000, and 8,000 Hz, and bone conduction at 500, 1,000, 2,000 and 4,000 Hz from participating volunteers. Audiological exclusion criteria were: conductive hearing loss above 15 dB in one or both ears measured at 500, 1,000, and 2,000 Hz; or asymmetrical hearing loss with differences in air conduction thresholds greater than 20 dB for at least two frequencies out of 500, 1,000, and 2,000 Hz. Tympanometry and speech audiometry were performed. Subjects were only included if the maximum speech recognition score and maximum intensity for speech recognition could be determined. Subjects with ear diseases that affect hearing thresholds and sensorineural hearing losses other than presbyacusis were excluded from the study. In general, subjects with a pathology that is reported to influence hearing were excluded according to an extended exclusion list designed by an international European consortium (see Supplementary Table S1; available online at http://www.interscience.wiley.com/jpages/1059-7794/suppmat). The subjects completed an extended questionnaire on the medical history and environmental exposure. After checking all of the exclusion criteria, 664 subjects were included. Selection of the better hearing ear and Z-score calculation ($Z_{high}$ and $Z_{low}$) were performed as described above.

### SNP Selection and SNP Identification

SNPs spread across the entire *KCNQ4* gene were selected from the dbSNP database (www.ncbi.nlm.nih.gov/SNP/) and the SNP consortium (http://snp.cshl.org/). In addition, exons and exon–intron boundaries were resequenced in 12 samples. Primers were designed upstream and downstream of each exon. Primers and PCR conditions are available upon request. PCR products were purified with a PCR purification kit (Amersham Biosciences, New York, NY; www.amersham.com). Sequencing reactions were performed according to the manufacturer's instructions using the DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences), and analyzed on an ABI PRISM 3100 Genetic analyzer (Applied Biosystems, Foster City, CA; www.appliedbiosystems.com). The resulting data were processed with Sequencing Analysis Software 3.7 (Applied Biosystems).

### Genotyping

Heterozygozity of selected SNPs was tested in 16 random samples with the SNaPshot (Applied Biosystems) detection method according to the manufacturer's instructions. Briefly, PCR products were purified with two hydrolytic enzymes: Exonuclease I and Calf Intestine Alkaline Phosphatase (CIAP) (Amersham Biosciences). Subsequently, a SNP-specific primer was hybridized to the denaturated PCR product and extended with a labeled base. Finally, the resulting products were purified with CIAP and the samples were analyzed with an ABI PRISM 3100 Genetic analyzer (Applied Biosystems). SNPs with heterozygosity values of 10% or more were included in the study.

For high throughput analysis, SNPs were genotyped with the Acycloprime-Fluorescence-Polarization (FP) SNP Detection System from Perkin Elmer (Wellesley, MA; www.perkinelmer.com) according to the manufacturer's instructions. Briefly, PCR reactions were purified with Exo-SAP-IT (USB, Cleveland, OH) in clean-up buffer (Perkin Elmer). Subsequently, hybridization of the SNP-specific primer and elongation with a labeled acyclo-dNTP took place. The resulting fluorescence was read on a Victor$^2$ 1420 multilabel counter (Perkin Elmer).

### Determining LD-Structure and Selection of Tag-SNPs

To determine the block structure, the coverage criteria of Patil et al. [2001] was used, whereby a block is defined as a region where the three most frequent haplotypes are representative for greater than 85% of the observed haplotypes. A total of 100 random independent samples were genotyped for the selected

SNPs. To get a general overview of the LD-structure of *KCNQ4*, we used LD-max, GOLD (www.sph.umich.edu/csg/abecasis/GOLD/docs/ldmax.html) [Abecasis and Cookson, 2000] and Haploview (www.broad.mit.edu/mpg/haploview). Using SNPHAP (www-gene.cimr.cam.ac.uk), haplotype frequencies were determined within small blocks of high LD, as determined by LD-max. Then, adjacent SNPs were successively added to the block, until the sum of the three most frequent haplotypes no longer reached 85%. Genotype data were also analyzed with Haploview to determine LD-blocks and LD between blocks, when this latter program became available. Within each block, tag-SNPs were identified with SNPtagger (www.well.ox.ac.uk/~xiayi/haplotype/) [Ke and Cardon, 2003]. Selected tag-SNPs, plus SNPs outside the blocks, were subsequently genotyped in all subjects. The non-tag-SNPs were not analyzed on the remainder of the samples.

### Individual Haplotype and Diplotype Determination

Individual haplotypes were determined with SNPHAP (www-gene.cimr.cam.ac.uk), which gives the most likely set of haplotypes and their likelihood for each individual. Diplotypes were reconstructed from the inferred haplotypes. In the two-way analysis of variance (ANOVA) tests on haplotypes and diplotypes, each individual was weighted by the likelihood of its haplotype or diplotype as given by SNPHAP [Sham et al., 2004]. Rare diplotypes were clumped into one category.

### Association Testing

Hardy-Weinberg equilibrium was checked for each individual SNP using SNPscorer. All data were analyzed using SPSS 12.0 for Windows (SPSS Inc., Chicago, IL; www.spss.com). Because the Z-scores were not normally distributed, a log-transformation was performed. We tested the association between the Z-score (for both high and low frequencies) and single SNPs, haplotypes, and diplotypes, respectively. A two-way ANOVA was used to account for sex differences.

All two-way ANOVA models were constructed step by step going backwards. First, a saturated model was fitted including main effects for sex and genotypes, and the interaction term sex*genotype. In case the interaction term was not significant, this term was omitted and a new model including only the main effects for sex and genotype was fitted. In case the interaction term was significant, one-way ANOVA was used to test females and males separately. This allowed us to detect effects of genes that influence hearing loss in males only or in females only. To enable two-way ANOVA for SNPs with a low minor allele frequency (MAF), we combined the rarest genotype (i.e., if $N < 10$) with the heterozygous genotype. In addition, rare haplotypes and diplotypes were clumped into one category. To test females and males separately, one-way ANOVA was used. Normality of the residuals homoscedasticity and homogeneity of variances were tested to check the validity of the final model. The cutoff value used for significance was 0.05, while P-values $< 0.1$ were indicative for a trend of association.

### Reference Sequences

The reference sequences used are the genomic sequence NT_004511 and the protein sequence NP_00491.

## RESULTS

For the study of a complex disorder like ARHI, a clear definition of the phenotype is crucial. We previously defined the Z-score, which is an age- and gender- independent value that quantitatively expresses how well a person hears given his age and sex [Fransen et al., 2004]. This allowed us to study ARHI as a continuous trait. In this study, we analyzed two phenotypes: $Z_{high}$ (the average of the Z-scores for 2,000, 4,000, and 8,000 Hz) and $Z_{low}$ (the average of the Z-scores for 250, 500, and 1,000 Hz). In a small pilot study using five SNPs of *KCNQ4* on 441 subjects belonging to Population 1, we detected a significant association between SNP15 (NT_004511:g.11257005C>T; NP_004691:p.Ala259Ala) in *KCNQ4* (Table 1) and $Z_{high}$ ($p = 0.009$). A total of 8% of the population was homozygous for the T-allele of this SNP. These subjects have better hearing thresholds than heterozygous subjects or subjects lacking the T-allele, which seems to indicate that the T-allele has a protective effect on the development of ARHI. Based upon these findings, we judged that *KCNQ4* was a good candidate gene and that it was worthwhile to further investigate its association with ARHI.

### Identification of SNPs in *KCNQ4*

From the 16 SNPs that were selected from the SNP database, 11 were polymorphic and were considered for further analysis. Resequencing the exons and exon–intron boundaries for extra SNPs in 12 random independent subjects resulted in the identification of six SNPs, SNP14–SNP18, SNP22 (NT_004511:g.11254837C>T to NT_004511:g.11257503A>G, NT_004511:g.11268746G>T; NP_004691:p.His455Gln) (Table 1), two of which were not present in the SNP databases, SNP16 (NT_004511:g11257098A>C) and SNP22. SNP22 was previously described as a polymorphism in exon 10 by Talebizadeh et al. [1999]. The resulting 13 SNPs were spread across the entire *KCNQ4* gene and separated by an average of 3 to 4 kb: SNP1–SNP5 (NT_004511:g.11220406C>T to NT_04511:g.11237168), SNP9 (NT004511:g.11244177A>T), SNP14–SNP18 (NT004511:g.11254837C>T to NT004511:g.11257509G>A; NP_00491:Thr293Thr), SNP22–SNP23 (NT_004511:g.11272952C>G) (Table 1, Fig. 2). For all 13 SNPs, SNaPshot and acycloprime-FP assays were optimized.

