Hereditary Hearing Impairment

Clinical and Genetic Aspects of DFNA3, DFNB8/10,
DFNX4, Muckle-Wells syndrome and Otosclerosis

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Colophon

Cover: Dorien Rouwenhorst
Print: Ipskamp, Enschede
ISBN: 978-90-9027323-5

Financial support for the publication of this thesis was provided by:
Advanced Bionics, ATOS Medical, Beter Horen, Carl Zeiss B.V., Cochlear, Daleco Pharma, Dos Medical B.V., EmiD Audiologische Apparatuur, Oticon Medical, Pentax, Specsavers.

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Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. mr. S.C.J.J. Kortmann,
volgens besluit van het college van decanen
in het openbaar te verdedigen
op dinsdag 12 maart 2013
om 13:30 uur precies

door

Nicole Johanna Dianne Weegerink
geboren op 21 november 1983
te Enschede

Promotoren

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Aan mijn ouders

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General introduction

Hereditary hearing impairment

Hearing impairment is the most common birth defect and the most prevalent sensorineural disorder in developed countries.[1] Hearing impairment forms a major social and economic burden. Approximately one in every 1000 newborns is deaf and one in 300 newborns has congenital hearing impairment of a lesser degree. Furthermore, an additional one in 1000 children becomes profoundly hearing impaired before adulthood.[2] In the general population, the prevalence of hearing impairment increases with age. Ten percent of the Western population has hearing impairment severe enough to impair communication.[2-4] Age-related hearing impairment (ARHI) or presbycusis is the most common sensory deficit in the elderly. Approximately 35% of individuals between 60 and 70 years demonstrate a pure-tone average threshold (PTA, average of the thresholds at 0.5, 1, 2 and 4 kHz) of 25 dB or more. In the age group between 70 and 80 years, as much as 50% of the individuals has a PTA threshold of 25 dB or more.[5] This increasing prevalence reflects the impact of genetic and environmental factors on the development of hearing impairment.[4]

Hearing impairment can be classified as genetic or nongenetic (acquired), prelingual or postlingual, and syndromic or nonsyndromic. The characteristics for an adequate clinical description of genetic hearing impairment are shown in table 1.[6-8] Hearing impairment is classified by type, onset, severity and frequency. Hereditary hearing impairment can be caused by a mutation in a single gene (monogenetic disorder, e.g. most forms of syndromic and early onset nonsyndromic hearing impairment) or can be a complex trait, influenced by an interplay between genetics and environment (e.g. otosclerosis and presbycusis).[6, 9, 10]

Clinical manifestation	*Characterization*	*Explanation*	
Type of HI	Conductive	Results from abnormalities of the external/outer ear and/or middle ear. Normal BC thresholds (<20 dB HL) and ABG >15 dB averaged over 0.5, 1 and 2 kHz.	
	Sensorineural	Cochlear	Results from malfunction of inner ear structures (i.e. cochlea). BC thresholds >20 dB HL and ABG <15 dB HL averaged over 0.5, 1 and 2 kHz.
		Auditory neuropathy	Results from damage or dysfunction at the level of the 8th cranial nerve, auditory brain stem or cerebral cortex.
	Mixed	Combination of conductive and sensorineural HI. BC thresholds >20 dB and ABG >15 dB averaged over 0.5, 1 and 2 kHz.	
Severity of HI	Mild	20-40 dB HL	
	Moderate	41-70 dB HL	
	Severe	71-95 dB HL	
	Profound	>95 dB HL	
Audiometric configuration	Low frequency ascending	>15 dB HL difference between the worst low frequency thresholds and the better high frequency thresholds.	
	Mid frequency U-shaped	>15 dB HL difference between the worst mid frequency thresholds and the better low and high frequency thresholds.	
	High frequency	Gently sloping: 15-29 dB HL difference between the mean of 0.5 and 1 kHz, and the mean of 4 and 8 kHz.	
		Steeply sloping: >30 dB HL difference between the above frequencies.	
	Flat	<15 dB HL difference between the mean of 0.25 and 1 kHz, the mean of 1 and 2 kHz, and the mean of 4 and 8 kHz.	
Frequency ranges	Low frequencies	≤0.5 kHz	
	Mid frequencies	>0.5 kHz and ≤2 kHz	
	High frequencies	>2 kHz and ≤8 kHz	
	Extended high frequencies	>8 kHz	
Symmetry of HI	Bilateral	<10 dB HL difference between the ears in at least two frequencies. Average over 0.5, 1 and 2 kHz of both ears >20 dB.	
	Unilateral	>10 dB HL difference between the ears in at least two frequencies. <20 dB HL average over 0.5, 1 and 2 kHz of the better ear.	
	Asymmetrical	> 10 dB HL difference between the ears in at least two frequencies. >20 dB HL average over 0.5, 1 and 2 kHz of the better ear.	
Age of onset	Prelingual	HI present before speech develops.	
	Postlingual	HI after the development of normal speech.	

Table 1. Characteristics for an adequate clinical description of hearing impairment.[8] ABG: air-bone gap; BC: bone-conduction; HI: hearing impairment; HL: hearing level.

Acquired hearing impairment

Approximately 25% of childhood hearing impairment is caused by environmental factors and commonly results from prenatal infections (TORCH; **t**oxoplasmosis, **o**ther infections (e.g. syphilis), **r**ubella, **c**ytomegalic virus and **h**erpes) or postnatal infections, particularly bacterial meningitis. Congenital cytomegalovirus infection is the most common cause of congenital acquired hearing impairment in developed countries.[4, 11] In the Netherlands, approximately 1000 children per year are born with congenital cytomegalovirus infection. Of these children approximately 180 children (0.1% of all newborns) will exhibit hearing impairment. In one of five children with bilateral profound hearing impairment, the cause of hearing impairment can be attributed to congenital cytomegalovirus infection.[12]

Acquired hearing impairment in adults is often multifactorial caused by both genetic and environmental factors (e.g. infections, acoustic or cerebral trauma, ototoxic drugs). Noise exposure is the most common environmental factor to attribute to acquired hearing impairment in adults. An individual's susceptibility to hearing impairment most likely reflect the environmental-genetic interaction.[4]

Prelingual and postlingual hearing impairment

Hearing impairment may begin before the development of speech (prelingual) or thereafter (postlingual). Prelingual hearing impairment is most frequently present at birth (congenital), but may start in early infancy before the acquisition of language. Often, prelingual hearing impairment is severe and stable. More than 50% of prelingual hearing impairment is genetic, most often autosomal recessive and nonsyndromic. The inheritance of prelingual nonsyndromic hearing impairment is estimated to be autosomal recessive in 75-85% of the cases, autosomal dominant in 20-25% of the cases and X-linked in 1-1.5% of the cases. (Figure 1) Mitochondrial inheritance is less than 1%.[6]

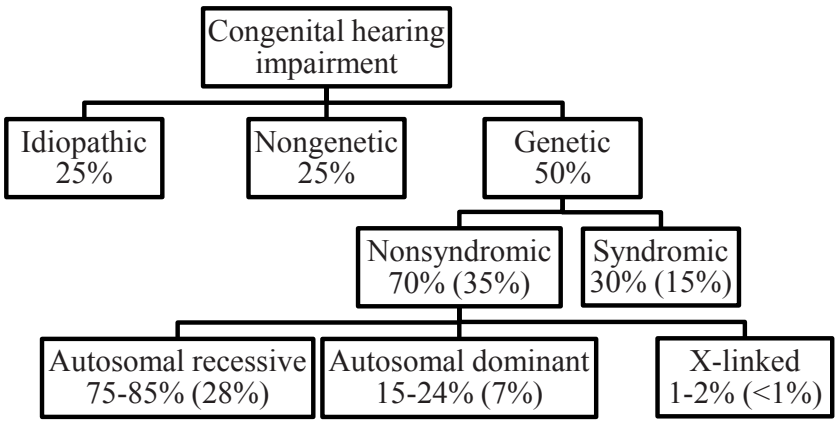


Figure 1. Causes of prelingual hearing impairment ≥ 40 dB in children. The percentages between parenthesis are the percentages in relation with congenital hearing impairment in general.

Postlingual hearing impairment is much more frequent than prelingual hearing impairment. Although postlingual hearing impairment has mostly a multifactorial inheritance, monogenetic forms exists with mainly autosomal dominant inheritance.[4]

Knowledge of the cause of hearing impairment can provide information on prognosis. Also, genetic counseling and risk assessment are dependent on the accurate determination of the specific genetic diagnosis. Furthermore, early identification of hearing impairment and timely intervention are essential for optimal cognitive development in children with prelingual hearing impairment. All children with a risk for hereditary hearing impairment should receive screening audiometry. Therefore, correct diagnosing of the specific cause of hearing impairment is essential.

Monogenic hearing impairment

Syndromic hearing impairment

Syndromic hearing impairment is characterized by hearing impairment in combination with other abnormalities. Important syndromic features are branchial cleft pits, cysts or fistulae, preauricular pits, telecanthus, heterochromia iridis, white forelock, pigmentary anomalies, high myopia, pigmentary retinopathy, goiter and

craniofacial anomalies. Most cases of monogenetic hearing impairment are nonsyndromic. Nevertheless, over 400 genetic syndromes with hearing impairment have been described. Syndromic hearing impairment accounts for up to 30% of prelingual hearing impairment.[6] However, the relative contribution of syndromic hearing impairment to all forms of hearing impairment is much smaller because of the impact of postlingual hearing impairment. Waardenburg syndrome is the most common type of autosomal dominant syndromic hearing impairment, followed by branchio-otorenal syndrome (BOR) and Stickler syndrome. Usher syndrome is the most common type of autosomal recessive syndromic hearing impairment, followed by Pendred syndrome, and Jervell and Lange-Nielsen syndrome. Alport syndrome and Mohr-Tranebjaerg syndrome demonstrate an X-linked pattern of inheritance. Mitochondrial mutations can also cause syndromic hearing impairment.[4]

Non-syndromic hearing impairment

More than 70% of prelingual hereditary hearing impairment is nonsyndromic.[13] The different loci for nonsyndromic hearing impairment are designated DFN (DeaFNess) and named after the mode of inheritance: DFNA (autosomal dominant), DFNB (autosomal recessive), DFNX (X-linked) and DFNY (Y-linked). Additionally, two modifier loci (DFNM) and one locus for auditory neuropathy (AUNA) have been documented. Otosclerosis loci are designated OTSC. The number following the designations reflects the order of mapping.[7]

The clinical manifestations and molecular genetics of autosomal dominant, autosomal recessive and sex-linked nonsyndromic hearing impairment are shown in table 2, table 3 and table 4, respectively.[7] Not all the causative genes of these loci are identified. Different mutations in the same gene can cause hearing impairment with distinctive modes of inheritance, for example DFNB1 and DFNA3 are caused by mutations in *GJB2* and *GJB6*, and DFNB21 and DFNA8/12 by mutations in *TECTA*. Furthermore, nonsyndromic and syndromic hearing impairment can be caused by different mutations in the same gene, for example DFNB18 and Usher syndrome 1C may be caused by mutations in *USH1C* gene, DFNB12 and Usher syndrome 1D by mutations in *CDH23* gene, DFNB4 and Pendred syndrome by mutations in *SLC26A4* gene, and DFNA6/14/38 and Wolfram syndrome by mutations in *WFS1* gene. Moreover, mutations in *MYO7A* can cause DFNB2, DFNA11 and Usher syndrome 1B.[4]

Autosomal dominant nonsyndromic hearing impairment				
Locus (OMIM number)	*Gene (OMIM number)*	*Gene expression in the cochlea*	*Age of onset*	*Audioprofile*
DFNA1 (124900)	*DIAPH1 (602121)*	Outer hair cells	Postlingual 1st decade	Moderate low frequency HI, progressive to profound flat HI
DFNA2 (600101) (612644)	*KCNQ4 (603537)*	Inner and outer hair cells, spiral ganglion	Postlingual 1st and 2nd decade	Mild-moderate high frequency HI, progressive to severe downsloping HI
	GJB3 (603324)	Spiral ligament, auditory nerve	Postlingual 4th decade	Mild-moderate high frequency HI, progressive, milder in females
DFNA3 (601544) (612643)	*GJB2 (121011)*	Supporting cells, spiral ligament	Pre- or postlingual childhood	Mild-moderate high frequency HI, progressive to moderate-severe downsloping HI
	GJB6 (604418)	Supporting cells, spiral ligament	Prelingual	Mild high frequency HI, progressive to profound downsloping HI
DFNA4 (600652)	*MYH14 (608568)*	Inner and outer hair cells, supporting cells, spiral ligament, stria vascularis, Reissner's membrane	Postlingual 1st and 2nd decade	Moderate flat to downsloping HI, progressive to severe-profound downsloping HI
	CEACAM16	-	Postlingual	Moderate progressive HI
DFNA5 (600994)	*DFNA5 (608798)*	Stria vascularis	Postlingual 1st decade	Mild high frequency HI, progressive to severe-profound downsloping HI
DFNA6/14/38 (600965)	*WFS1*[*1] *(606201)*	Inner and outer hair cells, supporting cells, spiral ligament, spiral ganglion, Reissner's membrane	Postlingual 1st and 2nd decade	Moderate low frequency HI, progressive to moderate-severe flat and sloping HI

DFNA7 (601412)	-	-	Postlingual 1st decade	Moderate high frequency HI, progressive to severe downsloping HI
DFNA8/12 (601543)	*TECTA (602574)*	Tectorial membrane	Pre- of postlingual 1st and 2nd decade	Mild mid frequency HI, progressive to moderate downsloping HI or stable HI
DFNA9 (601369)	*COCH (603196)*	Spiral ligament, basilar membrane	Postlingual 2nd decade	Mild high frequency HI, progressive to downsloping profound HI
DFNA10 (601316)	*EYA4 (603550)*	-	Postlingual 3rd and 4th decade	Mild flat to mid frequency HI, progressive to severe downsloping HI
DFNA11 (601317)	*MYO7A*[*2] (276903)*	Inner and outer hair cells	Postlingual 1st and 2nd decade	Mild flat or gently sloping HI, progressive to moderate-severe downsloping HI
DFNA13 (601868)	*COL11A2*[*3] (120290)*	Tectorial membrane	Postlingual 2nd to 4th decade	Mild mid frequency HI, progressive to moderate downsloping HI
DFNA15 (602459)	*POU4F3 (602460)*	Inner and outer hair cells	Postlingual 2nd to 3rd decade	Mild high frequency HI, progressive to moderate-severe downsloping HI
DFNA16 (603964)	-	-	Postlingual 1st decade	Fluctuating HI, moderate high frequency HI, progressive to severe-profound downsloping HI, relapsing sudden deafness with response to steroid therapy
DFNA17 (603622)	*MYH9*[*4] (160775)*	Inner and outer hair cells, spiral ligament, spiral ganglion	Postlingual 1st decade	Mild high frequency HI, progressive to moderate-profound downsloping HI
DFNA18 (606012)	-	-	Postlingual 1st decade	Moderate high frequency or flat to downsloping HI, progressive to severe-profound downsloping HI
DFNA19[24]	-	-	Prelingual	Mid frequency HI, progressive
DFNA20/26 (604717)	*ACTG1 (102560)*	Inner and outer hair cells	Postlingual 1st and 2nd decade	Mild-moderate high frequency HI, progressive to profound downsloping HI

DFNA21 (607017)	-	-	Postlingual 1st and 2nd decade	Mild mid frequency HI, progressive to moderate-severe downsloping HI
DFNA22 (606346)	*MYO6 (600970)*	Inner and outer hair cells	Postlingual 1st to 3rd decade	Mild flat, mid or high frequency HI, progressive to moderate-profound downsloping HI
DFNA23 (605192)	*SIX1*[5] *(601205)*	-	Prelingual	Brachiootic syndrome 3, moderate-profound high frequency or downsloping HI, stable
DFNA24 (606282)	-	-	Prelingual	Moderate-profound high frequency or downsloping HI, stable
DFNA25 (605583)	*SLC17A8 (607557)*	Inner hair cells	Postlingual 2nd to 6th decade	Mild high frequency, progressive to moderate downsloping HI
DFNA27 (612431)	-	-	Postlingual 1st and 2nd decade	Mild-moderate high frequency HI, progressive to severe-profound downsloping of flat HI
DFNA28 (608641)	*GRHL2/TFCP2L3 (608576)*	Supporting cells, stria vascularis, Reissner's membrane	Postlingual 1st to 3rd decade	Mild flat HI, progressive to moderate downsloping HI
DFNA30 (606451)	-	-	Postlingual 1st to 4th decade	High frequency HI, progressive to downsloping HI
DFNA31 (608645)	-	-	Postlingual 1st to 3rd decade	Mild mid or high frequency HI, progressive to moderate flat or downsloping HI
DFNA33 (614211)	-	-	Postlingual 2nd and 3rd decade	Mild high frequency HI, progressive to severe-profound downsloping HI
DFNA36 (606705)	*TMC1 (606706)*	Inner and outer hair cells	Postlingual 1st and 2nd decade	Mild-moderate high frequency HI, progressive to profound downsloping HI

DFNA39 (605594)	*DSPP*[*6] *(125485)*	-	Postlingual 2nd and 3rd decade	Dentinogenesis imperfecta, mild high frequency HI, progressive to moderate-severe downsloping HI
DFNA41 (608224)	-	-	Postlingual 2nd decade	Moderate flat-sloping HI, progressive to severe-profound sloping HI
DFNA43 (608394)	-	-	Postlingual 2nd and 3rd decade	Mild-moderate high frequency HI, progressive to severe-profound downsloping HI
DFNA44 (607453)	*CCDC50 (611051)*	Outer hair cells, stria vascularis, auditory nerve	Postlingual 1st decade	Mild-moderate low to mid frequency HI, progressive to profound flat or downsloping HI
DFNA47 (608652)	-	-	Postlingual 2nd and 3rd decade	Mild high frequency HI, progressive to moderate-severe downsloping HI
DFNA48 (607841)	*MYO1A (601478)*	Inner hair cells	Postlingual 1st and 2nd decade	Mild high frequency HI, progressive to moderate-severe downsloping HI
DFNA49 (608372)	-	-	Postlingual 1st decade	Moderate low or mid frequency HI, progressive to severe mid frequency HI
DFNA50 (613074)	*MIR96 (611606)*	Inner and outer hair cells	Postlingual 1st decade	Mild-moderate flat or downsloping HI, progressive to profound downsloping HI
DFNA51 (613558)	*TJP2 (607709)*	Inner and outer hair cells, supporting cells	Postlingual 3rd and 4th decade	Moderate high frequency HI (ski-slope/S-slope), progressive to profound downsloping HI
DFNA52 (607683)	-	-	Postlingual 2nd and 3rd decade	Mild high frequency HI, progressive to profound downsloping HI
DFNA53 (609965)	-	-	Postlingual 2nd decade	Mild high frequency HI, progressive to profound flat or downsloping HI

DFNA54[42]	-	-	Postlingual 1st and 2nd decade	Mild-moderate low frequency HI, progressive to moderate-severe sloping or flat HI
DFNA57[43]	-	-	Postlingual 1st decade	Mild low frequency HI, progressive to moderate flat HI
DFNA58[44]	-	-	Postlingual 2nd and 3th decade	Mild high frequency HI, progressive to moderate-severe flat or downsloping HI
DFNA59 (612642)	-	-	Prelingual congenital	Severe-profound downsloping HI, stable
DFNA60[45]	-	-	Postlingual 1st and 2nd decade	-
DFNA64 (614152)	*DIABLO/SMAC (605219)*	Inner hair cells	Postlingual 1st and 2nd decade	Mild-moderate flat HI, progressive to severe-profound flat or downsloping HI

Autosomal recessive nonsyndromic hearing impairment				
Locus (OMIM number)	*Gene (OMIM number)*	*Gene expression in the cochlea*	*Age of onset*	*Audioprofile*
DFNB1 (220290) (612645)	*GJB2 (121001)*	Supporting cells, spiral ligament	Prelingual congenital	Mild-profound downsloping or flat HI, usually non-progressive
	GJB6 (604418)	Supporting cells, spiral ligament	Prelingual congenital	Profound HI, usually non-progressive
DFNB2 (600060)	*MYO7A*[*1] *(276903)*	Inner and outer hair cells	Prelingual congenital	Profound HI
DFNB3 (600316)	*MYO15A (602666)*	Inner and outer hair cells	Prelingual congenital	Severe to profound HI, non-progressive
DFNB4 (600791)	*SLC26A4*[*2] *(605646)*	Supporting cells, spiral ligament, spiral ganglion	Pre- or postlingual childhood	Severe-profound HI, often progressive but could be non-progressive, Enlarged Vestibular Aqueduct (EVA)
DFNB5 (600792)	-	-	Prelingual congenital	Severe HI
DFNB6 (600971)	*TMIE (607237)*	-	Prelingual congenital	Severe to profound HI, non-progressive
DFNB7/11 (600974)	*TMC1 (606706)*	Inner and outer hair cells	Pre- or postlingual 1st decade	Severe to profound downsloping HI, progressive high frequency HI or non-progressive
DFNB8/10 (601072)	*TMPRSS3 (605511)*	Supporting cells, stria vascularis, spiral ganglion	Pre- or postlingual 1st and 2nd decade	Severe-profound downsloping HI, progressive high frequency HI (ski-slope) and non-progressive

DFNB9 (601071)	*OTOF (603681)*	Inner and outer hair cells	Prelingual congenital	Auditory neuropathy, usually severe-profound U-shaped HI, non-progressive, sometime temperature sensitive, relatively poor speech recognition
DFNB12 (601386)	*CDH23*[*1] *(605516)*	Inner and outer hair cells, Reissner's membrane	Prelingual congenital	Moderate-profound high frequency or downsloping HI, progressive to severe-profound downsloping HI or non-progressive
DFNB13 (603098)	-	-	Prelingual	Moderate-severe downsloping HI, progressive to severe-profound downsloping HI
DFNB14 (603678)	-	-	Prelingual	Profound HI
DFNB15/72/95 (601869)	*GIPC3 (608792)*	Inner and outer hair cells, spiral ganglion	Prelingual	Moderate-profound HI, non-progressive
DFNB16 (603720)	*STRC (606440)*	Inner and outer hair cells	Prelingual	Moderate-profound downsloping HI, non-progressive
DFNB17 (603010)	-	-	Prelingual	Profound HI
DFNB18 (602092)	*USH1C*[*1] *(605242)*	Inner and outer hair cells	Prelingual congenital	Severe to profound HI, non-progressive
DFNB20 (604060)	-	-	Prelingual congenital	Moderate-profound HI
DFNB21 (603629)	*TECTA (602574)*	Tectorial membrane	Prelingual	Moderate-profound flat or U-shaped HI, non-progressive
DFNB22 (607039)	*OTOA (607038)*	Between interdental cells and tectorial membrane	Prelingual	Moderate-profound HI, non-progressive

DFNB23 (609533)	*PCDH15*[*1] *(605514)*	Inner and outer hair cells, supporting cells, spiral ganglion	Prelingual	Severe-profound HI, non-progressive
DFNB24 (611022)	*RDX (179410)*	Inner and outer hair cells	Prelingual	Severe-profound HI, non-progressive
DFNB25 (613285)	*GRXCR1 (613283)*	Inner and outer hair cells, supporting epithelial cells	Pre- or postlingual 1st decade	Moderate-profound flat, U-shaped or downsloping HI, progressive or non-progressive
DFNB26 (605428)	-	-	Prelingual congenital	Severe-profound downsloping HI. HI is suppressed by dominant modifier DFNM1 in nonpenetrance patients.
DFNB27 (605818)	-	-	Prelingual	-
DFNB28 (609823)	*TRIOBP (609761)*	Inner and outer hair cells	Prelingual	Severe-profound HI, non-progressive
DFNB29 (614035)	*CLDN14 (605608)*	Inner and outer hair cells, supporting cells	Prelingual congenital	Severe-profound HI, non-progressive
DFNB30 (607101)	*MYO3A (606808)*	Inner and outer hair cells	Pre- or postlingual 2nd decade	Moderate-severe high frequency HI, progressive to severe-profound downsloping HI or non-progressive
DFNB31 (607084)	*WHRN*[*1] *(607928)*	Inner and outer hair cells	Prelingual	Profound HI
DFNB32/82 (608653) (613557)	*GPSM2 (609245)*	-	Prelingual congenital	Severe-profound flat or downsloping HI, non-progressive
DFNB33 (607239)	-	-	Prelingual early childhood	Severe HI

DFNB35 (608565)	*ESRRB (602167)*	Supporting cells, spiral ligament, stria vascularis, spiral ganglion, auditory nerve, Reissner's membrane	Prelingual	Severe-profound downsloping HI, non-progressive
DFNB36 (609006)	*ESPN (606351)*	Inner and outer hair cells	Prelingual	Profound HI
DFNB37 (607821)	*MYO6 (600970)*	Inner and outer hair cells	Prelingual congenital	Profound HI
DFNB38 (608219)	-	-	Prelingual	Profound flat HI
DFNB39 (608265)	*HGF (142409)*	-	Prelingual	Severe-profound downsloping HI
DFNB40 (608264)	-	-	Prelingual	Profound flat or downsloping HI
DFNB42 (609646)	*ILDR1 (609739)*	Inner and outer hair cells, supporting cells	Prelingual congenital	Severe-profound flat-downsloping HI, non-progressive
DFNB44 (610154)	-	-	Prelingual	Profound HI
DFNB45 (612433)	-	-	Prelingual	Profound HI
DFNB46 (609647)	-	-	Prelingual	Profound HI
DFNB47 (609946)	-	-	Prelingual	Profound downsloping HI
DFNB48 (609439)	-	-	Prelingual	Profound flat or downsloping HI

DFNB49 (610153)	*MARVELD2/TRIC (610572)*	Reticular lamina	Prelingual congenital	Moderate-profound U-shaped or downsloping HI, non-progressive
DFNB51 (609941)	-	-	Prelingual	Profound HI
DFNB53 (609706)	*COL11A2*[*3] *(120290)*	Tectorial membrane	Prelingual	Severe-profound flat or downsloping HI, non-progressive
DFNB55 (609952)	-	-	Prelingual	Profound flat HI
DFNB59 (610220)	*PJVK (610219)*	Spiral ganglion	Prelingual early childhood	Auditory neuropathy, severe-profound flat or downsloping HI, non-progressive, relative poor speech recognition
DFNB61 (613865)	*SLC26A5 (604943)*	Outer hair cells	Prelingual congenital	Severe-profound downsloping HI, non-progressive
DFNB62 (610143)	-	-	Prelingual	Profound flat or downsloping HI
DFNB63 (611451)	*LRTOMT/COMT2 (612414)*	Inner and outer hair cells	Prelingual congenital	Severe-profound downsloping HI, non-progressive
DFNB65 (610248)	-	-	Prelingual	Profound downsloping HI
DFNB66/67 (610212) (610265)	*LHFPL5 (609427)*	Inner and outer hair cells	Prelingual congenital	Severe-profound downsloping HI, non-progressive
DFNB68 (610419)	-	-	Prelingual	Profound HI
DFNB71 (612789)	-	-	Prelingual	Severe-profound flat or downsloping HI

DFNB73 (602522)	*BSND (606412)*	Stria vascularis	Prelingual congenital	Severe-profound flat HI, non-progressive, mild renal dysfunction
DFNB74 (613718)	*MSRB3 (613719)*	Inner and outer hair cells, spiral ganglion	Prelingual	Profound flat or downsloping HI
DFNB77 (613079)	*LOXHD1 (613072)*	Inner and outer hair cells	Postlingual 1st decade	Mild-moderate high frequency HI, progressive to severe-profound downsloping HI
DFNB79 (613307)	*TPRN (613354)*	-	Prelingual	Severe-profound flat or downsloping HI, usually non-progressive
DFNB81 (614129)	-	-	Prelingual	Severe-profound downsloping HI
DFNB83 (613685)	-	-	Prelingual	Profound HI
DFNB84 (613391)	*PTPRQ (603317)*	Inner and outer hair cells	Prelingual congenital	Moderate flat or downsloping HI, progressive to severe-profound flat or downsloping HI or non-progressive
DFNB85 (613392)	-	-	Prelingual	Profound HI
DFNB89 (613916)	-	-	Prelingual	Moderate-severe HI, non-progressive
DFNB91 (613453)	*SERPINB6 (173321)*	Inner hair cells	Postlingual 2nd decade	Moderate-severe downsloping HI, progressive
DFNB93[47]	-	-	Prelingual	Moderate-severe flat or U-shaped HI, non-progressive

Sex-linked nonsyndromic hearing impairment				
Locus (OMIM number)	*Gene (OMIM number)*	*Gene expression in the cochlea*	*Age of onset*	*Audioprofile*
DFNX1 (304500)	*PRPS1*[*1] (311850)*	Inner and outer hair cells, supporting cells, spiral ganglion	♂ pre- or postlingual – 1st decade ♀ postlingual – 5th decade	♂ Severe-profound flat HI, progressive ♀ Mild-moderate down- or sloping HI, could be asymmetrical or unilateral
DFNX2 (304400)	*POU3F4 (300039)*	Spiral ligament, Reissner's membrane	♂♀ Postlingual	♂♀ Stapes fixation, variable mixed or conductive HI, progressive to profound
DFNX3 (300030)	-	-	♂ prelingual - congenital ♀ postlingual – 3rd decade	♂ Profound downsloping HI ♀ Mild-moderate high frequency HI, non-progressive
DFNX4 (300066)	*SMPX (300226)*	Inner and outer hair cells, supporting cells	♂ postlingual – 1st decade ♀ postlingual – 2nd to 4th decade	♂ Moderate downsloping HI, progressive to profound downsloping HI ♀ Variable mild high frequency HI, progressive to moderate downsloping HI
DFNX5 (300614)	-	-	♂ Postlingual – 1st decade ♀ Postlingual – 2nd decade	♂♀ Auditory neuropathy, variable mild-profound flat or sloping HI, progressive, relatively poor speech recognition. Most women had normal hearing.
DFNY1 (400043)	-	-	♂ Postlingual – 1st decade	♂ Moderate-severe flat, U-shaped or downsloping HI, progressive

Table 2. Clinical manifestations and molecular genetics of autosomal dominant nonsyndromic hearing impairment. No clinical data are available for DFNA29, DFNA32, DFNA34, DFNA35, DFNA37, DFNA40, DFNA42, DFNA45, DFNA46, DFNA55, DFNA56, DFNA61, DFNA62 and DFNA63. The data are derived from OMIM[46] (see OMIM numbers in table). The genes marked with * are also involved in syndromic hearing impairment: 1. Wolfram syndrome, 2. Usher syndrome, 3. Stickler syndrome, 4. Epstein, Fletcher and Sebastian syndrome, 5. Branchio-oto-renal syndrome, 6. Dentinogenesis imperfecta. HI: hearing impairment.

Table 3. Clinical manifestations and molecular genetics of autosomal recessive nonsyndromic hearing impairment. No clinical data available for DFN19, DFNB34, DFNB41, DFNB43, DFNB50, DFNB52, DFNB54, DFNB56, DFNB57, DFNB58, DFNB60, DFNB64, DFNB69, DFNB70, DFNB75, DFNB76, DFNB78, DFNB80, DFNB86, DFNB87, DFNB88, DFNB90, DFNB92 and DFNB94. The data are derived from OMIM[46] (see OMIM numbers in table). The genes marked with * are also involved in syndromic hearing impairment: 1. Usher syndrome, 2. Pendred syndrome, 3. Stickler syndrome. HI: hearing impairment.

Table 4. Clinical manifestations and molecular genetics of sex-linked nonsyndromic hearing impairment. The data are derived from OMIM[46] (see OMIM numbers in table). The gene marked with * is also involved in syndromic hearing impairment: 1. Charcot-Marie-Tooth disease. HI: hearing impairment.

Autosomal dominant nonsyndromic hearing impairment

Autosomal dominant hearing impairment is frequently postlingual. The characteristic phenotype of a person with autosomal dominant nonsyndromic hearing impairment is progressive postlingual hearing impairment that begins in the second or third decade of life. However, stable congenital hearing impairment or an onset in the fourth decade of life or later can also be seen. (Table 2) The heterogeneity in autosomal dominant nonsyndromic hearing impairment is high with multiple genes implicated in the pathogenesis. However, the audioprofile can be distinctive and therefore useful in genetic testing of specific candidate genes. Furthermore, audioprofiling can be helpful in predicting the progression of hearing impairment in an individual with autosomal dominant nonsyndromic hearing impairment of known cause.[4, 14-19] An Age Related Typical Audiograms (ARTA) gives a comprehensive phenotype presentation and is therefore extremely useful in characterization of progressive DFNA types. Huygen et al. described the

construction of an ARTA from regression analysis (threshold on age) of age-related threshold data.[20] Unfortunately, mutations in different genes can lead to very similar phenotypes and therefore a similar ARTA. Additional distinguishing phenotypic features are then very important, for example speech recognition and vestibular function. Nevertheless, an ARTA can be used to compare the type of hearing impairment, the age of onset and the progression of hearing impairment in relation to the genotypes. An ARTA does not only help in selecting potentially interesting loci for linkage analysis or genes for mutation analysis, but it is also valuable for genetic and individual counseling.[20, 21] In addition, the program AudioGene[22] can perform automatic audioprofile analysis of the audiometrical data of an individual or a family.

Mutations in the same gene can cause very distinct phenotypes. One of these genes is *TECTA*. The encoded protein, α-tectorin, is one of the main noncollagenous proteins of the tectorial membrane, a ribbon-like strip of extracellular matrix that lies over the stereocilia of the hair cells and is critical for the mechanical transmission and amplification of sound. Missense mutations of *TECTA* cause autosomal dominant non-syndromic hearing impairment (DFNA8/12), whereas nonsense mutations cause autosomal recessive non-syndromic hearing impairment (DFNB21). (Table 2) The phenotype of DFNA8/12 depends on the domain and residue affected. The established genotype–phenotype correlations indicate that missense mutations in the zona pellucida domain and in the N-terminal region lead to mid-frequency sensorineural hearing impairment, whereas missense mutations in the zonadhesin region cause high-frequency sensorineural hearing impairment. If cysteine residues are affected hearing impairment is progressive; if other residues are affected hearing impairment is stable. The accurate genotype-phenotype correlations will lead to better diagnostic and prognostic information for patients with hereditary hearing impairment.[23-25]

Autosomal recessive nonsyndromic hearing impairment

Most cases of autosomal recessive inherited hearing impairment show prelingual severe to profound hearing impairment. (Table 3) Approximately 50% of autosomal recessive nonsyndromic hearing impairment in Mediterranean populations can be attributed to DFNB1, caused by mutations in *GJB2* (encoding connexin 26) and/or

GJB6 (encoding connexin 30). The remaining 50% of cases are attributed to mutations in numerous other genes, many of which have been found to cause hearing impairment in only one or two families.[4, 26-29] The prevalence of *GJB2* and *GJB6* mutations in the Netherlands is lower. The carrier frequency rate for *GJB2* mutations in the general United States population of northern European descent is approximately 1 in 33.[26] From the Dutch patients with recessive hearing impairment reported by Kemperman et al. 15,8% of the cases had mutations in the *GJB2* or *GJB6* gene.[30]

Sex-linked nonsyndromic hearing impairment

Sex-linked nonsyndromic hearing impairment is not very common, but can exhibit a wide range of clinical manifestations.[4] The clinical manifestations and molecular genetics of sex-linked nonsyndromic hearing impairment are shown in table 4.[7]

Mitochondrial nonsyndromic hearing impairment

The majority of mutations in mitochondrial genes cause a broad spectrum of maternally inherited multisystem disorders. However, specific mutations in *MT-RNR1* and *MT-TS1* can cause nonsyndromic hearing impairment by currently unknown mechanisms.[31] The phenotypic variation of these mutations is great with a highly variable penetrance of hearing impairment. Heteroplasmy is the main source of variation in severity of the hearing impairment. Unidentified genetic or environmental factors play also a role in the progression of the hearing impairment.[31] Furthermore, hearing impairment can be induced by administration of aminoglycosides in some individuals with specific mutations in *MT-RNR1*.[32]

Auditory neuropathy

Auditory neuropathy is a disorder in which the transmission of the auditory signals from the inner ear to the auditory nerve and auditory brainstem is distorted. Auditory neuropathy is characterized by normal outer hair cell function and disrupted inner hair cell function and/or auditory nerve function. The pure-tone levels of patients with auditory neuropathy can vary from normal to severely impaired. These patients also experience great difficulty in understanding speech, particularly in the presence of background noise. Auditory neuropathy can be congenital or acquired. Congenital auditory neuropathy is mostly genetic and may

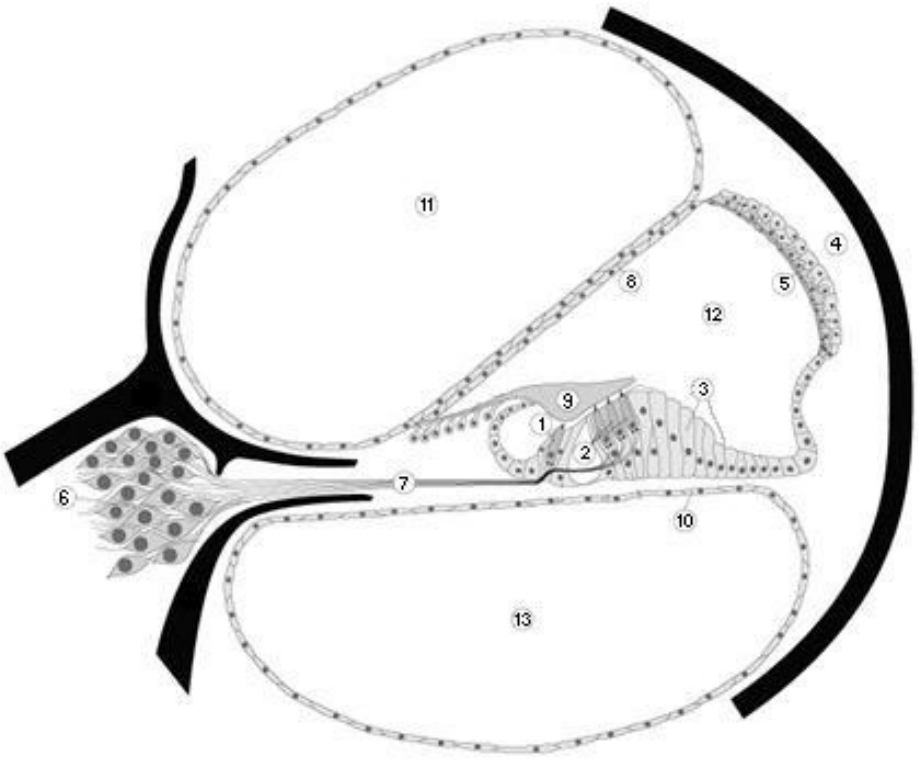
occur either isolated or in association with a syndrome. Approximately 40% of auditory neuropathy cases may have a genetic cause.[33] Autosomal dominant nonsyndromic auditory neuropathy can be caused by mutations in *DIAPH3*. The phenotype is variable with most frequently postlingual (1st decade) progressive profound hearing impairment.[34] Mutations in *OTOF*[35-37] and *PJVK*[38] can cause recessive nonsyndromic auditory neuropathy with prelingual onset and usually severe to profound hearing impairment. DFNX5 causes also auditory neuropathy with variable hearing impairment.[39]

Modifier genes

Modifier genes can act either as an enhancer or as a suppressor of hearing impairment. A dominant deafness modifier, designated DFNM1, has been demonstrated to cause nonpenetrance in family members who were homozygous for the DFNB26 haplotype. The location of DFNM1 is within the DFNA7 interval, suggesting that the DFNM1 suppressor phenotype and DFNA7 hearing impairment may be phenotypic variants of the same gene.[40]

Gene expression in the cochlea and gene function in the inner ear

Figure 2 shows the important structures of the cochlea.[48] The pathogenic mechanism of hearing impairment dependents on the involved gene. For example, DFNA8/12 (*TECTA* gene) and DFNA13 (*COL11A2* gene) related hearing impairment, originating from tectorial membrane abnormalities, exhibit intra-cochlear 'conductive' hearing impairment. Defects in the tectorial membrane result primarily in an attenuation of sound, whereas suprathreshold measures, such as otoacoustic emissions and speech perception in noise, are preserved rather well. The results of additional audiologic testing in DFNA8/12 and DFNA13 patients resembled the results found in patients with middle-ear conductive hearing impairment.[49, 50]



1. Inner hair cells
2. Outer hair cells
3. Supporting cells
4. Spiral ligament
5. Stria vascularis
6. Spiral ganglion
7. Auditory nerve
8. Reissner's membrane
9. Tectorial membrane
10. Basilar membrane
11. Scale vestibuli
12. Scale media
13. Scale tympani

Figure 2. Transverse section of the cochlea (available at hereditary hearing loss homepage; http://hereditaryhearingloss.org/main.aspx?c=.HHH&n=87131)

Most of the genes involved in nonsyndromic hereditary hearing impairment can be divided in different categories based on their function in the inner ear. One category of genes is involved in maintaining ion homeostasis in the inner ear. These genes mainly encode channels or ion pumps (e.g. *GJB2, GJB3, GJB6, GJA1, KCNQ4, SLC26A4, SLC26A5* and *WFS1*). Another category of genes encodes cytoskeletal components (e.g. *ACTG1, CCDC50, DIAPH1, DSPP, ESPN, RDX* and *TRIOBP*), adhesion proteins (e.g. *CHD23, PCDH15* and *TMHS*), motor proteins (e.g. *MYH9, MYH14, MYO1A, MYO3A, MYO6, MYO7A* and *MYO15A*) and scaffolding proteins (e.g. *WHRN* and *USH1C*). A

further group of genes encodes protein components of the extracellular matrix and the tectorial membrane (e.g. *COCH, COL11A2, OTOA, STRC* and *TECTA*). An additional category contains transcription factors involved in cochlear development (e.g. *ESRRB, EYA4, GRHL2, POU3F4* and *POU4F3*). MicroRNAs, such as *MIR96*, are noncoding regulatory RNAs that influence translation and stability of target mRNAs and form a separate group. Nevertheless, there are still numerous genes with unknown function.[48]

Treatment

In many types of hearing impairment, including hereditary forms, apoptosis of sensory hair cells is involved. In humans there is still no prove that damaged hair cells can regenerate spontaneously. Therefore it is likely that damaged hair cells cause permanent hearing deficits.[51] Specific inhibitors of apoptosis can prevent hair cell degeneration and are targets for therapies to preserve hearing.[52]

Reactive oxygen species, or free radicals, are released by damaged hair cells after a traumatic event and can initiate apoptosis of hair cells. Free radical scavengers can bind these free radicals and prevent or inhibit hair cell apoptosis. Thus, future therapies for preventing hearing impairment may include systemic or localized application of free radical scavengers.[52]

Replacement of hair cells, which are damaged by sound or ototoxic drugs has been demonstrated in birds. Supporting cell proliferation or transdifferentiation can replace the lost hair cells. Furthermore, hair cells of rats can be replaced after injury during neonatal development if epidermal (EGF) and/or transforming growth factor alfa (TGF-α) supplements were administered during the recovery process.[52]

Kawamoto used gene therapy to generate new hair cells in guinea pigs.[53] Some non-sensory cells in the immature inner ear of mice can differentiate into hair cells when the *Math1* gene (*Atoh1* gene in human) was introduced into these cells.[52, 54] In addition to hair cell development, substantial improvement in hearing thresholds in deaf mice was also demonstrated.[55] Furthermore, the lack of *Math1* in a knockout mouse resulted in no hair cell development.[56] Atoh1 is a potent transcription factor that induces the non-sensory cochlear cells to develop into new hair cells. However, plasticity and repair of damage during development do not usually persist into adulthood. Nevertheless, in vivo data indicate that non-sensory cochlear cells

maintain their competence to become new hair cells in mature animals. The ability to generate hair cells in the mammalian organ of Corti may lead to treatments for sensorineural hearing impairment caused by apoptosis of sensory hair cells.[52]

Regeneration of hair cells does not address the often ongoing hair cell loss caused by a genetic mutation in hereditary hearing impairment. Thus, the optimal solution for hereditary hearing impairment may be defective gene replacement. However, gene replacement therapy poses far greater challengers than other forms of cochlear gene therapy. To date, restoring hearing in hereditary hearing impairment with gene replacement therapy is not possible yet.[54]

Complex hearing impairment

Age related hearing impairment

Age related hearing impairment (ARHI), or presbycusis, is a complex trait, caused by an interplay between genetics and environment. The variation in ARHI is large, but little is known about the factors influencing the severity of hearing impairment. Part of this variation can be explained by medical conditions and by a different exposure to environmental factors, for example occupational noise. The importance of other environmental risk factors is less clear and often controversial. Some environmental factors are well-documented and clearly have an influence on hearing thresholds, but it is unclear to what extent they influence hearing at a later age.[5] Smoking can cause a significant dose-dependent increase in high-frequency hearing impairment. High body mass index (BMI) was also correlated with hearing impairment. Moderate alcohol consumption has probably a protective effect on hearing. These results suggest that a healthy lifestyle can protect against age-related hearing impairment.[57] Approximately half of the variance in ARHI is probably due to genetic factors, however, little is known about the precise genetic determinants.[5] Several genome-wide linkage studies and association studies on candidate genes for ARHI were performed to identify some of the genetic factors involved in ARHI.[10] The first locus on chromosome 8q24.13-q24.22 for ARHI was reported by Huyghe et al.[58] Two other studies identified the first susceptibility genes, *NAT2* and *KCNQ4*, for ARHI.[59, 60] Moreover, Van Eyken et al.[10] found an association between ARHI and *GSTT1* and *GSTM1* in the Finnish population and confirmed the previously reported correlation

with *NAT2* in the general European population. Furthermore, a strong association between ARHI and *GRHL2* was demonstrated and replicated by Van Laer et al.[61] Further research will be necessary to identify the causative variant(s) in these candidate genes.

Otosclerosis

Otosclerosis is also a complex disease caused by an interaction between environmental and genetic factors. In the following paragraphs, a detailed description of otosclerosis is given.

Epidemiology

Otosclerosis is one of the most common causes of adult-onset hearing impairment in the Caucasian population with a prevalence of 0.3-0.4%. The prevalence in blacks, Asians and Native Americans is much lower. The difference in occurrence of otosclerosis between races might be a reflection of differences in both genetic and environmental factors.[9, 62-64]

The histological form of otosclerosis is defined as an asymptomatic disease that can be identified only by morphological examination. Histological otosclerosis is far more common than clinical otosclerosis and is found in 10% of the Caucasian population.[64-66]

Several studies reported a higher prevalence of clinical otosclerosis in women with a female to male ratio of approximately 2:1. Histological studies of the temporal bone do not demonstrate a difference in sex ratio for histological otosclerosis.[64, 67] Besides a sex difference in prevalence, there is also a sex difference in the severity of hearing impairment caused by otosclerosis. Females had worse bone-conduction thresholds and developed more frequently sensorineural hearing impairment than males.[63, 68]

It has been demonstrated that during periods of endocrine change (e.g. pregnancy and puberty) otosclerosis may be initiated or progress in women, particularly with subsequent pregnancies. Recently, however, authors found no adverse effect of having children on hearing in otosclerotic women, not even with increasing numbers of pregnancies. Neither did breastfeeding affect the degree of hearing impairment.[69] Furthermore, no adverse effect of oral contraception use on otosclerosis could be demonstrated.[70] Nevertheless, it is well established that

oestrogens are critical regulators of the skeleton. Oestrogens are stimulators of osteocytic activity and may contribute to the ossification of otosclerotic foci.[71] Furthermore, estrogen possibly has a protective effect on hearing.[72] Despite the established participation of estrogens in osteocytic and osteoblastic function, their role in the pathogenesis of otosclerosis remains unsettled.[64] Otosclerosis is more likely to occur during childbearing ages and pregnancy could just be an incidental event.

