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Sanna Häkli

CHILDHOOD HEARING IMPAIRMENT IN NORTHERN FINLAND: PREVALENCE, AETIOLOGY AND ADDITIONAL DISABILITIES

UNIVERSITY OF OULU GRADUATE SCHOOL;
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Academic dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in Auditorium 6 of Oulu University Hospital, on 12 December 2014, at 12 noon

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Abstract

The purpose of this study was to determine the prevalence and aetiology of childhood hearing impairment (HI) in northern Finland and to evaluate the presence of additional disabilities among hearing impaired children. Such data would be valuable in guiding examinations and rehabilitation.

Study I consisted of 214 children with mild to profound HI ascertained prior to age 10 years. They belonged to the birth cohort spanning the years 1993–2002. The clinical data were collected from the patient records of the Oulu University Hospital. In studies II–III, mutations in mitochondrial DNA (mtDNA) and in the *WFS1* gene were determined in children with unknown aetiology of HI. Study IV is a prospective follow-up study examining the hearing of children with m.1555A>G mutation in mtDNA.

The prevalence of childhood HI was 2.3/1000 live births. Genetic causes were the most common (47%) aetiology of HI, while 16% of cases were acquired and 36% were unknown. Almost 40% of 214 children had one or more additional disabilities that adversely influenced their development or learning. The frequency of additional disabilities was not associated with the severity of HI. Children with acquired HI had additional disabilities more often (66%) than children with genetic or unknown aetiology of HI (44%).

Molecular analysis revealed that mutations in mtDNA and *WFS1* are rare causes of childhood HI. Three rare variants and the novel p.Gly831Ser variant were found in *WFS1*. The p.Gly831Ser variant may be a new member to the group of heterozygous *WFS1* mutations that lead to HI. One child harboured the pathogenic m.1555A>G mutation in *MT-RNR1*. In addition, eight rare variants and 13 polymorphisms were found in *MT-RNR1* or in *MT-RNR2*. Evaluation of m.990T>C suggested that this transition is a pathogenic rather than a neutral variant.

During a 7.8 year follow up of 19 children with m.1555A>G, HI was ascertained in 10 children (age range, 2.1–13.2 years at the end of the follow-up). Distinct phenotypes of HI were identified. Environmental factors contributing to the phenotype variation were not recognized. Because these children generally pass the newborn hearing screening, it is important to follow over time the hearing of children in families with the m.1555A>G mutation.

Keywords: additional disability, aetiology, child, epidemiology, hearing impairment, mitochondrial DNA, molecular genetics, prevalence, wolframin

Häkli, Sanna, Pohjoissuomalaisten lasten kuuloviat: esiintyvyys, etiologia ja lisäoireet.

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Tiivistelmä

Tämän työn tavoitteena oli tutkia lapsuusiän kuulovikojen esiintyvyyttä ja etiologiaa pohjoissuomalaisilla lapsilla sekä selvittää kuulovikaisilla lapsilla esiintyviä muita oireita. Tieto kuulovian etiologiasta ja mahdollisista muista oireista auttaa tutkimusten ja kuntoutuksen suunnittelussa.

Tutkimukseen osallistuvat lapset olivat syntyneet Pohjois-Suomessa vuosina 1993–2002. Osatyössä I kerättiin sairauskertomustiedot niistä lapsista, joiden kuulovika oli todettu ennen kymmenen vuoden ikää. Osatöissä II ja III määritettiin mitokondrion DNA:n ja tuman *WFS1*-geenin muutoksia lapsilla, joiden kuulovian etiologia oli tuntematon. Osatyössä IV seurattiin lasten kuuloa suvussa, jossa on todettu mitokondrion DNA:n mutaatio m.1555A>G.

Lapsuusiän kuulovikojen esiintyvyys oli 2,3 tuhatta vastasyntynyttä kohden. Kuulovian yleisin syy oli perinnöllinen (47 %). Hankinnaisia kuulovikoja oli 16 % ja etiologialtaan tuntemattomia 36 %. Lähes 40 %:lla 214 lapsesta oli kuulovian lisäksi yksi tai useampi muu oire, jonka arvioitiin vaikuttaneen haitallisesti lapsen kehitykseen tai oppimiseen. Muiden oireiden esiintyminen ei riippunut kuulovian vaikeusasteesta. Hankinnaiseksi luokiteltuihin kuulovikoihin liittyi enemmän muita oireita (66 %) kuin niihin kuulovikoihin, joiden syy oli perinnöllinen tai tuntematon (44 %).

Pohjoissuomalaisilla lapsilla mitokondrion DNA:n ja *WFS1*-geenin muutokset olivat harvinaisia kuulovian syitä. *WFS1*-geenissä todettiin kolme aikaisemmin tunnettua harvinaista ja yksi uusi geenimuutos. Tämän p.Gly831Ser-mutaation arvioitiin olevan heterotsygoottisena kuulovikaa aiheuttava muutos. Yhdellä lapsella todettiin mitokondrion DNA:n patogeeninen mutaatio m.1555A>G. Lisäksi *MT-RNR1*- ja *MT-RNR2*-geeneissä todettiin 13 polymorfiaa, jotka kuuluvat normaaliin vaihteluun ja kahdeksan harvinaista muutosta, joista m.990T>C-muutos on todennäköisesti kuulovikaa aiheuttava.

Seurantatutkimukseen osallistui 19 lasta, joilla oli m.1555A>G-mutaatio. Seuranta kesti 7,8 vuotta, ja sen aikana ilmaantui kuulovika 10 lapselle, joiden ikä tutkimuksen loppuessa oli 2,1–13,2 vuotta. Todetut kuuloviat olivat keskenään erilaisia. Vaihtelua selittäviä ympäristötekijöitä ei todettu. Lasten kuuloa on tärkeää seurata perheissä, joissa on m.1555A>G-mutaatio, koska lapset yleensä läpäisevät vastasyntyneen kuulonseulontatutkimuksen ja mahdollinen kuulovika kehittyy myöhemmin.

Asiasanat: epidemiologia, esiintyvyys, etiologia, kuulovika, lapsi, mitokondrio-DNA, molekyyligenetiikka, monioireisuus, wolframiini

To all hearing impaired children

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Oulu, October 2014 Sanna Häkli

Abbreviations

BEHL	better ear hearing level
CMV	cytomegalovirus
CSGE	conformation sensitive gel electrophoresis
GJB2	Gap junction beta 2
HI	hearing impairment
HmtDB	human mitochondrial database
LFSNHI	low-frequency sensorineural hearing impairment
mtDNA	mitochondrial DNA
NICU	neonatal intensive care unit
NHS	newborn hearing screening
OUH	Oulu university hospital
rRNA	ribosomal RNA
TEOAE	transient evoked otoacoustic emissions
tRNA	transfer RNA
USH	Usher syndrome
WS	Wolfram syndrome

List of original publications

This thesis is based on the following publications, which are referred throughout the text by their Roman numerals:

I Häkli S, Luotonen M, Bloigu R, Majamaa K, Sorri M (2014) Childhood hearing impairment in northern Finland, etiology and additional disabilities. Int J Pediatr Otorhinolaryngol 78(11):1852–6.

II Häkli S, Kytövuori L, Luotonen M, Sorri M, Majamaa K (2014) WFS1 mutations in hearing-impaired children. Int J Audiol 53(7):446–51.

III Häkli S, Luotonen M, Sorri M, Majamaa K (2014) Mutations in the two ribosomal RNA genes in mitochondrial DNA among Finnish children with hearing impairment. Manuscript.

IV Häkli S, Luotonen M, Sorri M, Majamaa K (2013) Audiological follow-up of children with the m.1555A>G mutation in mitochondrial DNA. Audiol Neurotol 18(1):23–30.

Contents

1 Introduction

The estimates for the prevalence of childhood hearing impairment (HI) vary depending on population, study design and the criteria used to define the degree of HI or age. The prevalence of congenital HI is estimated to be at least 1 per 1000 live births and the prevalence of childhood HI increases with age (Fortnum & Davis 1997, Fortnum *et al*. 2001, Mehl & Thomson 2002). Although the prevalence of congenital and early childhood HI is low, the consequences are important because childhood HI may adversely impact speech and language development. Early detection and rehabilitation of HI improves the outcome of hearing impaired children (American Academy of Pediatrics, Joint Committee on Infant Hearing 2007). Newborn hearing screening (NHS) programmes have enabled practitioners to find congenital HIs early. Even if a newborn has passed a hearing screening test, it is important to keep in mind that HI can arise and progress later in childhood.

Aetiologies of childhood HI are varied and have changed over recent decades. Aetiologies of HI can be broadly divided into genetic and non-genetic categories. Improvements in health care have decreased post-infectious HIs. At the same time, such improvements (i.e., neonatal intensive care practices) have led to increased survival of very premature newborns, and paradoxically, to a greater number of perinatal risk factors among hearing impaired children (Morzaria *et al*. 2004).

It has been estimated that genetic causes are the most common aetiology of childhood HI. Hereditary forms of HI can be classified by mode of inheritance into autosomal dominant or recessive, X-linked or mitochondrial HI. Autosomal recessive inheritance is the most common. In cases of autosomal recessive inheritance, both parents are asymptomatic mutation carriers with normal hearing, yet one or more children have HI. Advances in genetics have improved the chances of being diagnosed with genetic HI. However, such advances also indicate that molecular aetiology of HI is diverse. With the exception of gap junction beta 2 (*GJB2)* gene, no major HI genes have been identified (Petersen & Willems 2006).

Children with HI may have additional disabilities that are part of a syndrome or a comorbid condition. About 30–40% of hearing impaired children have been reported to have additional health problems that may adversely affect their development and learning (Birman *et al*. 2012, Fortnum & Davis 1997, Fortnum *et al*. 2002). These health problems may be ignored, although additional

disabilities could have even stronger effects on the outcome than HI itself. Therefore they are important to take into consideration in rehabilitation.

The aetiology of childhood HI is unknown in approximately 40% of cases (Korver *et al*. 2011, Morzaria *et al*. 2004). In the future, advances in diagnostics may decrease the proportion of unknown causes of HI. Knowledge of the aetiology of HI is valuable as it can help professionals make predictions regarding the progression of HI. Such knowledge is useful in guiding examinations, particularly with regard to additional disabilities and rehabilitation. Knowledge of the aetiology also helps with appropriate genetic counselling for HI.

The purpose of this study was to determine the aetiology and prevalence of childhood HI in northern Finland, to define the genetic aetiology and to evaluate the frequency of additional disabilities present among hearing impaired children.

2 Review of the literature

2.1 Definitions of hearing impairment

The type of HI can be categorized as conductive, sensorineural, or mixed, depending on the defect in the hearing organ. Sensorineural HI is the most common type of permanent HI and can be further divided into cochlear (sensory) and retrocochlear (neural) impairments. HIs can also be divided into central and peripheral impairments. The degree of HI is often classified according to definitions suggested by the European working group and based on pure tone average calculated over the frequencies 0.5, 1, 2 and 4 kHz. HI is classified according to the better ear hearing level (BEHL) as mild (21–39 dB), moderate (40–69 dB), severe (70–94 dB) or profound ($\geqslant$ 95 dB) (Stephens & Martini 1996). The onset time of childhood HI is classified as either prelingual or postlingual. Prelingual HI can be congenital or develop in early childhood before speech and language development.

The cause of HI is often considered to be either genetic or non-genetic. Non-genetic causes can be further divided as prenatal, perinatal or postnatal. Genetic HI can be chromosomal or monogenic and is most commonly inherited as an autosomal recessive or dominant trait. X-chromosomal or mitochondrial traits of inheritance are less frequent. In some cases, the cause of HI can be both genetic and acquired, such as mutations that confer susceptibility for aminoglycoside ototoxicity.

Mutations in a given gene can cause both syndromic and nonsyndromic forms of HI. The traditional classification of HI into syndromic and nonsyndromic forms is problematic, because additional disorders defining a syndromic state may arise later in childhood.

2.2 Prevalence of childhood hearing impairment

The prevalence of childhood HI varies between studies. Prevalence estimates differ primarily because of different HI definitions or because of different ages of the recruited children. Prevalence estimates also differ because of differences in health care systems, for example, vaccinations and treatment of infections.

In general, congenital, bilateral and at least mild HI has been reported in 1.1–1.54/1000 newborns (Mehl & Thomson 2002, Mehra *et al*. 2009, Morton &

Nance 2006). The prevalence of HI increases with age and among school age children, permanent HI has been reported in 1.65–2.7/1000 children (Darin *et al*. 1997, Fortnum *et al*. 2001, Fortnum *et al*. 2002, Morton & Nance 2006). In Finland, the prevalence estimates of childhood HI have remained stable for at least three decades at 1.2/1000 for at least moderate, (Mäki-Torkko *et al*. 1998) and 2.1/1000 for at least mild HI (Dietz *et al*. 2009, Vartiainen *et al*. 1997). An overview of previously reported prevalence estimates is shown in Table 1.

2.3 Age of ascertainment of hearing impairment

HI may have adverse effects on speech and language acquisition as well as social and emotional development. Early detection and intervention of HI improves the outcome of children. At least moderate HIs should be ascertained before the age of 3 months and intervention should be initiated before 6 months of age (American Academy of Pediatrics, Joint Committee on Infant Hearing 2007). In many studies, the age at ascertainment of HI has been reported to be higher than recommendations put forward in consensus guidelines (Dietz *et al*. 2009, Fortnum & Davis 1997, Mäki-Torkko *et al*. 1998). The only effective means of finding congenital HIs is a universal NHS (Mehl & Thomson 2002). Although congenital HIs can now be found earlier than before, in part due to NHS, it is important to keep in mind that some HIs are progressive, arise later in childhood, and cannot be detected by NHS.