### Determination of Block Structure

To determine the LD block structure, the 13 selected SNPs were genotyped on 100 independent samples from Population 1. However, LD-max and GOLD could not determine a clear block structure. Since it was known that a higher SNP density may result in a clearer block structure [Ke et al., 2004], we further saturated *KCNQ4* with 10 additional SNPs out of the SNP databases: SNP6–8 (NT_004511:g.11240018A>G to NT_004511:g.11243240C>T), SNP10–13 (NT_004511:g.11246340C>T to NT_004511_g.11253513 A>G), and SNP19–21 (NT_004511:g.11261497C>T to NT_04511:g.11267460A>G) (Table 1). After this, a SNP density of one SNP per 1 to 2 kb was reached for the 34-kb region comprising SNP5 to SNP22. The extra SNPs were genotyped on 100 samples and new analyses with LD-max and GOLD resulted in three clear blocks (Fig. 2). Block 1 encloses SNP8 to 11, and spans a region of 5 kb. Block 2 comprises SNP13 to SNP18 and measures 5 kb. Block 3 spans a region of 7 kb and contains SNP19 to 21 (Fig. 2). In addition, the genotype data were analyzed with Haploview. This analysis demonstrated LD between block 1 and block 2 (Fig. 2). Within each block, tag-SNPs were determined with SNPtagger and genotyped in the remaining samples of Population 1 (in total $N = 645$).

### Association Study of Population 1

Single-SNP association tests with $Z_{high}$ for each genotyped SNP resulted in two SNPs (SNP9 and SNP15), showing significant

TABLE 1. **Single SNP Association Tests for Population 1 Using Two-Way ANOVA for $Z_{high}$ and $Z_{low}$**

| SNP number | RS number | Systematic DNA name[a] | Systematic protein name[b] | p-value $Z_{high}$ | p-value $Z_{low}$ |
|---|---|---|---|---|---|
| SNP1 | rs2769256 | g.11220406C>T | NA | 0.078* | 0.978 |
| SNP2 | rs2769257 | g.11221160G>T | NA | 0.624 | 0.375 |
| SNP3 | rs709688 | g.11225103C>T | NA | 0.667 | 0.537 |
| SNP4 | rs823672 | g.11225258C>T | NA | 0.264 | 0.114 |
| SNP5 | rs823686 | g.11237168A>G | NA | 0.348 | 0.994 |
| SNP6 | rs1327891 | g.11240018A>G | NA | —[c] | —[c] |
| SNP7 | rs6675976 | g.11241157A>G | NA | 0.517 | 0.523 |
| SNP8 | rs4660466 | g.11243240C>T | NA | 0.593 | 0.387 |
| SNP9 | rs727146 | g.11244177A/T | NA | F = 0.004**; M = 0.295 (0.004[d,**]) | 0.202 |
| SNP10 | rs4660175 | g.11246340C>T | NA | —[c] | —[c] |
| SNP11 | rs878042 | g.11248106C>T | NA | 0.700 | 0.969 |
| SNP12 | rs2149034 | g.11249550A>T | NA | 0.183 | 0.016** |
| SNP13 | rs6661888 | g.11253513A>G | NA | —[c] | —[c] |
| SNP14 | rs13374844 | g.11254837C>T | NA | 0.305 | 0.195 |
| SNP15 | rs4660468 | g.11257005C>T | pAla259Ala | 0.049** | 0.336 |
| SNP16 | ss49840084 | g.11257098A>C | NA | 0.705 | 0.750 |
| SNP17 | rs12117176 | g.11257503A>G | pPro291Pro | 0.517 | 0.275 |
| SNP18 | rs12143503 | g.11257509G>A | pThr293Thr | 0.252 | 0.132 |
| SNP19 | rs3767938 | g.1123497C>T | NA | 0.059* | 0.281 |
| SNP20 | rs1571287 | g.11263702C>T | NA | 0.353 | 0.795 |
| SNP21 | rs1041239 | g.11267460A>G | NA | 0.084 | 0.573 |
| SNP22 | ss49840085 | g.11268746G>T | pHis455Gln | 0.820 | 0.702 |
| SNP23 | rs727334 | g.11272952C>G | NA | 0.064* | 0.568 |

[a]The following nucleotide reference sequence was used NT_004511.
[b]The following protein reference sequence was used NP_004691.
[c]No association studies were performed for these SNPs because an insufficient number of samples were genotyped.
[d]Two-way ANOVA sex*genotype interaction.
*p-values suggesting a trend for association ($p < 0.100$).
**Significant p-values ($p < 0.05$).
NA, not available.

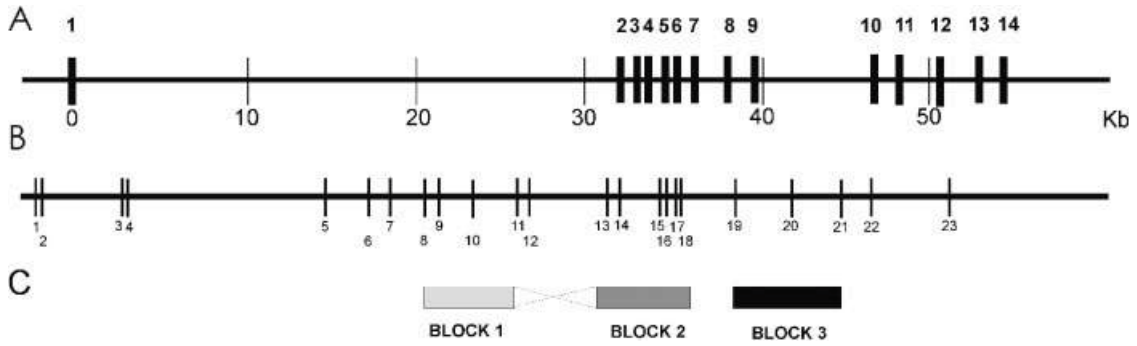


FIGURE 2. **A: Physical map of *KCNQ4*. Exons are represented by rectangles. B: Physical map of all genotyped SNPs for *KCNQ4*. C: Genotyping of 100 random independent samples resulted in three LD-blocks: block 1 from SNP8 to SNP11, block 2 from SNP13 to SNP18, and block 3 from SNP19 to SNP21. The dotted line indicates LD between block 1 and block 2.**

association with ARHI, and three SNPs (SNP1, SNP19, and SNP23), with a trend towards significance (Table 1). Statistical analysis of SNP15 resulted in a significant effect of the genotype on $Z_{high}$ ($p = 0.049$), which is still an indication for association as described above for our preliminary results (Fig. 3). For SNP9 we detected a significant sex*genotype interaction, which implies that the effect of the genotype is different between females and males ($p = 0.004$) (Table 1). One-way ANOVA resulted in a p-value of 0.004 within the female population, while the p-value for males did not reach significance ($p = 0.259$). Interestingly, for the female population the TT genotype has a disease-causing effect on ARHI only (Fig. 3). The results of the single-SNP association tests of the other SNPs are shown in Table 1. No other significant values were detected.

Single-SNP association tests with $Z_{low}$ for each genotyped SNP resulted in one SNP, SNP12, significantly associated with ARHI (Table 1). For SNP12, a moderately protective effect of the T allele in men and woman was demonstrated ($p = 0.016$). Subjects possessing the TT genotype hear better than subjects with the AA or AT genotype (Fig. 3).

