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The genetic causes of early onset hearing loss in Estonian children

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To my patients and their families

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by Roman numerals (I–V), and previously unpublished data:

I. Teek R, Kruustük K, Zordania R, Joost K, Reimand T, Möls T, Oitmaa E, Kahre T, Tõnisson N, Õunap K. Prevalence of c.35delG and p.M34T mutations in the *GJB2* gene in Estonia. Int J Pediatr Otorhinolaryngol 2010, 74(9); 1007–12.

II. Teek R, Oitmaa E, Kruustük K, Zordania R, Joost K, Raukas E, Tõnisson N, Gardner P, Schrijver I, Kull M, Õunap K. Splice variant IVS2–2A>G in the *SLC26A5* (Prestin) gene in five Estonian families with hearing loss. Int J Pediatr Otorhinolaryngol 2009; 73(1):103–7.

III. Puusepp H, Zilina O, Teek R, Männik K, Parkel S, Kruustük K, Kuuse K, Kurg K, Õunap K. 59 Mb microdeletion in chromosome band 17q22–q23.2 associated with tracheo-esophageal fistula and conductive hearing loss. Eur J Med Genet 2009; 52(1):71–4.

IV. Kalev I, Muru K, Teek R, Zordania R, Reimand T, Köbas K, Õunap K. LEOPARD syndrome with recurrent PTPN11 mutation Y279C and different cutaneous manifestations: two case reports and a review of the literature. Eur J Pediatr 2010; 169(4):469–73.

V. Teek R, Kruustük K, Zordania R, Joost K, Reimand T, Oitmaa E, Nelis M, Žilina O, Kahre T, Tõnisson N, Õunap K. Kuulmislanguse geneetilised põhjused Eesti lastel ning nendel leitud genotüübi ja fenotüübi omavaheline võrdlus. Eesti Arst 2010, accepted (in Estonian).

My contributions to the original articles are:

Publication I: I designed the study and led the clinical investigation of the patients; I conducted the collection of the data, the analysis and interpretation of the results and wrote / co-wrote the manuscript;

Publication II: I led the clinical investigation of patients and the clinical investigation of family members; I conducted / led the collection of the data; I conducted / led the analysis and interpretation of the results and wrote / co-wrote the manuscript;

Publication III: I conducted the specification of the phenotype; I conducted the analysis of the causes of hearing loss of the proband; I critically reviewed the manuscript;

Publication IV: I conducted the clinical investigation of the patient 1; I collected the samples and data; I critically reviewed the manuscript as one of the co-authors;

Publication V: I conducted the retrospective analysis of all clinical data and the investigation of the entire study group and wrote / co-wrote the manuscript;

ABBREVIATIONS
of HEARING LOSS STUDY GROUPS AND PATIENTS

(used in this cover text and not the original publications)

Group NB	Refers to the Study Group of Newborns born in Estonia in January 2005
Group $HL^{194/08}$	Refers to a Study Group of 194 children with the *SLC26A5* gene and hearing loss
Group HL^{233}	Refers to the overall Study Group of 233 children with early onset hearing loss
Group HL^{115}	Refers to a subset of Group HL^{233} of 115 children with *GJB2* mutations
Group HL^{96}	Refers to a subset of Group HL^{233} of 96 children with unknown etiologies of HL chosen for DNA analysis of congenital cytomegalovirus
Group HL^{73}	Refers to a subset of Group HL^{233} of 73 children with c.35delG homozygosity
Group $HL^{96/5}$	Refers to a subset of Group HL^{96} of 5 children with congenital cytomegalovirus
Group HL^{55}	Refers to a subset of Group HL^{233} of 55 children for whom chromosomal analysis was conducted
Group HL^{24}	Refers to a subset of Group HL^{233} of 24 children with unknown etiology of hearing loss who did not fit to any known dysmorphic syndrome
Group HL^{7}	Refers to a subset of Group HL^{233} of 7 children p.M34T homozygosity
Group HL^{S}	Refers to a subset of Group HL^{233} of 7 children with clinically confirmed syndromic HL
$Patient^{M1}$	Refers to a 10 year-old patient with the mutation m.1555A>G in the mitochondrial *12S r-RNA* gene
$Patient^{M2}$	Refers to a patient with the mutation 7472insC in the mitochondrial *tRNA-Ser* gene, but also carries the mutation 35delG in the *GJB2* gene

ABBREVIATIONS

A	Adenine
ABR	Auditory brainstem response
ADNSHL	Autosomal dominant non-syndromic sensorineural hearing loss
APEX	Arrayed primer extension assay
ARNSHL	Autosomal recessive non-syndromic sensorineural hearing loss
AS	Alport syndrome
ATG	Translational starting point
BOR	Branchio-oto-renal syndrome
bp	Base pair
C	Cysteine
CFTR	Cystic fibrosis transmembrane conductance regulator
CHL	Conductive hearing loss
CI	Confidence interval
CLS	*Café-au-lait* spots
CMA	Chromosomal microarray analysis
CMV	Cytomegalovirus
CT	Computed tomography
C_t	Cycle threshold
CVS	Chorionic villus sampling
dB	Decibel
DBS	Dried blood spots
DFN	*DFN* stands for **d**ea**fn**ess and ***1 (2, 3)*** designates the **first** (**second, third** etc) X-chromosomal locus identified in human
DFNA1	*DFN* stands for **d**ea**fn**ess and ***A1 (A2, A3)*** designates the **first** (**second, third** etc) autosomal **dominant** locus identified in human
DFNB1	*DFN* stands for **d**ea**fn**ess and ***B1 (B2, B3)*** designates the **first** (**second, third** etc) autosomal **recessive** locus identified in human
DVA	Dilated vestibular aqueduct
G	Guanine
GTG	G bands by Trypsin using Giemsa
HCM	Hypertrophic cardiomyopathy
HI	Hearing impairment
HIH	Hereditary impaired hearing
HL	Hearing loss
HWE	Hardy-Weinberg equilibrium
Hz	Hertz
I	Inactivating
kb	Kilobases
kHz	Kilohertz
LS	LEOPARD syndrome
ML	Multiple lentigines

MRI	Magnetic resonance imaging
mRNA	Mature ribonucleic acid
mtDNA	Mitochondrial desoxyribonucleic acid
NBHS	Newborn hearing screening
N	Normal or no mutation
NI	Non-inactivating
OAE	Otoacoustic emissions
OHC	Outer hair cells
p	Short arm of a chromosome
PCR	Polymerase chain reaction
PDS	Pendred syndrome
PTA	Pure tone average
PTP	Protein tyrosine phosphatase
q	Long arm of a chromosome
qPCR	Quantitative polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
SIDS	Sudden infant death syndrome
SNHL	Sensorineural hearing loss
SLC	Solute carrier
SNP	Single nucleotide polymorphisms
SSCP	Single-stranded conformation polymorphism
tRNA	Transfer ribonucleic acid
UNHS	Universal newborn hearing screening program
US	Usher syndrome
WS	Waardenburg syndrome
X	Other specified mutation in *GJB2* gene
Δ	Delta

1. INTRODUCTION

Hearing loss (HL) is a sensory disability affecting millions of people worldwide, and although not life-threatening can become a major drawback in social and professional life (Kemperman *et al.*, 2002).

References to hereditary HL date from the early 17th century "The deaf and dumb ought to abstain from marriage… for the good of commonwealth, because there is evidence they beget children like themselves…" (Cranefield and Federn, 1970; Toriello *et al.*, 2004). Autosomal recessive HL was apparently first noted in the sixteenth century by Schenck, who described multiple affected siblings with profound congenital HL who had normal parents (Toriello *et al.*, 2004). The relationship between HL and genetics has been recognized since the beginning of the 19th century (Ruben, 1991; Toriello *et al.*, 2004). In the second half of the 19th century, Sir William Wilde initiated systematic studies of hereditary impaired hearing (HIH). His research on congenital deafness was published in 1853 and included the observation that parental consanguinity increases recurrence risk for deafness, an insight that predated Mendel's laws of inheritance by 12 years. The research of Politzer (1882) supports Wilde's conclusions (Hone and Smith, 2001). Albrecht von Graefe in describing retinitis pigmentosa in three deaf-blind brothers (Toriello *et al.*, 2004), was the first to note the syndrome which later became broadly known as Usher's syndrome.

During the first decades of the 20th century, numerous detailed descriptions occurred of syndromic hearing impairment (HI), a designation applied to conditions in which HL co-segregates with other physical findings, whereas non-syndromic refers to HI in isolation (Hone and Smith, 2001). For the first half of the 20th century, geneticists argued about whether two, three, or perhaps four genes could explain the inheritance of deafness, and whether these genes were dominant or recessive (Nance, 2003). Localization and identification of genes for HIH started in the early 1990s (Tekin *et al.*, 2001b; Finsterer and Fellinger, 2005). The first locus mapped was that of DFNA1 (DFNA – autosomal dominant deafness locus) in a large Costa Rican family with autosomal dominant, nonsyndromic HIH in 1992 (Leon *et al.*, 1992; Bitner-Glindzicz, 2002; Finsterer and Fellinger, 2005). Astonishing progress was achieved during the last decade of the 20th century in identifying genes for deafness to the extant that any current account of this research must be regarded as a "work in progress" (Nance, 2003). Current research estimates that 1% of the 30,000–50,000 human genes are necessary for hearing, of which more than 120 independent genes have been identified as causes of HL (Finsterer and Fellinger, 2005).

The early detection of hearing impaired infants and children was emphasized in the late 1960s, which resulted in the implementation of hearing screening programs in the 1970s either as universal hearing screening or as targeted groups (Parving, 2007). Early detection of HL in newborns and infants by newborn hearing screening is necessary for early treatment. Undetected bilateral HL in a child causes delay in speech and cognitive development; normal speech and language, may not develop at all (Jakubikova *et al.*, 2009). A

newborn hearing screening program started in Estonia in 2004 and by 2009 88% of all newborns were included into the program.

Up to 2005, only the prevalence of HI in Estonia was studied. Uus and Davis (2000) conducted a retrospective study of permanent childhood HI in children born between 1985 and 1990 and resident in Estonia. Uus and David find the prevalence rate of all permanent HI for the birth cohort 1985–1990 was 172 per 100,000 live births (95 per cent (confidence interval (CI) 151–194) and that of congenital impairment was 152 per 100,000 (CI 134–170). The results of this investigation were comparable to other European studies, particularly the Trent study by Parker *et al*. (2000). The prevalence of congenital HI is in Estonia higher than the average in Europe (Uus and Davis, 2000).

The present study was initiated to establish the genetic causes of early onset HL among Estonian children using new molecular testing possibilities, to study genotype and phenotype correlations of our patients, to establish the prevalence of c.35delG and p.M34T mutations in *GJB2* gene among Estonian newborn population and to characterize rare genetic syndromic HL.

2. LITERATURE REVIEW

2.1. The epidemiology and frequency of HL

The global prevalence rate of children born with HL is approximately one to two per 1000 (Marazita *et al.*, 1993; Cryns *et al.*, 2004; Snoeckx *et al.*, 2005; Hilgert *et al.*, 2009b). The majority of deaf children are born to normal hearing parents (90–95%) and in most of these families there is no history of HL (Green *et al.*, 1999; Smith and Hone, 2003; Finsterer and Fellinger, 2005; Bayazit and Yilmaz, 2006). The incidence of severe to profound sensorineural hearing loss (SNHL) in young adults of 18 years is estimated to be six per 1000 (Billings and Kenna, 1999). About 4–6% of the global population is affected by HI (Estivill *et al.*, 1998; Gardner *et al.*, 2006) and genetic factors are likely to be important in all age groups (Bitner-Glindzicz, 2002). The prevalence of HL varies between countries and ethnic groups (Fortnum and Davis, 1997; Streppel *et al.*, 2000; Dietz *et al.*, 2009). Due to the high frequency and clinical impact of congenital HL, early detection has become an important public health issue (Cohn *et al.*, 1999). Since the beginning of the 2000s, universal newborn hearing screening programs have been developed and implemented in many developed countries (Hilgert *et al.*, 2009b). The rationale for these initiatives is based on the premise that early detection and intervention for children with HL maximizes opportunities for language and speech development, thereby facilitating the acquisition of normal social, cognitive and motor skills (Hilgert *et al.*, 2009b).

The etiology of HL is extremely heterogeneous. Identification of the etiology of SNHL in children facilitates management and provides important prognostic information (Morzaria *et al.*, 2004). While environmental factors such as congenital cytomegalovirus (CMV) infection, prenatal rubella infection, prematurity and meningitis are thought to be the cause of 40–50% of SNHL cases, the remainder are genetic and result from mutations involving any one of numerous loci (Marazita *et al.*, 1993; Norris *et al.*, 2006). The incidence of genetic HL is increasing because acquired impaired hearing from meningitis is decreasing as a consequence of improved prenatal and neonatal care, antibiotic therapy and vaccination programs (Marazita *et al.*, 1993; Smith and Hone, 2003; Finsterer and Fellinger, 2005; Yaeger *et al.*, 2006). Hilgert *et al.* (2009b) estimate genetic factors cause at least two-thirds of prelingual HL cases in developed countries and that environmental factors and unidentified genetic factors cause the remaining third. The most common environmental (non-genetic) cause of HL is congenital CMV infection (Kenneson and Cannon, 2007; Dietz *et al.*, 2009; Hilgert *et al.*, 2009b; Ludwig and Hengel, 2009). The overall birth prevalence is ~0.64%, but only about 10% of infected infants have non-specific symptoms at birth (Kenneson and Cannon, 2007; Hilgert *et al.*, 2009b). Congenital CMV infection is one of the most important causes of HL in young children after genetic mutations in connexin genes (Grosse *et al.*, 2008).

Genetic deafness is divided into syndromic and non-syndromic forms. The syndromic forms of deafness account for 30% of HL cases with genetic etiology and include several hundred deafness syndromes (Van Camp *et al.*, 1997; Pampanos *et al.*, 2002; Petersen and Willems, 2006; Hilgert *et al.*, 2009b). In most cases, genetic HL is monogenic and can be inherited in various ways. Localization and identification of genes for HIH started in early 1990s, when first locus was mapped for DFNA1 (Bitner-Glindzicz, 2002). Monogenic HL is an extremely heterogeneous trait, and over 100 mapped loci and 46 causally implicated genes have been identified up to now[1] (Van Camp and Smith, 2008). Most of genes are causative in only a small percentage of patients (Hilgert *et al.*, 2009a; Hilgert *et al.*, 2009b). Prelingual HL is transmitted via an autosomal recessive trait (75–80%), an autosomal dominant trait (10–20%), is X-linked (1–5%), or mitochondrial (0–20%) (Tekin *et al.*, 2001b; Petersen, 2002; Smith and Hone, 2003; Finsterer and Fellinger, 2005). The autosomal-recessive forms of HL are most common and usually more severe than the other forms and give rise to sensorineural deafness (Petersen and Willems, 2006). Mutations in the *GJB2* gene, which have been mapped to 13q11-q12 and encode the cap junction protein connexin 26 (MIM 121011), represent a major cause of pre-lingual, non-syndromic, recessive deafness, as they are responsible for as much as 50% of such cases in many populations (Guilford *et al.*, 1994; Pampanos *et al.*, 2002; Petersen and Willems, 2006). One specific mutation, c.35delG, accounts for the vast majority of the *GJB2* mutations detected in Caucasian populations and represents one of the most frequent disease-associated mutations identified so far (Petersen and Willems, 2006). Mutation p.M34T in the *GJB2* gene is reported to have a high frequency in the general Caucasian population, comparable to that of c.35delG (Green *et al.*, 1999; Roux *et al.*, 2004; Snoeckx *et al.*, 2005). High frequencies of *GJB2* mutations other than c.35delG have been reported in other ethnic groups: the c.167delT mutation is frequent in Ashkenazi Jews (Morell *et al.*, 1998; Green *et al.*, 1999; Kenneson *et al.*, 2002; Kokotas *et al.*, 2008; Hilgert *et al.*, 2009b), the 235delC in Japanese (Ohtsuka *et al.*, 2003; Kokotas *et al.*, 2008; Hilgert *et al.*, 2009b) and p.R143W in Africans (Brobby *et al.*, 1998) The etiology of mild, moderate, and severe, as well as unilateral HL is less well known (Nance *et al.*, 2006).

Genetic heterogeneity underlying syndromic and non-syndromic HL greatly complicates further genetic testing and diagnosis in small families and sporadic cases of HI (Bitner-Glindzicz, 2002). Although genetic testing provides better diagnostic methods, the proportion of children with SNHL of unknown origin is still high (Dietz *et al.*, 2009).

[1] http://hereditaryhearingloss.org

2.2. Classification of hearing loss

HL can be classified according to various criteria. Primarily HL is divided into acquired impaired hearing and HIH. Most frequently HIH is classified as syndromic and non-syndromic HL (Finsterer and Fellinger, 2005). Syndromic HL is associated with malformations of the external ear or other organs or with medical problems involving other organ systems. Non-syndromic HL does not have any associated visible anomalies of the external ear, nor are there any related medical problems; however, non-syndromic HL can be associated with abnormalities of either or both the middle ear and inner ear (Smith and Van Camp, 2007). HIH is classified also according to transmission via an autosomal dominant, autosomal recessive, X-chromosomal recessive, or maternal trait (Finsterer and Fellinger, 2005). In order to differentiate between the multiple nonsyndromic deafness loci, autosomal dominant loci are designated as DFNA, autosomal recessive loci as DFNB, and X-chromosomal loci as DFN (Tekin *et al.*, 2001b).

A secondary classification system divides HL according to onset before (prelingual) or after acquisition of speech (postlingual). Most prelingual cases with HIH follow a recessive segregation, whereas most cases of postlingual HIH follow an autosomal dominant trait (Bitner-Glindzicz, 2002; Finsterer and Fellinger, 2005; Petersen and Willems, 2006).

A third system classifies HL by location of defect – sensorineural, conductive, mixed HL and central audiotory dysfunction. Sensorineural HL (SNHL) results from malfunction of the inner ear structures, e.g. the cochlea (Smith and Van Camp, 2007). Nearly all genes for both recessive and dominant non-syndromic forms cause SNHL (Petersen and Willems, 2006). Conductive HL (CHL) results from the abnormalities of either or both the external ear and the ossicles of the middle ear (Smith and Van Camp, 2007). Conditions associated with middle ear pathology, e.g. chronic otitis media, poor Eustachian tube function, perforated eardrum, otosclerosis, ossicular chain malformations; external ear obstructions, e.g. cerumen, tumor, foreign body, otitis externa and rare inner ear pathologies, e.g. superior canal dehiscence, enlarged vestibular aqueduct, frequently cause CHL (Kelly *et al.*, 2008). Mixed HL is a combination of sensorineural and conductive HL. There are also many genetic syndromes associated with mixed SNHL and CHL, e.g. branchio-oto-renal syndrome (BOR), Goldenhar syndrome, Kallmann syndrome (Smith and Van Camp, 2007; Kelly *et al.*, 2008). Central auditory dysfunction results from damage or dysfunction at the level of the eighth cranial nerve, auditory brain stem, or cerebral cortex (Smith and Van Camp, 2007).

The frequency of HL is designated as low (<500 Hz), middle (501–2000 Hz) and high (>2000 Hz) (Smith and Van Camp, 2007).

Hearing is measured in decibels (dB). The severity of HI is graded by the degree of HL in the better ear as mild (21–40 dB), moderate (41–70 dB), severe (71–95 dB) and profound (greater than 95 dB). Profound HI is termed as deafness. The individuals with deafness are frequently members of a Deaf community and use Sign Language. As in other cultures, members are cha-

racterized by unique social and societal attributes. They consider themselves deaf, furthermore their deafness is not considered to be either a pathology or disease to be treated or cured (Cryns *et al.*, 2004; Smith and Van Camp, 2007).

2.3. Hereditary impaired hearing

HL is an etiologically heterogeneous trait with many known genetic and environmental causes (Nance, 2003). The causes of HL are numerous and, in a particular population, the relative contribution of genetic and environmental causes may be determined by social factors such as population structure and consanguinity, infection control and immunization, and provision of neonatal medical care (Bitner-Glindzicz, 2002). HIH traditionally has been distinguished from acquired HL by history and physical examination and complemented by a variety of tests to identify specific syndromes. HIH can be distinguished from acquired HL by otologic, audiologic, and psychical examination, complemented by family history and ancillary tests like temporal bone computed tomography (CT), urinalysis, thyroid function studies, ophthalmoscopy, and electrocardiography. Syndromic HL implies the co-inheritance of abnormalities of other organ systems while non-syndromic HL segregates as the only abnormality. The majority of congenital HIH is non-syndromic (Smith and Robin, 2002). Since non-syndromic HIH is almost exclusively caused by cochlear defects, affected patients suffer from SNHL (Petersen and Willems, 2006). In most cases, HIH is monogenic. Monogenic HL is an extremely heterogenous trait, with over 100 mapped loci and 46 causally implicated genes (Van Camp and Smith, 2008; Hilgert *et al.*, 2009b). In a majority of cases, 75–80%, prelingual HL is transmitted via an autosomal recessive trait; 10–20% via an autosomal dominant trait, 1–5% are X-linked (1–5%) and 0–20% are mitochondrial (Tekin *et al.*, 2001b; Petersen, 2002; Smith and Hone, 2003; Finsterer and Fellinger, 2005).

2.3.1. Nonsyndromic hearing impairment

2.3.1.1. *GJB2* gene

Autosomal-recessive forms of HL are the most common and are usually more severe than the other forms and cause sensorineural deafness (Petersen and Willems, 2006). The most important locus for nonsyndromic, autosomal-recessive deafness (DFNB1) was originally assigned to chromosome 13q11 by linkage analysis in two large consanguineous Tunisian families with prelingual, profound deafness in 1997 (Guilford *et al.*, 1994; Petersen and Willems, 2006). Mutations in the *GJB2* gene, which had been mapped to 13q11-q12 (Mignon *et al.*, 1996), were subsequently identified in three consanguineous Pakistan families with profound deafness genetically linked to 13q11 (Kelsell *et al.*, 1997).

Mutations in the *GJB2* gene, which encode the cap junction protein connexin 26 (MIM 121011), represent a major cause of pre-lingual, non-syndromic, recessive deafness, as they are responsible for as much as 50% of such cases in many populations (Guilford *et al.*, 1994; Kenneson *et al.*, 2002; Pampanos *et al.*, 2002; Petersen and Willems, 2006; Hilgert *et al.*, 2009b).

The *GJB2* gene has a single coding exon and the protein belongs to the family of connexins, which have been implicated in cap-junctional intercellular communication. Six connexin subunits bind together to form a hexamer (connexon) in the plasma membrane; each connexon associates with another connexon in an adjacent cell to form an intracellular channel. Multiple channels cluster in a specialized membrane region to form a gap junction (Kumar and Gilula, 1996; Petersen and Willems, 2006). Connexins are important for the recycling of potassium ions into the cochlear endolymph through the network of gap junctions. Ion homeostasis is essential for normal hearing, and mutations in several genes encoding connexins or ion channels lead to hereditary deafness. Mutations in the *GJB2* gene cause structural and functional defects in these gap junctions, leading to persistently high intracellular potassium concentration; this damages the mechanism that allows the quick response of ciliated cells to new auditory stimuli, consequently resulting in HL (Holt and Corey, 1999; Steel and Bussoli, 1999; Rabionet *et al.*, 2000a; Piatto *et al.*, 2005; Petersen and Willems, 2006).

Studies in mice with connexin ablations indicate that HI began at about the onset of inner ear function which corresponds to the 20^{th} week of gestation in humans (Pagarkar *et al.*, 2006).

According to the connexin deafness homepage, more than 100 different mutations for HL have been identified in *GJB2* gene (Ballana *et al.*, 2009). Some of these mutations are very frequent, others are extremely rare. These mutations occur at different frequencies across populations (Kenneson *et al.*, 2002; Hilgert *et al.*, 2009b). Hilgert *et al.* (2009b) conducted an extensive literature search and estimate that over 220 mutations have been reported in *GJB2* gene.