Table 1. Overview of previous reports on the prevalence of childhood bilateral hearing impairment (HI).

Author, year	Population	Children with HI (N)	Age of subjects at the time of data collection (years)	Degree of HI (BEHL) dB	Prevalence
Vartiainen, 1997	Finland	98	≤9	>25	2.1:1000
Fortnum, 1997	UK	487	5-10	≥40	1.3:1000 [1]
Darin, 1997	Sweden	86	6-10	>20	2.0:1000 [2]
Mäki-Torkko, 1998	Finland	253	4-23	≥40	1.2:1000
Uus, 2000	Estonia	248	8-13	≥40	1.7:1000
Nekahm, 2001	Austria	165	6-20	≥40	1.3:1000
Fortnum, 2001	UK	17 160	3	≥40	1.07:1000 [3]
			9-16	≥40	2.05:1000 [3]
Mehl, 2002	Colorado	291	Newborn	>35	1.1:1000
Dietz, 2009	Finland	92	≤7	≥20	2.1:1000

BEHL=better ear hearing level, [1] 409 congenital, prevalence 1.1:1000, [2] includes both unilateral and bilateral HI, [3] adjusted by capture-recapture

2.4 Aetiology

During the past several decades, the aetiologies of childhood HIs has changed in developed countries because of universal immunization programs, improved antibiotic treatments for meningitis, and improvements in neonatal intensive care. For example, chronic otitis media was once a common cause of permanent conductive or mixed HI but has almost disappeared in developed countries. Advances in molecular genetics suggest that the molecular aetiology of childhood HI is diverse yet, with the exception of *GJB2*, no major HI genes have been identified (Petersen & Willems 2006, Van Camp & Smith 2005). Hence, the aetiology of childhood HI is unknown in about 40% of cases (Korver *et al*. 2011, Morzaria *et al*. 2004). It is possible that recessive mutations are responsible for many of the HI cases that are currently classified as unknown in aetiology. Also, the prevalence of cytomegalovirus (CMV) infections may be underestimated in many studies because of challenges in diagnostics. In general, it is thought that the cause of childhood HI is genetic in 30–50% of cases and acquired in 14–30% of children (Korver *et al*. 2011, Morton & Nance 2006, Morzaria *et al*. 2004) (Table 2). The aetiologies of childhood HI differ greatly between studies depending on societies, health care systems and methodological differences of

study design. Genetic nonsyndromic, asphyxia and prematurity are more common aetiologies of HI than in earlier decades (Morzaria *et al*. 2004).

Table 2. Overview of previous reports on proportions of aetiologies in childhood hearing impairment.

Author, year	Population	Subjects (N)	Acquired (%)	Genetic (%)	Unknown (%)
Fortnum, 1997	UK	653	19.4	39.7	40.9
Uus, 2000	Estonia	248	29.4	36.3	34.3
Nekahm, 2001	Austria	165	31.0	33.0	36.0
Fortnum, 2002	UK	17 160	20.9	29.7	49.4
Deben, 2003	Belgium	190	30.6	36.8	32.6
Egeli, 2003	Turkey	162	37.3	41.4	20.0
Declau, 2008	Belgium	87	21.8 [1]	33.3	44.8
Dietz, 2009	Finland	92	14.0	46.0	40.0
Korver, 2011	Netherlands	185	29.7	38.9	24.3 [2]
Lammens, 2013	Belgium	277	37.8 [3]	30.2	29.1

[1] includes cytomegalovirus infections 10.3% and perinatal problems 11.5%

[2] in addition to these, miscellaneous causes 7.1%

[3] includes cytomegalovirus infection diagnostics and non-genetic malformations, 2.9% was classified as "other aetiology"

2.5 Non-genetic causes of childhood hearing impairment

2.5.1 Congenital infections

Infections during pregnancy or the perinatal period can damage the placenta and foetus. Infections causing HI often lead to visual and neurological dysfunction as well. Over the past several decades, the development of vaccines and the implementation of universal immunization programmes have helped to reduce the proportion of childhood HIs that are the result of prenatal infections. For example, congenital rubella infection is a rare disease in developed countries. In Finland, the nationwide rubella vaccination programme was started in 1975 and a combined vaccine against measles, mumps and rubella was adopted in 1982. Also, congenital syphilis infection can lead to sensorineural HI (Chau *et al*. 2009, George *et al*. 2009). All pregnant women in Finland are screened for syphilis and resulting infections can be treated with antibiotics. Congenital toxoplasmosis is a risk factor for childhood HI (American Academy of Pediatrics, Joint Committee

on Infant Hearing 2007). The incidence of HI associated with toxoplasmosis infection, however, is poorly known (Brown *et al*. 2009). Likewise, the incidence of HI in neonates exposed to Herpes simplex virus is not known (Westerberg *et al*. 2008).

Among non-genetic causes, CMV appears to be a significant cause of childhood HI. CMV infection has been reported to explain 8–25% of early childhood HIs (Avettand-Fenoel *et al*. 2013, Dahl *et al*. 2013, Grosse *et al*. 2008, Karltorp *et al*. 2012, Korver *et al*. 2009). The prevalence of congenital CMV infection at birth is estimated to be 0.64%. The majority of these infections are asymptomatic, with just 11% being symptomatic (Kenneson & Cannon 2007, Swanson & Schleiss 2013). CMV infection may be an overlooked cause of childhood HI in many studies. It has been estimated that about half of all children with symptomatic congenital CMV infection and between 8.5–21% with asymptomatic CMV infection will develop HI (Dollard *et al*. 2007, Foulon *et al*. 2008, Fowler & Boppana 2006). HI associated with CMV can be congenital or delayed in onset and its degree is variable (Foulon *et al*. 2008, Rosenthal *et al*. 2009).

2.5.2 Perinatal risk factors

The incidence of HI is higher among premature infants and neonates admitted to neonatal intensive care unit (NICU) than among healthy term-born children (Hintz *et al*. 2011, Johnson *et al*. 2009, Robertson *et al*. 2009). The relationship between various risk factors and HI among premature infants or infants in the NICU has been evaluated in many studies. There are several recognized risk factors for HI in the neonatal period, for example, a decrease in gestational age and low birth weight. Some studies have reported predictors of HI risk factors, including infant characteristics such as craniofacial anomalies, low Apgar scores, central nervous system or circulatory system conditions (i.e., asphyxia, intraventricular hemorrhage) or infections (Coenraad *et al*. 2010, Cone-Wesson *et al*. 2000, Lieu *et al*. 2013, Robertson *et al*. 2009). In recent studies, prolonged duration of mechanical ventilation and oxygen treatment have been reported as the most predictive risk factors for HI (Bielecki *et al*. 2011, Kountakis *et al*. 2002, Marlow *et al*. 2000, Martinez-Cruz *et al*. 2012, Robertson *et al*. 2009, van Dommelen *et al*. 2010). Ototoxic medication may be the most common risk factor for HI, but in recent studies, it has not been the most probable factor for patients with concentrations in the therapeutic range (Bielecki *et al*. 2011, de Hoog *et al*.

2003, van Dommelen *et al*. 2010). HI risk factors are also common risk factors for neurodevelopmental disorders (Johnson *et al*. 2009, Martinez-Cruz *et al*. 2012, Robertson *et al*. 2009, Synnes *et al*. 2012). Despite plenty of research in this area, it is impossible to conclude which risk factors are causal and which are simply indicators. HI is related to complications of premature birth such as asphyxia, prolonged neonatal illness and its subsequent management. At first, improved neonatal care resulted in fewer HIs, but in recent years, the increased survival of premature and very premature infants has led to more perinatal risk factors for HI. As the number of co-occuring risk factors increases, the probability of HI in infants also increases (Bielecki *et al*. 2011).

2.5.3 Meningitis

It is estimated that between 5–33% of bacterial meningitis cases result in HI (Edmond *et al*. 2010, Jit 2010). Being younger in age and having a more severe form of the disease are predictors of HI (Edmond *et al*. 2010, Roine *et al*. 2013). The severity of HI is variable. HI can be unilateral or bilateral (Karanja *et al*. 2014, Wellman *et al*. 2003). HI is believed to develop during the course of disease, but hearing threshold changes are also common after recovery (Richardson *et al*. 1998, Roine *et al*. 2014). As a result, individuals with meningitis are encouraged to have a hearing evaluation at least one month after admission (Roine *et al*. 2014).

Vaccinations and improvements in antibiotics have decreased the likelihood of meningitis as a cause of childhood HI (Roizen 2003). The introduction of vaccines against Haemophilus influenzae type B (1986 in Finland), and the more recent approval of pneumococcal vaccine (2010), have decreased the incidence of invasive bacterial diseases such as meningitis. A vaccine is also available for meningococcus, the third major pathogen of bacterial meningitis. However, the vaccine is used mainly to eliminate epidemic meningitis (Ruotsalainen *et al*. 2013).

2.5.4 Ototoxic medication and substances

Exposure to teratogenic substances or to ototoxic medication during pregnancy may damage the auditory system (Roizen 1999). Certain drugs used by pregnant woman, for example antiepileptic drugs, may harm a developing foetus (Adam *et al*. 2011). A few decades ago, thalidomide turned out to be a potent teratogen that

resulted in a variety of birth defects, some of which included anomalies of the ear and HI. Ototoxic medication can also cause HI postnatally. The most common ototoxic drugs used in developed countries are aminoglycoside antibiotics and cisplatin. Infants in the NICU are often treated with aminoglycosides. Later in childhood, exposure to cisplatin during the treatment of malignant diseases significantly increases the risk of HI. HI is prevalent among children with high-risk neuroblastoma (Landier *et al*. 2014).

Alcohol consumption during pregnancy can lead to foetal alcohol syndrome, where common consequences to the child include growth retardation, characteristic facial features and central nervous system anomalies or dysfunction (O'Leary 2004). Even though HI is not a common feature of foetal alcohol syndrome, it may be present among some children. Children with foetal alcohol syndrome may also have conductive HI caused by recurrent secretory otitis media, or sensorineural HI (Church & Abel 1998, Cohen-Kerem *et al*. 2007).

The Joint Committee on Infant Hearing 2007 position statement (American Academy of Pediatrics) identifies several risk factors for congenital, delayed onset or progressive childhood HI. These risk factors are as follows:

1. Caregiver concern regarding hearing, speech, language, or developmental delay
2. Family history of childhood HI
3. Neonatal intensive care of more than 5 days or any of these: ECMO, assisted ventilation, exposure to ototoxic medications, hyperbilirubinemia requiring exchange transfusion
4. In utero infections such as TORCH (Toxoplasmosis, Rubella, Cytomegalovirus, Syphilis, Herpes)
5. Craniofacial anomalies
6. Stigmata or other findings associated with a syndrome known to include HI
7. Syndromes or neurogenerative disorders associated with HI
8. Culture-positive postnatal infections associated with HI incl. meningitis
9. Head trauma that requires hospitalization
10. Chemotherapy

2.6 Genetic causes of childhood hearing impairment

Genetic HI is usually categorised by the mode of inheritance: autosomal recessive, autosomal dominant, X-linked and mitochondrial. The majority (70–80%) of

nonsyndromic HI cases are estimated to be autosomal recessive, with 10–20% autosomal dominant, 1% X-linked and 1% due to mitochondrial inheritance (Marazita *et al*. 1993, Morton 1991, Tomaski & Grundfast 1999, Van Camp & Smith 2005).

To date (August 2014), more than 120 genes have been identified as causing syndromic or nonsyndromic HI. Over 70 genes have been identified as cause nonsyndromic HI. Of these genes, there are 55 with recessive and 31 with dominant inheritance (some overlapped with recessive genes), 4 X-linked, and 2 mitochondrial (Van Camp & Smith 2005, Hereditary Hearing loss homepage). Mutations within a single gene have been found to result in different clinical phenotypes and different modes of inheritance. Several genes are involved in both recessive and dominant HI, as well as syndromic and nonsyndromic HI. Although gene mutations are present from the time of conception, genetic HI can develop in childhood or even adulthood. Advances in molecular genetics suggest that the aetiology of childhood HI is diverse yet, with the exception of *GJB2*, no major HI genes have been identified.

2.6.1 GJB2 gene

In 1997, the *GJB2* gene in chromosome 13q11 was the first gene to be identified in autosomal recessive HI (Kelsell *et al*. 1997). *GJB2* gene encodes Connexin 26 protein, which belongs to the transmembrane proteins, and has been implicated in gap junction intercellular communication. Connexins have important roles at least in potassium recycling and intercellular calcium signalling (Hoang Dinh *et al*. 2009).

Mutations in *GJB2* are the most common causes of childhood nonsyndromic sensorineural HI (OMIM 220290). Such mutations are present in 20–30% of cases (Chan & Chang 2014, Hutchin *et al*. 2005, Löppönen *et al*. 2003). The c.35delG mutation accounts for the majority of *GJB2* mutations. The carrier frequency of the 35delG mutation is 1.53% in northern Europe (Gasparini *et al*. 2000, Mahdieh & Rabbani 2009) and 1.28% in Finland (Löppönen *et al*. 2003). In addition to the c.35delG mutation, more than 100 other mutations in the *GJB2* gene have been identified as causing nonsyndromic HI (Ballana *et al*. 2005, The Connexins and deafness Homepage).