To perform a haplotype and diplotype-based association study, the most likely haplotype for each block was inferred for each subject. Weighted two-way ANOVA of the haplotypes and diplotypes vs. $Z_{high}$ for each of the three blocks gave no significant results (Tables 2 and 3). Similarly, no significant results were obtained for $Z_{low}$ when analyzing haplotypes and diplotypes for block 1 and block 3 (Tables 2 and 3). For block 2, however, a trend towards a significant sex*genotype interaction ($p = 0.060$) was

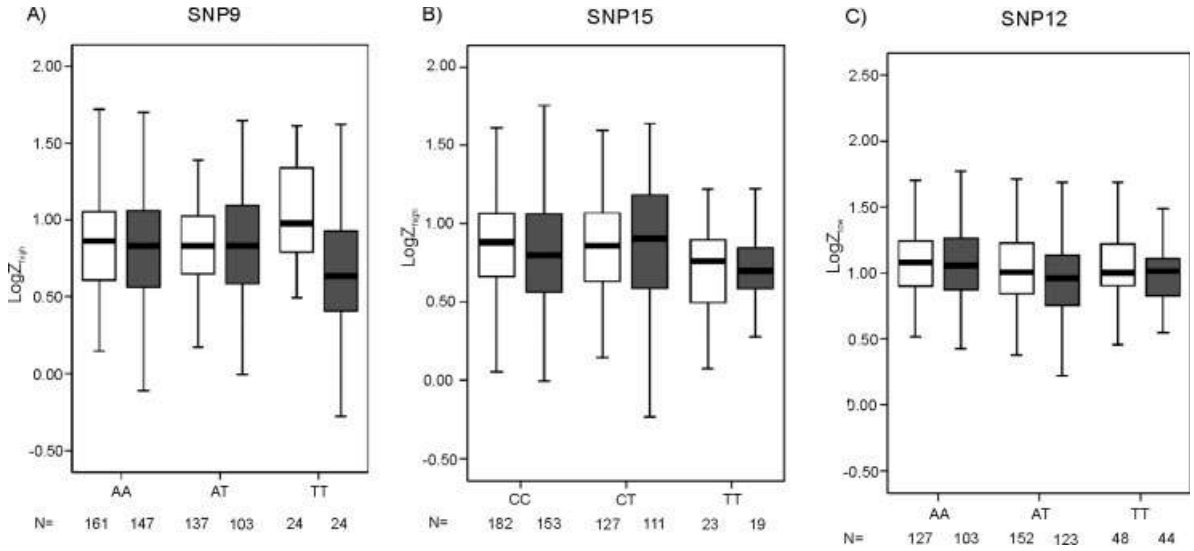


FIGURE 3. **Boxplots of significantly associated SNPs for females and males in Population 1. A,B: Boxplots of SNP9 and SNP15 for the high frequencies. C: Boxplot of SNP12 for the low frequencies. The sample size for each genotype for females (white boxes) and males (grey boxes) are indicated below each box. The upper flag is the 90th percentile (P90), the upper border of the box is P75, the bold line is P50, the lower border of the box is P25, and the lower flag is P10.**

TABLE 2. **Association Study for Haplotypes of Three *KCNQ4* Blocks for Population 1 Using Two-Way ANOVA for $Z_{high}$ and $Z_{low}$**

| Block | p-value $Z_{high}$ | p-value $Z_{low}$ |
|---|---|---|
| Block1 | 0.758 | 0.480 |
| Block2 | 0.715 | 0.397 |
| Block3 | 0.545 | 0.883 |

TABLE 3. **Association Study for Diplotypes of the Three *KCNQ4* Blocks for Population 1 Using Two-Way ANOVA for $Z_{high}$ and $Z_{low}$**

| Block | p-value $Z_{high}$ | p-value $Z_{low}$ |
|---|---|---|
| Block1 | 0.945 | 0.441 |
| Block2 | 0.173 | F = 0.017*,**; M = 0.986 (0.060[a,*]) |
| Block3 | 0.787 | 0.881 |

[a] Two-way ANOVA sex*genotype interaction.
*p-values suggesting a trend for association ($p < 0.100$).
**Significant p-values ($p < 0.05$).

TABLE 4. **Single SNP Association Tests for Population 2 Using Two-Way ANOVA $Z_{high}$ and $Z_{low}$**

| SNP number | RS number | p-value $Z_{high}$ | p-value $Z_{low}$ |
|---|---|---|---|
| SNP8 | rs4660466 | 0.981 | 0.543 |
| SNP9 | rs727146 | 0.495 | 0.021** |
| SNP11 | rs878042 | 0.170 | 0.162 |
| SNP12 | rs2149034 | 0.074* | 0.010** |
| SNP14 | rs13374844 | F = 0.106; M = 0.074* (0.008[a,**]) | 0.562 |
| SNP15 | rs4660468 | 0.153 | 0.092* |
| SNP18 | rs1214303 | F = 0.005**; M = 0.293 (0.009[a,**]) | 0.034* |

[a] Two-way ANOVA sex*genotype interaction.
*p-values suggesting a trend for association ($p < 0.100$).
**Significant p-values ($p < 0.05$).

detected upon analyzing the diplotypes (Table 3). A one-way ANOVA resulted in a p-value of 0.017 within the female population (males, $p = 0.986$).

### Association Study of Population 2

To further confirm the positive associations detected in Population 1, we genotyped the five tag-SNPs from the two associated blocks (SNP8, SNP9, SNP11 (NT_004511: g.11248106C>T), SNP15, and SNP18) along with two extra SNPs (SNP12 and SNP14) in an independent population (Population 2). Single SNP association testing for $Z_{high}$, using two-way ANOVA tests, revealed significant sex*genotype interactions for two SNPs (SNP14, $p = 0.008$ and SNP18, $p = 0.009$; Table 4). A separate one-way ANOVA for females and males resulted in p-values showing a trend towards significance for SNP14 (females, $p = 0.106$; males, $p = 0.074$), and significant p-values in females for SNP18 ($p = 0.005$; males, $p = 0.293$) (Table 4). Single SNP association testing for $Z_{low}$ resulted in three significant SNPs, SNP9 ($p = 0.021$), SNP12 (NT_004511: g.11249550A>T) ($p = 0.010$), and SNP18 ($p = 0.034$). The association with SNP12 confirms the findings in Population 1 (Table 1). A fourth SNP (SNP15) showed a trend towards significance ($p = 0.092$). If we look at the significant SNPs of Populations 1 and 2, it is remarkable that all these SNPs are located in the middle of the gene (Fig. 4).

Haplotypes and diplotype analysis did not lead to significant results (Tables 5 and 6).

## DISCUSSION

Up to now, only a single ARHI susceptibility gene has been identified in the human genome. One study found a significant association between ARHI and variations of N-acetyltransferase (NAT), an enzyme involved in the metabolism and detoxification of cytotoxic and carcinogenetic compounds [Unal et al., 2005]. Earlier attempts to identify genes associated with ARHI never led to significant results. Ates et al. [2005] studied the relationship between polymorphisms of glutathione-related anti-oxidant

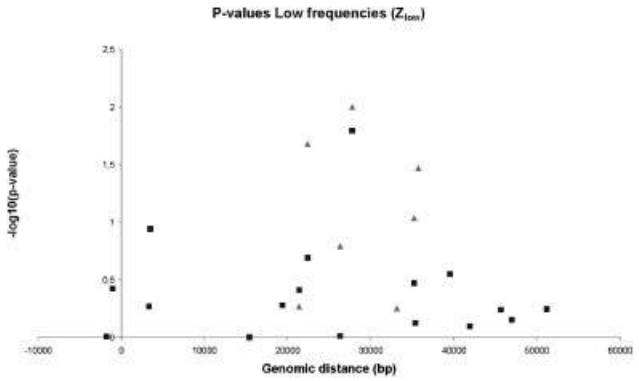


FIGURE 4. **Significance map for SNPs across *KCNQ4* for ARHI in Population 1 (squares) and Population 2 (triangles). The p-values were calculated for $Z_{low}$ and a -Log10 transformation was computed. The length of the gene is given in basepairs. The start codon of *KCNQ4* was chosen as the "0" setpoint.**

TABLE 5. **Association Study for Haplotypes of Two *KCNQ4* Blocks for Population 2 Using Two-Way ANOVA for $Z_{high}$ and $Z_{low}$**

| Block | p-value $Z_{high}$ | p-value $Z_{low}$ |
|---|---|---|
| Block1 | 0.422 | 0.166 |
| Block2 | 0.348 | 0.123 |