Pathogenesis

Otosclerosis is characterized by abnormal bone remodeling at specific sites of predilection confined to the endochondral layer of the otic capsule. The otic capsule is formed by three layers of bone, namely the endosteal layer next to the perilymphatic space, the intermediate endochondral layer with remnants of cartilage tissue, which are known as the 'globuli interossei', and the outer periosteal layer.[65, 67] The otic capsule is a unique structure and has almost completely absent growth and plasticity.[73]

Three types of otosclerotic lesions can be identified, namely cellular (spongiotic), fibrotic and sclerotic lesions. The early spongiotic phase may convert into the fibrotic phase and finally into the mature and inactive sclerotic phase.[74] The first histological sign of otosclerosis is resorption of bone around blood vessels by osteoclasts with secondary enlargement of perivascular spaces and intensive neovascularization.[67, 75] The hyperemic blood vessels of the adjacent promontory can be observed through the tympanic membrane as a red blush known as the 'Schwartze sign'.[64, 76] The Schwartze sign is closely associated with otosclerotic lesions extending to the promontory and is occasionally observed in cochlear otosclerosis. An objective increase in the blood flow to the promontory in patients demonstrating the Schwartze sign has been demonstrated using laser Doppler flowmetry.[77] As the otosclerotic focus expands, a central resorption space is formed containing a rich cellular content of monocytes, macrophages, multinucleated osteoclasts and osteoblasts (cellular spongiotic phase). Subsequently, new bone is formed characterized by dysplastic immature basophilic bone. In this fibrotic phase, osteoblastic activity leads to formation of new spongiotic trabecular bone,

distinctive from the surrounding normal lamellar bone. The extracellular matrix of the new spongiotic trabecular bone contains disoriented collagen fibrils and undergoes progressive fibrosis and calcification in the sclerotic phase. This reorganization of spongiotic trabecular bone leads to formation of relatively avascular and acellular dense sclerotic bone with a woven pattern.[67, 78] The different phases (otospongiosis and otosclerosis) can occur simultaneously and one does not necessarily precede the other.[76]

The activity of the otosclerotic foci can be classified from Grade I (most active) to Grade IV (inactive or healed) on the basis of cellularity, presence of osteoclasts and osteoblasts, degree of vascularization and the amount of extracellular collagen matrix.[65]

Etiology

Otosclerosis is a heterogeneous disease possibly with multiple etiologies. Several theories have been postulated, including collagen disorders[65, 79] (e.g. osteogenesis imperfecta[80, 81] and osteoporosis[82]), hormonal disorders (e.g. estrogen-induced hyperprolactinemia[83]), autoimmune diseases (e.g. antibodies to type II and IX collagen[79, 84], human leukocyte antigen (HLA) genotypes[65]), enzymatic disorders (e.g. increased diastrophic dysplasia sulfate transporter (DDST) activity[85]) and inflammatory disorders (e.g. persistent measles virus[86-88] and osteoprotegerin deficiency[89, 90]). Disturbances of various homeostatic functions have been associated with otosclerosis, for example prostaglandin overproduction, abnormal response to parathyroid hormone and therefore overproduction of alkaline phosphatase, and insufficient production of osteoprotegerin mediated through RANK and RANKL (receptor activator of nuclear factor κB and its ligament).[91, 92] Moreover, several genetic variations have been related to an increased risk for otosclerosis, for example certain bone morphogenetic proteins (BMPs) polymorphisms that result in an increased chondrogenesis.[67, 93, 94] Also, specific polymorphisms in angiotensin converting enzyme (ACE) and angiotensin (AGT) demonstrate an increased bone remodeling.[95-97] Transforming growth factor-β1 (TGF-β1) variants contribute to susceptibility to otosclerosis by modulating extracellular matrix production[67, 98, 99] and genetic variants of *COL1A1* cause disoriented collagen structures[99, 100]. However, despite the intensive research and the identification of a variety of factors involved

in the development of the disease, the etiology of otosclerosis is still not fully understood.[65, 97]

Otosclerosis is considered a heterogenetic disease. The heterogenetic heredity could explain the variable manifestations of the disease, that does not appear at the same age with the same progression and intensity in all patients. The role of heredity has originally been implied from demographic and epidemiologic observations and more recently from DNA analysis techniques. Epidemiological studies suggested an autosomal dominant inheritance with incomplete penetrance of approximately 40% and variable expression.[63, 64] Although a strong familial component exists, several studies have reported that sporadic otosclerosis represents 40-50% of all clinical cases.[63, 64, 76] Variable phenotypic expression within the same otosclerosis family and a high proportion of sporadic cases suggest the contribution of environmental factors in the etiology of otosclerosis.[64]

Genetic analysis

There are different methods to identify the genes involved in otosclerosis. In the past, otosclerosis genes were addressed by examining an association with a known gene complex or with a specific clinical factor, like eyes or hair color or blood group. All these studies failed to demonstrate evidence of linkage or cosegregation.

Candidate gene analysis is also one of the methods. These population-based studies investigate associations between genes and a disease, and require some understanding or hypothesis of the disease process to select possible candidate genes.[64, 67]

Linkage analysis focuses on large families segregating a disease to identify the causative gene. The location on a chromosome involved in the disease is identified by demonstrating a co-segregation between phenotype and genotype by analyzing several hundred to several thousand of genetic markers. The identification of the locus is the first step towards the identification of the gene itself. Large families with affected and unaffected individuals are needed for conclusive results. However, families large enough for genetic linkage study are rare, and in these families, factors like reduced penetrance and phenocopies complicate linkage analyse.[9, 63, 64, 67, 101]

Another method is a genome-wide association study. This approach does not depend on the selection of candidate genes that presume an understanding of

otosclerosis at the molecular level and does not need large families. It holds the promise of identifying genes and pathways that are causally related to otosclerosis but are not intuitively obvious at this time.[67, 101]

Otosclerosis loci and candidate genes

Genetic linkage studies have demonstrated the involvement of ten loci.[102-109] (Table 5) Details of *OTSC6* and *OTSC9* have not been published yet. Although these loci have been mapped, no causative genes have been identified. The identified loci include genes involved in collagen biosynthesis and metabolism, in the immune system, in cartilage and bone homeostasis, in growth suppression and in intercellular communication. To identify specific disease-causing genes, refinement of the candidate regions and mutation analysis of candidate genes is required. Identification and functional analysis of the causative genes and corresponding proteins may provide new insights into the molecular mechanisms of otosclerosis and may reveal targets for prevention and treatment of the disease.[64]

The *OTSC2* locus contains the T-cell receptor beta locus (*TRB* locus) and Schrauwen et al. implicated this as the causative gene in the *OTSC2* region. The human T-cell receptor (TCR) complex comprises integral membrane proteins with a fundamental role in the adaptive immune system. These proteins activate T cells in response to antigens presented by HLA molecules on antigen-presenting cells. A significant lower T-cell receptor-β (*TCR-β*) mRNA expression, a significant lower percentage of blood circulating *TCR-αβ*$^{+}$ T-cells and a significantly increased CD28^{null} population were detected in *OTSC2* patients compared with controls and patients with the complex form of the disease. These data suggest a disturbed T-cell development and ageing in *OTSC2* patients. Moreover, expanded populations of CD28^{null} T cells are related to autoimmune diseases. The cytotoxic capacities and the decreased susceptibility to immunoregulation of these T cells might facilitate or sustain chronic autoreactive immune responses. In otosclerosis, viral infections could activate the immune response. In conclusion, a genetic defect in the *TRB* locus causes disturbed T cell development and ageing, and potentially influences T cell reactivity toward unique structures within the otic capsule, leading to otosclerosis in *OTSC2* patients.[110]

Locus	*Location*	*Candidate genes and functions*
OTSC1 *102*	15q25-26	*Aggrecan;* non-collagenous component of extracellular matrix of cartilage.
OTSC2 *103, 111*	7q34-36	*TIF1a;* growth suppressor required for the growth-inhibitory activity of retinoic acid in bone remodeling. *PLOD3;* involved in collagen biosynthesis and metabolism by interfering with chondrocytic responses to TNFα-mediated stimuli. TRB locus; prominent role in immune system (recognition of antigens and subsequent activation of T cells).
OTSC3 *104, 112*	6q21.3-22.3	HLA locus; major histocompatibility complex (MHC) plays an important role in the immune system by presenting antigens to T cells. *COL1A1;* type I collagen, the major collagen component of bone. Also involved in osteogenesis imperfecta. *COL11A2;* type XI collagen, found in cartilage extracullar matrix and is important for the integrity and development of the skeleton. Also involved in DFNA13. *CDKN1A;* critical role in cellular response to DNA damage.
OTSC4 *105*	16q21-23.2	*Cadherin 1 and 3;* transmembrane proteins that mediate cell recognition and adhesion. Expressed in connective tissues, bone and cochlea. *COG4 and 8;* multiprotein complexes involved in Golgi structure and intracellular membrane trafficking. Expressed in the immune system. *Zink finger proteins;* multifunctional proteins with both transcriptional and posttranscriptional functions. Broad expression pattern including the inner ear, immune system and fibrous tissue. *DEAD family;* implicated in many cellular processes involving RNA, including cellular growth and division. Expressed in the immune system, cartilage and fibrous tissue.
OTSC5 *106, 113*	3q22-q24	*PCOLCE2;* involved in cartilage homeostasis and extracellular matrix function. *CHST2;* involved in cartilage homeostasis and extracellular matrix function. *ATP1B3;* Na,K-ATPase enzyme responsible for transport of sodium and potassium ions in most cells.
OTSC7 *107, 114, 115*	6q13-16.1	*COL12A1;* type XII collagen of the fibril-associated collagens with interrupted triple helices. *COL9A1;* type IX collagen of the fibril-associated collagens with interrupted triple helices. *TGF-β1;* involved in the chondrogenesis and bone remodeling of the otic capsule.
OTSC8 *108, 112*	9q13.1-21.11	*TJP2;* tight junction protein of the membrane-associated guanylate kinase (MAGUK) family that are involved in the organization of epithelial and endothelial intracellular function. *TRMP3;* cation-selective channel important for cellular calcium signaling, homeostasis and osteoclast activity. *KLF9;* regulation of cranial facial development.
OTSC10 *109, 116*	1q41-44	*TGF-β2;* important in bone formation and remodeling. *AGT;* angiotensinogen, which is involved in the renin-angiotensin-aldosterone-system (RAAS). RAAS is important in regulation of blood pressure and body-fluid homeostasis. AGT II also influences bone remodeling. During pregnancy, RAAS is activated and levels of AGT rise.

Table 5: Loci for otosclerosis derived from the Hereditary Hearing Loss Homepage. OTSC6 and OTSC9 are reserved.

Furthermore, a genome-wide association study suggested a strong association of otosclerosis and chromosome 7q22.1.[117] The Reelin gene (*RELN*) is located in this region. *RELN* encodes a secretory glycoprotein, which has a crucial role in the regulation of neuronal migration and positioning and in synaptic plasticity in brain development. Its known functions are difficult to relate to otosclerosis, making it unclear how this gene could be involved in the disease. However, two causative variants of *RELN* are suggested to be involved in otosclerosis.[118, 119] Furthermore, expression of RELN mRNA is found in human stapes footplates samples and in both mouse and human inner ear structures. RELN is expressed to a higher degree in osteocytes than osteoblasts. Although, the function of RELN is difficult to reconcile with our understanding of the pathogenesis of otosclerosis, it can help to reevaluate the molecular mechanisms that lead to this disease.[101, 117, 118]

Clinical symptoms

The characteristic clinical symptom of otosclerosis is progressive unilateral or bilateral conductive hearing impairment. The disease is bilateral in 70-80% of the patients and usually there is a symmetrical extension and distribution of otosclerotic foci, although, hearing impairment can be asymmetrical. The symptoms depend on the site of the otosclerotic foci.[64, 71, 76] The most common site is anterior to the oval window, followed by the round window niche, and the apical and medial cochlear wall, respectively. Other sites of predilection are posterior to the oval window, the posterior and anterior wall of the internal auditory canal, around the cochlear aqueduct and semicircular canals, and within the footplate.[73] The expansion of otosclerotic foci from the fissula ante fenestrum posteriorly towards the stapedial footplate causes gradual immobilization of the stapediovestibular joint and results in conductive hearing impairment, ranging from 0 to 50 dB.[120] Characteristically, hearing impairment is gradually progressive and first affects the low frequencies. The degree of conductive hearing impairment seems to be determined by the stage of stapedial footplate fixation.[120, 121] Low frequency hearing impairment is thought to be caused by the presence of highly cellular fibrous tissue that characterizes the spongiotic phase. As the pathologic changes progress to a stage of localized bony fixation of the anterior part of the footplate, it is thought to result in a moderate conductive hearing impairment spanning all frequencies with a gradual widening of

the air-bone gap. The hearing impairment increases to moderately severe when the diffuse bony ankylosis involves the entire circumference of the annular ligament, completely preventing the motion of the stapes.[121-123]
This progressive fixation in stapedial ankylosis is responsible for the decreased or absent reflexes seen on impedance testing.[124] The air-bone gap seems to be determined by narrowing and loss of the annular ligament. Bone-conduction thresholds and air-bone gaps are worse in cases with sclerotic lesions.[67, 76, 125]

A characteristic phenomenon in the audiogram of a patient with otosclerosis is the peak in the bone-conduction threshold at 2000 Hz. Carhart was the first to notice a notch in the audiometric curve with the largest depression at 2.000 Hz and this notch is named after him. The Carhart notch does not refer to a worse cochlear function at this frequency, but reflects the lacking mechanisms that are responsible for the transmission of vibrations of the surrounding bone to the middle ear. This transmission is still largely unclear, especially because the mechanisms influence each other. By one of these mechanisms, the vibrations of the surrounding bone are transferred to the ossicles. This mechanism is not present in otosclerosis because of the fixation of the ossicles. The direct transmission of the vibrations of the surrounding bone to the liquid in the cochlea is also limited because the fixated stapes suppresses these liquid movements. Thus, there is a weakening of the sound transmitted by the bone conductor, especially at frequencies of 2000 Hz for unknown reasons.

Anterior spread of fenestral otosclerotic foci leads to invasion of the cochlear endosteum and involvement of the stria vascularis, subsequently contributing to sensorineural hearing impairment.[126, 127] Although the cause of sensorineural hearing impairment is unknown, it may be related to the release of enzymes by remodeling of the bony labyrinth immediately surrounding the cochlea. Various enzymes have been found in the perilymph of otosclerosis patients.[76] These toxic enzymes interfere with the mobility of outer hair cells and can result in sensorineural hearing impairment.[128, 129] The literature provides conflicting information regarding the prevalence of sensorineural hearing impairment in patients with otosclerosis, but long-term follow-up studies suggest that about 10% of the patients with conductive hearing impairment develop sensorineural hearing

impairment. Cochlear otosclerosis can exists in the absence of conductive hearing impairment and is recognized as a separate entity, although isolated cochlear otosclerosis is a rare event.[9, 63, 125, 130]

The mean age of onset of clinical otosclerosis is in de third decade of life, but an age shift toward an older onset age has been reported. Some patients with otosclerosis exhibit hearing impairment in childhood whereas other patients as late as 60 years of age.[63, 101, 131, 132] Moreover, there is an increase in the prevalence of otosclerosis with age.[76] Approximately 90% of the patients are younger than 50 years at the time of diagnosis.[67]

Other clinical features besides hearing impairment are tinnitus and/or vertigo.[71] Tinnitus is a frequent symptom of otosclerosis, especially in patients with considerable sensorineural hearing impairment combined with stapedial footplate fixation. The prevalence of chronic tinnitus in otosclerosis ranges from 45% to 85%. Tinnitus is more frequently associated with mature (sclerotic) otosclerosis than with immature otosclerosis.[67, 133] The exact mechanism of this symptom is yet unknown.[120, 134, 135]

Otosclerosis has also been associated with an increased incidence of vestibular symptoms.[136] Ten to 24% of the patients with otosclerosis suffer from vertigo. Advancement of the otosclerotic foci in medial direction to the basal turn of the cochlea, the vestibule and the underlying otolitic organs is seen, and may lead to vestibular symptoms due to invasion and degeneration of the vestibular nerve endings, probably by changes in the biochemical composition of the endolymph. Loss of vestibular nerve fibers in clinical otosclerosis is directly related to the size of the lesion.[67, 137, 138] Vestibular symptoms can include transitory, recurrent, rotary, positional or spontaneous vertigo.[137] Spontaneous episodic vertigo is the most frequent manifestation. However, the vestibular symptoms, the frequency of occurrence, the duration of the vertigo attack and the intensity of the vestibular symptoms vary from patient to patient but also in time in the same patient.[136] A correlation with an increased incidence of benign paroxysmal positional vertigo (BPPV) has also been described. Otosclerosis is suggested to be responsible for the production of cupular deposits, however, an association between these deposits and vestibular symptoms could not be confirmed.[120, 137]

Vestibular involvement is more pronounced in patients with sensorineural hearing impairment and occurs more frequently in patients with sclerotic otosclerosis.[76] However, no consistent relationship could be demonstrated between the severity of vestibular symptoms and the results of caloric testing. Caloric and/or rotational hypoexcitability is most frequently seen in otosclerosis, followed by directional preponderance and positional nystagmus. Vestibular hyperreactivity is also possible and is presumably caused by labyrinth irritation.[139]

Radiological examination

In most cases of otosclerosis the diagnosis is based on clinical findings combined with the results of audiometry. However, the use of imaging in the detection of otosclerosis has increased with the development of spiral computed tomography (CT) scanners with high-resolution images. This type of CT scanners is at present the imaging modality of choice for the assessment of the osseous labyrinth, labyrinthine windows and cochlear capsule. In otosclerosis, high resolution CT gives the opportunity to assess the extent of the disease, and to confirm non-penetrance and/or cochlear involvement. In most cases, CT can detect the otospongiotic stage of the disease process, characterized by a hypodense lesion in the otic capsule. But large mature, sclerotic lesions of the otic capsule may go undetected by CT as they have the same density as normal bone tissue of the otic capsule.[140] Detection rates of surgically confirmed otosclerosis with presurgery CT of up to 91% have been reported.[141] The best method for the detection of otosclerotic foci on CT is to use sub-millimeter slice thickness and assess the images directly on a workstation. Although CT cannot replace histology in assessing microscopic lesions of the otic capsule, high-resolution CT scans are a valid tool that can be used to confirm, localize and determine the size of clinically suspected otosclerotic foci.[142]

The radiologic classification of otosclerosis has been proposed when the high resolution CT of the temporal bone was incorporated into the diagnostic work-up of hearing impairment. (Table 6) Fenestral and cochlear types of otosclerosis have been described. The fenestral type refers to hypodense lesions of demineralized bone adjacent to the oval window area and/or impinging in the stapedial footplate. The cochlear type is radiologically diagnosed as the presence of hypodense areas of

demineralization surrounding the cochlea, often described as 'double ring' or 'fourth turn' sign.[142]

Fenestral otospongiosis	Group 1	Otospongiosis limited to the fissula ante fenestram
	Group 2	Otospongiosis extends to at least half the diameter of the oval window niche and/or the cochleariform process
	Group 3	Otospongiosis extending over the entire diameter of the oval window niche
Cochlear otospongiosis	Group 1	Otospongiosis not exceeding the diameter of one cochlear turn
	Group 2	Between group 1 and 3
	Group 3	Spongiotic involvement of the entire otic capsule

Table 6. Classification of otospongiosis based on CT imaging.[142]

Je Shin et al. demonstrated that patients with and without a family history of otosclerosis had different radiologic expression of their disease. In the familial forms the lesions are more often detectable, bilateral and extensive, whereas fenestral otosclerosis occurs more often in sporadic forms of otosclerosis.[143]

The relationship between endosteal involvement and the degree of sensorineural hearing impairment has long been controversial, but after the use of more technically advanced CT scans, a positive correlation between CT findings and the severity of sensorineural hearing impairment could be established; the severity of sensorineural hearing impairment is correlated with the extension of the foci within the otic capsule. A pericochlear focus without extension to the endosteum is not sufficient to cause sensorineural hearing impairment. But when the endosteum is involved, sensorineural hearing impairment can be correlated with otosclerosis.[141]

Treatment

Stapes surgery

Conductive hearing impairment can be corrected by a hearing aid as well as by a surgical procedure. The basic surgical steps include disarticulating the incudostapedial joint, removal of stapes superstructure and opening up the stapes footplate. The surgical management for otosclerosis has evolved from total extraction of the footplate, the so called stapedectomy to a small hole in the posterior part of the stapes footplate, the stapedotomy.[144] In a stapedotomy, the

continuity of the ossicular chain is normally reestablished by positioning a piston, fixed to the long process of the incus and reaching into the vestibule through the footplate. The aim of stapes surgery is to restore the vibration of fluids within the cochlea.[66, 144]

Although the sensorineural component of the hearing impairment cannot be corrected, stapes microsurgery has proven to be highly successful to restore the normal conduction mechanism and can improve hearing thresholds by as much as 50 dB.[145] In the early postoperative period, the patients show a significant improvement in hearing thresholds, accompanied by a significant improvement in speech discrimination scores. The pure-tone thresholds for air- and bone-conduction intend to improve for approximately 2 years postoperatively and then deteriorate in a linear fashion. This deterioration in hearing impairment 2 years postoperatively is similar to the decline associated with presbycusis alone, meaning that stapedotomy has no demonstrable adverse effects on the cochlear function.[125, 146]

However there are complications of stapes surgery. In some instances, surgery results in no improvement in hearing or, even worse, in deterioration of hearing. Potential side effects of a stapedotomy include a change in sense of taste on the same side of the tongue, vertigo, perforation of the tympanic membrane and intolerance of very loud noises.[144] Compared to stapedectomy, stapedotomy results in equivalent pure-tone thresholds but better high frequency hearing and speech discrimination. Furthermore, stapedotomy reduces many of the complications of stapedectomy such as postoperative vertigo and sensorineural hearing impairment.[144, 147]

Pharmacological treatment

Although the treatment of hearing impairment associated with otosclerosis is well established, there is no curative therapy for otosclerosis. Surgical correction of the conductive hearing impairment is highly effective, whereas nonsurgical intervention has not yet proven to prevent or slow down the disease. Inadequate knowledge of the biological mechanisms triggering the otosclerotic process, limits the effective prevention and treatment options of otosclerosis.[148] Therapeutic strategies have been directed at suppression of bone remodeling. Furthermore, the candidate genes

of the otosclerosis loci and their pathological mechanisms provide possible treatment options.

Agents that suppress osteoclast recruitment and activation, such as sodium fluoride, bisphosphonates, monoclonal anti-TNF-α antibodies and short-term recombinant osteoprotegerin (OPG-Fc), were used to treat otosclerosis. Furthermore, vitamin D deficiency seemed to be present in some otosclerosis patients. Thus, vitamin D supplementation might be beneficial in these otosclerotic patients.[65] However, the efficacy of these therapeutic agents remains controversial.[78, 149, 150]

Many researchers investigated the effect of sodium fluoride on otosclerosis. High doses are probably needed for a positive effect, but may have serious side effects including multiple organ failure, dysostosis and spinal stenosis. Sodium fluoride is thought to inhibit toxic proteolytic enzymes and osteoclast activation, and consecutively osteolysis and disease progression.[65, 85] Moreover, there is conflicting evidence regarding the protective effect of a high fluoride concentration in drinking water. Epidemiological studies show that clinical otosclerosis is associated with areas that have low fluoride content in drinking water.[9, 65, 151] Furthermore, the deterioration in air- and bone-conduction thresholds in otosclerosis patients drinking fluoride poor water was more pronounced than that in patients drinking fluorinated water. However, the difference was not statistically significant.[152, 153]

Bisphosphonates are potent inhibitors of BMP synthesis. BMP are fundamental in bone remodeling; BMPs stimulate chondrogenesis, promote growth and act as inflammatory cytokines. There is some clinical evidence that bisphosphonates treatment is effective in the early stages of otosclerosis.[150]

Furthermore, an increased TNF-α production is supposed to be a trigger of focal bone resorption.[89, 154] TNF-α is a proinflammatory cytokine that plays a role in the osteolytic process, in the differentiation of osteocytes to osteoclasts or osteoblasts and in the intercellular communication between osteoclasts and osteoblasts. Administration of monoclonal anti-TNF-α antibodies (etanercept, infliximab, adalimumab) could be an option in the treatment of cochlear otosclerosis with sensorineural hearing impairment. However, more research is needed to be conclusive about the therapeutic use of TNF-α inhibitors in otosclerosis.[65, 129, 155]

OPG-Fc treatment could also have powerful anti-osteolytic effects, primarily in the early stages of otosclerosis, and preserve normal bone remodeling.[156] OPG is an inhibitory glycoprotein that blocks osteoclast formation and osteolysis, and induces the apoptosis of activated osteoclasts. Therefore, lack of OPG leads to osteoporosis while overexpression causes excessive bone formation or osteopetrosis. Furthermore, OPG-deficient mice demonstrated abnormal otosclerosis-like bone remodeling in the otic capsule. These mice exhibited progressive hearing impairment. However, active remodeling was seen throughout the entire skeleton of these mice and not all authors could confirm the stapes fixation histologically.[64, 67, 156] Coexpression of TNF-α and OPG mRNA have been demonstrated in otosclerotic stapes footplates, indicating the involvement of activated osteoclasts and inflammatory pathways. Increased expression of TNF-α could inhibit the protective functions of OPG on normal bone turnover in de otic capsule and may lead to extensive osteoclast activation and bone resorption.[67, 89, 156] Nevertheless, more research is needed to confirm these results.

There is no cure for the sensorineural component of otosclerosis, however, conventional hearing aids, bone anchored hearing aids (BAHA) and cochlear implantation are beneficial.[65, 66, 144, 148] BAHA can be an option in patients who are unable to benefit effectively from stapedotomy and/or conventional hearing aid rehabilitation. In contrast to stapedotomy, the risk of further hearing damage, tinnitus or vertigo is absent in BAHA surgery. Furthermore, BAHA surgery does not preclude patients from stapedotomy at a later stage. The advantage of the BAHA over conventional hearing aids is the better sound quality due to direct bone-conduction. Furthermore, the benefits of the BAHA could also be related to cosmetic or comfort improvements.[157] Cochlear implantation for patients with profound hearing impairment due to otosclerosis is also effective.[66, 158] Better performance was related to less severe signs of otosclerosis on CT scan, full insertion of the electrode array, little or no facial nerve stimulation and little or no need to switch off electrodes.[159] However, stapedotomy could also be effective in patients with profound hearing impairment. Stapedotomy can close the air-bone gap and improve the air-conduction thresholds to hearing levels that can be corrected with conventional hearing aid rehabilitation.[160]

Aim and outline of this thesis

The general aim of this thesis was to provide further insight into the phenotype of hereditary hearing impairment. This thesis focuses on genotype-phenotype studies in DFNA3, DFNB8/10, DFNX4, Muckle-Wells syndrome and otosclerosis (OTSC10).

Chapter 2 provides a detailed phenotypic description of three DFNA3 patients from two families. Mutation analyses revealed a p.Argl84Gln and a p.Arg75Trp mutation in *GJB2* in the these two families. The phenotypes were compared to previously described DFNA3 families.

Detailed phenotypic analyses of eight DFNB8/10 families are described in chapter 3. Differences in phenotypic effects of different *TMPRSS3* mutations could be distinguished. This different phenotypic expression of *TMPRSS3* mutations was also established for previously described *TMPRSS3* mutations.

In chapters 4.1 and 4.2, the clinical and genetic characteristics of a large Dutch DFNX4 family with a mutation in the *SMPX* gene are presented. The variable expression of hearing impairment in affected men and women was analyzed. The phenotype of the present family was compared to previously described DFNX4 families.

The phenotype of a Dutch family with Muckle-Wells syndrome (MWS) is described in chapter 5. Additional audiological testing has been performed to understand the pathogenesis of hearing impairment in MWS.

Otosclerosis is subject of chapter 6. Detailed analysis of audiometric data from a Dutch otosclerosis family, in which the disease is linked to OTSC10, are presented in chapter 6.1. The genetic analysis of this family is provided in chapter 6.2.

Chapter 7 provides the general discussion and conclusion. A summary of this thesis is described in chapter 8.

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2

Phenotypes of two Dutch DFNA3 families with mutations in *GJB2*

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Annals of Otology, Rhinology & Laryngology 2011; 120: 191-197

Abstract

In this paper we describe the phenotype of two Dutch DFNA3 families with mutations in the *GJB2* gene.

Two patients from family 1 and one isolated patient from family 2 were studied. The audiometric examination consisted of pure-tone and speech audiometry. Two patients underwent vestibular testing and high-resolution computed tomographic scanning of the temporal bone. Mutation analysis of *GJB2* and *GJB6* was performed.

All three patients had severe to profound sensorineural hearing impairment. Cochlear implantation was performed in two patients, and their phoneme recognition scores were good. Mutation analyses revealed a p.Argl84Gln mutation in *GJB2* in family 1 and a p.Arg75Trp mutation in *GJB2* in family 2. No mutations in *GJB6* were identified. Vestibular function tests and computed tomographic scans yielded normal findings in the examined subjects.

Severe to profound sensorineural hearing impairment was found in these DFNA3 patients and was well rehabilitated with cochlear implantation. A thorough genotype-phenotype correlation is difficult because of the small number of affected patients and the limited clinical data of these patients. More clinical data of DFNA3 families need to be published in order to create a reliable and precise phenotype characterization.

Introduction

Nonsyndromic autosomal dominant sensorineural hearing impairment, DFNA3, is caused by mutations in the *GJB2* (gap junction protein beta 2) gene or the *GJB6* (gap junction protein beta 6) gene, altering either connexin 26 or connexin 30, respectively. DFNA3 is characterized by childhood onset (prelingual or postlingual), progressive, moderate to severe high-frequency sensorineural hearing impairment. In addition, the hearing impairment in DFNA3 shows a variable severity and evolution in patients. The variability in phenotype can be explained by variable expression of the disease and can be related to genetic and/or environmental factors.[1] Furthermore, mutations in *GJB2* are also responsible for autosomal dominant syndromic hearing impairment with dermatologic features such as keratitis-ichthyosis-deafness, hystrix-like ichthyosis-deafness, Vohwinkel's syndrome and Bart-Pumphrey syndrome.[2, 3] So far, 12 mutations in *GJB2* (p.Trp44Cys, p.Trp44Ser, p.Thr55Asp, p.Pro58Ala, p.Arg75Gln, p.Arg75Trp, p.Argl43Gln, p.Metl63Leu, p.Alal71Thr, p.Aspl79Asn, p.Argl84Gln and p.Cys202Phe) have been reported in individuals with DFNA3.[1,3] The majority of these mutations have been shown to segregate in families and demonstrate ethnic predilections.[3]

Connexin 26 (Cx26) and connexin 30 (Cx30) are related transmembrane proteins that form gap-junctions (connexons). Serially arranged connexons of epithelial and connective tissue cells of the cochlea are important for recycling potassium ions that pass through sensory cells during auditory transduction. Connexin mutations may cause hearing impairment by several mechanisms, including interference with the proper oligomerization or intracellular transport of connexons, impairment of interactions between connexons in opposing cells and the formation of channels with altered permeation or gating properties.[4] In mice, these mechanisms disturb the homeostasis of cortilymph, because of impaired potassium transport by supporting cells, resulting in degradation of the organ of Corti.[5] In the inner ear, Cx26 is commonly coexpressed with Cx30. Mutations in the complex locus of DFNA3, which contains 2 genes (*GJB2* and *GJB6*), can result in a digenic pattern of inheritance of sensorineural hearing impairment.[6] Furthermore, certain mutant proteins can have dominant negative effects on Cx26 and Cx30 that are due to disrupted transfer of molecules.[7]

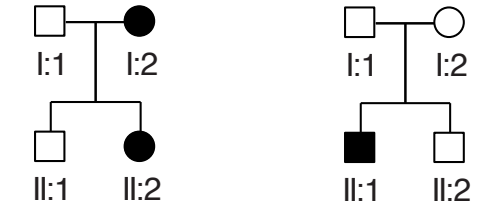
In the present article, we report the clinical characteristics of two Dutch DFNA3 families with a mutation in *GJB2.* The phenotype is compared with those previously published for other DFNA3 families.

Patients and methods

Patients

The study was approved by the local medical ethics committee of the Radboud University Nijmegen Medical Centre. The six patients included in this study signed an informed consent form. After informed consent, medical records and previous audiograms were traced.

The first family consisted of an affected mother and daughter (proband), and the second family of an affected son (proband). (Figure 1) Examination of the probands and their parents consisted of medical history, otoscopy, pure-tone audiometry and blood samples for linkage analysis. Clinically affected family members also underwent speech audiometry. The probands of both families underwent vestibulo-ocular examination and high-resolution spiral computed tomographic (CT) imaging of the temporal bones. In general, attention was paid to the presence of syndromic features such as skin disorders.



Family I *Family II*

Figure 1. Pedigrees of families 1 and 2. Square: male; circle: female; open symbol: unaffected; solid symbol: affected.

Audiometry and data analysis

Audiometric examination comprised conventional pure-tone audiometry in a sound-treated room according to common clinical standards. Air conduction thresholds were measured in decibels hearing level (HL) at 0.25, 0.5, 1, 2, 4 and 8 kHz, and bone

conduction thresholds were measured in decibels HL at 0.5, 1, 2, 4 and 8 kHz. The individual 95th percentile threshold values of presbycusis in relation to the patient's sex and age were derived for each frequency with ISO 7029.[8] Individuals were considered affected if the better-hearing ear showed all thresholds higher than the 95th percentile threshold values for presbycusis.

Individual longitudinal linear regression analysis of binaural mean air conduction threshold values on age was only performed in clinically affected persons with three or more consecutive measurements and an overall follow-up period of at least three years. The annual threshold deterioration (ATD) was calculated and the progression was considered significant if the 95% confidence interval did not include zero. The level of significance used in all tests was a p value of less than 0.05.

Speech audiometry was performed under the above-mentioned conditions by use of standard Dutch consonant-vocal-consonant word lists. The maximum phoneme recognition score (mean of both ears) was obtained from monaural performance-versus-intensity curves. After cochlear implantation, the words were presented through a loudspeaker at a fixed distance of 1 m from the patient at a normal conversational level of 70 dB sound pressure level (SPL). The subject responses were scored as the percentage of phonemes correct.

One family member underwent the Ewing distraction test for screening of his hearing. This test is based on expected responses; between 9 and 13 months, the infant will be able to localize sounds.[9] These tests were used at a time in which otoacoustic emissions (OAE) were not yet used in neonatal hearing screening.

Vestibulo-ocular examination

Two affected family members underwent vestibular and oculomotor tests. The test included evaluation of the vestibulo-ocular reflex using electronystagmography with computer analysis and saccadic smooth pursuit and optokinetic nystagmus responses. Vestibular stimulation comprised rotatory and caloric tests. The details and normal values have been described previously.[10]

Results

In both families, DNA sequencing of the coding region and splice sites of the *GJB2* gene identified two different pathogenic mutations. Genebank accession number NM_004004 is used as a reference sequence.

Family 1

In the proband of the first family, direct DNA sequencing identified a heterozygous change G→A at nucleotide 551, resulting in an arginine-to-glycine substitution at codon 184 (p.Arg184Gln). The chromatogram is shown in Figure 2a. The mother of the proband carried the same mutation. No pathogenic deletion in the *GJB6* gene was found on allele-specific polymerase chain reaction testing, so digenic inheritance of sensorineural hearing impairment is unlikely. A detailed family history did not reveal any other affected family members.

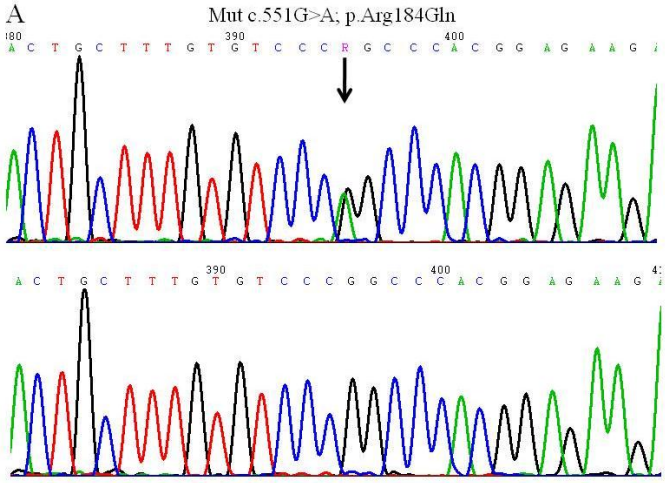


Figure 2. Chromatogram of the index patient of family 1. For comparison, wild-type sequence is added underneath.

The proband of the first family is a 6-year-old girl. When the child was 9 months of age, her mother first had doubts about her daughter's hearing. Audiometric evaluation at the age of 10 months showed bilateral sensorineural hearing

impairment. Reproducible responses were only seen at low frequencies with 80 to 90 dB stimulation. Furthermore, brainstem evoked response audiometry showed no reproducible responses for both ears with maximum stimulation (90 dB HL), indicating bilateral profound hearing impairment. At the age of 1 year hearing aids were adjusted and 1 year later the patient underwent cochlear implantation with a Nucleus device (Cochlear Ltd, Sydney, Australia). The implantation was uneventful and the implant was fully inserted. Three years after activation of the speech processor, the patient had a 70% phoneme score at 70 dB SPL. Unfortunately, compared to her peers, she is still far behind in speech and language development. Figure 3 shows the only available audiogram of the left (unimplanted) ear at the age of 5 years. The audiogram shows profound sensorineural hearing impairment. No skin disease or other clinical features were seen. No balance problems were described and vestibulo-ocular examination before cochlear implantation showed normal vestibular function. A CT scan of the temporal bone appeared normal.

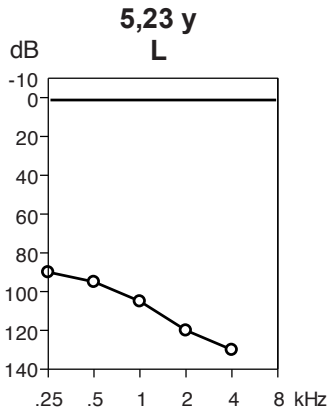


Figure 3. Audiogram of the left ear of the proband of family 1 at age of 5.23 years.

The mother of the proband also had profound sensorineural hearing impairment since the age of 3 years. Figure 4 shows the available audiograms of this individual; no remarkable deterioration of her hearing between the ages of 19 and 42 years was seen. Longitudinal regression analysis was performed and revealed only moderate significant progression at 1 and 2 kHz with ATD values of 0.4 and 0.3, respectively (data not shown). Unfortunately, no audiograms before the age of 19 years were

available, so possible progression of hearing impairment before this age cannot be ruled out. The maximum mean phoneme recognition scores at the ages of 19 and 37 years were 30%, and no deterioration of speech reception was noticed. This individual reported no vestibular symptoms and vestibulo-ocular examination was not performed. Furthermore, there was no evidence of skin disease.

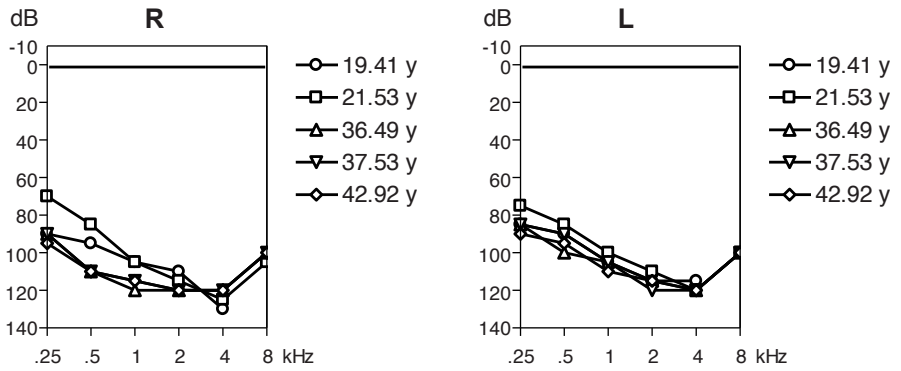


Figure 4. Audiograms of the mother of the proband of family 1 show air conduction thresholds at different ages for A) right ear and B) left ear.

Family 2

Direct sequencing of the DNA of the proband of the second family, a 6-year-old boy, showed a heterozygous C→T change at nucleotide 223, resulting in an arginine-to-tryptophan substitution at codon 75 (p.Arg75Trp). The chromatogram is shown in Figure 2B. Allele-specific polymerase chain reaction revealed no pathogenic deletion in the *GJB6* gene. Neither parent had this mutation in *GJB2*, so a de novo mutation is likely.

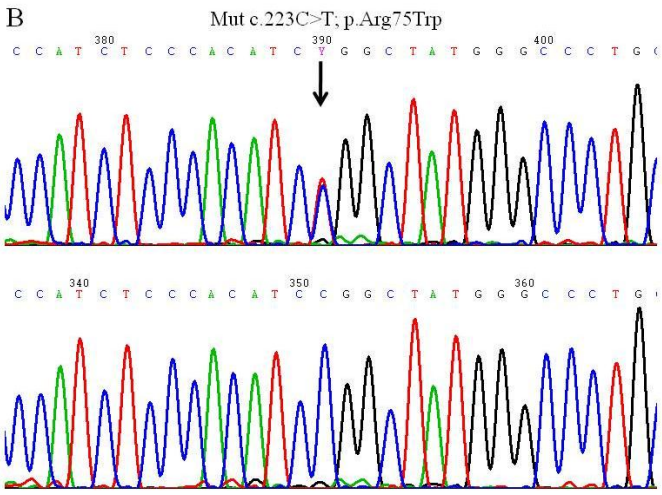


Figure 5. Chromatogram of the index patient of family 2. For comparison, wild-type sequence is added underneath.

The parents of the proband first noticed progressive hearing impairment at the age of 6 months. The results of the Ewing hearing test at 9 months of age were reported as normal. Audiometry evaluation at 17 months of age, however, showed no reproducible auditory responses and undetectable otoacoustic emissions. Brainstem evoked response audiometry (BERA) showed no responses at maximum stimulation (90 dB HL) and confirmed the presence of profound bilateral sensorineural hearing impairment. The patient's hearing aids were adjusted at the age of 2 years. Six months later a cochlear implant was implanted on the left side and at the age of 3,5 years the patient received a second cochlear implant on the right side (Nucleus, Cochlear). Both implantations were uneventful and complete insertion was obtained. The patient was tested 12 months after insertion of the second cochlear implant and showed speech reception scores of sound presented at 70 dB SPL of 76% on the left side, 90% on the right side and 85% for both cochlear implants together. The phoneme scores 24 months after insertion of the second cochlear implant were 93% on the left side, 87% on the right side, and 96% for both cochlear implants together. These results show that the patient's speech reception is still improving 24 months after insertion of the second cochlear implant. At 1 year after

bilateral cochlear implantation, his word usage was comparable to that of his peers; however, his sentence structure is not as good as that of his peers. Except for dryness, no skin abnormalities were noticed. No balance problems were reported and vestibulo-ocular examination at the ages of 3 and 4 years showed normal vestibular function. Hyporeflexia of the velocity-step responses on the left side was seen at 5 years of age (initial velocity of 24°/s and 50°/s for left and right directions, respectively). However, this was after cochlear implantation, so the value of this finding is probably limited. A CT scan of the temporal bone showed no abnormalities. No relatives exhibited sensorineural hearing impairment.

Discussion

This report presents the audiometry features of two small Dutch DFNA3 families with profound sensorineural hearing impairment. Mutation analysis revealed in the first family a p.Argl84Gln mutation and in the second family a p.Arg75Trp mutation in *GJB2.*

Family 1

The p.Arg184Gln mutation in *GJB2* has previously been described in a family from Ghana. Heterozygosity for p.Arg184Gln co-segregated with autosomal dominant profound sensorineural hearing impairment. None of the five affected family members showed skin alterations such as keratoderma.[11] Unfortunately, the audiometry data were limited, but seem to be in line with the audiometry data of the Dutch girl and her mother presented here.

Family 2

Janecke et al.[12] reported in 2001 the first p.Arg75Trp de novo mutation of the connexin 26 gene in a sporadic case of isolated profound hearing impairment. A 7-year-old Austrian boy with congenital profound sensorineural hearing impairment (mean: 105 dB HL for both ears) was assessed for cochlear implantation. CT findings of the temporal bone were normal. He had no skin disease or other clinical features.[12] Allowing for the limited information, the clinical data of the 7-year-old Austrian boy seem comparable with those of the 6-year-old Dutch boy presented here. Both had profound sensorineural hearing impairment with an indication for cochlear implantation.

The patients with the p.Arg75Trp mutation reported by Piazza et al.[13] were said to have profound hearing impairment. Remarkably, the patients with the same mutation reported by Feldmann et al.[14] had hearing impairment varying from moderate to severe.

The p.Arg75Trp mutation was previously observed in association with dominant hearing impairment and palmoplantar keratoderma in an Egyptian family. The affected father and daughter presented with congenital prelingual hearing impairment and diffuse thickening of the skin of the palms and soles. Audiometry confirmed bilateral profound sensorineural hearing impairment with no speech discrimination. The palmoplantar keratoderma developed during infancy and was characterized by diffuse hyperkeratosis with underlying erythema, peeling and deep fissures. The restricted audiological data of the affected Egyptian family members and of the present Dutch boy seem to be comparable. However, the Dutch boy has no skin lesions, except for dryness, but it is possible that the skin disease has not yet developed because of his young age. The exact onset age of palmoplantar keratoderma in the Egyptian family was not mentioned. The same report also described the p.Arg75Trp mutation in a control individual with no skin disease and unknown hearing status, suggesting that the p.Arg75Trp mutation may not be causative. However, in silico analysis with several prediction programs (SIFT: http://sift.jcvi.org/vvww/SIFT/; Polyphen: http://genetics.bwh.harvard.edu/pph/; AGVGD: http://agvgd.iarc.fr/agvgd) indicates the p.Arg75Trp mutation to be pathogenic. Even more convincing, the deleterious dominant negative effect of p.Arg75Trp on the function of gap junctions was demonstrated in the paired oocyte expression system,[15] as well as in communication-incompetent Hela cells.[16]

In 2009, Yuan et al.[17] described a p.Arg75Trp de novo mutation in a 15-year-old Chinese girl with sensorineural hearing impairment and palmoplantar keratoderma. Audiometric evaluation at the age of 15 years showed residual hearing levels. She developed thickening and peeling of the skin at the medial and lateral sides of her feet during infancy.[17] The audiometric configurations of this Chinese girl and of our Dutch boy were similar. However, the age of diagnosis in the Chinese girl was 15 years, whereas it was below 2 years in the present Dutch boy from family 2, so there might be a difference in age of onset. Again, the Dutch boy has no skin disease, but may develop palmoplantar keratoderma in the future.

Birkenhäger et al.[18] reported in 2010 a p.Arg75Trp de novo mutation in a German boy with severe hearing impairment and palmoplantar keratoderma. Hearing impairment was diagnosed at the age of 12 months. The summed action potentials in electrocochleography were negative, and the cochlear microphonics started at 90 dB on the right and at 110 dB on the left. CT scans of the temporal bone showed no morphological findings of the cochlea or vestibular apparatus. The German boy has been fitted with a cochlear implant.[18] The hearing impairments of the German boy and our Dutch boy seem to be similar. However, our Dutch boy has not (yet) been found to have palmoplantar keratoderma.

In conclusion, p.Arp75Trp is associated with both syndromic and nonsyndromic autosomal dominant hearing impairment. Patients with the identical p.Arg75Trp mutation exhibit a similar hearing impairment phenotype (from moderate to severe or profound bilateral sensorineural hearing impairment) and a wide range of cutaneous phenotypes. The variations in skin alterations associated with the p.Arg75Trp mutation may be due to the contribution of genetic background and environmental factors.[18] The p.Arg75Trp mutation could be a hotspot mutation, as this mutation has already been described in three other DFNA3 families. Furthermore, the majority of *GJB2* mutations associated with DFNA3 are described only in single families. Detailed audiometric findings have been reported for several other mutations in *GJB2* that cause DFNA3 and are outlined in the Table 1.[11-15, 17, 19-25]

Vestibular function

No vestibular evaluation was performed in previously described individuals with the p.Argl84Gln or the p.Arg75Trp mutation in connexin 26. Vestibular function tests performed by Denoyelle et al.[1] in subjects with other mutations in *GJB2* yielded normal findings. For the p.Thr55Asp mutation, vestibular failure has been reported to occur in affected individuals tested at ages 14 to 47 years.[21] In the future, we will repeat vestibular evaluation of the affected family members in our study to assess whether DFNA3 represents a progressive cochleovestibular disorder like DFNA9,[26-28] DFNA11[19] and DFNA15.[30, 31]

Mutation	*Reference*	*Audiogram*
p.Arg184Gln	Hamelmann et al. 2001[11]	Profound congenital sensorineural HI
p.Arg75Trp	Janecke et al. 2000[12] Piazza et al. 2005[13] Richard et al. 1998[15]	Profound sensorineural HI
	Feldmann et al. 2005[14]	Moderate to severe sensorineural HI
	Yuan et al. 2008[17]	Residual hearing levels
p.Trp44Cys	Tekin et al. 2001[27] Welch et al. 2007[28]	Severe to profound sensorineural HI
	Denoyelle et al. 2002[1]	Moderate to severe sensorineural HI starting at the high frequencies
p.Thr55Asp	Melchionda et al. 2005[19]	Severe to profound sensorineural HI
p.Arg143Gln	Löffler et al. 2001[29]	Moderate to severe sensorineural HI
p.Met163Leu	Matos et al. 2008[30]	Mild to moderate sensorineural HI
p.Cys202Phe	Morlé et al. 2000[31] Denoyelle et al. 2002[1]	Mild to moderate sensorineural HI
p.Asp179Asn	Primignani et al. 2003[32]	Mild to moderate sensorineural HI exclusively at the high frequencies

Table 1. Overview of mutations in *GJB2* gene causing DFNA3 and audiometric data reported for these mutations. HI: hearing impairment.