The HI phenotype caused by biallelic mutations in *GJB2* is highly variable. HI can be a profound congenital HI or a mild, progressive HI that presents later in childhood. The degree of HI is highly dependent on the genotype. For example, persons with homozygous c.35delG mutation have a more severe phenotype than

those with a non-c.35delG genotype (Chan & Chang 2014, Snoeckx *et al*. 2005). The HI caused by *GJB2* mutations is usually recessive and nonsyndromic, but some mutations in *GJB2* have been described as causing an autosomal dominant HI with skin manifestations (Petersen & Willems 2006).

2.6.2 Usher syndrome

Usher syndrome (USH) is an autosomal recessive disease characterized by sensorineural HI, retinitis pigmentosa, and often, vestibular dysfunction. It is estimated to be one of the most common forms of syndromic, recessive HI with a prevalence of 3–6% among individuals with severe to profound HI (Boughman *et al*. 1983, Kimberling *et al*. 2010). Kimberling *et al*. found the prevalence of USH to be 11.3% among hearing impaired children who were negative for *GJB2/6* mutations (Kimberling *et al*. 2010). The prevalence of USH ranges from 3.5 to 6.2 per 100 000 (Spandau & Rohrschneider 2002). The major clinical subtypes of Usher syndrome (USH type I, USH type II, and USH type III) are classified based on severity of the HI, presence or absence of vestibular dysfunction, and age at onset of retinitis pigmentosa. The most common subtype in Finland is USH type III, with progressive, usually postlingual HI, and variable vestibular dysfunction. The onset of vision symptoms usually occurs by the second decade of life (Pakarinen *et al*. 1995). Most Finnish patients with USH type III have the founder mutation p.Y176X in *CLRN1* gene. Otherwise, the genetic background of USH is heterogeneous and mutations in at least nine genes have been identified (Vastinsalo *et al*. 2013, Yan & Liu 2010).

2.6.3 SLC26A4 gene

Mutations in the *SLC26A4* gene are probably the second most frequent cause of autosomal recessive HI. These mutations are present in 1.6–14.3% of hearing impaired subjects (Du *et al*. 2014, Pourova *et al*. 2010, Siem *et al*. 2010). Mutations in *SLC26A4* can cause a broad spectrum of phenotypes ranging from Pendred syndrome (OMIM 274600) to nonsyndromic HI associated with enlarged vestibular aqueduct (OMIM 600791). Pendred syndrome is an autosomal recessive disorder characterized by goiter and sensorineural HI with inner ear malformations. Goiter usually manifests in early puberty or adulthood. Thyroid phenotype varies; euthyroid goiter or hypothyroidism are common. The perchlorate discharge test may be positive (Ladsous *et al*. 2014). Inner ear

malformations in Pendred syndrome range from isolated enlarged vestibular aqueduct to Mondini dysplasia, a malformation in which, in addition to enlarged vestibular aqueduct, the normal cochlear spiral of 2.5 turns is replaced by a hypoplastic coil of 1.5 turns. The HI in Pendred syndrome is usually congenital, severe to profound, or fluctuating and progressive (Coyle *et al*. 1996, Coyle *et al*. 1998, Goldfeld *et al*. 2005, Phelps *et al*. 1998). The clinical phenotype of nonsyndromic HI caused by *SLC26A4* gene mutations is typically prelingual, bilateral, progressive and fluctuating affecting mainly high frequencies. Some subjects have a HI and temporal bone anomalies (Abe *et al*. 1999, Campbell *et al*. 2001, Li *et al*. 1998).

2.6.4 WFS1 gene

Mutations in *WFS1* gene can cause both syndromic and nonsyndromic HI. Autosomal recessive Wolfram syndrome (WS) (OMIM #222300) is a rare neurodegenerative disease characterized by childhood-onset diabetes mellitus, optic atrophy, diabetes insipidus and sensorineural HI (Inoue *et al*. 1998, Strom *et al*. 1998). The prevalence of WS has been estimated to be 1/770 000 in the UK and 1/100 000 in North America (Barrett *et al*. 1995, Fraser & Gunn 1977). On average, diabetes mellitus presents at the age of six years (3 weeks–16 years) and optic atrophy at the age of eleven years (6 weeks–19 years). By age 14, three fourths of patients present with partial diabetes insipidus (Rigoli *et al*. 2011). HI is a common feature in WS patients, and is seen in approximately 62–78% of cases (Kumar 2010, Marshall *et al*. 2013). Subjects with WS typically have a HI in late childhood, but HI can also be the first symptom of WS and some affected individuals have a congenital profound HI (Aloi *et al*. 2012, Hansen *et al*. 2005, Marshall *et al*. 2013). HI in WS is usually progressive and affects mainly high frequencies, although low frequencies may be affected, too (Barrett *et al*. 1995, Cryns *et al*. 2003a, Rigoli *et al*. 2011).

Mutations in *WFS1* gene may also cause autosomal dominant low-frequency sensorineural HI (DFNA6/14/38, OMIM #600965) (Bespalova *et al*. 2001, Young *et al*. 2001). In fact, *WFS1* gene mutations account for most of the familial low frequency sensorineural HIs (Bespalova *et al*. 2001, Cryns *et al*. 2002, Lesperance *et al*. 2003). Nonsyndromic HI associated with *WFS1* mutations typically affects low frequencies first, but may later affect mid and high frequencies. HI progresses slowly to be moderate or severe, and the age of onset varies from early childhood to young adulthood (Bom *et al*. 2002, Brodwolf *et al*.

2001, Kunst *et al*. 1999, Lesperance *et al*. 2003). Dominantly inherited *WFS1* mutations are also responsible for HI, diabetes mellitus, and optic atrophy phenotype (OMIM #614296) (Eiberg *et al*. 2006, Hogewind *et al*. 2010, Rendtorff *et al*. 2011).

WFS1 gene on 4p16.1 encodes the endoplasmic reticulum protein wolframin. The function of wolframin in the inner ear is currently unknown, but wolframin is widely expressed in different cochlear cell types. Wolframin is suggested to be a transmembrane protein involved in the regulation of inner ear ion homeostasis, as maintained by the canalicular reticulum (Cryns *et al*. 2003b, Osman *et al*. 2003). The *WFS1* gene contains eight exons, of which the first is a non-coding exon. The majority of *WFS1* mutations are located in exon 8. WS mutations are typically inactivating, whereas mutations causing nonsyndromic HI are non-inactivating. Over 140 different mutations have been identified in WS and low frequency HI (Rigoli *et al*. 2011).

2.7 Mitochondrial hearing impairment

Mutations in mtDNA can cause either nonsyndromic or syndromic HI. The HI phenotype is typically sensorineural and progressive, affecting predominantly high frequencies. The age at onset of HI occurs mainly in young adulthood, but prelingual HI also exists (Kokotas *et al*. 2007).

2.7.1 Mitochondrial DNA

Mitochondria are small cytoplasmic organelles that are most concentrated in tissues with high energy demand. Mitochondria contain their own genome (mitochondrial DNA, mtDNA), but they are also dependent on nuclear DNA for all functions. Mitochondrial genome is a circular molecule of 16, 569 base pair encoding 13 mitochondrial proteins, 22 types of transfer RNA (tRNA) and 2 types of ribosomal RNA (rRNA). Mutations may occur in mtDNA and lead to mitochondrial disease (Ruiz-Pesini *et al*. 2007, Mitomap database). MtDNA mutations can be large rearrangements or mutations limited to a few base pairs, the majority of which are point mutations. Large rearrangements in mtDNA usually occur sporadically and lead mainly to multisystem disorders. Nonsyndromic HI is commonly caused by point mutations which are maternally inherited (Dimauro & Davidzon 2005, Kokotas *et al*. 2007). To date (August 2014), numerous mtDNA variants have been associated with syndromic HI and

over 40 variants with nonsyndromic HI (Ruiz-Pesini *et al*. 2007, Mitomap database). Pathogenic variants have been reported in different populations and on different haplotype backgrounds (Herrnstadt & Howell 2004). Certain mtDNA haplogroups or polymorphisms are thought to modulate the risk of certain diseases, or modulate the phenotype, such as HI. In addition, mtDNA haplogroups have been shown to be associated with age-related HI and with hereditary HI (Kato *et al*. 2012, Lu *et al*. 2010b, Manwaring *et al*. 2007). Numerous autosomal genes contribute to the function of mitochondria (Schapira 2012).

2.7.2 Mitochondrial DNA mutations and hearing impairment

MtDNA mutations are infrequently the cause of early childhood HI, but are more common at later ages. Among European subjects with postlingual, nonsyndromic HI, causative mtDNA mutations have been found in approximately 5% of patients (Jacobs *et al*. 2005, Marazita *et al*. 1993). The frequency of mtDNA pathogenic mutations among Japanese adult patients with maternally inherited HI is estimated to be 14.7% (Yano *et al*. 2014).

Cochlear damage is the predominant mechanism of HI in mitochondrial diseases, but retrocochlear dysfunction has also been reported. HI in mitochondrial diseases is commonly sensorineural, symmetric, progressive, and initially affects higher frequencies. The grade of HI can vary from mild to severe (Chinnery *et al*. 2000, Scaglia *et al*. 2006, Sue *et al*. 1998). HI mainly appears in young adulthood, but prelingual HI also exists (Kokotas *et al*. 2007). Sensorineural HI, alone or in conjunction with other symptoms, is a common finding among patients with mutations in mtDNA (Chennupati *et al*. 2011, Zwirner & Wilichowski 2001).

2.7.3 Syndromic hearing impairment

HI is frequently seen as a clinical sign of systemic neuromuscular disorders caused by mtDNA mutations. The most well-known mtDNA mutation associated with multisystem disease and HI is the m.3243A>G mutation in the *MTTL1* gene encoding $tRNA^{Leu(UUR)}$. The prevalence of m.3243A>G mutation in Finland is reported to be 16.3–18.4/100 000 (Majamaa *et al*. 1998, Uusimaa *et al*. 2007). Classic clinical features of the syndrome of mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS, OMIM #540000) include childhood-onset neurological deficits, lactic acidosis, seizures, short stature,

myopathy, and normal early development (Pavlakis *et al*. 1984). In recent studies, a more variable phenotype has been recognized. HI is one of the most common features in subjects carrying m.3243A>G (Uusimaa *et al*. 2007).

Maternally inherited diabetes and deafness (MIDD) is also a phenotype caused by defects in mtDNA. Many mtDNA variants have been linked with this phenotype, including m.3243A>G (Murphy *et al*. 2008). Patients with mitochondrial multisystem disorders, such as Kearns-Sayre syndrome, NARP (neurogenic weakness, ataxia and retinitis pigmentosa) or MERFF syndrome (myoclonic epilepsy/mitochondrial encephalomyopathy and ragged red fibers), may also present with HI as a part of the syndrome (Kokotas *et al*. 2007).

2.7.4 Nonsyndromic hearing impairment

Two mitochondrial genes, *MT-TS1* encoding tRNA Ser and *MT-RNR1* encoding 12S rRNA are commonly associated with nonsyndromic HI. The most frequently reported mutations in these genes are m.1494C>T and m.1555A>G in *MT-RNR1* gene and m.7445A>G and m.7511T>C in *MT-TS1* gene (Ruiz-Pesini *et al*. 2007, Mitomap database). The pathogenicity in these mutations has been confirmed by functional studies (Levinger *et al*. 2001, Li *et al*. 2004c, Qian & Guan 2009, Xing *et al*. 2007, Zhao *et al*. 2005). These mutations have been reported among hearing impaired subjects in different populations and on different haplotype backgrounds. The phenotype of HI is usually nonsyndromic and progressive. The degree of HI varies from mild to severe and the age of onset varies from early childhood to adulthood. Some families with m.7445A>G are combined with palmoplantar keratoderma (Jacobs *et al*. 2005, Xing *et al*. 2007, Zheng *et al*. 2012). In addition to these four variants, many other mtDNA variants have been reported in association with nonsyndromic HI (Table 3). Some of these variants have also been found in control populations and the pathogenicity of them has remained elusive.

Table 3. Mitochondrial DNA mutations and variants reported in association with nonsyndromic hearing impairment according to Mitomap database.

Gene	RNA	Mutation	Mutation	Mutation
MT-TF	tRNA Phe	636A>G		
MT-RNR1	12S RNA	669T>C	961T>G	1291T>C
		735A>G	990T>C	1310C>T
		801A>G	1005T>C	1331A>G
		827A>G	1027A>G	1374A>G
		839A>G	1095T>C	1452T>C
		960 insC	1116A>G	1494C>T[1]
		960 delC	1180T>G	1517A>C
		961 delT	1192C>T	1537C>T
		961 insC(n)	1192C>A	1555A>G[1]
		961T>C	1226C>G	
MT-TW	tRNA Trp	5568A>G		
MT-TC	tRNA Cys	5780G>A		
MT-CO1		7443A>G	7444G>A	
MT-TS1 precursor	tRNA SerUCN precursor	7445A>C	7445A>T	7445A>G[1]
MT-TS1	tRNA SerUCN	7456A>G	7505A>C	7511T>C[1]
		7462C>T	7510T>C	
MT-TH	tRNA His	12201T>C		
MT-TS2	tRNA SerAGY	12236G>A		

[1] Pathogenicity confirmed, Mitomap (www.mitomap.org/MITOMAP)

MT-RNR1

The m.1555A>G mutation in the *MT-RNR1* gene is the first and the most common mtDNA mutation responsible for both aminoglycoside-induced and nonsyndromic HI (Prezant *et al*. 1993). The prevalence of m.1555A>G in the general population is 1/385 to 1/500 (Bitner-Glindzicz *et al*. 2009, Rahman *et al*. 2012, Vandebona *et al*. 2009). Among European patients with HI, the prevalence

of m.1555A>G is 0.4–2.6% (Kokotas *et al*. 2009, Kupka *et al*. 2002, Lehtonen *et al*. 2000). Higher prevalence of m.1555A>G mutation has been reported among Spanish and Asian patients (Estivill *et al*. 1998, Lu *et al*. 2010a, Malik *et al*. 2003).