TABLE 6. **Association Study for Diplotypes of Two *KCNQ4* Blocks for Population 2 Using Two-Way ANOVA for $Z_{high}$ and $Z_{low}$**

| Block | p-value $Z_{high}$ | p-value $Z_{low}$ |
|---|---|---|
| Block1 | 0.494 | 0.139 |
| Block2 | 0.168 | 0.304 |

enzymes and ARHI, but no increased risk for ARHI could be demonstrated. In another study no strong association between *DFNA5* and ARHI could be detected [Van Laer et al., 2002]. In the current study, we analyzed the effects of polymorphisms in the *KCNQ4* gene on ARHI in two independent Caucasian populations. KCNQ4 is involved in recycling potassium in the inner ear and *KCNQ4* mutations are known to cause monogenic hearing loss [Kubisch et al., 1999]. Moreover, Coucke et al. [1999] described a monogenic KCNQ4 family with a pattern of hearing loss very similar to ARHI. This makes *KCNQ4* an excellent candidate gene for ARHI. Genes identified for ARHI in mice, like *CDH23*, are also good candidate genes [Noben-Trauth et al., 2003]. Nevertheless, it is likely that many different genes will contribute to ARHI in humans, and in our opinion, *KCNQ4* can be regarded as a very strong candidate susceptibility gene.

We have genotyped a total of 23 SNPs in a first population and looked for association with two distinct ARHI phenotypes. $Z_{high}$ was studied because it captures the frequencies typically affected by ARHI [International Organisation of Standardisation, 2000]. $Z_{low}$ represents the frequencies for which the highest heritability was detected [Gates et al., 1999], which suggests an important genetic contribution for the phenotype. Three SNPs were significantly associated with either $Z_{high}$ or $Z_{low}$. Two of these, SNP9 ($Z_{high}$) and SNP12 ($Z_{low}$), were located in the first intron of *KCNQ4* in a region conserved across species (Vista; http://genome.lbl.gov/vista/index.shtml). This might indicate the presence of a regulatory region or an internal promoter of *KCNQ4*. Promoter prediction programs predicted an internal promoter in intron 1 (www.genomatix.de). SNPs residing in these conserved regions might therefore exert an effect on *KCNQ4* expression levels.

The remaining associated SNP, SNP15 ($Z_{high}$), was located in exon 5 and did not cause an amino acid change in the protein. This exonic SNP might be present in an exonic splicing enhancer (ESE) [Caputi et al., 2002], or it could lead to cryptic splicing. However, since we expect small changes in expression levels or protein function to be responsible for complex diseases, we do not consider these latter hypotheses very likely. The six transmembrane segments encoded by exon 1 to 7 are crucial for *KCNQ4* functioning, and cryptic splicing and the usage of ESEs would probably have a major effect on protein functioning. Still, we cannot rule out that some connecting pieces between the transmembrane segments might lose or gain a few amino acids without having a large effect on the function of the channel. Another hypothesis is that the three SNPs are in LD with yet unidentified causative variants. All significant SNPs are located in the middle of the gene in a 13-kb region ranging from intron 1 to exon 6, which indicates that the causative variant for ARHI is probably located within this region.

Four different *KCNQ4* splice variants, which differ by the alternative usage of exons 9–11 or the complete lack of these three exons, have been demonstrated in mice [Beisel et al., 2005]. Some of these variants are tissue restricted. In the inner ear, multiple splice forms are present that are distributed differently in the various cell types in the cochlea. Beisel et al. [2005] noticed a quantitative difference in expression pattern along the length of the cochlea from base to apex, a different spatiotemporal regulation, and a different regulation of the splice variants. In other words, particular splice variants are more expressed in the apex, while other variants are more expressed in the base. In addition, it was shown that the expression of *KCNQ4* increases with age. Beisel et al. [2005] suggested that an increasing load of mutated or defective protein may lead to progressive cellular dysfunction. Therefore, and in view of our current findings, a possible explanation for the relationship between *KCNQ4* and ARHI could be that a rare inner ear specific *KCNQ4* splice variant forms KCNQ4 channels with altered electrophysiological characteristics. Aging might increase the expression of this variant, resulting in ARHI.

SNP9 and SNP18 are positively associated with high frequency ARHI in Population 1 and Population 2, respectively, but only for the female population. In addition, the diplotypes of block 2 show a similar pattern for low frequency ARHI in Population 1. Previous studies showed that males are more affected by ARHI than females [Davis, 1994; Gates et al., 1999]. Our results might suggest that *KCNQ4* is more involved in ARHI in women than in men. In the case of SNP9, which is present in a putative regulatory region or an internal promoter, an explanation for our results might be in the effect of the thyroid hormone (TH) on the expression of *KCNQ4*. Knipper et al. [2003] located three thyroid response elements (TREs) upstream of the promoter of *KCNQ4* [Knipper et al., 2003]. The secretion of TH hormone is regulated by female sex hormones [Adlersberg and Burrow, 2002], in particular the estrogens. Changes in estrogen and free TH levels present in postmenopausal women might cause the difference in hearing loss between males and females for SNP9 [Hultcrantz et al., 2006], as the effect of SNP9 on *KCNQ4* expression levels might be TH-dependent.

Nowadays, correcting for multiple testing using a Bonferonni correction is considered too strict. Therefore, we applied the false discovery rate (FDR) method [Sabatti et al., 2003] to evaluate the significance of our association results in Populations 1 and 2. When correcting for multiple testing, none of the previously associated SNPs remained significant (data not shown). Nevertheless, correcting for multiple testing remains a matter of debate among genetic epidemiologists. Some of them even doubt whether a correction is really necessary for association studies within a single gene, and argue that replication in different populations is more important than having very low p-values [Neale and Sham, 2004]. Therefore, despite the borderline significance, in our opinion, our results are of value because we have been able to replicate our findings in two independent populations. There also exists a lot of criticism on the candidate gene approach, which frequently leads to spurious results. A major problem with association studies is that many studies fail to replicate in subsequent association studies [Lohmueller et al., 2003]. Non-replication might result from study population differences or real biological differences [Tabor et al., 2002]. We succeeded in replicating our ARHI association study. However, not all SNPs associated in the first population resulted in significant associations in the second population. Only one SNP (SNP12) was associated in both populations. However, to confirm an association of a gene with a disease, it is not necessary that identical SNPs lead to significant results in both independent populations [Neale and Sham, 2004]. Both populations might harbor different associated SNPs within the same gene.

The differences between the two populations under study might partly be explained by the fact that these populations were collected differently. For instance, the inclusion criteria for the second population were slightly more strict than for the first population. In addition, the first population was not a real random population. Some people were included in the study through clinical practice, others through posters or oral advertisement, and some were spouses of people attending the clinic. This can cause skewness towards more severely affected participants that have probably been exposed to some environmental factor that remained unrecognized. The second population was more random because all subjects were collected through population registries. This difference in how participants were included in the study could have created a bias. However, including the origin of the samples of Population 1 as covariate into the statistical analysis did not have an effect on the results (data not shown). We believe that the most important difference between the two populations might be the age-range, which was 40 to 80 years for the first population, while it was 55 to 65 years for the second population. Therefore, possible early or late effects of *KCNQ4* on ARHI would be lost in the latter population. We did calculate whether there could be a possible effect of age on the disease (data not shown) but did not find any indications that early or late effects of *KCNQ4* on ARHI did indeed exist.

Finally, the differences obtained between the two populations might result from the complexity of the disease itself. Usually, when performing association studies, it is assumed that each gene and each environmental factor contribute individually to the risk of the disease. In reality, genes interact with each other and with environmental factors. This could explain why, even among the most replicated susceptibility genes for diseases other than ARHI, many negative studies have been published [Ober, 2005]. Therefore, association studies for complex diseases should take into account interactions between genes and between genes and environment [Ober, 2005]. Our study of the involvement of *KCNQ4* on the development of ARHI might also benefit from allowing such interactions.