Given the high prevalence of DFNB1 deafness, molecular testing for *GJB2* mutations has become the standard of care for the diagnosis of patients with non-syndromic HI of unknown cause (del Castillo *et al.*, 2005).

Dominant mutations in *GJB2* have been reported predominantly in Caucasians and cause both autosomal dominant non-syndromic HL (ADNSHL) and syndromic HL associated with diverse skin disorders. Skin disorders are very heterogeneous and include diffuse palmoplantar keratodermahyperkeratosis, Vohwinkel syndrome and Keratitis–Ichthyosis/ Deafness syndrome (Richard *et al.*, 2002; de Zwart-Storm *et al.*, 2008). The particular phenotype appears to depend both on the type of mutation and its location (Hilgert et al., 2009a; Hilgert et al., 2009b).

2.3.1.1.1. c.35delG mutation

One specific mutation, the c.35delG mutation, accounts for the vast majority of the *GJB2* mutations detected in Caucasian populations and represents one of the

most frequent disease-associated mutations identified so far (Denoyelle *et al.*, 1997; Estivill *et al.*, 1998; Petersen and Willems, 2006). The c.35delG mutation consists of a deletion of a guanine (G) in a sequence of six Gs extending from position 30–35 leading to a frameshift and premature stop codon at nucleotide 38 (Denoyelle *et al.*, 1997; Zelante *et al.*, 1997; Petersen and Willems, 2006). A carrier frequency of the c.35delG mutation as high as 3.5–4.0% has been detected in Italian and Greek populations, implying that deafness due to homozygosity for this mutation could affect as many as one in 2500 newborns in these populations (Estivill *et al.*, 1998; Antoniadi *et al.*, 1999). The carrier frequency of the c.35delG mutation in southern Europe and in the Mediterranean region is thus higher than the carrier frequency of the major ΔF508 mutation of the *cystic fibrosis transmembrane conductance regulator* (*CFTR*) gene causing cystic fibrosis (Gasparini *et al.*, 2000; Petersen and Willems, 2006).

The prevalence of c.35delG mutation is now known to be high in the Mediterranean area. Lucotte shows in meta-analysis that of 5826 healthy subjects, the mean prevalence of c.35delG mutation is 1 in 49. The highest carrier rates were found in Napoli (1:25), Greece (1:28), Sardinia (1:29) and Corsica (1:30) (Lucotte, 2007). See Table 1 for the prevalence of c.35delG mutation in European populations.

Table 1: Carrier frequencies of c.35delG and p.M34T mutations in *GJB2* gene in Europe

Country	**c.35delG**	**p.M34T**
Austria (Janecke *et al.*, 2002) (Frei *et al.*, 2002)	1:60–1:110	–
Belgium (Gasparini *et al.*, 2000)	1:190	–
Belgium (Storm *et al.*, 1999)	1:40	–
Bulgaria (Gasparini *et al.*, 2000)	1:157	–
Croatia (Sansovic *et al.*, 2005)	1:68	–
Czech Republic (Gasparini *et al.*, 2000)	1:48.7	–
Denmark (Gasparini *et al.*, 2000)	1:47.5	–
Finland (Lopponen *et al.*, 2003) (Pastinen *et al.*, 2001)	1:43–1:63	1:26
France (Brittany) (Gasparini *et al.*, 2000)	1:96	–
France (Gasparini *et al.*, 2000)	1:200	–
France (Corsica) (Lucotte and Pinna, 2003)	1:30	–
France (Perpignan)(Mercier *et al.*, 2005)	1:51	–
France (Montpellier) (Mercier *et al.*, 2005)	1:65	–
France (Marseilles) (Mercier *et al.*, 2005)	1:31	–
France (Toulon) (Mercier *et al.*, 2005)	1:66	–
France (Grasse) (Mercier *et al.*, 2005)	1:53	–
France (Roux *et al.*, 2004)	1:66	1:43
Germany (Gasparini *et al.*, 2000)	1:50	–
Greece (Antoniadi *et al.*, 1999)	1:28.2	–
Greece (Gasparini *et al.*, 2000)	1:33	–
Holland (Gasparini *et al.*, 2000)	1:44.5	–
Italy (Napoli) (Estivill *et al.*, 1998)	1:25	–

Country	c.35delG	p.M34T
Italy (Gasparini *et al.*, 2000)	1:32	–
Italy (Sardinia) (Gasparini *et al.*, 2000)	1:29.5	–
Italy (Genoa) (Lucotte, 2007)	1:129	–
Italy (Sicily) (Lucotte, 2007)	1:34	
Lebanon (Beirut) (Mustapha *et al.*, 2001)	1:43	–
Malta (Gasparini *et al.*, 2000)	1:36	–
Norway (Gasparini *et al.*, 2000)	1:190	–
Portugal (Gasparini *et al.*, 2000)	1:45	–
Slovenia (Gasparini *et al.*, 2000)	1:182	–
Spain (Gasparini *et al.*, 2000)	1:40	–
Spain (Lucotte, 2007)	<1:149	–
Spain (Rabionet *et al.*, 2000b)	1:43	–
Sweden (Hederstierna *et al.,* 2005)	1:66	–
Turkey (Gasparini *et al.*, 2000)	1:37.5	–
Turkey (Ankara) (Tekin *et al.*, 2001a)	1:78	–
UK and Ireland (Houseman *et al.*, 2001)		1:25.2
United Kingdom (Gasparini *et al.*, 2000)	1:119	–
Estonia (this study)	**1:22**	**1:17**

Research of Caucasian populations show the mutation c.35delG is responsible for an average of 70% of *GJB2* deafness alleles (Snoeckx *et al.*, 2005; Hilgert *et al.*, 2009a) and that the proportion of c.35delG among *GJB2* deafness alleles ranges from 58% to 95% (Zelante *et al.*, 1997; Denoyelle *et al.*, 1999; Janecke *et al.*, 2002; Pampanos *et al.*, 2002; Cryns *et al.*, 2004; Roux *et al.*, 2004; Kalay *et al.*, 2005; Hilgert *et al.*, 2009a) (Table 2).

Table 2: Proportion of c.35delG mutation among patients with nonsyndromic, pre-lingual, SNHL and *GJB2* mutations by population.

Country/ Reference	% of *GJB2* mutations among patients with HL	% of c.35delG homosygosity among *GJB2* genotypes
France (Denoyelle *et al.*, 1999)	39.8	71.4
Italy (Murgia *et al.*, 1999)	39.6	76.3
Italy/Spain (Estivill *et al.*, 1998)	36.8	88.0
USA (Kelley *et al.*, 1998)	34.5	69.8
Israel (Sobe *et al.*, 2000)	33.3	44.1
Lebanon (Mustapha *et al.*, 2001)	33.3	94.0
Greece (Pampanos *et al.*, 2002)	33.3	90.1
Italy/Spain (Rabionet *et al.*, 2000b)	31.6	82.0
Spain/Cuba (del Castillo *et al.*, 2002)	30.6	
USA (Prasad *et al.*, 2000)	27.3	54.2
Austria (Loffler *et al.*, 2001)	18.8	53.7
USA (Kenna *et al.*, 2001)	18.2	11.0

Country/ Reference	% of *GJB2* mutations among patients with HL	% of c.35delG homosygosity among *GJB2* genotypes
UK (Mueller *et al.*, 1999)	14.8	80.4
Germany (Gabriel *et al.*, 2001)	14.3	61.5
Australia (Wilcox *et al.*, 2000b)	13.5	30.4
Japan (Fuse *et al.*, 1999)	10.0	0.0
Korea (Park *et al.*, 2000)	8.2	0.0
Oman (Simsek *et al.*, 2001)	0.0	0.0
Estonia (this study)	**31**	**75**

The relative contribution of the *GJB2* gene to non-syndromic, pre-lingual deafness varies from 0%–39.8% (Table 2) in the populations studied, demonstrating genetic heterogeneity, but some of the studies were based on small numbers of patients, and also the ascertainment criteria and mutation screening methods differed between the studies (Petersen and Willems, 2006).

2.3.1.1.2. p.M34T mutation

The mutation p.M34T was one of the first connexin mutations to be associated with deafness (Kelsell *et al.*, 1997; Skerrett *et al.*, 2004), consistent with the study by White *et al*. (1998). Functional studies of *in vitro* expression systems support the hypothesis of dominant negative effect (White *et al.*, 1998; Skerrett *et al.*, 2004; Snoeckx *et al.*, 2005). Further genetic observations identified individuals with mild to moderate prelingual HL who were homozygous for p.M34T, suggesting it is a recessive *GJB2* allele (Cucci *et al.*, 2000; Houseman *et al.*, 2001; Skerrett *et al.*, 2004).

p.M34T is a missense, non-inactivating mutation, located in transmembrane domain (Rabionet *et al.*, 2000b; Picciotti *et al.*, 2009) but research has yet to provide a clear, unambiguous, effect of the mutation. Most research agree the p.M34T allele is an autosomal recessive pathogenic mutation in presence of other *GJB2* mutations or in homozygous condition (Cohn and Kelley, 1999; Cucci *et al.*, 2000; Houseman *et al.*, 2001; Janecke *et al.*, 2002; Lopponen *et al.*, 2003; Snoeckx *et al.*, 2005; Azaiez *et al.*, 2007), whereas other studies state that this variant is not pathogenic (Green *et al.*, 1999; Griffith *et al.*, 2000; Marlin *et al.*, 2001; Feldmann *et al.*, 2004; Snoeckx *et al.*, 2005). Snoeckx *et al.* (2005) hypothesize that the p.M34T allele is simply a normal variant and that the frequent presence of p.M34T in patients with impaired hearing may be coincidental to high carrier rate of the mutation in the population. If p.M34T is indeed a polymorphism, individuals with the c.35delG/p.M34T genotype are carriers of only one *GJB2* mutation (c.35delG), and their HL must be caused by other unidentified mutations in *GJB2* or by other genes (Snoeckx *et al.*, 2005).

Snoeckx *et al.* (2005) studied 16 individuals with p.M34T homozygosity, of whom most had mild HI, only one patient had moderate and two patients severe HI, the median $PTA_{0.5,1,2kHz}$ was 30 dB. All individuals with c.35delG/p.M34T genotype had mild to moderate HL, with a median $PTA_{0.5,1,2kHz}$ of 34 dB (Snoeckx *et al.*, 2005). Dietz *et al.* (2009) in a study of 42 children with HIH,

finds six children in four families with homozygous p.M34T mutation with mild SNHL and noticed the high rate of homozygous p.M34T mutation among children with HIH. The degree and type of SNHL in the patients of these two studies show quite specific features, suggesting that the p.M34T mutation is indeed pathogenic (Snoeckx *et al.*, 2005; Dietz *et al.*, 2009). Most recent research also regards p.M34T as a pathological variant of connexin 26 associated with HL (Houseman *et al.*, 2001; Bicego *et al.*, 2006; Dietz *et al.*, 2009), however, there is an argument that the genotype-phenotype correlation of the p.M34T mutation needs further investigation (Lopponen *et al.*, 2003; Dietz *et al.*, 2009).

p.M34T represents high carrier rates across populations (see Table 1). p.M34T is even more frequent than c.35delG, but despite this high prevalence in the general population, deaf individuals homozygous for p.M34T mutation have seldom been described (Cucci *et al.*, 2000; Marlin *et al.*, 2001; Lopponen *et al.*, 2003; Roux *et al.*, 2004), and some studies were unable to identify either p.M34T homozygotes or p.M34T/c.35delG compound heterozygotes (Green *et al.*, 1999; Roux *et al.*, 2004). One possible explanation is the high fatality among p.M34T homozygous individuals; fewer p.M34T homozygous children than expected survive (Cohn and Kelley, 1999). The lower frequency of p.M34T, compared with c.35delG, in the patient sample may reflect reduced penetrance or possible ascertainment bias toward more-severe HL, since individuals with mild HL are less likely to see an otorhinolaryngologist for audiologic or genetic testing (Snoeckx *et al.*, 2005).

2.3.1.1.3. Other mutations in *GJB2* gene

High frequencies of *GJB2* mutations other than c.35delG have been reported in other ethnic groups:

- c167delT mutation among Ashkenazi Jews (Morell *et al.*, 1998; Green *et al.*, 1999; Kenneson *et al.*, 2002; Kokotas *et al.*, 2008; Hilgert *et al.*, 2009b);
- 235delC mutation among Asian populations (Fuse *et al.*, 1999; Liu *et al.*, 2002; Ohtsuka *et al.*, 2003; Apps *et al.*, 2007; Kokotas *et al.*, 2008; Hilgert *et al.*, 2009b);
- p.R143W mutation among isolated African subgroups (Brobby *et al.*, 1998; Fuse *et al.*, 1999; Hamelmann *et al.*, 2001);
- W24X mutation among Spanish/Roman (gypsy) and Indian populations (Maheshwari *et al.*, 2003; Minarik *et al.*, 2003; Alvarez *et al.*, 2005; Apps *et al.*, 2007).

These mutations have been estimated to account for 70–91% of recessive HI amongst each of these ethnic groups.

According to the Connexin-deafness homepage[2], over 100 different *GJB2* mutations have been reported (Ballana et al., 2009). Some of those mutations are very frequent while others really rare (Kenneson *et al.*, 2002; Petersen and Willems, 2006; Hilgert *et al.*, 2009b).

[2] http://davinci.crg.es/deafness/

2.3.1.1.4. Genotype-phenotype correlations in *GJB2* mutations

The most common phenotype associated with autosomal recessive *GJB2* mutations in DFNB1, characterized by profound, prelingual SNHL, although interfamilial and intrafamilial variability in the degree and progression of HL has been recorded (Tekin *et al.*, 2001b; Finsterer and Fellinger, 2005). Children with homozygous *GJB2* mutations commonly have congenital HL detected by neonatal hearing screening tests.

Snoeckx *et al.* (2005) carried out the largest study to date involving a cross-sectional analysis of *GJB2* genotype and audiometric data from 1531 individuals from 16 different countries with autosomal recessive, mild-to-profound, nonsyndromic deafness. The stufy identified 83 mutations, 47 were classified as non-inactivating (NI), e.g. missense mutations, and 36 as inactivating (I), e.g. premature stop codons. By classifying mutations this way, the authors defined three genotype classes:

a) **Biallelic inactivating (I/I) mutations**: 1183 (77.3%) of the 1531 individuals studied segregated two inactivating mutations that represented 64 genotypes (36% of all genotypes found). The degree of HI in this cohort was profound in 59%-64% of individuals, severe in 25%-28%, moderate in 10%-12% and mild in 0%-3%;
b) **Biallelic non-inactivating (NI/NI) mutations**: 95 (6.2%) of the 1531 individuals studied segregated two non-inactivating mutations that represented 42 different genotypes (24% of all genotypes found). The degree of HI was mild in 53% of individuals and severe to profound in 20% of individuals;
c) **Compound heterozygous inactivating/non-inactivating (I/NI) mutations**: 253 (16.5%) of the 1531 individuals studied, segregated one inactivating and one non-inactivating mutation that represented 71 different genotypes (40% of all genotypes found). The degree of HI was profound in 24%–30% of individuals and severe in 10%-17% of individuals (Snoeckx *et al.*, 2005; Smith and Van Camp, 2008).

Scatter diagrams were constructed to show the binaural mean pure tone average (PTA) at 0.5, 1, and 2 kHz ($PTA_{0.5,1,2kHz}$) for each person within each genotype class; using individuals homozygous for the c.35delG allele as a reference group:

a) **I/I genotypes**: Only two genotypes differed significantly from the c.35delG homozygote reference group:

i) Individuals doubly heterozygous for *GJB2* c.35delG and del(*GJB6*-D13S1830) had significantly greater HI (median $PTA_{0.5,1,2\ kHz}$ = 108 dB; $p<0.0001$);
ii) Individuals who are *GJB2* compound heterozygotes for c.35delG/IVS1+1G→A had significantly less HI (median $PTA_{0.5,1,2kHz}$ = 64 dB; $p<0.0001$).

b) I/NI genotypes: Nine genotypes differed significantly from the c.35delG homozygote reference group:

i) One *GJB2* compound heterozygous genotype, c.35delG/p.R143W, showed significantly greater HI;
ii) Eight genotypes that had significantly less HI were *GJB2* compound heterozygotes, of which three are:
   – c.35delG/V37I (median $PTA_{0.5,1,2\ kHz}$ = 40 dB; p<0.0001);
   – c.35delG/p.M34T (median $PTA_{0.5,1,2kHz}$ = 34 dB; p<0.0001);
   – double heterozygotes – *del(GJB6)-D13S1830*/*GJB2* p.M34T (median $PTA_{0.5,1,2kHz}$ = 25 dB; p<0.0001).

Five other genotypes are not shown because they are represented by a small number of individuals (n<5) or a large variation in the threshold (SD>25 dB).

c) NI/NI genotypes: Three genotypes differed significantly from the c.35delG homozygote reference group in having less HI:

– p.M34T homozygotes (median $PTA_{0.5,1,2\ kHz}$ = 30 dB, p < 0.0001);
– V37I homozygotes (median $PTA_{0.5,1,2\ kHz}$ = 30 dB, p < 0.0001);
– p.M34T/V37I compound heterozygotes (median $PTA_{0.5,1,2\ kHz}$ = 23 dB, p < 0.0001) (Snoeckx *et al.*, 2005; Smith and Van Camp, 2008).

Snoeckx *et al.* (2005) find that the inactivating mutations of *GJB2* are associated with HL to a greater degree of than non-inactivating mutations. Several of the common genotypes were associated with mild-to-moderate HL, which suggests that complete *GJB2* mutation screening, including IVS1+1G→ A and del(*GJB6-D13S1830*), should be offered to all children with non-syndromic HL, regardless of severity (Snoeckx *et al.*, 2005).

2.3.1.2. *GJB6* gene

The number of deaf people carrying a single *GJB2* mutation is higher than expected and a search for other mutations in or near *GJB2* has led to the identification of two large deletions: del(GJB6-D13S1830) and del(GJB6-D13S1854) (del Castillo *et al.*, 2002).

The *GJB6* gene (MIM 604418) was mapped to chromosome 13q12, in DFNB1 locus, and encodes connexin-30, another component of the gap junction networks of the cochlea (del Castillo *et al.*, 2005). Connexin-26 and connexin-30 are the major connexins expressed in the cochlea, where they co-localize, from heteromeric gap junctions (Ahmad *et al.*, 2003) and play a role in cochlear homeostasis (Zhao *et al.*, 2006; Rodriguez-Paris and Schrijver, 2009). *GJB6* is unique because of its chromosomal localization within 50 kb of *GJB2* (Snoeckx *et al.*, 2005).

In a multicentre study, Del Castillo *et al*. (2003) show that the del(GJB6-D13S1830) mutation is most frequent in Spain, France, the United Kingdom, Israel, and Brazil (5.9–9.7% of all DFNB1 alleles); less frequent in the USA, Belgium, and Australia (1.3–4.5% of all DFNB1 alleles) and very rare in southern Italy (Del Castillo *et al.*, 2003). The deletion was also found in other

studies in the USA and Germany, but not in Austria, Turkey or China (del Castillo *et al.*, 2005). Although the finding of the del(GJB6-D13S1830) mutation provided an explanation for the HI in as many as 30–70% affected *GJB2* heterozygotes in some populations, it has become evident that other DFNB1 mutations remain to be identified in most countries (Del Castillo *et al.*, 2003; del Castillo *et al.*, 2005).

The Δ(*GJB6*-D13S1830) deletion is the second most frequent (after the c.35delG mutation in *GJB2*) genetic cause of nonsyndromic prelingual HI in the Spanish population (del Castillo *et al.*, 2002).

2.3.1.3. *SLC26A5* (prestin) gene

The mammalian inner ear consists of the cochlea and vestibule (Liu *et al.*, 2003). Cochlear hair cells are non-neuronal epithelial cells that transduce acoustic signals. The outer and inner hair cells of the mammalian cochlea perform different functions (Zheng *et al.*, 2000). Outer hair cells (OHC) are sensory cells of the mammalian cochlea, which change their length in response to variations in membrane potential. The transmembrane protein prestin is highly and almost exclusively expressed in the OHC. Prestin is a member of the solute carrier (SLC) gene family 26, which encodes anion transporters and related proteins (Finsterer and Fellinger, 2005). The restricted expression of prestin in the OHC makes it a strong candidate for an association with human deafness. The human *SLC26A5* gene (OMIM number +604943), which encodes prestin, contains 21 exons (GeneID 375611, genome build 36.3). The most abundant adult mRNA isoform of the gene in cochlea, isoform a, is spliced from 20 exons (Liu *et al.*, 2003). In humans, a single nucleotide change – IVS2–2A>G (NM_198999.1:c.-53–2A>G) – in the second intron of the *SLC26A5* gene, has been reported in association with HL (Liu *et al.*, 2003; Tang *et al.*, 2005). There are only two prior reports about IVS2–2A>G in humans (Liu *et al.*, 2003; Tang *et al.*, 2005). However, a relatively high frequency of heterozygosity for this sequence change was observed in affected subjects, suggesting the possibility of a semi-dominant influence of the mutation (Liu *et al.*, 2003).

2.3.1.4. *SLC26A4* (pendred) gene

Pendred syndrome (PDS) as one of the most common types of autosomal recessive syndromic deafness is caused by mutations in *SLC26A4* (previously known as *PDS*) and characterized by sensorineural deafness and goiter. Classically, the deafness is congenital and the thyromegaly becomes apparent after the second decade (Campbell *et al.*, 2001; Smith and Robin, 2002).

Mutations in *SLC26A4* also cause autosomal recessive nonsyndromic SNHL (ARNSHL) at the DFNB4 locus. By definition, individuals with DFNB4 do not have thyromegaly, since no other abnormal physical findings co-segre-

gates with their HL. The HL is associated with temporal bone abnormalities, which range from isolated enlargement of the vestibular aqueduct (dilated vestibular aqueduct, DVA) to Mondini dysplasia. Both abnormalities can be diagnosed by either CT or magnetic resonance imaging (MRI) (Campbell *et al.*, 2001; Smith and Robin, 2002).

SLC26A4 encodes a transmembrane protein pendrin, which functions as a transporter of chloride and iodide and is expressed in the thyroid gland, inner ear, and the kidney (Scott *et al.*, 1999; Petersen and Willems, 2006). Functional studies show that mutations associated with PDS have complete loss of chloride and iodide transport, while mutant alleles in patients with DFNB4 are able to transport both iodide and chloride, although at a much lower level than wild-type pendrin (Scott *et al.*, 2000; Petersen and Willems, 2006). In order to explain the associated temporal bone abnormalities, a hypothesis is that *SLC26A4* controls fluid homeostasis in the membranous labyrinth, which in turn affects development of the bony labyrinth (Campbell *et al.*, 2001; Petersen and Willems, 2006).

Recent data suggest that the major genetic cause of DVA and Mondini dysplasia is mutation in *SLC6A4*. Because simplex cases include both genetic and non-genetic causes of DVA and Mondini dysplasia, mutation screening of *SLC6A4* is clinically useful to establish a diagnosis of Pendred syndrome/ DFNB4 and makes accurate genetic counseling possible. By identifying the subset of individuals carrying *SLC6A4* deafness-causing mutations, a more precise delineation of the clinical phenotype is also possible (Campbell *et al.*, 2001; Smith and Robin, 2002).

2.3.1.5. Mitochondrial mutations

The first genetic defect causing non-syndromic SNHL was detected in 1993 and was a mitochondrial mutation. Various mitochondrial DNA (mtDNA) mutations, causing progressive, nonsyndromic, symmetric bilateral HIH, have since been identified (Fischel-Ghodsian, 1999; Finsterer and Fellinger, 2005).