The age of onset and severity of HI varies widely among subjects and families with m.1555A>G. Moreover, many mutation carriers have normal hearing (Bitner-Glindzicz *et al*. 2009, Rahman *et al*. 2012). The common phenotypic features of m.1555A>G include sensorineural, often progressive, and bilaterally symmetric HI with sloping audiogram configuration. In most cases the HI has been found postlingually, but prelingual HI has also been reported (Matsunaga *et al*. 2005, Usami *et al*. 1997).

The m.1494C>T mutation in *MT-RNR1* may also cause aminoglycoside-induced and nonsyndromic HI (Rodriguez-Ballesteros *et al*. 2006, Wang *et al*. 2006, Zhao *et al*. 2004). This mutation is located in the same conserved region of 12S rRNA as m.1555A>G (Zhao *et al*. 2005). The prevalence of the m.1494C>T mutation is lower than that of the m.1555A>G mutation (Kokotas *et al*. 2007, Lu *et al*. 2010a). The phenotype of HI is variable, but similar to that described in patients with m.1555A>G.

Aminoglycoside ototoxicity

The m.1555A>G and m.1494C>T mutations account for the majority of mtDNA mutations related to aminoglycoside ototoxicity cases. In addition to these, m.1095T>C and mutations at position 961 have been associated with aminoglycoside-induced deafness (Guan 2011). Aminoglycosides, such as streptomycin, tobramycin, netilmycin and gentamycin, are a group of antibiotics, which are in developed countries used mainly in hospitalized patients or in patients with tuberculosis. In developing countries, aminoglycosides are often used to treat common infections such as bronchitis and otitis media. Premature infants in NICUs are widely exposed to aminoglycosides. Given that preterm infants are more likely to have HI than full-term neonates, there is increasing concern surrounding how mtDNA mutations, administration of aminoglycosides and the noisy environment of a NICU may impact the auditory system, particularly during this sensitive period of auditory development (Zimmerman & Lahav 2013).

It has been estimated that 2–5% of patients treated with aminoglycosides develop a HI (Guan 2011, Moore *et al*. 1984, Rybak 1986). The HI may occur

anywhere from 3 days to 3 months after the administration of the aminoglycosides. After aminoglycoside exposure, the HI is usually severe or profound. Age at the time of drug administration has been reported to correlate with the severity of HI (Lu *et al*. 2010a, Zhao *et al*. 2004). Ototoxicity occurs in both dose-dependent and idiosyncratic fashion. Aminoglycosides concentrate in the perilymph and the endolymph of the inner ear. Aminoglycosides can affect both vestibular and auditory systems. Effects of aminoglycosides are usually irreversible, however the exact mechanisms are still unclear. The m.1555A>G mutation makes the human mitochondrial rRNA structurally more similar to the bacterial ribosomal RNA, which is the target of aminoglycoside antibiotics (Fischel-Ghodsian 2005, Qian & Guan 2009).

Modifying factors

Aminoglycoside exposure is the most common modifying factor for HI associated with 12S rRNA mutations. However, in the absence of aminoglycosides, the HI phenotype, age of onset and the penetrance of HI are widely variable, which indicates the involvement of other modifying factors in the phenotypic expression of m.1555A>G mutation carriers.

Phenotype differences may be due to an interaction between the mtDNA mutation and a variation in an autosomal gene. Linkage analysis has suggested a role for a locus on chromosome 8p23.1, but no modifier genes have been identified (Bykhovskaya *et al*. 2001, Finnilä & Majamaa 2003). More recently, the *TRMU* gene has been suggested to modify the expression of m.1555A>G (Guan *et al*. 2006, Yan *et al*. 2006). Other candidates for nuclear modifying genes have reported to be *TFB1M*, *MTO1* and *GTPBP3*. The protein products of these genes participate in 12S rRNA or tRNAs modification, which is important for mitochondrial protein synthesis (Luo *et al*. 2013).

In contrast to many mtDNA mutations, which are frequently heteroplasmic, the mtDNA point mutations causing nonsyndromic HI (including m.1555A>G) are primarily found in homoplasmy. Only some families have been reported to carry heteroplasmic mutations (Ballana *et al*. 2008, del Castillo *et al*. 2003, el-Schahawi *et al*. 1997, Zhu *et al*. 2014). The phenotype variability with m.1555A>G cannot be completely explained by the heteroplasmy level.

Previous reports suggest that mtDNA haplotypes or certain additional mtDNA mutations may modulate the HI phenotype of the m.1555A>G or

m.1494C>T mutations, but contrary reports exist (Li *et al*. 2004b, Lu *et al*. 2010b, Torroni *et al*. 2003).

2.8 Additional disabilities among hearing impaired children

Children with HI may have additional disabling conditions, either as part of a syndrome or a comorbid condition. About 30–40% of children with HI have been reported to have additional health or developmental problems which may affect their development and learning (Fortnum & Davis 1997, Fortnum *et al*. 2002, Holden-Pitt & Albertorio 1998, Van Naarden *et al*. 1999, Wiley *et al*. 2011). Additional disabilities are most commonly grouped under the following headings: 1) cognitive deficit or developmental delay or learning difficulties, 2) visual impairment, 3) motor impairment, 4) craniofacial anomalies 5) other anomalies or other systemic disorders. Most commonly, these disabilities are reported to be either intellectual or developmental (Birman *et al*. 2012, Chilosi *et al*. 2010, Van Naarden *et al*. 1999).

Hearing impaired children with additional disabilities are extremely heterogeneous, and the variability in both the severity of disability and the outcomes is broad. About half of all hearing impaired children with additional disabilities have more than one disability (Chilosi *et al*. 2010, Fortnum & Davis 1997, Nikolopoulos *et al*. 2008). The presence of additional disabilities is a significant predictor of outcome in children with HI (Ching *et al*. 2013, Cruz *et al*. 2012, Huttunen 2008). The total number of disabilities has been reported to have a strong correlation with the outcome (Nikolopoulos *et al*. 2008). Cognitive functioning has been reported to be a particularly strong predictor of outcome (Edwards 2007, Meinzen-Derr *et al*. 2010).

The frequency of additional disabilities is higher among cases with HI resulting from intrauterine infections and pre- or perinatal issues than among individuals with unknown or hereditary HIs (Chilosi *et al*. 2010, Fortnum & Davis 1997, Karltorp *et al*. 2014). The risk factors for acquired aetiology of HI, such as prenatal infections and perinatal problems, are also risk factors for developmental problems (Johnson *et al*. 2009, Lee *et al*. 2005, Robertson *et al*. 2009).

Most studies on additional disabilities in hearing impaired children have been focused on children with a cochlear implant or children with severe or profound HI (Beer *et al*. 2012, Birman *et al*. 2012, Chilosi *et al*. 2010, Edwards 2007). Only a few studies have included children with mild HI and additional

disabilities. Additional disabilities are important to consider because they appear to be as common among children with mild HI as those with at least moderate HI (Chilosi *et al*. 2010, Van Naarden *et al*. 1999, Wiley *et al*. 2011).

3 Aims of the study

The purpose of this study was to determine the prevalence and aetiology of childhood HI among a ten-year birth cohort in northern Finland, and to evaluate the frequency of additional disabilities among hearing impaired children. In cases where the aetiology of HI is unknown, molecular analysis of selected genes was carried out. Genes in mtDNA, as well as the *WFS1* gene, were examined primarily because mutations here may lead to a phenotype resembling a mitochondrial disease.

The specific aims of this study were:

1. To evaluate the prevalence and aetiology of HI among children in northern Finland and to analyse the frequency of additional disabilities among children with HI.
2. To determine the presence of mutations in *WFS1* among children with HI.
3. To determine the presence of mutations and variants in *MT-RNR1* and *MT-RNR2* genes among children with HI.
4. To describe the audiological phenotype and the progression of HI among children with the m.1555A>G mutation in mtDNA.

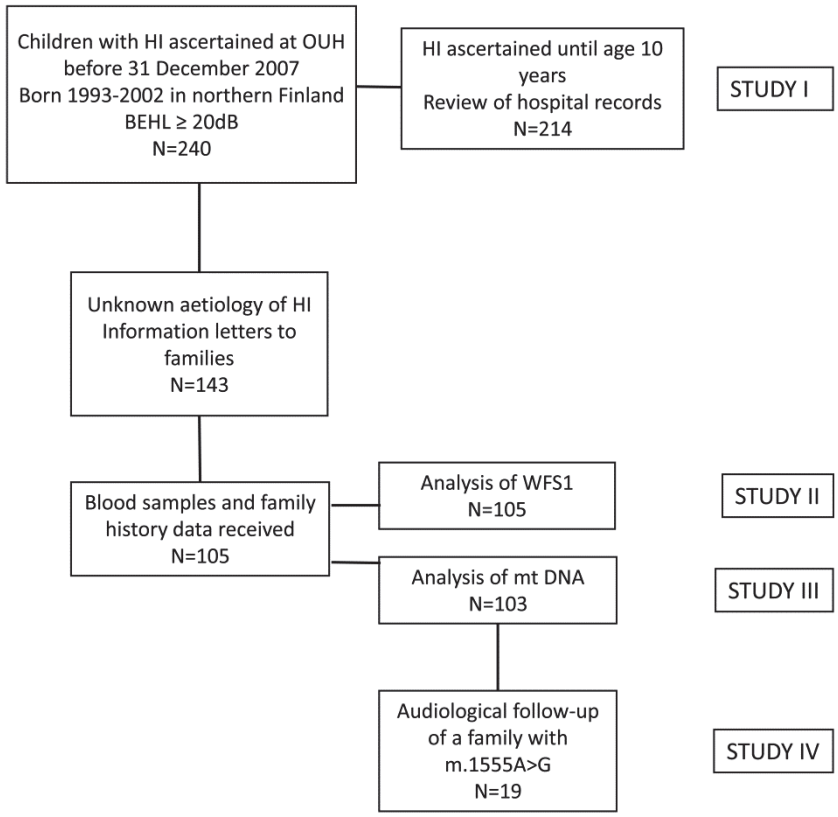

Fig. 1. Study profile.

4 Subjects and methods

4.1 Subjects

The subjects of this study were hearing impaired children born in Northern Finland during the ten year period from January 1st 1993 to December 31st 2002. The follow-up of the cohort was continued until 31st December 2007. Review of records at Oulu University Hospital (OUH) revealed 240 children with HI. Among the 240 hearing impaired children, the aetiology of HI for 143 children was unknown. Blood samples were obtained from 105 (Study II) or 103 (Study III) unrelated children with unknown aetiology of HI (Figure 1). Children under study here had a syndromic or nonsyndromic, bilateral, mild to profound sensorineural HI. The syndromic features of these patients are described in Study I. In studies II and III, 102 children were the same. All children with a nonsyndromic HI were negative for mutations in the coding exon of the *GJB2* gene. Study I included 214 children whose HI was ascertained before age 10 years. The uptake area of the OUH includes all of northern Finland, including the provinces of Northern Ostrobothnia and Lapland. OUH is the tertiary care hospital that serves this population by providing advanced medical treatment, including diagnostics of early childhood HIs and rehabilitation of children.

Control subjects included 285 blood donors in Study II and 99 blood donors in Study III. The donors, as well as their mothers, were required to be free of the common manifestations of mitochondrial diseases such as diabetes mellitus, HI and neurological ailments. The donors and their mothers had to be born in the province of Northern Ostrobothnia. The age of the donors was 41 ± 12 years (mean ± standard deviation; range 18–64 years) indicating with relative certainty that all control subjects had received a hearing screening at school and in the case of men, at the entry of military service.

The subjects of Study IV consisted of a large Finnish pedigree with the m.1555A>G mutation. In this pedigree, three sisters had non-progressive HI affecting high frequencies only. Together, these three women had 19 children (9 girls and 10 boys) who were recruited into the study in 2003 or at birth (IV: Figure 1). All children and their mothers carried the m.1555A>G mutation. None of the children had been exposed to aminoglycosides or other ototoxic medications. The three women had normal pregnancies and deliveries that were without any complications. Thirteen children underwent and passed a universal

NHS using transient otoacoustic emissions (TEOAEs). The growth and early development of these nineteen children were normal. None of the children had a history of noise exposure.

The study protocol was approved by the Ethics Committee, OUH (I–IV), and the Finnish Red Cross (II–III). Written informed consent was obtained from all patients or their parents.