In conclusion, this study detected a significant association between *KCNQ4* and ARHI in two independent populations. However, except for one SNP (SNP12), different SNPs were positively associated in both populations. Nevertheless, these SNPs are all located in the same 13-kb region in the middle of the *KCNQ4* gene. This indicates that the causative variants for ARHI are probably located within this region. Future association and functional studies of *KCNQ4* will contribute to the identification of the causative SNP for ARHI.

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# Discussion

**Discussion**

Untill now, hearing impairment genes have been identified mainly by linkage analysis that provides the initial localization of the gene at study to perform the positional cloning or positional candidate gene approach. Today, more sophisticated techniques such as the use of Single Nucleotide Polymorphisms (SNPs) and linkage disequilibrium are used to identify complex genetic types of hearing impairment. All these genetic analyses have in common that the hearing impairment in each individual has to be recognized and characterized correctly. The phenotype has to be studied carefully to be able to study the genotype of hereditary hearing impairment. Therefore clinical and audiological characterisation of hearing impairment is crucial in the multi-disciplinary approach of hereditary hearing impairment.

Chapter 2 describes cases in which immediate diagnostic genetic analyses can be performed when the phenotype of a deafness trait is suggestive for a specific deafness gene. Some of the reported deafness genes are even used in screening protocols such as *GJB2* gene. More than 80 different deafness causing allele variants of *GJB2* have been reported.(1) However one single mutation termed 35delG seems to predominate in populations of European descent. The *GJB2* gene encodes the connexin 26 (CX26) protein. Connexins oligomerize to hexameric hemi channels called "connexons," which are present in the plasma membrane, where they can bind with connexons from adjacent cells to form functional gap junctions.(2) In the cochlea, CX26-containing gap junctions are though to play a role in K+ homeostasis.(3) The hearing impairment in persons with *GJB2* mutations ranges from mild to profound and is not progressive.(4;5). Truncating mutations of GJB2 are associated with a greater degree of hearing impairment than non-truncating mutations.(6) Variants of the *GJB2* gene account for up to 50% of cases of autosomal recessive nonsyndromic hearing impairment. Considering also the fact that this gene is rather small with only one coding exon makes it a very useful in screening protocols. Nevertheless, there is a big gap between fundamental genetic research on hereditary hearing impairment and clinical otology. Although many deafness genes have been identified, only in few cases genetic analysis provides evidence for a medical diagnosis of hereditary hearing impairment. Especially in late onset deafness, environmental factors have a disturbing effect for making the diagnosis of hereditary deafness.

In chapter 3 describes the clinical and audiological characterization of sensorineural hearing loss in a large Belgian family. The trait was localised to the DFNA22 locus by linkage analysis. This locus has been described before in an Italian kindred and is associated with a mutation in the *MYO6* gene that encodes for myosin VI, a member of the myosin super-family.(7) Myosins are motor proteins that use hydrolysis of ATP to move on F-actine through which they convert chemical energy into mechanical energy. In the first reported DFNA22 family, a missense mutation has been identified in exon 12 of the *MYO6* gene on chromosome 6q13, which causes in non-syndromic progressive hearing impairment without vestibular dysfunction.(7) The second family with another missense mutation in the *MYO6* gene is reported to have progressive late onset autosomal dominant hearing impairment combined with cardiac hypertrophy. (8) In this thesis we report a third family with mid-frequency, progressive sensorineural hearing loss linked to DFNA22 without vestibular dysfunction or cardiac hypertrophy. Thorough clinical and audiological study could not accelerate genetic research. Classical linkage analysis and a time consuming genome scan were necessary to link this family to DFNA22. Though genetic analysis has localized the gene to a region of chromosome 6q13-6q14.1, which contains a known deafness gene *MYO6*, DNA sequencing of the coding region did not reveal a mutation. Although a mutation outside the coding region could be responsible for the deafness, it is also possible that another gene in this

region is responsible for the deafness trait in this family. The genetic search is ongoing. The success of phenotype characterization to discern affected and unaffected family members is proven by a high LOD score. This result is not always that obvious because often unrecognized phenocopies have an interfering effect on LOD score calculations.

One of the more frequently encountered autosomal dominant loci is DFNA2. At this locus on chromosome 1p34, two deafness genes have been identified: the GJB3 gene that encodes connexin 31, a gap junction protein, and the KCNQ4 gene that encodes the subunits of a voltage-gated potassium channel.(9;10) Both genes presumably play a role in recycling potassium ions from the hair cells to the endolymph.(11) Clinically the *KCNQ4* gene is well studied. Therefore a successful genotype phenotype correlation study led to the discovery of the fifth Dutch DFNA2 family. In this study of chapter 4, time consuming linkage analysis was skipped and mutation analysis was immediately applied based on only audiometric and clinical analysis. The ARTA supported the hypothesis for a *KCNQ4* gene mutation that was confirmed genetically. All clinically affected family members were found to be carriers of the W276S missense mutation in exon 5 of the *KCNQ4* gene. Refined phenotypic features confirmed previously described phenotypes of DFNA2 comprising progressive, high-frequency sensorineural hearing impairment. The hearing loss involved with KCNQ4 mutations resembles Age-Related Hearing Impairment (ARHI). Therefore KCNQ4 has been postulated as a good candidate gene for ARHI.

In chapter 5 the phenotype of the *CDH23* gene on chromosome 10 is studied, which can be responsible for non-syndromic autosomal recessive deafness in DFNB12 as well as for syndromic deafness in Usher syndrome type 1D. This allelic heterogeneity in which different phenotypes originate from different alleles of the same gene is called allelism.(12)
Three different clinical types of Usher syndrome are known. Usher syndrome Type I is characterized by congenital, profound deafness associated with vestibular areflexia and retinitis pigmentosa. Heterogeneity also affects subset of Usher Syndrome type I in which seven genes or loci have been identified as producing the same syndrome. Usher syndrome Type II is characterized by moderate to severe sensorineural hearing impairment, intact vestibular responses, and retinitis pigmentosa. Here again three loci are mapped for Usher syndrome type II. Usher syndrome Type III is characterized by progressive hearing impairment, variable vestibular function, and retinitis pigmentosa.(13)
The protein Cadherin 23 is involved in Usher syndrome type 1D (USH1D) and DFNB12. Cadherin 23 is a transmembrane protein with 27 extracellular cadherin repeats, a transmembrane domain, and a cytoplasmic domain. It is encoded by the *CDH23* gene, which consists of 69 exons.(14;15) Cadherins are important for cell-to-cell contact and the organization of the extra cellular matrix. Binding of calcium ions to these proteins is essential for linearization, rigidification, and dimerization of the cadherin molecules.(16;17)
In chapter 5 the findings of audiovestibular and ophthalmologic examinations in four families with mutations in the *CDH23* gene are reported. This study shows that recessive missense mutations in the *CDH23* gene lead to a milder phenotype causing DFNB12 than splice-site mutations that cause Usher syndrome type 1D. Splice-site mutations cause significantly more severe hearing impairment than in DFNB12 and in addition also cause retinitis pigmentosa and vestibular areflexia. However, abnormal bilateral flecks, suggestive for lipofuchsine accumulation can also be observed in DFNB12 patients. Non syndromic, autosomal recessive sensorineural hearing loss associated to DFNB12 produces a moderate to profound hearing loss, usually non progressive and an onset in childhood. Although the DFNB12 patients could not be diagnosed with retinitis pigmentosa they did reveal ophthalmologic observations that were pathological. This finding might suggest that there is a relative continuum between the

phenotypes of DFNB12 and USH1D. This continuum might even be larger than we suspect at this moment when we consider that the *CDH23* gene in mice have been reported as modifier genes for Age-Related Hearing Impairment (ARHI). (18) Modifier genes alter, most often quantitatively, the expression of another gene. This can also be a protective phenomenonfor a certain trait. Together these modifier genes and protective alleles provide important glimpses into the molecular and cellular basis for the functional networks that provide robustness and homeostasis in complex biological systems.(19) The correlation between the genotypes and phenotypes can be studied from different angles to reveal the interaction between both.