Mitochondrial mutations are present in less than 1% of the children with pre-lingual deafness (Marazita *et al.*, 1993) but are more frequent at a later age. In Caucasian populations, at least 5% of post-lingual, non-syndromic HI is caused by known mtDNA mutations, representing the most frequent cause of HL after the c.35delG mutation in the *GJB2* gene encoding connexin 26 (Jacobs *et al.*, 2005). In oriental populations the frequency might even be higher (Kokotas *et al.*, 2007).

Mitochondrial genes encoding *12S ribosomal RNA* (rRNA) and the *transfer RNA (tRNA)* genes have been found to be particularly associated with non-syndromic HL (Kokotas *et al.*, 2007).

The A1555G and other mutations in the mitochondrial *12S r-RNA* gene are perhaps the best examples of preventable forms of genetic deafness. These mutations are associated with sensitivity to aminoglycosides, and lead to

deafness if gene carriers are exposed to these antibiotics (Nance *et al.*, 2006). HL associated with aminoglycoside ototoxicity is bilateral and severe to profound, occurring within a few days to weeks after administration of any amount (even a single dose) of an aminoglycoside antibiotic such as gentamycin, tobramycin, amikacin, kanamycin, or streptomycin (Bates, 2003; Pandya, 2007). Once HL appears, HL is irreversible but not progressive. HL associated with the 1555A>G mutation results from hair cell dysfunction and hence is cochlear in nature (Bravo *et al.*, 2006; Pandya, 2007). Although the expression of the mutation is variable, cochlear alterations are present in all carriers of mutation A1555G (Bravo *et al.*, 2006). Some carriers develop HL later in life without aminoglycoside exposure, possibly reflecting genetic modifiers. These mutations are common causes of deafness in Asia where aminoglycosides were used without restraint in the past (Nance *et al.*, 2006).

2.3.1.6. Other genes

The most frequent genes implicated in autosomal recessive non-syndromic HL are *GJB2,* which is responsible for more than half of cases, followed by *SLC26A4, MYO15A, OTOF, CDH23* and *TMC1*. For each of these genes, at least 20 mutations have been reported (Hilgert *et al.*, 2009b).

a) Mutations in the *MYO15A* gene cause congenital severe-to-profound HL at the DFNB3 locus. All 28 identified mutations have been found by linkage analysis in consanguineous families, most of which originate from Pakistan (Nal *et al.*, 2007; Hilgert *et al.*, 2009b). It is likely the number of ARSNHL-causing *MYO15A* mutations is higher, as the gene is large (66 exons) and mutation analysis is rare if complementary linkage analysis has not been performed (Hilgert *et al.*, 2009b).

b) *OTOF* gene mutations cause prelingual, profound ARSNHL, which initially may be accompanied by auditory neuropathy in about half of cases with biallelic *OTOF* mutations (Rodriguez-Ballesteros *et al.*, 2008; Hilgert *et al.*, 2009b). Auditory neuropathy is characterized by the presence of otoacoustic emission (OAE) responses in absence of auditory brainstem responses (ABR). However, as the HL progresses, OHC function is lost, and so is the OAE response. As *OTOF* mutations have been suggested as the major cause of auditory neuropathy, mutation screening of *OTOF* should be considered when OAE responses are present in the absence of ABR responses (Rodriguez-Ballesteros *et al.*, 2008; Hilgert *et al.*, 2009b).

c) Mutations in *CDH23* gene cause both Usher syndrome (US) type 1D and moderate-to-profound progressive ARSNHL at the DFNB12 locus. Genotype-phenotype studies suggest that missense mutations or in-frame alterations cause ARSNHL, while truncating mutations cause USH1D. No single *CDH23* mutation predominates as a cause of either USH1d or ARSNHL (Hilgert *et al.*, 2009b).

d) *TMC1* gene mutations are one of the more frequent causes of ARSNHL in consanguineous populations. Twenty-one different mutations have been reported in 33 consanguineous families, only one of which was Caucasian. All reported cases show a similar phenotype characterized by prelingual severe-to-profound HL (Hilgert *et al.*, 2009b).

In contrast to ARSNHL, where mutations in two genes are frequently found, none of the genes causing autosomal dominant non-syndromic HL (ADNSHL) is a frequent cause of HL. Based on this summary, *WFS1, KCNQ4, COCH* and *GJB2* mutations are slightly more frequent in comparison to the other reported genes. In addition, *WFS1, COCH* and *TECTA* mutations cause HL with a recognizable phenotype (Hilgert *et al.*, 2009b).

e) Mutations in *WFS1* gene cause both autosomal dominant low-frequency SNHL at the DFNA6/14/38 locus and Wolfram syndrome, characterized by autosomal recessive HL, diabetes mellitus, diabetes insipidus and optic atrophy (Kumar, 2009). In addition, *WFS1* may also play a role in the susceptibility to diabetes mellitus and possibly also psychiatric disorders, although its exact role in these disorders needs to be determined (Cryns *et al.*, 2003; Hilgert *et al.*, 2009b). HL caused by dominant *WFS1* mutations is very characteristic, only affecting the low frequencies and rising to normal hearing in the high frequencies. With increasing age, hearing in the high frequencies is lost and the audioprofile flattens. In families segregating LFSNHL, *WFS1* mutations are found in 30–80% of pedigrees, depending on whether the families are pre-selected by linkage analysis prior to WFS1 mutation analysis (Cryns *et al.*, 2002; Lesperance *et al.*, 2003; Fukuoka *et al.*, 2007).

***f) KCNQ4* gene** has been identified as a disease-causing gene at the DFNA2 locus. Twelve different mutations have been reported to date (10 missense mutations and 2 deletions), and a genotype–phenotype correlation has been proposed (Topsakal *et al.*, 2005; Kamada *et al.*, 2006; Hilgert *et al.*, 2009b). Missense mutations are believed to exert a dominant-negative effect by which the mutant protein interferes with the normal channel subunit. These mutations cause HL beginning at a young age and affecting all frequencies. Both deletions, which are proposed to exert a pathogenic effect through haploinsufficiency, cause a milder phenotype, have an older age of onset and affect only the high frequencies (Hilgert *et al.*, 2009b).

g) Seven *COCH* gene mutations have been reported, all of which are missense mutations. This gene encodes for cochlin, a major constituent of the inner-ear extracellular matrix (Grabski *et al.*, 2003; Finsterer and Fellinger, 2005). Six of these mutations cause a phenotype characterized by progressive late-onset HL with vestibular impairment (Kemperman *et al.*, 2005). The late onset and the parallel auditory and vestibular decline make this phenotype very recognizable. One mutation, 1625G>T, causes earlier onset HL, vestibular dysfunction and abnormal ocular motor responses (Street *et al.*, 2005). The P51S mutation is a common cause of late onset cochleovestibular impairment in Belgian and Dutch populations through a founder effect (Fransen *et al.*, 2001; Hilgert *et al.*, 2009b).

h) *TECTA* gene mutations cause both autosomal dominant mid-frequency HL and high-acid substitution. Mutations in the zona pellucida domain of atectorin cause mid-frequency HL, mutations in the zona frequency HL. A genotype–phenotype correlation has been established that is defined by the protein domain in which the mutation occurs and the nature of the amino adherens domain cause high-frequency HL and cysteine (C) replacing substitutions cause progressive HL (Pfister *et al.*, 2004; Hilgert *et al.*, 2009b).

2.3.2. Syndromic hearing impairment

Over 400 genetic syndromes that include HL have been described. If 'deafness' is used a key search word of the London Dysmorphology Database, the result is a list of 633 genetic syndromes and conditions (Winter and Baraitser, 2007). Syndromic HI may account for up to 30% of prelingual deafness, but its relative contribution to all deafness is much smaller, reflecting the occurrence and diagnosis of postlingual HL (Smith and Van Camp, 2007). Some discussion about hereditary syndromic deafness is valuable where an accompanying disorder is not clinically obvious or has a later onset. HL is often detected before the manifestations of other organ system pathology, and a young child with one of these syndromes might be incompletely diagnosed with non-syndromic HL (Friedman and Griffith, 2003).

Although genetic syndromes such as Waardenburg (WS), US, and Jervell and Lange-Nielsen are noted in textbooks to be relatively common in population with HL, in reality these syndromes represent a small proportion of this total population. Nowadays many children with HL and deafness are mainstreamed with hearing children, thought this make more difficult to investigate of children with HL and deafness. In clinical practice this means that many children with HL never have a diagnostic assessment beyond audiometric testing (Billings and Kenna, 1999; Kenna *et al.*, 2001).

Performing a syndrome diagnosis of every individual with HI is significant, as Bitner-Glindzicz (2002) explains:

- The individual and family need to be monitored for known complications and associations of the syndrome, such as heart, renal or eye disease;
- Inheritance may be clearly defined for many syndromic causes of deafness even the gene is unknown;
- Molecular testing, which may confirm the diagnosis, may be available for many of the more common syndromes.

Syndromic HL is the most frequently categorized by the mode of inheritance (Smith and Van Camp, 2007).

2.3.2.1. Waardenburg syndrome (WS)

WS is the most common type of autosomal dominant syndromic HL (Smith and Van Camp, 2007). The syndrome described so precisely by Waardenburg in 1951 (Waardenburg, 1951), was reported by earlier researchers, e.g. van der Hoeve in 1916, Mende in 1926 and Klein in 1947 (Pantke and Cohen, 1971; Gorlin *et al.*, 2001). WS consists of variable degrees of SNHL and pigmentary abnormalities of the skin, hair (white forelock), and eyes (heterochromia iridis). Because affected individuals may dye their hair, the presence of a white forelock should be specifically sought in the history and physical examination. Four types are recognized – WS I, WS II, WS III, and WS IV – based on the presence of other abnormalities. WS I (OMIM 193500) and WS II (OMIM 193510, 600193, 606662, 608890, 611584) share many features but have an important phenotypic difference: WS I is characterized by the presence of dystopia canthorum (i.e., lateral displacement of the inner canthus of the eye) while WS II is characterized by its absence. In WS III, upper-limb abnormalities are present, and in WS IV, Hirschsprung disease is present. Recent reports have suggested that the frequency of HL in WS I is 58–75% and in WS II, 78–91% (Newton, 1990; Liu *et al.*, 1995; Oysu *et al.*, 2000; Toriello *et al.*, 2004). The extent of HL is quite variable, ranging from no measurable clinical loss to severe congenital unilateral or bilateral SNHL (Hageman, 1977; Hildesheimer *et al.*, 1989; Newton, 1990; Toriello *et al.*, 2004). Bilateral HL is more common (Newton, 1990; Toriello *et al.*, 2004). Mutations in *PAX3* gene cause WS I and WS III. Mutations in *MITF* gene cause some cases of WS II. Mutations in *EDNRB*, *EDN3*, and *SOX10* genes cause WS IV (Smith and Van Camp, 2007).

2.3.2.2. Branchio-oto-renal (BOR) syndrome

BOR syndrome (OMIM 113650) is the second most common type of autosomal dominant syndromic HL (Smith and Van Camp, 2007). The term BOR was first used in 1975 by Melnick *et al.* (1975) to refer to patients with branchial cleft, fistulas, or cysts; otologic anomalies, including malformed pinnae, preauricular pits or sinuses, and HL; and renal anomalies of various types. Many other features have subsequently been noted. BOR syndrome prevalence is about 1:40,000 and it is thought to occur in about 2% of profoundly affected children (Fraser *et al.*, 1980; Smith and Van Camp, 2007). Penetrance of BOR syndrome is high, but expressivity is extremely variable (Smith and Van Camp, 2007). HL has been reported in about 75% of cases. CHL is found in 30%, SNHL in 20%, and mixed HL in 50% of patients with BOR syndrome. Age of onset of HL varies from early childhood to young adulthood. HL may also be progressive (Toriello *et al.*, 2004). In approximately 40% of families segregating a BOR phenotype, mutations in the *EYA1* gene can be identified; in a few other families mutations have been found in *SIX1* (Ruf et al., 2004), consistent with the known interaction of EYA1 and SIX1 proteins in transcription regulation (Smith and Van Camp, 2007).

2.3.2.3. Stickler syndrome

Stickler syndrome is a quite common type of autosomal dominant syndromic HL (Smith and Van Camp, 2007). Printzlau and Andersen (2004) estimate that the incidence of Stickler syndrome among neonates is approximately 1:7,500–1:9,000. The syndrome of flat midface, ceft palate, high myopia with retinal detachment and cataracts, HL, and arthropathy with generally mild spondyloepiphyseal dysplasia was described by Stickler and coworkers in 1965–1967 (Stickler *et al.*, 1965; Toriello *et al.*, 2004). The syndrome is now known as Stickler syndrome I type (OMIM 108300), and constitutes 75% of Stickler syndromes (Gorlin *et al.*, 2001; Toriello *et al.*, 2004). A combination of eye findings, HL, cleft palate, marfanoid build, and bone changes are characteristic features of the I type syndrome (Toriello *et al.*, 2004). Stickler syndrome II type (OMIM 604841), representing another 25%, is characterized by midface hypoplasia, anteverted nostrils, small mandible, SNHL (40%) or mixed HL (30%), and joint pain (50%). Height is not reduced and there is mild myopia (Gorlin *et al.*, 2001; Toriello *et al.*, 2004). Progressive SNHL has been noted in 60% of Stickler syndrome type I patients and in 90% of Stickler syndrome type II (Toriello *et al.*, 2004). Mutations affecting one of four genes (*COL2A1*, *COL9A1, COL11A1*, and *COL11A2*) have been associated with Stickler syndrome; because a few families with features of Stickler syndrome are not linked to any of these four loci, mutations in other genes may also cause the disorder (Robin *et al.*, 2009). About 70% of cases of Stickler syndrome type 1 are due to mutations in the *COL2A1* gene (Gorlin *et al.*, 2001). Mutations of the *COL11A1* gene lead to either or both Stickler syndrome II type and Marshall syndrome (Snead and Yates, 1999; Gorlin *et al.*, 2001). Non-ocular 'Stickler-like' syndromes were mapped near the *COL11A2* gene (Brunner *et al.*, 1994; Gorlin *et al.*, 2001). These syndromes have been collectively called oto-spondylo-megepiphyseal dysplasia and while the symptoms resemble Stickler syndrome, midface hypoplasia is more expressed and patients are small, have large epiphyses and lack eye involvement (Gorlin *et al.*, 2001).

2.3.2.4. LEOPARD syndrome (LS)

LS (OMIM 151100) is a rare autosomal dominant multisystemic disorder with full penetrance and variable expressivity (Gorlin *et al.*, 1969). Nevertheless, LS diagnostic clues are cutaneous manifestations, including multiple lentigines (ML) and *café-au-lait* spots (CLS), hypertrophic cardiomyopathy (HCM) and deafness (Digilio *et al.*, 2006). Although LS seems to be under diagnosed or misdiagnosed due to its mild features and the absence of lentiginosis, about 200 patients have so far been reported worldwide (Sarkozy *et al.*, 2008). SNHL has been observed in 15–25% of patients with LS. There is marked degree of HL in affected individuals, but mostly the HL is mild. HL is usually of childhood onset, but can develop during adulthood (Toriello *et al.*, 2004). LS is mainly

caused by missense mutations in the *PTPN11* gene, encoding SHP-2, a cytoplasmic protein tyrosine phosphatase (PTP), which regulates intracellular signalling for several growth factors, cytokine and hormone receptors that control distinct developmental processes (Tartaglia *et al.*, 2001). However, mutations in the *RAF1* (Pandit *et al.*, 2007) and *BRAF* (Koudova *et al.*, 2009; Sarkozy *et al.*, 2009) genes have recently been described as being associated with a LS phenotype. In more than 85% of LS cases, a heterozygous missense mutation is detected in *PTPN11* exons 7, 12 or 13. So far only 11 heterozygous *PTPN11* mutations have been reported in LS patients (Y279C, Y279S, A461T, G464A, T468M, T468P, R498W, R498L, Q506P, Q510E, Q510P) (Sarkozy *et al.*, 2008).

2.3.2.5. Usher syndrome (US)

US is the most common type of autosomal recessive syndromic HL (Smith and Van Camp, 2007). US is characterized by retinitis pigmentosa and SNHL (Toriello *et al.*, 2004). The German ophthalmologist Albrecht von Graefe described the association of congenital HL and progressive pigmentary dystrophy of retina in 1858, and Charles Usher, a British ophthalmologist, provided extensive clinical documentation of affected individuals. In particular, Usher emphasized the autosomal recessive pattern of inheritance in 1914 (Keats and Corey, 1999). The epidemiological studies indicate that the average prevalence of US among the deaf population is 10%. Three types of US are recognized based on the degree of HI and result of vestibular function testing: US I type (OMIM 276900, 276904, 601067, 612632, 602083, 602097, 606943, 605242) is characterized by congenital severe-to-profound HL with development of retinitis pigmentosa by age 10 years and absent vestibular responses. In US II type (OMIM 605472, 276901, 611383) there is usually stable congenital moderate HL in the low frequencies sloping to severe or profound HL in the higher frequencies with onset of retinitis pigmentosa from the mid-teens to early 20's and normal vestibular responses. US type III (OMIM 276902) patients have progressive HL with progressive vestibular involvement and variable severity of retinitis pigmentosa (Smith *et al.*, 1994; Kimberling and Moller, 1995; Keats and Corey, 1999; Toriello *et al.*, 2004). The recognition of US is difficult in infants and young children. Nevertheless, five of the nine Usher genes also cause non-syndromic HL (Hilgert *et al.*, 2009b). The DNA analysis for 614 mutations in the 9 genes linked to US (*CDH23, MYO7A, PCDH15, Harmonin, SANS, Usherin, VLGR1, USH3A* and *Whirlin*) is available in Estonia by Asper Biotech[3] (Cremers *et al.*, 2007).

[3] http://www.asperbio.com

2.3.2.6. Pendred syndrome (PDS)

PDS (OMIM 274600) is the second most common type of autosomal recessive syndromic HL (Toriello *et al.*, 2004). Although the first recorded case may well be that of Mondini in 1791, the syndrome is eponymously named for Pendred's report in 1896 of a pair of sisters with goiter and profound congenital HL (Toriello *et al.*, 2004). The syndrome is characterized by congenital severe-to-profound SNHL and euthyroid goiter. Goiter is not present at birth and develops in early puberty (40%) or adulthood (60%). Delayed organification of iodine by the thyroid can be documented by a perchlorate discharge test. The deafness is associated with an abnormality of the bony labyrinth (Mondini dysplasia or dilated vestibular aqueduct) that can be diagnosed by CT examination of the temporal bones. Vestibular function is abnormal in the majority of affected individuals (Smith and Van Camp, 2007). HL is severe in more than 50% of patients. Speech development is generally poor because of the early severe HL (Toriello *et al.*, 2004). Mutations in *SLC26A4* are identified in about 50% of multiplex families. Such genetic testing is appropriate for individuals with Mondini dysplasia or an enlarged vestibular aqueduct and progressive HL (Smith and Van Camp, 2007). Early studies reported that PDS accounted for up to 7.5% of congenital deafness (Fraser, 1965), but contemporary studies suggest that the prevalence of PDS is lower (Park *et al.*, 2003); mutations of the *SLC26A4* gene are also a cause of nonsyndromic HL (DFNB4) (Smith and Van Camp, 2007).

2.3.2.7. Jervell and Lange-Nielsen syndrome

Jervell and Lange-Nielsen syndrome (OMIM 220400, 612347) is the third most common type of autosomal recessive syndromic HL (Smith and Van Camp, 2007). In 1957, Jervell and Lange-Nielsen described profound congenital SNHL, electrocardiographic abnormalities characterized by a long QT interval, repeated syncopal episodes, and sudden unexplained death in Norwegian children (Jervell and Lange-Nielsen, 1957; Toriello *et al.*, 2004). The syndrome consists of congenital deafness and prolongation of the QT interval as detected by electrocardiography [the abnormal QTc (c=corrected) is greater than 440 msec]. Affected individuals have syncopal episodes and may have sudden death. Although a screening electrocardiogram is not highly sensitive, it may be suitable for screening deaf children. High-risk children (i.e., those with a family history that is positive for sudden infant death (SIDS), syncopal episodes, or long QT syndrome) should have a thorough cardiac evaluation. Mutations in two genes have been described in affected individuals: *KCNQ1* and *KCNE1* gene (Sakagami *et al.*, 1991; Neyroud *et al.*, 1997; Steel and Bussoli, 1999).

2.3.2.8. Alport syndrome (AS)

AS is characterized by progressive SNHL of varying severity, progressive glomerulonephritis leading to end-stage renal disease, and variable ophthalmologic findings (i.e., anterior lenticonus) (Smith and Van Camp, 2007). The syndrome was first described by Alport in 1927 (Alport, 1927). Classic AS represents a defect in type IV collagen. AS is now known to represent a genetically heterogeneous group of at least six different disorders that exhibit unique ultrastructural and antigenic abnormalities in basement membranes due to defective type IV collagen (Toriello *et al.*, 2004). Autosomal dominant (OMIM 104200), autosomal recessive (OMIM 203780), and X-linked (OMIM 301050) forms are described. X-linked inheritance accounts for about 85% of cases (*COL4A5, COL4A6, FACL4, AMMERC1* genes), and autosomal recessive inheritance accounts for about 15% of cases (*COL4A3* and *COL4A4* genes) (Toriello *et al.*, 2004). Autosomal dominant inheritance has been reported occasionally. HL usually does not manifest before the age of ten years (Smith and Van Camp, 2007).

2.4. The identification of new rare loci of HL

During the last fifteen years, major achievements have been made in detecting new deafness genes. These achievements have far outpaced translation of this knowledge to improved clinical care. The cap created reflects the fact that diagnostic tests are still performed using the classical sequencing technology. These automatic DNA sequencers are very useful but are limited by slow throughput, which makes extensive sequencing of all known deafness-causing genes very expensive and time consuming. For this reason, only a very small set of genes is routinely screened for mutation with result that in a large percentage of individuals with HL, no genetic cause is identified (Hilgert *et al.*, 2009b).

An additional problem for extended diagnostic screening is that most genes for ARSNHL lead to congenital severe-to-profound HI that is indistinguishable between different genes. The similarity of phenotype makes it impossible to predict the genetic cause based on audioprofiling for almost all cases. This situation highlights the urgent need for new DNA sequencing technologies which allow complete screening of all deafness genes in a quick and cost-effective way (Hilgert *et al.*, 2009b).

Another option is chromosomal microarray analysis (CMA), which is increasingly utilized for genetic testing of individuals with unexplained developmental delay/intellectual disability, autism spectrum disorders, or multiple congenital anomalies (Hochstenbach *et al.*, 2009; Miller *et al.*, 2010). CMA-based cytogenetics affords the capacity to examine the whole human genome on a single chip with a resolution as high as a few hundred base pairs (Pinkel *et al.*, 1998).

2.5. Acquired HL

Congenital acquired or later acquired SNHL may be caused by prenatal, perinatal or postnatal environmental factors. Prenatal etiology includes rubella, cytomegalovirus (CMV) infection, measles, toxoplasmosis, herpes zoster, varicella zoster, syphilis, diabetes mellitus, alcohol, drugs. Perinatal etiologies include prematurity, asphyxia, anoxia, meconium aspiration, hyperbilirubinaemia, prolonged neonatal intensive care treatment, drugs. Postnatal etiology includes meningitis, sepsis, aminoglycosides, trauma, chemotherapy, measles, varicella zoster, herpes zoster (Morzaria *et al.*, 2004; Dietz *et al.*, 2009).