Cochlear implantation

Our results indicate that cochlear implantation in DFNA3 patients provides satisfactory speech reception and seems to be a promising treatment option. Furthermore, bilateral cochlear implantation resulted in further improvement of speech reception in our patient group.

The relatively good speech reception of *GJB2* patients can be explained by the fact that *GJB2* mutations do not affect the spiral ganglion cells stimulated by the cochlear implant. Normal cognitive function could also play a role. The word score 3 years after cochlear implantation of patients with *GJB2*-related hearing impairment reported by Sinnathuray et al.[32] was 92% (range, 79% to 100%). This score is better than the phoneme score of our Dutch girl of family 1. Three years after activation of the speech processor, she had a phoneme score of 70% at 70 dB SPL. Furthermore, phoneme scoring gives higher percentage values than word scoring, making the difference in speech reception even greater. No phoneme score was available for our Dutch boy of family 2 from 3 years after cochlear implantation; however, his

phoneme score at 24 months after implantation was already comparable to the word score reported by Sinnathuray et al.[32]

Conclusion

It is remarkable that the majority of *GJB2* mutations in autosomal dominant hearing impairment are described in only single small families or simplex cases. Moreover, the available clinical data of affected individuals are usually limited. There is no doubt that a thorough genotype-phenotype analysis of DFNA3 requires more data on DFNA3 families with several *GJB2* mutations. Long-term clinical data could also be useful in counseling of patients and their family members. Furthermore, our results indicate that cochlear implantation in DFNA3 patients could be a promising treatment option.

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3

Genotype–phenotype correlation in DFNB8/10 families with *TMPRSS3* mutations

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JARO 2011; 12: 753-766

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Abstract

In the present study, genotype-phenotype correlations in eight Dutch DFNB8/10 families with compound heterozygous mutations in *TMPRSS3* were addressed. We compared the phenotypes of the families by focusing on the mutation data.

The compound heterozygous variants in the *TMPRSS3* gene in the present families included one novel variant, p.Val199Met, and four previously described pathogenic variants, p.Ala306Thr, p.Thr70fs, p.Ala138Glu and p.Cys107Xfs. In addition, the p.Ala426Thr variant, which had previously been reported as a possible polymorphism, was found in one family.

All affected family members reported progressive bilateral hearing impairment, with variable onset ages and progression rates. In general, the hearing impairment affected the high frequencies first, and sooner or later, depending on the mutation, the low frequencies started to deteriorate, which eventually resulted in a flat audiogram configuration. The ski-slope audiogram configuration is suggestive for the involvement of *TMPRSS3*.

Our data suggest that not only the protein truncating mutation p.T70fs has a severe effect but also the amino acid substitutions p.Ala306Thr and p.Val199Met. A combination of two of these three mutations causes prelingual profound hearing impairment. However, in combination with the p.Ala426Thr or p.Ala138Glu mutations, a milder phenotype with postlingual onset of the hearing impairment is seen. Therefore, the latter mutations are likely to be less detrimental for protein function. Further studies are needed to distinguish possible phenotypic differences between different *TMPRSS3* mutations.

Evaluation of performance of patients with a cochlear implant indicated that this is a good treatment option for patients with *TMPRSS3* mutations as satisfactory speech reception was reached after implantation.

Introduction

Autosomal recessive nonsyndromic hearing impairment (arNSHI) is the most common type of inherited hearing impairment, accounting for approximately 80% of inherited prelingual hearing impairment. The phenotype associated with nonsyndromic recessive hearing impairment is usually prelingual, non-progressive and severe to profound. Autosomal recessive inheritance is rare in nonsyndromic hearing impairment with postlingual onset.[1]

Already 72 loci have been described to be involved in arNSHI and 40 causative genes have been identified thus far, indicating enormous genetic heterogeneity. However, there is little knowledge about the contribution of the different genes to arNSHI in the European population. *GJB2* mutations are a frequent cause of arNSHI, as in most populations; however, the relative contribution varies per country. Other genes reported to be relatively important for arNSHI in populations of western European origin are *TMC1*, *OTOF* and *CDH23*.[2]

Mutations in the transmembrane protease serine 3 (*TMPRSS3*, OMIM 605511) gene on chromosome 21q22 are responsible not only for arNSHI with a prelingual onset (DFNB10, OMIM 605316)[3] but also for postlingual (DFNB8, OMIM 601072)[4] arNSHI. *TMPRSS3* mRNA has been detected in the spiral ganglion, the entire epithelium that supports the cells of the organ of Corti and in low levels in the stria vascularis, but was not detected in sensory hair cells with in situ hybridization.[5] However, in a more recent study by Guipponi et al.[6], expression of the TMPRSS3 protein was shown in the inner hair cells of the organ of Corti and in the cell bodies of the spiral ganglion neurons. The function of TMPRSS3 in the auditory pathway is currently poorly understood, but it has been hypothesized that lack of TMPRSS3 activity results in hearing impairment because of an increased sodium concentration in the endolymph by insufficient ENaC activation.[5] However, individuals with pseudohypoaldosteronism type 1, which are homozygous for null alleles of ENaC subunits, demonstrate no hearing impairment.[7] This indicates that hearing impairment associated with *TMPRSS3* mutations cannot be explained by inactive ENaC. Involvement of TMPRSS3 in the proteolytic cleavage of proneurotrophins could play a role.[5] Proneurotrophins function in the development and maintenance

of inner ear innervation.[8] The serine protease plasmin, which belongs to the same family as TMPRSS3, has been shown to cleave the neurotrophic factor BDNF.[5, 9]

So far, mutations in *TMPRSS3* have mainly been identified in families from Asian and Mediterranean countries, and did not seem to contribute substantially to prelingual arNSHI in the Caucasian population.[10] However, in the present study, we demonstrate that mutations in *TMPRSS3* are a common cause of progressive arNSHI with a childhood onset in the Dutch population. We characterized the hearing impairment in eight Dutch families with compound heterozygous mutations in *TMPRSS3* and described genotype–phenotype correlations. The present family E was previously described as family 2 in a report on autosomal recessive progressive high-frequency hearing impairment with childhood onset.[11]

Patients and methods

Patients

The pedigrees of eight families with arNSHI were constructed. (Figure 1)[11] After informed consent had been obtained from the participating family members, audiograms were obtained to establish the hearing impairment phenotype of these families. The study was approved by the medical ethics committee of the Radboud University Nijmegen Medical Centre, the Netherlands.

Examination of the family members included a medical history guided by a questionnaire, otoscopy, pure-tone audiometry and drawing blood samples for DNA isolation. Some of the affected family members underwent speech audiometry, vestibulo-ocular examination and high-resolution spiral computed tomography imaging of the temporal bones. Furthermore, concomitant disease, use of medication and any other possible causes of acquired hearing impairment were ruled out. Previous medical records and audiograms were traced for individual longitudinal analysis. The previously published audiograms of family E were also included.[11]

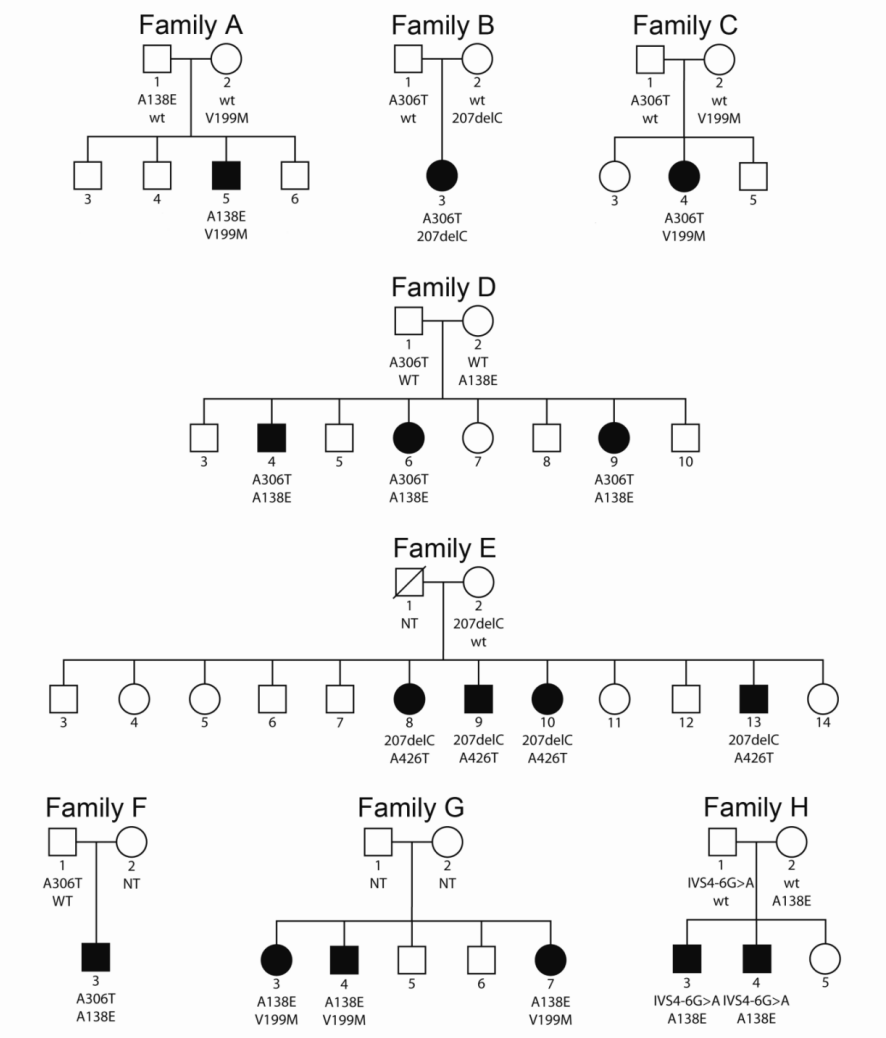


Figure 1. Pedigrees of the Dutch families with autosomal recessive hearing impairment and segregation of the *TMPRSS3* mutations. Family E is previously described by Cremers et al.[11] All unaffected sibs were either carrier of one mutant allele or of two wild-type alleles. Square: male; circle: female; open symbol: clinically unaffected; solid symbol: clinically affected; slash: deceased individuals. NT not tested.

Linkage analysis

Genomic DNA of all participating individuals was extracted from peripheral blood lymphocytes according to standard protocols. Families D and E were selected for linkage analysis. For family D, the unaffected parents, and three affected and five

unaffected siblings were genotyped using the Illumina 6k single nucleotide polymorphism (SNP) array according to the manufacturer's protocol. Similarly, for family E, the unaffected mother, and four affected and five unaffected siblings were genotyped. Multipoint linkage analysis was performed with GeneHunter version 2.1r5 in the Easy-Linkage software package.[12] An autosomal recessive mode of inheritance with a penetrance of 95% and a disease allele frequency of 0.001 were used for limit of detection (LOD) score calculations.

Sequence analysis and prediction of effects of mutations on protein structure

Amplification of all coding exons and flanking intronic sequences by PCR was performed on 40 ng of genomic DNA with Taq DNA polymerase (Roche). Primer sequences and PCR conditions are available in electronic supplementary material Table S1. PCR fragments were purified using NucleoFast 96 PCR plates (Clontech) according to the manufacturer's protocol. Sequence analysis was performed with the ABI PRISM Big Dye Terminator Cycle Sequencing V3.1 Ready Reaction kit and the ABI PRISM 3730 DNA analyzer (Applied Biosystems).

For segregation analysis of the identified *TMPRSS3* mutations in the families, restriction digestion could be performed. Relevant exons were amplified and PCR products were purified as described for sequencing. Digestion of the PCR products with restriction enzymes was performed according to the manufacturer's protocol and restriction fragments were analyzed on 2% agarose gels. Primer sequences and restriction enzymes are listed in Table S1. The same approach was used for testing the occurrence of the c.595G9A and c.1276G9A variants in Dutch control individuals. As reference sequence, NM_024022 was used.

The effects of the amino acid substitutions in *TMPRSS3* on its structure were analyzed using the Project HOPE web server (http://www.cmbi.ru.nl/hope).[13] The exact 3D structure of *TMPRSS3* is unknown; therefore, HOPE built a model based on the homologous structure protein data bank entry 1z8g.[14] The model was built using an automatic script in the Yasara and WHAT IF Twinset.[15, 16]

Audiometry and data analysis

Audiometric examination comprised conventional pure-tone audiometry in a sound-treated room. Air-conduction (AC) thresholds were measured in decibel hearing level (HL) at 0.25, 0.5, 1, 2, 4 and 8 kHz; bone-conduction thresholds were measured

in decibel HL at 0.5, 1, 2, 4 and 8 kHz. The individual 95th percentile threshold values of presbycusis in relation to the patient's sex and age were derived for each frequency using the ISO 7029 method. Individuals were considered affected if the best hearing ear showed thresholds beyond the 95th percentile threshold values for presbycusis at three frequencies or more. Binaural mean air-conduction threshold values were calculated for each frequency.

Individual longitudinal regression analysis of binaural mean air-conduction threshold values on age was performed for individual 10 of family E because the audiometric data of this individual were most comprehensive.[11] The regression coefficient (slope) was called annual threshold deterioration (ATD), expressed in decibels per year. Progression was significant if the 95% confidence interval of the ATD did not include zero.

Speech audiometry was performed under the aforementioned conditions using standard Dutch monosyllabic consonant–vocal–consonant word lists. The maximum phoneme recognition score (mean percentage correct for both ears) was retained from the monaural performance versus intensity curves. These maximum phoneme recognition scores were analyzed in relation to age and to pure-tone average (mean value for both ears) at frequencies of 1, 2 and 4 kHz ($PTA_{1,2,4\ kHz}$). Cross-sectional analysis was performed using linear regression analysis to determine the local average slope, called deterioration rate in the score-against-age plot and deterioration gradient in the score-against-$PTA_{1,2,4\ kHz}$ plot. A previously described group of subjects with only presbycusis was used as the reference group.[17]

Vestibulo-ocular examination

Nine of 16 affected individuals (A5, B3, C4, E8, E9, E10, E13 and G3) underwent vestibular and ocular motor tests. The test included evaluation of the vestibulo-ocular reflex using electronystagmography with computer analysis, and saccadic, smooth pursuit and optokinetic nystagmus responses. Vestibular stimulation comprised rotatory and caloric tests. Details and normal values have been described previously.[18]

Genotype-phenotype correlations

We compared the phenotype of the present families by focusing on the genotype to study whether the mutations differ in severity. The phenotypes of the different

families were compared when at least one identical mutation in *TMPRSS3* was present. Therefore, the effect of the second mutation on the phenotype in these families could be determined. All family members with the same compound heterozygous mutations were taken together, for example, families A/G and D/F. The threshold values of each family member were compared to the threshold values of every family member of the other family. We compared the thresholds at all frequencies in relation to age.

Evaluation of the effect of CI on phoneme recognition

Nine patients underwent cochlear implantation (CI). Speech audiometry was performed in a quiet environment using standard monosyllabic Dutch word lists after cochlear implantation. Words were presented through a loudspeaker at a fixed distance of 1 m from the patient at a normal conversational level of 70 dB sound pressure level (SPL). Subject responses were scored as the percentage of phonemes correct. As a reference, the test results after 1 year of use in postlingually implanted adults were used. The first reference group (n = 70) was implanted with a CII/HR90K device of Advanced Bionics® and the second reference group (n = 65) with a Nucleus 24RCA of Cochlear®. A mean phoneme score of 64% (SD 23%) was demonstrated in the first reference group and the second group (n = 65) showed a mean phoneme score of 72% (SD 18%).[19]

Results

Compound heterozygous TMPRSS3 mutations in Dutch arNSHI patients

Linkage analysis was performed for two arNSHI families, D and E, with progressive hearing impairment. For family D, five regions were found with a maximum LOD score of ~1.82. (Figure S1) Two of the regions contained a known deafness gene, namely, *GRXCR1* on chromosome 4 and *TMPRSS3* on chromosome 21. Mutations in *GRXCR1* were excluded in a previous study.[20] For family E, three regions with suggestive linkage were found with a LOD score of ~2.42. One of those regions harbored *TMPRSS3*. In both families, the LOD score for the *TMPRSS3* region reached the theoretical maximum LOD score.

Thus, mutation analysis was performed for all coding exons and exon–intron boundaries of *TMPRSS3*. In both families, compound heterozygous sequence variants

were detected. (Table 1) The variants c.413C9A, c.916G9A and c.207delC have been described before to be pathogenic in families with arNSHI. The c.1276G9A variant is present in the SNP database (http://www.ncbi.nlm.nih.gov/sites/SNP; rs56264519). However, this SNP has not been validated. We did not identify this variant in 590 Dutch control alleles and, importantly, the resulting amino acid change Ala426Thr has been shown to affect protein function.[21] We therefore considered the Ala426Thr substitution to be pathogenic. All four *TMPRSS3* mutations co-segregated with the hearing impairment in the families. (Figure 1)

To investigate the contribution of *TMPRSS3* mutations in progressive hearing impairment, we screened a panel of 22 unrelated supposed arNSHI patients with progressive hearing impairment; compound heterozygous mutations were found in four of them, belonging to families A, B, C, and F. (Figure 1 and Table 1) The novel variant, c.595G9A, leading to the substitution of a methionine for a valine at position 199 of the protein (p.Val199Met) was present in two patients. This variant was not found in 590 Dutch control alleles. In summary, we identified compound heterozygous *TMPRSS3* mutations in 6 of 24 families (25%) with progressive arNSHI.

To evaluate the prevalence of *TMPRSS3* mutations in unselected patients with (putative) arNSHI in the Netherlands, we screened a panel of 212 index patients for whom *GJB2* mutations had been excluded as the cause of their hearing impairment. Compound heterozygous mutations were detected in two of these index patients (Table 1), which segregated with the hearing impairment in the corresponding families G and H. (Figure 1) Hearing impairment was found to be progressive in these families as well.

Large variation in age of onset

The clinical characteristics and genotypes of the patients are listed in Table 1. For none of the affected family members, there was evidence of other causes of hearing impairment, and otoscopy was normal. All affected individuals reported progressive bilateral hearing impairment. Some individuals had prelingual hearing impairment, whereas other individuals were not aware of being hearing impaired until in their late teens, which indicates postlingual hearing impairment. (Table 1)

Family	*Family research number*	*Patient*	*Age of onset*	*Phenotype*	*Mutation*	*Protein change (predicted)*	*Protein domain*
A	W06-357	5	5 years	High-frequency hearing impairment with normal thresholds of the low frequencies until at least 12 years of age	c.413C>A c.595G>A	p.Ala138Glu p.Val199Met	SRCR SRCR
B	W06-418	3	Prelingual (2 years)	Downsloping audiogram configuration with impairment of the low frequencies at a very young age	c.207delC c.916G>A	p.Thr70fs p.Ala306Thr	truncation after TM serine protease
C	W06-445	4	Prelingual (2 years)	Downsloping audiogram configuration with impairment of the low frequencies at a very young age	c.595G>A c.916G>A	p.Val199Met p.Ala306Thr	SRCR serine protease
D	W02-068	4	7 years	Downsloping audiogram configuration with deterioration of the low frequencies after 11 years of age	c.413C>A c.916G>A	p.Ala138Glu p.Ala306Thr	SRCR serine protease
		6	16 years	High-frequency hearing impairment with normal thresholds of the low frequencies until at least 19 years of age			
		9	18 years				
E	W07-0050	8	17 years	Mainly high-frequency hearing impairment with deterioration of the low frequencies after approximately 30 years of age	c.207delC c.1276G>A	p.Thr70fs p.Ala426Thr	truncation after TM serine protease
		9	12 years				
		10	7 years				
		13	17 years				

F	W03-357	3	10 years	High-frequency hearing impairment with mild decline of the low frequencies at the age of 22 years	c.413C>A c.916G>A	p.Ala138Glu p.Ala306Thr	SRCR serine protease
G	W08-1756	3	15 years	Flat audiogram configuration with thresholds of about 110 dB at 49 years of age	c.413C>A c.595G>A	p.Ala138Glu p.Val199Met	SRCR SRCR
		4	33 years	Downsloping audiogram configuration with impairment of the low frequencies starting after 44 years.			
		7	21 years				
H	W10-1160	3	5 years	Downsloping audiogram configuration with deterioration of the low frequencies at 23 years of age	c.413C>A c.323-6G>A*	p.Ala138Glu p.Cys107fs	SRCR SRCR
		5	4years	High-frequency hearing impairment with normal threshold of the low frequencies until at least 16 years of age			

Table 1. Overview of the clinical characteristics and genotypes in the eight families. Family E is previously described by Cremers et al.[11]

* notation of the IVS4-6G>A mutation with NM_024022 as a reference sequence.

Imaging of temporal bones

Computed tomography (CT) of the temporal bone was performed in ten individuals (A5, C4, D4, E8, E9, E10, E13, F3, G3 and H3), mainly as part of a preoperative CI selection procedure. Magnetic resonance imaging (MRI) was accomplished in individual B3. The CT scans and MRI showed normal middle and inner ear structures in all cases.

Vestibulo-ocular examination

Although vestibular symptoms were not reported, testing of vestibular function in three affected individuals (A5, E8 and E9) revealed mild hyperreflexia of the velocity step responses with time constants between 28 and 32 seconds (normal limit: 26 s). Furthermore, individuals G3 and H3 showed significant hyporeflexia of the velocity step responses with time constants between 3 and 9 s for both nystagmus directions. Caloric tests revealed borderline bilateral caloric weakness in individual G3 and normal caloric functioning in individual H3. None of the relative prevalences of the abnormal vestibular findings was above chance level. The tests of vestibular function in four additional affected individuals (B3, C4, E10 and E13) demonstrated no abnormalities. The vestibulo-ocular examination in all nine affected individuals was performed before cochlear implantation. None of the patients reported by Bonne-Tamir et al.[3] showed signs of vestibular involvement. However, formal vestibular tests were not performed. Information on the vestibular function of the other *TMPRRS3* families previously reported were not available.

Typical ski-slope audiogram configuration

A representative selection of the available pure-tone audiograms of all 16 affected individuals are shown in Figure 2. The thresholds in families B and C (gray audiograms in Figure 2) are higher than or similar to the thresholds in the other families, at a much younger age. This is in accordance with the reported prelingual hearing impairment in these families. Most frequently, a down-sloping audiogram configuration was observed, indicating high-frequency hearing impairment. Remarkable is the typical ski-slope audiogram configuration for patients with postlingual hearing impairment, as seen in, for example, individuals A5, F3, D6 and H3. However, flatter audiogram configurations, close to residual hearing, were also observed (individuals D4, E8, E9, E10, E13 and G3), but only at ages above 28 years.

The families with postlingual hearing impairment initially presented with hearing impairment of the high frequencies which was followed by an increase of the low- and mid-frequency threshold values with advancing age.

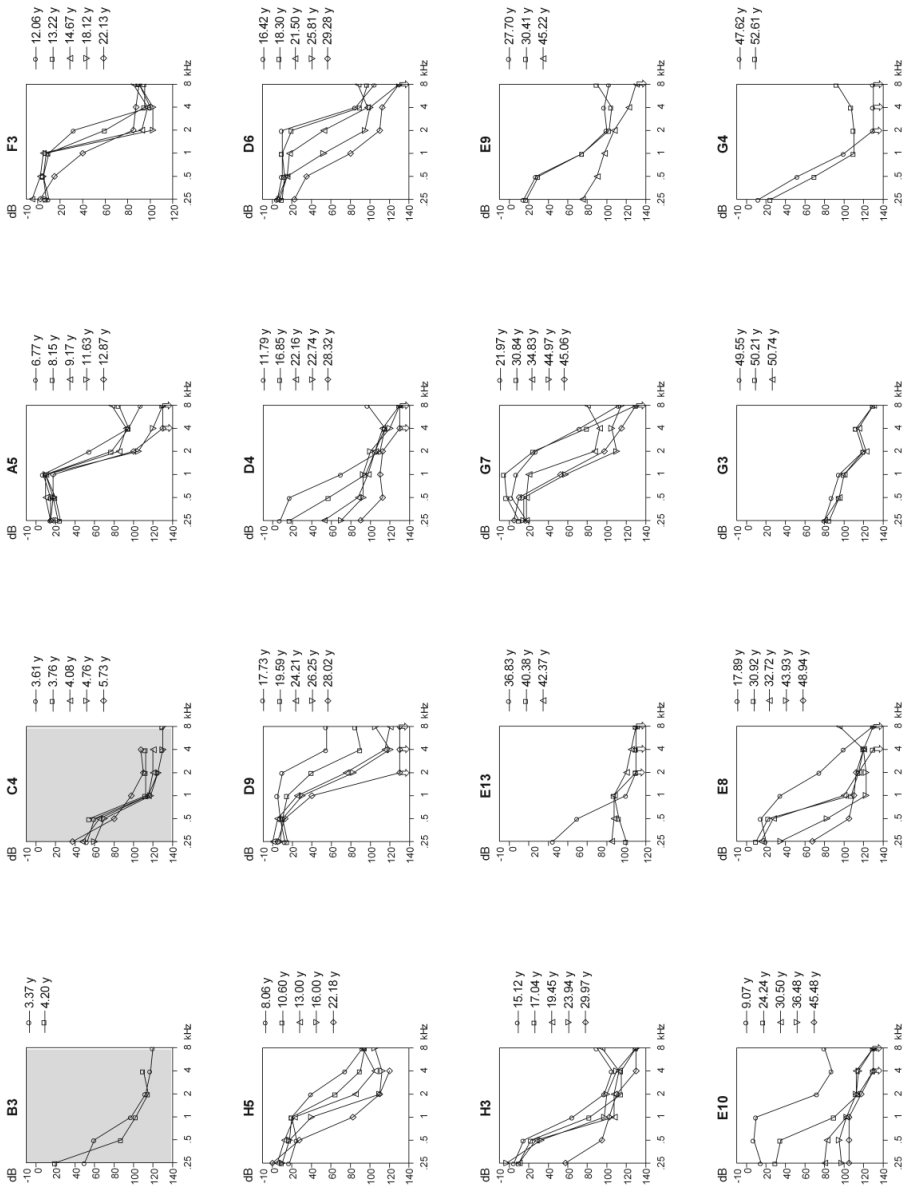


Figure 2. Selection of binaural mean air-conduction threshold values of clinically affected family members at different ages, ordered by age (from top left to bottom right) at last visit.[11] Family number and sequence number are above each audiogram. Gray background, relatively poorer thresholds.

The thresholds in individual D4 are exceptionally high at the low and mid-frequencies at ages between 21 and 28 years, as compared with the thresholds of individuals D6 and D9. The threshold of individual G3 at these frequencies are also remarkably high in comparison to the thresholds of individuals G4 and G7 at the same ages. So far, there is less variation in thresholds in families E and H at matching ages. However, it is not clear at this point whether the hearing impairment in individual H4 will show the same progression as the hearing impairment of his brother H3 until the age of 29 years.

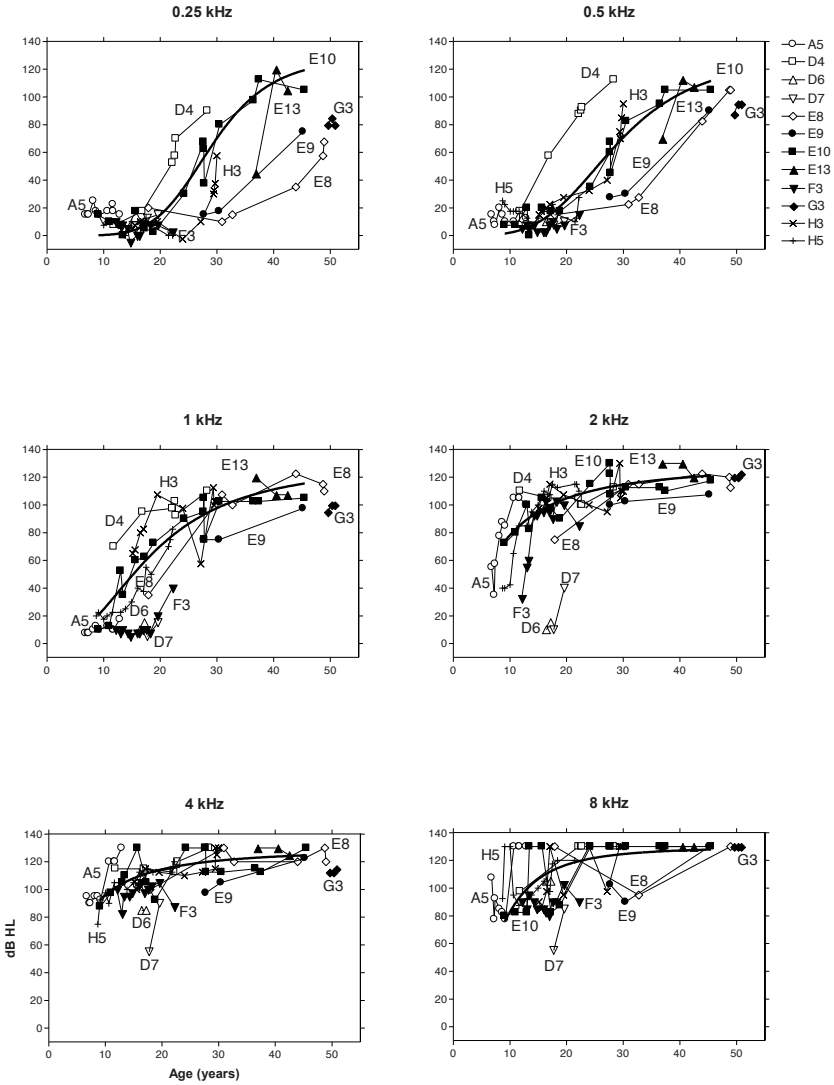
Progressive bilateral hearing impairment at all frequencies

Regression analysis for individual E10 demonstrated a significant deterioration of threshold levels with advancing age for all frequencies. The longitudinal threshold data of individual E10 covered approximately 36 years (from age 9 to 45 years). Figure 3a shows the threshold data for all frequencies separately. A nonlinear regression model of a dose-response curve with a variable slope fitted far better to the data than a linear regression model (comparative data not shown); the fitted curves are included in Figure 3a. The maximum local slope (ATD) was about 6 dB/year at 0.25 kHz at ages between 25 and 35 years. This maximum ATD can also be derived from the age related typical audiogram (ARTA; Figure 3b); the threshold at 0.25 kHz increases from about 40 dB at 25 years to about 95 dB at 35 years. Figure 3 illustrates that most of the progression at the higher frequencies, in the absence of a pronounced congenital hearing impairment, already occurred before the age of 10 years. If that indeed was the case in individual E10, the threshold levels at around 9 years of age would suggest an average progression of about 10 dB/year at 2–8 kHz. (Figure 3a-b)

Figure 3a also shows all the longitudinal threshold data of individuals A5, D4, D6, D7, E8, E9, E13, F3, G3, H3 and H5 (postlingual DFNB8 patients; see below). The available audiograms of individual A5 were measured between 6 and 13 years of age. The hearing impairment in this individual indeed demonstrated progression only at 2–8 kHz, which is on the order of about 10 dB/year. Figure 3a also demonstrates that the longitudinal data for individual E10 are, more or less, representative for the whole group of DFNB8 patients in the present collection of families. The sigmoidal regression curve for individual E10 takes a fairly median

position; however, some of the other individuals are represented by threshold values that indicate major deviations from that position. (Figure 3a)

a.



b.

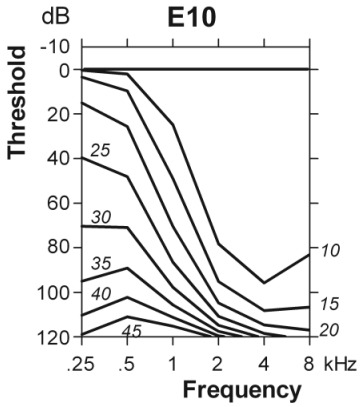


Figure 3. **a** Longitudinal individual measurements of individuals A5, D4, D6, D7, E8, E9, E10, E13, F3, G3, H3 and H5 for each frequency separately (different symbols for each individual).[11] A dose-response curve with a variable slope could be obtained for the longitudinal data of individual E10 for each frequency (bold line). **b** ARTA derived from a longitudinal regression analysis of mean AC threshold levels of individual E10. Italics indicate age in years.

Relatively good speech recognition scores

Figure 4 shows the available single-snapshot measurements of the phoneme scores in the affected members of families A, D, E, F, G, and H. For the more severely affected families B and C (see below), we only had one score for individual C4. Speech recognition in the patients with a *TMPRSS3* mutation was severely impaired, even at young ages. The 50% score was attained at the age of 25 years, whereas in the presbycusis group, this score was attained at 89 years. At approximately 55 years of age, there was no remaining speech recognition. The $PTA_{1,2,4\ kHz}$ levels at which speech recognition scores of 50% were attained in the *TMPRSS3* and the presbycusis patients were 95 and 87 dB, respectively. Therefore, the *TMPRSS3* patients tended to have somewhat higher scores than the presbycusis patients at similar $PTA_{1,2,4\ kHz}$ levels. The deterioration rate in the score-against-age plot was 1.6% per year and the deterioration gradient in the score-against-$PTA_{1,2,4\ kHz}$ plot was 2.1% per decibel. The presbycusis group showed a higher deterioration rate (3.3% per year) and a lower deterioration gradient (1.1% per decibel). Compared with matching scores of the other family members, the speech recognition score of individual C4 was observed at a younger age and a higher $PTA_{1,2,4\ kHz}$ level. (Figure 4)

Unfortunately, speech recognition scores were not reported for previously described families with *TMPRSS3* mutations.

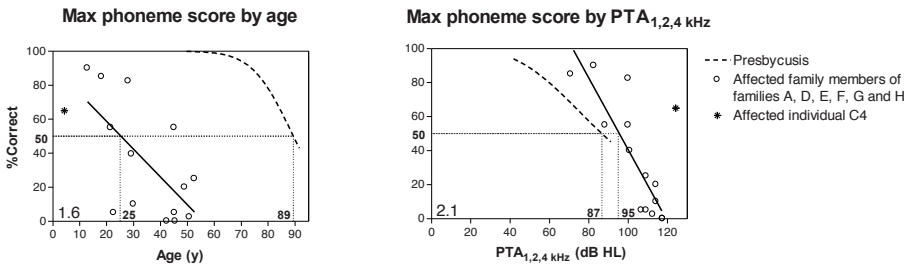


Figure 4. Single-snapshot measurements of the affected family members of binaural mean phoneme recognition scores against age (left) and against binaural mean pure-tone average at 1, 2 and 4 kHz (right).[11] The solid regression line covers the cross-sectional analysis. The dotted curve represents presbycusis and was previously established for patients with presbycusis. See text for the meaning of the figures, and the straight horizontal and vertical lines.

Genotype–phenotype correlations

In the present eight families, four different missense mutations and two frameshift mutations were detected. Four of these mutations were recurrent and present in two to four of the eight families. To study whether the mutations differ in severity, we compared the phenotypes of the families with one identical mutation in *TMRPSS3*. This allows a comparison of the effect of the second mutation in these families. For example, patients in families B and E have the p.Thr70fs mutation in common (Table 1); therefore, a comparison of the phenotypic effect of the p.Ala306Thr mutation in family B and the p.Ala426Thr mutation in family E is possible. Thresholds in family B at age 3–4 years are poorer than those in family E at ages between 9 and 30 years, but fairly similar to those in family E at age 24–37 years and better than those in family E at age 36–49 years. (Figure 2) This implies that threshold levels similar to those shown by family B in the first decade of life are unlikely to be found in family E before the third or fourth decade of life and that poorer levels are only likely to be found in family E from the fourth to the fifth decade of life onwards. This suggests that the p.Ala426Thr mutation has a milder effect than the p.Ala306Thr mutation.

The same procedure was repeated for the other mutations. The p.Ala306Thr mutation has a fairly similar phenotypic effect as p.Val199Met (families A/G versus D/F) and p.Cys107fs (families D/F versus family H), whereas p.Val199Met has a fairly similar effect as p.Thr70fs (family B versus C) and p.Cys107fs (families A/G versus family H): this suggests that also p.Ala306Thr and p.Thr70fs must have fairly similar phenotypic effects. Both p.Ala426Thr and p.Ala138Glu have milder phenotypic effects than p.Ala306Thr (family B versus E and family C versus families A/G, respectively); this suggests that p.Ala426Thr and p.Ala138Glu have fairly similar phenotypic effects. Furthermore, p.Ala138Glu has a milder effect than p.Val199Met (family C versus families D/F) and also a milder effect than p.Thr70fs (family B versus families D/F).

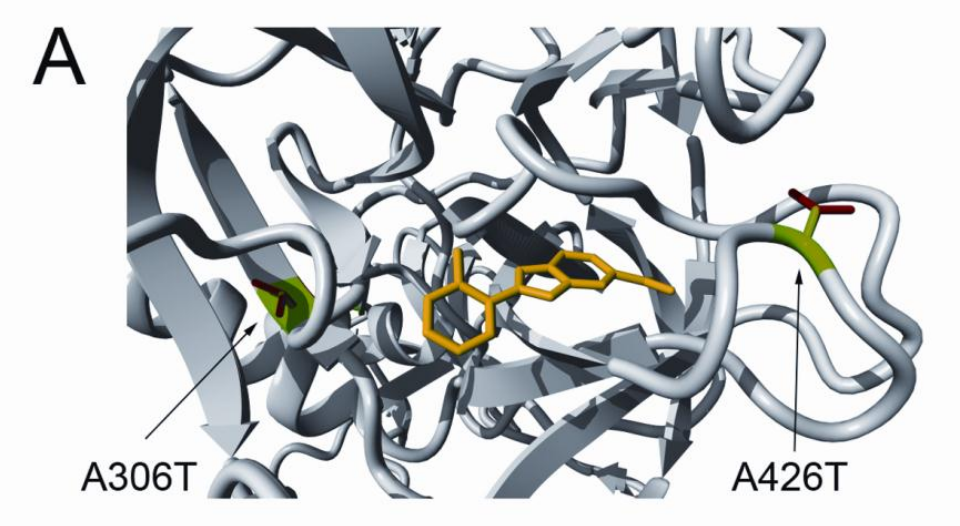
The thresholds of individuals D4 and G3 were higher compared with the thresholds of their sibs, and therefore individuals D4 and G3 were excluded from the genotype-phenotype assessment. Nevertheless, the classification of mutations did not change when individuals D4 and G3 were included. In line with these considerations and the levels of the thresholds in the various families, we suggest that the mutations in *TMPRSS3* can be classified in mild and severe mutations. Our classification of *TMPRSS3* mutations is in accordance with the two types of hearing impairment phenotypes:

1. DFNB10: a severe congenital or early childhood type with prelingual hearing impairment (families B and C) caused by the presence of two severe mutations
2. DFNB8: a later-onset progressive but initially milder type with postlingual hearing impairment (families A/G, D/F and E) caused by the presence of one mild and one severe mutation

Predicted effects of amino acid substitutions on TMPRSS3 structure

Genotype–phenotype correlations in the present study suggest that the amino acid substitutions p.Ala138Glu and p.Ala426Thr have a less severe effect as compared with p.Val199Met and p.Ala306Thr. Both substitutions p.Ala306Thr and p.Ala426Thr are within the serine protease domain. These changes result in the substitution of a larger residue for a conserved amino acid, predicted to result in the destabilization of the protein. The difference in severity of the effect of the two mutations might well be explained by the location of the substituted residue. The

p.Ala306Thr is located close to one of the active residues, Asp304, and therefore this substitution can be predicted to directly disturb the function of the serine protease domain. Moreover, the alanine at position 426 is predicted to be semi-buried in the protein and the side chain of threonine is only slightly bigger than the alanine side chain. Therefore, there could be enough space for the side chain of the threonine at this position. (Figure 5a) Both substitutions p.Ala138Glu and p.Val199Met occur within the scavenger receptor cysteine-rich (SRCR) domain, which is thought to be involved in interactions with extracellular molecules. Also, these two mutations affect evolutionary conserved residues and substitute a larger amino acid for the wild-type residue, which is predicted to cause steric clashes with other residues and hence to destabilize the protein. (Figure 5b) The 3D modeling could not directly explain the difference in the effect of p.Ala138Glu and p.Val199Met mutations, since the exact function and binding partners of the SRCR domain are still unknown.



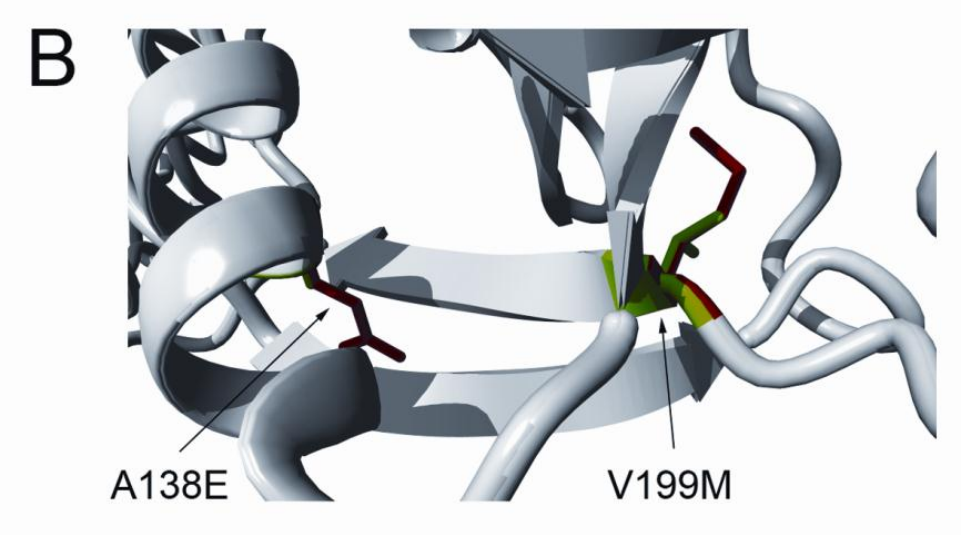


Figure 5. Molecular modeling for *TMPRSS3* missense mutations. Graphic representation of the effect of the p.Ala306Thr and p.Ala426Thr mutations in the serine protease domain (A) and of the p.Ala138Glu and p.Val199Met in the SRCR domain (B). The wild-type residues are depicted in green, while the mutant residues are shown in red. The yellow structure represents a substrate for the serine protease domain (figure made using the model for TMPRSS and YASARA).

Good speech reception after cochlear implantation

Eight family members underwent cochlear implantation in our hospital. Implantation was uneventful and the implant was fully inserted on the left side in all cases. Individuals B3, C4 and H3 were implanted with a Nucleus Freedom (Cochlear), individuals E8 and G3 with a Nucleus Contour CI24R (Cochlear), and individuals E9, E10 and E13 with a Clarion AB-5100H (Advanced Bionics). Individual 4 of family D was implanted in another hospital with a Nucleus CI24M (Cochlear) and complete insertion was reported. Phoneme and word scores were unavailable for individuals D4 and H3. Individual H3 was implanted less than a year ago. The available phoneme and word scores of sound presented at 70 dB SPL 1 year after activation of the speech processor are presented in Table 2. We compared these results with the results of our two reference groups 12 months after use. The phoneme and word scores in the seven patients were above the average phoneme and word scores of our two reference groups. The mean phoneme score of 84.1%

(SD = 5.4) is significantly higher than the phoneme score of reference group 1 (p = 0.0001) and reference group 2 (p = 0.0005). Elbracht et al.[22] also reported good results with bilateral cochlear implantation, but unfortunately, there were no phoneme or word scores available to compare with our results.

Individual	*Preoperative*	*Postoperative*	
	Phoneme score	*Phoneme score*	*Word score*
B3	-	91%	-
C4	-	80%	-
D4	5%	-	-
E8	20%	89%	75%
E9	5%	76%	60%
E10	0%	82%	58%
E13	0%	83%	62%
G3	2.5%	88%	68%
H3	10%	-	-

Table 2. Available phoneme and word scores preoperative and one year after activation of the speech processor of the cochlear implant. Sound was presented at 70 dB sound pressure level.

Discussion

This report presents the clinical and genetic analyses of eight Dutch DFNB8/10 families with compound heterozygous mutations in *TMPRSS3*. (Table 1) Our study suggests genotype-phenotype correlations for the detected mutations.

Phenotype of TMPRSS3 mutations

The reported age of onset and severity of hearing impairment in the present families showed a wide variation. Also within the families, variation was seen. This is frequently demonstrated in hereditary hearing impairment, however mainly in the dominantly inherited forms. At a young age, *TMPRSS3*-associated hearing impairment was more pronounced at the high frequencies, and sooner or later, depending on the mutation, thresholds for the low frequencies deteriorated,

eventually resulting in a flatter audiogram configuration (i.e. residual hearing). Prelingual as well as postlingual hearing impairments were reported. A ski-slope audiogram configuration was indicative for postlingual hearing impairment.

Twenty-two families with a mutation in *TMPRSS3* had been reported previously.[3, 4, 10, 22-29] (Table 3) Since the clinical data provided for most of these *TMPRSS3* families are very limited, a thorough comparison with our data is not possible. However, the available clinical data seem to be in line with those of the present families. All families with the *TMPRSS3* mutations described in the literature showed either severe to profound prelingual (DFNB10) or postlingual (DFNB8) progressive bilateral sensorineural hearing impairment.[3, 4, 10, 22-28]

Genotype-phenotype correlations

In the homozygous state, the previously described *TMPRSS3* mutations lead to either postlingual progressive (DFNB8) or prelingual severe to profound (DFNB10) hearing impairment (Table 3), with one exception, namely the p.Pro404Leu mutation.[25, 26] Although, Wattenhofer et al.[26] described an age of detection of hearing impairment of 6-7 years in a family with a homozygous p.Pro404Leu mutation, the average threshold levels (0.5-4 kHz) at that age were already 85-99 dB. Since members of this family were reported to have had a normal hearing until the age of detection, it can be concluded that hearing impairment in this family exhibited a very fast progression in childhood. Wattenhofer et al.[26] did not report on the speech development of the affected family members and hearing impairment might still be classified as prelingual, which is defined as a delayed speech development. Hearing impairment in the second family with a homozygous p.Pro404Leu mutation was reported to be congenital and severe to profound, and therefore probably prelingual.[25] Based on these data, we conclude that p.Pro404Leu is likely to be a severe mutation. Modifying genetic factors may explain the difference in onset between the two families with this mutation.

Family	*Mutation*	*Protein domain*	*Age of*	*Phenotype*
Pakistani family[4]	**p.Cys107fs**[30]	SRCR	10-12 years	By the age of 14-16 years: profound bilateral hearing impairment of all frequencies. The maximum threshold: 105 dB at 1.000 Hz.
British family[28]	**Ala138Glu**	SRCR	5 years	Progressive moderate to severe downsloping hearing impairment.
German family[22]	**Arg216Cys**; *Ala306Thr*	Serine protease	6 years	Severe to profound hearing impairment. Progression to profound hearing impairment by about 20 years.
Turkish family[26]	*Pro404Leu*	Serine protease	6-7 years	Severe to profound hearing impairment.
Palestinian family[3]	*insertion of β-satellite repeats*[30]	Serine protease	Congenital	Severe to profound hearing impairment. Minimum threshold: >75-80 dB. No progression.
Pakistani family[29]	*Glu330fs*	Serine protease	Prelingual	Severe to profound bilateral hearing impairment.
Pakistani families[27]	*Thr70fs*	Truncation after TM	Congenital	Profound hearing impairment.
Spanish family[26]	*Thr70fs*	Truncation after TM	Prelingual	Severe to profound hearing impairment.