4.2 Hearing measurements

To exclude possible secretory otitis media, pneumatic otoscopy, otomicroscopy and/or tympanometry were performed. Possible secretory otitis media was treated before hearing assessment. Hearing of infants was examined using TEOAEs (EZ SCREEN, ILO V6, Otodynamics, Hatfield, United Kingdom) and sound field audiometry. The click-evoked or frequency-specific auditory brainstem responses (CHARTR EP, GN Otometrics, Taastrup, Denmark) were recorded when needed. Chloral hydrate was used to sedate infants and young children during auditory brainstem response testing. Pure tone audiometry, using an AURICAL PLUS audiometer (GN Otometrics, Taastrup, Denmark), was performed as early as the child was co-operative. Air-conduction pure tone thresholds were measured at 0.125, 0.25, 0.5, 1, 2, 3, 4, 6 and 8 kHz if possible. Audiology assistants who were trained to examine children performed the measurements in a sound-insulated cabin.

4.3 Definition of hearing impairment

Data from the 214 children (Study I) were collected retrospectively from the electronic patient records of the OUH. The age of ascertainment, the risk factors for, and the aetiology of HI were recorded. Information on clinical examinations, including the first and the most recent audiogram as well as co-occuring disabilities, was recorded. The degree and the type of HI and the audiogram configuration were determined on the basis of the most recent audiogram. According to definitions recommended by the EU expert group (Stephens & Martini 1996), the degree of HI was based on average thresholds calculated over the frequencies 0.5, 1, 2 and 4 kHz in the better hearing ear. The degree of HI according to BEHL was classified as mild (21–39 dB), moderate (40–69 dB), severe (70–94 dB), and profound ($\geqslant$95 dB).

If no hearing could be measured at a given frequency, the value of 130 dB was used in calculations per the recommendation of the British Society of Audiology (1988). The first reliable audiological examination at the Department of Audio-Phoniatrics of the OUH was considered to be the time of ascertainment of HI, as well as the basis of prevalence figures. Reliable audiograms were not obtained for 16 children. In these cases, hearing level was evaluated on the basis of sound field audiometry and auditory brainstem responses. The type or symmetry of HI could not be determined in some children because exact thresholds could not be measured. Audiogram configurations were calculated based on air conduction thresholds, as recommended by the EU expert group (Stephens & Martini 1996), with the exception of the less stringent definition for the flat configuration (i.e. < 15 dB difference between the mean of 0.25 and 0.5 kHz, the mean of 1 kHz and 2 kHz, and the mean of 4 kHz and 8 kHz). If the threshold for 8 kHz was not available, classification was based on the 4 kHz threshold only. The less stringent definition was chosen because previous research indicates that the more stringent definition of flat audiograms leads to a high proportion of unclassified audiograms (Sorri *et al*. 2000). Deterioration of at least 15 dB in the pure tone average calculated over the frequencies 0.5, 1, 2 and 4 kHz within a 10 year period was used to indicate progressive HI. The HI was defined as asymmetric if the difference between thresholds was more than 10 dB in at least two adjacent frequencies.

4.4 Definitions of aetiology and additional disabilities (I)

The HI was classified as being hereditary in aetiology if either a genetic cause was identified in the course of clinical diagnostics or there was a positive family history. A permanent HI ascertained in childhood or young adulthood in at least one first-degree relative was indicative of a positive family history. The HI was classified as acquired in cases where a known pre- or postnatal cause was identified or three or more perinatal risk factors could be discovered and the family history was negative. Perinatal risk factors included gestational age $\leqslant$ 32 weeks, birth weight <1500g, exposure to ototoxic medication, assisted ventilation for more than 5 days, hyperbilirubinemia requiring exchange transfusion, or Apgar score $\leqslant$ 6 at 5 minutes. The aetiology of HI was classified as unknown if none of the above-mentioned aetiologic factors could be identified. An identified cause was given priority over the presence of risk factors or a positive family history. The HI was classified as syndromic or presumably syndromic if other

remarkable clinical features were found in addition to HI. Clinical features included malformations of the ear or malformations or dysfunction of other organs. Having a significant developmental delay associated with HI was classified as a syndromic or presumably syndromic HI. The HI was classified as nonsyndromic if there were no anomalies or comorbid conditions associated with HI.

Subjects were classified as having an additional disability if they had previously received a diagnosis of significant developmental disorder (i.e. an intellectual disability, global developmental delay, vision or motor impairment, epilepsy or neuropsychiatric disorder). Minor anomalies were defined as conditions where clinical evidence showed no adverse influences on development or where no extra rehabilitation was required.

4.5 Molecular methods and data analysis (II–IV)

4.5.1 DNA extraction and polymerase chain reaction

A blood sample was obtained from children with an ascertained HI and genomic DNA was extracted using the QIAamp Blood Kit (Qiagen, Hilden, Germany). The primer pairs used for the polymerase chain reaction were designed to cover the coding exons of the genes or previously published primers were used. The template DNA was amplified by polymerase chain reaction under specific conditions and the amplified fragments were used for sequencing, restriction fragment analysis or conformation-sensitive gel electrophoresis (CSGE). More detailed descriptions can be found in the original Studies II–IV.

4.5.2 Analysis of sequences and sequence variants

Screening for mutations and polymorphisms in the *MT-RNR1* and *MT-RNR2* genes (Study III) was performed using CSGE (Finnilä *et al*. 2000). For CSGE, 12 pairs of primers were used to amplify the mtDNA fragments by polymerase chain reaction. Samples were electrophoresed through a polyacrylamide gel, visualized with an UV transluminator, and photographed. Fragments were sequenced if they differed in motility in CSGE. In addition, exon 8 in *WFS1* gene (Study II), *MT-TS1* gene and D-loop (Study III), were sequenced. Sequencing was carried out using the BigDye Terminator v1.1 Cycle Sequencing Kit and the ABI PRISM

3130xl Genetic Analyzer (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, U.S.A.). The primers used for sequencing can be found in papers II–III. Mutations m.1555A>G and m.3243A>G were detected by restriction fragment analysis (Study III–IV) and seven common alleles in *POLG1* were screened by restriction fragment analysis or by allele-specific amplification (Study IV).

4.5.3 Haplogroup analysis

A phylogenetic network, based on the D-loop sequences, was constructed in Study III by using a reduced-median algorithm (Bandelt *et al*. 1995). MtDNA haplogroups were identified on the basis of informative variants (Finnilä *et al*. 2001). The frequencies of mtDNA haplogroups among children with HI and among the control subjects were compared using an exact test of population differentiation as implemented in Arlequin version 3.5.1.3. (Excoffier & Lischer 2010).

4.5.4 Data analysis

In Study II, variants in *WFS1* gene were annotated according to the recommendations of the Human Genome Variation Society (www.hgvs.org). The annotations were based on the reference sequences NM_006005.3 and NP_005996.2 (GeneID 7466, GenBank). With the exception of p.Glu776Val and p.Gly831Ser, the frequencies of the various *WFS1* alleles in control subjects have been reported elsewhere (Kytövuori *et al*. 2013). Comparisons of the allele frequencies and deviations from Hardy-Weinberg equilibrium were calculated using Arlequin version 3.5.1.3. (Excoffier & Lischer 2010).

Pathogenicity of the variants in *WFS1* gene was predicted by using four algorithms, including PolyPhen-2 (Adzhubei *et al*. 2010), SIFT (Ng & Henikoff 2001), SNAP (Bromberg & Rost 2007), and MutationTaster (Schwarz *et al*. 2010). These algorithms predict whether a variant will have a pathogenic or neutral effect in a protein. The predictions are based on information about the conservation of the protein sequence, sequence divergence, and the physical properties of amino acids.

In Study III, mtDNA variants were identified by comparing the obtained sequences with the revised Cambridge reference sequence. The Mitomap database and the Human Mitochondrial database (HmtDB) (Attimonelli *et al*. 2005,

Rubino *et al*. 2012), were used to evaluate variant frequencies and to compare frequencies in different haplogroups. A variant was defined as being rare if its frequency was ⩽ 0.2% in either of the databases. Variants with frequencies > 0.2% were regarded as common polymorphisms (Wang *et al*. 2012). The conservation of the nucleotides at variant sites located in the *MT-RNR-1* and *MT-RNR-2* genes was assessed. The alignments were performed using the Clustal Omega-Multiple Sequence Alignment tool (http://www.ebi.ac.uk/Tools/msa/clustalo/). For this alignment, the species and reference sequences were chosen according to the proposed consensus panel of 12 organisms (Yarham *et al*. 2012).

4.6 Audiological examination and follow-up (IV)

The hearing of the 19 children with the m.1555A>G mutation was examined prospectively. The follow-up period extended from September 2003 to May 2011. The total follow-up time was 98.9 years (range 2.1–7.8 years). With the exception of one child (IV: Figure 1, subject IV-2 in family B), hearing turned out to be normal at the first examination. Thus, it was possible to pinpoint the onset of HI, as well as follow its possible progression throughout childhood.

Parents of the 19 children completed a questionnaire pertaining to their child's previous medical care and clinical examinations. In addition, the medical records of these children were reviewed. All children underwent an annual otorhinolaryngological examination and, in ten cases, hearing was followed since birth. Tympanometry was performed if necessary. If the hearing thresholds were normal at the age of eight years, follow-ups were discontinued. Penetrance of HI was calculated as the proportion of affected matrilinear relatives out of all matrilinear relatives. The rate of the progression of the HI was calculated as the change in $BEHL_{(0.5\text{-}4kHz)}$ during the entire follow-up time. Hearing was considered to be impaired if the threshold in at least one frequency exceeded 20 dB.

4.7 Statistical methods

Statistical analyses were done using IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp. Released 2011. Armonk, NY). The Chi-Squared test was applied for the prevalence between additional disabilities and either the degree of HI or different aetiologies (significance level $p<0.05$). Confidence intervals (95% CI) were used when appropriate.

5 Results

5.1 Hearing impairment (I)

During the 10-year period under study here, 91,835 children were born in northern Finland. A permanent HI was ascertained in 214 children (117 boys, 55%) by the age of ten years. The prevalence of any bilateral childhood HI was 2.3 per 1000 live births (95% CI; 2.0, 2.7). The prevalence of at least moderate HI was 1.1/1000 live births (95% CI; 0.92, 1.4). The mean age at the time of ascertainment of mild HI was 5.9 years, while that of moderate HI and severe or profound HI was 3.7 years and 1.5 years, respectively.

Among study participants, HI was sensorineural in 71.0% (n=152), conductive in 12.1% (n=26) and mixed in 4.7% (n=10). For 26 participants (12.2%), their HI was unclassified or central type. HI was symmetric in 62.6% of participants (n=134) and asymmetric in 29.0% of participants (n=62). Eighteen participants (8.4%) could not be classified as either symmetric or asymmetric because reliable audiometric testing could not be performed. HI was nonsyndromic in 53.3% of children (n=114) and syndromic or presumably syndromic in the remaining children. The majority of children had a mild or moderate HI (Table 4).

Table 4. Type and degree of hearing impairment among 198 children. Figures for right (R) and left (L) ear are shown separately.

Degree	Sensorineural	Conductive	Mixed	Not Determined[1]	All
	R/L	R/L	R/L	R/L	N (%)
Mild	67/66	18/19	4/4	6/6	190 (48.0)
Moderate	57/58	6/7	5/3	4/4	144 (36.4)
Severe	9/9	1/1	2/2	0/0	24 (6.0)
Profound	19/19	0/0	0/0	0/0	38 (9.6)
All	152/152	25/27	11/9	10/10	396 (100.0)

[1] reliable audiometric testing could not be performed

At minimum, two audiograms were obtained from 182 children. The mean time between the first and the most recent audiogram was 3.6 years (median 3.5). Fifteen children presented with a progressive HI during a median follow-up of 4.9 years. Among these children, one had USH, one had the m.1555A>G mutation in mtDNA, and two had received cisplatin treatment for a malignant disease. The aetiology of HI could not be determined in the remaining 11 children.

Configurations could be determined for 375 audiograms (I: Table 2). The most common configuration was high-frequency gently sloping, which accounted for 24% of the audiograms. Ninety-four audiograms (25%) remained unclassified, but according to clinical judgement, 45 were high-frequency gently or steeply sloping, 21 were flat, five were low-frequency ascending and 23 were mid-frequency U-shaped.

5.2 Aetiology (I)

The aetiology of HI among the 214 hearing impaired children was considered to be genetic in 101 children (47.2%), acquired in 35 (16.4%) and unknown in 78 (36.4%). For the 114 children (53.3%) with nonsyndromic HI, the most common cause was a mutation in *GJB2* (Table 5). Sequencing of *GJB2* was carried out in 123 children (57.5%), including all children with nonsyndromic HI. Homozygous c.35delG was the most prevalent mutation in the *GJB2* gene and was found in 11 children. In addition, the homozygous p.M34T mutation was found in four children, while three were compound heterozygotes with respect to p.([M34T];[V37I]) and one with respect to p.([M34T];[R143W]). In total, pathogenic *GJB2* mutations were found in 19 children which accounted for 16.7% of nonsyndromic HIs. For 100 children (46.7%) with syndromic or presumably syndromic HI, chromosomal aberration was the most common cause. Twenty children had a combination of three or more perinatal risk factors. Detailed aetiologies are shown in Table 6.

Table 5. Aetiology of nonsyndromic hearing impairment among 114 children born in northern Finland.

Aetiology	Aetiology	N	%
Genetic		57	50.0
Autosomal recessive	GJB2 mutation	19	
	Positive family history pattern	11	
Autosomal dominant	Positive family history pattern	13	
Maternal	m.1555A>G mutation	2	
Nonspecified	Positive family history	12	
Acquired		11	9.6
Perinatal	≥ 3 perinatal risk factors, negative family history	8	
Postnatal	Meningitis	1	
	Chemotherapy	2	
Unknown	Negative family history, ≤ 2 perinatal risk factors	46	40.4
Total		114	100.0

Table 6. Aetiology of syndromic or presumably syndromic hearing impairment among 100 children born in northern Finland.