The most common environmental (non-genetic) cause of HL is congenital CMV infection (Hilgert *et al.*, 2009b). Although the overall birth prevalence is 0.64%, only 11% of infected infants have non-specific symptoms at birth, and the definition of symptomatic varies between studies (Kenneson and Cannon, 2007). Neonatal signs of congenital CMV infection include jaundice, hepatosplenomegaly, petechiae, microcephaly, and chorioretinitis. There are also many less common non-specific symptoms of congenital CMV infection. CMV is the most common potentially disabling perinatal infectious disease. In 10–15% of infants with congenital CMV infection, who are asymptomatic at birth, and all neonates with symptoms of congenital CMV, infection causes persistent problems to develop, of which the most common are neurologic impairment, SNHL and decreased vision (van der Knaap *et al.*, 2004). HL is a common sequel to congenital CMV infection.Grosse *et al.* (2008) found that HL occurs in 30–40% of children symptomatic at birth with congenital CMV and in 5–10% of children with asymptomatic infections. More than 67% of cases of HL among children who were congenitally infected with CMV develop HL months or years after birth and may therefore be missed by a hearing screening at birth (Fowler *et al.*, 1999; Ludwig and Hengel, 2009). Fowler *et al.* (1999) estimate that universal screening of hearing in neonates will detect less than half of all SNHL caused by congenital CMV infection. Congenital CMV is an important but underestimated cause of HL (Grosse *et al.*, 2008). Several authors found that children with congenital CMV have a risk of up to 22% of developing SNHL (Barbi *et al.*, 2006b; Foulon *et al.*, 2008a, b). HL can be either unilateral or bilateral and vary from mild to profound. Children with symptomatic infection have HL at an earlier age and with greater severity than children with asymptomatic infection (Fowler *et al.*, 1999). Grosse *et al.* (2008) estimate that congenital CMV infection is one of the most important causes of HL in young children after genetic mutations in connexin genes.

The gold-standard to detect congenital CMV infection at birth is viral culture or polymerase chain reaction (PCR) from urine or saliva within the first two weeks of life (Ludwig and Hengel, 2009). Retrospective diagnosis of congenital infection is possible by PCR detection of the CMV DNA in dried blood spots (DBS) stored on perinatal Guthrie cards (Barbi *et al.*, 1996; Barbi *et al.*, 2006a; Vauloup-Fellous *et al.*, 2007). The DBS test is simpler, faster and less costly than viral isolation; in addition the samples can be safety stored for

long periods, so diagnosis can be made even after several years (Barbi *et al.*, 2000; Barbi *et al.*, 2006a).

2.6. Newborn hearing screening

Early diagnosis of bilateral SNHL prevents poor language skills and the development of speech delay (Morzaria *et al.*, 2004). Researchers estimate that more than 80% of HL in children is either congenital or acquired in the neonatal period (Johnson *et al.*, 2005; Jakubikova *et al.*, 2009). The first step for early detection of HL in newborns in any country is universal newborn hearing screening program (UNHS) (Jakubikova *et al.*, 2009). UNHS has lowered the age of detection of HL. The early detection of hearing impaired infants and children was initially emphasized in the late 1960s, which resulted in the implementation of hearing screening programs in the 1970s either as universal hearing screening or as targeted groups (Parving, 2007).

Two methods are used in newborn hearing screening: OAE and automated ABR. In most European countries UNHS uses a two-stage OAE program (Johnson *et al.*, 2005; Jakubikova *et al.*, 2009). In Estonia, a three-stage UNHS is used: Stage 1 – OAE at the age of 3–4 days in a maternity hospital; Stage 2 after a negative result, OAE is repeated at the age of one month in a maternity hospital, and Stage 3, after a second negative result either OAE or automated ABR occurs at the age of 3 months in the Otorhinolaryngology Department.

In babies who are diagnosed with severe-to-profound HL in the absence of other abnormal findings on physical examination, the single best next diagnostic test is mutation screening for deafness at DFNB1 locus. This screening should include DNA sequencing of *GJB2* and mutation testing for the two large *GJB6*-containing deletions, as *GJB2* mutations cause about half of all genetic HL cases (Hilgert *et al.*, 2009b).

2.7. Genetic counseling of patient with HL

An understanding of the attitudes of normally hearing parents (90–95% of the cases) of deaf children is necessary for optimal counseling strategies. The majority of these parents approves of genetic testing and believes that the test should be offered prenatally (Brunger *et al.*, 2000). The vast majority of these parents have inaccurate beliefs about their own and children's recurrence risk, irrespective of whether or not they have undergone genetic testing. One-third of these parents believe that the absence of a *GJB2* mutation generally excludes heredity of their children's deafness. They do not understand that HIH has a complex, heterogeneous background. Consequently both pre-test and post-test genetic counseling should be provided (Finsterer and Fellinger, 2005).

Genetic diagnosis, carrier detection, and reproductive risk counseling can be provided only for a limited number of affected individuals, since mutation screening is available for only limited number of genes. The identification of further genes will not only improve early diagnosis but may also offer future opportunities for a rational therapy. With the discovery of new technologies, genetic testing for HIH may become a routine service of otolaryngologists (Finsterer and Fellinger, 2005).

Establishing a genetic diagnosis has been shown to be beneficial to parents as it alleviates parental guilt and anxiety, makes accurate recurrence change counseling possible, and provides prognostic information. For example, the parents of a child with *GJB2*-related deafness have a 25% recurrence risk to have another child with the same *GJB2* genotype. Furthermore, if their first child has mild-to-moderate HL and they do have a second hearing-impaired child, there is a 66% chance that the second child will have mild-to-moderate HL and a 34% change that the HL will be more severe. If their child has severe-to-profound HL and receives a cochlear implant, several studies have shown that the parents can expect their child to have an excellent outcome (Connell *et al.*, 2007; Hilgert *et al.*, 2009b).

The deaf population can be broadly divided into those individuals who identify themselves with the Deaf community and those who do not, making the provision of genetic counseling to this population particularly challenging. Individuals in the Deaf community identify themselves with the Deaf culture. As with other cultures, members are characterized by unique social and societal attributes and a unique language, Sign Language. They do not consider themselves to be hearing "impaired", nor do they feel that they have a hearing "loss". Rather, they consider themselves deaf. Their deafness is neither a pathology nor a disease to be treated or cured. If a Deaf individual seeks genetic counseling, sessions will be hampered without the aid of a skilled interpreter. The objectives of the Deaf individual may seem foreign, since they view deafness as a distinguishing characteristic and not as a handicap, impairment, or medical condition requiring "treatment," "cure," or "prevention." For example, just as a normal-hearing couple might "prefer" to have a normal-hearing child, a Deaf couple might "prefer" to have a deaf child. This preference does not preclude Deaf individuals from having an interest in learning about the cause of their deafness and obtaining information on medical, educational, and social services, rather than information about prevention, reproduction, or family planning. As in all genetic counseling, it is important for the counselor to identify, acknowledge, and respect each family's questions, concerns, and fears (Smith and Robin, 2002).

Prenatal diagnosis for some forms of HIH is technically possible by analysis of DNA extracted from foetal cells obtained by amniocentesis usually performed at about 15–18 weeks' gestation or chorionic villus sampling (CVS) at about ten to 12 weeks' gestation. The deafness-causing allele(s) of a deaf family member must be identified before prenatal testing can be performed. Requests for prenatal testing for conditions such as HL are not common.

Accurate interpretation of a positive prenatal test result is difficult, because in some cases the presence of the mutation does not predict the severity of HL. Differences in perspective may exist among medical professionals and within families regarding the use of prenatal testing, particularly if the testing is being considered for the purpose of pregnancy termination rather than early diagnosis. Although most centers would consider decisions about prenatal testing to be the choice of the parents, careful discussion of these issues is appropriate (Pandya, 2007; Smith and Van Camp, 2007).

3. AIMS OF THE PRESENT STUDY

The aims of the present study were:

1. To establish the prevalence of the c.35delG and p.M34T mutations in the *GJB2* gene among the Estonian newborn population.
2. To determine the prevalence of mutations in *GJB2, GJB3, GJB6, SLC26A4* and two the mitochondrial genes (*12S rRNA* and *tRNA-Ser*) in Estonian children with hearing loss.
3. To study the genotype and phenotype correlations in patients with *GJB2* gene mutations.
4. To identify the IVS2–2A>G sequence change in the *SLC26A5* (Prestin) gene in Estonian individuals with hearing loss and in their family members.
5. To characterize rare genetic syndromic hearing loss.
6. To evaluate the occurrence of small submicroscopic chromosomal rearrangements in children, when hearing loss is one of the main complaints.
7. To evaluate the incidence of CMV infection in Estonian children with hearing loss using molecular analysis.

4. MATERIAL AND METHODS

4.1. Study subjects

4.1.1. Study group of newborn children

We screened a cohort of 998 samples from anonymous newborns which were partially dissociated (Group NB). These neonates were born consecutively in January 2005, throughout Estonia. We screened these samples for the presence of the two *GJB2* mutations, c.35delG and p.M34T. DNA was obtained from anonymous samples of DBS on Guthrie cards, which Estonia's newborn screening program for detecting cases of phenylketonuria and congenital hypothyreosis had collected. In order to facilitate interpretation of geographical gradients, we separated the birthplaces of the selected newborns into three distinct regions of Estonia: northern, western and south-eastern (see Figures 1 and 2).

4.1.2. Study group of children with HL

The overall study group consisted of 233 probands (children ranging in age from 0–18 years) who were referred to genetic counselling from January 2000 to March 2009 from the whole of Estonia, with early or childhood onset HL as a main complaint (Group HL^{233}). The diagnosis of HL was confirmed by audiologists in the hearing centres of Estonia. All the probands in Group HL^{233} were selected from children who were referred to an oto-rhino-laryngologist due to a suspicion of HL or were selected from the newborn hearing screening (NBHS) program.

The NBHS program started in Estonia in 2004 and by 2009 88% of all newborns were included in the program. In Estonia we have three-stage UNHS (see section 2.6 for details). All children whose HI with a $PTA_{0.5–4kHz}$ is less than 40 dB in the better ear should be identified by UNHS. The definition of the degree and type of HI was based on the most recent audiogram available. The severity of HI was graded by the degree of HL in the better ear as mild (21–40 dB), moderate (41–70 dB), severe (71–95 dB) and profound (greater than 95 dB).

All children in Group HL^{233} were evaluated in one of two tertiary education hospitals, Tallinn Children's Hospital for northern and western Estonia and Tartu University Hospital for south eastern Estonia. In all cases of HL, family histories were collected, focusing particularly on the potential occurrence of HL in multiple generations. The clinical examination was performed with particular attention to dysmorphic features including growth parameters, facial phenotype, external ears, neck, skin, hair, eyes and digits, to exclude syndromic causes of

HL. In the case of 55 children (Group HL^{55}), a geneticist decided that regular chromosomal analysis should be performed from peripheral blood lymphocytes.

All probands in Group HL^{233} were tested between 2005 and 2009 with an arrayed primer extension (APEX) assay (Asper Biotech, Tartu, Estonia) (Gardner *et al.*, 2006) in the Department of Genetics of the United Laboratories of Tartu University Hospital. Thirty-two patients were analyzed before 2005 for c.35delG mutation in *GBJ2* gene by PCR analysis; if the patients were homozygous for mutation c.35delG, APEX array analysis was not performed.

From 126 probands whose etiology of HL remained unknown after DNA testing with the APEX method, 96 patients were chosen for the DNA analysis of CMV infection from neonatal screening cards - blood stored on Guthrie cards – (Group HL^{96}). Since the neonatal screening program for the whole of Estonia for phenylketonuria started in 1993, neonatal screening cards were available for 85 (88.5%) of Group HL^{96}.

From 121 probands with HL of unknown etiology (normal results in APEX array and CMV testing) we selected 24 children who, in addition to HL, had subtle facial dysmorphism, and either or both a failure to thrive and developmental or behavioral problems and who did not fit to any known dysmorphic syndrome (Group HL^{24}). For exclusion of syndromic etiology we used the London Dysmorphology Database (Robin M. Winter, 2007).

4.1.3. Patients with syndromic HL

We selected, from another research study entitled "The genetic causes of mental retardation in Estonia" (Puusepp, 2009), one case with dysmorphic phenotype and early onset HL from the non-specific mental retardation group of 11 patients. This patient was investigated further to discover the exact aetiology of their hearing phenotype.

One patient with clinical suspicion of LS and HL was later excluded from our patients' group (see 4.1.2.), as their HL was not confirmed by automated ABR investigation. However, this patient was studied further for the *PTPN11* gene mutation, which causes LS. This patient is described as a part of this study (Section 5.6.1).

4.2. Methods

4.2.1. APEX assay for SNHL

An EDTA blood sample (2–5 ml) was taken for DNA analysis for all children in Group HL^{233}. DNA was extracted from peripheral blood leukocytes using the standard salting-out method.

DNA samples were tested with an APEX assay (Asper Biotech, Tartu, Estonia) (Gardner *et al.*, 2006). This microarray is capable of simultaneous

evaluation of 199 mutations: several connexin genes (*GJB2, GJB6, GJB3*), mutations in two SLC26 anion transport genes (*SLC26A4* and *SLC26A5*), and mutations in two mitochondrial genes (*12S rRNA* and *tRNA-Ser*). The APEX assay is based on a versatile platform technology and is a robust, cost-effective, sensitive and easily modifiable assay. This assay validated patients' genomic DNA samples with confirmed mutations and also with 45-mer synthetic DNA templates. A complete description of this APEX assay including a list of the 198 mutations covered is published by Gardner *et al*. (2006). All the c.35delG and p.M34T mutations we found were confirmed by PCR analysis, as described below. All other APEX detected mutations were confirmed by sequence analysis. All investigated mutations are given in Supplementary Table 1, Publication I.

4.2.2. PCR analysis for c.35delG and p.M34T mutations in *GJB2* gene

Group NB was tested for *GJB2* gene c.35delG and c.101T>C (p.M34T) mutations using PCR amplification and restriction fragment length analysis. Genomic DNA was extracted from whole blood using the standard salting-out method. c.35delG mutation analysis was adapted from Wilcox *et al*. (Wilcox *et al.*, 2000a; Wilcox *et al.*, 2000b). GJB2 c.35delG mutation was detected with 5'-GCTGGTGGAGTGTTTGTTCACACCCGC-3' forward and 5'-TCTTTTC CAGAGCAAACCGC-3', the forward primer containing a mismatch base and creating an MvaI restriction site for the c.35G allele amplification product. c.101T>C (p.M34T) mutation was detected with 5'-CCCTGTTCTGTCC TAGCTAGTGATT-3' forward and 5'-CTCCTTTGCAGCCACAACGAT GAT-3' reverse primers, the reverse primer containing a mismatch base and creating a BclI restriction site for the p.M34 allele amplification product. The *GJB2* gene two mutation sites were amplified in separate reactions in a BioRad PTC200thermal cycler (Bio-Rad Laboratories[4]) as follows: 3 minutes 30 seconds initial denaturation, 36 cycles of 35 seconds at 95°C, 40 seconds at 62°C, 40 seconds at 72°C followed by 7 minutes final elongation at 72°C. 30µl PCR amplification reactions consisted of 3µl 10x PCR buffer, 2.5mM $MgCl_2$, 12pmoles of both amplification primers, 3µl of 2mM dNTPmix, 5 units of Hot Start Taq DNA polymerase (Fermentas, Inc., Vilnius, Lithuania) and 100ng genomic DNA. The amplification products were digested with MvaI (c.35delG) and BclI (p.M34T) (Fermentas, Inc.) and analysed in agarose gel electro-phoresis.

[4] http://www.bio-rad.com/

4.2.3. Cytogenetic investigation

Cytogenetic investigation was carried out using peripheral blood lymphocyte culture analysis. The chromosomes were prepared from 72-hour cultures and banded by using standard GTG (G bands by Trypsin using Giemsa) banding technique. The chromosomes were classified according to the International System for Human Cytogenetic Nomenclature (ISCN, 1995).

4.2.4. Illumina analysis

In order to detect the copy number changes in the selected patients of Group HL[233], whole-genome genotyping was performed, using the Illumina HumanCytoSNP-12 version 1.0 BeadChip or Human370CNV-Duo BeadChips (Illumina Inc., San Diego, CA, USA[5]).

1) Illumina HumanCytoSNP-12 version 1.0 BeadChip includes 301,232 markers that cover the whole genome with median spacing of 6.2 kb, and includes SNPs targeting all regions of known cytogenetic importance.
2) HumanCNV370-Duo has coverage of 52,167 markers from 14,000 CNV regions of the genome, with coverage of 90 Mb of DNA. More than 318,000 tag SNP markers have been selected using the International HapMap Project Phase I and II data, so that each HumanCNV370-Duo BeadChip genotypes more than 370,000 loci. The median SNP spacing is 5.0 kb (7.9 kb mean).

The signal intensity (log R ratio) and allelic composition (allelic frequency) of genotyped markers were analyzed with Illumina GenomeStudio version 2009.1 and Illumina KaryoStudio version 1.0.3 software (Illumina, Inc.). In addition, QuantiSNP version 1.0 (Colella *et al.*, 2007) was used to confirm the copy number variations found with Illumina software packages, and also to find new copy number changes.

The found regions were compared with the reference sample-set (1000 population-based samples from Estonia (Nelis *et al.*, 2009) to exclude population specific variations.

4.2.5. Real time PCR analysis

Quantitative PCR (qPCR) study was applied to confirm the detected aberrations and to verify if these are inherited or not. Analysis was performed on a 7900HT Real-Time PCR system (Applied Biosystems) using SYBR-Green based detection. Primers were designed using web-based service qRTDesigner 1.2[6]. For confirmation of each aberration, a total of eight primer pairs were designed: four outside the deletion and four within the deleted area. To eliminate non-

[5] http://www.illumina.com/
[6] http://bioinfo.ut.ee/gwRTqPCR/

specific variations, such as differences in DNA input amounts or presence of PCR inhibitors, C_t values were normalized using the C_t values of a reference region (from chromosome 1) with a known copy number. Analysis was performed as a relative quantification using the Pfaffl method (Pfaffl, 2001) for calculation.

4.2.6. CMV PCR analysis

DNA was extracted from a Guthrie test card specimen using QIAamp DNA Mini Kit (50) (Cat. No. 51304) using the manufacturer's instructions (Qiagen). The CMV copy number was established by Real-Time PCR method using artus CMV Kit CE (Qiagen)[7].

4.2.7. Statistical analysis

We used the FREQ procedure and Fisher's Exact test of the SAS system, Release 8.2 (Sas Institute, 1999) to analyze the distribution of genotypes among Groups NB and HL^{233} and for the distribution of genotypes in the three regions of Estonia. We used the Maximum Likelihood (ML) to estimate the probability of p.M34T and c.35delG alleles under the Hardy-Weinberg equilibrium (HWE) assumption. On this basis, we calculated the probabilities of different genotypes. Based on the HWE assumption, the observed and expected frequencies of genotypes among Groups NB and HL^{233} with specific symptoms were evaluated. P-values (probability) were calculated using multinomial probability distribution with the ML estimates for allele probabilities in Group HL^{233} and in Group NB's sample.

4.2.8. Ethical considerations

This study was approved by the Ethics Committee on Human Research at the University of Tartu. Informed consent was obtained from the parents or legal guardians of the children, none of whom refused to participate in this study.

[7] http://www.qiagen.com/

5. RESULTS AND DISCUSSION

5.1. *GJB2* gene

5.1.1. Study group of newborn children (Publication I)

We tested blood samples from 998 anonymous Estonian neonates from general population (Group NB). We detected 58 heterozygotes for the p.M34T mutation, and zero p.M34T homozygotes, indicating a carrier frequency of 1:17 (Table 3). We also found 45 heterozygotes and two homozygotes for the c.35delG mutation, giving a carrier frequency 1:22 (Table 3). Two of Group NB were found to be compound heterozygotes for the c.35delG and p.M34T mutations. Using this data, the theoretical prevalence of c.35delG homozygotes by HWE should be 1:1936, and the prevalence of p.M34T homozygotes should be 1:1156. In Group NB, we identified four individuals having pathogenic genotypes (two c.35delG homozygotes and two c.35delG/p.M34T compound heterozygotes). Phenotypically, one of the c.35delG homozygotes had moderate SNHL and the other had severe SNHL. One of the c.35delG/p.M34T compound heterozygotes had mild SNHL; the other did not pass the OAE testing in one ear, but later testing revealed no HI.

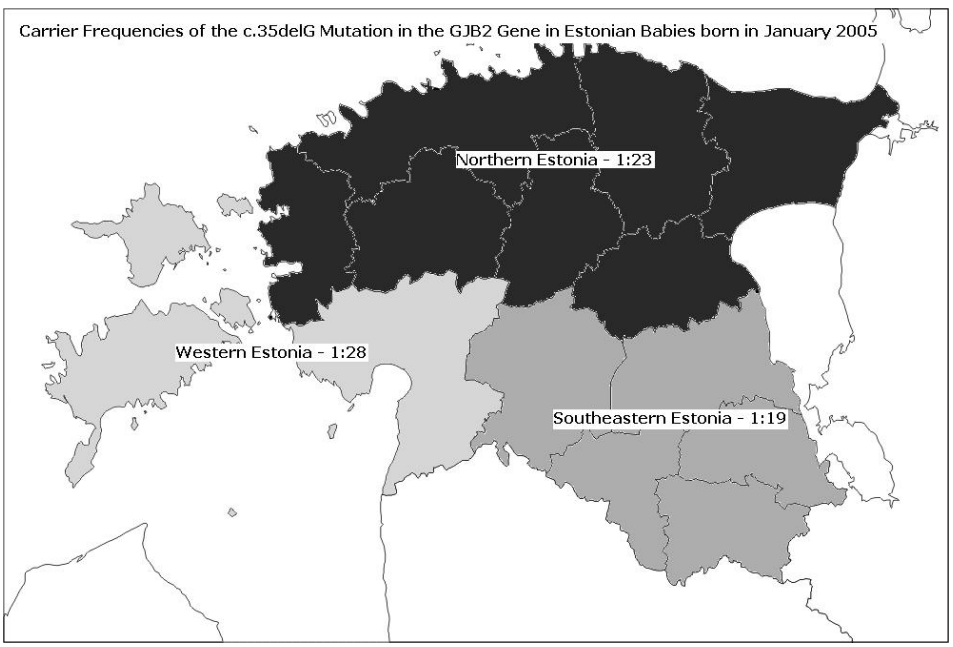

Figure 1: The carrier frequency of c.35delG mutation among Estonian children born in the Northern, Western and Southeastern Regions of Estonia in January 2005

Table 3: Data of the screening of *GJB2* mutations c.35delG and p.M34T of newborns throughout Estonia in January 2005.

Region	Newborns screened	c.35delG heterozygotes	p.M34T heterozygotes
Northern	608	26	36
Western	111	4	10
South-eastern	279	15	12
Estonia	**998**	**45**	**58**
Heterozygote frequency		**1:22**	**1:17**

We analyzed the distribution of c.35delG and p.M34T mutation carriers in the three regions of Estonia (Northern, Western, and Southeastern). The highest proportion of c.35delG mutation carriers occurred in Southeastern Estonia (1:19) and the lowest in Western Estonia (1:28), but this difference was not statistically significant (P = 0.98) (Figure 1). The frequency of p.M34T mutation carriers was highest in Western Estonia (1:11) and lowest in South-eastern Estonia (1:23), but this difference was also not significant (P = 0.19) (Figure 2). We also tested for HWE of the genotypes within the general Estonian population. There was no evidence to suggest that the Estonian baby population violates HWE (P > 0.25 and P > 0.4, for c.35delG and p.M34T alleles, respectively).