Greek family[10]	*Thr70fs; Asp103Gly*	TM; LDLRA	Prelingual	Severe to profound hearing impairment.
Newfoundland family[27]	*Thr70fs; c.782+8insT**	TM; serine protease	Prelingual	Severe to profound hearing impairment.
Pakistani family[24]	*Arg109Trp*	SRCR	Congenital	Profound hearing impairment.
Pakistani families[24, 27]	*Cys194Phe*	SRCR	Congenital	Profound hearing impairment.
Palestinian family[23]	*Cys194X*	Serine protease	Prelingual	Bilateral hearing impairment.
Turkish family[26]	*Arg216Leu*	Just before serine protease	Congenital-1.5 years	Severe to profound hearing impairment.
Tunisian family[25]	*Trp251Cys*	Serine protease	Congenital	Severe to profound hearing impairment. Minimum threshold: >70 dB at 0.5-2 kHz.
Turkish family[26]	*Gln398X*	Truncation of serine protease	Congenital	Severe to profound hearing impairment.
Tunisian family[25]	*Pro404Leu*	Serine protease	Congenital	Severe to profound hearing impairment. Minimum threshold: > 70 dB at 0.5-2 kHz.
Pakistani families[24, 27]	*Cys407Arg*	Serine protease	Congenital	Profound hearing impairment.

Table 3. Overview of the literature on mutations in the *TMPRRS3* gene. The grey rows represent families with postlingual hearing impairment (DFNB8) and the blank rows the families with prelingual hearing impairment (DFNB10). The mutations in bold are the mutations with a mild effect on the phenotype and mutations in italic are the mutations with a more severe effect on the phenotype.

Both in the present and previously described families, the severe classified mutations p.Ala306Thr, p.Val199Met and p.Thr70fs result in postlingual hearing impairment when present in combination with mutations only described to be associated with postlingual hearing impairment (p.Arg216Cys, p.Ala138Glu and p.Ala426Thr mutations). Prelingual hearing impairment is seen by us and others when the p.Ala306Thr, p.Val199Met and p.Thr70fs mutations are present in the compound heterozygous state with other mutations associated with prelingual hearing impairment (p.Thr70fs and p.Ala306Thr). However, there is an inconsistency in this classification of the c.323-6G9A (p.Cys107fs) mutation. According to our classification, the c.323-6G9A mutation is (relatively) severe, which means that a homozygous c.323-6G9A mutation could be expected to result in prelingual (DFNB10) hearing impairment. However, a homozygous c.323-6G9A mutation has been described to be the underlying cause of postlingual (DFNB8) hearing impairment by Veske et al.[4] When the Berkeley Drosophila Genome Project Splice Site Prediction Program (http://www.fruitfly.org/seq_tools/splice.html) was used, the c.323-6G9A mutation is predicted to introduce a novel splice acceptor site with a score of 0.96 (on a scale from 0 to 1) in addition to the normal splice acceptor site (score of 0.94). Therefore, both normal and abnormal splicing may occur in relative amounts of transcripts that vary between individuals, which may then lead to phenotypic variation. Testing this hypothesis on patient samples is not possible since the level of transcription of *TMPRSS3* is low in blood cells.

We can conclude that our study, combined with previous data, suggests the classification of *TMPRSS3* mutations into relatively mild and severe, which are associated with DFNB8 (postlingual hearing impairment) or DFNB10 (prelingual hearing impairment), respectively. Furthermore, our study suggests that compound heterozygosity for a mild and severe mutation leads to postlingual hearing impairment. The intrafamilial variation indicates that these data do not allow prediction of the phenotypic outcome for individual cases. Analysis of more families is necessary to confirm our conclusions and to address whether a subclassification of the mutations associated with DFNB8 is possible. Also, other (epi)genetic and nongenetic factors are likely to influence the severity of the phenotype. This has to be considered especially for families with a single affected individual. For the

families of this type in our study, these factors did not seem to have a major effect since the phenotype corresponded to the previously reported phenotype of patients with the same mutations.

Conclusion

In patients with progressive hearing impairment and a possible autosomal recessive mode of inheritance, *TMPRSS3* mutations should be considered. The ski-slope audiogram configuration is suggestive for mutations in this gene. The age of onset and the rate of progression are variable. Our analyses suggest that mutations in *TMPRSS3* can probably be classified as mild and severe mutations according to their phenotypic effect. Furthermore, our results demonstrate that cochlear implantation is a good treatment option for patients with *TMPRSS3* mutations since satisfactory speech reception was observed.

Acknowledgement

We thank the families for their participation. This work was financially supported by the Heinsius Houbolt foundation, RNID (GR36), Fonds NutsOhra (Project SNO-T-0702-102), EUROHEAR (LSHG-CT-2004-512063), The Oticon Foundation (09–3742), and ZonMW (40-00812-98-09047). We would like to thank Mrs. W. van Drunen for support in collecting families.

Supplemental table and figure

Fragment	Oligonucleotides	Size (bp)	Annealing Temperature (°C)	
Primers for PCR and sequence analysis				Enzyme
TMPRSS3 exon 2	Forward: accgtatgaccaagatgcac Reverse: tctagggaagtgcaggtgtc	361	57	
TMPRSS3 exon 3	Forward: tagagaatgtgccccttgg Reverse: tgctgggatgagagggg	365	57	
TMPRSS3 exon 4	Forward: ggggacagttgttagtgttgc Reverse: aagggtcagggttggcttc	249	57	
TMPRSS3 exon 5	Forward: tgcctatggtctcagggttc Reverse: cgttaaagcacccaatagtgc	286	57	
TMPRSS3 exon 6	Forward: acatcccccatgtacaatcc Reverse: catcacaaatccagcaggtg	293	57	
TMPRSS3 exon 7	Forward: gaccaatgttgagttcagcc Reverse: agccacattgtccaggatac	674	57	
TMPRSS3 exon 8	Forward: cccttgcagcacttgtcttag Reverse: cttctcaccacccaaagcag	395	57	
TMPRSS3 exon 9	Forward: ttcaggatacctgaggtcaatg Reverse: caactgatgccaacaccaac	400	57	
TMPRSS3 exon 10	Forward: tcctcagaggcagaagcatag Reverse: cccatgggaacatcacaatg	279	57	
TMPRSS3 exon 11	Forward: tgttgcgacacaccagagag Reverse: cttgagcaaatttcttctccac	400	57	
TMPRSS3 exon 12	Forward: gtccacagaaagcaatctcg Reverse: agcacaagcgtctgacacc	380	57	
TMPRSS3 exon 13	Forward: gtcatcatgttggacggatg Reverse: agacccctggagagaaaacc	663	57	
Primers for PCR of products used for restriction analysis				
TMPRSS3 exon 4	Forward: gaaacaggctgctgacagg Reverse: cagctcgatacacttaaaggatg	204	57	MaeIII
TMPRSS3 exon 5	Forward: tgcctatggtctcagggttc Reverse: cgttaaagcacccaatagtgc	286	57	MwoI
TMPRSS3 exon 7	Forward: gtgtgacctcatcctcatgg Reverse: agagtgatgggacatcatgg	483	57	PmlI
TMPRSS3 exon 9	Forward: tttccctgttggacaatcc Reverse: gcaaatcctcttgaaacaaag	186	57	MslI
TMPRSS3 exon 12	Forward: gtccacagaaagcaatctcg Reverse: agcacaagcgtctgacacc	380	57	HhaI

Table S1. Primer sequences, PCR conditions and restriction enzymes. Reference sequence: NM_024022 transmembrane protease, serine 3 isoform 1.

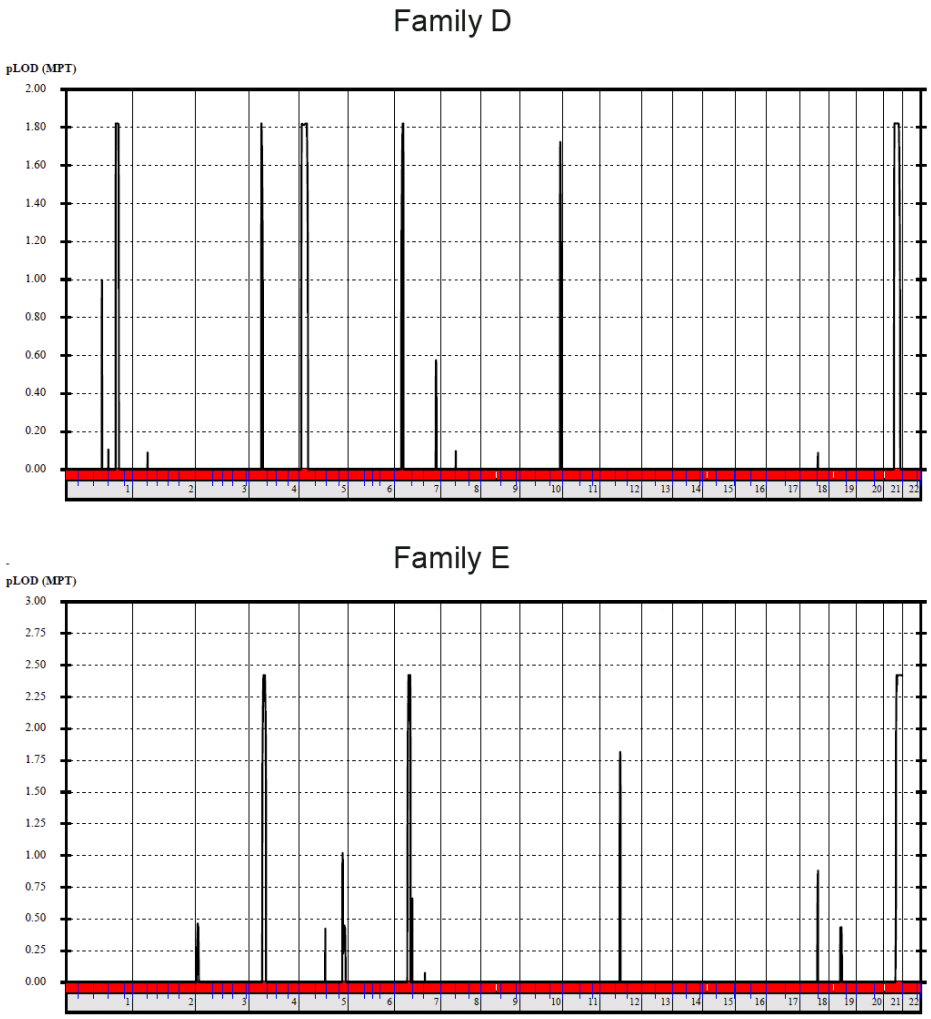


Figure S1. Result of linkage analysis for families D and E. Multipoint linkage analysis was performed with GeneHunter version 2.1r5 in the EasyLinkage software package. An autosomal recessive mode of inheritance with 95% penetrance and a disease allele frequency of 0.001 were used for linkage analysis.

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4.1

Variable degrees of hearing impairment in a Dutch DFNX4 (DFN6) family

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Hearing Research 2011; 282: 167-177

Abstract

In the present study, we investigated the audiometric characteristics of a large Dutch DFNX4 family with a p.Glu72X mutation in the *SMPX* gene. Sixty family members participated in this study and examination consisted of medical history, otoscopy, pure-tone and speech audiometry. Linkage and mutation analysis revealed a pathogenic mutation in the *SMPX* gene.

All 25 mutation carriers exhibited hearing impairment, except one woman aged 25 years. The men (n = 10) showed more severe hearing impairment than the women (n = 14) and already at a younger age. The age of onset according to history was 2-10 years (mean: 3,3 years) in men and 3-48 years (mean: 26,4 years) in women. In the men, severe threshold deterioration mainly occurred during the first two decades of life, especially at the higher frequencies. The women showed milder threshold deterioration, and more pronounced across-subjects and individual inter-aural variation, especially at 2-8 kHz. Longitudinal linear regression analysis demonstrated significant progression of at least two frequencies in five individuals (3 men and 2 women).

The speech recognition scores of the mutation carriers with hearing impairment were decreased at relatively young ages compared to a reference group of patients with only presbycusis, especially in men. However, all these patients tended to have better speech recognition scores than the presbycusis patients at matching $PTA_{1,2,4\ kHz}$ levels.

This study demonstrates the phenotypic heterogeneity in this large family with an X-linked pattern of inherited sensorineural hearing impairment. The men showed more severe hearing impairment at a younger age with more pronounced progression during the first two decades of life, while women demonstrated less severe hearing impairment with more gradual progression, and a wider variation in age of onset, degree of hearing impairment and inter-aural asymmetry in thresholds.

Introduction

Hearing impairment is the most common birth defect and the most prevalent sensorineural disorder in developed countries.[1] More than 50% of prelingual hearing impairment has a genetic cause and more than 70% of hereditary hearing impairment is nonsyndromic.[2] Only 1-5% of cases with nonsyndromic hereditary hearing impairment exhibit X-linked inheritance. In syndromic hearing impairment X-linked inheritance is seen far more often.[3]

X-linked nonsyndromic hearing impairment (DFNX) can be either pre- or postlingual with an age of onset varying from congenital (DFNX2 and DFNX3) to childhood (DFNX4). The type of hearing impairment is sensorineural, except for DFNX2 that shows mixed hearing impairment. In most patients hearing impairment is progressive and severely affects all frequencies.[4]

DFNX4, formerly designated DFN6, was first documented as bilateral high-frequency hearing impairment in a Spanish family with ten affected male and seven affected female individuals. Hearing impairment in male mutation carriers started between the age of five and seven years, and progressed before adulthood to severe or profound hearing impairment across all frequencies. Female carriers demonstrated more variable expression and incomplete penetrance of about 70%. Seven out of ten female carriers exhibited moderate to severe hearing impairment at the high frequencies with an onset in the fourth decade of life. Vestibular function was reported to be normal.[5]

The DFNX4 locus mapped to Xp22 in a 15 cM interval.[6] Recently, a nonsense mutation (p.Gly59X) in the *SPMX* gene was identified in the previously described patients from the Spanish DFNX4 family.[6, 7] Another nonsense mutation (p.Glu37X) in *SMPX* was detected in a large German DFNX4 family.[7] Here, we report the clinical features of a large Dutch DFNX4 family with a c.214G>T (p.Gly72X) mutation in the *SMPX* gene. A more detailed description of the genetic analysis of this family is reported elsewhere.[8]

Patients and methods

Patients

A Dutch family with sensorineural hearing impairment was studied and the pedigree was constructed. (Figure 1) The pattern of inheritance suggested X-linked inheritance. After informed consent, the family was clinically as well as genetically studied. The study was approved by the local medical ethics committee of the Radboud University Nijmegen Medical Centre, the Netherlands.

All participating family members were invited to the outpatient clinic. Examination of the participants included medical history guided by an questionnaire, otoscopy and pure-tone audiometry. A number of mutation carriers with hearing impairment also underwent speech audiometry and vestibulo-ocular examination. Attention was paid to the presence of syndromic features. Furthermore, concomitant disease, the use of medication and any other possible cause of acquired hearing impairment were identified. Previous medical records and audiograms were retrieved for individual longitudinal analyses.

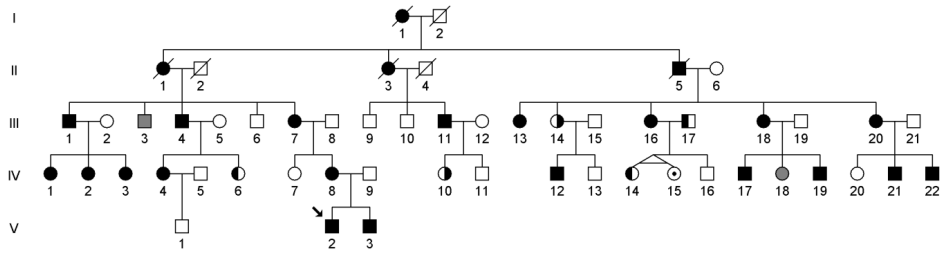


Figure 1. Pedigree of a Dutch family with X-linked inheritance of sensorineural hearing impairment. Square: male; circle: female; open symbol: no hearing impairment; solid symbol: hearing impairment; half-shaded symbol: unilateral hearing impairment; grey symbol: phenocopy; slash: deceased individuals. Person IV:15 is indicated as a non-manifesting mutation carrier.

Pure-tone audiometry

Audiometric examination comprised conventional pure-tone audiometry in a sound-treated room according to common clinical standards. Air-conduction (AC) and bone-conduction (BC) thresholds were measured in dB hearing level (HL) at 0.25,

0.5, 1, 2, 4 and 8 kHz. BC thresholds were measured to exclude conductive hearing impairment. The individual 95th percentile (P95) threshold values of presbycusis in relation to the patient's sex and age were derived for each frequency using the ISO 7029 method.[9] Individuals were considered affected if at least one ear showed threshold values beyond the P95 threshold for presbycusis at three or more frequencies.

Regression analysis of audiometric data

Analysis of audiometric data was performed on AC threshold values of mutation carriers with hearing impairment. The key analysis parameter for threshold-on-age regression analysis was the binaural mean AC threshold. The men showed non-linear progression of hearing impairment and an arbitrary equation of a saturation hyperbola was used for non-linear regression analysis using Prism software (GraphPad, San Diego):

$$Y = B_{max} * ((age - onset)/(K_d + (age - onset)))$$

where Y = threshold (dB HL), Bmax = saturation level (dB HL), onset = onset age (y); Kd is age (y) at half saturation for onset = 0, for onset > 0 half saturation occurs at (age – onset) = Kd and thus age = onset + Kd. We also used the option offered by Prism to find out which of the two different regression models, in this case the present non-linear model and a simple linear regression model, fits best to a given set of data. In addition, evaluation of the distribution of residuals from regression around the fitted line or curve was performed to evaluate whether these showed a pseudo-random distribution rather than any systematic deviations. Progression of hearing impairment was more gradual in women and linear regression analysis was applied. The regression coefficient (slope) is called annual threshold deterioration (ATD), expressed in dB per year. Age Related Typical Audiograms (ARTAs) were derived for men and women by using the results of regression analyses as described by Huygen et al.[10]

Individual longitudinal linear regression analysis of binaural mean AC threshold values on age was only performed in hearing impaired mutation carriers with five or more consecutive measurements and an overall follow-up period of at least five

years. Again, the ATD was calculated. Progression was significant if the 95% confidence interval did not include zero.

Across-subjects variation was appreciated from the threshold-on-age regression plots. Individual inter-aural (I-A) variation was evaluated by using the I-A standard deviation (SD) in AC threshold for each separate frequency. Plots of the SD against age were inspected to assess whether this type of variation depended on age. Furthermore, we studied the parameter behavior of the individual I-A difference in more detail by using the absolute value of the I-A differences |Difference|. This difference was plotted against the individual binaural mean threshold for each frequency separately. The across-subjects mean and the SD of the individual I-A difference were calculated (GraphPad, San Diego).

Speech audiometry and data analysis

Speech audiometry was performed in a sound-treated room according to common clinical standards using standard Dutch monosyllabic consonant-vocal-consonant word lists. The maximum phoneme recognition score (mean value averaged for both ears) was obtained from monaural performance versus intensity curves. These maximum phoneme recognition scores were analyzed in relation to age and to pure-tone average (mean value for both ears) at 1, 2, and 4 kHz ($PTA_{1,2,4\ kHz}$). Cross-sectional analysis was performed using linear regression analysis and nonlinear regression analysis (a sigmoidal dose-response curve with a variable slope) to fit the phoneme recognition scores of the mutation carriers with hearing impairment. The average tangent slope around the inflection point of the curve was called deterioration rate in the score-against-age plot and deterioration gradient in the score-against-$PTA_{1,2,4\ kHz}$ plot. A previously described group of subjects with only presbycusis (P50) was used as a reference group.[11]

Vestibulo-ocular examination and data analysis

Five of the 24 mutation carriers with hearing impairment underwent vestibular and ocular motor tests including evaluation of the vestibulo-ocular reflex, using electronystagmography with computer analysis, and saccadic, smooth pursuit and optokinetic nystagmus responses. Vestibular stimulation comprised rotatory and caloric tests. Test conditions and normal values have been described previously.[12]

Results

Family members

A five-generation pedigree was established for the present family, suggesting an X-linked pattern of inheritance. (Figure 1) We identified 28 affected family members by history and 24 of them were alive. Sixty family members participated in this study. Next generation sequencing (NGS) of X-chromosomal genes was performed and a nonsense mutation, c.214G>T, in exon 4 of the *SPMX* gene was detected. This mutation introduces a premature stop codon and is predicted to result in a truncated protein after residue 71 (p.Glu72X). Furthermore, the mutation segregated with the hearing impairment in the family. Genetic analysis identified 25 mutation carriers.[8]

Individuals III:3 and IV:18 showed hearing impairment but did not carry the mutation and were excluded from further regression analyses. Hearing impairment of individual III:3 was less severe than usual in this family and started much later, at an age of 60 years. Therefore, his hearing impairment may have been caused by reported noise exposure. The audiogram of individual IV:18 showed a mild threshold elevation at 0.25-1 kHz. The cause of her hearing impairment could not be identified. Individual IV:15 showed no signs of hearing impairment, but did carry the mutation. She was 25 years of age at the last visit and may still develop hearing impairment.

The mutation carriers with hearing impairment showed no evidence of other causes of hearing impairment. Otoscopy was normal in all family members. Most affected family members reported bilateral progressive hearing impairment. First symptoms of hearing impairment were reported by men (n = 10) at ages ranging from 2 to 10 years with a mean onset age of 3.3 years. Women (n = 14) reported first symptoms of hearing impairment at ages ranging from 3 to 48 years with a mean onset age of 26.4 years.

Vestibular function

Two of the 24 mutation carriers with hearing impairment (individuals IV:3 and IV:8) reported varying vestibular symptoms, including dizziness and instability, especially in the dark. The other family members mentioned no vestibular symptoms. Vestibular examination in individuals IV:3, IV:8 and V:2 revealed no abnormalities at

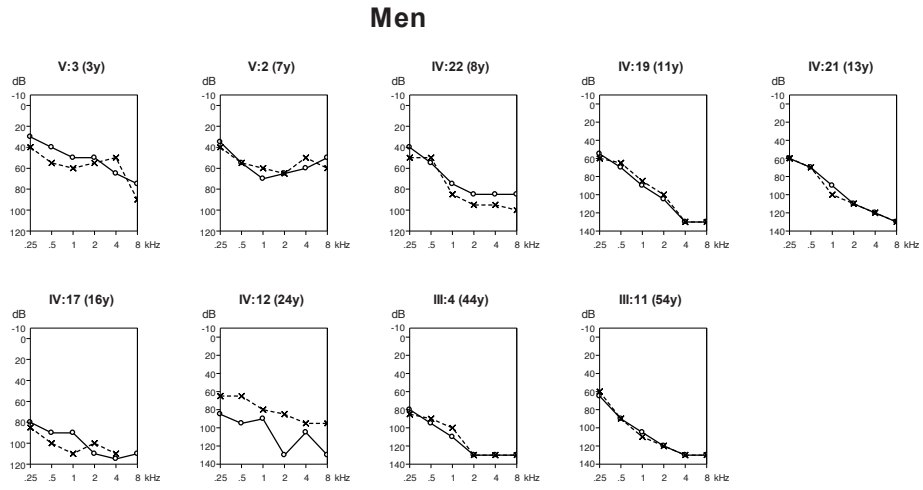
the ages of 35, 38 and 7 years, respectively. Caloric tests in individual III:7 at age 62 years revealed asymmetric responses with right unilateral weakness, combined with normal rotatory test results. Individual III:11 demonstrated bilateral caloric weakness and hyporeflexia on rotatory testing at age 55 years.

Pure-tone audiograms

The most recent pure-tone audiograms of mutation carriers with hearing impairment are shown in Figure 2. The men had more severe hearing impairment than the women, already at a younger age. Individual audiograms of the left and right ear in men were fairly symmetric, except for individual IV:12. All frequencies were affected and a downsloping audiogram configuration was seen in the majority of cases. Fairly flat audiogram configurations were also seen (individuals IV:17 and V:2). (Figure 2a)

The women showed more pronounced across-subjects variation in audiogram configuration, as well as in degree of hearing impairment. Furthermore, a number of women demonstrated a large individual I-A variation in AC threshold (individuals III:7, IV:1, IV:3, IV:10 and IV:14). The women IV:6, IV:10 and IV:14 exhibited close to normal thresholds for the right or left ear at the age of 32 years, at the ages of 25-28 and 5-26 years, respectively. (Figure 2b and 3) A downsloping threshold configuration was again most frequently observed. Fairy flat audiogram configurations were also seen (individuals III:14 and III:16). (Figure 2b)

a.



b.

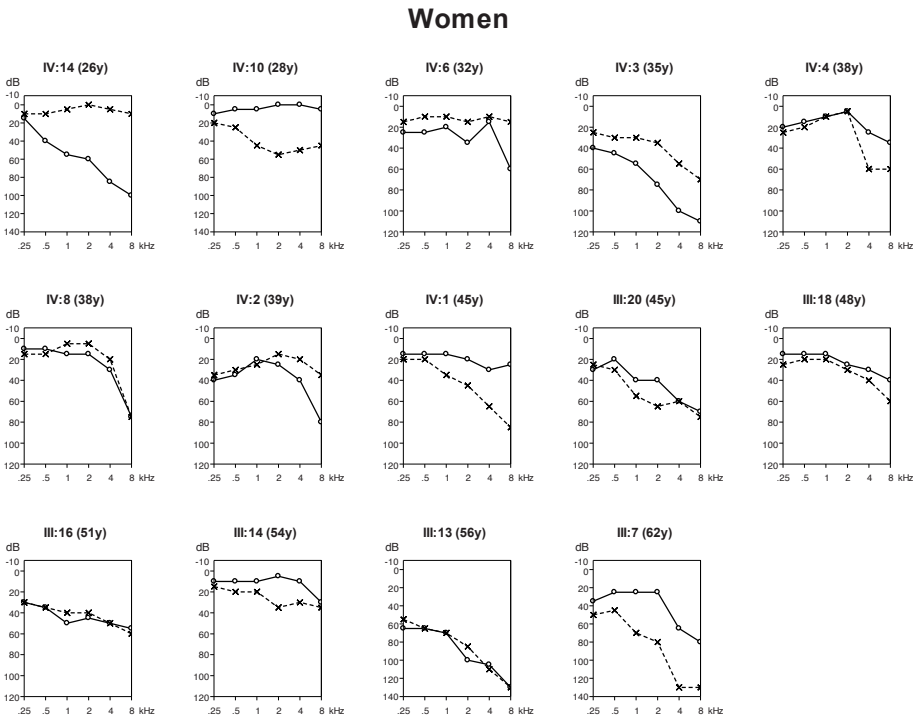


Figure 2. Last-visit audiograms of clinically affected male (a) and female (b) participants carrying the mutation, ordered by age (from top left to bottom right) at last visit. Shown are the AC threshold levels for the right (open circles and solid line) and for the left (crosses and dashed line) ears. Above each audiogram: pedigree number, age in years (*y*).

Progression of hearing impairment

All threshold data of mutation carriers with hearing impairment, combining individual longitudinal and single-snapshot measurements, are plotted against age in Figure 3. Again, more severe hearing impairment at younger age is visible in men when compared to women. The degree of across-subjects variation in threshold was clearly smaller in male family members than in female family members. (Figure 3). In men, major progression in threshold occurred during the first two decades of life, especially at higher frequencies. Longitudinal threshold data for some men clearly suggested non-linear progression during this age interval. (Figure 3) Threshold progression in women was milder than in men. (Figure 3)

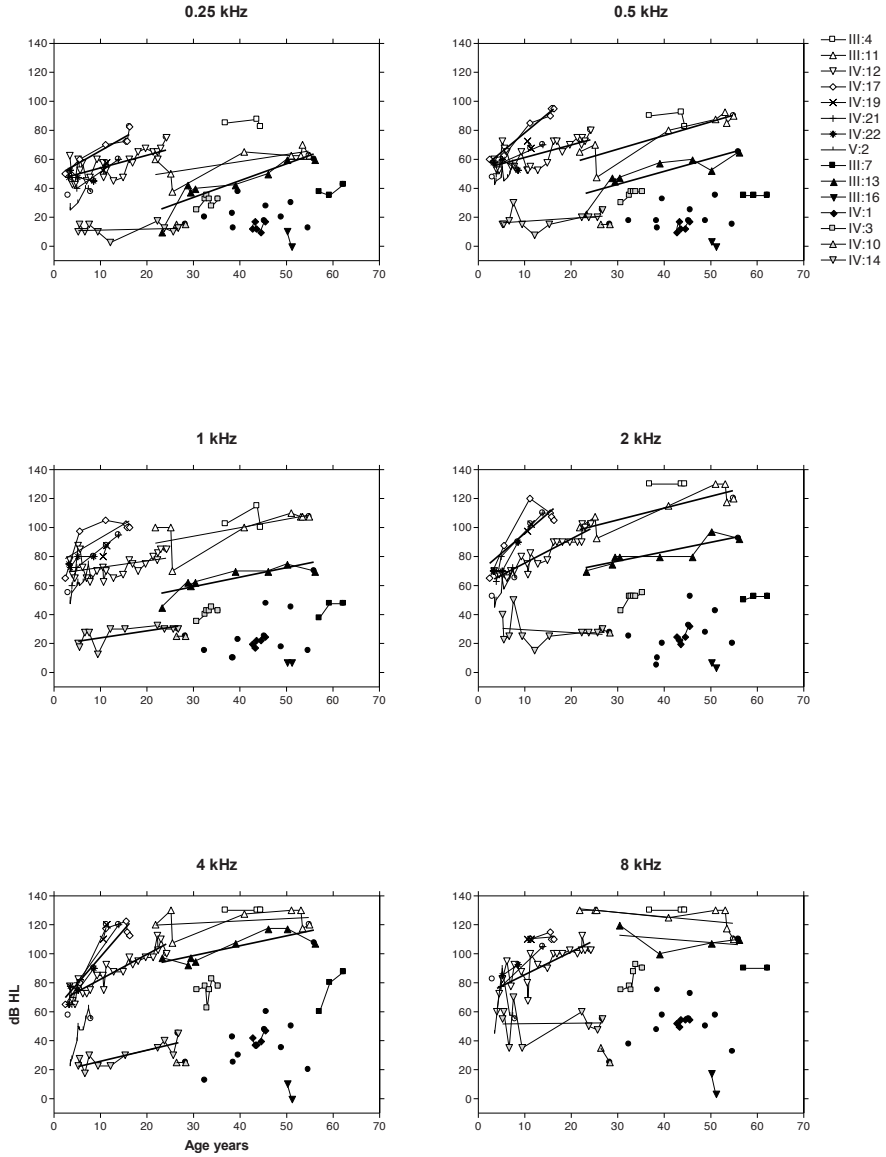


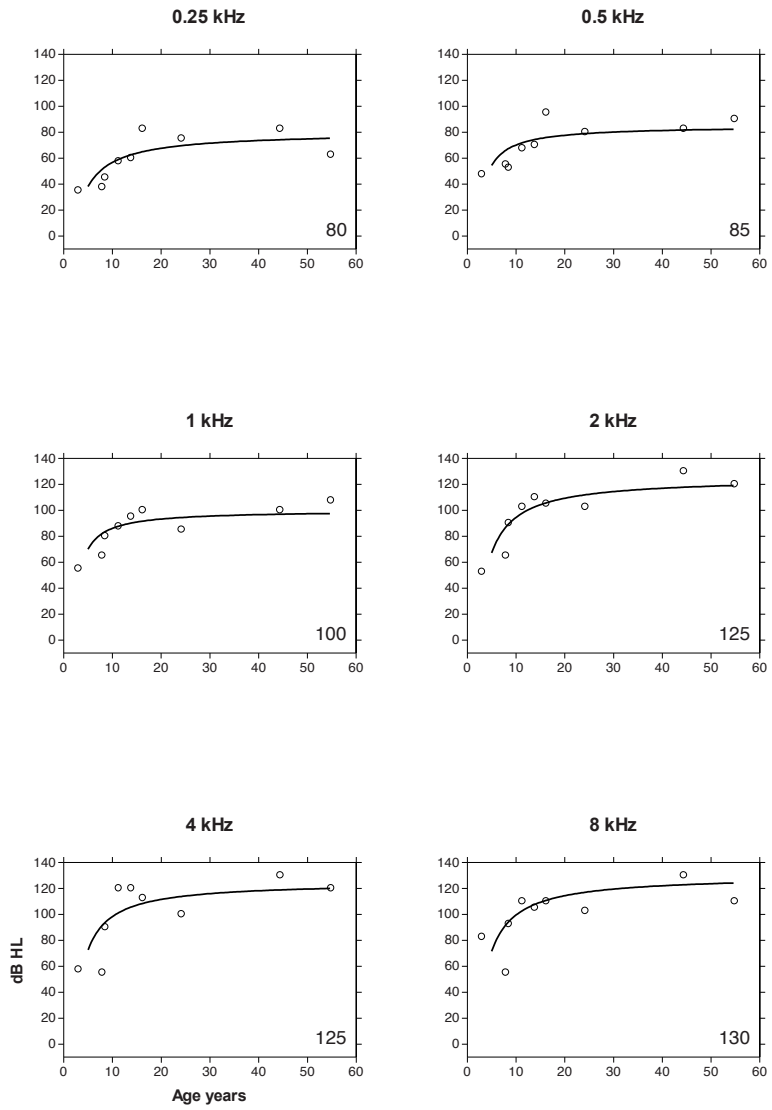
Figure 3. Mixed single-snapshot and longitudinal individual measurements (binaural mean AC threshold) of men (open symbols) and women (solid black and shaded symbols) are shown in relation to age for each frequency separately. Open circles are the most recent measurements of men and dots are the most recent measurements of women. For the longitudinal measurements (n > 1), the different symbols are shown in the symbol key. The regression lines fitted to the individual longitudinal measurements are also included. A bold line indicates significant progression.

Longitudinal regression analysis of audiometric data of individuals III:11, III:13, VI:12, IV:14 and IV:17 revealed significant progression for all individuals at at least two frequencies. (Figure 3) The significant ATD values for the men (individuals III:11, IV:12 and IV:17) ranged from 0.8 to 3.7 dB/year. The high frequencies showed the highest ATD values. For the women (individuals III:13 and IV:14) the significant ATD values ranged from 0.5 to 1.2 dB/year. This progression rate is approximately the same as the progression rate of all women combined. (Figure 4b)

Figure 4 shows the results of cross-sectional non-linear regression analysis for men and linear regression analysis for women of binaural mean AC threshold on age. (Figure 4) Presumably, the large across-subjects variation in threshold in women prohibited the demonstration of significant progression in the linear regression analysis. It is noteworthy that in women the threshold range around the regression line was wider than the threshold range covered by age-related progression, especially at the higher frequencies. (Figure 4b) The ATD values of women ranged from 0.5 to 1.3 dB/year, with the highest values at the high frequencies.

In order to obtain stable non-linear regression results for men, we decided to prefix the B_{max} values. Reasonable fits, as judged by eye, were obtained using values increasing from 80 dB at 0.25 kHz to 130 dB at 8 kHz, as is shown in the separate panels of Figure 4a. The initial curvature of the saturation hyperbolas appeared to fit in a satisfactory way when the supposed onset age was fixed at 2 years and the K_d values were fitted at each frequency (to values of 1-3 years) by the non-linear regression program. The results of the test (data not shown) comparing between the fit produced by the present non-linear regression model and a simple linear regression model favoured the non-linear model at each frequency. The nonlinear model generally produced the smaller residual sum of squares at a higher R-squared value (0.59-0.79, as opposed to 0.35-0.58) at 0.25-4 kHz. Systematic inspection of the residuals from regression showed that residuals from linear regression were distributed along the line in a systematic, clearly non-random way, whereas the residuals from non-linear regression were neatly distributed in pseudo-random fashion along the regression curve (data not shown).

a.


Men
0.25 kHz
0.5 kHz
1 kHz
2 kHz
4 kHz
8 kHz
80
85
100
125
125
130
dB HL
Age years

b.

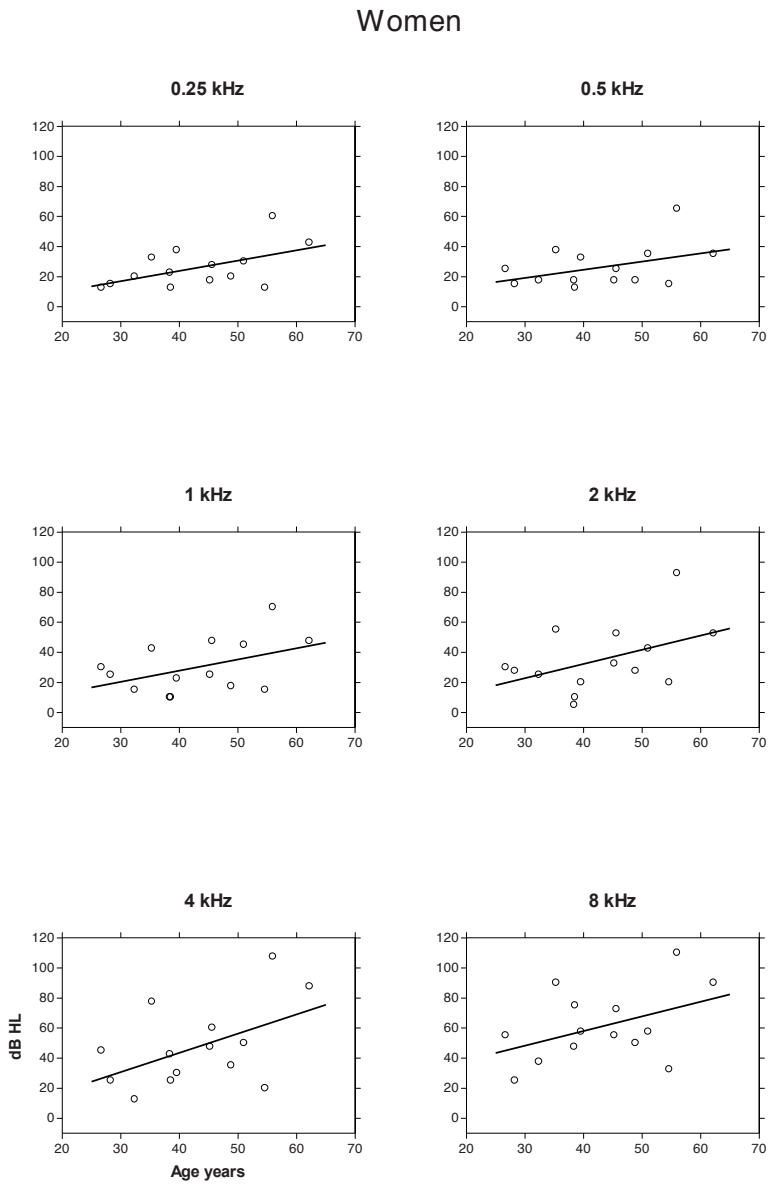


Figure 4. Single-snapshot (open circles) threshold values (binaural mean AC threshold measured at last visit) of men (a) and women (b) are shown in relation to age for each frequency separately. The line in left panels represents the results of the cross-sectional non-linear regression analysis in men (a) and the line in the right panels represent the results of the cross-sectional linear regression analysis in women (b) for each frequency. The number in the lower right corner of each panel of the men is the prefixed B_{max} level (dB HL).

In Figure 5 the ARTA for men and women are depicted. Even before the age of five years, the threshold values of all frequencies were severely elevated in the men. The ARTA for men illustrate the substantial progression in the first two decades of life, mainly at higher frequencies. (Figure 5a) The more gradual progression in women is also illustrated by their ARTA. Predicted threshold values showed slightly more progression at the high frequencies than at the low frequencies. (Figure 5b)

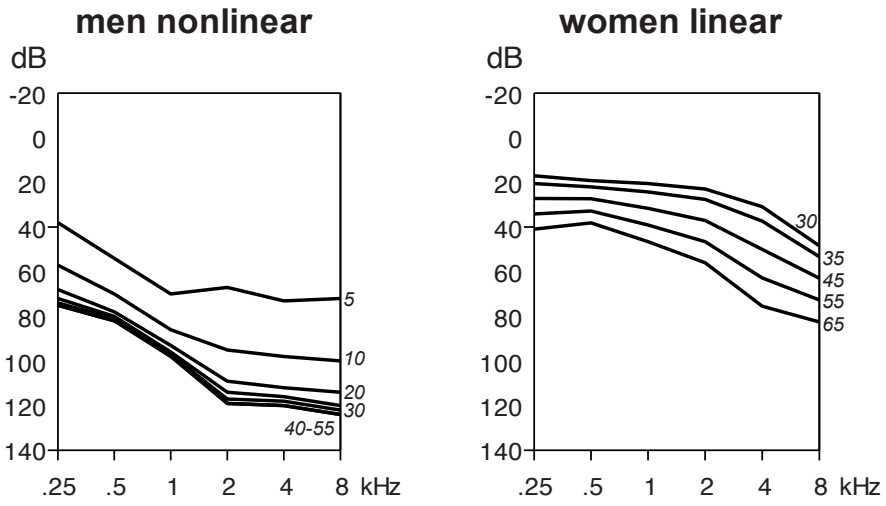


Figure 5. Age-related typical audiograms (ARTAs) derived from cross-sectional regression analysis of mean AC threshold values for men (a: non-linear fit) and women (b: linear fit). Numbers in italic indicate age in years.

Variation in hearing impairment

In Figure 6, the individual I-A SDs of the AC thresholds are plotted against age at 0.5 and 4 kHz for men and women separately. The plots for 0.25 kHz and 1 kHz (not shown) were fairly similar to those for 0.5 kHz, and the plots for 2 kHz and 8 kHz (not shown) were fairly similar to the plots for 4 kHz. None of the plots of I-A SD against age, i.e. those shown in Figure 6, but also those not included in this Figure, showed any substantial tendency for significant regression on age in across-subjects evaluation. However, in individual longitudinal analyses, men IV:12 and IV:17 showed significant progression in I-A SD at 0.25-1 kHz and 0.5-1 kHz, respectively, whereas woman IV:14 showed significant progression in I-A SD at all frequencies except for 0.25 kHz. (shown in part Figure 6) In men IV:12 and IV:17, this

progression in SD was associated with significant, substantial threshold progression. (Figure 3) Serial audiograms of woman IV:14 (data not shown) demonstrated that from age 5-26 years the left ear never showed any substantial hearing impairment, whereas the right ear developed a gradual increasing impairment. (Figure 2b only shows age 26 years) In the two men IV:12 and IV:17 asymmetry in hearing threshold indeed increased somewhat with increasing age, but to a lesser extent. (Figure 2a only shows last visit)

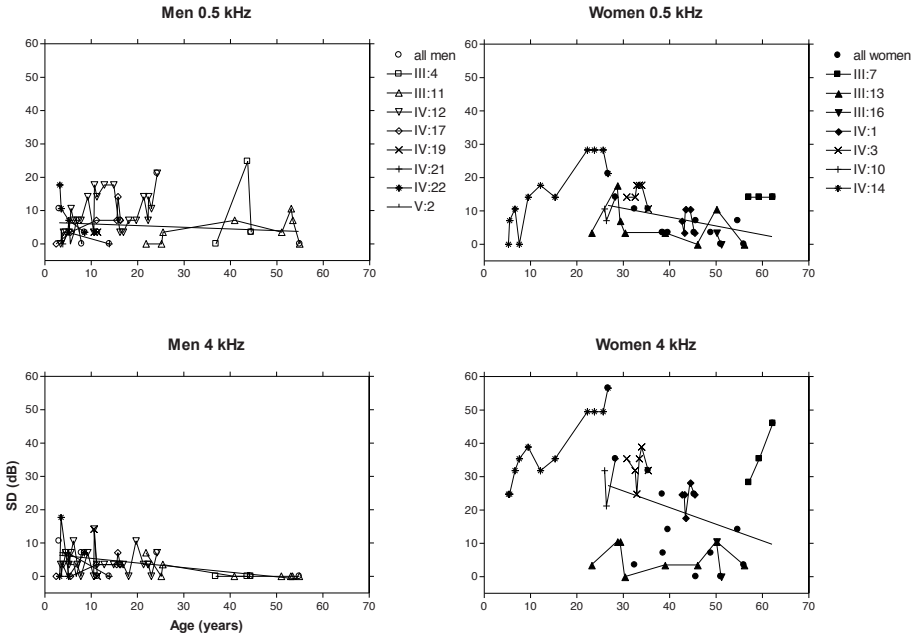


Figure 6. Individual inter-aural (I-A) standard deviation (SD) of AC threshold values plotted against age at the frequencies 0.5 kHz and 4 kHz for men (left panels) and women (right panels) separately. Open circles are the most recent measurements of men and dots are the most recent measurements of women. For the longitudinal measurements (n > 1), different symbols are used for each family member, as shown in the symbol key.

Instead of the individual I-A SD, the I-A difference might have been plotted in Figure 6. It should be realized, however, that this would have influenced only the scale of the graphs, because |I-A Difference| = $SD(2)^{1/2} \approx 1.414$ SD. Nevertheless, we thought it could be worthwhile to study into more detail parameter behavior of the I-A

difference (more simply, |Difference|) and see whether statistical tests comparing between men and women could be performed. Figure 7 shows plots of the individual |Difference| against the individual binaural mean threshold for similar panels as in Figure 6 (men vs. women at 0.5 kHz and 4 kHz). The data have now been limited to the last-visit threshold measurements pertaining to the cross-sectional analyses. Linear regression analysis demonstrated that at all frequencies (data not shown for 0.25, 1, 2, and 8 kHz) there was no significant regression of individual |Difference| on individual binaural mean threshold in men or women. Regression of the parameters mean |Difference| and SD(|Difference|) on binaural mean threshold could therefore be ignored.

The bottom panel of Figure 7 shows that in men neither the across-subjects mean |Difference| or the across-subjects SD(|Difference|) depended significantly, as demonstrated by linear regression analysis on audio frequency. The across-subjects mean |Difference| and SD(|Difference|) varied, independently from audio frequency, at the various frequencies between 4 and 13 dB (mean 8.3 dB) and between 3 and 10 dB (mean 5.3 dB), respectively. In contrast, the women showed a systematic, significant (S) increase in both the across-subjects mean |Difference| (from 8 to 32 dB) and the across-subjects SD(|Difference|) (from 4 to 27 dB) with increasing audio frequency. Using Student's t tests, including Welch's correction if Bartlett's test disclosed unequal variances, the across-subjects mean |Difference| appeared to be significantly greater for women than for men at 2-8 kHz. Bartlett's test demonstrated that the across-subjects SD(|Difference|) was significantly greater for women than for men at 1-8 kHz. Thus it appeared that not only the degree of individual asymmetry in threshold between the ears was, on average (i.e. the across-subjects mean |Difference|), substantially greater in women, but also the across-subjects variation in this asymmetry (i.e. SD(|Difference|) was substantially greater in women as compared to men.