Aetiology	N	%
Genetic	44	44.0
Autosomal recessive		
Usher	5	
IOSCA [1]	1	
Segregation pattern	4	
Autosomal dominant		
Treacher Collins	2	
BOR	1	
Leopard syndrome	1	
Segregation pattern	2	
Other syndromic / hereditary unclassified	8	
22q11.2-deletion syndrome	1	
CHARGE syndrome [2]	1	
Oculo-auriculo-vertebral spectrum [3]	5	
Landau-Kleffner	1	
Chromosomal		
Down syndrome	5	
Turner syndrome	2	
Other chromosomal abnormalities	5	
Acquired	24	24.0
Prenatal infections		
Cytomegalovirus	3	
Toxoplasma	1	
Perinatal risk factors ≥3 ,	20	
negative family history		
Unknown aetiology	32	32.0
Negative family history,		
≤ 2 perinatal risk factors		
Total	100	100.0

[1] IOSCA, infantile-onset spinocerebellar ataxia

[2] CHARGE, coloboma, heart, atresia choanae, retarded growth and development, ear anomaly/deafness

[3] includes Goldenhar syndrome and craniofacial microsomia

5.3 Additional disabilities (I)

Among the 214 children with HI, 101 (47.2%) had other minor or major disabilities (Table 7). Seventeen children (7.9%) had minor anomalies that did not affect their developmental or learning skills, while 84 children (39.3%) had

one or more additional disabilities with the potential to adversely affect their development or learning. An intellectual disability was verified in 36 children (16.8%). Fifty-three children with additional disabilities had more than one disability. The frequency of additional disabilities was 35.8% in children with mild HI and 32.8% in children with moderate or severe HI (p = 0.78; Chi-Squared test) (I: Table 5). In addition, 23/35 children (65.7%) with acquired HI had additional disabilities, whereas 78/179 children (43.6%) with genetic or unknown aetiology had additional disabilities (p = 0.035; Chi-Squared test).

Table 7. The frequency of additional disabilities in 101 hearing impaired children from northern Finland. The percentages are shown from the total sample of 214 children.

Type of disability	N	%
Intellectual disability	36	16.8
Developmental delay, neurocognitive conditions[1]	21	9.8
Motor impairment	23	10.8
Visual deficits	22	10.3
Craniofacial anomalies	24	11.2
Other anomalies	16	7.5

[1]Neurocognitive conditions include epilepsy, attention deficit/hyperactivity disorder, and autism spectrum disorders

5.4 Role of *WFS1* in childhood hearing impairment (II)

Sequencing of the last coding exon of *WFS1* revealed eight synonymous and nine nonsynonymous variants among 105 hearing impaired children. The nonsynonymous variants included five common polymorphisms and four rare variants including p.Gly831Ser (c.2491G>A), a novel nonsynonymous variant. The allele frequency of the common polymorphism p.[His456] (c.1367G>A) was 1.9-fold higher among children with HI than among control subjects. No significant differences were found in the allele frequencies of the variants between the cases and controls (II: Table 1).

In order to examine the pathogenicity of the four rare variants, audiological examination of the family members and mutation segregation analysis was carried out (II: Table 2) and Figure 2. In addition, the remaining coding exons of the probands were sequenced in order to exclude WS. The p.Glu385Lys (c.1153G>A) variant was detected in families A and B. After exclusion of the probands, none of the five siblings with p.Glu385Lys had HI. Among the five siblings with homozygous reference allele, one subject had HI. This finding

indicates that p.Glu385Lys does not segregate with HI. Non-segregation was also observed in family C which harboured variant p.Glu776Val (c.2327A>T). In family E, three siblings with p.Gly674Arg (c.2020G>A) had a moderate nonsyndromic HI and three siblings with homozygous reference allele had normal hearing. However, two siblings and their father also harboured p.Gly674Arg but had normal hearing. Analysis of all exons of *WFS1* from the mother did not suggest compound heterozygosity among the two discordant siblings.

In family D, the proband harboured p.Gly831Ser. She had a profound sensorineural HI with measurable thresholds only at the lowest frequencies. Her HI was ascertained at the age of 1.6 years and she received a cochlear implant at the age of 3.5 years. In addition, she had growth hormone deficiency and exceptionally poor language development, but was otherwise healthy. Her mother and three siblings had normal hearing, while her father had a left-sided 30-dB dip at 3 kHz and 35-dB dip at 4 kHz. None of her family members had the p.Gly831Ser variant which suggests that it arose de novo and is possibly pathogenic.

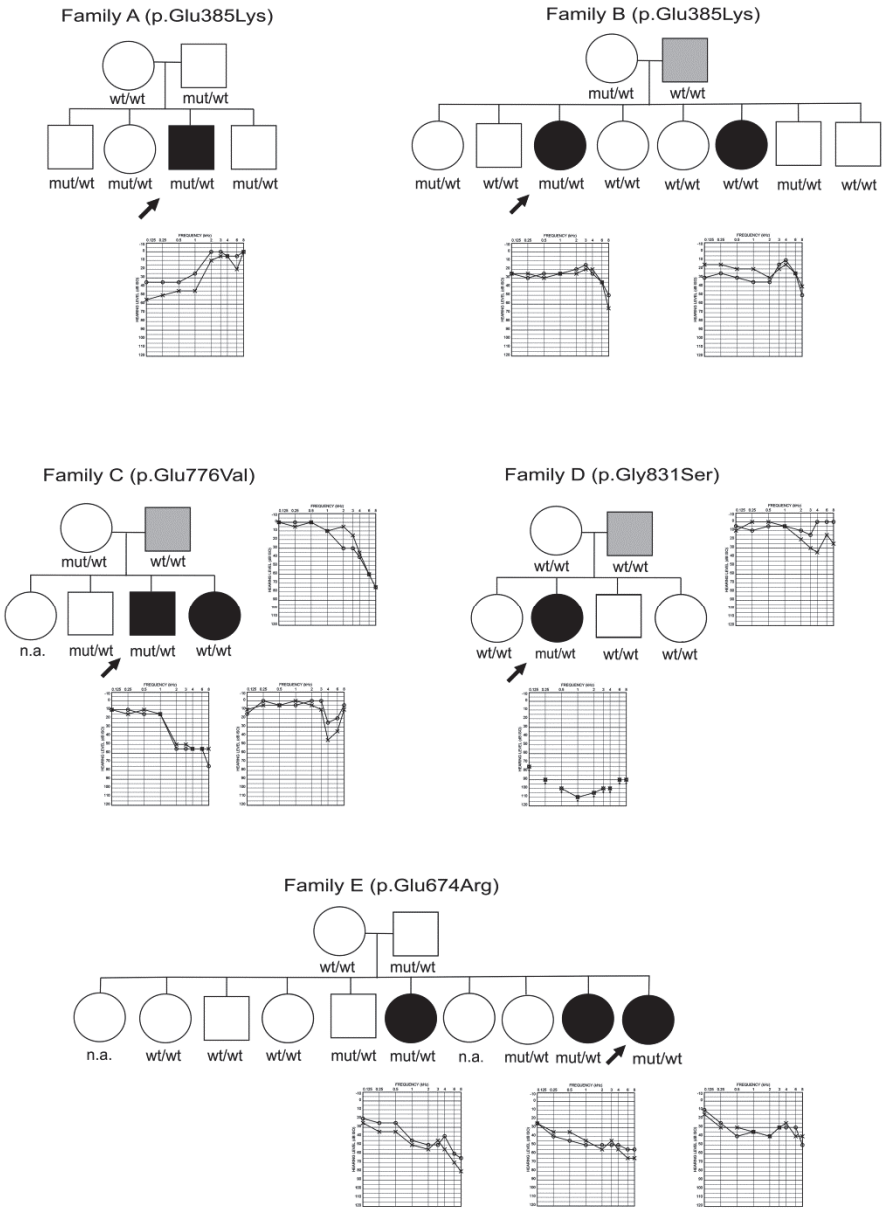

Fig. 2. Rare WFS1 variants and audiological phenotypes in five pedigrees. White symbol, normal hearing; black symbol, hearing impairment; grey symbol, one-sided hearing impairment; mut, mutation; wt, wild type variant; n.a., not applicable. Arrow indicates the proband.

5.5 MtDNA and childhood hearing impairment (III)

5.5.1 Haplogroup analysis

In Study III, D-loop sequences were used to infer mtDNA haplogroups and haplotypes in the 103 children with HI. The frequencies of mtDNA haplogroups was not different from those in the general population of the province of Northern Ostrobothnia ($p = 0.78$, exact test of population differentiation). The 103 children belonged to 66 haplotypes, 24 of which were present in the phylogenetic network of Finnish mtDNA (Finnilä *et al*. 2001). The remaining 42 haplotypes differed from the nearest neighbour by at least one substitution (III: Figure 1).

5.5.2 Mutations and variants in mtDNA

Among the 103 children whose aetiology of HI was unknown, one child harboured m.1555A>G mutation in *MT-RNR1* gene. All children were negative for m.3243A>G and mutations in the *MT-TS1* gene. The child with m.1555A>G had a sensorineural HI ascertained at the age 4.8 years. By the age of 10.2 years her HI was considered to be severe. She received a cochlear implant at the age of 11.3 years. Her mother, along with five of her eight siblings had a HI. In total, four children born in Northern Finland between the years 1993–2002 were ascertained with a HI and m.1555A>G.

Analysis of *MT-RNR1* and *MT-RNR2* sequences revealed 21 variants in addition to m.1555A>G. A variant was considered to be rare if its frequency was $\leqslant$0.2% in the Mitomap or HmtDB. Variants with frequencies >0.2% were regarded as common polymorphisms (Wang *et al*. 2012). Consequently, we identified eight rare variants and 13 polymorphisms. Among the 13 polymorphisms, five were at a frequency greater than 5%, four were at a frequency 1–5% and four were at a frequency >0.2% and <1% (III: Table 1).

The eight rare variants were present among the sequences in Mitomap, HmtDB or our own files. Comparison of the sequences indicated that m.740G>A, m.896A>G, m.1341C>T, and m.2405C-CC were strictly haplogroup-specific (Table 8). The remaining four rare variants (m.958C>T, m.990T>C, m.2098G>A, and m.2445T>C) have been found in various haplogroups. The positions m.958 and m.2445 were not evolutionary conserved (III: Table 3). Children harbouring these variants belonged to haplogroups U5b and U5a, which have previously been assigned to these variants. The position m.2098 was rather conserved. However,

the child with m.2098G>A belonged to haplogroup H1, which was also the case in 29/33 sequences in the databases. This finding suggests that m.2098G>A is a haplogroup H1 associated variant. Finally, the m.990T>C variant occurred in subhaplogroup V2 and the position m.990 was rather highly conserved (III: Table 3).

Table 8. Clinical features of nine hearing impaired children with rare variants in *MT-RNR1* and *MT-RNR2*.

Variant	Sex	Degree of SNHI	Other symptoms	Age at diagnosis (years)	Family history	Haplogroup	Haplogroup in HmtDB or Mitomap
740G>A	Girl	Severe	Nonsyndromic	5	Negative	Z1a	Z1a
740G>A	Boy	Moderate	Intellectual disability Short stature Renal dysplasia	10	Negative	Z1a	Z1a
896A>G	Girl	Mild		8	Negative	U5b	U5b
958C>T			Intellectual disability Cleft palate Congenital hypothyreosis				U5b,M5a,M5b,M7d
2445T>C							U5a,U5b,D1,H1,L2
990T>C	Girl	Profound[1]	Nonsyndromic	5	Negative	V2	L3,D4,V2,H1,H3,H4
1341C>T	Girl	Mild	Nonsyndromic	11	Negative	U5b	U5b
2098G>A	Girl	Severe	Intellectual disability Hydrocephalus Spastic triplegia	3	Negative	H1	H1,K2,J2
2405c-cc	Boy	Mild	Nonsyndromic	7	Negative	U4d	U4d
2405c-cc	Girl	Moderate	Nonsyndromic	5	Positive dominant	U4d	U4d
2445T>C	Boy	Severe	Nonsyndromic	3	Negative	U5a	U5a,U5b,D1,H1,L2

SNHI sensorineural hearing impairment, [1] mild conductive HI on one side

5.6 Audiological follow-up of children with m.1555A>G (IV)

The hearing of 19 children in three nuclear families of a pedigree with a m.1555A>G mutation was prospectively followed across a period of 7.8 years. At the end of the follow-up, ten children had HI and nine children had normal hearing. Three children had a HI at high frequencies only, six had a moderate, progressive HI and one had a severe, progressive HI (IV: Table 1). The median age at first examination was 0.3 years (range, 0.0–6.0 years) and ten children were audiologically followed since their birth. The mean follow-up time was 5.2 years. The median age of onset of HI was 3.7 years (range, 1.6–5.4 years). At the end of the follow-up period, the median age of the children was 6.5 years (range, 2.1–13.2 years). Based on at least two audiograms completed during follow-up, the change in $BEHL_{0.5\text{-}4kHz}$ varied from 0.0 dB to 12.4 dB per year (median 3.7 dB) among the eight children with HI. There were no differences between the children in terms of environmental factors such as viral infections or exposure to antibiotics.