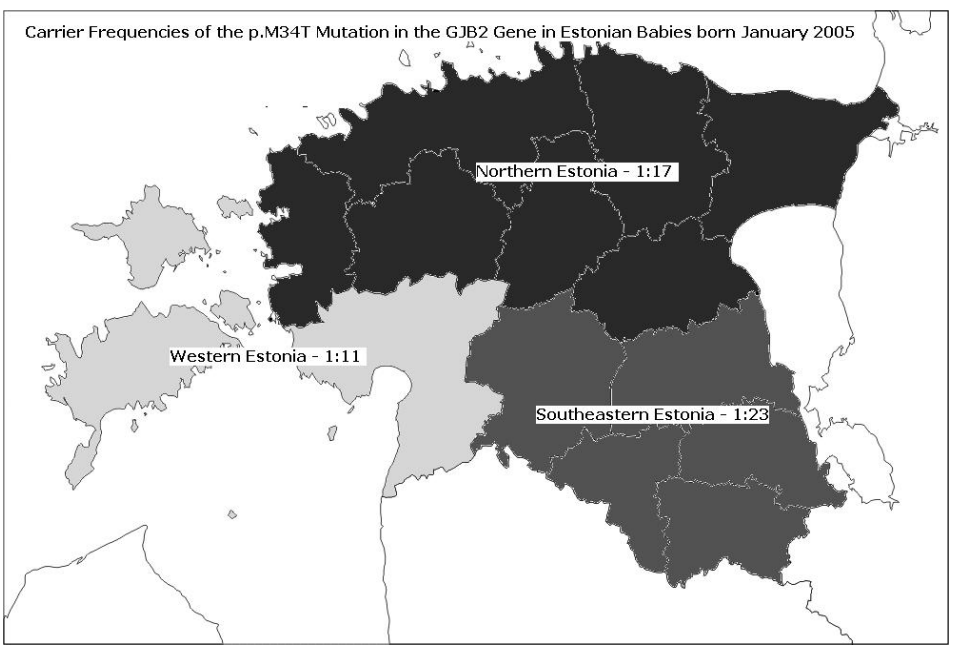

Figure 2: The carrier frequency of p.M34T mutation among Estonian children born in the Northern, Western and Southeastern Regions of Estonia in January 2005

In our study, the carrier frequency of c.35delG among Group NB was 1:22 (4.5%). This result is in accordance with a previous study by Gasparini *et al.* (2000) (see Table 1) who studied the c.35delG mutation in a total of 3270 unrelated, random subjects from 17 European populations, including Estonians (n=113). The carrier frequency for c.35delG in central and northern Europe varied between 1:200 for France and 1:22.5 for Estonia (Gasparini *et al.*, 2000), which is the highest prevalence found in European populations (Mahdieh and Rabbani, 2009).

The high frequency of the mutant allele c.35delG can be explained by genetic drift. Historically, Estonia has experienced periods with significantly reduced inhabitants, the most recent being at the beginning of the 18th century. There are only limited data available about the carrier rates of c.35delG in countries neighboring Estonia. Nevertheless, (Pastinen *et al.*, 2001) analyzed 2147 people in different regions of Finland, and found the carrier frequency of the c.35delG mutation to be between 1:43 and 1:63. Anichkina *et al.* (2001) investigated 569 individuals from five ethnic groups in Russia for the c.35delG mutation, and found the average carrier frequency to be 1:46.7. There is no published data of the c.35delG carrier frequency for the whole of Russia or Latvia.

The prevalence of the p.M34T mutation in Estonia has not previously been studied. We have found the carrier frequency for p.M34T mutation to be 1:17 (5.9%), which is even higher than the frequency of c.35delG mutation (4.5%). The frequency of the p.M34T allele in Estonia is the highest among European countries (Houseman *et al.*, 2001; Lopponen *et al.*, 2003; Roux *et al.*, 2004) (see also Table 1).

5.1.2. Study group of children with early onset HL (Publication I)

We screened 233 probands with early onset HL (Group HL^{233}) and found 115 patients (49%) with *GJB2* gene mutation on at least one allele (Group HL^{115}). The genotypes of Group HL^{233} are listed in Table 4, and include 73 (31%) who were homozygous for c.35delG mutation, 7 (3%) who were homozygous for the p.M34T mutation, and 5 (2%) who had c.35delG/p.M34T compound heterozygosity. There were 22 c.35delG heterozygous patients, of whom 12 had a mutation other than p.M34T and in 10 the mutation in the other allele remained unidentified (Table 4). Thus, c.35delG may be a coincidental finding as APEX array covers over 99% of mutations which are described in *GJB2* gene (Snoeckx *et al.*, 2005), see also Supplementary Table 1, in Publication I.

Table 4: *GJB2* genotypes of 233 Estonian patients (Group HL^{233}) with early onset HL

Genotype	No. of cases	% of cases	c.35delG/X genotype	No. of cases	p.M34T/X genotype	No. of cases
c.35delG/c.35delG	73	31	p.R143W	4	p.R143W	1
p.M34T/p.M34T	7	3	c.-3201G>A	4	c.-3201G>A	1
c.35delG/p.M34T	5	2	c.167delT	1	p.L90P	1
c.35delG/X	22	9.5	c.312del14	2		
p.M34T/X	8	3.5	p.E47X	1		
N/N	118	51	unknown	10	unknown	5
Total	**233**	**100**	**Total**	**22**	**Total**	**8**

X = other specified mutation in *GJB2* gene, N = normal or no mutation in *GJB2* gene found. "Unknown" denotes either possible mutant alleles other than those explicitly shown in Table 1 or a normal allele.

The mutation found most frequently among patients with early onset HL is c.35delG, which was detected in 173 chromosomes (or 75% of all *GJB2* deafness alleles) (Table 5). The second most common alteration, p.M34T, is significantly less frequent among patients with early onset HL (found in 12% of all *GJB2* deafness alleles). All other *GJB2* mutations were found rarely (0.5–2%) (Table 5).

We screened Group HL^{233} for 107 different mutations in the *GJB2* gene. In 115 of these children, we identified a *GJB2* mutation on at least one allele (Group HL^{115}). The most common mutation, c.35delG, was identified in at least one allele in 100 children. The c.35delG mutation is the main cause of early childhood onset HL in Estonian children, and accounts for 75% of *GJB2* alleles. This proportion is similar to that reported in studies of other Caucasian populations as ranging between 58% and 95% (Zelante *et al.*, 1997; Denoyelle *et al.*, 1999; Janecke *et al.*, 2002; Cryns *et al.*, 2004; Roux *et al.*, 2004; Kalay *et al.*, 2005; Snoeckx *et al.*, 2005; Putcha *et al.*, 2007; Hilgert *et al.*, 2009a), see also Table 2.

Amongst Group HL^{233}, we found seven children (Group HL^{7}) who were homozygous for p.M34T (3%), who all had mild HL (Figure 3). The overall incidence of the p.M34T allele among the investigated chromosomes was 5.8%. In our study, we observed that all of Group HL^{7} had at least mild HL in the better ear (Figure 3), which supports the first hypothesis that p.M34T mutation is pathogenic. The comparison of frequencies of p.M34T homozygous phenotype between Group HL^{7} and the general population confirm this hypothesis as well.

Other than c.35delG and p.M34T, we identified six other *GJB2* mutations. We detected p.R143W in five alleles, c.-3201G>A in five alleles, c.312del14 in two alleles, c.167delT in one allele, p.E47X in one allele, and p.L90P in one allele (Tables 4 and 5). In European populations, *GJB2* mutations other than c.35delG are relatively rare (Petersen and Willems, 2006). Whereas the p.R143W mutation is frequent in African populations, it is rare in Caucasian populations (Snoeckx *et al.*, 2005). Snoeckx *et al*. (2005) also report that compound heterozygosity for c.35delG/p.R143W results in more severe HL, and that c.35delG/c.-3201G>A results in less severe HL than c.35delG homozygosity. The c.-3201G>A mutation is rarely found (0.75% of all *GJB2* deafness alleles) in Caucasian populations (Snoeckx *et al.*, 2005), and was also rare in our study population (2%). Only a few research studies have reported cases of the c.312del14 mutation (Denoyelle *et al.*, 1999; Pandya *et al.*, 2003; Mani *et al.*, 2009).

Table 5: The percentage of mutations in the *GJB2* gene in Estonians with early onset HL (Group HL^{115}); the percentage among *GJB2* deafness alleles among the study group of patients (Group HL^{233})

Mutation	No. of chromosomes	% of *GJB2* deafness alleles in Group HL^{115} (230 chromosomes)*	% of allele in Group HL^{233} (466 chromosomes)**
c.35delG	173	75	37.1
p.M34T	27	12	5.8
p.R143W	5	2	1.1
c.-3201G>A	5	2	1.1
c.312del14	2	1	0.4
c.167delT	1	0.5	0.2
p.E47X	1	0.5	0.2
p.L90P	1	0.5	0.2
Unknown	15*/251**	6.5	53.9

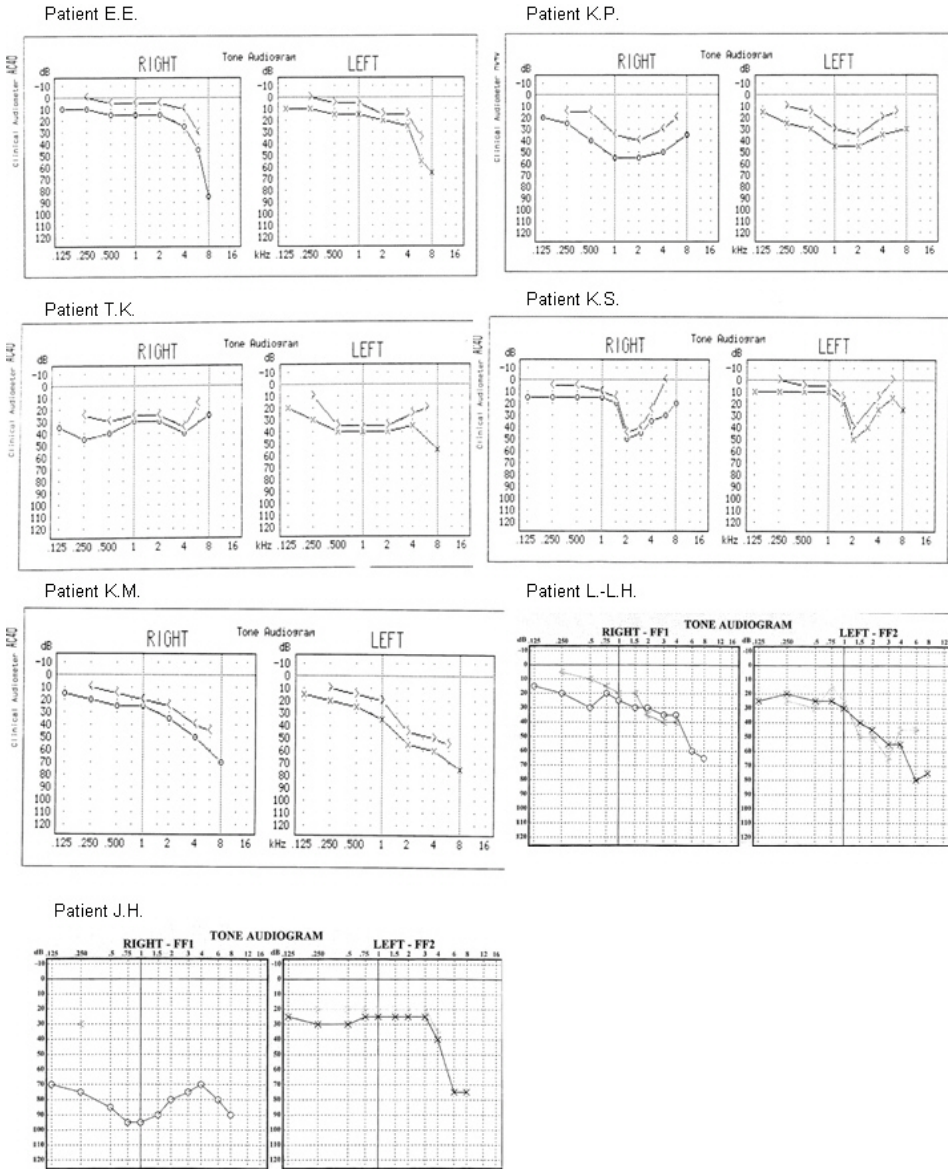

Figure 3: Audiograms from seven Estonian children (Group HL[7]) with early onset HL found to be homozygous for the p.M34T mutation in the *GJB2* gene.

5.1.3. The genotype and phenotype correlation in patients with *GJB2* gene mutations (Publications I and V)

In order to perform a distribution of degree of HL in different biallelic *GJB2* genotypes the severity of HI was defined by the degree of HL in the better ear: average thresholds between 21–40 dB were considered as a mild HI, 41–70 dB as moderate HI, 71–95 dB as severe HI, and greater than 95 dB as profound deafness, see GENDEAF, the European Thematic Network on Genetic Deafness[8] (Cryns *et al.*, 2004).

The most common phenotype associated with c.35delG homozygosity showed significantly more severe HI with respect to other *GJB2* genotypes (Cryns *et al.*, 2004). A patients group with c.35delG homozygosity is often used as a reference or 'standard' group, because the group is well defined and is present, in most of the reported studies of *GJB2* mutations, as the cause of HI (Snoeckx *et al.*, 2005). Therefore in our results the other genotypes of *GJB2* mutations are compared with respect to c.35delG/c.35delG genotype.

Table 6 shows the distribution of the degree of HL in different *GJB2* genotypes in our study, including *GJB2* heterozygotes, where the mutation in second allele was not found. Whereas the APEX assay covers 99.1% of known *GJB2* mutations, we can assume that this finding in children with the *GJB2* heterozygosity was occasional and their HL is caused by other reasons. In one of these children, the 7472insC mutation in mitochondrial *tRNA-Ser* gene was identified, which is responsible for early onset HL (Hutchin *et al.*, 2001).

The most prevalent genotype c.35delG/c.35delG was detected in 73 probands for whom the most frequent degrees of HI were profound (50.7%) of patients, followed by severe HI (27.4%) and moderate (20.6%). We did not find any children with c.35delG homozygosity and mild HL in Group HL[233]. Our results are in accordance with previous studies, where the majority patients with c.35delG homozygosity have severe and profound HL, some c.35delG homozygotes have only moderate HL, and in a few patients the HL is only mild (Snoeckx *et al.*, 2005; Hilgert *et al.*, 2009b). The absence of mild HL in Estonian children with c.35delG homozygosity may be due to small size of the study group.

[8] http://www.gendeaf.org

Table 6: Degree of HL for patients with *GJB2* mutations (n=233)

Genotype	No of cases (%)	No (%) of cases with mild HL	No (%) of cases with moderate HL	No (%) of cases with severe HL	No (%) of cases with profound HL	No (%) of cases with unspecified HL
c.35delG/c.35delG	73 (31.3)	–	15 (21.9)	20 (27.4)	37 (50.7)	–
p.M34T/p.M34T	7 (3.0)	7 (100)	–	–	–	–
c.35delG/p.M34T	5 (2.2)	1(20)	2 (40)	–	1(20)	1(20)
c.35delG/p.R143W	4 (1.7)	–	–	–	4 (100)	–
c.35delG/c.-3201G>A	4 (1.7)	1 (25)	–	2 (50)	–	1 (25)
c.35delG/c.312_325del14	2	–	–	2 (100)	–	–
c.35delG/c.167delT	1	–	–	1(100)	–	–
c.35delG/p.E47X	1	–	1(100)	–	–	–
c.35delG/ -	9 (3.9)	2 (22.2)	1(11.1)	1(11.1)	3 (33.3)	2 (22.2)
c.35delG/ - *	1	–	–	–	–	1(100)
p.M34T/p.R143W	1	–	1(100)	–	–	–
p.M34T/ c.-3201G>A	1	–	–	1(100)	–	–
p.M34T/p.L90P	1	1(100)	–	–	–	–
p.M34T/ -	4 (1.7)	–	2(50)	–	1(25)	1(25)
p.M34T/ - **	1	1(100)	–	–	–	–

*HL is due to mitochondrial mutation 7472insC in *tRNA-Ser* gene
**HL is due to congenital CMV

While the effect of the p.M34T mutation continues to be debated, we found seven children with HL and p.M34T homozygosity (Group HL[7]). Most patients with p.M34T/p.M34T genotype have mild HL (Snoeckx *et al.*, 2005) and indeed all the children in Group HL[7] had mild HL. Figure 3 shows the audiogram profiles of Group HL[7], the shapes of which, despite the entire group having mild HL, are significantly different. The detailed description of their phenotype appears in Table 7.

The compound heterozygotes for c.35delG and p.M34T showed different degrees of HL among five patients (see Figure 4). One patient had mild HL, two had moderate and one had profound HL; the degree of HL in one patient with c.35delG/p.M34T genotype was not specified. Patients with p.M34T/p.M34T genotype have milder HL compared to individuals with c.35delG/p.M34T genotype (Snoeckx *et al.*, 2005), but in our study there were only small group of children with these genotypes. We found three more compound heterozygotes for p.M34T – mutations p.R143W (c.427C>T), c.-3201G>A (IVS1+G>A) and p.L90P (c.269T>C) in the second allele.

Table 7: Degree and type of HL and audiogram profiles for seven patients (Group HL[7]) with p.M34T/p.M34T genotype

No	Degree of HL	Type of HL	Profile of audiogram
1	Normal hearing through 3 kHz with a moderate to severe high frequency HL	Sensorineuralis	High-frequency
2*	Mild to moderate	Mixed	U-shaped
3	Mild	Sensorineuralis	Flat
4	Mild to moderate	Sensorineuralis	U-shaped
5	Mild	Sensorineuralis	High frequencies gently sloping
6	Right: mild HL through 4 kHz with a moderate at the high frequencies Left: mild HL through 1 kHz with a moderate at the high frequencies	Sensorineuralis	Descending
7	Right: severe to profound Left: mild HL through 3 kHz with high frequency sensorineuralis deficit	Sensorineuralis	Right: U-shaped Left: high frequency

* mixed HL is not characteristic to *GJB2* related HL; in this case the conductive component is due to recurrent purulent otitis complications.

The c.35delG/p.R143W genotype is one of few genotypes giving significantly greater HI than the c.35delG/c.35delG reference group, based on data of 10 patients from a group of 1531 (0.65%) (Snoeckx *et al.*, 2005). In our study group we found four children (1.7%) with compound heterozygosity for c.35delG and p.R143W, all of whom had profound HL similar to the patients in Snoeckx et al's study. We found also four children with c.35delG/c.-3201G>A genotype, who had less HL compared to c.35delG/c.35delG genotype. Among the c.35delG heterozygotes, we found three more *GJB2* mutations in the second allele: c.312_325del14 (312del14), c.167delT (p.L56fs) and p.E47X (c.139G> T). The mutation c.167delT has been reported among Ashkenazi Jews (Morell *et al.*, 1998). The p.E47X is mutation occurs in Caucasian populations in 1.4% of all *GJB2* deafness alleles, (Snoeckx *et al.*, 2005), but in Group HL[233] only one patient (0.4%) had p.E47X. The 312del14 mutation is rarely reported, and there are only a few cases in research reports (Denoyelle *et al.*, 1999; Pandya *et al.*, 2003; Mani *et al.*, 2008). Nevertheless, we did find two probands with compound heterozygosity for c.35delG and c.312_325del14.

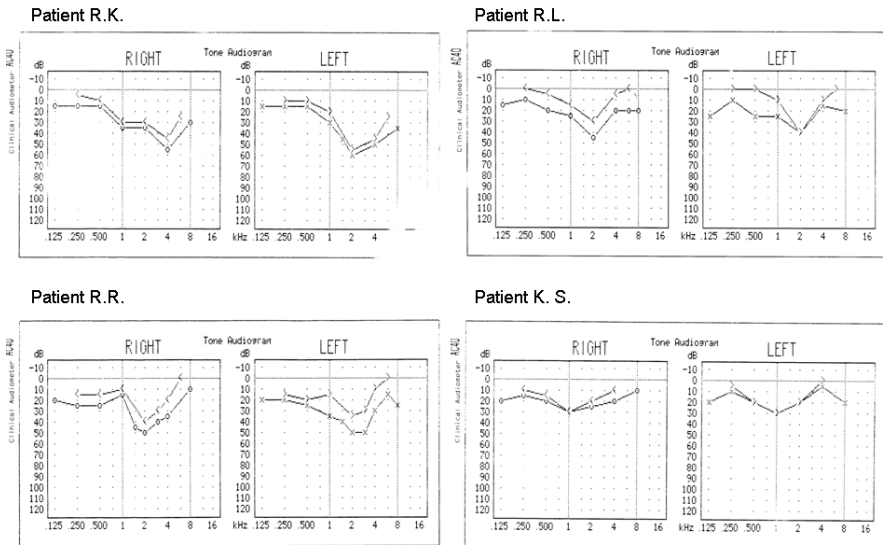

Figure 4: Audiograms from four children with early onset HL found to be compound heterozygous for c.35delG and p.M34T mutations in the *GJB2* gene.

5.2. *GJB3* and *GJB6* gene (Publications I and V)

Two large deletions in the *GJB6* gene, del(GJB6-D13S1830) and del(GJB6-D13S1854), are usually found in compound heterozygosity with a *GJB2* coding mutation and cause HL that is significantly worse than most other *GJB2* mutations, possibly because expression of both copies of *GJB2* and one copy of *GJB6* is abolished (Hilgert *et al.*, 2009b). The del(*GJB6*-D13S1830) deletion is the second most frequent genetic cause of nonsyndromic prelingual HL, after the 35delG mutation in *GJB2*, in Spain's population (del Castillo et al., 2002). We did not found any children with *GJB6* mutations in Group HL[233]. However, the del(GJB6-D13S1830) mutation is the most frequent in Spain, France, the United Kingdom, Israel, and Brazil (5.0–9.7% of all DFNB1 alleles); occurs less frequently in the USA, Belgium, and Australia (1.3–4.5% of all DFNB1 alleles) (Del Castillo *et al.*, 2003; Marlin *et al.*, 2005), and has not yet been found in Austria, Turkey, or China (del Castillo *et al.*, 2005). Nevertheless, patients with HL should be tested for *GJB2* mutations and especially *GJB2* heterozygotes as the next investigation for *GJB6* mutations.

Mutations in the *GJB3* gene have been pathologically linked to nonsyndromic autosomal dominant or recessive HL and erythrokeratoderma variabilis without HL (Richard *et al.*, 1998; Alexandrino *et al.*, 2004; Finsterer and Fellinger, 2005). We investigated Group HL[233] for five mutations in the *GJB3* gene: two autosomal dominant (R180X, E183K) and three autosomal recessive mutations (141del Ile, I141V and P223T). We did not find any occurrences of

GJB3 mutations in our study among the patients with HL – it shows a low prevalence of mutations in the *GJB3* gene occurs in Estonia. Mutations in *GJB3* have originally been reported as the cause of autosomal dominant non-syndromic HL (DFNA2) in Chinese patients (Xia *et al.*, 1998) and also among patients with to autosomal recessive nonsyndromic HL (Liu *et al.*, 2000). Variations in the *GJB3* gene have also been linked to nonsyndromic HL in Brazilian patients (Alexandrino *et al.*, 2004). A previous study shows that whereas mutations in the *GJB3* gene are cause of HL in Chinese population, their prevalence is non-existent or extremely low in European populations and Caucasians in general (Liu *et al.*, 2009).

5.3. Two mitochondrial genes (*12S rRNA* and *tRNA-Ser*) (Publication V)

Among Group HL[233] we found two patients (0.9%) whose HL is caused by a mutation in the mitochondrial DNA.

The first patient was 10 years old and had the mutation m.1555A>G in the mitochondrial *12S r-RNA* gene (Patient^{M1}). Patient^{M1} had had two weeks of treatment in the neonatal intensive care unit due to respiratory distress. Unfortunately data about the treatment were not made available. Patient^{M1}had moderate bilateral SNHL (Figure 5). The second patient referred to genetic consultation at the age of two years and had a family history for deafness in three generations (Patient^{M2}). Patient^{M2}'s HL was diagnosed at the age of one year by using subjective investigation visual reinforcement audiometry and the HL degree was mild-to-moderate. Patient^{M2}'s HL was caused by the mutation 7472insC in the mitochondrial serine *tRNA-Ser* gene, but Patient^{M2} also carried the mutation 35delG in the *GJB2* gene. Both parents of Patient^{M2} had profound HL; the mother had 7472insC mutation in mitochondrial serine *tRNA-Ser* gene, and the father was c.35delG homozygote in *GJB2* gene.