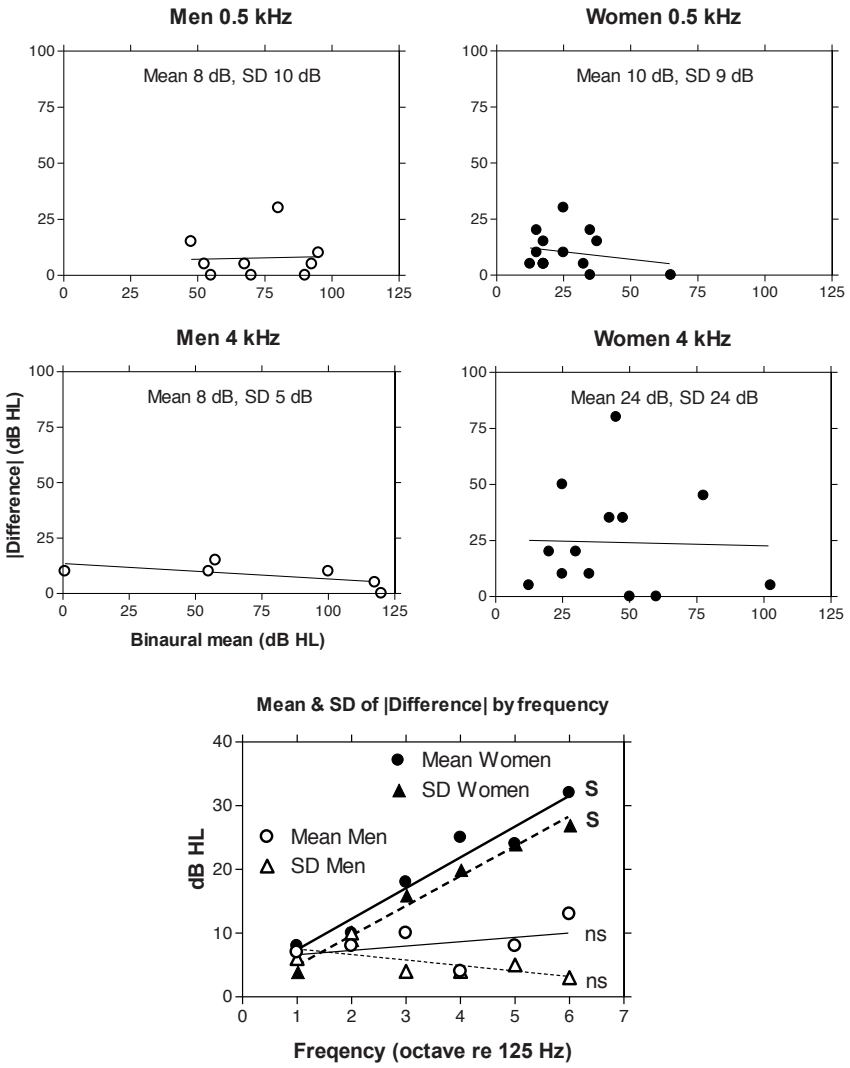


Figure 7. Upper 4 panels: individual inter-aural (I-A) difference in threshold (|Difference|) at 0.5 kHz (top row of panels) and 4 kHz (second row of panels) plotted against the individual binaural mean threshold at the last visit for men (circles in left panels) and women (dots in right panels) separately. The values for the across-subject mean |Difference| and SD(|Difference|) are indicated. Bottom panel: across-subjects mean |Difference| and SD(|Difference|), including the values indicated for 0.5 and 4 kHz in the upper 4 panels, plotted against audio frequency for men (open circles and triangles) and women (dots and filled triangles). The lines in each panel are the straight lines resulting from linear regression analysis (data not shown). S: significant regression (bold line); ns: non-significant regression.

Speech recognition

Figure 8 shows the available single-snapshot measurements of phoneme scores of all the hearing impaired male mutation carriers (except for IV:2, IV:6 and IV:8) and female mutation carriers (except for III:4, IV:17, IV:21 and V:3). Speech discrimination was severely decreased at a relatively young age in men. The women had far better speech discrimination scores than men, but their scores were substantially lower than those of presbycusis patients at matching ages. The 50% speech recognition score in men was attained at the age of 33 years, whereas in the presbycusis group this score was attained at 89 years. In women all scores were well above 50% but the 90% score was attained at 50 years of age, compared to 74 years in presbycusis patients. The men remarkably never showed scores as high as 90%. (Figure 8, left panel)

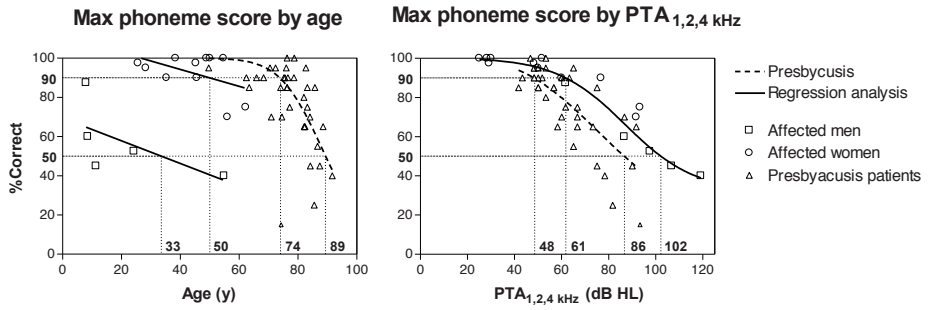


Figure 8. Single-snapshot measurements of maximum phoneme recognition scores (binaural means) plotted against age (left) and binaural mean pure-tone average at 1, 2, and 4 kHz (right). The continuous lines are the regression curves fitted to these measurements (left panel: lower linear regression line for men, upper linear regression line for women; right panel: common non-linear regression curve for men and women). The dashed curves represent the presbycusis data, previously established for patients with presbycusis. The dotted lines mark the 50% and 90% score levels (bold scores along Y axis) attained at the ages (left panel) or PTA levels (right panel) indicated by bold scores along X axis. Square: male family member; circle: female family member; triangle: presbycusis patient.

Quite remarkably, male and female mutation carriers had relatively good speech recognition scores in relation to their $PTA_{1,2,4\ kHz}$ levels. The relatively high speech recognition scores in women seemed to align with the lower scores in men, as

judged from their positions relative to the common regression curve. The 90% score was attained at a $PTA_{1,2,4\ kHz}$ level of 61 dB in the DFNX4 patients (men and women combined), whereas this score was already attained at a $PTA_{1,2,4\ kHz}$ level of 48 dB in presbycusis patients. The 50% scores were attained at $PTA_{1,2,4\ kHz}$ levels of 102 dB and 86 dB in DFNX4 and in presbycusis patients, respectively. (Figure 8, right panel) The deterioration rate in the score-against-age plot was about 0.6% per year for men and 0.4% per year for the women. The deterioration gradient in the score-against-$PTA_{1,2,4\ kHz}$ plot was approximately 1.0% per dB for men and women combined. The presbycusis group showed a higher deterioration rate (3.3%/y) and approximately the same deterioration gradient (1.1%/dB).

Discussion

This report presents the clinical features of a Dutch DFNX4 family with a p.Glu72X mutation in *SMPX*. All 25 mutation carriers demonstrated hearing impairment, except for one female.

DFNX4 families

Del Castillo et al. described the first DFNX4 family and were the first to emphasize the difference in hearing impairment between men and women. Affected men showed mainly high-frequency hearing impairment with an onset age of 5-7 years. Hearing impairment became severe to profound and involved all frequencies before adulthood. Affected women demonstrated an age of onset in the fourth decade of life, with the earliest manifestation of hearing impairment at 30 years of age. Hearing impairment manifested as bilateral moderate high-frequency hearing impairment. Comparison of the 3 audiograms published by Del Castillo et al. with the present audiograms at matching ages, indicated by the ARTA in Figure 5(a, b), shows a fair similarity in both men and women.[5, 6]

The German DFNX4 family is described by Huebner et al.[7] The men exhibited postlingual hearing impairment with an age of onset of 3-7 years. Moderate hearing impairment of especially the high frequencies progressed with age to affect all frequencies. The onset of hearing impairment in female carriers was in the second to third decades of their lives and hearing impairment progressed to severe hearing

loss in 10-15 years. Asymmetrical hearing impairment was seen in a number of women. Imaging of temporal bones showed no abnormalities.[7]

Del Castillo et al.[6] reported incomplete penetrance of 70% in the female carriers. The penetrance in the present family was 93% (14/15). Nevertheless, the unaffected female mutation carrier (individual IV:15) in the present family was only 25 years of age at last visit and might still develop hearing impairment because only five of the fourteen affected females reported hearing impairment before the age of 25 years.

The audiometric characteristics of the present DFNX4 family is largely similar to those of the previously described DFNX4 families, however, subtle differences in age of onset and rate of progression of hearing impairment exist. The presence of different mutations in the present family and the previously described Spanish DFNX4 family might explain the possible differences in phenotype, but also other genetic and environmental factors might be involved.[5-7]

Variation of hearing impairment

In the present family the men showed more severe hearing impairment than the women at a younger age. Figure 3 clearly shows that, not depending on any specific regression model, there were substantial differences in the degree of and the progression in hearing impairment between the men and the women. In addition, the women showed more pronounced across-subjects and I-A variation than the men at 1-8 kHz and 2-8 kHz, respectively. X-inactivation in women might explain such findings.

X-inactivation in mammals is a process by which one of the two copies of the X-chromosome present in females is inactivated. Such inactivation occurs randomly, but once a given X-chromosome is inactivated it will remain inactive in that cell and its descendants.[13] This may well explain the occurrence of unilateral hearing impairment in some women of the present family. In affected men, all cells have the mutated allele active and consequently the men show more severe and more similar clinical features of the disease.

Good speech recognition performance

The DFNX4 patients of the present family tended to have better speech recognition scores than the presbycusis patients at matching $PTA_{1,2,4\ kHz}$ levels. DFNA2[11, 14], DFNA5[15], DFNA11[16, 17] and DFNA15[18, 19] have been documented as autosomal

dominant hearing impairment disorders with relatively good speech recognition scores. Schraders et al.[8] hypothesised that *SMPX* is important in the development and/or maintenance of sensory hair cells. Huebner et al.[7] suggested that the long-term maintenance of mechanically stressed inner-ear cells critically depends on SPMX function because response to physical force is a characteristic feature of the protein. Further studies are required to establish the pathogenic pathway of DFNX4.
It is particularly appealing that for the combination of speech recognition scores of both men and women one, common dose-response curve could be fitted to describe the relationship between the performance of speech recognition and the degree of hearing impairment. (right panel of Figure 8) For the lower scores pertaining to the men, the underlying source of variation in the degree of impairment clearly was age-dependent progression. For the higher scores pertaining to the women, X-inactivation apparently constituted a substantial source of variation in the degree of impairment, next to age-dependent progression. From Figure 4b the observation could be made that in the women the threshold range covered by scatter around the regression line was wider than the threshold range covered by age-related progression, especially at the higher frequencies. This may indicate X-inactivation as a major source of variation in pure-tone threshold, and it is not unlikely that this also entails most of the variation in speech recognition performance in the women.

Normal vestibular function

Only two of the five tested family members demonstrated reduced vestibular responses, namely individuals III:7 and III:11 at age 62 and 55 years, respectively. Del Castillo et al.[5] and Huebner et al.[7] reported normal vestibular function, but it is unclear whether formal testing was performed. At present there is no evidence that DFNX4 is a progressive cochleovestibular disorder, such as DFNA9 and DFNA11[20], as well as DFNA15[19, 21, 22]. In situ hybridization in the mouse demonstrated *SMPX* expression in the inner ear, presumably in the sensory epithelium of the vestibular organs.[8] More DFNX4 patients of various ages should be tested to assess whether vestibular dysfunction is a feature of DFNX4.

Remarkable progression

It is noteworthy that the men affected by the present mutation in *SMPX* showed remarkable progression in hearing impairment in the first two decades of their lives

and thereby attained severe to profound levels of hearing impairment at a relatively young age. (Figure 5a) Among the DFNA loci, only DFNA20/26 caused by *ACTG1* mutations and DFNA36 caused by *TMC1* mutation exhibited fairly similar degrees of progression and impairment (in both men and women).[23, 24]

Conclusion

The present study describes the phenotype of a DFNX4 family with a c.214G>T (p.Glu72X) mutation in *SMPX*. The phenotypic heterogeneity was remarkable and was probably related to X-inactivation in female mutation carriers. Men showed severe hearing impairment at a young age with more pronounced progression during the first two decades of life, whereas women demonstrated mild to severe hearing impairment with more gradual progression and a higher, variable age of onset. The across-subjects and I-A variations in thresholds were more pronounced in women than in men, especially at 2-8 kHz. Speech recognition in men and women was remarkably well preserved.

The phenotype of the present family is largely similar to the phenotype of the previously described DFNX4 families. However, subtle differences in onset age and rate of progression of hearing impairment seem to exist and could be caused by the different mutations in *SMPX*. However, a thorough genotype-phenotype analysis of DFNX4 requires more data on DFNX4 families harbouring different *SMPX* mutations.

Acknowledgement

This study was supported by a grant from the Heinsius Houbolt foundation and by a grant from the INTERREG IV A-program Germany - the Netherlands.

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4.2

Next-generation sequencing identifies mutations in the small muscle protein, X-linked, (*SMPX*) as a cause of progressive hearing impairment

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American Journal of Human Genetics 2011; 88: 628-634

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Abstract

In a Dutch family with an X-linked postlingual progressive hearing impairment, a critical linkage interval was determined to span a region of 12.9 Mb flanked by the markers DXS7108 and DXS7110. This interval overlaps with the previously described DFNX4 locus and contains 75 annotated genes. Subsequent next-generation sequencing (NGS) detected one variant within the linkage interval, a nonsense mutation in *SMPX*. *SMPX* encodes the small muscle protein, X-linked (SMPX).

Further screening was performed on 26 index patients from small families for which X-linked inheritance of nonsyndromic hearing impairment (NSHI) was not excluded. We detected a frameshift mutation in *SMPX* in one of the patients. Segregation analysis of both mutations in the families in whom they were found revealed that the mutations cosegregated with hearing impairment.

Although we show that *SMPX* is expressed in many different organs, including the human inner ear, no obvious symptoms other than hearing impairment were observed in the patients. *SMPX* had previously been demonstrated to be specifically expressed in striated muscle and, therefore, seemed an unlikely candidate gene for hearing impairment. We hypothesize that SMPX functions in inner ear development and/or maintenance in the IGF-1 pathway, the integrin pathway through Rac1, or both.

Hereditary nonsyndromic hearing impairment (NSHI) is genetically extremely heterogeneous, as is illustrated by the currently associated genes, numbering more than 50, and the large number of loci for which the gene harboring the causative mutation(s) is still elusive (Hereditary Hearing Loss Homepage).[1] This hampers DNA diagnostics and adequate mutation-based genetic counseling. Inheritance patterns of monogenic NSHI can be (in order of decreasing prevalence) autosomal recessive, autosomal dominant, X-linked or mitochondrial, and digenic inheritance has also been indicated.[2, 3] Age-related hearing loss is a complex disorder, although variants in genes involved in monogenic forms of NSHI have been found to be among the genetic factors.[4] Genes in which variation is associated with deafness have a wide variety of functions and have contributed significantly to our understanding of the molecular biology of hearing.[1, 5] Because of this functional diversity, bioinformatics tools such as ENDEAVOUR or Prospectr have been of limited value in the positional cloning of deafness genes.[6] Currently, next-generation sequencing (NGS) is an excellent strategy for identification of disease-causing variants.[7, 8]

In the present study, we identified mutations in the gene encoding the small muscle protein, X-linked (SMPX [MIM 300226]) as a cause of nonsyndromic hearing impairment by using a two-step strategy of linkage analysis and NGS.

This study was approved by the medical ethics committee of the Radboud University Nijmegen Medical Centre and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all subjects or, in case of children, from their parents.

Affected subjects of a large, five-generation family (W08-1701) of Dutch origin presented with postlingual progressive hearing impairment. (Figure 1) An X-linked pattern of inheritance was suggested by the absence of male-to-male transmission and the fact that hearing impairment developed earlier and was more severe in men than in women.

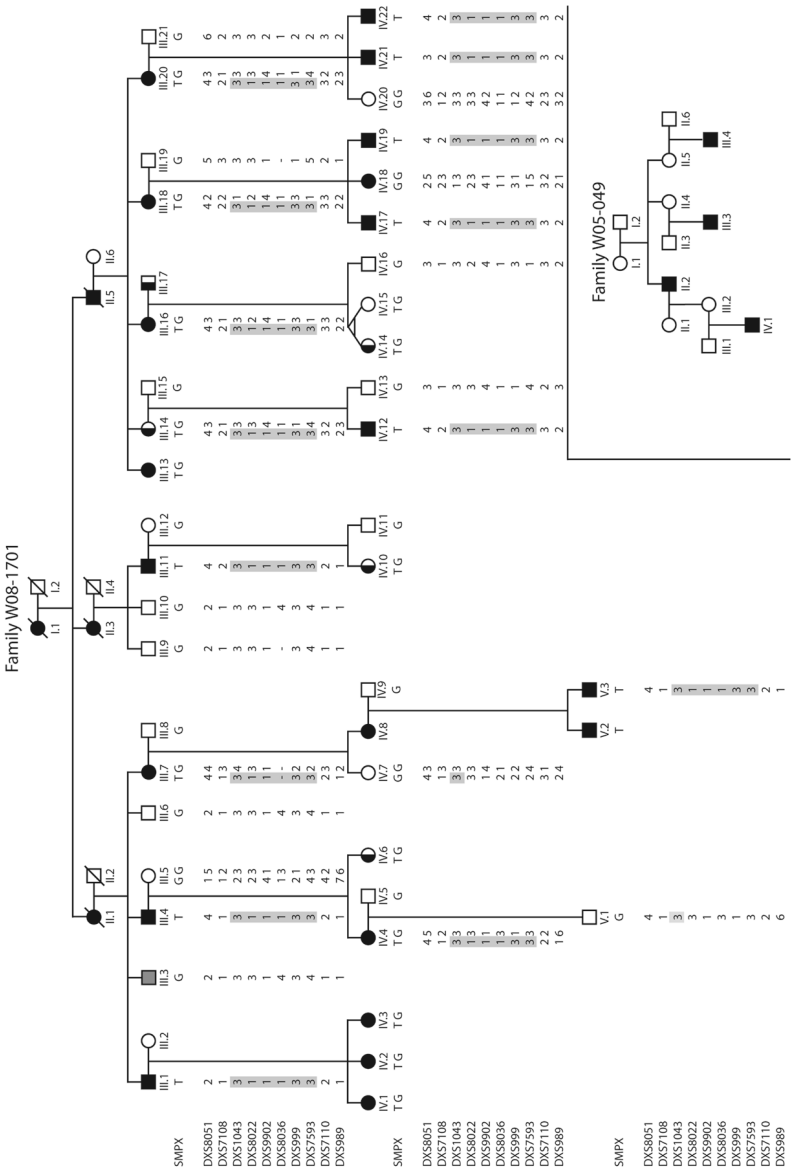
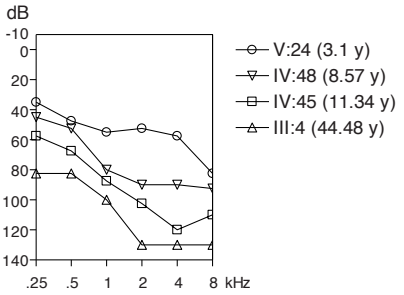


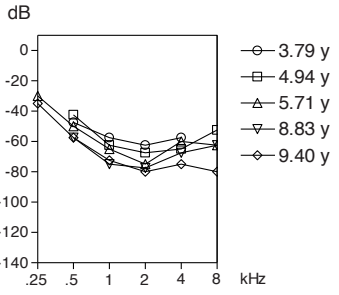
Figure 1. Pedigrees and genetic analysis. Only those family members of the large pedigree who were relevant for the study are depicted. The haplotype associated with the hearing impairment is indicated by the gray bar. In individual V:1, allele 3 of marker DXS1043 might be derived from the affected haplotype. The segregation of the c.214G>T is presented above the haplotypes. Family W08-1701 is of Dutch origin and family W05-049 is of Netherlands Antilles' origin. The following symbols are used: black squares: affected males; black circles: affected females; half-shaded circles: females with unilateral hearing impairment; half-shaded square: male with unilateral hearing impairment; gray shaded square: male with hearing impairment less severe than the other affected males.

The majority of affected family members reported bilateral, (slowly) progressive hearing impairment. Pure-tone audiometry and otoscopy were performed for all depicted individuals by standard procedures. There was no evidence for nongenetic causes of hearing impairment except for individual III:3, who reported noise exposure. Also, hearing impairment in this individual was less severe and had a late onset at about age 60. The reported age at which hearing impairment was first noticed was 2–10 years old for men (with a mean of 3.3 years old) and 3–48 years old for women (with a mean of 28.2 years old). In males, the largest increase of threshold values occurred in the first two decades and progression to profound hearing impairment was already seen in the second decade in one of the affected males. (Figure 2) Hearing impairment in women exhibited a large inter-individual variation with regard to the severity and also with regard to interaural variation. (Figure 2) Brainstem Evoked Response Audiometry (BERA) for the proband, individual V:2, revealed normal waveform responses at an intensity level of 45 dB. There was no indication of conductive hearing impairment. Furthermore, pure-tone audiometry never revealed a persistent air-bone gap or pseudoconductive hearing impairment in any of the affected family members. (Figure S1). A more detailed description of the audiometric evaluation of the family will be reported elsewhere.

A. Family W08-1701 men



B. Family W05-049 individual IV:1



A. Family W08-1701 women

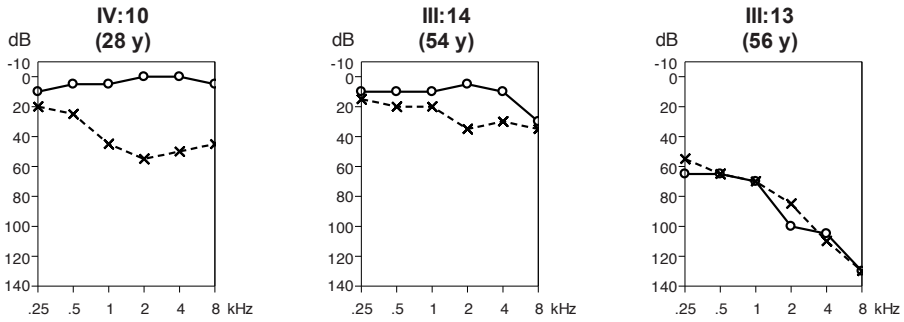


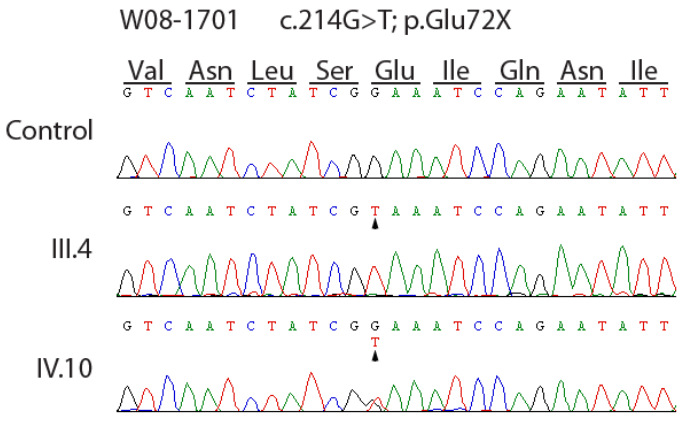
Figure 2. Audiometric characteristics of the families. (A) Representative audiograms (air-conduction) of affected men (showing the means of thresholds of the left and right ears) and women (thresholds of left (x) and right ear (o) shown separately) of family W08-1701 demonstrate progressive hearing loss in males within the first two decades and the variability of the hearing loss in females. (B) Representative audiograms of individual IV:1 from family W05-049 at different ages (means of thresholds of the left and right ears are shown). Pure-tone audiometry was performed in a sound-treated room according to current clinical standards. y is an abbreviation for years.

Genetic studies in this family were initiated by linkage analysis for the known X-chromosomal NSHI loci, recently renamed DFNX1-5 (Hereditary Hearing Loss Homepage).[9-13] Twenty-eight individuals from this family were genotyped for microsatellite markers from the loci DFNX1 (MIM 304500), DFNX3 (MIM 300030), and DFNX4 (MIM 300066). (Table S1) After exclusion of DFNX1 and DFNX3, evidence of linkage with the disease was found for marker DXS8022, derived from the DFNX4 locus that was previously described as DFN6 for a family with similar audiometric features.[12] Subsequent genotyping of nine additional markers defined a critical region of 12.9 Mb flanked by the markers DXS7108 and DXS7110. (Figure 1) In this region, chrX:10,192,226-23,111,851, 75 genes have been annotated (UCSC Genome Browser, hg19). We calculated two-point LOD scores with SuperLink version 1.6 in the Easy-Linkage software package by using the genotypes of males only.[14, 15] Penetrance was assumed to be 99%, and a disease allele frequency of 0.001 was employed for the calculations. Individual III:3 was included in the calculations as an individual with an unknown phenotype. A significant maximum

LOD score of 4.87 (θ = 0.000) was obtained for marker DXS8022; LOD scores are presented in Table S2.

Three candidate genes, *PRPS2* (MIM 311860), *SHROOM2* (MIM 300103) and *GPM6B* (MIM 300051), were selected for a mutation search by conventional Sanger sequencing because of homology with a known deafness gene or expression in the inner ear, but no pathogenic variant was identified. Subsequently, we performed targeted enrichment by using the Agilent SureSelect Human X Chromosome Kit and single-read 76 nt NGS on the Illumina GAII sequencer for individual III:4.[16] In total, 28,363,277 reads were obtained, of which 23,339,533 could be mapped, and 95.1% of the targeted bases were covered at least 10-fold. After analysis of the sequence data with in-house-developed tools and filtering of the predicted sequence variants against dbSNP, the 1000 Genome Project, and 200 Danish control individuals,[17] two variants remained, chrX:117960412T>G and chrX:21755734C>A (base-pair positions according to the NCBI37/hg19 assembly of the human genome), and only the latter was located within the critical region. This variant, c.214G>T, is located in exon 4 of *SMPX* (NM_014332.1) and introduces a premature stop codon predicted to result in a truncated protein after residue 71 (p.Glu72X). The presence of this candidate disease-causing variant was confirmed by Sanger sequencing in the affected males III:1, III:4 and V:2 and a female carrier, IV:10. (Figure 3)

A



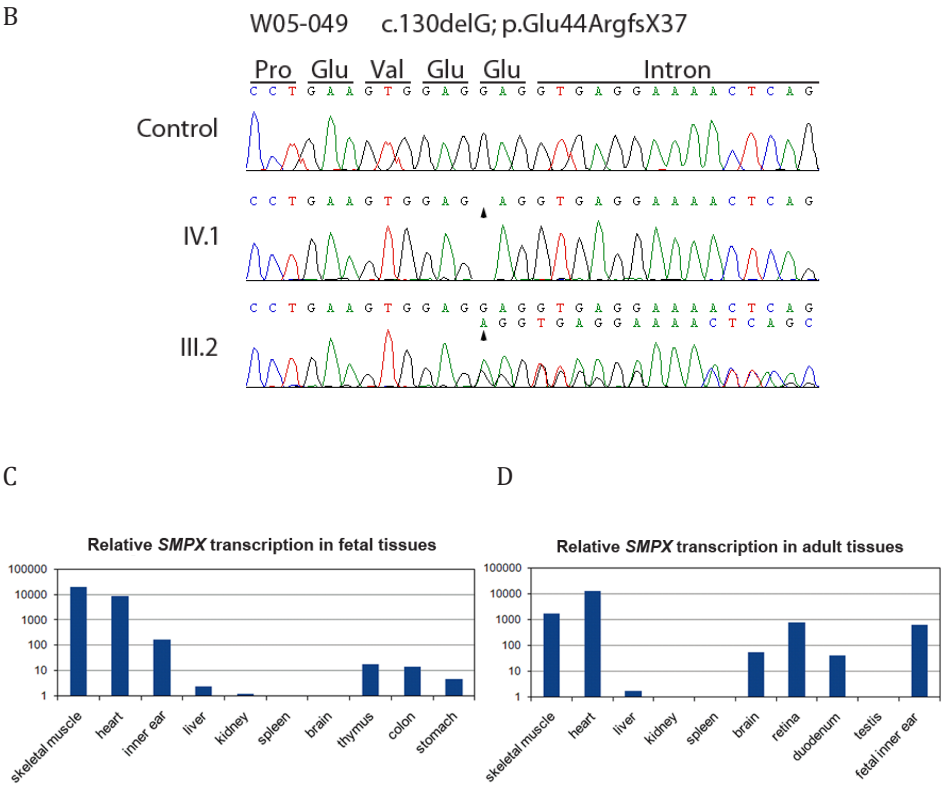


Figure 3. Mutation and expression analysis of *SMPX*. (A and B) Partial *SMPX* sequence chromatograms are shown for normal controls (upper panels), affected males (middle panels), and female carriers (bottom panels). The predicted amino acid changes and the surrounding amino acids are indicated above the sequence. Mutated nucleotides are marked by an arrowhead. As a reference, we employed sequence NM_014332.1 by using the first ATG translation initiation codon for numbering of the nucleotide change. (C and D) Relative *SMPX* mRNA expression as determined by quantitative PCR in fetal (C) and adult (D) human tissues. Because this was performed for adult and fetal tissues in two separate experiments, fetal inner ear was included in both for comparison.

The c.214G>T transversion removes a restriction site for Hpy188I. Therefore, we performed restriction digestion of exon 4 amplicons according to the manufacturer's protocol (New England Biolabs) to test all family members and ethnically matched controls for the presence of the mutation. DNA fragments were analyzed on 2% agarose gels. (Figure S2). None of the 172 control individuals carried the mutation,

whereas in the family, the mutation was found to fully cosegregate with hearing impairment in males, as expected from the linkage analysis, and individual III:3 indeed did not carry the mutation. (Figure 1) Therefore, his hearing impairment might well be caused by the reported noise exposure. For females the mutation also coincides with the mutation-carrying haplotype, indicating that individual IV:18 is either a phenocopy or a genocopy. She has a mild increase in thresholds in the pure-tone audiogram for the frequencies 0.25–1 kHz. On the other hand, individual IV:15 shows no signs of hearing impairment but does carry the mutation as does her twin sister (monozygotic). The latter exhibits unilateral hearing impairment. Both sisters were 25 years old at the last visit in the clinic and because the age at onset for females from this family is variable (3–48 years), individual IV:15 could well develop hearing impairment in the coming years.

To investigate the involvement of *SMPX* in other families with hearing impairment, we performed Sanger sequencing of the three protein-coding exons (2–4) and the flanking intronic sequences in 26 index patients of small families for which X-linked inheritance of NSHI was not excluded. Sequence analysis was performed as described,[18] and primer sequences and conditions for PCR amplification are provided in Table S3. In one of the index patients (individual VI:1 of family W05-049, Figure 1), a deletion of a single base pair, c.130delG, was found in exon 3. This variant leads to a frameshift and a premature stop codon, p.Glu44ArgfsX37. The patient's mother (III:2 in Figure 1) was found to carry the deletion as well. No DNA samples from other family members were available for testing. The mutation was not detected in 129 Dutch control individuals who are not ethnicity matched because the family is of Netherlands Antilles' origin. Audiograms of the index patient are presented in Figure 2B. Hearing impairment was first suspected around the age of 4 and has progressed since then. (Figure 2) No air-bone gap was detected (Figure S2) and BERA revealed normal waveform responses at 65 dB. The proband's mother (III:2 in Figure 1) did not report any signs of hearing impairment at her last visit at clinic at the age of 38. Also, no hearing impairment was reported for obligate female carriers in the previous generations.

In an independent study of two additional families with X-linked hearing impairment, two different truncating mutations have been detected in *SMPX*. The

family in which the DFNX4 locus was determined was one of these.[12] The results are presented in the accompanying paper by Huebner et al. in this issue.[19]

Because of its high and preferential expression in striated muscle, *SMPX* was not an obvious candidate for NSHI.[20, 21] As a first step to identify the function of SMPX in the inner ear, we analyzed *SMPX* transcription by RT-PCR on RNA isolated from human inner ear of an embryo at 8 weeks gestation. Indeed, *SMPX* mRNA could easily be amplified and sequence verified (data not shown). Further evidence for *SMPX* expression in the inner ear is provided by RNA in situ hybridization in the mouse embryos at 14.5 days of gestation (Eurexpress database assay 006968 and Genepaint assay DC27). In addition to being present in developing muscle, Smpx transcripts are present in a region that presumably corresponds to the developing sensory epithelium of the vestibular organs. Immunohistochemistry with an Smpx antibody on sections of an adult mouse's organ of Corti revealed staining in different cell types, including Böttcher cells, root cells, pillar cells and interdental cells of the limbus spiralis. Low levels of staining were detected in hair cells.[19] Transcription of *SMPX* was further addressed by quantitative PCR (qPCR) on cDNA derived from various fetal and adult human tissues as described previously.[18] (Table S3) In Figure 3 the relative amounts of *SMPX* transcripts in the tissues as compared to that in the spleen (set to 1) are depicted. The housekeeping gene *GUSB* (MIM 611499) was used as a reference gene. *SMPX* mRNA levels were highest in both fetal and adult skeletal muscle and heart, which is in agreement with previous studies.[20, 22] No transcripts were detected in fetal brain or in adult kidney and spleen tissues. Importantly, relatively high *SMPX* transcript levels are detected in fetal inner ears, which supports the involvement of *SMPX* in X-linked NSHI. Retinas derived from adult humans also exhibits a relatively high level of *SMPX* transcripts. Despite the indications for significant expression levels of *SMPX* in heart skeletal muscles and retinas, no clear symptoms indicating an adverse effect of a truncating *SMPX* mutation in these tissues are reported by affected individuals from family W08-1701. Although one of the males reported mild muscle injury upon heavy exercise, a causative link to a defective SMPX remains to be determined by detailed testing of muscle function. No information is available for family W05-049. For heart and skeletal muscle, functional redundancy was already indicated by studies of a

conditional knock-out allele of *Smpx* in mice.[22] This conditional knockout allele appeared to be null because immunoblot analysis revealed no detectable Smpx protein. However, the knockout mice displayed no overt developmental or structural deficits in their skeletal muscles or hearts, suggesting a genetic or functional redundancy.

Smpx, previously also called Csl, is proline-rich and was described to contain a nuclear localization signal, two casein kinase II phosphorylation sites, and a proline, glutamic acid, serine and threonine-rich (PEST) sequence that suggests Smpx undergoes rapid degradation.[20] From late-fetal to neonatal stages of murine cardiac- and skeletal-muscle development onward, Smpx becomes associated with the costameres.[22] Costameres are subsarcolemmal protein assemblies at the sarcomere-sarcolemma attachment sites.[23] Three actin-associated costameric protein complexes have been distinguished: the focal adhesion-type complexes, the spectrin-based complex and the dystrophin-based complex, all of which tether molecules that control, among other processes, mechanoreception and cytoskeletal remodeling.[24] Smpx is likely to be part of the actin-associated complex because it was found, upon expression in mouse myoblasts, to influence actin turnover and to induce lamellipodia in a Rac1-dependent manner.[22, 25] Furthermore, Smpx colocalizes with focal adhesion proteins at the membrane of these lamellipodia, suggesting a link to integrin signaling.[25] Interestingly, both Rac1 and integrins (α8β1) are essential for normal cochlear development and function.[26, 27] Conditional inactivation of *Rac1* (MIM 602048) leads to a shortened cochlea and abnormal cellular organization of the sensory epithelium. Furthermore, planar cell polarity of cochlear hair cells and the morphogenesis of the hair bundle are affected.[26] Integrin (type α8β1) was found to be essential for normal hair-bundle development and/or maintenance, and colocalizes with its ligand fibronectin and the integrin-regulated focal adhesion kinase in the apical region of developing vestibular hair cells.[27] On the basis of all these data, we hypothesize that Smpx functions in the development and/or maintenance of the sensory hair cells.

A second link between SMPX and cochlea development and function is provided by insulin-like growth factor-1 (IGF-1) (MIM 147440). Smpx modifies cell shape and promotes myocyte fusion when expressed in C2C12 mouse myogenic cells in an IGF-

1 dependent manner.[22] *Igf-1*-deficient mice have multiple cochlear abnormalities, including an abnormal differentiation, a reduced survival of spiral ganglia neurons and an abnormal tectorial membrane.[28, 29] *MEF2* (MIM 600663) has been indicated to be a target gene of IGF-1 in the mouse cochlea in both the sensory cells and the spiral ganglia neurons. Interestingly, the IGF-1-mediated increase of MEF2 activity in myoblasts is augmented by Smpx.[22] Furthermore, the consensus sequence for MEF2 binding is present twice in the highly conserved 50 upstream region of *SMPX*.[20] *IGF1* mutations in humans cause syndromic, severe to profound and congenital or very early-onset sensorineural hearing impairment (MIM 608747).[30-32] This inner ear phenotype is more severe than that in the families included in this study.

In-depth studies are required for the discernment of which cell types and pathways in the cochlea are affected by mutations in *SMPX*. Fast deterioration of hearing in the first decades of life, as seen in family W08-1701, has been reported previously for patients with mutations in a number of genes, including *ACTG1* (MIM 102560) encoding the cytoskeletal γ-1-actin.[33, 34] Interestingly, this is thought to be the major cytoskeletal actin in costameres.[35]

In conclusion, this study identifies *SMPX* as a gene in which variation is associated with X-linked deafness and illustrates that NGS is instrumental in the efficient identification of disease-causing variants in unexpected genes. Our results will contribute to adequate mutation-based genetic counseling of patients and their relatives. Because females can be affected, although generally not in childhood, *SMPX* should also be considered in small pedigrees with dominantly inherited hearing impairment and those pedigrees in which X-linked inheritance cannot be excluded. Further analysis of the function of SMPX will provide insights into inner ear development and function, and SMPX might well be a player in the regulation of stereocilia development and/or maintenance.

Acknowledgments

We thank the families for their participation in this study. We would like to thank Suzanne Granneman for her contribution to the linkage analysis; Anne-Katrin Emde, Sean O'Keeffe and Wei Chen for their help with establishing the bioinformatic tools;

Claudia Langnick for construction of the single-end Illumina sequencing library; Corinna Jensen and Melanie Bienek for NGS. The work was financially supported by the Heinsius Houbolt Foundation, the INTERREG IV A-program Germany-the Netherlands, The Oticon Foundation (09-3742), the Netherlands Genomics Initiative (40-41009-98-9073), and ZonMW (40-00812-98-09047).

Supplemental tables and figures

Locus	*Marker*	*Marshfield position*
DFNX1	DXS8048	66.58 cM
	DXS1230	67.12 cM
	DXS6797	67.12 cM
DFNX3	DXS8039	30.84 cM
	DXS1036	33.54 cM
DFNX4	DXS8022	22.18 cM

Table S1. Microsatellite Markers from the Loci DFNX1, DFNX3, and DFNX4.

Marker	*Marshfield position*	*Recombination fractions (θ)*								
		0.00	*0.01*	*0.05*	*0.10*	*0.20*	*0.30*	*0.40*	Z_{max}	θ_{max}
DXS8051	17.29 cM	-13.69	-1.00	0.60	1.11	1.27	0.99	0.51	1.28	0.171
DXS7108	18.37 cM	0.30	1.29	1.77	1.81	1.56	1.13	0.58	1.81	0.084
DXS1043	18.37 cM	-0.15	-0.16	-0.14	-0.12	-0.09	-0.05	-0.02	0.00	0.500
DXS8022	22.18 cM	4.87	4.79	4.47	4.05	3.16	2.17	1.08	4.87	0.000
DXS9902	22.18 cM	1.66	1.64	1.52	1.37	1.06	0.72	0.36	1.67	0.000
DXS8036	22.72 cM	1.90	1.86	1.72	1.55	1.18	0.80	0.39	1.90	0.000
DXS999	23.26 cM	2.16	2.12	2.00	1.84	1.46	1.03	0.53	2.16	0.000
DXS7593	25.97 cM	4.31	4.24	3.95	3.58	2.79	1.92	0.94	4.31	0.000
DXS7110	29.22 cM	0.22	1.22	1.72	1.78	1.52	1.03	0.45	1.79	0.087
DXS989	29.76 cM	-1.01	0.02	0.62	0.82	0.84	0.66	0.37	0.87	0.154

Table S2. Two-point LOD scores between polymorphic markers on Xp22 and the disease gene.

Fragment	*Oligonucleotides*	*Size (bp)*	*Annealing Temperature (°C)*
	Primers for PCR and sequence analysis		
SMPX exon 2	Forward: aatatatggccagtgaaaggg Reverse: agctaggagtgaacaatcgc	245	58
SMPX exon 3	Forward: cttcacaacgattactgtctcag Reverse: ctcccttgtcctggatagc	273	58
SMPX exon 4	Forward: ctcaacaacacaagggacag Reverse: cttaaattgaaggcacctgg	418	58
	Primers for qPCR		
SMPX exon 3-4	Forward: aatgtactcctgaagtggagg Reverse: tggatttccgatagattgactg	113	NA
GUSB	Forward: agagtggtgctgaggattgg Reverse: ccctcatgctctagcgtgtc	80	NA

Table S3. Sequences of primers for amplification of exons, intron-exon boundaries and transcripts of *SMPX.* NA: not applicable. As reference sequence NM_014332.1 and NT_167197.1 were employed.

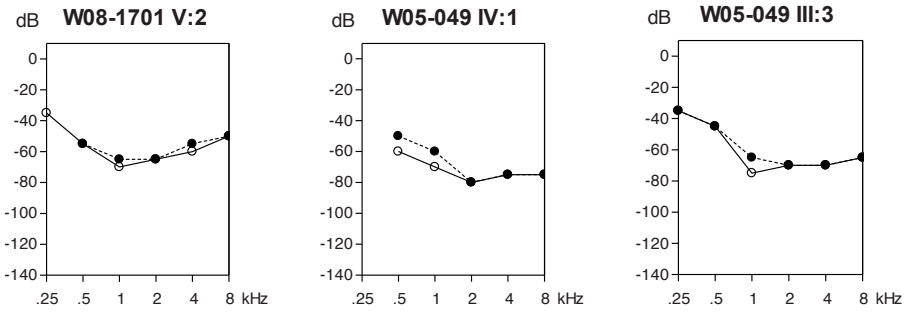


Figure S1. Representative audiograms of air- and bone-conduction thresholds of the right ear, with open circles indicating the air-conduction thresholds and filled circles the bone-conduction thresholds. No air-bone gaps were detected.

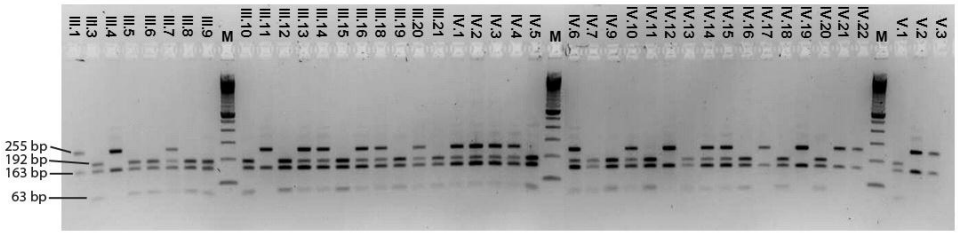


Figure S2. Segregation analysis of the c.214G>T *SMPX* mutation in family W08-1701. Pedigree numbers as in Figure 1 of the main manuscript. The c.214G>T transversion removes a restriction site for *Hpy188I* (New England Biolabs). Therefore restriction digestion of exon 4 amplicons was performed according to the manufacturer's protocol. The exon amplicon is 418 base pairs (bp) in length. *Hpy188I* digestion of a wildtype product results in fragments of 192 bp, 163 bp and 63 bp, while digestion of a mutant fragment results in fragments of 255 bp and 163 bp. A wildtype restriction pattern is seen for individuals III:3, III:5, III:6, III:8, III:9, III:10, III:12, III:15, III:19, III:21, IV:5, IV:7, IV:9, IV:11, IV:13, IV:16, IV:18, IV:20 and V:1. A mutant restriction pattern is seen for individuals III:1, III:4, III:11, IV:12, IV:17, IV:19, IV:21, IV:22, V:2 and V:3. A heterozygous digestion pattern (wildtype + mutant bands) is seen for individuals III:7, III:13, III:14, III:16, III:18, III:20, IV:1, IV:2, IV:3, IV:4, IV:6, IV:10, IV:14 and IV:15. M: 100 bp DNA ladder.

Web Resources

The URLs for data presented herein are as follows:
1000 Genome Project, http://www.1000genomes.org/
Eurexpress, A Transcriptome Atlas Database for Mouse Embryo,
http://www.eurexpress.org/ee/
Genepaint, a Digital Atlas of Gene Expression Patterns in the Mouse,
http://www.genepaint.org
Hereditary Hearing Loss Homepage, http://hereditaryhearingloss.org/
Online Mendelian Inheritance in Man, http://www.ncbi.nlm.nih.gov/Omim
UCSC Human Genome Browser, Build hg19, March 2006, http://www.genome.ucsc.edu

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5

Audiometric characteristics of a Dutch family with Muckle-Wells syndrome

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Hearing Research 2011; 282: 243-251

Abstract

In this paper, we describe the audiometric and vestibular characteristics of a Dutch family with Muckle-Wells syndrome (MWS). Examination of all family members consisted of pure-tone audiometry, otoscopy and genetic analysis. In addition, a selected group underwent speech audiometry, vestibulo-ocular examination, acoustic reflex testing, tests assessing loudness scaling, gap detection and difference limen for frequency, and speech perception in noise. Linear regression analyses were performed on the audiometric data.

Six clinically affected family members participated in this study and all were carriers of a p.Tyr859His mutation in the *NLPR3* gene. Most affected family members reported bilateral, slowly progressive hearing impairment since childhood. Hearing impairment started at the high frequencies and the low- and mid-frequency threshold values deteriorated with advancing age. Annual threshold deterioration (ATD) ranged from 1.3 to 1.9 dB/year with the highest values at the lower frequencies. Longitudinal linear regression analysis demonstrated significant progression for a number of frequencies in five individuals. Speech recognition scores were clearly affected. However, these individuals tended to have higher speech recognition scores than presbycusis patients at similar $PTA_{1,2,4\ kHz}$ levels. The loudness growth curves were steeper than those found in individuals with normal hearing, except for one family member (individual IV:6). Suprathreshold measurements, such as difference limen for frequency (DL_f), gap detection and particularly speech perception in noise were within the normal range or at least close to data obtained in two groups of patients with a so-called conductive type of hearing loss, situated in the cochlea.

Hearing impairment in MWS is variable and shows resemblance to previously described intra-cochlear conductive hearing impairment. This could be helpful in elucidating the pathogenesis of hearing impairment in MWS. Other associated symptoms of MWS were mild and nonspecific in the present family. Therefore, even without any obvious syndromic features, MWS can be the cause of sensorineural hearing impairment, especially when combined with (mild) skin rash and musculoskeletal symptoms. An early diagnosis of MWS is essential to prevent irreversible damage from amyloidosis. The effect of IL-1β inhibitors on hearing

impairment is more controversial, but an early start of treatment seems to be essential. Therefore, our results are of importance in patient care and counseling.

Introduction

Muckle-Wells syndrome (MWS; OMIM 191900) is an autoinflammatory disease that belongs to the inherited cryopyrin-associated periodic fever syndromes (CAPS), including familial cold autoinflammatory syndrome (FCAS; OMIM 120100) and the chronic infantile neurologic cutaneaous and articular (CINCA; OMIM 607115) syndrome.[1] MWS is a rare autosomal dominant disorder characterized by intermittent episodes of fever, urticarial rash, and muscle and joint pains (arthralgias or arthritis). Furthermore, the syndrome is associated with late-onset progressive sensorineural hearing impairment. In general, episodic fever occurs at irregular intervals every few weeks, lasting 12-36 h before resolving spontaneously. The onset age of clinical symptoms is variable and precipitating factors cannot always be identified.[2, 3] In addition, prolonged inflammation can lead to deposition of proteins, especially in the kidney, and secondary amyloidosis (type AA) can occur in a subset of patients, leading to nephropathy.[4-7] Blood and cerebral fluid analysis can also demonstrate signs of inflammation during acute outbursts of the disease.[2, 5]

MWS arises from mutations in a single gene: *NLRP3* (OMIM 606416) located on chromosome 1q44,[8] which encodes a protein called cryopyrin. This protein consists of several distinct motifs, including a pyrin domain, a central nucleotide binding site domain (NBS; NACHT subfamily) and a C-terminal domain containing seven leucin-rich repeats (LRR).[1, 9, 10] Cryopyrin is part of the multiprotein inflammasome complex, the formation of which is triggered by "cellular danger" including infection and metabolic dysregulation.[11, 12] The NLRP3 inflammasome activates caspase 1, leading to the processing and secretion of the pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18.[13] Mutations in *NLRP3* are thought to cause abnormal formation of the inflammasome complex and inappropriate production of active IL-1β, possibly due to defective self-inhibition by the mutant cryopyrin protein.[14] Recent studies in mice carrying mutations in the *Nlrp3* gene indicated that IL-1β indeed has a pivotal role in the CAPS disease spectrum and that it leads to Th17 cell-dominant immunopathology in autoinflammation.[15-17]

The importance of IL-1β in the pathogenesis of MWS is further confirmed by the effectiveness of treatment with IL-1 inhibitors.[18] IL-1 inhibitors can control the

symptoms of systemic inflammation in patients with MWS.[14, 19] However, the effect on hearing impairment remains uncertain.[20]

Two other autoinflammatory disorders, FCAS and CINCA, are also caused by mutations in the *NLRP3* gene.[1, 9, 21, 22] The overlapping symptoms among these different syndromes indicate a continuum in severity of the disease,[9, 23-25] with CINCA syndrome being the most severe, FCAS the mildest and MWS the intermediate phenotype.[26] The majority of these mutations were missense mutations occurring in exon 3, which encodes the central NBS domain, indicating that this domain is crucial to cryopyrin function. However, there is no apparent correlation between disease severity and the particular domain in which the mutation occurs, the specific residue mutated or the conservation of amino acids.[27, 28]

In the present paper, audiometric characteristics of a Dutch MWS family with a novel mutation in *NLRP3* are presented. As it is yet unknown how MWS affects the cochlea, a broad set of audiological tests was administered to assess cochlear function in some detail.