All children and their mothers carried the m.1555A>G mutation, yet the fathers did not. Screening of the *POLG1* gene revealed a heterozygous p.R722H allele in five subjects in family B including the father and four children. Two of these children had normal hearing and two had a moderate, progressive HI.

There were three girls and three boys in family A (ages, 3.5–9.3 years) at the completion of follow-up. Two girls had normal hearing. One girl and one boy had a HI that appeared to be nonprogressive during the follow-up period, with a steeply sloping audiogram configuration. The configuration was quite similar to those of the three mothers in the third generation. The second boy had a progressive HI and the third boy had a recent ascertainment of HI, which at present is of undefined type. For these four children, their HI was ascertained between the ages of 2.3 and 5.4 years (IV: Table 1) and Figure 3.

In family B, there were two girls and seven boys (ages, 2.1–13.2 years) at the completion of follow-up. Three children had normal hearing, while four had a HI that affected mainly high frequencies but extended to other frequencies, too (IV: Table 1) and Figure 3. For these children, their HI was ascertained between the ages of 3.0 and 5.2 years. All of the children were fitted with hearing aids. The first child (IV-2) already exhibited a moderate HI at her first examination (age 4.8 years). Her HI progressed to severe during the follow-up period and she received a cochlear implant at the age of 11.3 years. The youngest son (IV-9) in family B

was considered to have a moderate or severe HI when he was 1.6 years old. His TEOAEs were normal at the age of two months, but were absent five months later. However, auditory brainstem responses (click-evoked, 1 kHz, 2 kHz, 4 kHz) could be recorded at 70-80 dB for the right ear and at 60 dB for the left. All of the four girls in family C had normal hearing.

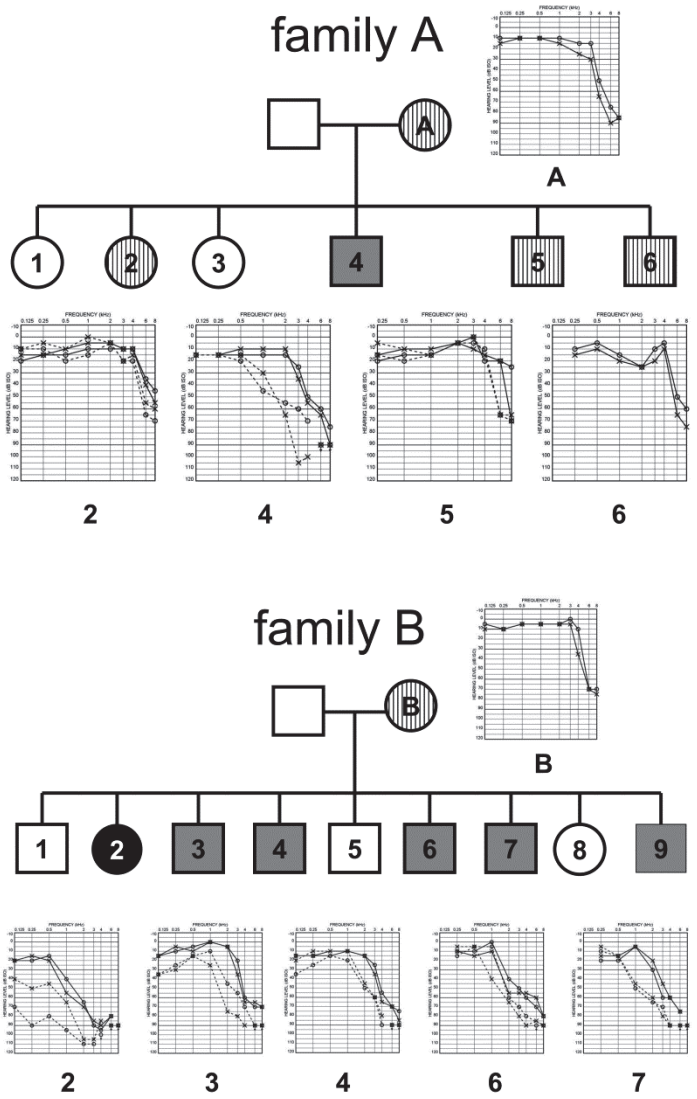

Fig. 3. Audiograms of family A and family B. The audiogram in the upper row, mother belonging to generation III; audiograms in the lower row, the children in generation IV. The estimated hearing thresholds of child 9 in family B are based on auditory brainstem responses and sound field audiometry. White symbol, normal hearing; striped symbol, hearing impairment (HI) at high frequencies; grey symbol, progressive HI; black symbol, severe HI.

6 Discussion

6.1 Hearing impairment (I)

The prevalence of all childhood HI in the present study was slightly greater than 2/1000 children. The prevalence of at least moderate HI was slightly more than 1/1000 children. Both of these figures are in line with prevalence estimates from previous studies in Finland (Dietz *et al*. 2009, Mäki-Torkko *et al*. 1998). Although the prevalence of congenital and early childhood HI is low, the consequences of this impairment are important because childhood HI may significantly influence speech and language development (Kennedy *et al*. 2006, Pimperton & Kennedy 2012).

Early detection and rehabilitation of HI improve outcomes pertaining to speech and language development among hearing impaired children (American Academy of Pediatrics, Joint Committee on Infant Hearing 2007). In this study, the mean age at ascertainment of at least moderate HI was 3.0 years. For severe or profound HI, the mean age at HI ascertainment was 1.5 years. Unfortunately, results from the three consecutive 10-year periods did not indicate any decrease in the ascertainment age. It has been shown (Mehl & Thomson 2002) that the only effective means to detect congenital HIs is a universal NHS, which was implemented in OUH in 2002, and in other hospitals in northern Finland from 2002–2004. The influence of NHS and the decrease in the ascertainment age may be evident in later cohorts.

In the current cohort, approximately 40% of the audiograms from hearing impaired children were classified as high-frequency sloping configuration. HI was asymmetric in one third of children. These findings are similar to previous studies, where children have been shown to have substantially different audiogram configurations than adults, whose audiograms are more often (50–90%) high-frequency sloping (Hannula *et al*. 2011, Pittman & Stelmachowicz 2003). Asymmetric HIs have also been reported to be more common and the degree of asymmetry more extensive among children than adults (Pittman & Stelmachowicz 2003). These special features of the HIs need to be considered when fitting hearing aids and when deciding on uni– or bilateral cochlear implantation for children.

6.2 Non-genetic causes of childhood hearing impairment (I)

The causes of childhood HIs are more diverse than those for adults. The aetiology of childhood HI is thought to be genetic in 30–50% of cases, acquired in 14–30% of cases and unknown in about 40% of cases (Korver *et al*. 2011, Morton & Nance 2006). Universal immunization programs and improved antibiotic treatments (e.g., for meningitis) have decreased post infectious HIs. In the present study, the aetiology of HI was defined to be acquired in 16% of children, which is in line with recent findings (Korver *et al*. 2011). Improvements in neonatal intensive care have increased the rate of survival for premature and very premature newborns. About 13% of hearing impaired children had three or more perinatal risk factors and negative family history. In a review of recent studies, asphyxia and prematurity were found to be more common aetiologies of childhood HI than in past years, which may be due to the increased survival of premature and very premature newborns (Morzaria *et al*. 2004).

Only two children with intrauterine CMV infection were found in this sample. In recent studies, CMV infection has been found to be the second most frequent cause of early childhood HI in developed countries after *GJB2* mutations (Avettand-Fenoel *et al*. 2013, Karltorp *et al*. 2012). The prevalence of CMV infections in this study may be underestimated because of non-diagnosed cases. It is possible that at least some of the unknown cases were caused by CMV infection. In the future, more attention should be paid to diagnosing congenital CMV infections and determining which cases require medical treatment.

6.3 Genetic causes of childhood hearing impairment

In the present study, 47% of childhood HIs were genetic, which is slightly more than the 30% reported in recent reviews (Korver *et al*. 2011, Morzaria *et al*. 2004). Studies on molecular genetics of HI have shown that the molecular aetiology of childhood HI is diverse. With the exception of *GJB2*, no major HI genes have been identified. In this study, *GJB2* gene sequencing was carried out in 123 children, including all children with nonsyndromic HI. A pathogenic mutation in *GJB2* was found in 19 children, resulting in a similar frequency to that reported one decade ago in the same area (Löppönen *et al*. 2003).

6.3.1 WFS1 gene in childhood hearing impairment (II)

In Study II, the presence of mutations in exon 8 in *WFS1* gene were determined. Among children with HI, nine nonsynonymous variants, five common polymorphisms and four rare variants including p.Gly831Ser (c.2491G>A), a novel nonsynonymous variant, were identified. Mutation segregation and prediction analysis, as well as audiological examination of the family members were done. The p.Glu385Lys and p.Glu776Val variants were not associated with HI. The p.Gly674Arg variant in family E did not segregate with HI. Recently, p.Gly674Arg has been reported in trans with p.Val540del in a WS patient (Aloi *et al*. 2012) and in trans with p.Pro504Leu in another WS patient. However, an unaffected daughter in one family was homozygous for p.Gly674Arg suggesting that this variant is a polymorphism (Gomez-Zaera *et al*. 2001). Interestingly, two other variants at this position, p.Gly674Glu and p.Gly674Val, have been reported in two Dutch families with low-frequency sensorineural HI (LFSNHI) (Cryns *et al*. 2002, Pennings *et al*. 2003). The pathogenicity of the rare variant p.Gly674Arg remains unclear.

One child with a profound HI had the p.Gly831Ser variant in *WFS1*, which has not been reported before. There are two previous reports on c.2492G>A (p.Gly831Asp) mutation in two families with LFSNHI. In one family the heterozygous p.Gly831Asp mutation was found in the proband with moderate to severe bilateral LFSNHI. The proband´s aunt and her daughter also harboured p.Gly831Asp but the age of onset of their LFSNHI was not reported (Bespalova *et al*. 2001). The proband of the second family had an early-onset LFSNHI. Unfortunately, other members of this family were not examined (Cryns *et al*. 2002). The clinical features of our patient with p.Gly831Ser differed from those in the two previously reported families by having an early-onset, profound HI.

The p.Gly831Ser variant occurred in a heterozygous form in the present proband. The family data suggested that this variant might be a de novo mutation, however the possibility of false paternity was not excluded due to ethical reasons. Previously, heterozygous *WFS1* variants have been associated with early-onset profound HI in six families (Eiberg *et al*. 2006, Hansen *et al*. 2005, Hogewind *et al*. 2010, Rendtorff *et al*. 2011), but only p.His313Tyr in two families has been verified as a de novo mutation (Hansen *et al*. 2005). In these six families, there were 11 subjects with the *WFS1* mutation and a HI. All of these individuals developed optic atrophy between the ages of 6–41 years (median 11 years), whereas the present proband (age 19 years) does not have visual problems. At this

point in time, whether the p.Gly831Ser variant is in fact a new heterozygous *WFS1* mutation that leads to HI and optic atrophy cannot be determined with certainty.

WFS1 gene mutations seem to be a rare cause of HI among Finnish children. The phenotype of HI caused by *WFS1* mutations varies widely. Still, the most prevalent HI phenotype might be low-frequency HI with dominant inheritance. In these instances, *WFS1* gene mutation screening should be considered.

6.3.2 MtDNA variants in childhood hearing impairment (III)

Among 103 hearing impaired children, neither m.3243A>G nor *MT-TS1* gene mutations were found. One child harboured a m.1555A>G mutation in *MT-RNR1* gene (See chapter 6.3.3). In addition to a m.1555A>G mutation, eight rare variants and 13 polymorphisms were found in *MT-RNR1* and *MT-RNR2* genes.

It is difficult to determine the pathogenicity of novel mtDNA variants because mtDNA has a high amount of variation and most changes are neutral polymorphisms. Pathogenic variants have usually been reported on different haplotype backgrounds (Herrnstadt & Howell 2004) and they occur at conserved sites of mtDNA. The rare variant sequences were compared with those reported in Mitomap or HmtDB, and found that five rare variants (m.740G>A, m.896A>G, m.1341C>T, m.2098G>A, and m.2405c-cc) were deemed haplogroup-specific polymorphisms rather than pathogenic mutations. The remaining three variants (m.958C>T, m.990T>C, and m.2445T>C) were present in more than one haplogroup in Mitomap or HmtDB sequences. For these variants, conservation analysis was carried out because evolutionary conservation at such a variant site supports pathogenic potential, while non-conservation at the site suggests that the variant is a homoplasy. Hence, m.958C>T and m.2445T>C occurred at non-conserved sites at mtDNA and were considered homoplasic variants, while m.990T>C occurred at a conserved site and was deemed to be unclassified in terms of its pathogenic potential.

The m.990T>C variant has been reported to be associated with HI (Konings *et al*. 2008). The mtDNA from the child with m.990C>T in this study belonged to haplogroup V2, while the 11 sequences with m.990T>C deposited in the Mitomap database belonged to four haplogroups or eight subhaplogroups. These findings suggest that this variant (m.990T>C) has emerged several times in human history. The nucleotide position m.990 in stem 20 of 12SrRNA is rather highly conserved suggesting that m.990T>C is a pathogenic rather than a homoplasic variant.

Unfortunately, detailed clinical features or mtDNA haplogroup data were not reported from the child with HI and m.990T>C variant (Konings *et al*. 2008). The presence of m.990T>C variant in various haplogroups, in combination with the rather high degree of conservation at this site, suggests that this transition is a pathogenic rather than a homoplasic neutral variant. Identification of further patients with m.990T>C should help in determining the pathogenic potential of this variant.