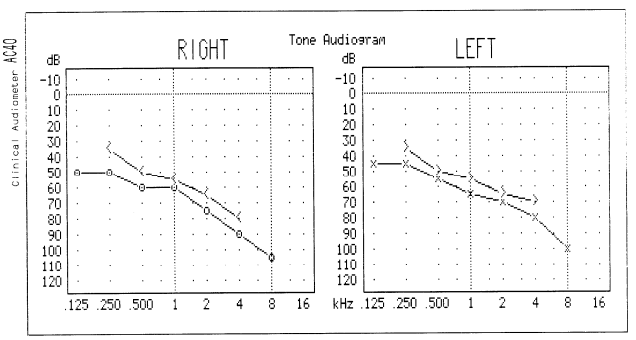

Figure 5: Audiogram from Patient^{M1} with moderate bilateral SNHL and the mutation m.1555A>G in mitochondrial *12S r-RNA* gene

The mutation m.1555A>G is the most common mitochondrial mutation associated with HL, found in many families worldwide (Prezant *et al.*, 1993; Usami *et al.*, 1997; Estivill *et al.*, 1998b; Li *et al.*, 2004; Jacobs *et al.*, 2005; Young *et al.*, 2005; Mkaouar-Rebai *et al.*, 2010). The mutation m.1555A>G can be found in 0.6–2.5% of the Caucasian clinical population with nonsyndromic SNHL (Xing *et al.*, 2007). SNHL for this mutation may be triggered by the use of aminoglycosides, and may also occur without exposure to these drugs (Van Camp and Smith, 2000; Xing *et al.*, 2007). The penetrance of mutation m.1555A>G differs between families; in the absence of aminoglycosides, the clinical phenotype may be variable even among family members (Prezant *et al.*, 1993; Estivill *et al.*, 1998b; Xing *et al.*, 2007). We found only one patient with the mutation m.1555A>G and the mutation's characteristically low HL among Group HL^{233}. Nevertheless, there may be carriers of the m.1555A>G mutation among adults with late onset HL, but because genetic HL is thought to be early onset and severe or profound they are rarely referred to genetic consultation for their HL.

The mutation 7472insC in the *tRNA-Ser* gene occurs more frequently in European populations than others (Hutchin *et al.*, 2001). Most individuals carrying mutation 7472insC have progressive SNHL, accompanied occasionally by one of a variety of widespread neurological diseases including ataxia, dysarthria, and myoclonic seizures (Xing *et al.*, 2007). The 7472insC mutation alone is usually sufficient to cause HL, and when present in very high levels can also lead to neurological dysfunction (Hutchin *et al.*, 2001; Xing *et al.*, 2007). However, Jacobs *et al.* (2005) have found two cases in patients with 7472insC mutation and SNHL as the only feature. In Group HL^{233} we found one child with 7472insC mutation in *tRNA-Ser* gene. Neurological symptoms in a patient and their mother with profound HL and carrying the same mutation are not known, therefore we can not state whether or not the 7472insC mutation, without neurological signs, is the cause of HL in this family.

5.4. *SLC26A5* (prestin) gene (Publication II)

The study group consisted of 194 Estonian children with HL (Group $HL^{194/08}$), as we summarized and published the data of the *SLC26A5* gene analyses in 2008.

We identified a heterozygous IVS2–2A>G change in the *SLC26A5* gene in four of Group $HL^{194/08}$ (2.1%) with early onset HL and in five of 68 family members (Table 8). We did not find any homozygosity for this splice variant in the probands or in their family members. We described those nine individuals from five different families.

Table 8: Heterozygous IVS2–2A>G change in the *SLC26A5* gene in four probands and five family members

No	**Family, patient**	**Age**	**Hearing status**	**Genotype**
1	Family 1, I-2*	25 years	Normal hearing	Heterozygous IVS2–2A>G in *SLC26A5* Heterozygous IVS1+1G>A mutation in *GJB2* gene
2	Family 2, mother	28 years	Severe HL since 5 years of age	Heterozygous IVS2–2A>G in *SLC26A5*
3	Family 2, daughter	3 years	Normal hearing	Heterozygous IVS2–2A>G in *SLC26A5*
4	Family 3, son	17 years	Early onset severe HL	Heterozygous IVS2–2A>G in *SLC26A5*
5	Family 4, III-2**	4 months	Early onset severe HL	Homozygous mutation c.35delG in *GJB2* gene Heterozygous IVS2–2A>G in *SLC26A5*
6	Family 4, II-2**	32 years	Normal hearing	Heterozygous mutation c.35delG in *GJB2* gene Heterozygous IVS2–2A>G in *SLC26A5*
7	Family 4, I-2**	53 years	Normal hearing	Heterozygous mutation c.35delG in *GJB2* gene Heterozygous IVS2–2A>G in *SLC26A5*
8	Family 5, son with Down syndrome	2 years	Early onset moderate HL	Heterozygous IVS2–2A>G in *SLC26A5*
9	Family 5, father	46 years	Normal hearing	Heterozygous IVS2–2A>G in *SLC26A5*

1.1. Family 1

In this family there are two children with severe HL. A clinical geneticist evaluated and screened the two children with the APEX microarray. In both children, compound heterozygous mutations in the *GJB2* gene, which encodes connexin 26, were present. Their genotype was c.35delG/IVS1+1G>A. The parents were also tested and received an audiology evaluation as well. Mild HL and compound heterozygosity for mutations c.35delG and V37I in the *GJB2* gene was established in the mother. The 25-year-old father has normal hearing, and carried IVS1+G>A in the *GJB2* gene and IVS2–2A>G in the *SLC26A5* gene (Figure 6).

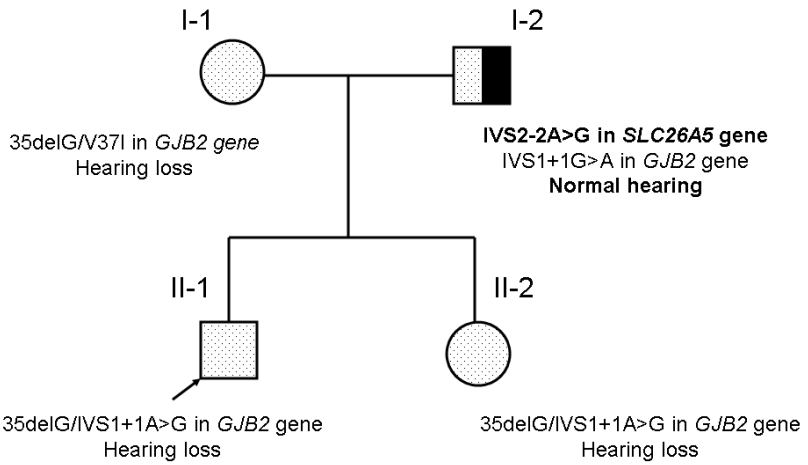

Figure 6: The pedigree for Family 1.

1.2. Family 2

The adult proband in this family is affected with severe HL. After acute tonsillitis at the age of five years the parents noticed that their child had HI. Hearing problems in earlier years had not been noticed. The HL has been progressive. This individual has a three-year-old daughter, who has normal hearing. Both mother and daughter are heterozygous for IVS2–2A>G in the *SLC26A5* gene.

1.3. Family 3

The proband in this family had severe, early onset SNHL, but was only evaluated by a clinical geneticist at the age of 17 years (Table 8). APEX screening identified only one heterozygous nucleotide change: IVS2–2A>G in the *SLC26A5* gene. The parents were not available for testing.

1.4. Family 4

The proband of this family did not pass the neonatal hearing screening tests by transient evoked OAE. HL was definitively diagnosed at the age of four months. Using the APEX microarray we determined that the patient was homozygous for mutation c.35delG in the *GJB2* gene. In addition, IVS2–2A>G in the *SLC26A5* gene was detected (Figure 7, III-2). In this case HL is clearly explained by homozygosity for the c.35delG mutation, which causes a frameshift and is one of the most common and unambiguously pathogenic

mutations in the *GJB2* gene (Estivill *et al.*, 1998). The proband's mother (II-3) was heterozygous for c.35delG and the father (II-2) was heterozygous for the mutations c.35delG in the *GJB2* gene and for IVS2–2A>G in *SLC26A5*. The father is 32 years old and has normal hearing. He has one brother with early onset HL. The brother was homozygous for c.35delG. As expected, the paternal grandparents each carried the c.35delG allele. The 53 year-old paternal grandmother (I-2) also carried the IVS2–2A>G change in *SLC26A5*. She had no clinical complaints about her hearing.

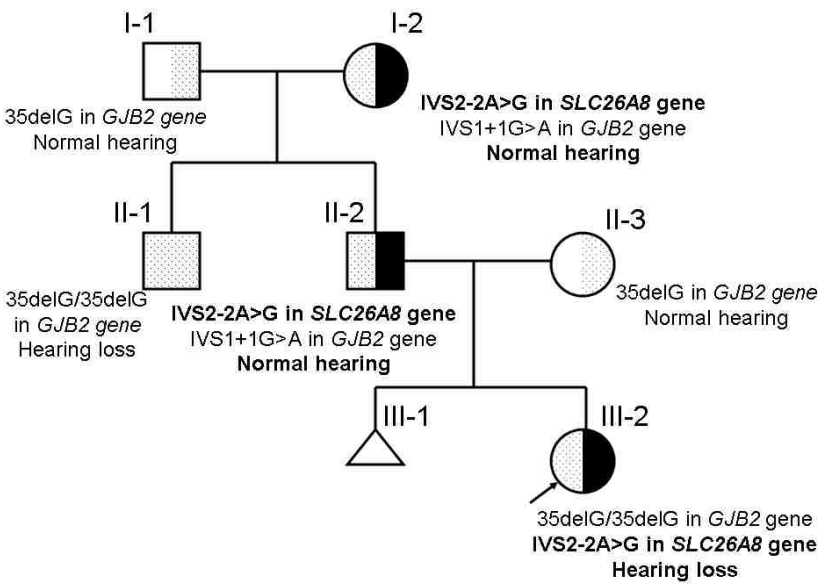

Figure 7: The pedigree for Family 4

1.5. Family 5

The proband in the fifth family was a two-year-old boy with Down syndrome and early-onset moderate HL. This patient's HL may be associated with Down syndrome. We identified the IVS2–2A>G change in the *SLC26A5* gene and tested the parents with the APEX array, as well. The 46-year-old father was also heterozygous for IVS2–2A>G and his hearing is, by history, normal.

In our study we applied the APEX microarray method to patients with HL. In four probands (2.1%) of Group $HL^{194/08}$, we identified a heterozygous IVS2–2A>G change in the *SLC26A5* gene (Prestin gene). One of these probands, however, is also homozygous for the c.35delG mutation in the *GJB2* gene and a second patient has Down syndrome, which is also associated with HI. Therefore, in those two cases, the etiology of HL is probably not associated with the IVS2–2A>G sequence change in the *SLC26A5* gene. This change is likely to be

coincidental. Based on patients and findings we can conclude that the mutation does not seem to be a dominant one. In other words, carrying one copy of this mutation does not cause HL. A more likely reason is that *SLC26A5* mutations are inherited in an autosomal recessive way and that two mutations are necessary for a phenotype to occur.

There are only two research papers about the IVS2–2A>G mutation in *SLC26A5* gene in humans (Liu *et al.*, 2003; Tang *et al.*, 2005). Liu *et al.* (2003) studied 220 probands and identified two who were homozygous for IVS2–2A>G and seven who were heterozygous (3.2%). The degree of HL was highly variable and the onset of HL is heterozygous for IVS2–2A>G in ages ranging from birth to 35 years (Liu *et al.*, 2003). In Group HL$^{194/08}$, we found five individuals aged 3, 25, 32, 46 and 53 years old with IVS2–2A>G mutation, all of whom presently have normal hearing.

Tang *et al.* (2005) identified the heterozygous IVS2–2A>G mutations in 4 of 74 HI subjects of Caucasian, Hispanic or uncertain ethnicity (5.4%) and 4 of 150 controls (2.7%); they did not identify any homozygous subjects. Only one of the four HI carriers reported a history of HL in a parent. These data suggest that heterozygosity may not, by itself, be sufficient to cause HL.

In addition to the IVS2–2A>G mutation, the c.449G>A (R150Q) missense mutation in the *SLC26A5* gene has been recently described to be potentially associated with mild to moderate non-syndromic HL (Toth *et al.*, 2007). These two mutations in *SLC26A5* are, so far, the only ones with potential clinical importance. Surprisingly, there are also no entries of the coding for single nucleotide polymorphisms (SNP) in the *SLC26A5* gene in the *Entrez Gene* database[9].

We cannot fully exclude that due to the methodological limitations of previous studies, some alterations in the *SLC26A5* gene have been missed. In their initial study, (Liu *et al.*, 2003) used an indirect method, single-stranded conformation polymorphism (SSCP), to discover mutations. The second study focused only on the sequencing of the known splice acceptor site where the IVS2–2A>G mutation is located (Tang *et al.*, 2005). Thus full gene sequencing may provide more mutations and insights into the contribution of the SLC26A5 gene to the etiology of SNHL in humans.

In mice, research shows that the targeted deletion of the *SLC26A5* gene reduces cochlear sensitivity (Liberman *et al.*, 2002) and eliminates both frequency selectivity and OHC somatic electromotility (Cheatham *et al.*, 2004). Cheatham *et al.* (2005) also studied the cochlear function in mice with only one copy of the *SLC26A5* gene, but in contrast to Liberman *et al.* (2002) their results show near normal cochlear physiology and protein levels in adolescent heterozygous mice, which seems to imply that a single copy of the mutated prestin gene does not cause HL. Liu *et al.* (2003) have also studied alternative splicing of *SLC26A5* mRNA. Their results show the presence of at least four distinct isoforms, differing in the exons located in 3' region of the gene. In all the known isoforms, protein coding starts in exon 3. *In silico*, Tang *et al.* (2005)

[9] http://www.ncbi.nlm.nih.gov/

have predicted the presence of five additional splice acceptor sites in the second intron of the *SLC26A5* gene. None of the mRNA isoforms using these alternative splice sites have been described so far. Still, low-levels of mRNA from mutated alleles might partially explain the ambiguity of the results of the clinical importance of IVS2–2A>G mutation in previously published papers, as well as in our study.

A slightly similar discrepancy exists between the predicted and actual clinical severity of the known IVS1+1G>A splice donor site mutation of the *GJB2* gene. None of the functional mRNA should be present with both the truncating c.35delG mutation and the splice site IVS1+1G>A mutations. Despite this, the phenotype of c.35delG/IVS1+1G>A compound heterozygotes is significantly less severe, compared to c.35delG homozygotes (Snoeckx *et al.*, 2005). In the *GJB2* gene, ATG is located in the exon 2 and the splice site mutation of the 5' noncoding exon appears to preserve low-level functioning of the gene.

In conclusion, we can say that in 2.1% of investigated individuals with early onset HL in Estonia, we found heterozygous IVS2–2A>G mutation in the *SLC26A5* gene. We did not identify any homozygous individuals for this sequence change among Group $HL^{194/08}$. Our data supports the hypothesis that heterozygosity for the IVS2–2A>G mutation in the *SLC26A5* gene may not be, by itself, sufficient to cause HL. Further studies are needed to clarify and refine the IVS2–2A>G allele frequencies in various ethnic groups and examine the role of the IVS2–2A>G nucleotide substitution, as well as other SLC26A5 changes, in HL.

5.5. *SLC26A4* (pendred) gene (Publication V)

We found five patients from Group HL^{233} with heterozygous mutation in *SLC26A4* gene and their genotype and phenotype are shown in Table 9.

Table 9: Patients with heterozygous mutations in *SLC26A4* gene: their age, genotype and phenotype.

No.	Age	Mutation in *SLC26A4* gene	Degree of HL	Localisation
1	10 years	L597S / –	profound	unilateral
2	2 years	L597S / –	severe	bilateral
3*	8 years	F335L / –	profound	bilateral
4	1 year	L597S / –	unspecified	bilateral
5**	1 year	R409H / –	unspecified	unspecified

* Patient also has heterozygous mutation p.M34T in *GJB2* gene

** Patient also has heterozygous mutation c.35delG in *GJB2* gene

Mutations in *SLC26A4* gene are the second most frequent cause of autosomal recessive nonsyndromic HL (ARNSHL). The associated phenotypic spectrum ranges from PDS at one extreme to isolated nonsyndromic HL with enlarged vestibular aqueduct at the other (DFNB4 locus) (Hilgert *et al.*, 2009b). To date, more than 150 *SLC26A4* mutations have been identified in patients with PDS, DFNB4 or enlarged aqueduct (Kopp *et al.*, 2008; Anwar *et al.*, 2009). The most prevalent mutations tend to be specific to one or a few related ethnic groups (Park *et al.*, 2003; Tsukamoto *et al.*, 2003; Guo *et al.*, 2008). In northern Europe, four mutations (L236P, T416P, E384G and IVS8+1G>A) are found quite frequently (Coyle *et al.*, 1998; Hilgert *et al.*, 2009b). It can be challenging to distinguish between PDS and nonsyndromic HL with EVA because the goiter phenotype is usually not seen until adolescence and in some families, affected siblings can be discordant for thyroid disease (Reardon *et al.*, 2000; Hilgert *et al.*, 2009b).

As the five children in HL^{233} have the heterozygous mutation in *SLC26A4* gene only in one allele and we cannot link those findings with the children's HL. Therefore we could not confirm any HL caused by mutations in *SLC26A4* gene.

5.6. Syndromic HL (Publication V)

The main aim of our study was to investigate the genetic causes of early onset non-syndromic HL. We selected individuals, preferably children, with non-syndromic HL, with the view of following testing with an APEX assay. However, after careful clinical investigation, syndromic HL was clinically suspected in seven (3%) of Group HL^{233} and in some syndromic HL was also confirmed. The genetic dysmorphic syndromes diagnosed in these seven children (Group HL^{S}) are shown in Table 10.

Syndromic HI may account for up to 30% of pre-lingual deafness, but its relative contribution to all deafness is much smaller, reflecting the occurrence and diagnosis of post-lingual HL (Smith and Van Camp, 2007). Though many syndromes such as WS, US, and Jervell and Lange-Nielsen are noted in text-books as being quite common in the population with HL, in reality these syndromes represent a small proportion of this total population (Billings and Kenna, 1999; Kenna *et al.*, 2001). Billings and Kenna studied 301 children with HL, of whom known syndromes were identified in 12.3% patients (Billings and Kenna, 1999).

Table 10: The syndromic HL among Estonian children (Group HL^S)

Syndrome	No. of patients	Degree of HL	Type of HL
Waardenburg syndrome II type	1	severe	bilateralis sensorineuralis
Waardenburg syndrome II type	1	moderate	bilateralis sensorineuralis
Stickler syndrome II type	1	moderate	bilateralis sensorineuralis
Klippel-Feili syndrome	1	severe	bilateralis sensorineuralis
Kearns-Sayre syndrome	1	moderate	bilateralis sensorineuralis
Usher syndrome	1	moderate	bilateralis sensorineuralis
Goldenhar syndrome	1	mild	bilateralis conductive

Genetic dysmorphic syndromes have a broad spectrum of symptoms, which can diagnosed by clinical evaluation but for which confirmation by DNA analysis is not possible. We were able, after extensive clinical examination and exclusion of all other common etiological factors for early onset HL, to diagnose the genetic syndromes of Group HL^S. We diagnosed two patients with WS II type and one patient each with Stickler syndrome II type and US, which are the most prevalent syndromes in patients with HL. WS has a broad spectrum of symptoms and is, therefore, clinically difficult to distinguish common familial pigmentary abnormalities without HL from the WS II type. We had a few more patients with suspicions of WS and BOR syndromes, but their clinical features were very mildly expressed, which made even a clinical diagnosis difficult. We found only one patient with US. The absence of US in Group HL^{233} is probably due to the relatively late development after the age of ten years of one of Usher's most important symptoms, pigmentretinitis. Patients in Group HL^{233} were referred for genetic consultations at earlier ages and therefore the etiology of their HL remained unknown.

We excluded from Group HL^{233} a patient who was referred for genetic consultation due to HL and a suspicion of LS. Although the HL diagnosis was not later confirmed, and at the moment the patient does not have HL, the LS diagnosis was confirmed by DNA analysis. This patient is described in section 5.6.1.

5.6.1. LEOPARD syndrome – case report (Publication IV)

Our patient was 4 year-old boy, who was referred to genetic consultation due to a suspicion of LS. At the age of 3 years HL was suspected, but the degree of HL was not determined. The patient was the only child in this family. Patient was born at term with a birth weight of 3220 g and birth length of 49 cm. Psychomotor development in the patient's first year was normal. At the age of 4 years, his height and weight were both in the 25th percentile and his head circumference was in the 50th percentile. His psychomotor development was according to his age. The mothers' main complaint was the child's hyperactive behaviour. The patient had a facial phenotype with a prominent forehead; he had a short

neck and a wide torso. Lentigines had been present at birth, but their intensive growth began at the age of 2–4 years. Multiple dark lentigines (>50) were located mainly on the face and the upper part of the torso; the oral mucosa was spared. Cardiac investigation showed mild mitral valve insufficiency. Molecular analysis revealed Y279C mutation in *PTPN11* gene. A secondary (repeat) investigation for identifying HL showed normal hearing. The suspicion of HL at the younger age was probably due to the hyperactive behaviour and faulty collaboration in investigations. As the patient did not have HL, we excluded him from our study group. This patient is described in detail in Publication IV.

This case is good example of the complicated diagnostics of syndromal HL when HL, on of the main symptoms, is missing. However, the patient will be followed for possible future HL. In this case, we had the opportunity to confirm the diagnosis of LS by DNA analysis. Had we had to rely on a clinical diagnosis, with the absence of HL, we would not have confirmed the LS diagnosis on clinical grounds only.

5.7. Chrosomal rearrangements

5.7.1. Results of regular karyotyping (Publication V)

Among Group HL^{233}, chromosomal analysis was conducted for 55 (23.6%) patients (Group HL^{55}). The indication for chromosomal analysis was decided during clinical evaluation by a clinical geneticist. The four chromosomal abnormalities diagnosed in Group HL^{55} by regular karyotyping are shown in Table 11.

Table 11: The chromosomal abnormalities found in regular karyotyping in children with HL (Group HL^{55}) and additional medical problems or symptoms

No.	Karyotype	Degree of HL	Type of HL
1	46,XY,t(2;7)(q21;q32)	unspecified	unspecified
2	46,XY,t(3;8)(q25;q24.1)	moderate	bilateral sensorineuralis
3	46,XY,t(6;7)(p21.1;q36)	moderate	bilateral sensorineuralis
4	47,XY,+21 Down syndrome	moderate	bilateral sensorineuralis

We had one patient with Down syndrome in Group HL[55]. The parents were referred to genetic consultation in order to learn the recurrence risk for isolated HL for future children. We performed the APEX analysis and identified the IVS2–2A>G change in the *SLC26A5* gene. However, the patient's HL may be associated with Down syndrome. See Section 5.4, *Family 5* for more detail and Publication II (Family 5) for the exact detail.

In the three patients of Group HL[55] who had a balanced chromosomal aberration, we performed CMA using Human370CNV-Duo BeadChips to detect submicroscopic chromosomal rearrangements in the breakpoint region; we did not find any abnormalities. A search in the Hereditary Hearing Loss Homepage (Van Camp and Smith, 2008) showed some loci for autosomal dominant HL – DFNA, located to the broken chromosomal region in two patients.

The first patient in Table 11 had karyotype 46,XY,t(2;7)(q21;q32); and DFNA50 is located to region 7q32, the gene is unknown (Modamio-Hoybjor *et al.*, 2004). The third patient in Table 11 had karyotype 46,XY,t(6;7)(p21.1;q36); DFNA13 is located to 6p21 and the gene is *COL11A2* (Brown *et al.*, 1997; McGuirt *et al.*, 1999), DFNA21 to 6p21 (Kunst *et al.*, 2000) and DFNA31 to 6p21.3 (Snoeckx *et al.*, 2004) – in last two loci, the genes are not known. However, the patients may have some rearrangements in the breakpoints, but any relevant studies are not available for us at the present, therefore the possible changes are not detectable and not known to us. Further studies are needed for these three patients with chromosomal translocations.