Patients and methods

Patients

A Dutch family (n = 15) with autosomal dominant sensorineural hearing impairment (W07-1001) was studied. The pedigree is shown in Figure 1. After informed consent had been obtained from the participating family members, a family investigation was performed. The study was approved by the local medical ethics committee of the Radboud University Nijmegen Medical Centre, the Netherlands.

The examination of all family members included medical history guided by a questionnaire, otoscopy, pure-tone audiometry and collection of blood samples for genetic analysis. Clinically affected family members also underwent speech audiometry. Vestibulo-ocular examination was performed in three clinically affected family members. Furthermore, concomitant disease, the use of medication and any other possible cause of acquired hearing impairment were ruled out. Previous medical records and audiograms were traced for individual longitudinal analysis.

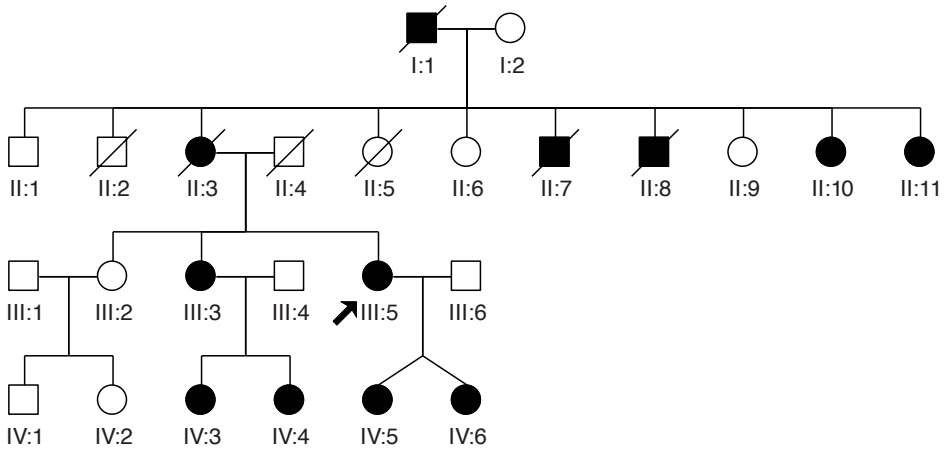

Figure 1. Pedigree of a Dutch family with Muckle-Wells syndrome. Square: male; circle: female; open symbol: clinically unaffected; solid symbol: clinically affected; slash: deceased individuals.

Linkage analysis

Genomic DNA of all participating individuals was extracted from peripheral blood lymphocytes according to standard protocols. Microsatellite markers flanking the *NLRP3* gene, more specifically D1S2836, D1S2215 and D1S2682, were genotyped under standard PCR conditions and were analyzed on an ABI Prism 3730 Genetic Analyzer with the GeneMapper program according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). Multipoint linkage analysis was performed with GeneHunter version 2.1r5 in the EasyLinkage software package. An autosomal dominant mode of inheritance with a penetrance of 100% and a disease allele frequency of 0.001 were used for LOD score calculations.

Mutation analysis

Amplification of all coding exons and flanking intronic sequences by polymerase chain reaction (PCR) was performed on 40 ng of genomic DNA with Taq DNA polymerase (Roche, Indianapolis, USA). Primer sequences and PCR conditions are available in Table S1. PCR fragments were purified by using NucleoFast 96 PCR plates (Clontech, Mountain View, CA, USA) according to the manufacturer's protocol. Sequence analysis was performed with the ABI PRISM Big Dye Terminator Cycle Sequencing V3.1 Ready Reaction kit and the ABI PRISM 3730 DNA analyzer (Applied

Biosystems, Foster City, CA, USA). NM_004895.4 and NT_167186.1 were used as reference sequences.

The segregation of the c.2575T>C transition in the family and presence of this transition in healthy controls was tested via an amplification refractory mutation system (ARMS) approach; primer sequences are provided in Table S1.

Pure-tone audiometry and data analysis

Audiometric examination comprised conventional pure-tone audiometry in a sound-treated room according to common clinical standards. Air-conduction (AC) and bone-conduction (BC) thresholds were measured in dB hearing level (dB HL) at 0.25, 0.5, 1, 2, 4 and 8 kHz. Bone-conduction was measured to rule out conductive hearing impairment. The individual 95th percentile (P95) threshold values of presbycusis in relation to the patient's sex and age were derived for each frequency using the ISO 7029 method.[29] Individuals were considered affected if the best hearing ear showed thresholds at three or more frequencies beyond the P95 for presbycusis.

Analyses of audiometric data were performed on the data pertaining to the mutation carriers of the present family. Binaural mean AC threshold values were calculated for each frequency. All binaural mean AC threshold values of the six affected family members were included to establish a trend line for the progression of hearing impairment with advancing age for each frequency separately. The regression coefficient (slope) was called annual threshold deterioration (ATD), expressed in dB per year. Age Related Typical Audiograms (ARTA) were drawn by using age-related threshold data derived from the results of the linear regression curves as described by Huygen et al.[30]

Individual longitudinal linear regression analysis of binaural mean AC threshold values on age was only performed in clinically affected persons with three or more consecutive measurements and an overall follow-up period of at least three years. Again, the ATD was calculated. Progression was significant if the 95% confidence interval did not include zero.

Speech audiometry and data analysis

Speech audiometry was performed under above-mentioned conditions using standard Dutch consonant-vocal-consonant word lists. The maximum phoneme

recognition score was obtained from monaural performance versus intensity curves and represents the mean phoneme recognition score of both ears. These maximum phoneme recognition scores were analyzed in relation to age and to pure-tone average (mean value for both ears) at 1, 2 and 4 kHz ($PTA_{1,2,4\ kHz}$). Cross-sectional analysis was performed using linear regression analysis. The age of onset and the onset level were defined at a recognition score of 90% in cross-sectional performance versus age and performance versus impairment plots, respectively. The average slopes were called deterioration rate and deterioration gradient, respectively. A previously described group of subjects with only presbycusis (P50) was used as a reference group. The phoneme recognition scores in this group had been fitted using nonlinear regression analysis based on a dose-response curve with variable slope.[31]

Additional audiological testing

Five affected family members (III:3, III:5, IV:4, IV:5 and IV:6) were also evaluated with additional audiological tests, namely loudness scaling, gap detection, difference limen for frequency and speech perception in noise, as described previously by Plantinga et al.[32] Loudness scaling was performed at 0.5 and 2 kHz using a 7-point categorical scale.[33] The slope of the loudness versus stimulus level graph was used as outcome measure. Gap detection was measured with gated white noise at 0.5 kHz and 2 kHz. Difference limen for frequency (DL_f) discrimination was measured with frequency-modulated pure-tones ranging from 0.1% to 5% presented at the individual listener's most comfortable level at 0.5 kHz and 2 kHz. Speech perception in noise was measured with short, everyday Dutch sentences.[34] Speech reception threshold (SRT) was measured with an adaptive procedure. All tests were performed with headphones on the ear with the best hearing, at the patient's most comfortable listening level (except for loudness scaling). The mean outcomes were compared to those of normal-hearing individuals as well as to those of patients with autosomal dominant nonsyndromic sensorineural hearing impairment originating from tectorial membrane abnormalities, namely patients with DFNA8/12 (*TECTA* gene)[32] and DFNA13 (*COL11A2* gene).[35]

Vestibulo-ocular examination and data analysis

Three affected family members (III:3, III:5 and IV:3) also underwent vestibulo-ocular examination. Vestibular and ocular motor tests included evaluation of the vestibulo-ocular reflex, using electronystagmography with computer analysis, and evaluation of saccadic, smooth pursuit and optokinetic nystagmus responses. Vestibular stimulation comprised rotatory and caloric tests. Details and normal values have been described previously.[36]

Results

Symptoms of the family members

A four-generation pedigree was established for the present family, in which hearing impairment segregated in a pattern that suggested autosomal dominant inheritance. (Figure 1) Twelve family members were affected, four of whom only by history. Fifteen family members participated in this study. No informed consent could be obtained from individuals II:10 and II:11.

Clinically affected family members (individuals III:3, III:5, IV:3, IV:4, IV:5 and IV:6) showed no evidence of other causes of hearing impairment. Most affected family members reported bilateral, slowly progressive hearing impairment. First symptoms of hearing impairment were reported at ages ranging from 4 to 25 years (mean subjective age of onset: 12 years).

The proband of the present family (individual III:5) reported intermittent episodes of headache, urticarial rash and joint pains in addition to her hearing impairment. Furthermore, blood and cerebral fluid analysis showed signs of an inflammatory process. These symptoms in combination with progressive sensorineural hearing impairment raised the suspicion of MWS. More detailed history revealed also frequent conjunctivitis and hypoesthesia with tingling sensations of her hands. Furthermore, Anakinra (interleukin-1 receptor antagonist) treatment resolved her symptoms and normalized her erythrocyte sedimentation rate. When specifically asked for, the other affected family members (individuals III:3, IV:3, IV:4, IV:5 and IV:6) also reported other symptoms besides hearing impairment, such as urticarial rash, joint pains, conjunctivitis and tingling sensations of their hands. However, these symptoms were less severe than the symptoms reported by the proband.

No relation with cold temperature could be demonstrated and FCAS was considered to be unlikely. Furthermore, gradual hearing impairment is not a common symptom of FCAS. Severe inflammatory damage, for example joint deformities, is frequently seen in CINCA, but not in de present family. Moreover, the present family members had no mental or cognitive disorders and no gradual loss of eyesight, which is often present in CINCA. Therefore, CINCA was also considered not likely.

Genetic analysis

Genotyping of microsatellite markers was performed to test the segregation of the *NLRP3* locus with the disease phenotype in the present family. As shown in Figure S1 the markers segregated perfectly with the disease, yielding a maximum multipoint LOD score of 2.99. Mutation analysis in individual III:3 revealed a heterozygous nucleotide substitution, c.2575T>C in exon 6 of the *NLRP3* gene leading to the substitution of histidine for tyrosine at position 859 of the protein (p.Tyr859His). The mutation co-segregated with the disease in the family and was not found in 114 ethnically matched controls.

Vestibular function

Variable vestibular symptoms were reported by three of the six affected family members (individuals III:3, IV:3 and III:5), including dizziness and instability, especially in the dark. Evaluation of vestibular function in individual III:3 at age 47 years revealed no abnormalities. Individual IV:3 at age 21 years showed remarkable hyporeflexia of velocity-step responses with time constants of 7 and 10 s for both nystagmus directions. Furthermore, caloric testing revealed bilateral caloric weakness. Vestibular function tests in individual III:5 at age 44 years showed hyporeflexia in the rotatory tests, however, caloric testing revealed no abnormalities. More patients with MWS should be tested to assess whether vestibular dysfunction indeed can be part of MWS.

Pure-tone audiograms

A representative selection of available pure-tone audiograms of the six clinically affected family members is shown in Figure 2. Pure-tone audiometry never revealed a persisting air-bone gap in any of the family members. The individual audiograms of the left and right ear were fairly symmetric and within limits of 20 dB. Therefore,

mean values of binaural AC thresholds were calculated. However, some inter-individual variation in audiometric configuration, as well as in the degree of hearing impairment was observed. Most frequently, high-frequency hearing impairment was observed with a down-sloping audiogram configuration. Flat audiogram configurations were, however, also seen. In general, hearing impairment started at the high frequencies, the low- and mid-frequency threshold values deteriorated with advancing age. A downsloping audiometric configuration applies to the audiograms at young ages (IV:3, IV:4, IV:5 and IV:6). More flat audiometric configurations are mainly seen at more advanced ages (III:3 and III:5). High-frequency hearing impairment appeared to start even before the age of 5 years in individual IV:4. (Figure 2) Before the age of 30 years, the low-frequency threshold values started to deteriorate, resulting in low-frequency threshold values in the range of 60-80 dB at 40 years of age. High-frequency threshold values deteriorated to about 100 dB at the age of 40 years. (Figure 2)

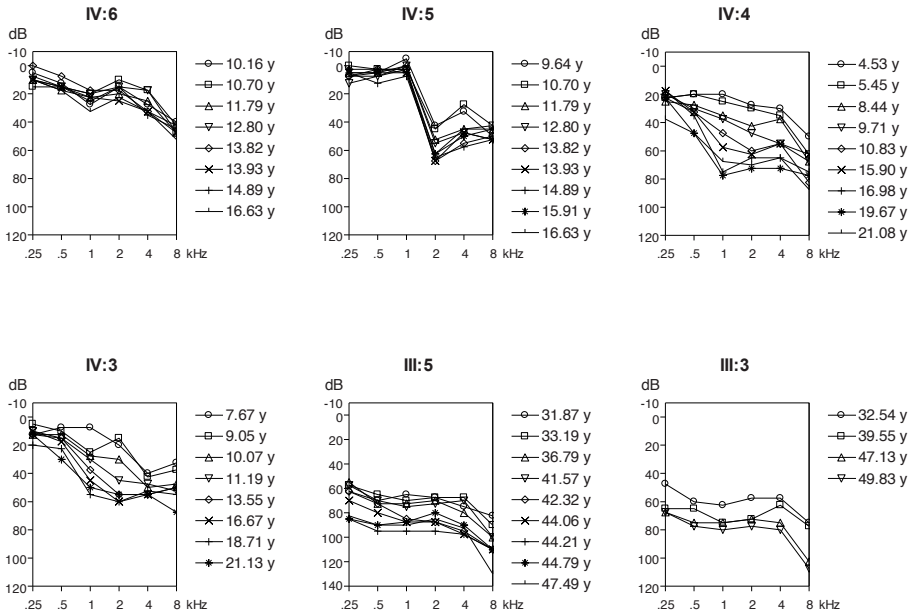

Figure 2. Selection of binaural mean air-conduction threshold values of six clinically affected family members at different ages, ordered by age (from top left to bottom right) at last visit.

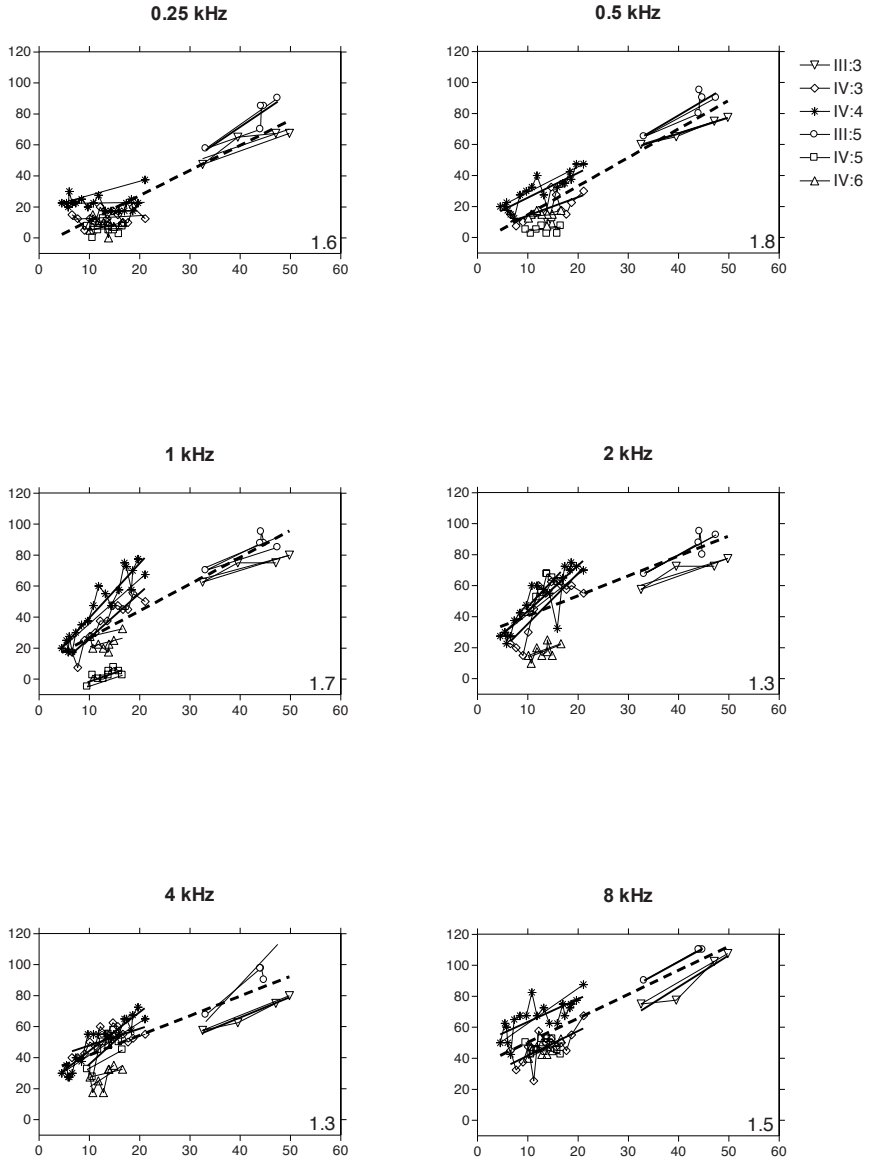

Figure 3. Mixed single-snapshot and longitudinal individual measurements with connection lines (different symbols for each family member) of affected family members are shown for each frequency separately. The black dashed line (overall 'trend line') represents the cross-sectional linear regression analysis. The number in the lower right of each panel represents the average threshold deterioration value in dB/year derived from cross-sectional linear regression analysis. The regression lines fitted to the individual longitudinal measurements are also included (straight lines). A bold line indicates significant progression.

Progression of hearing impairment

All threshold data of the examined individuals are plotted against age in Figure 3. The individual regression lines are included and an overall trend line could be established for each frequency. ATD values ranged from 1.3 to 1.8 dB/year with the highest values at the lower frequencies. The trend lines seemed to provide a reliable estimation of the overall progression in this family. (Figure 3)

All affected family members showed a slowly progressive type of sensorineural hearing impairment. However, longitudinal regression analysis of audiometric data revealed significant progression for all individuals at some frequencies, except for individual IV:6. (Figure 3) This is probably because the available audiograms covered only a relatively short age range. The longitudinal regression analyses and the trend lines showed fairly similar progression rates with advancing age. (Figure 3)

The ARTA derived from the (dashed) overall trend line in Figure 3 is shown in Figure 4. Even before the age of ten years, the threshold values at the high frequencies were substantially affected. However, the threshold values at the low frequencies showed more progression than the threshold values of the high frequencies with advancing age.

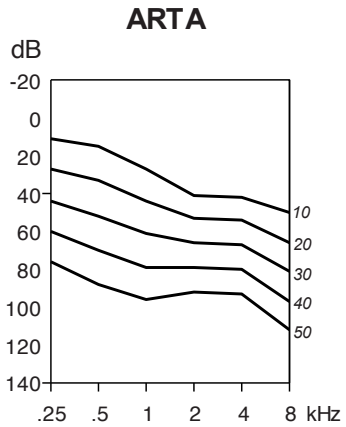

Figure 4. Age-related typical audiograms (ARTA) derived from the (dashed) overall trend lines in Figure 3. The italic numbers indicate age in years.

Speech recognition

Figure 5 shows the available single-snapshot measurements of the phoneme scores of the affected family members examined. Speech recognition was remarkably well preserved in the present family. The age of onset (X90) was 35 years with a deterioration rate of 0.5% per year, whereas the onset age in presbycusis patients was 74 years with a deterioration rate of 3.3% per year. The speech recognition scores related to the level of hearing impairment in the present family members appeared to be better than those of the presbycusis patients at similar levels of hearing impairment. (Figure 5, right panel) The 90% recognition scores were found at a $PTA_{1,2,4\ kHz}$ level of 71 dB and 48 dB in the affected family members and the presbycusis patients, respectively. The deterioration gradient in the score-against-$PTA_{1,2,4\ kHz}$ plot was approximately 0.3% per dB, compared to the deterioration gradient of 1.1% per dB in the presbycusis patients.

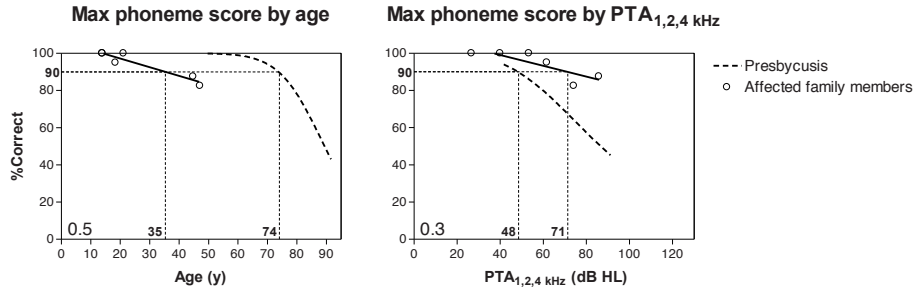

Figure 5. Single-snapshot measurements of maximum phoneme recognition scores against age (left) and against mean pure-tone average at 1, 2, and 4 kHz (right). The solid lines are the linear regression curves fitted to these measurements. The dotted curves represent the P50 presbycusis line, previously established for patients with presbycusis.

Figure 6 shows speech perception scores in noise for sentences in relation to PTA at 2 and 4 kHz. Reference data were obtained from Smoorenburg,[37] who studied a large group of subjects with moderate to severe sensorineural hearing impairment. Individual data points of the present family members are displayed in the Figure, as well as the best fit through the data of Smoorenburg[37] with its two standard deviations region. The lower the score, the better. Even for patients with severe to profound hearing impairment (individuals III:3 and III:5), speech recognition in

noise scores were remarkably good and significantly better than those of the control patients. This suggests that indeed, speech perception is better than expected when related to hearing thresholds.

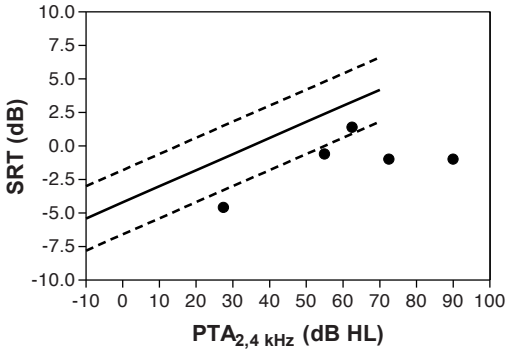

Figure 6. Speech Reception Thresholds (SRT) in noise for sentences in relation to PTA at 2 and 4 kHz. The symbols are the data for the present family members. The straight line represents the average SRT values in noise as a function of the average pure tune thresholds at 2 and 4 kHz. The dashed lines represents two times the standard deviation of this regression curve.[37]

Additional audiological measurements

Table 1 shows the mean results (and standard deviations) of loudness scaling, gap detection and difference limen for frequency experiments at 2 kHz. For comparison, results of normal hearing individuals, DFNA8/12 patients and DFNA13 patients taken from previous studies are included.[32, 35] In several affected family members, tests at 0.5 kHz did not reveal remarkable results because the thresholds at 0.5 kHz were close to normal. Therefore, it was decided to disregard the results of the tests at 0.5 kHz. Loudness growth curves of affected family members showed steeper slopes than that of the individuals with normal hearing. The mean loudness growth slopes of DFNA8/12 and DFNA13 patients are also steeper than the loudness growth curves of the normal hearing individuals, and comparable to those of the present MWS patients. The mean gap detection result of the affected family members was close to normal and comparable to those reported for DFNA8/12 patients. The DFNA13 patients demonstrated a poorer result on gap detection testing. Compared to individuals with normal hearing, who achieve a DL_f of approximately 0.3% in

response to a 2 kHz tone, the mean performance of the present family members was clearly poorer, also in comparison with that of the DFNA13 patients. The DL_f of the DFNA8/12 patients was fairly similar to those of the present patients. Unfortunately, the number of MWS patients (n = 5) and DFNA8/12 patients (n = 5) included in additional audiological testing was very small. Furthermore, the results of the MWS patients, DFNA8/12 patients and DFNA13 patients showed a wide variation. Nevertheless, it could be concluded that the results of the present MWS patients are comparable to the results of DFNA13 patients and mainly to the results of DFNA8/12 patients with intra-cochlear conductive hearing impairment.[32, 35]

Patients	*Loudness growth in loudness category/dB (SD)*	*GAP detection at 2 kHz in ms (SD)*	*DL_f at 2 kHz in % (SD)*
MWS	0.08 (0.03)	4.2 (3.5)	1.2 (0.7)
Normal hearing	0.05	4 (0.5)	0.3 (0.3)
DFNA8/12	0.09 (0.02)	4.4 (2.5)	1.1 (1.0)
DFNA13	0.07 (0.02)	6.6 (4.0)	0.5 (0.3)

Table 1. Results of loudness scaling, GAP detection and difference limen for frequency (DL_f) at 2 kHz of the five affected family members (individuals III:3, III:5, IV:4, IV:5 and IV:6), normal hearing individuals, DFNA8/12 patients and DFNA13 patients. SD: standard deviation.

Discussion

This report presents the audiometric characteristics and genetic analysis of an MWS family with a c.2575T>C mutation in the *NLRP3* gene. In the present family, a heterozygous missense mutation, p.Tyr859His, was identified in exon 6 of *NLRP3*, which encodes the LRR domain of the cryopyrin protein. This is a novel mutation, but the previously described p.Tyr859Cys mutation affects the same amino acid.[38, 39] This amino acid is highly conserved throughout evolution. Several studies have revealed the importance of the LRR domain in the proper functioning of NLPR3[10, 40-42] and Jéru et al.[43] demonstrated the mild functional effects of the p.Tyr859Cys mutation by structural analysis.[44]

Hearing impairment in MWS

Sensorineural hearing impairment is one of the diagnostic criteria of MWS, but the degree of hearing impairment can be very variable. Mild hearing impairment is described but also profound hearing impairment. The severity of hearing impairment depends on the age of the patient and on the moment that treatment with an IL-1 inhibitor was started.[45] Hearing impairment is progressive and more pronounced at the high frequencies, but may involve all frequencies with advancing age. Usually hearing impairment starts in the second decade of life, but also onset in early childhood as well as midlife onset have been described.[3, 45, 46] Large variation is found in hearing impairment phenotype between and within CAPS families. Nevertheless, hearing impairment of the present family seemed relatively severe and started already at a young age (mean subjective onset age: 12 years, range: 4-25 years). In the second decade of life, most family members required a hearing aid. This was also demonstrated by the ARTA in Figure 4. Because of the good speech recognition scores, the present family members are not appropriate candidates for cochlear implantation.

Phenotypic heterogeneity in CAPS

The large intra-familiar phenotypic heterogeneity of CAPS suggests possible involvement of modifier genes and environmental factors in expression of the phenotype. This variable expression is also seen in the present family. Individuals III:3, IV:3, IV:4, IV:5 and IV:6 reported, besides hearing impairment, only mild symptoms, whereas individual III:5 showed a more severe autoinflammatory response. Kuemmerle-Deschner et al.[47] demonstrated that female patients presenting with hearing impairment have the highest likelihood of manifesting severe symptoms of MWS and should be considered a high-risk group. However, the affected women in the present family do not demonstrate other severe symptoms besides hearing impairment.

Pathogenesis of hearing impairment in MWS

Muckle and Wells reported in 1962 the results of postmortem examinations of temporal bones of two patients with MWS and progressive hearing impairment since childhood. In both these patients degeneration of the cochlear nerve, the organ of Corti and the vestibular sensory epithelium was demonstrated. These findings

may have been caused by postmortem autolysis or by ischemia due to vascular amyloid deposits. However, amyloid deposits were not detected anywhere in the temporal bone sections.[2] Furthermore, in the present family hearing impairment was already present at young age, in the absence of amyloidosis. Speech recognition was relatively good compared to the severity of hearing impairment in the present family and this suggests sparing of the cochlear nerve. Unfortunately, speech recognition scores were not reported for previously described families with MWS. Taken together, this suggests that the degenerative changes of the cochlear nerve and organ of Corti described by Muckle and Wells have been caused by postmortem autolysis and cannot be the main cause of hearing impairment in MWS.[2] Hypofunction of the vestibular labyrinth demonstrated in some family members of the present family could, however, be caused by degeneration of the vestibular sensory epithelium.

Muckle and Wells also demonstrated ossification of the basilar membranes in patients with MWS. Since ossification did not occur in other parts of the temporal bone, an otosclerotic pathogenesis was excluded.[2] The cause of hearing impairment in MWS is still unknown, but the basilar membrane could be involved in the pathogenesis of hearing impairment, as is the case in the pathogenesis of Alport syndrome.[48-50] The structurally defective basement membrane in Alport syndrome probably provides inadequate adhesion between the organ of Corti and the underlying basilar membrane. It is suggested that basilar membrane motion is not properly adjusted by the outer hair cells and this inappropriate tuning probably results in sensorineural hearing impairment by interfering with cochlear micromechanics.[50] Furthermore, pathology at the level of the basilar membrane could be responsible for the good speech recognition scores seen in the present family, which is also found in Alport syndrome.[48, 49] Results of the audiometric evaluation also indirectly support the hypothesis of improper motion of the basilar membrane in patients with MWS. Suprathreshold measures such as DL_f and gap detection were within the normal range or at least close to data obtained in two groups of patients with known abnormalities of the tectorial membrane.[32, 35] The speech perception in noise scores of all family members were better than those of the control patients. These findings demonstrate remarkably good cochlear function even in severe hearing loss and contrasts to results often found in sensorineural

hearing impairment that results from loss of outer and/or inner hair cells (e.g. presbycusis, noise induced hearing loss). In conclusion, our results indicate that MWS related hearing impairment might be considered as a cochlear conductive hearing impairment, similar to hearing impairment in DFNA8/12 and DFNA13.[32, 35] Decreased motion transmission may be a common factor to the hearing deficits in Alport syndrome and the type of mid-frequency hearing impairment found in DFNA8/12 and DFNA13. In the latter two the loss of transmission occurs between the tectorial membrane and the outer hair cells, whereas in the former it occurs at the level between the basilar membrane and the organ of Corti. In both conditions the proper function of outer hair cell motion is jeopardized.

However, hearing impairment in the present family members III:3 and III:5 was more severe than the 60-70 dB threshold values reported in Alport syndrome,[48] which may indicate the influence of additional causative factors. Inflammatory processes in the cochlea and leptomeninges are probably also contributing to sensorineural hearing impairment in MWS.[51] Improvement of hearing impairment with Anakinra therapy also suggests the contribution of a local inflammatory response caused by IL-1β secretion.[52] Unfortunately, the expression of NLRP3 in the inner ear is not known, but this information could be helpful to elucidate the pathogenesis of hearing impairment.

Treatment with IL-1 inhibitors

The proband of the present family has been treated with Anakinra 100 mg daily subcutaneously for the last year. This treatment has controlled the clinical and serologic symptoms of an active inflammatory process, however her hearing impairment has not improved. The other family members have only recently started treatment with IL-1 inhibitors. Kuemmerle-Deschner et al.[20] described the effects of Anakinra treatment on hearing in ten MWS patients. Audiometric thresholds improved by 10-30 dB in the 0.25-4 kHz range to normal hearing levels in one patient, aged 15 years at start of therapy. One adult of 44 years of age had gradual improvement. Hearing worsened in two other adult patients, while treated with Anakinra. In the six remaining patients, hearing stabilized.[20] Rynne et al.[53] also reported improvement of 15-30 dB in the frequency range of 0.25-4 kHz after 18 weeks of Anakinra therapy. The patient started with Anakinra therapy at the age of

59 years, after 15 years of progressive hearing impairment.[53] In addition, Mirault et al.[54] described a case of complete recovery of hearing impairment in a 22-year-old patient, who had been hearing impaired since the age of 12 years. After 3 months of therapy, threshold values improved from 50 dB to approximately 10 dB.[54] The patient described by Dalgic et al.[55] showed improvement of threshold values of about 20 dB after 2 months of Anakinra treatment. Audiometric evaluation at the age of 13 years revealed sensorineural hearing impairment and the patient started with Anakinra therapy one year later.[55] Moreover, Yamazaki et al.[52] described an 8-year-old patient with asymmetrical hearing impairment. Threshold values improved to approximately 10 dB for both ears after 3 months of treatment.[52] Nevertheless, there are also numerous reports of no significant improvement in threshold values after treatment with Anakinra.[19, 26, 43, 56,] An accurate early diagnosis before the occurrence of irreversible hearing impairment seems to be crucial for the possibility of stopping deterioration or even improve hearing with Anakinra treatment.

Conclusion

The present study describes a Dutch family with MWS caused by a p.Tyr859His mutation in the *NLRP3* gene. Hearing impairment was progressive and more pronounced at the high frequencies, but involved all frequencies with advancing age. Despite the severe hearing impairment, speech recognition was remarkably good, even in noise.

The cause of hearing impairment in MWS is not yet understood, but the basilar membrane of the cochlea may be involved in the pathogenesis of hearing impairment. The present data suggest that hearing impairment in MWS can be characterized as an intracochlear conductive hearing impairment. This could be helpful in elucidating the pathogenesis of hearing impairment in MWS.

An early diagnosis of MWS is essential to possibly prevent profound hearing impairment and irreversible damage from amyloidosis. Treatment with the IL-1β inhibitors has proven to be effective in reducing the symptoms of systemic inflammation. The effect on hearing impairment is more controversial, but an early start of treatment seems to be essential. Therefore, our results are important in patient care and counseling.

In every patient with sensorineural hearing impairment in combination with skin rash and musculoskeletal symptoms, MWS should be considered. However, these symptoms can be mild and nonspecific, as was the case in the present family. Therefore, the diagnosis of MWS can be easily missed.

Acknowledgement

This study was supported by a grant from the Heinsius Houbolt foundation and by a grant from the INTERREG IV A-program Germany-the Netherlands.

Supplemental table and figure

Fragment	*Oligonucleotides* *Primers for PCR and sequence analysis*	*Size (bp)*	*Annealing Temperature (°C)*
NLRP3 exon 1	Forward: gctggtcttgaattcctcag Reverse: tttaaaagtcttccttccactcac	589	58
NLRP3 exon 2	Forward: gaaatgctcccaaccagac Reverse: agtatggccaagttacccag	385	56
NLRP3 exon 3_1	Forward: attctcggcacctttcctac Reverse: gaggaagaggattctggagg	650	58
NLRP3 exon 3_2	Forward: tgtgacacagaggagcctg Reverse: aaggaagaagacgtacaccg	544	58
NLRP3 exon 3_3	Forward: tcctcttcaccatgtgcttc Reverse: agtaggaggtcctctcctgg	564	56
NLRP3 exon 3_4	Forward: agacgtgacagtccttctgg Reverse: tctcaaacagacagtggtgg	632	56
NLRP3 exon 4	Forward: ggcatttctctgaactggtg Reverse: tggtcctgaagatctttctcc	360	60
NLRP3 exon 5	Forward: caggtgtgttctgatgctttc Reverse: acactcactgaccgcaatg	505	66
NLRP3 exon 6	Forward: tagagcttgtgtccactccc Reverse: taccttcagctctgcctgac	403	58
NLRP3 exon 7	Forward: tgagagaggacgaggcac Reverse: tgatcctgtaacaaggcaaac	397	58
NLRP3 exon 8	Forward: ttagtcctgtgctcctgtgc Reverse: aggcccaacctaatcttgag	387	60
NLRP3 exon 9	Forward: tgtgtggagtttagggaaatg Reverse: gtcggcaagctctcttctc	347	60
Wildtype exon 6 c.2575T>C	Forward: attgagcaccagccattccctgaccagactat Forward: attgagcaccagccattccctgaccagactac Reverse: ccctcaacaggcaattgggctgcac	387	60

Table S1. Sequences of primers for amplification of exons, intron-exon boundaries and transcripts of *NLPR3.* NM_004895.4 and NT_167186.1 were used a reference sequences.

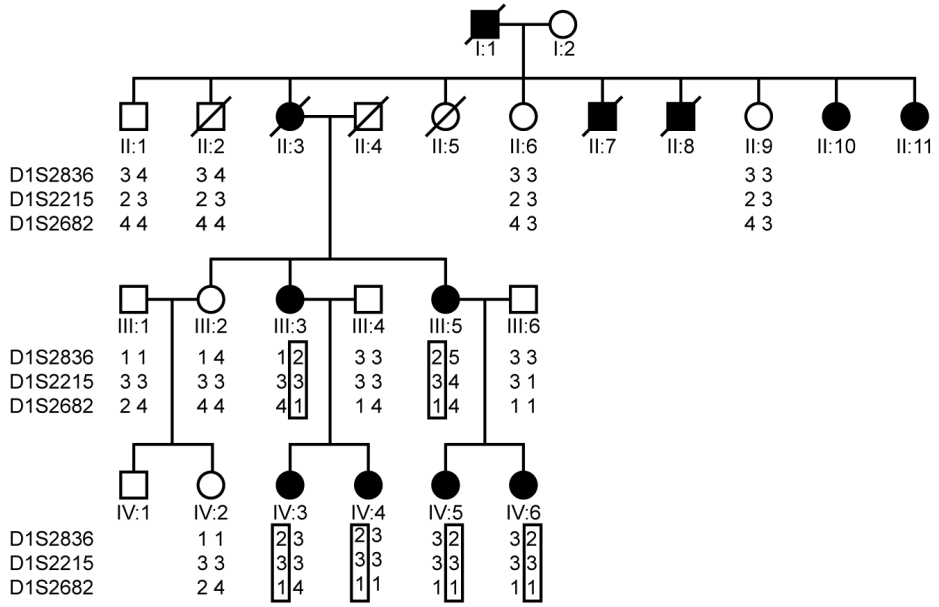

Figure S1. Pedigree and genetic analysis. The haplotype associated with the Muckle-Wells syndrome is indicated with a box. Square: male; circle: female; open symbol: clinically unaffected; solid symbol: clinically affected; slash: deceased individuals.

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6.1

Phenotype of the first otosclerosis family linked to OTSC10

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The Laryngoscope 2011; 121: 838-845

Abstract

In the present study, we report the audiometric findings in the first otosclerosis family linked to OTSC10. A family study in a large otosclerosis family was performed and a pedigree was constructed. Examination of all family members consisted of medical history guided by a questionnaire, pure-tone audiometry, otoscopy and collection of blood samples for genetic linkage analysis. In addition, a selected group underwent stapedial reflex measurements and tympanometry. Cross-sectional as well as longitudinal analyses of audiometric data were performed.

Eleven family members were identified as clinically affected and were all carriers of the disease haplotype. Twelve clinically unaffected family members carried the disease haplotype as well. Cross-sectional analyses of clinically affected family members showed no significant progression of air-conduction (AC) thresholds, bone-conduction (BC) thresholds and air-bone gap (ABG) levels with increasing age. Longitudinal regression analyses in one family member revealed significant deterioration of AC thresholds at all frequencies. The BC thresholds showed a significant increase with advancing age at 0.5 kHz, 2 kHz and 4 kHz. A significant progression of ABG was seen at 8 kHz.

The intersubject variation, in terms of age of onset, level of progression and audiogram configuration was remarkable, probably due to reduced penetrance and variable expression of the disease. Long-term audiometric data in one patient, however, were useful to demonstrate progression of hearing impairment.

Introduction

Otosclerosis is a unique form of bone dysplasia exclusively affecting the temporal bone. The pathologic process is characterized by abnormal bone remodeling at specific sites of predilection confined to the endochondral layer of the otic capsule.[1]
Otosclerosis can be divided into a histologic and clinical type. Histologic otosclerosis is defined as asymptomatic disease that can only be discovered in histologic temporal bone sections. Clinical otosclerosis is characterized by progressive conductive hearing impairment due to stapes ankylosis. Histologic otosclerosis is about 10 times more common than clinical otosclerosis and is found in 10% of the Caucasian population. In other races, the prevalence is much lower. The disease is bilateral in 70% to 80% of patients usually with a symmetrical extension and distribution of otosclerotic foci. The symptoms depend on the site of the otosclerotic foci. The most common site is anterior to the oval window (fissula ante fenestram), followed by the round window niche and the apical and medial cochlear wall, respectively. Other sites of involvement are posterior to the oval window, the internal auditory canal, around the cochlear aqueduct, around the semicircular canals and within the footplate.[1, 2]
Furthermore, cochlear otosclerosis is recognized as a separate entity characterized by progressive sensorineural hearing impairment (SNHI) due to otosclerotic involvement of the cochlea.[1, 3] The literature provides conflicting information regarding prevalence and cause of SNHI in patients with otosclerosis, but long-term follow-up studies suggest that about 10% of patients with conductive hearing impairment develop SNHI. Cochlear otosclerosis can exist in the absence of conductive hearing impairment, although isolated cochlear otosclerosis is rare.[4, 5]
The diagnosis of otosclerosis is in most cases based on clinical findings combined with audiometry results. However, the development of spiral computed tomography (CT) scanners with high-resolution images has increased the detection of otosclerosis and is a valid tool to confirm, localize and determine the size of clinically suspected otosclerotic foci. In most cases, CT can detect otospongiotic foci, characterized by hypodense lesions in the otic capsule. However, sclerotic foci may not be detected by CT, because these foci have the same density as normal bone tissue of the otic capsule.[6]

Conductive hearing impairment can be corrected by a hearing aid as well as by stapedotomy. Stapes microsurgery has proven to be highly successful in restoring the normal conduction mechanism and can improve hearing thresholds by as much as 50 dB.[7]

Otosclerosis is considered to be a multifactorial disease, caused by both genetic and environmental factors. However, rare monogenic forms of the disease also exist and several studies have reported that sporadic otosclerosis represents 40% to 50% of all clinical cases. Phenotype-genotype studies showed evidence for autosomal dominant inheritance with incomplete penetrance by approximately 40%.[8, 9] Genetic linkage analysis has demonstrated the presence of nine loci,[10] of which loci 6 and 9 are reported to the Human Genome Organization nomenclature committee, but have not been published yet. (Table 1) The large number of different loci reflects the genetic heterogeneity of this disorder. Furthermore, the variable phenotypic expression of the genetic forms of otosclerosis suggests the contribution of environmental factors to its etiology.[8, 9] Several theories have been postulated, including a viral etiology, disturbances of bone remodeling, disorders of endocrine and immune systems, and connective tissue disorders. However, despite intensive research and identification of a variety of factors involved in the development of the disease, the etiology of otosclerosis is still not fully understood.[2]

Locus	*Location*	*Reference*	
		Clinical	*Genetic*
OTSC 1	15q25-26	Not available	Tomek et al., 1998
OTSC 2	7q34-q36	Declau et al. 2007	Van den Bogaert et al., 2001
OTSC 3	6q21.3-22.3	Ali et al. 2007	Chen et al., 2002
OTSC 4	16q21-23.2	Brownstein et al. 2006	Brownstein et al., 2006
OTSC 5	3q22-q24	Pauw et al. 2006	Van den Bogaert et al., 2004
OTSC 6	Reserved		
OTSC 7	6q13-16.1	Iliadou et al. 2005; Pauw et al. 2007	Thys et al., 2007
OTSC 8	9q13.1-9q21.11	Ali et al. 2007	Ali et al., 2008
OTSC 9	Reserved		

Table 1. Loci for otosclerosis derived from the Hereditary Hearing Loss Homepage (http://hereditaryhearingloss.org).

To identify the disease-causing genes in the loci, refinement of candidate regions and mutation analysis of candidate genes is required. Cloning and completing functional analysis on causative genes and their related proteins may provide new insights into the molecular mechanisms of otosclerosis and may reveal targets for prevention and treatment of the disease.[8, 9] Genotype-phenotype correlation studies on each otosclerosis locus could be helpful to distinguish possible differences in clinical behavior. This article presents the clinical characteristics of the first family linked to OTSC10.

Patients and methods

Patients

A large multigenerational Dutch family with otosclerosis was studied and its pedigree was constructed. (Figure 1) After informed consent had been obtained from the 51 participating family members, a family study was performed. The study was approved by the local medical ethics committee of the Radboud University Nijmegen Medical Centre, the Netherlands.

Medical history was taken from all participants guided by the questionnaire, paying special attention to concomitant disease, use of medication and other possible causes of acquired hearing impairment. Medical examination included otoscopy, pure-tone audiometry and collection of blood samples for linkage analysis. Otoscopy was performed to rule out other causes of conductive hearing impairment, such as excessive ear wax, signs of previous otitis media and tympanic membrane perforation. Five family members had additional stapedial reflex evaluation. Previous medical records and audiograms were traced for individual longitudinal analysis.

Family members who previously had a stapedotomy for otosclerosis were considered to be affected. In nonoperated family members, the clinical diagnosis of otosclerosis was considered when at least several of the following criteria were met: presence of conductive or mixed hearing impairment, air-bone gap (ABG) ≥20 decibels (dB) averaged across 0.5 to 2 kHz, absent stapedial reflexes and normal otoscopy.

This family was previously investigated by genetic linkage analysis, which revealed linkage to the OTSC10 locus on chr1q41-44 (data not shown).

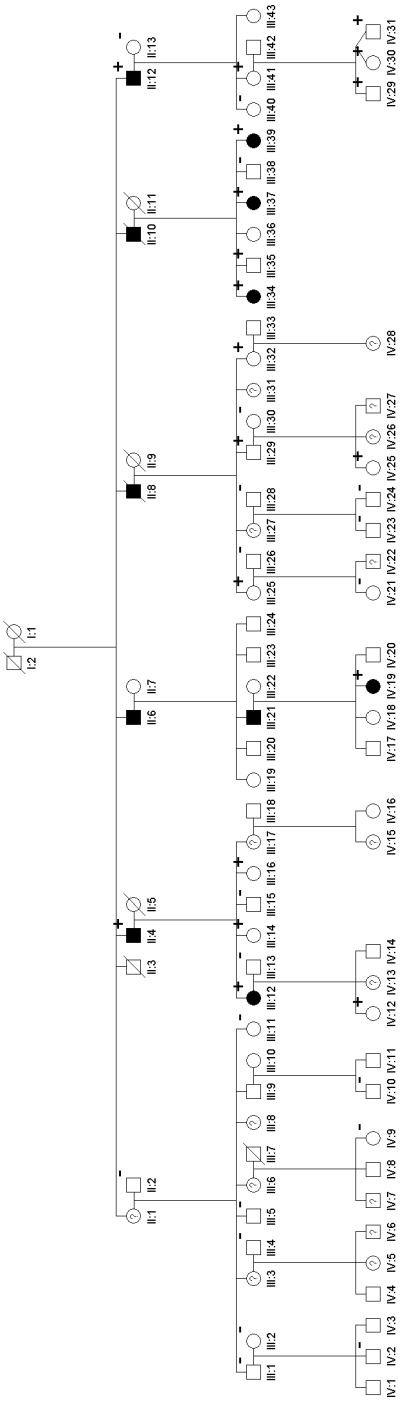


Figure 1. Pedigree of the Dutch OTSC10 family. Square: male; circle: female; slash: deceased individual; open symbol: clinically unaffected; solid symbol: clinically affected; + : genetically affected; - : genetically unaffected; ? : unclear genetic status.

Audiometry and data analysis

Audiometric examination comprised conventional pure-tone audiometry in a sound-treated room according to common clinical standards. Air-conduction (AC) thresholds were measured in dB hearing level (HL) at 0.25, 0.5, 1, 2, 4 and 8 kHz, and bone-conduction (BC) thresholds were measured in dB HL at 0.5, 1, 2, 4 and 8 kHz. The individual 95th percentile threshold values of presbycusis in relation to the patient's sex and age were derived for AC thresholds at each frequency using the ISO 7029 method.[11]

The most recent audiogram or last audiogram before stapedotomy of the clinically and genetically affected family members were included in the analysis. Only one ear per individual was included to avoid bias of the cross-sectional analysis because some individuals had unilateral otosclerosis. In case of bilateral otosclerosis, the nonoperated ear or ear with poorest hearing was included.