In addition to the eight rare variants, m.961T>G variant was found. This variant has been found to be associated with HI in previous studies (Li *et al*. 2004a, Turchetta *et al*. 2012). The mtDNA of our patient belonged to haplogroup H11 and this was also the case for 62 sequences in Mitomap. These data and previous considerations (Rydzanicz *et al*. 2010) suggest that m.961T>G is a haplogroup-specific variant rather than a pathogenic mutation.

An increased number of rare polymorphisms have been reported among Finnish adult patients with sensorineural HI. Furthermore, increased sequence variation in mtDNA as a possible genetic risk factor for HI has been proposed (Lehtonen *et al*. 2003). Interestingly, among the 103 children with HI, there was one individual with three rare variants (m.896A>G, m.958C>T and m.2445T>C). This individual also belonged to haplogroup U5b. A search in the HmtDB database revealed that a motif consisting of m.896A>G, m.958C>T and m.2445T>C can be found in only one sequence of Finnish origin. The phenotype of this subject is not known and thus, it cannot be determined whether the motif is a rare haplogroup U5b signature or whether it contributes to syndromic HI.

6.3.3 m.1555A>G (III-IV)

Among 103 hearing impaired children, one harboured a m.1555A>G mutation. Her HI was ascertained at age 4.8 years and progressed to severe by age 10.2 years. Her mother and five of her eight siblings had a HI. They all carried m.1555A>G, as did their matrilineal relatives. In Study IV, the hearing of 19 children, including the above mentioned girl and her siblings, was examined prospectively during a period of 7.8 years in three nuclear families of m.1555A>G pedigree. All of the 13 children who had been screened with TEOAEs as neonates passed the examination, including the child who developed a moderate or severe HI by the age of 1.5 years. In some families, the HI has been suggested to be congenital (Casano *et al*. 1998, Matthijs *et al*. 1996). However, results from the present study indicate that the children with m.1555A>G were

born with normal hearing and HI developed later in childhood. These findings suggest that children with m.1555A>G can pass the NHS but still develop the HI in later life. Therefore, it is essential to follow all children with a known m.1555A>G regularly throughout childhood, even if they pass the NHS.

Among subjects with m.1555A>G, both the severity of HI and the age of onset varied within and between generations. In the third generation, there were two HI phenotypes (i.e. a profound, progressive HI and a mild, stable, high-frequency HI). Interestingly, the HI phenotype appeared to be even more variable in the fourth generation. The follow-up of audiograms revealed a progressive HI in six children. A progressive HI has previously been identified in cases both with and without aminoglycoside exposure, but the HI is suggested to be milder in nonexposed cases (Usami *et al*. 1997). In contrast, the present study showed that the HI can be progressive and severe even in the absence of aminoglycoside exposure. Despite a lack of exposure in this family, it is important to avoid aminoglycosides or other ototoxic medications in patients with m.1555A>G mutation.

The HI phenotype, age at onset and HI penetrance have been found to be highly variable in patients with the m.1555A>G. Different nuclear genetic or environmental factors such as noise, viral infection, drug or stress may modify the HI phenotype (Guan 2011). Therefore, it was particularly interesting that all four children in family C had normal hearing, whereas various phenotypes of HI were found in families A and B. Parents were prospectively interviewed and reported in detail all infections and medications particular to their children. There were no environmental factors that could have modified the development or the progression of the HI. Differences in the phenotypes of the m.1555A>G mutation carriers could possibly be due to an interaction between the mtDNA mutation and a variation in an autosomal gene. A chromosome 8 locus (Bykhovskaya *et al*. 2001, Finnilä & Majamaa 2003) and, more recently, the *TRMU* gene (Guan *et al*. 2006, Yan *et al*. 2006) have been suggested to modify the expression of the m.1555A>G. In the future, advances in molecular genetics will probably resolve the nuclear genetic factors that modify the phenotype of m.1555A>G mutation carriers.

6.4 Additional disabilities among hearing impaired children (I)

The traditional classification of HI into syndromic and nonsyndromic forms is problematic, especially in childhood because additional disabilities can arise later.

Children with a HI may have other disabling conditions that are part of a syndrome or the result of a comorbid condition. In the present study, almost 40% of the hearing impaired children had one or more additional disabilities with the potential to affect their development or learning. This estimate corresponds well to that of earlier published reports (Birman *et al*. 2012, Fortnum & Davis 1997, Fortnum *et al*. 2002, Van Naarden *et al*. 1999). Interestingly, the frequency of additional disabilities was similar, regardless of the degree of HI. These findings are in line with previous reports, however, other work has primarily focused on children with severe or profound HI. Only a few studies have examined children with mild HI and additional disabilities (Chilosi *et al*. 2010, Van Naarden *et al*. 1999). Children with moderate or profound HI are more likely than children with mild HI to be evaluated by a developmental paediatrician (Wiley *et al*. 2011). Because additional disabilities seem to be common irrespective of the degree of HI, it is important to carry out a comprehensive examination and rehabilitation even when the HI is mild.

In this study, additional disabilities were also examined in different aetiological groups of HI. Children with acquired HI had additional disabilities more often than those with genetic or unknown aetiology of HI. These findings are in line with those recently reported (Chilosi *et al*. 2010) and point to the increased survival of very premature infants with multiple problems. Previous studies have also suggested that prenatal infections and perinatal problems are risk factors for both acquired HI and developmental disabilities (Johnson *et al*. 2009, Lee *et al*. 2005, Robertson *et al*. 2009).

6.5 General discussion

Public health care systems and child welfare clinics are accessible to every child in Finland. Both hearing and developmental concerns are thoroughly screened, which consistently enables even mild childhood HIs and developmental disabilities to be found. OUH provides advanced medical treatment for the people in the Northern Ostrobothnia Hospital District and northern Finland. Diagnostics of early childhood HIs and rehabilitation of hearing impaired children in northern Finland has been concentrated in OUH. Therefore, in this study, the ascertainment of hearing impaired children in northern Finland is close to complete. A slight departure from complete ascertainment occurred because the follow-up of some children in the birth cohort was only 5 years.

The hearing measurements of all children were performed by audiology assistants with special training in paediatric audiometry. At the time of ascertainment of HI, pneumatic otoscopy was performed, and HIs due to secretory otitis media were not included in this study. Molecular genetic studies were carried out in a laboratory with more than two decades of experience conducting molecular studies on human samples. All analyses were performed under the supervision of trained personnel.

The Audio-Phoniatric Department of OUH has a long history of providing comprehensive examinations of hearing impaired children. Clinical practice provides speech and language assessment for children with HI. Also, questionnaires from kindergarten or from school are completed regularly. Because of retrospective data collection and probable missing information, it is possible that additional disorders among hearing impaired children were even greater than findings from this study. The proportion of syndromic or presumably syndromic HIs was 47% in this study. Defining syndromic HI is challenging, especially in childhood. Many symptoms can be subclinical or appear later, and as a result, some nonsyndromic HIs may be defined as syndromic at a later point in time. It is important to take additional disorders into consideration when planning rehabilitation for hearing impaired child, particularly because they are common in both mild and severe HIs.

Genetic causes were the most frequent (47%) aetiology of childhood HI, but unknown causes were also common. The definition of genetic HI was based in many cases on positive family history data. In cases of autosomal recessive inheritance, the hearing impaired child can be the only family member to have HI. The number of genetic HIs may be underestimated because recessive inheritance is common in childhood HIs.

The research group has considerable experience analysing molecular aetiologies of mitochondrial diseases. Therefore, mtDNA and the *WFS1* gene were chosen to further define genetic aetiology of childhood HIs. Mutations in mtDNA and in the *WFS1* gene were found to be rare causes of HI among children in northern Finland. If the molecular analysis had been made for the complete mtDNA and for all the exons of *WFS1*, additional information might have been received. The number of patients for molecular genetic studies aimed at determining the presence of mutations in mtDNA and WFS1 gene was rather small. However, the clinical features and family history of patients were well known. This cohort presents molecular aetiology of childhood HI in northern Finland. As a result of geographical, cultural and genetic isolation, some

hereditary diseases are almost entirely absent in our population, whereas some are clustered here. Therefore, the molecular genetic results cannot be generalized.

Results from the follow-up study indicated that children with m.1555A>G mutation were born with normal hearing, and HI developed later in childhood. Therefore, it is essential to follow the hearing of children in known m.1555A>G families, even if they pass the NHS. Because aminoglycosides are a known contributory factor for HI among m.1555A>G mutation carriers, m.1555A>G mutation screening should be considered prior to aminoglycoside treatment.

The genetic background of childhood HI is heterogeneous, and genetic diagnostics in clinical work is challenging. The proportion of unknown HIs is still rather high in the present study, as well as those previously conducted. One shortcoming of this study was the lack of CMV diagnostics. At least some of the unknown HIs were undoubtedly caused by CMV infection. Also, genetic testing for USH was not widely performed, mainly due to clinical practices. If genetic testing would have been done, some more USH patients might have been ascertained. This also concerns many other genes which have been identified as causes of HI. If the next generation techniques would have been used in this research, more valuable information could have been received.

Mutations in the same gene can result in different phenotypes and in different modes of inheritance. Several genes, for example *WFS1* gene, are involved in both recessive and dominant HI, and in both syndromic and nonsyndromic HI. Typically, and also in mitochondrial disorders, the phenotype of HI is widely variable. In some cases of childhood HI, certain phenotypes can give reason to specific aetiological investigations. In cases with HI and retinitis pigmentosa, analysis of USH-associated genes is reasonable. Or, in cases with HI and enlarged vestibular aqueduct, analysis of the *SLC26A4* gene makes sense. Moreover, in cases with matrilinear inheritance, mtDNA mutations should be analysed and, for example, in cases with dominant inheritance and LFSNHI, *WFS1* gene analysing should be considered.

In the future, advances in diagnostics (e.g., targeted next-generation sequencing methods) can provide a remarkable opportunity to identify variants known to be associated with HI genes. Also, molecular investigations and functional studies are needed to confirm the pathogenicity of novel or rare variants. Thus, in the future, the proportion of unknown causes of HI may decrease. Also, NHS now enables the detection of congenital HIs earlier than before. Knowledge of the aetiology of HI is valuable as it can help professionals make predictions regarding the progression of HI. Such knowledge is useful in

guiding examinations, particularly with regard to additional disabilities and rehabilitation. Knowledge of the aetiology also helps with appropriate genetic counselling for HI. Early detection of HI and growing knowledge of the aetiologies, as well as, awareness of possible additional disabilities, makes it possible to focus rehabilitation and to improve the outcomes among hearing impaired children.

7 Conclusions

The prevalence and aetiology of childhood HI was determined among ten-year birth cohort in northern Finland. The following conclusions can be drawn from this research:

1. The prevalence of any bilateral childhood HI was 2.3 per 1000 live births. It has remained unchanged in northern Finland through three decades. Genetic causes were the most common (47%) aetiology of HI, while 16% of cases were acquired and 36% were unknown. Almost 40% of 214 children had one or more additional disabilities that adversely influenced their development or learning. The frequency of additional disabilities was not associated with the severity of HI. Children with acquired HI had additional disabilities more often (66%) than children with genetic or unknown aetiology of HI (44%). It is essential to take additional disabilities into consideration in rehabilitation.
2. We identified four rare variants in exon 8 of *WFS1*. One of the variants was the novel, heterozygous p.Gly831Ser mutation, which was found to be de novo in the family. The p.Gly831Ser variant may be a new member to the group of heterozygous *WFS1* mutations that lead to HI and optic atrophy. Regardless, *WFS1* mutations appear to be a rare cause of childhood HI in northern Finland.
3. Mutations in mtDNA were a rare cause of childhood HI. The m.1555A>G mutation was found in one child, but neither m.3243A>G nor mutations in *MT-TS1* were found. Eight rare mtDNA variants were detected including m.990T>C that was deemed to be unclassified in terms of its pathogenic potential. Identification of further patients with m.990T>C and functional studies should help to determine the pathogenic potential of the variant.
4. Follow-up of children with m.1555A>G revealed that these children were born with normal hearing, but half of them developed a HI during the follow-up period of 7.8 years (age range, 2.1–13.2 years at the end of the follow-up). Distinct phenotypes of HI were identified. Environmental factors contributing to the phenotype variation were not recognized. Because these children generally pass the NHS, it is important to follow over time the hearing of children in families with the m.1555A>G mutation.

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Original publications

I Häkli S, Luotonen M, Bloigu R, Majamaa K, Sorri M (2014) Childhood hearing impairment in northern Finland, etiology and additional disabilities. Int J Pediatr Otorhinolaryngol 78(11):1852–6.

II Häkli S, Kytövuori L, Luotonen M, Sorri M, Majamaa K (2014) WFS1 mutations in hearing-impaired children. Int J Audiol 53(7):446–51.

III Häkli S, Luotonen M, Sorri M, Majamaa K (2014) Mutations in the two ribosomal RNA genes in mitochondrial DNA among Finnish children with hearing impairment. Manuscript.

IV Häkli S, Luotonen M, Sorri M, Majamaa K (2013) Audiological follow-up of children with the m.1555A>G mutation in mitochondrial DNA. Audiol Neurotol 18(1):23–30.

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1257. Lahti, Anniina (2014) Epidemiological study on trends and characteristics of suicide among children and adolescents in Finland

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