5.7.2. Results of genome array analysis

Finally we selected out 24 patients who, in addition to early onset HL, had subtle facial dysmorphism, and either or both a failure to thrive and developmental or behavioral problems. In order to detect copy number changes in selected patients, we performed CMA. We found three potentially pathogenic regions in three separate patients with the loss of one allele. All the findings with potentially clinical significance were confirmed by qPCR analysis.

The data of one patient (Case 4) from a previous research study (Puusepp, 2009), was added to this section (see 5.7.2.2) as this patient was further investigated to find out the exact etiology of his phenotype as a part of this study.

5.7.2.1. Case 1

A 8.5-year-old girl (A-7364): she has mild SNHL and a peculiar facial phenotype – palpebral fissures slant up, hypertelorism, prominent glabella, broad nasal bridge, hypoplastic alae nasi, cupid bow shape mouth, high palate and dysmorphic ears (Figure 8a, b). She has moderate mental retardation. At the age of 2 years mild supravalvular pulmonary stenosis was diagnosed. Family history: there is no occurrence of HL or dysmorphism in the other members of this family. However, her three siblings have mild developmental problems; three other siblings are presently normal.

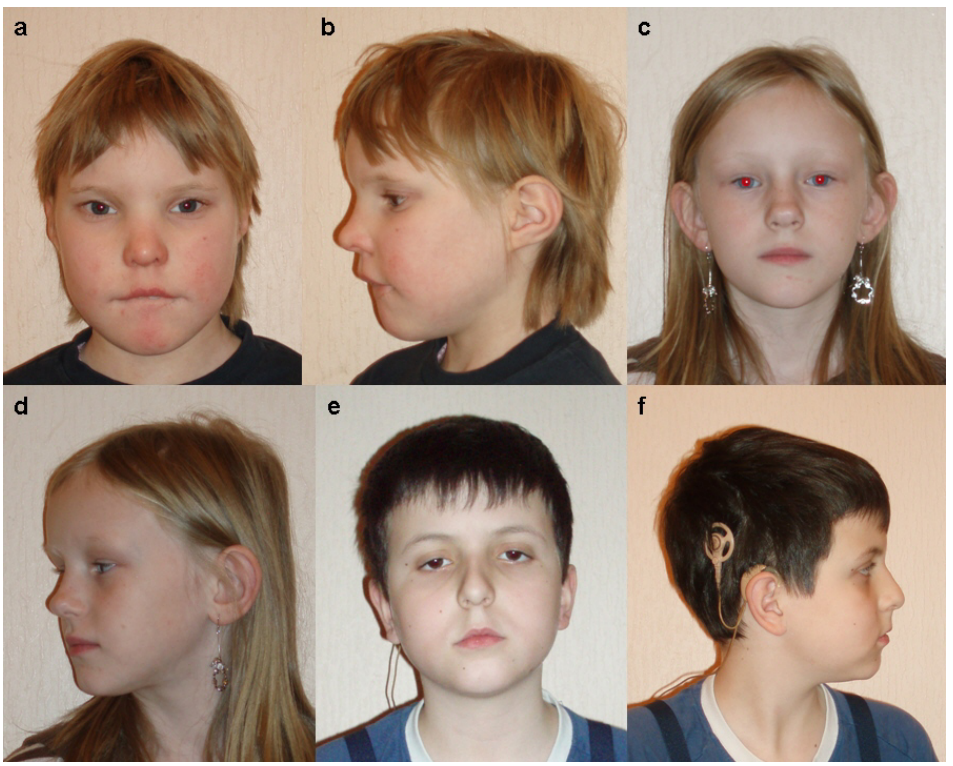

Figure 8: (**a, b**) a facial phenotype of Case 1 (A-7364); (**c, d**) a facial phenotype of Case 2 (A-4140); (**e, f**) a facial phenotype of Case 3 (A-3925).

CMA identified ~2.94-Mb size deletion in chromosomal region 12q13.3-q14.1 (55604593–58543784 bp), which probably occurred *de novo* (Figure 9a). The positions (bp) of primers for qPCR validation analysis of 12q deletion are given in Figure 10a. This deletion was not found in her mother. The father was not available for investigation.

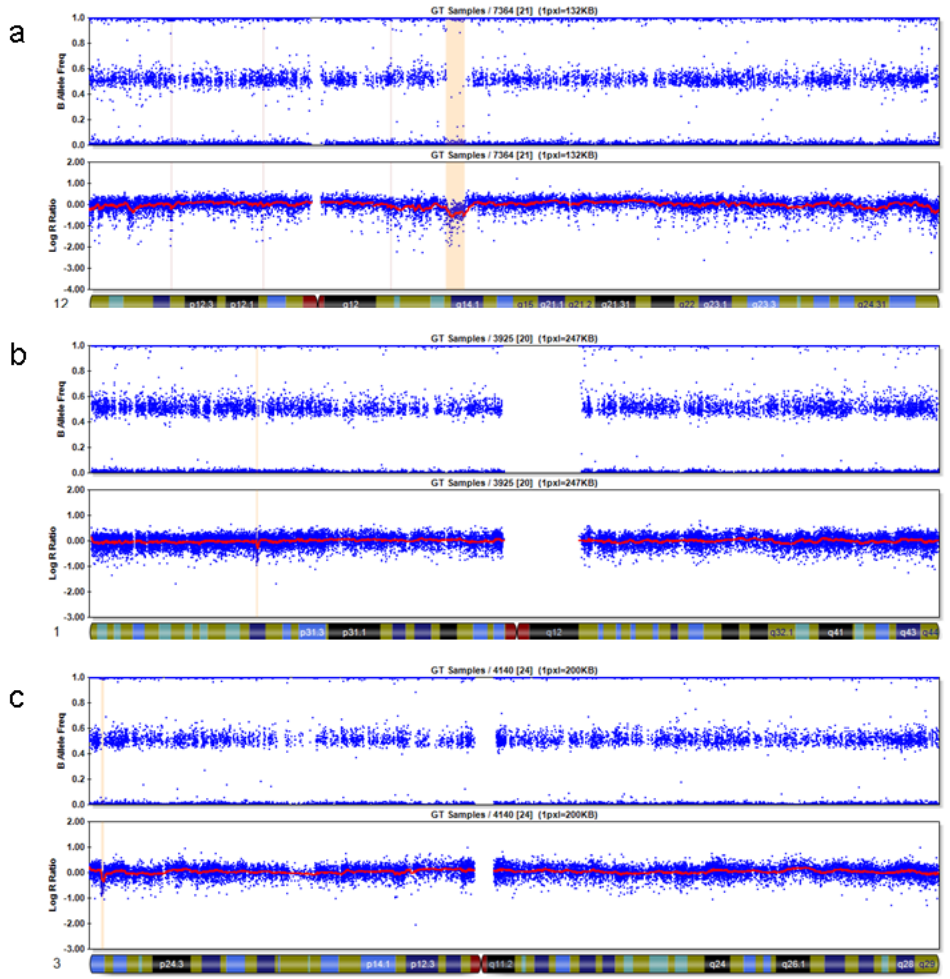

Figure 9: (**a)** CMA results of Case 1 (A-7364) with 12q13.3-q14.1 microdeletion (~2.94-Mb size); (**b**) CMA results of the case 2 (A-4140) with 3p26.2 microdeletion (~0.74-Mb size); (**c**) CMA results of the case 3 (A-3925) with 1p33 microdeletion (~0.54-Mb size).

Nonsyndromic autosomal dominant HL locus, DFNA48 is located to chromosome 12q13-q14 (D'Adamo *et al.*, 2003). The *MYO1A* gene, which is located within DFNA48 locus, is the first myosin I family member found to be involved in causing HL. Donaudy *et al.* (2003) identified one nonsense and six missense mutations in *MYO1A* gene in unrelated patients who were affected by SNHL of variable degree, usually ranging from moderate to severe but never profound. In some families the autosomal-dominant pattern of transmission with either or both variable penetrance and expression was documented. The *MYO1A* gene has a cochlear expression and is considered to play a major role in hair-cell

function (Donaudy *et al.*, 2003). Therefore we can conclude that the haplo-insufficiency of the *MYO1A* gene is responsible for the development of SNHL in our patient 1.

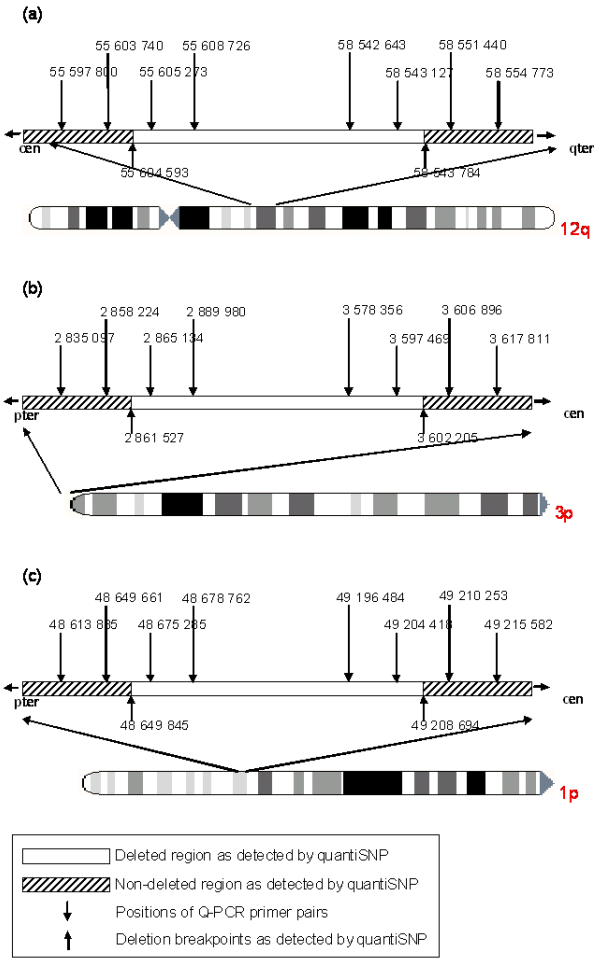

Figure 10: Positions (bp) of primers for qPCR validation analysis (**a**) Case 1 (A-7364), (**b**) Case 2 (a-4140) and (**c**) Case 3 (A-3925).

There are numerous genes located in the deleted area 12q13.3-q14.1, therefore, it is very complicated to say which of them could contribute to the development of MR and pulmonary stenosis in the patient 1. Menten *et al*. (2007) reported a new 12q14 microdeletion syndrome causing short stature, MR and osteopoikilosis. However, the causative microdeletion is located more distally and has no overlap with one found in our patient 1. However, there are two cases

reported in Decipher Database[10], who had larger deletion in this region and in one case with deletion 12q13.3-q14.2 a similar heart anomaly – pulmonary stenosis is also reported.

5.7.2.2. Case 2

A 10-year-old girl (A-4140): she has mild SNHL, failure to thrive (-2 SD) and a mildly dysmorphic face – small eyes, upslanted palpebral fissures, high palate, protruding ears (Figures 8c, 8d). In addition mild thorax deformation, long fingers and long toes were also noted.

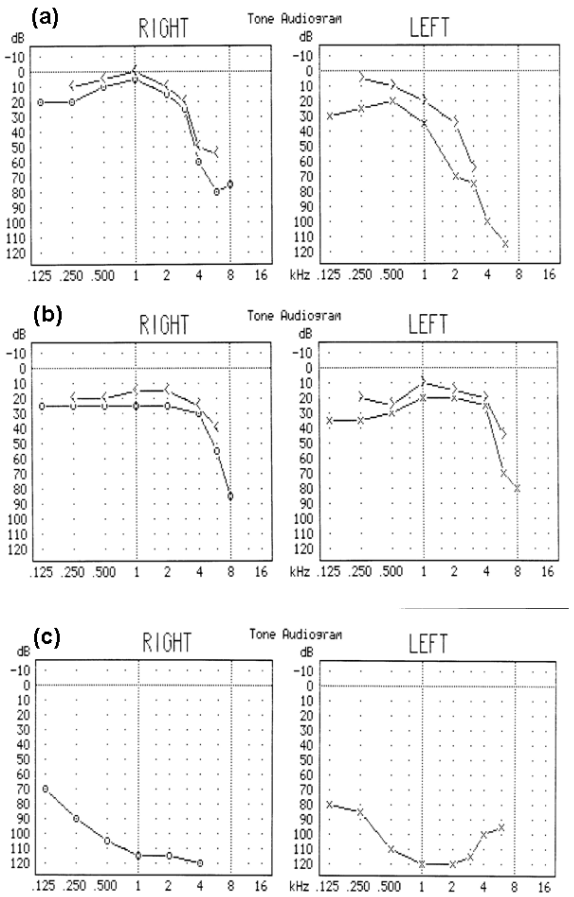

Figure 11: Audiograms from (**a**) Case 2 (A-4140) and (**b**) her mother; (**c**) Case 3 (A3925).

[10] https://decipher.sanger.ac.uk/application/

SNHL was diagnosed at the age of 5 years. She had normal hearing sensitivity on the low and middle frequencies and severe HL at high frequency in the right ear; mild deficits at low and middle frequencies and severe-to-profound hearing deficits at high frequency in the left ear (Figure 11a). The patient initially went to a regular school, but after one year she left to attend a school for children with the special needs of hearing and speech.

Family history: Patient's mother also has a hearing deficit, which developed in adult age. She has bilateral steeply sloping (mild to severe) SNHL (Figure 11b). The hearing of the patient's father was normal.

CMA was identified ~0.74-Mb size deletion in 3p26.2 region (2861527–3602205 bp), which she inherited from her father (Figure 9b). The positions (bp) of primers for qPCR validation analysis of 3p deletion are given in Figure 10b.

CMA identified ~0.74-Mb size deletion in 3p26.2 region (2.86–3.6 Mb) in patient 2, which she inherited from her father. To date, no genetic locus in the 3p25-pter region has been linked to dominant or recessive HL in humans (Van Camp and Smith, 2010). However, bilateral SNHL has been found in some but not all cases of 3p- syndrome, suggesting the possibility that it is due to loss of a critical gene in band 3p25 (McCullough *et al.*, 2007). This critical area can be even 3p26.3-p25, as only one case among seven analyzed by McCullogh *et al*. (2007) had no deletion in area 3p26.3 by marker analysis. *ATP2B2* gene (ATPase, Ca++ transporting, plasma membrane, 2) was located in 3p26-p25 region. Functional genomic analysis of the mouse homolog, *Atp2b2*, has demonstrated its possible role in hearing: mice heterozygous for a functional null *Atp2b2* allele show moderate, bilateral SNHL - a hearing phenotype similar to that seen in 3p- patients (McCullough and Tempel, 2004). It has not been shown to cause human HL, but was shown to have modifier effect, increasing the severity of HL caused in the first instance by mutations in other genes such as autosomal-dominant *MYO6* gene or autosomal-recessive *CDH23* gene (Schultz *et al.*, 2005; McCullough *et al.*, 2007). The precise localization of *ATP2B2* gene is not known, presently its location is assumed very near to the deleted area in our patient 2 at NCBI[11]. Therefore, is possible that the autosomal-dominant HL in this patient and her mother is caused by the mutation in some other autosomal-dominant gene (for example *MYO6* gene), but the haploinsufficiency of *ATP2B2* gene causes more severe clinical phenotype in the patient 2 in comparison to her mother.

3p deletion syndrome is a rare contiguous-gene disorder and characterized by developmental delay, growth retardation, and dysmorphic features (Schinzel, 2001). The majority of distal 3p deltions are terminal deletions, only very few interstitial deletions are described (Shuib *et al.*, 2009). Our patient 2 had growth retardation, dysmorphic facial features and mild SNHL, but normal mental development. It has been suggested that loss of *CNTN4* and *CRBN* gene contributes to MR in 3p- syndrome cases (Dijkhuizen *et al.*, 2006). Fernandez *et al.* (2004) demonstrated disruption of a single copy of *CNTN4* is sufficient to

[11] http://www.ncbi.nlm.nih.gov/

confer key aspects of the 3p deletions syndrome phenotype, including developmental delay. In our patient 2 both those gene are partially or totally deleted, but she has normal mental development. Moreover, her father who is also carrying this deletion, is normal too. Therefore, we assume that defects of some other genes in 3p25-p26 region should play a causative role in the etiology of MR. Our assumption is supported by Roohi *et al.* (2009) who showed that loss of *CNTN4* is not sufficient to cause a 3p deletion syndrome phenotype but, in combination with other autistic susceptibility alleles, could contribute to autistic spectrum disorders. Shuib *et al*. (2009) have recently suggested that *SRGAP3* gene may be the major determinant of MR on 3p deletion syndrome. Another explanation is variable penetrance of 3p deletions, which creates challenges in genetic counseling, as it was described by Pohjola *et al.* (2010) and as we saw in our case.

5.7.2.3. Case 3

A 10-year-old boy: in his first year, he was investigated in a children's neurology unit on several occasions due to delayed psychomotor development. Additionally, he had muscular hypotonia and paresis of *n. facialis* on the right side. At the age of 11 months bilateral profound SNHL was diagnosed (Figure 11c). At the age of 6 years our examination revealed a mildly dysmorphic face – downslanted palpebral fissures, widely spaced teeth, small mouth and thin upper lip (Figures 8e, 8f). Other dysmorphic features were lentigines, fifth finger clinodactyly, sandal gap, the clinodactyly of the toes, joints laxity and inverted nipples. Patient also had muscular hypotonia and vertigo. Family history: There are no other family members with hearing problems.

CMA analysis identified 0.54-Mb size deletion in 1p33 region (48649845–49208694 bp) (Figure 9c). The positions (bp) of primers for qPCR validation analysis of 1p33 deletion are given in Figure 10c. Analysis of DNA samples from A3925's father and his mother's sister revealed that the deletion in the patient appeared *de novo*.

The *SLC5A9* gene (solute carrier family 5, sodium/glucose cotransporter, member 9) is located in 1p33 region. The *SLC5A9* gene possibly acts as a Mannose/1,5-anhydro-D-glysitol/fructose transporter in the intestine and kidney and is required mainly for protein glycosylation (Tazawa *et al.*, 2005). It is known that some other anion exchangers (for example SLC26 gene family) are multifunctional and play intriguing roles in normal physiology and human pathophysiology including in pathophysciology of HL (Mount and Romero, 2004). Therefore, we assume that the haploinsufficiency of *SLC5A9* gene may play role in the development of profound SNHL in our patient. Three other genes are located in deleted area of patient 3 - *SPATA6* (spermatogenesis associated 6), *AGBL4* (ATP/GTP binding protein-like 4) and *BEND5* (BEN domain containing 5) gene, but by present knowledge we are not able to find a connection between the loss of one copy of those genes and patient 3 phenotype.

5.7.2.4. Case 4 (Publication III)

The patient was a 7-year-old boy with multiple congenital anomalies and developmental delay. He was born by normal vaginal delivery at term, with normal birth weight and length, but had microcephaly (-2 SD). He was the first child born in this family. The first evaluation by a clinical geneticist was performed at the age of 2 weeks in an intensive care unit. Chromosomal analysis showed an apparently balanced karyotype 46,XY,inv(9)(p12q13). At the age of two months he was placed in institutional care. At the age of 20 months Feingold syndrome (microcephaly-oculo-digital-esophageal-duodenal syndrome, OMIM 164280) or a still unidentified small chromosomal aberration were considered as differential diagnoses. At the age of 7 years, our clinical evaluation revealed normal stature, poor weight gain (-2.5 SD), severe microcephaly (-5 SD) and trigonocephaly. He had facial dysmorphism, symphalangism, contractures of large joints, hyperopia, strabismus, bilateral conductive HL, genital abnormality, psoriasis vulgaris and tracheo-esophageal fistula. He had profound mental retardation with stereotypic movements. The patient was initially investigated as a child with SNHL. Later investigations enabled the type of HL to be identified as conductive and this knowledge helped us to define the genotype-phenotype correlation.

Analysis with CMA detected a 5.9 Mb deletion in chromosome band 17q22–q23.2 with breakpoints between 48,200,000–48,300,000 bp and 54,200,000–54,300,000 bp (according to NCBI 36). The aberration was confirmed by real-time quantitative PCR analysis. Only eight cases involving deletions of chromosome 17 in the region q22–q24 have been reported previously.

Haplo-insufficiency of the *NOG* gene has been implicated in the development of conductive HL, skeletal anomalies including symphalangism, contractures of joints, and hyperopia in this patient and may also contribute to the development of either or both tracheo-esophageal fistula and esophageal atresia. Detailed information (including Figures) is available in Publication III.

5.8. Cytomegalovirus PCR analysis (Publication V)

In Group HL^{96}, consisting of probands whose etiology of HL remained unknown after DNA testing with the APEX method, we conducted DNA analysis of CMV infection from neonatal screening cards (blood stored on Guthrie cards). The neonatal screening cards were available for 85 (88.5%) of Group HL^{96}. We found positive results in five patients (2%) - Group $HL^{96/5}$ (see Table 12). Therefore HL in Group $HL^{96/5}$ is due to congenital CMV infection.

The most common environmental (non-genetic) cause of HL is estimated to be congenital CMV infection (Hilgert *et al.*, 2009b) with overall birth prevalence at 0.64%. Diagnosis is difficult, as only 11% of infected infants have non-specific symptoms at birth, and the definition of symptomatic varies between studies (Kenneson and Cannon, 2007). HL is a common sequela of

congenital CMV infection: (Grosse *et al.*, 2008) found that HL occurs in 30–40% of children symptomatic at birth with congenital CMV and in 5–10% of children with asymptomatic infections. More than two thirds of children congenitally infected with CMV develop HL only months or years after birth; HL may, therefore, be missed by a hearing screening at birth (Fowler *et al.*, 1999; Ludwig and Hengel, 2009). The patients in Group $HL^{96/5}$ had, in addition to HL, further medical problems. One patient, for example, initially had a suspected metabolic disease due to white matter lesions, which made the diagnosis more complicated. We did not found decreased vision among the five patients with congenital CMV infection. However, it was significant that MRI investigations found white matter lesions in two patients of Group $HL^{96/5}$. In investigating the etiology of patients with non-progressive abnormal white matter lesions and HL, molecular analysis of CMV DNA using blood stored on Guthrie cards can be helpful.