Cross-sectional analysis was performed for each frequency separately using linear regression analysis. The regression coefficient (slope) was called annual threshold deterioration (ATD), expressed in dB per year. Progression was significant if the 95% confidence interval of the ATD did not include zero. According to binomial distribution statistics, progression was only rated as significant if it occurred at two (or more) out of six frequencies. Individual longitudinal linear regression analysis of AC thresholds, BC thresholds and ABG levels on age was only performed in clinically affected persons with three or more consecutive measurements and an overall follow-up period of at least five years. Again, the ATD was calculated and the progression was significant if the 95% confidence interval did not include zero. The level of significance used in all tests was $p < 0.05$.

Results

Pedigree and general findings

A five-generation pedigree was established for the present family, demonstrating an autosomal dominant pattern of inheritance with incomplete penetrance. (Figure 1)

A CT scan of the temporal bones was performed in one family member at the age of 44 years (individual III:37). Individual III:37 underwent, at the age of 39 years, a stapedotomy for otosclerosis of her right ear and four years later for otosclerosis of

her left ear. The CT scan showed demineralization at the fissula ante fenestram on both sides.

Eleven clinically affected family members (II:4, II:6, II:8, II:10, II:12, III:12, III:21, III:34, III:37, III:39 and IV:19) were identified. Three of them (II:12, III:37 and III:39) previously underwent stapedotomy. Individual III:34 underwent middle ear inspection and the presence of otosclerosis was confirmed. In three other family members (II:4, III:12 and IV:19), the clinical diagnosis was based on the diagnostic criteria. We did not succeed in obtaining audiograms from individuals II:6, II:8, II:10 and III:21, but according to history and their position in the pedigree, these family members were considered as affected. (Figure 1) Some of the clinically unaffected family members showed a deviating audiogram according to sex and age, but did not meet the clinical criteria for otosclerosis and their hearing impairment had probably another cause. Therefore, a total of 11 affected family members were identified for genetic linkage analysis. This analysis revealed a new locus for otosclerosis, OTSC10, localized to chromosome 1q41-44 (data not shown).

All 11 clinically affected family members had the linked haplotype and no phenocopies were identified. In addition, 12 clinically unaffected family members (mean age: 39 years; range: 22–56) had the disease haplotype. The genetic status in 16 family members was uncertain because these individuals carried only part of the linked haplotype. In total, we identified 23 genetically affected family members.

The male:female ratio was 6:5 for clinically affected individuals and 10:13 for individuals with the disease haplotype. The mean subjective onset age of hearing impairment was 31 years (range: 16–51; standard deviation [SD] ± 14.1), and the mean age at first surgery was 38 years (range: 31–49; SD ± 6.9).

Audiometric analysis

The seven most recent or last audiograms before stapedotomy of the 11 clinically affected individuals are shown in Figure 2. Of four clinically affected family members, no audiogram could be retrieved. The characteristic audiogram of a patient with otosclerosis shows a dip in the BC threshold at 2 kHz, creating the typical Carhart notch. In general, the ABG is maximal at the lower frequencies and decreases with increasing frequency but may be minimal in association with the Carhart notch.

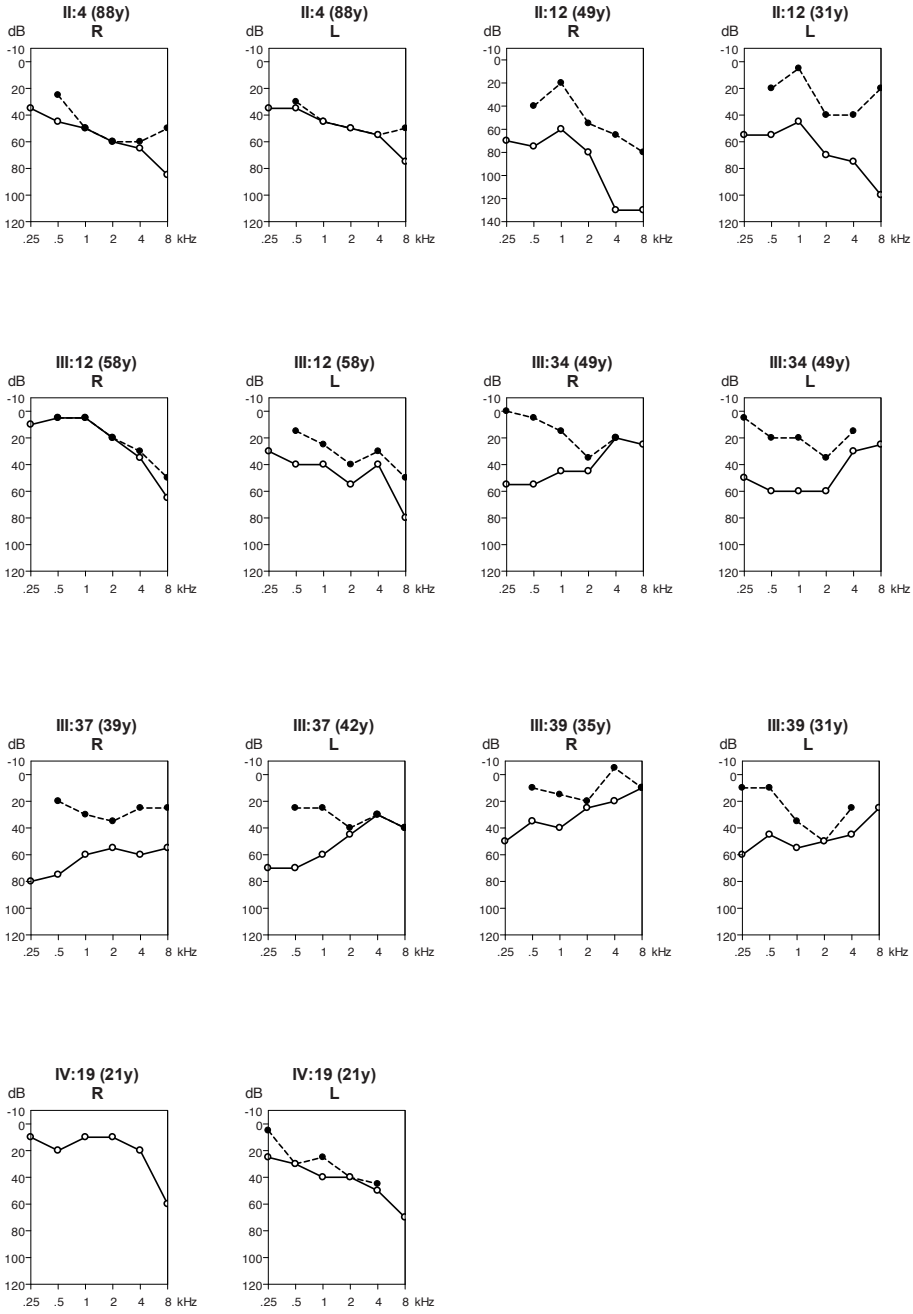


Figure 2. Seven available individual audiograms of the eleven clinically and genetically affected family members. Air-conduction threshold: open circles and solid line; bone-conduction threshold: dots and dashed line. Above each audiogram are the pedigree number, age in years (*y*) and right (R) or left (L) ear.

Most of the audiograms show the audiogram configuration typical for clinical otosclerosis, except for the audiogram of the left ear of individual IV:19, which is not typical for otosclerosis. This audiogram shows mixed hearing impairment (MHI) with a conductive component at the lower frequencies. In combination with absent reflexes of the left ear, otosclerosis was considered very likely. The audiograms of the right ear of individuals III:12 and IV:19 were considered to be normal in relation to age and sex, and therefore excluded from further regression analysis. Individual II:4 was identified as a carrier of the disease haplotype because of his position in the pedigree; his daughter (individual III:12) was diagnosed with otosclerosis. The most recent audiogram shows SNHI in both ears and cochlear otosclerosis was considered because no other cause of SNHI could be found. (Figure 2)
Twelve clinically unaffected family members carried the affected haplotype for OTSC10. Although none of these carriers met the criteria for clinical otosclerosis, some of them did show hearing impairment that was clinically relevant for their age, but probably has another etiology.

The right ear of individuals II:4, II:12 and III:37, and the left ear of individuals III:12, III:34, III:39 and IV:19 were included in the cross-sectional regression analysis. The cross-sectional regression analysis on the threshold data of the clinically affected mutation carriers did not reveal significant progression with advancing age of AC, BC and ABG levels at any frequency. Therefore, Figure 3 shows the mean audiograms for AC, BC and ABG levels.

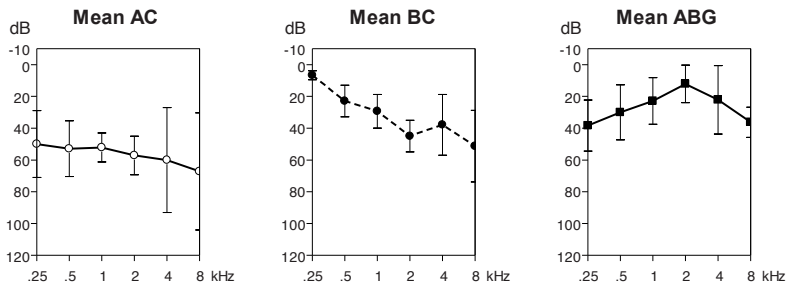
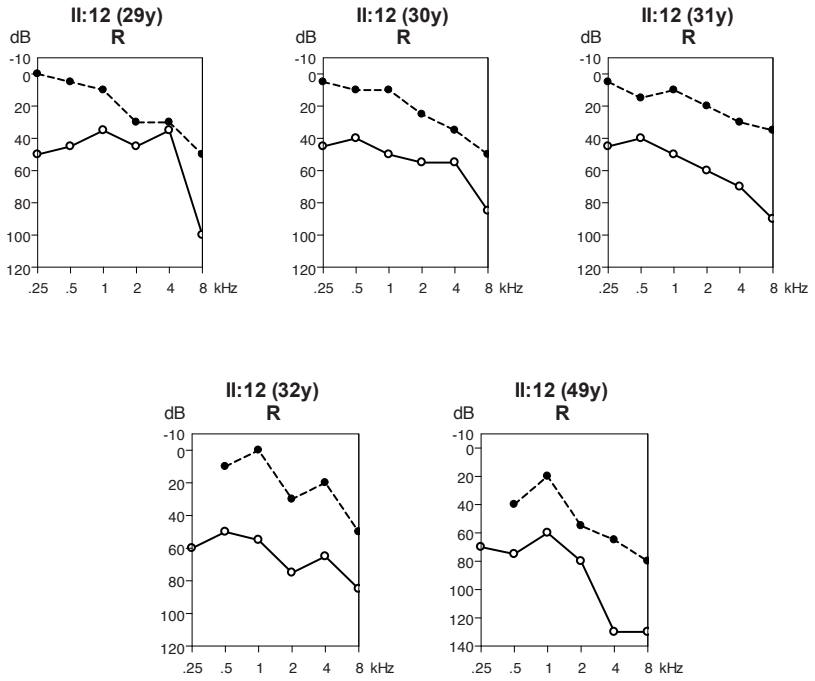


Figure 3. Average threshold data based on audiometry results from individuals II:4, II:12, III:12, III:34, III:37, III:39 and IV:19 (ages 21–58 years). Air-conduction threshold: open circles and solid line; bone-conduction threshold: dots and dashed line; air-bone gap threshold: solid squares and line.

Sufficient longitudinal audiometric data before surgery were only available for individual II:12 and covered 20 years (range: 29–49 years). He underwent in 1964, at the age of 31 years, stapes-replacing surgery for otosclerosis of his left ear and in 1982 (at 49 years) for otosclerosis of his right ear. Since 1992, he has worn hearing aids in both his ears. A selection of the available audiograms of this individual (right ear) are shown in Figure 4 and demonstrate the deterioration of his hearing. Mainly, the AC threshold increased with advancing age. The BC threshold did not deteriorate substantially until the age of 49 years. The ABG at 49 years is smaller at the low frequencies than the ABG at 32 years, but this may be due to errors of measurement. (Figure 4)



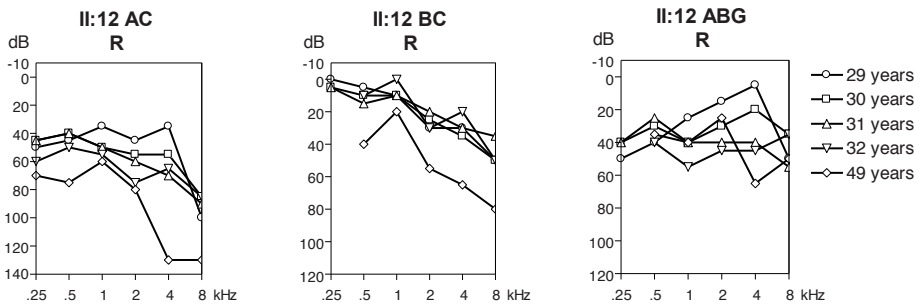


Figure 4. Selection of the available presurgery audiograms of the right ear of individual II:12. The third row shows the air-conduction (AC), bone-conduction (BC), and air-bone gap levels of the right ear of individual II:12 at different ages in the same figure. AC threshold: open circles and solid line; BC threshold: dots and dashed line. Above each audiogram are the pedigree number, age in years (y) and right (R) ear.

Linear regression analysis revealed significant progression of the AC threshold with advancing age for all frequencies. The progression of the BC levels was significant at 0.5 kHz, 2 kHz and 4 kHz. However, this can be caused by incorrect measurement of the BC threshold at the age of 49 years. The ABG did not show significant progression at two or more frequencies; progression was only significant at 8 kHz (data not shown).

Individuals II:12, III:37 and III:39 had stapes-replacing surgery for otosclerosis in one or both ears. Figure 5 shows the AC and ABG levels of these individuals before and 4 to 6 months after surgery. As expected, the AC threshold improved and the ABG diminished, and all the patients reported an improvement of their hearing after surgery. To calculate the preoperative ABG, we used the postoperative bone-conduction levels. We evaluated the effect of stapedotomy on AC and ABG levels a few months (4 to 6 months) after surgery. Later, the ongoing otosclerotic process may impair the results of stapedotomy,[12] making it difficult to evaluate the results of surgery solely.

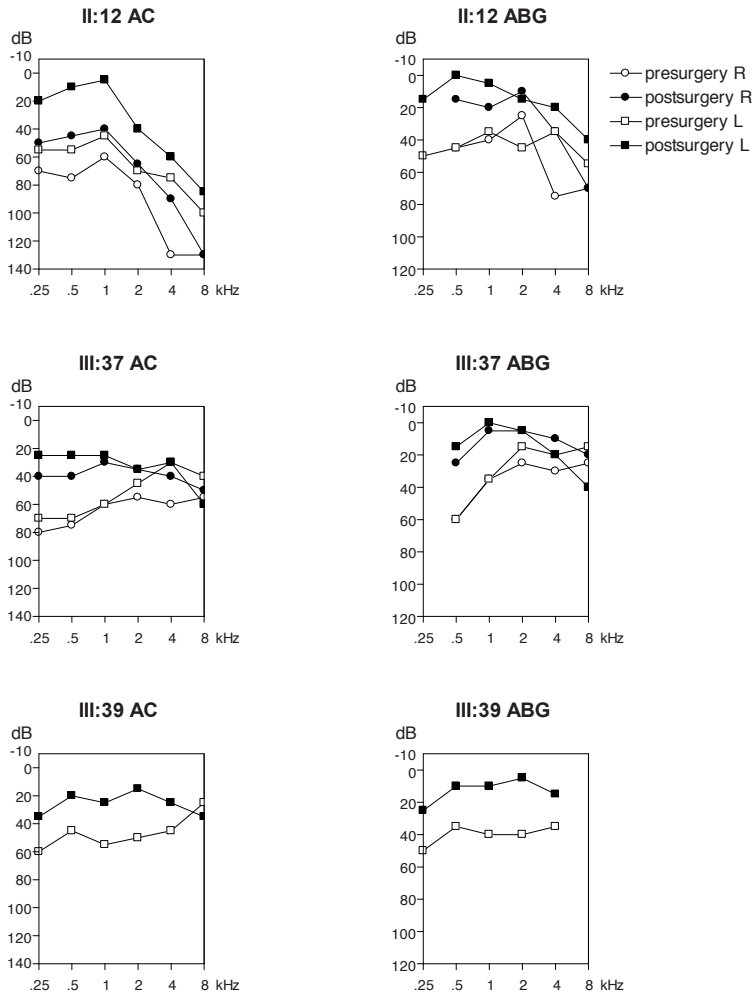


Figure 5. Air-conduction (AC) and air-bone gap (ABG) levels of individuals II:12, III:37 and III:39 before and 4 to 6 months after stapedotomy. Presurgery threshold right ear: open circles; postsurgery threshold right ear: dots; presurgery threshold left ear: squares; postsurgery threshold left ear: solid squares.

Discussion

This report presents the clinical description of the first otosclerosis family linked to OTSC10 on chromosome 1q41-44.

Reduced penetrance

The present family harbors eleven patients with clinically confirmed otosclerosis and all of them carry the disease haplotype. An additional twelve clinically unaffected family members were found to carry the disease haplotype. Furthermore, sixteen family members had an uncertain genetic diagnosis. This would imply a penetrance of 48% (11 of 23) for all ages. Reduced penetrance is not uncommon in otosclerosis and the majority of otosclerosis studies support an autosomal dominant mode of inheritance with reduced penetrance of 25% to 45%, which is presumed to depend on age.[8, 9] Ninety percent of new cases of otosclerosis are diagnosed before the age of 50 years.[9] As a consequence, no definitive diagnosis of clinically unaffected family members could be given to individuals below this age. Nine of the twelve clinically unaffected family members with linkage to OTSC10 are under 50 years of age and may still develop clinical otosclerosis. Three other clinically unaffected family members have ages of 52 to 56 years. They might still develop the disease or never express it due to other modifying genetic and environmental factors. The penetrance above 50 years of age in this family is 64% (7 of 11). In general, the chance to develop clinical otosclerosis for a child with an affected parent is 24% (50% x 48%).

Clinical otosclerosis

The reported age of onset in clinically affected family members ranged from 16 to 51 years, with a mean onset age of 31 years. The age of onset reported in literature ranges from 20 to 40 years.[1, 2, 9] The mean onset age lies within the reported age of onset for clinical otosclerosis.

Clinical otosclerosis is, according to literature, more common in females than in males with a male:female ratio of approximately 1:2. However, histologic studies do not confirm the skewed sex ratio.[2, 13] The male:female ratio of the present family was 1:1 for the clinically affected individuals and 10:13 for individuals with the disease haplotype. We have no explanation for the difference between the male:female ratio

of clinical otosclerosis in the present study and the ratio found in literature. The unusual sex ratio of clinical otosclerosis is not fully understood. It has been implicated that during periods of endocrine change (e.g., pregnancy and puberty), otosclerosis can be initiated or progress in women, particularly with multiple pregnancies. However, some authors found no deleterious effect of having children on hearing in women with otosclerosis. Neither did breastfeeding affect the degree of hearing impairment.[14] Nevertheless, it is well established that sex steroid hormones are critical regulators of bone metabolism and growth.[2] In the present family, only one woman (individual III:37) experienced more tinnitus during pregnancies. The tinnitus disappeared after stapedotomy. The role of hormonal imbalance and the exact mechanism remains inconclusive. Otosclerosis mainly occurs during childbearing ages, therefore it is possible that pregnancy is just an intercurrent event and not the trigger of disease.

Radiological imaging of the temporal bones

High-resolution CT imaging of individual III:37 revealed otospongiotic foci at the fissula ante fenestram on both sides. Detection rates of otospongiotic foci in surgically confirmed otosclerosis with a CT scan prior to surgery of up to 91% have been reported in literature.[15] The best method for detection of otosclerotic foci on CT is the use of submillimeter slice thickness and the assessment of the images directly on the workstation.[6] After the use of more technically advanced CT scans, a significant positive correlation between the size of the ABG and the extension of the otosclerotic lesion on CT could be established.[6, 15] Furthermore, there is also a relationship between degree of SNHI and level of extension in the endosteum of a pericochlear focus.[15]

Progression of hearing impairment

Based on the presumed natural history of otosclerosis, the clinical phenotype must include progression during at least a number of years prior to surgery. This was confirmed by the longitudinal audiometric data of individual II:12. The progressive conductive hearing impairment stabilized around the third decade with ABG levels of 50 dB and later a progressive sensorineural component developed. Conversely, cross-sectional analysis revealed no significant progression of hearing impairment. Analysis of the audiometric data of one family member may better reflect the natural

course of progression of hearing impairment in otosclerosis than the results of the cross-sectional analysis of all the affected family members because of the occurrence of large variability of individual thresholds and onset ages. This prominent intersubject variation of the audiograms prohibited significant results in cross-sectional linear regression analyses and only a trend could be appreciated. Furthermore, the small number of clinically affected individuals and the lack of audiometric data on four clinically affected individuals (II:6, II:8, II:10 and III:21) also weakens the cross-sectional results. The variable audiogram configurations and variable progression of hearing impairment were also demonstrated in previously described otosclerosis families. (Table 2)

Conclusion

The intersubject variation, in terms of age of onset, level of progression and audiogram configuration, was remarkable, probably due to reduced penetrance of 48% and variable expression of the disease. Long-term audiometric data in one patient, however, were useful to demonstrate progression of hearing impairment. There is no doubt that additional, similar studies are needed to be able to distinguish possible phenotypic differences between different genetic types of otosclerosis. Phenotype-genotype correlation studies may also help to elucidate the pathophysiology of otosclerosis.

The identification of the involved genes will help to elucidate the pathophysiology of otosclerosis at a molecular level and may provide possible targets for prevention, diagnosis and therapy of this disease. However, currently the value of genetic testing for otosclerosis is mainly of research interest. Furthermore, insight into the natural course of the various phenotypes of otosclerosis may provide the opportunity for a proper evaluation of the efficacy of current and future therapies.

Acknowledgements

This work was supported by grants from the Heinsius Houbolt Foundation and the Flemish FWO (GN13807N).

Locus	*Age of Onset (in years)*	*Type of HI*	*Audiometric Data*
OTSC 1	-	CHI, MHI	Age-dependent development of sensorineural component superimposed on CHI. No age-related increase of CHI.
OTSC 2	-	CHI, MHI	Quite variable audiogram configurations. Linear regression analysis: limited progression with age.
OTSC 3	Mean: 29 (range: 18–40)	CHI, MHI	-
OTSC 4	F: late 20s–early 30s M: late 30s–early 40s	CHI, MHI, SNHI	Progressive HI beginning in the late 20s to 40s. Large variability between affected individuals in age at onset, type of HI, shape of audiogram and symmetry of HI. Little correlation between age and severity.
OTSC 5	-	CHI, MHI, SNHI	Cross-sectional linear regression: no significant increase of AC, BC or ABG. Longitudinal linear regression: deterioration of AC in the younger patient.
OTSC 7	± 10 as CHI but soon MHI	CHI, MHI, SNHI	Multiple linear regression: age-independent but frequency-specific ABG. Progressive BC across all frequencies but with frequency-specific progression rate. More prominent increase of BC at the higher frequencies.
	Mean: 28.8 (range: 18–45; SD ± 9.1)	CHI, MHI	Cross-sectional linear regression: no significant progression of AC, BC and ABG. Longitudinal linear regression: significant increase in AC, BC and ABG. Progressive CHI stabilized around third decade with ABG of 50 dB, later on progression of SNHI.
OTSC 8	Mean: 34 (range: 14–45)	CHI, MHI, SNHI	-
OTSC 10	Mean: 31 (range: 16–51; SD ± 14.1)	CHI, MHI, SNHI	Cross-sectional linear regression: no significant progression of AC, BC and ABG. Longitudinal linear regression: significant deterioration of AC at all frequencies, significant progression of BC at 0.5 kHz, 2 kHz and 4 kHz, significant increase of ABG at 8 kHz.

Table 2. Phenotypes of the different otosclerosis loci. HI: hearing impairment; CHI: conductive hearing impairment; MHI: mixed hearing impairment; SNHI: sensorineural hearing impairment; AC: air-conduction; BC: bone-conduction; ABG: air-bone gap; F: female; M: male; SD: standard deviation.

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6.2

A new locus for otosclerosis, OTSC10, maps to chromosome 1q41-44

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Clinical genetics 2011; 79: 495-497

Abstract

Otosclerosis is a common form of hearing loss characterized by a disordered bone remodeling in the otic capsule. The abnormal bone remodeling can result in conductive hearing loss due to fixation of the stapes footplate. Although its aetiology remains unknown, otosclerosis can be considered a complex disease with rare monogenic forms. Linkage analysis in large families segregating autosomal dominant otosclerosis has led to the identification of 7 loci (OTSC1-5, 7-8). However, none of the corresponding genes has been identified, with exception to the OTSC2 region, where evidence was found that *TCRB* is the disease causing gene.

In this study a new large Dutch otosclerosis family with autosomal dominant inheritance was investigated. After exclusion of the known loci, a genome scan was performed, which localized the gene on chr1q41-44 with a maximum LOD score of 3.3. This locus, named OTSC10, has a candidate region of 26.1Mb, which contains 306 genes/gene predictions. This new gene localization confirms the strong genetic heterogeneity of otosclerosis, as until now almost every new large family maps to a different locus. As no mutation for monogenic otosclerosis has been identified yet, this represents another opportunity to identify the first one.

Otosclerosis is a hearing disorder which is associated with disordered bone remodeling in the otic capsule. The bone remodeling can lead to conductive, mixed or sensorineural hearing loss, as a result of stapes footplate fixation or cochlear involvement, respectively. Although its etiology remains unknown, otosclerosis can be considered a complex disease with rare monogenic forms. Linkage analysis in large families segregating autosomal dominant otosclerosis has led to the identification of seven loci (OTSC1–5, 7–8). None of the corresponding genes has been identified, but in the OTSC2 region indications have been found that suggest *TCRB* as the causative gene.[1]

In this study, a new large Dutch otosclerosis family with autosomal dominant inheritance was investigated. (Figure 1) All family members were ascertained through the Department of Otorhinolaryngology of the Radboud University Nijmegen Medical Centre (The Netherlands). The examination of family members included the following: a questionnaire for medical history, pure-tone audiometry and otoscopy. Stapedial reflex measurements were done in case of suspicion of otosclerosis. Otoscopic examination was performed to rule out other middle ear pathology. When immobility of the stapes was confirmed during stapes replacing surgery, patients were considered affected. In non-operated persons, patients were considered affected when several of the following criteria were met: presence of conductive or mixed hearing impairment, air-bone gap (ABG) ≥ 20 decibels (dB) averaged across 0.5-2 kHz, absent stapedial reflexes and normal otoscopy. Informed consent was obtained from all family members.

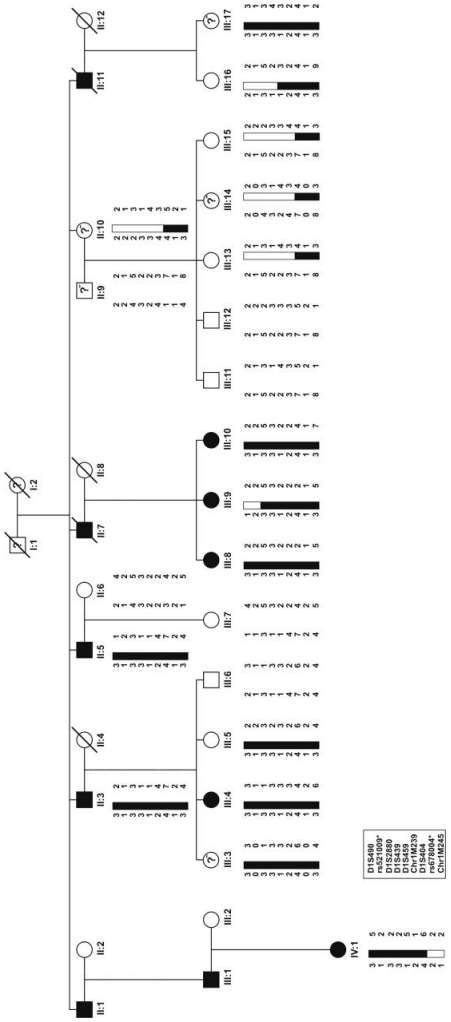

Figure 1. Pedigree of the Dutch family used to localize OTSC10 to chr1q41–44. The haplotypes are given below the symbols and the linked haplotype is shown in black. The two recombinant SNPs (marker rs521009 proximally (individual III:9) and rs678004 distally (individual IV:1)), genotyped by the Illumina cyto-12 beadchip, are indicated with an asterisk. Individuals III:3 and III:14 were not included in the genome scan. A question mark (?) indicates individuals with an uncertain diagnosis. ?- indicates individuals with hearing impairment abnormal for their age and sex (ISO 7029 standards), but the criteria for otosclerosis were not met. Although the hearing loss probably had a different cause, they were considered of uncertain diagnosis in the calculations of the LOD scores. The presence of otosclerosis was confirmed during surgery in individuals II:5, III:8, III:9 and III:10. In the remaining individuals, II:3, III:4 and IV:1, the clinical diagnosis was based on the diagnostic criteria and their position in the pedigree.

DNA was extracted from blood using standard procedures. Microsatellite markers were analyzed that are located in or near the known loci and the OTSC10 region. Information on all markers was taken from the NCBI (National Center for Biotechnology Information) database (http://www.ncbi.nlm.nih.gov/). Markers Chr1M239 and Chr1M245 are not in this database and were developed on the basis of the human genome sequence (Human genome build 37) (Table S1). One of the polymerase chain reaction (PCR) primers was synthesized with an M13 sequence at the 5' end. PCR amplification was carried out using standard conditions. A fluorescently labeled M13 primer was included in the PCR reaction, thus labeling the PCR product. Capillary electrophoresis and pattern visualization were performed using an ABI 3130XL automatic DNA sequencer (Applied Biosystems Inc., Foster City, USA). Allele sizes were determined using the GeneMapper V3.7 software (Applied Biosystems Inc., Foster City, USA).

A genome scan was performed using the HumanCytoSNP-12 BeadChips v2.0 (Illumina Inc, San Diego, USA), following the manufacturer's instructions. Individuals III:3 and III:14 were not included in the genome scan, but were included for the confirmation of linkage and reconstruction of the haplotypes. Fluorescent data was imported into Beadstudio version 3.3 for genotype calling (Illumina Inc, San Diego, USA).

For the SNP-based genome search, a subset of 6,000 informative SNPs were selected with an average inter-SNP distance of 500 KB. Multipoint linkage analysis was performed using SimWalk 2 version 2.91.[2] Prior to linkage analysis, unlikely genotypes were set to missing using the mistyping option implemented in Simwalk2. Data manipulation to obtain the input files for SimWalk 2 was performed using Mega 2, version 4.2.[3] As SimWalk2 uses a non-deterministic algorithm to calculate LOD scores, the LOD scores may fluctuate somewhat between runs. Therefore, in the non-excluded regions the analysis was repeated multiple times using different random number seeds and the mean LOD scores were calculated.

Autosomal dominant inheritance was assumed with a disease frequency of 0.0001. Because of the high variability in the onset of hearing loss for otosclerosis,[4, 5] linkage analysis was performed assuming age dependent liability classes with penetrance values of 0.5 and 0.9 for age groups > 55 and < 55 years, respectively. The cut-off

value of 55 was chosen because the age of onset for the confirmed patients in the family varied from 16 to 51 years. Recombination frequencies were assumed to be equal in both females and males, and the phenocopy rate was set at 0.

A copy number variant (CNV) analysis was done using the data from the HumanCytoSNP-12 BeadChips. Intensities were normalized against a reference panel of 120 HapMap samples. An in-house developed CNV analysis tool was used to detect aberrations (http://medgen.ua.ac.be/cnv/), applying a combination of 3 Hidden-Markov-Model algorithms (QuantiSNP, PennCNV and VanillaICE).[6-8] In addition, the coding regions and intron–exon boundaries of selected genes were amplified by a standard PCR reaction. Direct sequencing of the PCR products was performed on forward and reverse strands using an ABI 3130XL sequencer with the BigDyes Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems Inc.).

The known otosclerosis loci and the *NOG* gene (noggin) were excluded for linkage in this family by analysis of at least three informative microsatellite markers in each region. Linkage to the *NOG* gene was investigated, because it is involved in syndromic cases of stapes fixation.[9] Subsequently, a genome wide screen was performed. Multipoint linkage analysis revealed only one region with a LOD score above 1 and 89% of the genome could be excluded (LOD<−2). This region is localized on chr1q41–44 and was confirmed by analysing 27 extra microsatellite markers. Multipoint linkage analysis showed a maximum LOD score of 3.3. Figure 1 shows the reconstructed haplotypes of a subset of the analyzed markers in all family members. This locus, named OTSC10, has a candidate region of 26.1 Mb and contains 306 genes/gene predictions based on the latest draft of the human genome sequence (Build 37.1).

Thirty-seven of these genes are reported to be expressed in the inner ear and 62 in bone tissue.[10, 11] The complete OTSC10 region was screened for copy number variants (CNVs) on the basis of the SNP microarray, but we could not detect any aberration in the linked region in the patients (data not shown). In addition, two interesting genes in the region (transforming growth factor beta 2 (*TGF-β2*) and angiotensinogen (*AGT*)) were selected for mutation analysis by DNA sequencing. These genes were selected because of their important role in bone remodeling and on the basis of previously found associations with otosclerosis.[12-14] All exons and

intron–exon boundaries were sequenced in two affected individuals, but no pathogenic variant could be identified in both genes.

Reduced penetrance is common in otosclerosis and has been estimated to be around 40% on average,[15] although this can vary greatly between families. The families linked to the earlier reported loci also illustrate a reduced and diverse penetrance.[16-22] In addition, otosclerosis has a varying age of onset, which is usually between 16 and 50 years, but development at later ages is also possible.[23] Both these facts suggest that the presence of other modifier factors, such as environment factors and/or modifier genes. Reduced penetrance and a varying age of onset is also present in this family. (Figure 1) The mean age of onset was 31 years, but this ranged between 16 and 51 years. Individuals III:5, III:13, III:15 and III:16 have a negative clinical diagnosis, but have inherited the disease haplotype or might carry the disease-causing mutation because they have inherited part of the disease haplotype. Their ages range from 52 to 57 years, so they might still develop the disease, but it is also possible that they will never express it as a result of the interference of additional genetic or environmental factors.

In conclusion, we were able to identify a new locus for otosclerosis on chr1q41–44 (OTSC10). This new gene localization confirms the strong genetic heterogeneity of otosclerosis, as until now almost every new large family maps to a different locus. Currently, there is little knowledge on the pathological processes involved in otosclerosis. Identification of the first gene for otosclerosis will undoubtedly be an important step towards new insights into the pathogenic mechanisms involved in the disease.

Acknowledgements

We thank all the family members for their cooperation. This work was supported by grants from the Flemish FWO (GN13807N) and the Heinsius Houbolt Foundation. In addition, we would like to thank Geert Vandeweyer for the development of the CNV analysis tool and assistance in CNV analysis and Kathleen Vanderstraeten for further technical assistance. I. S. is a postdoctoral fellow of the Flemish FWO.

Supplemental table

MarkerID	*Primer*	*Repeat*	*Position (NCBI Build 37, hg19)*
Chr1M239	Forward: AGAGAATTGCCTCCCTTTCC Reverse: CATCAGGTCTGGAACACAGG	CA repeat	chr1:239461688+239461884
Chr1M245	Forward: TAAGCCACAAGCAGCCAAG Reverse: AATATCTGTCACCCCAAAACG	CA repeat	chr1:245430887+245431134

Table S1. Information on analyzed markers.

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7

Discussion and conclusion

Discussion and conclusion

The aim of this thesis was to contribute to our knowledge on genotype-phenotype correlations in hereditary hearing impairment. In the chapters of this thesis the clinical features of DFNA3, DFNB8/10, DFNX4, Muckle-Wells syndrome (MWS) and otosclerosis (OTSC10) families have been described in relation to their genotypes. Detailed audiometric analyses of these families have been performed and were compared with previously described families in literature.

Genotype-phenotype correlations

Identification of the genetic defects underlying hearing impairment gives the opportunity to determine genotype-phenotype correlations. Establishing genotype-phenotype correlations is very important for establishing accurate diagnostic and prognostic information, and for genetic counseling. Identification of the mutation confirms the genetic cause of the hearing impairment and provides an opportunity to estimate the chance that other family members will be affected as well. The causative gene, the type of mutation, variants in other genes (modifier genes) and/or environmental factors may all contribute to the type of hearing impairment and the variation within and among families. Moreover, information on the genetic defect can exclude or indicate syndromic forms of hearing impairment. Associated symptoms in syndromic hearing impairment can therefore be recognized and treated in time, for example in MWS.

In chapter 2, two small Dutch DFNA3 families with profound sensorineural hearing impairment are described. Mutation analysis revealed a p.Argl84Gln and a p.Arg75Trp mutation in *GJB2* in the two families, respectively. Recently, Huang et al. described two DFNA3 patients of two different families carrying the same p.Arg184Gln mutation in *GJB2*. Both patients demonstrated bilateral profound sensorineural hearing impairment. The audiogram configuration was flat with pure tone thresholds of approximately 100 dB. Temporal bone CT scans showed no inner ear abnormalities. Moreover, neither patient had skin problems.[1] The phenotype is consistent with the previously reported DFNA3 families carrying the p.Arg184Gln mutation; prelingual severe to profound sensorineural hearing impairment.[1-6]

The p.Arg75Trp mutation in *GJB2* can cause autosomal dominant nonsyndromic hearing impairment (DFNA3) as well as syndromic hearing impairment with dermatologic features.[7] Patients with this mutation exhibit a similar hearing impairment phenotype (moderate to severe or profound bilateral sensorineural hearing impairment) and a wide range of cutaneous phenotypes. The variations in skin alterations associated with the p.Arg75Trp mutation may be due to the contribution of genetic background and environmental factors.[6, 8]

Previous reports have attempted to address *GJB2* genotype-phenotype correlations for recessive mutations, but only a few associations have been recognized, probably because of the large number of genotypes and the small number of affected individuals in most series. Nevertheless, mutations causing an early protein truncation can be concluded to cause profound hearing impairment, whereas missense mutations were found to have a more variable effect.[9-12] Truncating *GJB2* mutations include nonsense, splice and frameshift mutations and result in defective protein synthesis.[13] The pathogenicity of missense mutations depends on many factors, including the position and the nature of the substitution in the protein. Because of the complex structure and function of gap junctions, it is extremely difficult to predict pathogenicity of some missense mutations.[10] The variability in phenotype may reflect the effect of modifier genes and/or nongenetic factors that lead to incomplete penetrance and variable expression.[10, 14]

Detailed phenotypic analyses of eight DFNB8/10 families are described in chapter 3. Our analyses suggest that mutations in *TMPRSS3* can be classified as mild and severe mutations according to their phenotypic effect. Recently, Lee et al. demonstrated six *TMPRSS3* mutations in ten consanguineous Pakistani families. Four novel variants, of which three missense (p.Glu104Lys, p.Ala256Val and p.Cys425Arg) and one nonsense (p.Glu104Stop), were identified. Additionally, the previously reported deletion c.207delC (p.Thr70fs) was identified in one family and the previously reported p.Cys407Arg mutation was found in five families. All affected family members demonstrated bilateral severe to profound hearing impairment affecting all frequencies. Unfortunately, the age of onset of hearing impairment is not reported by Lee et al. and therefore, these mutations cannot be classified as relatively mild or severe, resulting in DFNB8 (postlingual hearing impairment) or

DFNB10 (prelingual hearing impairment), respectively.[15, 16] The deletion c.207delC and the p.Cys407Arg mutation have previously been shown to cause severe to profound prelingual (DFNB10) hearing impairment. Analysis of more DFNB8/10 families is necessary to confirm our classification of *TMPRSS3* mutations into relatively mild and severe and to address whether a subclassification of the mutations associated with DFNB8 is possible.

TMPRSS3 is a type II transmembrane serine protease, consisting of a transmembrane domain located near the N-terminus, a low density lipoprotein receptor A domain which binds calcium and low density lipoprotein, a scavenger receptor cysteine-rich domain that is involved in protein-protein interaction and a C-terminal serine protease domain for which the prototype is chymotrypsin. *TMPRSS3* mutations are found in all functional domains and all tested mutations disrupt the proteolytic activity of TMPRSS3.[15, 17, 18] The disruption of the proteolytic activity of TMPRSS3 is tightly correlated with the pathogenesis of hearing impairment. The low-density lipoprotein receptor A domain and the scavenger receptor cysteine-rich domain are involved in interactions with extracellular molecules. To date, it is unknown how mutations in these domains affect the proteolytic activity of TMPRSS3. It may be possible that these domains are necessary for proper folding or assembly of the catalytic domain (structural stability) or protease substrate recognition and binding.[15, 19] Further research is needed to establish whether a correlation exists between disease severity and the particular domain in which the mutation occurs, the specific residue mutated or the conservation of amino acids.

The establishment of thorough genotype-phenotype correlations is constrained by the limited clinical data reported and by the small number of patients in most studies. There is no doubt that more clinical data of more patients with hereditary hearing impairment need to be published in order to create a more reliable and precise phenotype characterization. An Age Related Typical Audiograms (ARTA) gives a comprehensive phenotype presentation and can be used to compare the type of hearing impairment, the age of onset and the progression of hearing impairment in relation to the genotype. Therefore, an ARTA can be helpful in selecting potentially interesting loci for linkage analysis or genes for mutation analysis and

can be valuable for individual counseling.[20] However, intra-familial phenotypic variation for a specific genotype is not visible in an ARTA and prediction of the phenotypic outcome for individual cases should be made with caution. Moreover, mutations in different genes can lead to a very similar ARTA. In that case, additional distinguishing phenotypic features are very important, for example additional audiometric test results. The program AudioGene can perform automatic audioprofile analysis of the audiometrical data of an individual or a family.[21] Very similar phenotypes of different genotypes and limited clinical data of some genotypes limit the genotypic predictions of the programs. Nevertheless, in some cases the program was able to predict the correct causative loci or gene.[22, 23]

Hearing impairment associated with DFNA3 (OMIM 601544) is more easily to recognize because prelingual onset of hearing impairment is rare in autosomal dominant nonsyndromic hearing impairment, which is frequently postlingual and progressive. DFNA8/12 (OMIM 602574), DFNA19, DFNA23 (OMIM 605192), DFNA24 (OMIM 606282) and DFNA59 (OMIM 612642) are other types of autosomal dominant nonsyndromic hearing impairment reported to cause early childhood onset of hearing impairment. In DFNA3 all frequencies are severely affected, in contrast to primarily the mid-frequencies or high-frequencies in DFNA8/12, DFNA19, DFNA23 and DFNA24. DFNA59 causes congenital, bilateral, non-progressive, severe-to-profound sensorineural hearing impairment, similar to hearing impairment seen in DFNA3.[24] Especially in combination with skin abnormalities, DFNA3 should be considered. However, it is not always possible to distinguish DFNA3 from DFNA59 based on the phenotype. Moreover, the causative gene for DFNA59 has not yet been identified and routine DNA diagnostic screening is not possible for DFNA59.[24]

In autosomal recessive nonsyndromic hearing impairment postlingual progressive hearing impairment is not very common. Many patients with autosomal recessive inherited hearing impairment show prelingual severe to profound hearing impairment. Postlingual onset of hearing impairment is seen in DFNB4 (OMIM 600791), DFNB7/11 (OMIM 600974), DFNB8/10 (OMIM 601072), DFNB25 (OMIM 613285), DFNB30 (OMIM 607101), DFNB77 (OMIM 613079) and DFNB91 (OMIM 613453). The most common form of inner ear abnormality, namely enlarged

vestibular aqueduct (EVA), is associated with mutations in *SLC26A4* (DFNB4). The characteristic clinical findings include fluctuating and often progressive sensorineural hearing impairment, and vestibular symptoms in a minority of patients. Radiological examination can confirm the presence of EVA. Hearing impairment associated with DFNB25 has a very early postlingual onset and is stable. DFNB7/11, DFNB8/10, DFNB30, DFNB77 and DFNB91 patients demonstrate progressive severe to profound downsloping hearing impairment with a later postlingual onset. In addition, the ski-slope audiogram configuration is suggestive for DFNB8.

TMC1 (DFNB7/11), *TMPRSS3* (DFNB8/10), *MYO3A* (DFNB30), *LOXHD1* (DFNB77) and *SERPINB6* (DFNB91) are all expressed in the inner and/or outer hair cells and are essential for their normal function.[18, 25-29] Fasquelle et al. developed a *Tmprss3* deficient mouse model. Homozygous mutant mice exhibited severe hearing impairment. In situ hybridization localized *Tmprss3* mRNA in sensory hair cells in the cochlea and the vestibule, in the supporting cells of the organ of Corti and in lesser degree in the spiral ganglion cells. Histological examination showed degeneration of the organ of Corti in these adult mice after initial normal development. Cochlear hair cell degeneration started at the onset of hearing in the basal turn and progressed very rapidly toward the apex within two days. Otoacoustic emissions were absent in the mice with a homozygous *Tmprss3* mutation, indicating outer hair cell damage. These results suggest that Tmprss3 is essential in the functional maturation of cochlear hair cells. Both inner and outer hair cells degenerated following the same pattern, suggesting that Tmprss3 function is equally important in both hair cell types. Moreover, homozygous mutant mice exhibited normal development of ganglion neurons at early postnatal stages, but later on progressive loss of ganglion neurons occurred. The stria vascularis of these mutant mice showed normal histology and physiology.[18] Degeneration of hair cells, after initial normal development, could result in the postlingual onset of hearing impairment in DFNB8. Mutations in *TMC1, LOXHD1* and *SERPINB6* also cause hearing impairment by progressive hair cell degeneration.[26, 28, 29] A common pathogenetic pathway may be responsible for hearing impairment in these postlingual progressive autosomal recessive hearing disorders, but further research is needed to unravel the molecular pathogenesis.

A clear-cut genotype-phenotype correlation would enhance straight-forward DNA diagnostic screening. Although the intra-familial variation in hereditary hearing impairment is high, selective DNA diagnostic screening is already possible for some patients with specific phenotypic characteristics, for example skin symptoms, a ski-slope audiogram configuration or low- or mid-frequency hearing impairment. Unfortunately, the presence of specific phenotypic characteristics is no guarantee for identification of the genetic defect, because this genetic defect can be in a novel deafness gene. There are probably many of these deafness genes yet to be identified. For example, we identified compound heterozygous *TMPRSS3* mutations in 25% of the families with progressive autosomal recessive nonsyndromic sensorineural hearing impairment. Since progressive hearing impairment with an autosomal recessive mode of inheritance is not common, yet unknown deafness genes are likely to be involved in these remaining families. Whole exome sequencing in these families would be a good tool to identify these genes. Moreover, genetic characterization of families with hereditary hearing impairment can give important information about the contribution of specific hearing impairment causing genes in the Dutch population and can be helpful to develop a cost-effective strategy for routine DNA diagnostics in patient care.

Hearing rehabilitation

More elaborate audiometric testing than only pure-tone audiometry and speech perception, of patients with hereditary hearing impairment may be helpful to predict the outcome of hearing rehabilitation with a hearing aid or a cochlear implant. However, there is currently insufficient knowledge to select the best rehabilitation method in different forms of hereditary hearing impairment. Knowledge about the pathogenesis of hearing impairment may provide important information for adequate hearing rehabilitation. Insight in differences in inner ear function can be useful to determine the type of sound amplification in hearing aid rehabilitation.