Otosclerosis is clinically and audiologically studied in chapter 6. The aetiology is not fully understood but both genetic and environmental factors are assumed to be involved.(20) An important clinical aspect in this study was to discern the disease from age related deterioration of hearing that is though to be physiological. Since 1998 five genes have been localised for autosomal dominant forms of otosclerosis but none of them have been identified.(21) Otosclerosis is an isolated disorder of bone homeostasis of the otic capsule in the middle ear that can cause a conductive as well as a sensorineural hearing loss (SNHL). The disease is characterised by resorption of healthy bone tissue and subsequent formation of abnormal bone tissue, a process referred to as otospongiosis. During this process a fixation of the stapes occurs by focal bone formation around the foramen ovale that generates a conductive hearing loss.(22;23) The disease exclusively occurs in the human otic capsule and therefore animal models are not available. On the other hand the conductive hearing loss can be corrected with microsurgery. Stapedotomy is a clinical procedure that is often performed which makes it difficult to find a population with no therapeutic intervention to phenotype the natural evolution of this disease, to help understand its aetiology. The study in chapter 6 demonstrates that there is a significant sensorineural component in otosclerosis patients planned for stapedotomy, which is worse than age-related hearing loss (ARHL) by itself. Deterioration rates of air conduction and bone conduction thresholds have been reported which can be helpful in clinical practice but might also guide the characterization of allegedly different phenotypes for familial and sporadic otosclerosis. Sensorineural hearing loss because of otosclerosis has been hypothesized for a long time but in this thesis it is demonstrated with statistical analyses on audiological data. In families with more members suffering from conductive hearing loss there are often also members with sensorineural hearing loss. Perhaps otosclerosis should be regarded as a pleiotropic disease causing otosclerotic foci at random which can either involve or spare the stapes. It might even be considered to regard all hearing impaired family members, whether of perceptive or conductive nature, as affected individuals in linkage analysis. Possibly this approach can lead to the discovery of more otosclerosis loci and reveal the path to the first genes causing otosclerosis.

Age-Related Hearing Impairment (ARHI) is another type of SNHL of unknown aetiology. There has long been a misconception that hearing impairment is an inevitable part of ageing rather than a preventable disease. Today ARHI is also considered a complex genetic trait where again both genetic and environmental factors are presumed to play a role. While the first genetic variant associated with ARHI is still to be identified, there is substantial literature about environmental and medical risk factors leading to ARHI. The study presented in chapter 7 is part of an ongoing multicenter study into the genetic and environmental causes of ARHI across 7 European countries. This study has been primarily designed to find genetic variants leading to ARHI, whereby environmental and medical causes are nuisance factors. To minimize non-genetic influences on ARHI, many medical conditions with a possible or proven role in hearing impairment or subjects with ear diseases other than presbyacusis, had to be excluded based on clinical and audiological examination. The study of the phenotype of

ARHI is consistent with the scope of this thesis, especially the application of the Z-score conversion. The latter is an age and sex independent quantification of ARHI that not only facilitates genetics analyses but can also serve to study environmental factors of ARHI. The preliminary results on the Belgian sample of this European study in chapter 7 confirm that gunfire noise exposure is a risk factor for ARHI. However we could not substantiate this for occupational noise because the sample consisted of highly screened healthy cases with very little exposure. On the other hand there were enough smokers in the sample to prove a significant deterioration on hearing caused by smoking in both sexes. Moreover a significant interaction was found between noise exposure and smoking, with noise exposure and cigarette smoking having a more-then additive effect. Alcohol consumption seemed to improve hearing in males, whereas in females it seems to decline. There where alcohol abuse can lead to increased hearing loss, a protective effect of moderate alcohol consumption on hearing has been noted before in some studies but this does not exactly explain our findings. A probable bias is comprised in our questionnaire which actually contains data on self-report alcohol consumption. Over-presentation in men and denial of drinking in women is possible. Despite the limitations of our study population, we were able to analyze several potential ARHI environmental risk factors. This study only explains a minute fraction of the variance found in ARHI and more is to be expected from the study of interactions between genetic risk factors. Also preliminary results of the first genetic results on the Belgian sample are presented in chapter 8. These analyses have to be correlated to similar analyses in the entire European sample of the ARHI consortium.

The completion of the human genome project has lead to the discovery of millions of genetic variants (single-nucleotide polymorphisms or SNPs). These SNPs are ubiquitous in the human genome, and are held responsible for phenotypic variation between individuals. As mentioned before, the phenotype of ARHI resembles DFNA2. Therefore the *KCNQ4* gene is a candidate gene for ARHI. The collected clinical sample has been analysed to study SNPs in a candidate susceptibility gene, *KCNQ4*, for ARHI. This study itself is beyond the scope of this thesis. However, audiometric and clinical analysis of this sample is exactly consistent with the objectives of this thesis. The calculation of Z-scores for high and low frequencies have quantified ARHI. Especially the division of the Z-score to higher (Z-high) and lower (Z-low) is another example of novel developments in phenotype characterisation. The Z-high was studied because it captures the frequencies typically affected by ARHI. The Z-low represents the frequencies for which the highest heritability was detected, which suggests an important genetic contribution for the phenotype.(24) Three SNPs were significantly associated with either Z-high or Z-low. Two of these, SNP9 (Z-high) and SNP12 (Z-low), were located in the first intron of KCNQ4 in a region conserved across species. This might indicate the presence of a regulatory region or an internal promotor of KCNQ4. Promoter prediction programs predicted an internal promoter in intron 1. The SNPs residing in these conserved regions might therefore exert an effect on KCNQ4 expression levels. The remaining associated SNP, SNP15 (Z-high), was located in exon 5 and did not cause an amino acid change in the protein. A possible explanation for the relationship between KCNQ4 and ARHI could be that a rare inner ear specific KCNQ4 splice variant, forms KCNQ4 channels with altered electrophysiological characteristics. Aging might increase the expression of this variant, resulting in ARHI.

Since the last three years only 3 new autosomal dominant and 28 new autosomal recessive loci for non syndromal HHI have been reported. On the other hand much more studies have revealed functions and protein products of already known deafness genes. In other words the gap between fundamental genetic research on hereditary hearing impairment and clinical

otology should be closing with functional studies. The challenge is to provide clinical applications of the knowledge on these deafness genes. Another recent research focus is how deafness genes interact with each other or perhaps with environmental factors. Within this focus again phenotype determination will provide an important foundation for genetic studies. Phenotype determination covers more than simply performing a hearing test as shown over the separate chapters in this thesis. Clinical examination remains the basis of audiological and statistical assessment of hearing impairment. In conclusion, researchers interested in hereditary hearing impairment should master several techniques and methods to phenotype the deafness in the individual case to improve the success of genetic studies to discover the genotype.

In conclusion:

- This thesis indicates that thorough clinical and audiological investigation can:

    1. Sometimes provide enough information for immediate genetic testing in as diagnostic context (Review chapter 2)

    2. Facilitate genetic linkage analyses (DFNA22 study in chapter 3)

    3. Facilitate and even speed up genetic research (DFNA2 study in chapter 4)

    4. Test and justify genetic classification of clinically different disorders (DFNB12-USH1D study in chapter 5)

- In this context a thorough clinical and audiological description is reported for:

    1. Otosclerosis, (chapter 6)
        a. To facilitate genetic research for this complex genetic trait
        b. To facilitate clinical counselling concerning surgical intervention

    2. Presbyacusis.
        a. To report environmental risk factors for hearing loss (chapter 7)
        b. To facilitate genetic research on ARHI (*KCNQ4* study in chapter 8)

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# Samenvatting

## Samenvatting

### Erfelijke Doofheid: Een klinisch audiologische benadering

Slechthorendheid is een belangrijke zintuigstoornis dat een significante handicap impliceert. Ongeveer 35 procent van mensen tussen 65 en 70 jaar hebben meer dan 25 dB gehoorverlies terwijl 1 op 1000 kinderen doof worden geboren in onze gemeenschap. De etiologie van slechthorendheid is vaak multi-factorieel en omvat erfelijke- en verworven factoren. Bekende oorzaken van verworven doofheden zijn onder andere; lawaaitrauma, ototoxiciteit en infecties (o.a. middenoorontsteking, rotsbeenontsteking, hersenvliesontsteking). Erfelijke doofheid kan soms worden veroorzaakt door 1 afwijkend gen. In de laatste 15 jaar zijn vele vormen van monogene, door 1 gen veroorzaakte, vormen van erfelijke doofheid ontdekt aan de hand van genkoppelings studies op grote slechthorende families. Indien de doofheid gepaard is met andere symptomen, zoals blindheid spreekt men van syndromale doofheid. Het Usher syndroom bijvoorbeeld omvat doof-blindheid terwijl het Pendred syndroom een combinatie is van doofheid en schildklierdysfunctie. Naast monogene doofheid bestaan er ook complex genetische vormen van slechthorendheid. Hierbij komt een bepaalde genetische voorgeschiktheid tot expressie indien het uitgelokt wordt door een zekere omgevingsfactor. Complex genetische aandoeningen kunnen ook veroorzaakt worden door een samenspel van verschillende genen. Lawaai geinduceerde slechthorendheid, ouderdomsslechthorendheid en otosclerose zijn voorbeelden van complex genetische doofheid.