Table 12: Five patients with HL due to CMV infection

No	Age	Degree of HL	Type of HL	Karyotype	Additional findings
1	3.5 years	profound	bilateral sensorineuralis	46,XX	white matter lesions
2	9 years	moderate	bilateral sensorineuralis	–	–
3	13 years	profound	unilateral sensorineuralis	46,XY	white matter lesions
4	14 years	mild	unilateral sensorineuralis	46,XX	p.M34T heterozygosity in *GJB2* gene
5	9 months	moderate	bilateral sensorineuralis	–	–

6. CONCLUSIONS

1. The prevalence of mutation c.35delG heterozygosity in the *GJB2* gene among the Study Group of Estonian newborns (Group NB) is 1:22 (4.5%). This result is in accordance with a previous study including 113 Estonian subjects by (Gasparini *et al.*, 2000). The prevalence of mutation p.M34T heterozygosity in *GJB2* gene in Group NB is 1:17 (5.9%), which is even higher than the carrier frequency of c.35delG mutation. The prevalence of the carrier frequencies of the mutations c.35delG and p.M34T in the *GJB2* gene in Estonia is the highest of results found in European populations.
2. In the subset Study Group HL^{115} (equating to 49% of the overall Study Group HL^{233} of patients with early onset HL), we found a mutation in at least one allele of the *GJB2* gene. Seventy-three (31% of Group HL^{233}) were homozygous for the c.35delG mutation, seven (3% of Group HL^{233}) were homozygous for the p.M34T mutation, and five (2% of Group HL^{233}) had c35delG/p.M34T compound heterozygosity.
3. The most common *GJB2* gene mutations in Estonian children (both Group HL^{233} and Group HL^{115}) with early onset HL were c.35delG and p.M34T, with c.35delG accounting for 75% of *GJB2* alleles and p.M34T accounting for 12% of *GJB2* alleles. Another six identified mutations in *GJB2* gene occurred rarely (0.5–2%).
4. We did not find any children in Group HL^{233} with either *GJB3* or *GJB6* gene mutations. Mutations in the *GJB3* and *GJB6* genes (including del(*GJB6*-D13S1830) deletion) do not belong, as in other countries, to the frequent genetic causes of early onset HL in Estonian children.
5. We found five patients among the overall Study Group HL^{233} with heterozygous mutation in *SLC26A4* gene. As they have the heterozygous mutation in *SLC26A4* gene in only one allele, we cannot link this finding with children's HL.
6. The prevalence of HL due to mitochondrial mutations is low in Estonia, as it is in other European countries. We found only two children ($Patient^{M1}$ and $Patient^{M2}$) with mitochondrial mutations (0.9%). $Patient^{M1}$ had a mutation m.1555A>G in mitochondrial *12S r-RNA* gene and $Patient^{M2}$ had a mutation 7472insC in mitochondrial serine *tRNA-Ser* gene.
7. The most prevalent genotype c.35delG/c.35delG was connected to profound HL (50.7%), followed by severe HL (27.4%) and moderate HL (20.6% of patients). We did not find any children with c.35delG homozygosity and mild HL. Our results are in accordance with previous research, in which the majority of patients with c.35delG homozygosity have severe and profound HL, some c.35delG homozygotes have only moderate HL, and only in a few cases the HL is mild.
8. Study Group HL^{7}, patients with the second most frequent genotype – p.M34T homozygotes – all had mild HL, which is in accordance with previous research data.

9. We identified the IVS2–2A>G change in one allele of the *SLC26A5* gene in four probands (2.1%) with early onset HL and in five unaffected family members from five families. We did not find any homozygosity for this splice variant. Our data support the hypothesis that heterozygosity for the mutation IVS2–2A>G in *SLC26A5* gene may not, by itself, be sufficient to cause HL.
10. We confirmed six syndromes with HL in seven patients, Study Group HL^{S} equating to 3% of the overall Study Group HL^{233}. The types and frequencies of the six syndromic HL were: Waardenburg syndrome type II in two cases, and Stickler syndrome type II, Klippel-Feil syndrome, Kearns-Sayre syndrome, Usher syndrome and Goldenhar syndrome in one case each. The proportion of syndromic HL was lower in Group HL^{233} than 12–30% noted in previous research. This result may be due to two reasons. First, the main aim of the study was to investigate the genetic causes of non-syndromic HL; secondly Estonian children with early onset HL are referred to genetic consultation at an early age, before the clinical features of syndromic HL are fully developed. The latter reason is supported by two confirmed cases of LEOPARD syndrome in whom one of the main symptoms – HL – had not yet developed.
11. Among the overall Study Group HL^{233} were 24 children with an unknown etiology of HL (Group HL^{24}) all of whom, in addition to early onset HL, had subtle facial dysmorphism, and either of both a failure to thrive and developmental or behavioral problems. We conducted chromosomal microarray analysis (CMA) on the patients of Group HL^{24} and found in each of three separate patients, three regions with the loss of one allele. These three patients were:

    An 8.5 year-old girl has peculiar facial phenotype, developmental delay, mild SNHL and ~2.94-Mb size deletion in chromosomal region 12q13.3–q41.1. She has haplo-insufficiency of cochlear-expressed *MYO1A* gene (DFNA48).

    A 10-year-old girl with subtle facial dysmorphism and mild SNHL has ~0.74-Mb size deletion in 3p26.2 region, which she inherited from her father. The *ATP2B2* gene was located in 3p26-p25 region. Other research has not shown this to cause human HL, but has been shown to have a modifier effect, increasing the severity of HL caused in the first instance by mutations in other genes.

    A 10-year-old patient has severe SNHL, subtle facial dysmorphism and 0.54-Mb size deletion in 1p33 region. Research has yet to identify an SNHL locus in this area.
12. DNA analysis of congenital CMV infection conducted retrospectively on neonatal screening Guthrie cards of Study Group HL^{96} produced a positive result in five patients Group $HL^{96/5}$ (2% of Group HL^{233}). This result is in accordance with previous research, which estimates that the most common environmental (non-genetic) cause of HL is congenital CMV infection.

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SUMMARY IN ESTONIAN

Varajase algusega kuulmislanguse geneetilised põhjused Eesti lastel

Pärilik ehk geneetiline kuulmislangus

Kuulmislangus (KL) on kõige sagedasem pärilik sensoorne haigus. Ligikaudu 70 miljonit inimest kogu maailmas kannatab KL all, mis häirib oluliselt nende suhtlusvõimet ja vähendab tunduvalt toimetulekut iseseisvas elus. Varajase ehk kõne-eelse KL esinemissagedus arvatakse olevat ligikaudu 1–2 juhtu 1000 lapse kohta (Marazita *et al.*, 1993; Cryns *et al.*, 2004; Snoeckx *et al.*, 2005; Hilgert *et al.*, 2009b). Kuni 60%-l juhtudest on tegemist päriliku ehk geneetilise KL-ga (Finsterer and Fellinger, 2005).

Geneetiline KL jagatakse sündroomseks ja mittesündroomseks vormiks. (Finsterer and Fellinger, 2005). Sündroomse vormi korral esineb patsiendil lisaks KL-le väliskõrva nähtav anomaalia või teiste elundisüsteemide patoloogia, kus elundite haaratus põhjustab erinevaid meditsiinilisi probleeme. Mittesündroomse ehk isoleeritud vormi korral ei esine KL-ga patsiendil väliselt nähtavaid arenguanomaaliaid ega teiste elundisüsteemide patoloogiat (Smith and Van Camp, 2007).

Päriliku KL-ga seotud geenide kaardistamise ja identifitseerimisega alustati XX. sajandi 90-ndate aastate esimesel poolel. Autosoom-dominantseid (AD) lookuseid tähistatakse DFNA, kus DFN on lühend inglisekeelsest sõnast *deafness* ja A tähistab autosoom-dominantset KL-st. Autosoom-retsessiivseid (AR) lookuseid tähistatakse DFNB, kus DFN pärineb sõnast *deafness* ja B tähistab autosoom-retsessiivset KL-st. Esimene DFNA1 lookus kaardistati 1992. aastal ja esimesed AR KL-ga seotud geenid isoleeriti 1997. aastal (Petersen and Willems, 2006). Tänapäeval arvatakse, et normaalse kuulmisprotsessiga on seotud umbes 1% inimese geenidest, s.o. 300–500 geeni, KL põhjustajana on hetkel kirjeldatud kõigest 120 geeni (Finsterer and Fellinger, 2005). Kuigi kahekümnenda sajandi viimasel dekaadil identifitseeriti kiiresti suur hulk KL-ga seotud geene, tuleb sellesse siiski suhtuda kui senini pooleliolevasse töösse, kus on teada vaid umbes 1/3 kõikidest KL-ga seotud geenidest (Nance, 2003; Finsterer and Fellinger, 2005; Teek *et al.*, 2007).

Mutatsioon c.35delG *GJB2* geenis

Vaatamata suurele AR- tüüpi pärilikku KL-st põhjustavate geenide arvule on kindlaks tehtud, et enamus AR mittesündroomse KL juhtudest (58–88%) on seotud 13. kromosoomi regioonis q11–q12 asuva DFNB1 lookusega ning on tingitud mutatsioonist *GJB2* geenis (MIM 121011). *GJB2* geen kodeerib nn tühimikku ühendavat valku (*gap junction protein*) – konneksiin 26 (Guilford *et*

al., 1994). *GJB2* geenis esinev c.35delG lugemisraami nihet põhjustav mutatsioon on Euroopas populatsioonides kõige sagedamini esinev pärilikku KL põhjustav mutatsioon (Gasparini *et al.*, 2000). c.35delG kandluse esinemissagedust erinevatel Euroopa rahvastel on põhjalikult uuritud ning arvatakse, et c.35delG on kõige sagedamini esinev haigusseoseline mutatsioon (Denoyelle *et al.*, 1997; Estivill *et al.*, 1998; Petersen and Willems, 2006). Kirjanduse andmetel esineb kõige kõrgem c.35delG kandlus Vahemere maades ja Lõuna-Euroopas (Gasparini *et al.*, 2000; Petersen and Willems, 2006). Samas leiti 2000. aastal Gasparini ja kaasautorite poolt tehtud erinevaid Euroopa riike hõlmavas c.35delG kandluse uuringus, et Eestis on c.35delG kandlus 1:22,5-le, mis on kõrgeim Euroopas (Gasparini *et al.*, 2000; Petersen and Willems, 2006).

DNA diagnostikas KL testimisel kasutatakse kõige enam *GJB2* geenis asuva mutatsiooni c.35delG analüüsi, mida leitakse umbes 30%-l KL-ga patsientidel (Gasparini *et al.*, 2000; Snoeckx *et al.*, 2005).

Mutatsioon p.M34T *GJB2* geenis

Esimeste isoleeritud *GJB2* geeni mutatsioonide seas oli ka aminohappe asendusmutatsioon p.M34T. Kirjanduses vaieldakse jätkuvalt p.M34T patogeensuse üle. Sageli käsitletakse p.M34T mutatsiooni kui AR patogeenset mutatsiooni, mille toime avaldub siis kas koos teiste *GJB2* geeni koosesinemisega või homosügootsena (Cohn and Kelley, 1999; Cucci *et al.*, 2000; Houseman *et al.*, 2001; Janecke *et al.*, 2002; Lopponen *et al.*, 2003; Snoeckx *et al.*, 2005; Azaiez *et al.*, 2007), teiste autorite arvates ei ole p.M34T patogeenne mutatsioon (Green *et al.*, 1999; Griffith *et al.*, 2000; Marlin *et al.*, 2001; Feldmann *et al.*, 2004; Snoeckx *et al.*, 2005). Mutatsiooni p.M34T esinemissagedust nii üldpopulatsioonis kui ka KL-ga patsientide hulgas on tunduvalt vähem uuritud kui c.35delG esinemist. Mitmete tööde juures on ära märgitud, et mutatsiooni p.M34T kandlus on üldpopulatsioonis kõrge, teinekord isegi kõrgem võrreldes mutatsiooni c.35delG kandlusega (Cucci *et al.*, 2000; Marlin *et al.*, 2001; Lopponen *et al.*, 2003; Roux *et al.*, 2004). Samas KL-ga isikute hulgas on p.M34T homosügootsus või liitheterosügootsus harvaesinev (Green *et al.*, 1999; Roux *et al.*, 2004). Antud probleemi olemuse selgitamiseks ei ole praeguseni ühtset seisukohta.

Töö eesmärgid

1. Määrata *GJB2* geeni mutatsioonide c.35delG ja p.M34T esinemissagedus Eesti vastsündinute populatsioonis.
2. Teha kindlaks *GJB2, GJB3, GJB6, SLC26A4* geenide ja kahe mitokondriaalse geeni (*12S rRNA* and *tRNA-Ser*) mutatsioonide esinemissagedus Eesti KL-ga laste hulgas.

3. Kirjeldada *GJB2* geeni mutatsioonidega patsientide genotüübi ja fenotüübi vahelisi seoseid.
4. Uurida *SLC26A5* (prestiini) geenis mutatsiooni IVS2–2A>G esinemist Eesti KL-ga isikute ja nende pereliikmete hulgas.
5. Iseloomustada harvaesinevaid KL-ga seotud sündroome.
6. Kirjeldada väikeste submikroskoopiliste kromosomaalsete ümberkorralduste esinemist lastel, kelle peamiseks kaebuseks/probleemiks on KL.
7. Määrata molekulaarse analüüsiga kaasasündinud tsütomegaloviirus-infektsiooni (CMV) esinemine Eesti KL-ga lastel.

Uurimisgrupid ja uurimismeetodid

Mutatsioonide c.35delG ja p.M34T esinemissagedus Eesti vastsündinute populatsioonis

Uuringugrupis oli 998 anonüümset vastsündinut, kes olid järjestikku sündinud Eesti erinevates piirkondades ühe kuu jooksul (jaanuar 2005). Uuringuks kasutasime kuivatatud vereplekke Guthrie kaartidelt (vastsündinute skriining-programm fenüülketonuuria ja kaasasündinud hüpotüreoosi suhtes). Uurisime kuivatatud vereplekkidest eraldatud vastsündinute DNA-d kahe Eestis sagedamini esineva KL-st põhjustava *GJB2* geenis asuvate c.35delG and p.M34T mutatsioonide suhtes. Geograafilise gradiendi määramiseks jagasime Eesti kolmeks piirkonnaks: Põhja, Lääne ja Kagu Eestiks.

Varajase algusega KL-ga laste uuringugrupp

Selles uuringugrupis oli 233 last vanuses 0–18 aastat üle Eesti, kellel oli diagnoositud lapseea-algusega KL. Patsiendid olid esmalt pöördunud nina-kõrva-kurguarsti konsultatsioonile kas vastsündinute KL skriininguuringu mitteläbimise tõttu või hiljem tekkinud kahtlusega KL esinemisele. Uuringugruppi kuulusid lapsed, kellel KL diagnoos kinnitus ja nad suunati edasi geneetiku konsultatsioonile ajavahemikus 1. jaanuar 2000 kuni 31.märts 2009. Põhja-Eesti piirkonnas toimus geneetiku konsultatsioon Tallinna Lastehaiglas ja Lõuna-Eestis Tartu Ülikooli Kliinikumis. Kõikidel juhtudel täpsustati perekonna anamnees ja sugupuu koostamisel pöörati tähelepanu just KL esinemisele mitme põlvkonna jooksul. Sündroomse KL diagnostikaks märgiti kliinilisel läbivaatusel üles kasvu parameetrid ja fenotüübis esinevad tunnused elundkondade kaupa.

Kõigile uuringugrupi patsientidele tehti:

a) DNA analüüs seitsmes erinevas geenis (*GJB2, GJB3*, *GJB6*, *GJA1*, *SLC26A4, SLC26A5, 12S-rRNA* ja *tRNA-Ser* geen) paikneva 199 mutatsiooni suhtes APEX (*arrayed primer extension* – praimerekstensioon oligonukleotiidmaatriksil) meetodil (Asper Biotech, Tartu) Tartu Ülikooli Ühendlabori geneetikakeskuses (Gardner *et al.*, 2006). Kakskümmend kolm patsienti olid uuritud enne 2005. aastat *GJB2* mutatsiooni c.35delG suhtes PCR analüüsiga ja neil oli leitud mutatsiooni c.35delG homosügootsus. Seetõttu nendele patsientidele APEX analüüsi ei tehtud. Teistel enne 2005. aastat uuritud patsientidel, kaasa arvatud mutatsiooni c.35deG heterosügoodid, tehti hiljem juurde APEX analüüs.
b) Kliinilise näidustuse alusel tehti kromosomaalne analüüs perifeerse vere lümfotsüütide kultuurist 55 patsiendile.
c) Patsientide hulgast, kelle KL etioloogia jäi ikkagi ebaselgeks, valiti välja 96 last kaasasündinud tsütomegaloviirusinfektsiooni (CMV) DNA analüüsi teostamiseks vastsündinute skriiningkaartidelt (kuivatatud vereplekid Guthrie kaartidel). Kuna Eesti vastsündinute skriiningprogramm fenüülketonuuria suhtes algas 1993. aastast, siis testkaardid olid kättesaadavad 96-st väljavalitud patsiendist 85-l.
d) Allesjäänud ebaselge etioloogiaga KL-ga laste hulgast valisime välja 24 patsienti, kellel esines lisaks KL-le näo düsmorfism, arengus mahajäämus või käitumishäired ning kellel me ei diagnoosinud KL-ga seotud sündroomi. Antud 24-l patsiendil tegime kogu genoomi genotüpiseerimise analüüsi, kasutades selleks HumanCytoSNP-12 versiooni 1.0 või Human370CNV-Duo (Illumina Inc., San Diego, CA, USA; www.illumina.com). Sündroomse KL diferentsiaaldiagnostikas kasutasime Londoni Düsmorfoloogia andmebaasi (Winter and Baraitser, 2010).

Uuringu peamised tulemused

1. Mutatsiooni c.35delG kandluse esinemissagedus Eesti vastsündinute populatsioonis oli 1:22 (4,5%). See tulemus vastab eelnevalt 113-l Eesti isikul läbiviidud uuringu tulemustele. Mutatsiooni p.M34T kandlus Eestis oli 1:17 (5,9%), mis on kõrgem kui mutatsiooni c.35delG kandlus. Mutatsioonide c.35delG ja p.M34T kandluse esinemissagedus Eestis on kõrgeim võrreldes teiste Euroopa rahvastega.
2. Varajase KL-ga patsientide hulgast (233 patsienti) leidsime vähemalt ühes *GJB2* geeni alleelis mutatsiooni 115-l (49%), kellest 73 patsienti (31%) olid homosügootsed c.35delG mutatsiooni ja seitse (3%) homosügootsed p.M34T mutatsiooni suhtes ning viiel patsiendil (2%) esines mutatsioonide c.35delG /p.M34T liitheterosügootsus.

3. Kõige sagedamini esinesid Eesti KL-ga laste hulgas *GJB2* geeni mutatsioonidest c.35delG ja p.M34T. Mutatsioon c.35delG esines 75% ja mutatsioon p.M34T 12% *GJB2* alleelidest. Ülejäänud kuus *GJB2* geeni mutatsiooni esinesid harvem (0,5–2%).
4. Me ei leidnud oma uuringugrupis ühtegi last *GJB3* või *GJB6* mutatsioonidega. Seega nagu ka mitmetes teistes riikides, ei kuulu *GJB3* ja *GJB6* geeni mutatsioonid Eestis sagedaste varajase algusega KL geneetiliste põhjuste hulka.
5. Me leidsime viis patsienti, kellel esines heterosügootne mutatsioon *SLC26A4* geenis. Kuna nendel oli mutatsioon vaid *SLC26A4* geeni ühes alleelis, ei saa me antud leidu lastel esineva KL-ga seostada.
6. Mitokondriaalsetest mutatsioonidest põhjustatud KL on Eestis madala esinemissagedusega, samad andmed on ka teiste Euroopa riikide kohta. Me leidsime oma uuringugrupist vaid kaks last, kellel esines mitokondriaalne mutatsioon (0,9%). Ühel lapsel oli mutatsioon m.1555A>G mitokondriaalses *12S r-RNA* geenis ja teisel lapsel mutatsioon 7472insC mitokondriaalses *tRNA-Ser* geenis.
7. Kõige enam levinud genotüübiga – c.35delG/c.35delG patsientidel esinesid sagedamini väga raske (50,7%) ja raske (27,4%) KL ning kolmandal kohal oli keskmine KL (20,6%). Mutatsiooni c.35delG homosügootide hulgas ei olnud meil ühtegi kerge KL-ga last. Meie uuringutulemused on vastavuses kirjanduse andmetega, kus enamusel c.35delG homosügootsusega patsientidel kirjeldatakse väga rasket ja rasket KL (Cryns *et al.*, 2004). Mõnedel patsientidel on keskmine KL ja üksikjuhtudel on varasemalt leitud ka kerget KL-st
8. Sageduselt teine genotüüp oli p.M34T/p.M34T ja kõikidel antud genotüübiga uuritud lastel esines kerge KL nii nagu ka leitud kirjanduse andmetel.
9. Neljal KLga probandil (2,1%) ja nende viiel tervel pereliikmel kokku viiest perekonnast leidsime *SLC26A5* geeni ühes alleelis mutatsiooni IVS2–2A>G. Me ei leidnud ühtegi antud mutatsiooni homosügooti. Meie töö tulemused toetasid ka varem kirjanduses esitatud arvamust, mille kohaselt IVS2–2A>G heterosügootne mutatsioon *SLC26A5* geenis ei ole üksinda piisav KL väljakujunemiseks.
10. Kliiniliste uuringute põhjal diagnoosisime seitsmel patsiendil (3%) sündroomset KL-st. Kahel patsiendil kinnitus Waardenburgi sündroomi II tüübi diagnoos ja ühel patsiendil vastavalt Stickleri sündroomi II tüübi, Klippel-Feili sündroomi, Kearns-Sayre sündroomi, Usheri sündroomi või Goldenhari sündroomi diagnoos. Meie uuringugrupis oli sündroomse KL osakaal madalam kui kirjanduse andmetel (12–30%). See võib olla tingitud meie eesmärgist uurida peamiselt mittesündroomse KL geneetilisi põhjuseid. Samuti pöörduvad varajase algusega KL-ga lapsed geneetiku konsultatsioonile esimestel eluaastatel, kuid selles eas ei ole sündroomse KL kliinilised tunnused veel välja kujunenud. Seda toetavad ka kaks LEOPARD sündroomi molekulaardiagnostiliselt kinnitatud juhtu, kus KL kui antud

sündroomi üks peamistest sümptomitest, ei olnud veel patsientidel välja kujunenud.

11. Valisime KL-ga laste grupist 24 patsienti, kelle KL-se etioloogia oli eelnevate uuringute järgselt endiselt ebaselgeks jäänud, kellel esines lisaks KL-le düsmorfne välimus, arengus mahajäämus ja/või käitumishäired ning kellel ei saanud diagnoosida sündroomset KL-st. Uurisime antud patsiente kasutades ülegenoomset genotüpiseerimist Illumina geenikiipidega. Kolmel patsiendil (12%) leidsime 3 erinevat deleteerunud kromosoomipiirkonda, mis võivad olla seotud antud patsientidel esinevate kliiniliste sümptomitega. Ühel 8,5 aasta vanusel tüdrukul esinesid omapärane näo fenotüüp, arengus mahajäämus, kerge sensorineuraalne KL (SNKL) ja ~2,94-Mb suurune deletsioon 12q13.3-q41.1 kromosomaalses piirkonnas. Arvestades leidu oli patsiendil kohleas ekspresseeruva *MYO1A* geeni (DFNA48) haploinsuffitsentsus. 10-aastasel tüdrukul esinesid vähene näo düsmorfism ja kerge SNKL ning ~0,74-Mb suurune deletsioon 3p26.2 kromosomaalses piirkonnas, mille ta oli pärinud oma isalt. 3p26-p25 kromosomaalsesse piirkonda lokaliseerub *ATP2B2* geen, mida ei teata küll otseselt KL põhjustajana, küll aga seostatakse antud geeni modifitseeriva efektiga, kus see mõjub teiste geenide poolt põhjustatud KL raskema fenotüübi väljakujunemisele. Kolmas patsient oli 10-aastane poiss, kellel esines raske SNKL, vähene näo düsmorfism ja 1p33 piirkonnas ~0,54-MB suurune deletsioon. Teadaolevalt ei ole antud piirkonda lokaliseeritud ühtegi SNKL tekkega seotud olevat lookust.
12. DNA analüüsi kaasasündinud CMV infektsiooni esinemise suhtes leidsime viiel patsiendil (2%) positiivse tulemuse. Antud tulemus on kooskõlas kirjanduse andmetega, kus kõige sagedasemaks omandatud (ehk mittegeneetilise) KL põhjustajaks peetakse just kaasasündinud CMV-infektsiooni. Kaasasündinud CMV infektsiooni on kerge retrospektiivselt testida, kasutades selleks vastsündinute Guthrie kaarte.

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PUBLICATIONS

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Teadustegevus

Kokku on ilmunud 7 publikatsiooni ja 8 ettekannet rahvusvahelistel konverentsidel.

Teadustöö on olnud seotud järgmiste valdkondadega: kuulmislanguse geneetiliste põhjuste väljaselgitamine, kliiniline geneetika ja düsmorfoloogilised sündroomid.

Eesti Nooremarstide Ühenduse ja Eesti Inimesegeneetika Ühingu liige.

DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS

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