The cause of hearing impairment in MWS is not yet understood, but the basilar membrane of the cochlea may be involved in the pathogenesis of hearing impairment, as is the case in Alport syndrome.[30-32] Muckle and Wells demonstrated

ossification of the basilar membranes in patients with MWS.[33] Furthermore, pathology at the level of the basilar membrane could explain the good speech recognition scores seen in the present MWS family and in previously reported families with Alport syndrome.[31, 32] Results of the audiometric evaluation of the present MWS family also indirectly support the hypothesis of improper motion of the basilar membrane in patients with MWS. Suprathreshold measurements, particularly speech in noise, were preserved rather well and it seems that a defect in the basilar membrane results primarily in an attenuation of sound by a shift in the operation point of the outer hair cells with near intact function at high levels. This preservation of stimulus fine structure at higher levels is also found in conductive middle ear hearing impairment. This contrasts to results often found in sensorineural hearing impairment, that results from loss of outer and/or inner hair cells (e.g. presbycusis, noise induced hearing loss). In conclusion, our results indicate that MWS related hearing impairment might be considered as a intra-cochlear 'conductive' hearing impairment, similar to hearing impairment in DFNA8/12 and DFNA13.[34, 35]

Decreased motion transmission may be a common causative factor in the hearing deficits in Alport syndrome and in the type of mid-frequency hearing impairment found in DFNA8/12 and DFNA13. In the latter two the loss of transmission occurs between the tectorial membrane and the outer hair cells, whereas in the former it occurs at the level between the basilar membrane and the organ of Corti. Reduced sound transduction results in sound attenuation and in all conditions the proper stimulation of outer hair cell motion is jeopardized. Because of the affected threshold sensitivity with little suprathreshold consequences in MWS, it is possible to successfully rehabilitate hearing in MWS patients with hearing aid amplification. The MWS patients described in chapter 5 also demonstrated effective hearing rehabilitation with a hearing aid. As long as sounds are presented hard enough, the results of speech recognition will be good in MWS patients. Moreover, given the good speech recognition scores of the MWS patients, cochlear implantation is not a suitable treatment option for these patients.

Most DFNX4 patients are also not appropriate candidates for cochlear implantation because of the good speech recognition scores of these patients. The presence of

Smpx in hair cells and supporting cells of the murine cochlea indicates its role in the inner ear.[36, 37] Moreover, SMPX is associated with the cytoskeleton like many other hearing impairment causing genes encoding actin or actin-binding proteins, motor proteins of the myosin family or proteins that are otherwise linked to the cytoskeleton.[38, 39] SMPX is probably involved in the response to mechanical force. Therefore, Huebner et al. suggested that the long-term maintenance of mechanically stressed inner ear cells critically depends on SMPX function.[36] SMPX might also contribute to actin turnover and length regulation in stereocilia, as these features are closely regulated by extrinsic biomechanical forces.[40] However, expression studies failed to demonstrate localization of Smpx in the stereocilliar bundles of the inner and outer hair cells.[36] Despite these indications for the function of SMPX in the inner ear, the exact pathogenic mechanism of hearing impairment in DFNX4 is not fully understood and further studies are required to determine the best hearing rehabilitation method.

Cochlear implantation has become a common treatment for patients with profound hearing impairment. Results of cochlear implantation are highly variable, depending on numerous factors, such as onset age of hearing impairment, age at implantation, duration of implant use and probably etiology of hearing impairment.[41] Neural and/or central damage to the auditory system probably gives poorer outcomes after cochlear implantation than hearing disorders that primarily affect the hair cells, like in many types of hereditary nonsyndromic hearing impairment.[42] Cochlear implants by-pass the hair cells in the cochlea and stimulate the auditory nerve fibers directly.[43]

Several studies have investigated the auditory performance after cochlear implantation for patients with and without *GJB2* mutations, but the overall results are inconclusive.[44-62] Nevertheless, all the studies demonstrated good results of cochlear implantation in patients with *GJB2* related hearing impairment and these patients can be expected to perform on average. However, cutaneous manifestations associated with DFNA3 can pose a major challenge to successful cochlear implantation. Eczematous dermatitis constitutes a risk factor for wound healing and skin necrosis.[63-65]

Successful rehabilitation with cochlear implants is possible in patients with *GJB2* related hearing impairment because *GJB2* mutations do not affect the spiral ganglion cells stimulated by the cochlear implant. Histopathologic examination of temporal bones associated with *GJB2* related hearing impairment revealed preservation of spiral ganglion cells.[66, 67] Propst et al. performed electrically evoked compound action potential testing of the auditory nerve in patients with a cochlear implant and demonstrated consistent spiral ganglion cell survival throughout the length of the cochlea in the patients with *GJB2* related hearing impairment when compared to patients with non-*GJB2* related hearing impairment.[68]

Evaluation of performance of *TMPRSS3* patients with a cochlear implant indicated that this is a good treatment option for these individuals, as satisfactory speech reception was reached after implantation.[69] Degeneration of ganglion neurons in *Tmprss3* deficient mice has been demonstrated and this could result in disappointing results after cochlear implantation. However, ganglion degeneration occurred after degeneration of hair cells and could be secondary to the pathogenic changes in the inner ear.[18] Therefore, it might well be important not to postpone cochlear implantation too much after it is indicated based on the severity of hearing impairment. More research is needed to establish the effect of ganglion neuron degeneration on the speech reception after cochlear implantation.

Before progression of hearing impairment in the low frequencies, *TMPRSS3* patients seem suitable candidates for an Electric Acoustic System (EAS) to rehabilitate the high frequencies. However, considering the progression for the low frequencies, EAS is less suitable for DFNB8/10 patients. Therefore, mutations in *TMPRSS3* should be excluded before considering an EAS in patients with a ski-slope audiogram configuration and a possible recessive mode of inheritance.

Cochlear implantation with a hypo-traumatic electrode (Nucleus 422, Cochlear) is a good treatment option for DFNB8/10 patients. This cochlear implant preserves residual hearing at the lower frequencies and enables electro-acoustic stimulation with a hearing aid. In general, DFNB8 patients have relatively low threshold levels at the low-frequencies, but progression of hearing impairment occurs. When electro-acoustic stimulation becomes insufficient, the hypo-traumatic electrode can take over and stimulate the low-frequencies as well.

Therapeutic interventions of hearing impairment

Genotype-phenotype correlations can provide additional insight in the involved pathogenic mechanism of hearing impairment. Understanding of the pathogenic processes will not only provide important information for rehabilitation but may also reveal important targets for prevention and treatment. If the causative genes of hearing impairment are not identified, many molecular pathways might remain elusive. Additional audiometric evaluation can also contribute to the localization of the defect in the inner ear and thereby the identification of the defective protein. Defects at particular parts in the inner ear can result in specific phenotypic characteristics. The knowledge obtained by genetic studies may also contribute to the development of novel gene-specific or mutation-specific therapeutic approaches.

Besides the disturbed function of the basiliar membrane, inflammatory processes in the cochlea and leptomeninges probably also contribute to sensorineural hearing impairment in MWS as well.[70] The inflammation in the inner ear could also be the cause of the basilair membrane dysfunction. Ahmadi et al. demonstrated cochlear enhancement on fluid attenuated inversion recovery magnetic resonance imaging (FLAIR-MRI) in patients with MWS. Cochlear inflammation was significantly associated with hearing impairment.[71]

MWS is caused by gain of function mutations in the *NLRP3* gene encoding cryopyrin.[72] Cryopyrin is involved in the assembly of the inflammasome, a multiprotein complex implicated in innate immunity. The inflammasome activates the caspase-1 enzyme, which in turn cleaves pro-IL-1β and pro-IL-18 into their active proinflammatory forms, IL-1β and IL-18.[72, 73] The binding of IL-1β to its receptor initiates a cascade of signals resulting in early inflammatory responses. The balance between IL-1β and its receptor is essential in regulation of proinflammatory and anti-inflammatory responses.[74] In MWS, cryopyrin-mediated inflammasome and caspase-1 activation might cause inappropriate sustained secretion of inflammatory cytokines including IL-1β. The overproduction of activated IL-1β can cause chronic aseptic meningitis and probably subsequent increased permeability for cytokines between perilymph and cerebrospinal fluid via the highly porous modiolus. These cytokines can stimulate the spiral ligament fibrocytes to produces additional

mediators, that might induce an uncontrolled chronic inflammation responsible for cochlear dysfunction and therefore hearing impairment.[75] However, hearing impairment is also observed in MWS patients without detectable aseptic meningitis.[71] This suggests that *NLRP3* mutations might result in unregulated local production of cytokines, such as activated IL-1β, and chronic inflammatory responses within the cochlea. Local production of proinflammatory cytokines in the spiral ligament, stria vascularis and spiral ganglion neurons after acoustic trauma to the cochlea is also demonstrated by Fuijoka et al.[76] Improvement of hearing impairment by therapy with IL-1 inhibitors in some studies also suggests the contribution of a local inflammatory response caused by IL-1β secretion.[77-83] Treatment with IL-1 inhibitors has also proven to be effective in reducing the symptoms of systemic inflammation.[82-85] Nevertheless, an early diagnosis of MWS is essential for an immediate start of treatment to prevent irreversible damage from amyloidosis and possibly profound hearing impairment.[81] Currently, the expression pattern of NLRP3 in the inner ear is not known, but this information could be helpful to elucidate the pathogenesis of hearing impairment.

Therapeutic interventions that block the expression of mutated copies of *GJB2* or *GJB6* genes and thereby prevent the dominant-negative mechanism could rehabilitate hearing impairment. Overexpression of Cx26 in the cochlea of Cx30 deficient mice could prevent hearing impairment by avoiding hair cell death in these mice. These results suggest that the function of Cx30 is not essential for normal hearing.[86] Together with Cx26, Cx30 is probably required for producing sufficient quantities of gap junctions in the cochlea, for the initiation and maintenance of the endocochlear potential.[87] In the early cochlear development, Cx26 is the only gap junction protein detected in many key supporting cells in the organ of Corti. Qu et al. demonstrated severe hearing impairment in Cx26 deficient mice, regardless of whether Cx30 was over-expressed. The essential developmental functions of Cx26 required for normal hearing is unique and not replaceable by Cx30.[88] Overexpression of Cx26 and its effect on hearing is not yet investigated in humans.

The OTSC10 locus contains 306 genes/gene predictions. This new locus confirms the strong genetic heterogeneity of otosclerosis, as the disease gene in almost every new large family maps to a different locus. The identification of the involved genes will

help to elucidate the pathophysiology of otosclerosis at a molecular level and may provide possible targets for prevention, diagnosis and therapy of this disease. Furthermore, insight into the natural course of the various phenotypes of otosclerosis may provide the opportunity for a proper evaluation of the efficacy of current and future therapies. Two interesting genes in the region, transforming growth factor beta 2 (*TGF-β2*) and angiotensinogen (*AGT*), were selected for mutation analysis by DNA sequencing. These genes were selected because of their important role in bone remodeling and on the basis of previously found associations with otosclerosis.[89-91]

The first gene, *TGF-β2*, plays an important role in the chondrogenesis of the otic capsule.[92] Members of the transforming growth factor-β (TGF-β) superfamily, including TGF-bs, bone morphogenetic proteins (BMPs) and activins, are fundamental in the process of bone remodeling. The related *TGF-β1* has been demonstrated to be associated with otosclerosis. TGF-β1 contributes to the embryonic development of the otic capsule. In early stages, the otic epithelium produces TGF-β1 to stimulate the chondrogenesis and to promote growth. Later on, TGF-β1 selectively inhibits this process to allow capsular modeling. In human otosclerotic bone cell cultures, *TGF-β1* can modify the expression of glycosaminoglycan (GAG), fibronectin and collagen of the extracellular matrix.[92] The variant T263I of *TGF-β1* is biologically more active by inducing transcription of the TGF-β1 receptor gene. This variant decreases the susceptibility to otosclerosis by inhibiting osteoclast differentiation and activation in the first otospongiotic phase of otosclerosis. This variant has been found to be under-represented in otosclerosis patients.[89, 93, 94] Thus, *TGF-β1* may be involved in the pathogenesis of otosclerosis by modulating extracellular matrix production.[95] Camurati-Engelmann disease (CED) or progressive diaphyseal dysplasia is also caused by mutations in *TGF-β1*. CED is very rare sclerosing bone dysplasia characterized by a rapid bone turnover. Some of the CED patients demonstrated stapes fixation at stapes surgery. Therefore, some researchers suggested that otosclerosis could be part of the CED phenotype.[96, 97] Furthermore, inhibition of extracellular matrix in cell cultures is possible with a novel inhibitor of the TGF-type I receptor kinase activity: SB-431542. A concern with kinase inhibitors is nonspecific toxicity caused by inhibition of important but unrelated kinases.[98] Furthermore, neutralizing antibodies against TGF-β1 can

prevent the accumulation of extracellular matrix in kidneys and lungs of rodents.[99-101] Applicability of TGF-β1 inhibitor or antibodies in humans has still to be investigated.

The second gene, *AGT*, encodes the protein angiotensinogen which is cleaved by the enzyme renin and angiotensin converting enzyme (ACE) to generate the physiologically active enzyme angiotensin II (AT-II). This renin-angiotensin-aldosterone system (RAAS) is important in the regulation of blood pressure and body-fluid homeostasis, but AT-II can also influence bone remodeling. Furthermore, the plasma levels of angiotensin (AGT) rise during pregnancy and the RAAS is activated. This might explain why hearing impairment in otosclerosis often manifests or progresses during pregnancy. Genetic variants in *AGT* and *ACE* were previously found to be associated with otosclerosis, although the results were contradictory and could not be replicated in another study. A genetic variant of *AGT* has been associated with higher plasma AGT concentrations and a genetic variant of *ACE* with increased enzymatic activity of ACE. These variants were demonstrated to be over-represented in otosclerosis patients.[90, 91, 95, 97, 102-104] ACE-inhibitors and AT-II receptor inhibitors are used primarily for the treatment of hypertension and congestive heart failure. These drugs are interesting candidate drugs for treating otosclerosis as well. Further research is needed to determine the efficacy of such therapeutic treatments.[105]

Unfortunately, DNA sequencing of the coding region of these two candidate genes in the OTSC10 region did not reveal a causative mutation in the present otosclerosis family.[103] However, introns and regulatory sequences of these genes were not sequenced and the involvement of these genes in the present otosclerosis family cannot be completely excluded.

Future perspectives and general considerations

Hearing impairment is the most common birth defect and the most prevalent sensorineural disorder in developed countries. Moderate or more severe hearing impairment has a negative impact on speech, language and cognitive development.[106] Therefore, universal newborn hearing screening is very important for early identification and management of hearing impairment in infants.[107] However, children with mild, progressive or later onset hearing impairment will be

missed with the newborn hearing screening.[108, 109] Nevertheless, mild bilateral sensorineural hearing impairment might also have a negative impact on school performance and early intervention, and speech and language monitoring are recommended.[110] Importantly, the underlying cause is not addressed with the newborn hearing screening. More than 50% of childhood sensorineural hearing impairment is caused by genetic factors and some of the involved genes are associated with mild and/or progressive hearing impairment.[111] Regular audiometric evaluation is necessary for infants who are at risk for progressive or later onset hearing impairment. Long-term follow-up of these children might provide insight into the development of hearing impairment associated with specific gene mutations. Knowledge of individual genes involved in hearing impairment has already been used successfully in patient treatment, management and genetic counseling. In the future, newborn genetic screening may be helpful in identifying groups of children with an increased risk of developing sensorineural hearing impairment. However, the discussion with regard to the ethical issues and cost-effectiveness of this type of genetic screening have to take place.[112]

Unfortunately, genetic counseling is still problematic, mainly because of the large number of genes involved in hereditary hearing impairment. In the majority of cases the question on the cause of hearing impairment cannot be answered and no proper counseling can be given. However, with the use of whole exome sequencing by next-generation sequencing (NGS) techniques, it is possible to obtain high-quality nucleic acid sequence data of whole exomes in a cost effective manner and in short period of time.[113] NGS can identify disease causing genes in rare genetic disorders with even a limited number of patients samples. The sensitivity, specificity and reproducibility of NGS in hearing impairment is high, however, interpretation of the functional effect of the large amount of DNA variants is challenging. Whole exome sequencing by NGS can be used as a diagnostic test in hearing impairment.[114, 115] However, its applicability in patient care is still limited. Whole exome sequencing by NGS can lead to the discovery of new deafness genes. Especially for these novel deafness genes, but also for some known deafness genes, there is insufficient phenotypic information available. Therefore, it is currently not always possible to provide satisfactory prognostic information. More phenotypic data need to be collected for

adequate counseling, especially because of the variation in phenotype associated with different defects in a specific gene or even with the same genetic defect. The creation of an elaborate database for the association of clinical data and the variant identified in the exome would be valuable. In the future, improved applications of NGS may help define genetic profiles of patients and contribute to personalized medicine.[113]

In conclusion, describing large groups of patients with hereditary hearing impairment is, next to accurate genetic counseling of patients, also very important for the discovery of new deafness causing genes, for the development of routine DNA-diagnostics of hereditary hearing impairment and for understanding the (dys)function of the inner ear. In the future, novel treatment strategies like drug therapy, hair cell regeneration or gene therapy may be introduced in clinical practice. For the development of gene-specific or mutation-specific treatment strategies in the future, it is essential to identify the genetic defects in patients with hereditary hearing impairment. Moreover, for accurate evaluation of these treatment strategies on restoring hearing and/or preventing hearing impairment, elaborate genotype-phenotype correlations are necessary. At present, genotype-phenotype correlations are mainly used for counseling of family members with hereditary hearing impairment.

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8

Summary / Samenvatting
List of abbreviations

Summary

In chapter 1 of this thesis a general introduction of hereditary hearing impairment with special attention for otosclerosis is provided.

Chapter 2 provides a detailed phenotypic description of three DFNA3 patients from two families. Mutation analyses revealed a p.Argl84Gln mutation in *GJB2* in family 1 and a p.Arg75Trp mutation in *GJB2* in family 2. No mutations in *GJB6* were identified. All three patients had severe to profound sensorineural hearing impairment. Vestibular function tests and computed tomographic scans yielded normal findings in the examined subjects. Cochlear implantation was performed in two patients, and their phoneme recognition scores were good.
A thorough genotype-phenotype correlation is difficult because of the small number of patients and the limited available clinical data. More clinical data of DFNA3 families need to be obtained in order to create a reliable and precise phenotype characterization.

Detailed phenotypic analyses of eight DFNB8/10 families are described in chapter 3. The compound heterozygous variants in the *TMPRSS3* gene in the present families included one novel variant, p.Val199Met, and four previously described pathogenic variants, p.Ala306Thr, p.Thr70fs, p.Ala138Glu and p.Cys107Xfs. In addition, the p.Ala426Thr variant, which had previously been reported as a possible benign polymorphism, was found in one family. All affected family members reported progressive bilateral hearing impairment, with variable onset ages and progression rates. In general, the hearing impairment affected the high frequencies first, and sooner or later, depending on the mutation, the low frequencies started to deteriorate which finally resulted in a flat audiogram configuration. The ski-slope audiogram configuration is suggestive for the involvement of *TMPRSS3*. In patients with progressive hearing impairment and a possible autosomal recessive mode of inheritance, *TMPRSS3* mutations should be considered. Evaluation of performance of patients with a cochlear implant indicated that this is a good treatment option for patients with *TMPRSS3* mutations as satisfactory speech reception was observed after implantation.

Our analyses suggest that mutations in *TMPRSS3* can be classified as mild and severe according to their phenotypic effect. Additional studies are needed to further distinguish possible phenotypic differences between different *TMPRSS3* mutations.

In chapters 4.1 and 4.2, the clinical and genetic characteristics of a large Dutch DFNX4 family are presented. Next-generation sequencing detected one variant within the linkage interval, a novel c.214G>T nonsense mutation (p.Glu72X) in *SMPX*. All 25 mutation carriers exhibited hearing impairment, except one woman aged 25 years. The age of onset according to history was 2-10 years (mean: 3,3 years) in men and 3-48 years (mean: 26,4 years) in women. Men showed more severe hearing impairment at a younger age with more pronounced progression during the first two decades of life, especially at the higher frequencies, while women demonstrated less severe hearing impairment with more gradual progression and a wider variation in age of onset, degree of hearing impairment and inter-aural asymmetry in thresholds, especially at 2-8 kHz. Longitudinal linear regression analysis demonstrated significant progression of at least two frequencies in five individuals (3 men and 2 women). Speech recognition in men and women was remarkably well preserved.
The phenotype of the present family is largely similar to the phenotype of the previously described DFNX4 families. However, subtle differences in onset age and rate of progression of hearing impairment seem to exist and might be caused by the different mutations in *SMPX*. However, a thorough genotype-phenotype analysis of DFNX4 requires more data on DFNX4 families harbouring different *SMPX* mutations. Moreover, the phenotypic heterogeneity in females was remarkable which is probably related to random X-inactivation in female mutation carriers.

The phenotype of a Dutch family with Muckle-Wells syndrome (MWS) caused by a novel p.Tyr859His mutation in the *NLRP3* gene is described in chapter 5. Most affected family members reported bilateral, slowly progressive hearing impairment since childhood. Hearing impairment started at the high frequencies and the low- and mid-frequency threshold values deteriorated with advancing age. Annual threshold deterioration ranged from 1.3 to 1.9 dB/year with the highest values at the lower frequencies. Longitudinal linear regression analysis demonstrated significant progression for a number of frequencies in five individuals. Speech

recognition scores were clearly affected. However, these individuals tended to have higher speech recognition scores than presbycusis patients at similar $PTA_{1,2,4\ kHz}$ levels. The loudness growth curves were steeper than those found in individuals with normal hearing, except for one family member (individual IV:6). Suprathreshold measurements, such as difference limen for frequency, gap detection and particularly speech perception in noise were within the normal range or at least close to data obtained in two groups of patients with a so-called conductive type of hearing loss, situated in the cochlea.

Hearing impairment in MWS is variable and shows resemblance to previously described intra-cochlear conductive hearing impairment. The cause of hearing impairment in MWS is not yet understood, but the basilar membrane of the cochlea might be involved in the pathogenesis of hearing impairment.

In every patient with sensorineural hearing impairment in combination with skin rash and musculoskeletal symptoms, MWS should be considered. However, these symptoms can be mild and nonspecific, as was the case in the present family. Therefore, the diagnosis of MWS can be easily missed. Unfortunately, an early diagnosis of MWS is essential to prevent irreversible damage from amyloidosis. Treatment with the IL-1β inhibitors has proven to be effective in reducing the symptoms of systemic inflammation. The effect on hearing impairment is more controversial, but an early start of treatment seems to be essential.

Otosclerosis is subject of chapter 6. Detailed analysis of audiometric data from a Dutch otosclerosis family in which the disease is linked to OTSC10, are presented in chapter 6.1. The genetic analysis of this family is provided in chapter 6.2. After exclusion of the known loci, a genome scan was performed which localized the gene to chr1q41-44 with a maximum LOD score of 3.3. This locus, named *OTSC10*, is 26.1Mb in size and contains 306 genes. Eleven family members were identified to be clinically affected and they were all carriers of the disease haplotype. Twelve unaffected family members carried the disease haplotype as well. Cross-sectional analyses of affected family members showed no significant progression of air-conduction (AC) thresholds, bone-conduction (BC) thresholds, and air-bone gap (ABG) levels with increasing age. Longitudinal regression analyses in one family member revealed significant deterioration of AC thresholds at all frequencies. The

BC thresholds showed a significant increase with advancing age at 0.5 kHz, 2 kHz and 4 kHz. A significant progression of the ABG was seen at 8 kHz. The progressive conductive hearing impairment in this family member stabilized around the third decade with ABG levels of 50 dB, and later a progressive sensorineural component developed.

The intersubject variation, in terms of age of onset, level of progression and audiogram configuration was remarkable, probably due to reduced penetrance and variable expression of the disease. Long-term audiometric data in one patient, however, were suitable to demonstrate progression of hearing impairment. There is no doubt that additional, similar studies are needed to be able to distinguish possible phenotypic differences between different genetic types of otosclerosis. Genotype-phenotype correlation studies may also help to elucidate the pathophysiology of otosclerosis at a molecular level and may provide possible targets for prevention, diagnosis and therapy of this disease. Furthermore, insight into the natural course of the various phenotypes of otosclerosis may provide the opportunity for a proper evaluation of the efficacy of current and future therapies.

Samenvatting

In hoofdstuk 1 van dit proefschrift wordt een algemene inleiding over erfelijke slechthorendheid en otosclerose gegeven.

Hoofdstuk 2 geeft een gedetailleerde beschrijving van het fenotype van drie DFNA3 patiënten uit twee families. Mutatie-analyse toonde een p.Argl84Gln mutatie in *GJB2* in familie 1 en een p.Arg75Trp mutatie in *GJB2* in familie 2. Mutaties in *GJB6* werden niet geïdentificeerd. Alle drie de patiënten hadden ernstig tot zeer ernstig perceptief gehoorverlies. Vestibulaire functie testen en computertomografie (CT) scans gaven normale bevindingen in de onderzochte familieleden. Twee patiënten kregen een cochleair implantaat en hun foneemscores waren goed.
Een gedetailleerde genotype-fenotype correlatie is moeilijk vast te stellen vanwege het kleine aantal DFNA3 patiënten en de beperkte klinische gegevens van deze patiënten. Meer klinische gegevens van DFNA3 families zijn nodig om een betrouwbare en nauwkeurige karakterisering van het fenotype te kunnen geven.

Hoofdstuk 3 geeft gedetailleerde fenotypische beschrijvingen van acht DFNB8/10 families. De mutaties in het *TMPRSS3* gen in deze families betreffen een nieuwe mutatie, p.Val199Met, en vier eerder beschreven pathogene mutaties, p.Ala306Thr, p.Thr70fs, p.Ala138Glu en p.Cys107Xfs. Bovendien werd de p.Ala426Thr variant, die eerder is beschreven als een mogelijk polymorfisme, aangetoond in een familie. Alle aangedane familieleden hadden een bilaterale slechthorendheid met een variabele beginleeftijd en een variabele progressie van het gehoorverlies. In het algemeen zijn de gehoordrempels van de hoge frequenties als eerste toegenomen. Vroeger of later, afhankelijk van de mutatie, zullen ook de gehoordrempels van de lage frequenties verslechteren, uiteindelijk resulterend in een vlak audiogram. Het audiogram met een ski-hellingconfiguratie is suggestief voor betrokkenheid van *TMPRSS3*. Bij patiënten met een progressief gehoorverlies van de hoge tonen en een mogelijke autosomaal recessieve overerving, moeten *TMPRSS3* mutaties worden overwogen als oorzaak. Evaluatie van de resultaten van patiënten met een cochleair implantaat geven aan dat dit een goede behandelingsoptie is voor patiënten met *TMPRSS3* mutaties, aangezien na implantatie een goed spraakverstaan werd waargenomen.

Onze analyses suggereren dat mutaties in *TMPRSS3* waarschijnlijk geclassificeerd kunnen worden als milde of ernstige mutaties. Aanvullende studies zijn nodig om mogelijk kleinere verschillen in het fenotype voor verschillende *TMPRSS3* mutaties te onderscheiden.

In de hoofdstukken 4.1 en 4.2, zijn de klinische en genetische kenmerken van een grote Nederlandse DFNX4 familie gepresenteerd. Met next-generation sequencing van alle genen op het X-chromosoom is de c.214G>T nonsense-mutatie (p.Glu72X) in *SMPX* aangetoond. Alle 25 mutatiedragers waren slechthorend, met uitzondering van een 25-jarige vrouw. De subjectieve beginleeftijd van het gehoorverlies was 2-10 jaar (gemiddeld: 3,3 jaar) voor mannen en 3-48 jaar (gemiddeld: 26,4 jaar) voor vrouwen. De mannen hadden al op jonge leeftijd een ernstig gehoorverlies met meer uitgesproken progressie van vooral de hoge frequenties tijdens de eerste twee decennia. Bij vrouwen daarentegen was de slechthorendheid minder ernstig met meer geleidelijke progressie en een grotere spreiding in beginleeftijd, in ernst van het gehoorverlies en in interaurale verschillen in drempels, in het bijzonder bij 2-8 kHz. Longitudinale lineaire regressie-analyse toonde een significante progressie van het gehoorverlies voor tenminste twee frequenties in vijf personen (3 mannen en 2 vrouwen). Het spraakverstaan van mannen en vrouwen was opmerkelijk goed.
Het fenotype van de huidige familie komt grotendeels overeen met het fenotype van de eerder beschreven DFNX4 families. Echter, subtiele verschillen in beginleeftijd en progressie van het gehoorverlies bestaan en kunnen worden veroorzaakt door de verschillende mutaties in *SMPX*. Voor een uitvoerige genotype-fenotype analyse van DFNX4 zijn meer gegevens van DFNX4 families met verschillende *SMPX* mutaties nodig. Bovendien is de heterogeniteit van het fenotype bij vrouwen opmerkelijk. Dit wordt waarschijnlijk veroorzaakt door X-inactivatie in vrouwelijke mutatiedragers.

Het fenotype van een Nederlandse familie met het Muckle-Wells syndroom (MWS), veroorzaakt door een nieuwe p.Tyr859His mutatie in het *NLRP3* gen, is beschreven in hoofdstuk 5. De meeste aangedane familieleden vermelden bilaterale, langzaam progressieve slechthorendheid sinds kinderleeftijd. Het gehoorverlies begon bij de hoge frequenties en met het toenemen van de leeftijd verslechterden ook de gehoordrempels van de lage en midden frequenties. Het gehoorverlies nam toe met 1,3 tot 1,9 dB per jaar, met de grootste achteruitgang voor de lage frequenties.

Longitudinale lineaire regressie-analyse toonde een significante progressie van het gehoorverlies voor een aantal frequenties in vijf personen. Spraakverstaanscores van de aangedane familieleden waren duidelijk verminderd. Echter, de familieleden hadden hogere spraakverstaanscores dan presbyacusispatiënten bij vergelijkbare $PTA_{1,2,4\ kHz}$ waarden. De luidheidsgroeicurven van de aangedane familieleden waren steiler dan de curven van personen met een normaal gehoor, met uitzondering van één familielid (individu IV: 6). Bovendrempelige metingen, zoals detectie van het verschil in frequentie, detectie van pauze of stilte tussen twee tonen en in het bijzonder spraakverstaan-in-ruis waren binnen het normale bereik en vergelijkbaar met de resultaten van twee groepen patiënten met een zogenaamd geleidingsverlies gelegen in de cochlea.

Het gehoorverlies in MWS is variabel en toont gelijkenis met het eerder beschreven intracochleair conductief gehoorverlies. De oorzaak van het gehoorverlies in MWS is nog niet duidelijk, maar het basilair membraan in de cochlea is mogelijk betrokken bij de pathogenese van slechthorendheid.

In elke patiënt met perceptief gehoorverlies in combinatie met huiduitslag en spier- en gewrichtsklachten moet MWS worden overwogen. Echter, deze symptomen kunnen mild en aspecifiek zijn, zoals het geval was in deze MWS familie. Derhalve kan de diagnose MWS gemakkelijk gemist worden, terwijl een vroege diagnose essentieel is voor het voorkomen van irreversibele schade ten gevolg van amyloïdose. Behandeling met IL-1β remmers is bewezen effectief voor het verminderen van de symptomen van ontsteking. Het effect op de slechthorendheid is meer controversieel, maar een vroege start van de behandeling lijkt van essentieel belang.

Otosclerose is het onderwerp van hoofdstuk 6. Een uitgebreide analyse van de audiometrische gegevens van een Nederlandse otosclerose familie, met genetische koppeling met het OTSC10 locus, is gepresenteerd in hoofdstuk 6.1. De genetische analyse van deze familie is beschreven in hoofdstuk 6.2. Na het uitsluiten van koppeling met bekende loci, werd een genoomscan uitgevoerd, waarbij het gen voor deze familie gelokaliseerd werd op chr1q41-44 met een maximale LOD score van 3.3. Dit locus, genaamd OTSC10, is 26.1Mb groot en bevat 306 genen. Elf familieleden werden geïdentificeerd als klinisch aangedaan en deze familieleden

waren allemaal drager van het otosclerose veroorzakende haplotype. Twaalf niet aangedane familieleden waren eveneens drager van het haplotype dat otosclerose kan veroorzaken. Cross-sectionele analyses van de audiometrische gegevens van de aangedane familieleden toonden geen significante progressie van de luchtgeleidingsdrempels, beengeleidingsdrempels en air-bone gap (ABG) met toenemende leeftijd. Longitudinale regressie-analyses van de audiometrische gegevens van één aangedaan familielid gaf wel een significante progressie van de luchtgeleidingsdrempels over alle frequenties. De beengeleidingsdrempels toonden een significante toename met de leeftijd bij 0,5 kHz, 2 kHz en 4 kHz. Een significante progressie van de ABG werd vastgesteld bij 8 kHz. Het progressieve conductieve gehoorverlies stabiliseerde rond het derde decennium met een ABG van 50 dB en later ontwikkelde zich een progressieve perceptieve component.

De interindividuele variatie wat betreft de beginleeftijd, de mate van progressie en de audiogramconfiguratie was opmerkelijk. Waarschijnlijk wordt deze variatie veroorzaakt door de verminderde penetrantie en de variabele expressie van de ziekte. Longitudinale audiometrische gegevens van een patiënt zijn bruikbaar om de progressie van het gehoorverlies te bepalen. Er is geen twijfel mogelijk dat aanvullende, soortgelijke, studies nodig zijn om mogelijke fenotypische variaties tussen de verschillende genetische typen van otosclerose te kunnen onderscheiden. Genotype-fenotype studies kunnen ook bijdragen aan de ontrafeling van de pathofysiologie van otosclerose op moleculair niveau en kunnen mogelijke aanknopingspunten voor preventie, diagnose en therapie van deze ziekte aan het licht brengen. Bovendien kan inzicht in het natuurlijke beloop van de verschillende fenotypen de mogelijkheid bieden tot het verrichten van een goede evaluatie van de effectiviteit van huidige en toekomstige therapieën.

List of abbreviations

ABG	air-bone gap
AC	air-conduction
ACE	angiotensin converting enzyme
AGT	angiotensin
ARHI	age-related hearing impairment
ARMS	amplification refractory mutation system
arNSHI	autosomal recessive nonsyndromic hearing impairment
ARTA	age-related typical audiogram
AT-II	angiotensin II
ATD	annual threshold deterioration
ATP	adenosine triphosphate
AUNA	auditory neuropathy
BAHA	bone-anchored hearing aid
BC	bone-conduction
BERA	brainstem evoked response audiometry
BMI	body mass index
BMP	bone morphogenetic protein
BOR	branchio-otorenal syndrome
BPPV	benign paroxysmal positional vertigo
CAPS	cryopyrin-associated periodic fever syndromes
CED	Camurati-Engelmann disease
CHI	conductive hearing impairment
CI	cochlear implant
CINCA	chronic infantile neurologic cutaneous and articular
CNV	copy number variant
CT	computed tomography
Cx26	connexin 26
Cx30	connexin 30
dB	decibel
DDST	diastrophic dysplasia sulfate transporter
DFN	deafness
DFNA	autosomal dominant nonsyndromic hearing impairment
DFNB	autosomal recessive nonsyndromic hearing impairment
DFNM	modifier gene locus for nonsyndromic hearing impairment
DFNX	X-linked nonsyndromic hearing impairment
DFNY	Y-linked nonsyndromic hearing impairment
DL_f	difference limen for frequency
DNA	deoxyribonucleic acid
EAS	electric acoustic system
EGF	epidermal growth factor
FCAS	familial cold autoinflammatory syndrome
FLAIR	fluid attenuated inversion recovery
GAG	glycosaminoglycan
GJB2	gap junction protein beta 2

GJB6	gap junction protein beta 6
HI	hearing impairment
HL	hearing level
HLA	human leukocyte antigen
I-A	inter-aural
IGF-1	insulin-like growth factor-1
IL	interleukin
LOD	limit of detection
LRR	leucin-rich repeats
MAGUK	membrane-associated guanylate kinase
MHC	major histocompatibility complex
MHI	mixed hearing impairment
MRI	magnetic resonance imaging
MWS	Muckle-Wells syndrome
NBS	nucleotide binding site
NGS	next generation sequencing
NSHI	nonsyndromic hearing impairment
OAE	otoacoustic emissions
OPG-Fc	short-term recombinant osteoprotegerin
OTSC	otosclerosis
PCR	polymerase chain reaction
PEST	proline glutamic acid, serine and threonine-rich
PTA	pure tone average
RAAS	rennin-angiotensin-aldosterone-system
RANK	receptor activator of nuclear factor κ B
RANKL	receptor activator of nuclear factor κ B ligand
RNA	ribonucleic acid
S	significant
SD	standard deviation
SNHI	sensorineural hearing impairment
SNP	single nucleotide polymorphism
SPL	sound pressure level
SRCR	scavenger receptor cysteine-rich
SRT	speech reception threshold
TCR	T-cell receptor
TGF	transforming growth factor
TMPRSS3	transmembrane protease serine 3
TORCH	toxoplasmosis, other infections (e.g. syphilis), rubella, cytomegalovirus, herpes
TRB	T-cell receptor beta locus

Dankwoord

Curriculum Vitae

List of publications

Dankwoord

En dan nu het meest gelezen hoofdstuk van dit proefschrift! Promoveren doe je zeker niet alleen. Zonder hulp van vele anderen was dit proefschrift nooit tot stand gekomen. Graag wil ik afsluiten met mijn woorden van dank aan de vele mensen die hebben bijgedragen aan dit proefschrift.

Ten eerste wil ik alle familieleden, die vrijwillig hebben meegewerkt aan dit onderzoek, bedanken voor hun enthousiaste deelname. Hartelijk dank voor jullie hulp bij het opstellen van de uitgebreide familiestambomen. Velen van jullie hebben ontelbare kilometers afgelegd om in Nijmegen 'de familiedag' bij te wonen. Zonder jullie had ik dit proefschrift niet kunnen schrijven.

Dr. H.P.M. Kunst, beste Dirk, dank voor jouw enorme vertrouwen in mij en alle kansen die je mij hebt gegeven! Jij gaf mij de mogelijkheid om een wetenschappelijke stage over erfelijke slechthorendheid te volbrengen en dankzij jou heb ik het onderzoek kunnen voortzetten in een promotietraject. De manier waarop jij mij de afgelopen jaren hebt begeleid, heb ik vanaf het begin als zeer prettig ervaren. Altijd kon ik met jou overleggen. Ik begrijp nog steeds niet waar je de tijd vandaan haalde! Jouw commentaren en adviezen waren verhelderd en aanmoedigend. Maar ik kon ook bij jou terecht met mijn frustraties als ik het even niet meer zag zitten. Vooral jouw opportunisme en loyaliteit zijn bewonderenswaardig. Ik wil je hartelijk danken voor jouw steun en inspanningen, niet alleen met betrekking tot dit proefschrift!

Prof. dr. J.M.J. Kremer, beste Hannie, jij bent een grote steun voor mij geweest. Jij hebt mij geïntroduceerd in de wondere wereld van de genetica. Jouw kritische commentaren hebben een belangrijke bijdrage geleverd aan de inhoud van dit proefschrift. Jij wist mij te inspireren en te stimuleren met jouw opbouwende kritieken. Enorm bedankt voor je hulp, begeleiding en vriendschap! Ik vind het een eer om jouw promovendus te zijn.

Prof. dr. C.W.R.J. Cremers, beste professor, ons eerste gesprek over een familie met erfelijke slechthorendheid kan ik mij nog goed herinneren. Dit gesprek bevestigde mijn enthousiasme voor de KNO. Ik heb bewondering voor uw visie, inzet en begeleiding. Uw uitgebreide ervaring als promotor heeft mij zeer geholpen bij de

succesvolle afronding van dit proefschrift. Ook tijdens de familiedagen was u altijd bereid om mee te helpen. U wist mij elke keer weer te verbazen met de snelle correctie van de stukken. Veel dank voor alles!

Dr. P.L.M. Huygen, beste Patrick, jouw wetenschappelijke kennis heeft een essentiële bijdrage geleverd aan deze promotie. Het was mij een genoegen om samen te brainstormen over de statistische analyses. Dankzij jou is mijn statistische kennis significant toegenomen! Geweldig dat jouw deur altijd open stond en ik altijd even binnen kon lopen. Ik hoop dat nog velen van jouw unieke kwaliteiten en creativiteit mogen genieten. Hartelijk bedankt voor jouw betrokkenheid en fijne gezelschap!

Dr. R.J.E. Pennings en dr. R.J.C. Admiraal, beste Ronald en Ronald, dankbaar heb ik de afgelopen jaren gebruik gemaakt van jullie otogenetische kennis en ervaring. Bedankt voor jullie begeleiding, maar vooral voor de plezierige samenwerking! Ik waardeer jullie oprechte interesse en belangstelling enorm. Geweldig dat ik altijd op jullie steun kon rekenen!

Prof. dr. G. van Camp en dr. I. Schrauwen, beste Guy en Isabelle, hartelijk dank voor de fijne samenwerking die heeft geleid tot de hoofdstukken 6.1 en 6.2. In zekere zin waren wij afhankelijk van elkaars data. Bedankt voor de onmisbare genetische inbreng. Ik bewonder de onderzoeksresultaten die jullie hebben bereikt en ik ben trots dat ik daaraan heb mogen bijdragen. Bedankt voor jullie hulp! Ik wens jullie beiden enorm veel succes met jullie verdere carrières.

Beste medewerkers van de afdeling antropogenetica, Margit Schraders, Jaap Oostrik, Lies Hoefsloot en Saskia ver der Velde, bedankt voor alle analyses van de genetische data. Ik heb onze samenwerking altijd als zeer prettig ervaren. Beste Margit, in het bijzonder bedankt voor de uiterst aangename besprekingen van de vele families en het goede overleg tijdens het schrijven van de artikelen. Jouw continue inzet en ijver werkten aanstekelijk. Beste Saskia van der Velde, ik wil je in het bijzonder bedanken voor de goede logistieke organisatie van de bloedmonsters. We vormden een goed team!

Beste medewerkers van het audiologisch centrum, bedankt voor jullie bereidheid om op zaterdagen de audiologische onderzoeken te verrichten. De vele uitgebreide meting bij patiënten waren essentieel voor dit proefschrift. Tijdens de familiedagen

kon ik altijd op jullie rekenen. Dank voor jullie inzet! Beste Ad Snik en Joop Leijendeckers, bijzonder dank voor jullie bijdrage aan de psychofysica testen. Ondanks de drukke agenda's wisten jullie altijd op korte termijn nog tijd vrij te maken voor overleg. Jullie inbreng was zeer waardevol. Bedankt voor de vele nuttige adviezen en de prettige samenwerking!

Beste medewerkers van het vestibulair onderzoek, Andy Beynon en Karin Krommenhoek, dank voor jullie bijdrage. Het spreekt voor zich dat de vestibulaire resultaten een meerwaarde aan de artikelen geven. Bedankt voor jullie inzet en medewerking! Andy, bedankt voor jouw enthousiaste uitleg en de fijne gesprekken over het vak!

Dr. A. Simon, beste Anna, bedankt voor je inbreng aan hoofdstuk 5. Jouw resultaten waren niet alleen een interessante toevoeging aan het artikel, maar nog veel meer van belang voor de patiënten van deze familie. Bedankt voor jouw betrokkenheid en fijne samenwerking!

Beste KNO poli verpleegkundigen, dank voor jullie hulp tijdens de familiedagen. Dankzij jullie verliep de bloedafname bij patiënten probleemloos. Bedankt voor de inzet en gezelligheid tijdens de familiedagen!

Beste medewerkers van het KNO stafsecretariaat en de KNO poli administratie, hartelijk dank voor alle ondersteunde werkzaamheden! Veel patiënten betekent ook veel papierwerk en een strakke organisatie. Bedankt voor jullie hulp, interesse en de prettige gesprekken!

Beste leden van de manuscriptcommissie, prof. dr. F.P.M. Cremers, prof. dr. C.B. Hoyng en prof. dr. I.J.M. Dhooge, hartelijk dank dat u het manuscript heeft willen beoordelen.

Beste arts-assistenten KNO, dank voor de collegialiteit en plezierige samenwerking. Beste Robbert-Jan Miserus, dank voor jouw steun tijdens de vele kopjes koffie. Jij was altijd bereid mee te denken en te discussiëren. Ik denk met veel genoegen terug aan deze tijd. Bedankt voor je betrokkenheid! Heel veel succes met jouw opleiding tot audioloog.

Beste collega's van de Allergologie Praktijk Arnhem, dank voor het welkome ontvangst, de prettige samenwerking en de fijne sfeer. Ik heb ontzettend veel geleerd over verscheidene aspecten van de medische wereld. Ik had deze gezellige en leerzame tijd voor geen goud willen missen! Beste Ad Jansen, jij stond altijd voor mij klaar met opbouwende kritiek. Er was altijd ruimte voor discussie. Maar wat mij het meest is bijgebleven is jouw vermogen om te relativeren en alles weer in de juiste context te plaatsen. Fijn om altijd op jouw steun te kunnen vertrouwen!

Beste staf van de Neus-, Keel- en Oorheelkunde van het Universitair Ziekenhuis te Gent, bedankt voor het in mij gestelde vertrouwen en voor de kans die jullie mij hebben gegeven om mijn opleiding tot Neus-, Keel- en Oorarts in Gent te volgen.

Beste arts-assistenten NKO, bedankt voor jullie steun, gezelligheid en collegialiteit. Door jullie warme onthaal voel ik mij helemaal thuis in Gent! Dank voor alle leuke dingen die we samen hebben beleefd.

Lieve vrienden, dank voor alle leuke momenten. Velen van jullie heb ik de afgelopen maanden minder frequent gezien, maar ik hoop dat we nog lang van het leven mogen genieten. Het is fantastisch om jullie om me heen te hebben en gezellig te kletsen over niet-medische zaken. Bedankt voor alles wat jullie voor mij betekenen!

Lieve schoonfamilie, Leo en Ria, Michel en Marie-Louise, Dorien en Daniel, dank voor jullie getoonde interesse. Het is wellicht niet altijd duidelijk geweest waar ik nou eigenlijk mee bezig was, maar jullie belangstelling is altijd gebleven. Bij jullie vind ik altijd een warm thuis en ik ben blij dat Joy en ik regelmatig gebruik mogen maken van jullie gastvrijheid. Ik weet dat ik altijd op jullie kan rekenen. Dorien, de omslag is echt fantastisch! In het bijzonder bedankt voor het geweldige ontwerp.

Lieve Denise en Derk Jan, dank voor jullie oprechte meeleven de afgelopen jaren. Jullie betekenen ontzettend veel voor mij! Denise, bedankt voor je correcties van de Nederlandse teksten. Ik ben trost op je en blij om je zus te zijn! Bedankt voor alles!

Lieve papa en mama, het valt niet in woorden uit te drukken hoe waardevol ik jullie onvoorwaardelijke liefde, steun en vertrouwen vind. Ik ben ontzettend dankbaar voor alle kansen die jullie mij hebben gegeven. Jullie hebben mij altijd gestimuleerd

mijn mogelijkheden ten volle te benutten. Zonder jullie was ik niet wie en waar ik nu ben! Met trots draag ik dit boekje aan jullie op.

Lieve Joy, de laatste woorden in dit boekje zijn voor jou. Als er iemand is die mij het meest heeft gesteund dan ben jij dat wel! Onbeschrijfelijk lief dat je er altijd voor mij bent. In dit proefschrift zitten heel wat kostbare uurtjes die eigenlijk voor jou bestemd waren… Bedankt voor je geduld en begrip! Jouw steun en liefde waren essentieel voor de succesvolle afronding van dit proefschrift. Ik hoop dat we nog vele jaren samen kunnen doorbrengen en ik kijk enorm uit naar de toekomst samen met jou!

Curriculum Vitae

Nicole Weegerink werd op 21 november 1983 geboren te Enschede. Aan het Assink Lyceum te Haaksbergen haalde zij in 2001 haar HAVO eindexamen en in 2003 haar VWO eindexamen. In datzelfde jaar begon zij met de studie Geneeskunde aan de Radboud Universiteit te Nijmegen. Tijdens haar coschappen in the UMC St Radboud groeide haar enthousiasme voor de Keel-, Neus- en Oorheelkunde. In 2009 volgde een onderzoeksstage naar erfelijke slechthorendheid op de afdeling KNO in het UMC St Radboud onder leiding van dr. H.P.M. Kunst. Deze stage resulteerde in een aanstelling als arts-onderzoeker op de KNO afdeling. Onder leiding van dr. H.P.M. Kunst, prof. dr. H. Kremer en prof. dr. C.W.R.J. Cremers werd het onderzoek naar erfelijke slechthorendheid voortgezet, wat uiteindelijk heeft geleid tot het tot stand komen van dit proefschrift. Tijdens de afronding van haar proefschrift werkte zij als arts-assistent op de Allergologie Praktijk Arnhem. In oktober 2012 is zij gestart met de opleiding tot Keel-, Neus- en Oorarts in het Universitair Ziekenhuis te Gent.

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* These authors contributed equally to this work

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