Dit proefschrift stelt als doel verschillende vormen van erfelijk gehoorverlies klinisch en audiologisch te kenmerken om genetisch onderzoek naar de etiologie van de slechthorendheid mogelijk te maken. De methodiek om verschillende typen van slechthorendheid zodanig van elkaar te onderscheiden ter voorbereiding van genetisch onderzoek is belangrijk en wordt fenotyperen genoemd. Fenotype beschrijving is noodzakelijk voor het achterhalen van een ziekteveroorzakende mutatie, wat genotyperen wordt genoemd. Uiteraard ontstaat dan de mogelijkheid om genotype-fenotype correlaties te leggen voor bekende vormen van doofheid.

Hoofdstuk 1 geeft een algemene inleiding over de anatomie van het oor en fysiologie van het horen. Hier is ook een sectie gewijd aan problemen en valkuilen die bestaan bij fenotyperen van erfelijke doofheid. Horen varieert o.a. naargelang geslacht en leeftijd. Bij familiale doofheid is het belangrijk om met klinische en audiologische methoden slechthorende familieleden te onderscheiden van goedhorenden. Dit onderscheid is van wezenlijk belang voor de genotypering. Gangbare strategieën voor genetisch onderzoek naar erfelijke doofheid worden hier ook toegelicht.

Hoofdstuk 2 beschrijft een overzicht van verschillende vormen van erfelijke doofheid waarbij in sommige gevallen de klinische en audiologische presentatie, direct het aanvragen van genetisch onderzoek kan maatstaven. Dit kan belangrijke diagnostische implicaties hebben en patiënten voorlichting enorm vergemakkelijken. Er bestaan verschillende standaard protocollen voor het screenen van bijvoorbeeld mutaties in het GJB2 gen. Ondanks dat er meer dan 80 verschillende mutaties in dit gen al beschreven zijn is het gemakkelijk en zinvol om dit systematisch te onderzoeken bij vroeg kinderlijke doofheid. Enerzijds is dit gen relatief klein en gemakkelijk genetisch te controleren en anderzijds domineert in Europa één specifieke mutatie getypeerd als 35delG. Het GJB2 gen codeert voor een eiwit dat onderdeel uitmaakt van een zogeheten connexine verbinding in gap junctions tussen 2 naburige cellen. Deze verbindingen spelen een belangrijke rol in signalisatie en celhomeostase van cellen in o.a. het slakkenhuis. Het gehoorverlies dat gepaard gaat met mutaties in het GJB2 gen varieert

in ernst en wordt vaak in de kindertijd ontdekt. Andere eveneens genetisch goed beschreven doofheidsgenen zijn in de dagelijkse kliniek minder goed te screenen. Genetisch onderzoek staat nog te ver van de dagelijkse oorheelkundige praktijk. Toch blijft kennis van de verschillende fenotypen van erfelijke doofheid cruciaal voor de dagelijkse praktijk.

In hoofdstuk 3 wordt de klinische en audiologische beschrijving van een grote Belgische familie met autosomaal dominante doofheid gerapporteerd. Deze studie van het fenotype heeft geleidt tot het lokaliseren van de genetische afwijking op locus DFNA22 op chromosoom 6q13-6q14.1. Op deze locus is al eerder doofheid beschreven in een Italiaanse familie, waarbij een mutatie in het MYO6 gen de slechthorendheid veroorzaakt. Het MYO6 gen codeert voor myosine VI dat een motor proteïne is die door hydrolyse van ATP chemische energie omzet in mechanische energie. Bij de tweede familie met een mutatie in dit gen is ook hypertrofie van de hartspier opgemerkt. Snell's walzer muizen vormen het analoge dierenmodel van dit gen en deze vertonen naast doofheid ook hypofunctie van het vestibulair stelsel. In de hier gerapporteerde Belgische familie dat is gekoppeld aan DFNA22, is echter alleen mid-frequent progressief gehoorverlies geobjectiveerd zonder evenwicht- of hartafwijkingen . Genetisch onderzoek heeft de gekende mutaties in deze locus uitgesloten. Theoretisch zou het kunnen dat het doofheid veroorzakend gen buiten deze locus ligt maar de bekomen hoge LOD scores maken deze kans klein. Het genetische onderzoek is nog lopende en mogelijks betreft het hier een nieuwe mutatie in de DFNA22 locus.

Een van de meest frequent aangetaste loci voor autosomaal dominante doofheid is DFNA2. Deze locus op chromosoom 1p34 omvat twee doofheidsgenen. Enerzijds het GJB3 gen dat codeert voor het connexin 31 eiwit en anderzijds het KCNQ4 gen dat codeert voor subunits van een voltage afhankelijk Kalium kanaal. Beide eiwitten vormen onderdeel van verschillende structuren die een rol spelen in de Kalium homeostase van haarcellen. Het KCNQ4 gen is klinisch en genetisch goed bestudeert. Dit heeft ertoe geleidt dat een vijfde DFNA2 familie zeer snel gegenotypeerd kon worden. Hoofdstuk 4 beschrijft een familiestudie. Het fenotype in deze familie omvat progressief, hoog frequent perceptief gehoorverlies dat met behulp van ARTA (Age Related Typical Audiograms) veel gelijkenissen vertoonde met andere DFNA2 families. Tijdrovend koppelingsanalyse werd vermeden en direct mutatieanalyse in het KCNQ4 gen werd opgestart voor gekende mutaties. Alle klinisch aangetaste familieleden bleken drager te zijn van de W276S missense mutatie in exon 5 van het KCNQ4 gen.

In hoofdstuk 5 wordt het fenotype van het CDH23 gen bestudeerd. Deze kan zowel verantwoordelijk zijn voor syndromale doofheid in het Usher syndroom type 1D alsook voor niet syndromale autosomaal recessieve doofheid in DFNB12. Dit fenomeen waarbij verschillende fenotypen hun oorsprong vinden in verschillende allelen van hetzelfde gen wordt ook wel allelisme genoemd. Locus DFNB12 is gekenmerkt door autosomaal recessieve matig tot ernstig gehoorverlies dat vroeg ontstaat in de kinderjaren maar geen progressie kent. Het Usher syndroom wordt klinisch onderverdeeld in 3 subtypen waarbij elk type retinitis pigmentosa vertoont dat tot slechtziendheid leidt. Hiernaast wordt Usher type I gekenmerkt door aangeboren ernstige doofheid met vestibulaire areflexie. Usher syndroom Type II is gekenmerkt door milder gehoorverlies en vestibulair intacte reflexen terwijl Usher syndroom type III variabele vestibulaire reflexen vertoond met progressief gehoorverlies. In hoofdstuk 5 wordt het fenotype van Usher syndroom type 1D en DFNB12 vergeleken. Deze studie toont dat recessieve missense mutaties in het CDH23 gen leiden tot een milder fenotype dat DFNB12 veroorzaakt dan splice-site mutaties die het Usher syndroom type 1D veroorzaken. De splice-site mutatie veroorzaken niet alleen tegelijkertijd retinitis pigmentosa en

vestibulaire areflexie maar ook het gehoorverlies is ernstiger in Usher 1D patiënten dan in DFNB12 patiënten. Ophthalmologisch onderzoek in DFNB12 patiënten toont geen retinitis pigmentosa maar wel werden abnormale flecks, mogelijks suggestief voor lipofuchsine opstapeling, geobjectiveerd. Deze bevindingen suggereren dat er wellicht een relatief continuüm is tussen Usher Syndroom type 1D en DFNA12.

In hoofdstuk 6 wordt het fenotype bestudeerd van een complex genetische vorm van slechthorendheid. Otosclerose is een aandoening van het otisch kapsel in het menselijke middenoor dat zowel perceptief als transmissief gehoorverlies kan veroorzaken. De etiologie van otosclerose is niet gekend maar genetische en omgevingsfactoren spelen vermoedelijk een rol. Een belangrijke uitdaging in de studie van het fenotype van otosclerose in hoofdstuk 6 bestond eruit om deze ziekte te onderscheiden van ouderdomsgebonden slijtage van het gehoor. Otosclerose veroorzaakt een resorptie van gezond bot weefsel en vervolgens wordt abnormaal bot aangemaakt, wat ook wel otospongiosis wordt genoemd. Tijdens dit proces worden kleine foci van bot gevormd die de stijgbeugel kunnen fixeren en transmissief gehoorverlies veroorzaken. Foci in en rondom het slakkenhuis veroorzaken anderzijds perceptief gehoorverlies wat moeilijk te onderscheiden is van ouderdomsslijtage van, eveneens in het slakkenhuis gelegen haarcellen. Hiernaast is het moeilijk om een populatie te verzamelen waarbij de natuurlijke evolutie van otosclerose bestudeerd kan worden omdat de patiënten met deze aandoening vaak al heelkundig behandelt zijn. Stapedotomie is thans een gangbare therapeutische tussenkomst voor stijgbeugelverkalking. Hoofdstuk 6 bestudeert preoperatieve audiometrische data van heelkundig bevestigde otosclerose patiënten. Deze studie wijst op het bestaan een wezenlijke perceptief component in otosclerose dat significant verschilt van de te verwachten leeftijdsgebonden achteruitgang van het gehoor. Tevens is de progressiesnelheid van het perceptief en transmissief gehoorverlies berekend en kan dit in de praktijk dienen correcte timing voor stapedotomie. Perceptief gehoorverlies als gevolg van otosclerose is een goed gekend fenomeen echter alhier wordt het bewezen aan de hand van statistische analysen op audiologische data. Mogelijks kan deze studie een nieuwe discussie op gang brengen naar de vermeende indeling van otosclerose in familiale en sporadische gevallen. In vele relatief grote families met transmissief gehoorverlies door otosclerose zijn er ook vaak familieleden met enkel perceptief gehoorverlies die buiten de koppelingsstudie worden gesteld. Indien otosclerose als een pleiotrope aandoening wordt gezien die otosclerotische foci veroorzaak al dan niet leidend tot stijgbeugelverkalking, dan kunnen familieleden met perceptief gehoorverlies ook in de koppelingsstudie worden opgenomen. Mogelijks kan deze aanpak de complex genetische etiologie van otosclerose iets ontrafelen.

Slechthorendheid bij toenemende leeftijd werd vaak als onoverkomelijk beschouwd terwijl echter de laatste jaren ook risicofactoren voor slechthorendheid uitvoerig worden bestudeerd. Ook bij ouderdomsslechthorendheid of presbyacusis vermoed men een complex genetische etiologie waarbij zowel genetische alsook omgevingsfactoren van invloed zijn. Reeds verschillende omgevingsfactoren zijn gerapporteerd als risicofactor voor presbyacusis maar er zijn nog geen oorzakelijke genen beschreven. De studie in hoofdstuk 7 is onderdeel van een lopende studie naar genetische en omgevingsfactoren van ouderdomsslechthorendheid onder de naam ARHI (Age-Related Hearing Impairment) over 7 Europese landen. Hierbij valt de studie van het fenotype van presbyacisus, vooral de toepassing van de Z-score, volledig bonnen de doelstellingen van dit proefschrift. De Z-score is een conversie gebaseerd op de ISO7029 normen en laat een quantificatie van presbyacusis toe dat onafhankelijk is van geslacht en leeftijd. De Z-score beschrijft het verschil tussen gemeten gehoordrempels en verwachtte gehoordrempels gedeeld door de standaard deviatie. Een negatieve Z-score wijst op een beter gehoor dan verwacht voor leeftijd en geslacht en vice versa. Met behulp van deze

Z-score kunnen correlaties gelegd worden met verschillende risicofactoren. De voorlopige Belgische resultaten van de ARHI studie bevestigen dat onbeschermd lawaai blootstelling en vuurwapengebruik risico's vormen voor slechthorendheid. Roken bleek eveneens een risicofactor voor slechthorendheid, waarbij een combinatie van roken en lawaaiblootstelling wijzen op een meer dan additief schadelijk effect op horen. Alcohol consumptie bij mannen correleerde met een beter gehoor terwijl alcoholgebruik bij vrouwen correleerde met een slechter gehoor. Deze analysen zijn uitgevoerd op gegevens van vragenlijsten waarbij deelnemers zelf de hoeveelheid van alcoholgebruik hebben aangeduid. Overvloedig gebruik kan zijn aangeduid door mannen terwijl vrouwen eerder te bescheiden hebben geantwoord. Andere studies wijzen op een verslechtend effect van overmatig alcoholgebruik op het gehoor terwijl matig alcoholgebruik een beschermend effect kan hebben. Dit kon niet worden bevestigd met onze dataset. De ARHI studie is hoofdzakelijk gericht op het vinden van genetische varianten die mogelijks ouderdomsslechthorendheid kunnen verklaren. Hierbij zijn veel medische aandoeningen die tot slechthorendheid kunnen lijden vervat in de exclusie criteria om een zo gezond mogelijke studiepopulatie te bekomen. Ondanks deze beperking voor het bestuderen van omgevingsfactoren zijn toch een aantal risicofactoren voor slechthorendheid bevestigd en zullen worden getoetst aan de volledige studiepopulatie over 7 deelnemende landen.

In hoofdstuk 8 worden eveneens de voorlopige Belgische genetische resultaten van de ARHI studie gerapporteerd. Het fenotype van ouderdomsslechthorendheid lijkt veel op het fenotype van DFNA2, beide kennen namelijk een progressieve afname van de hoge frequenties in de tijd. Op basis van audiologische overeenkomsten werd het KCNQ4 gen beschouwd als kandidaat gen voor ouderdomsslechthorendheid. Deze studie kent de introductie van de Z-score voor lage en hoge frequenties afzonderlijk. De Z-high score werd bestudeerd vanwege het feit dat presbyacusis zich presenteert op de hoge frequenties. De Z-low score werd bestudeerd omdat voorgaande studies hogere heretabiliteit tonen voor lagere frequenties. Twee verschillende SNPs (Single Nucleotide Polymorphisms) toonden significante associatie met de Z-high score (SNP 9 en 15) en één SNP met de Z-low score (SNP12). Twee SNPs (9 en 12) waren gelegen in de eerste intron van het KCNQ4 gen en de andere SNP (15) was gelokaliseerd in exon 5. Ook deze bevindingen zullen worden getoetst aan de gehele ARHI studiepopulatie over 7 deelnemende Europese landen.

In conclusie:
Dit proefschrift wijst uit dat grondig klinisch en audiologisch onderzoek;

- Aanvragen van direct diagnostisch genetisch onderzoek kan ondersteunen (hoofdstuk2)
- Onmisbaar is voor genetisch koppelingsonderzoek (hoofdstuk 3)
- De efficiëntie van genetisch onderzoek kan verhogen (hoofdstuk 4)
- Genetische classificaties kan toetsen aan het fenotype (hoofdstuk 5)

In dezelfde context wordt een audiologische en klinische beschrijving gerapporteerd voor;

- Otosclerose (Hoofdstuk 6)
  - Voor het faciliteren van genetisch onderzoek in de toekomst
  - Voor het faciliteren van klinische counseling
- Ouderdomsslechthorendheid
  - Voor het rapporteren van risicofactoren voor gehoorverlies (Hoofdstuk 7)
  - Voor het faciliteren van genetisch onderzoek van presbyacusis (Hoofdstuk 8)

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