NONSYNDROMIC HEARING LOSS OF GENETIC ORIGIN IN HUNGARY: FROM MOLECULAR BIOLOGY TO CLINICAL DATA

PhD Thesis

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List of publications related to the thesis

I. A. L. Nagy, R. Csáki, F. Tóth, R. Sepp, M. Csanády Sr., M. Csanády Jr., Gy. Tálosi, J. Klem, K. Kovács, J. Jóri, J. G. Kiss: Possibilities of early detection of hearing disturbances of genetic origin. Proceedings of the 8th International Congress of The Mediterranean Society of Otology & Audiology, 17 - 21 May 2006 Dubrovnik, Medimond. Srl. Bologna, Italy Pp:15-18 (2006).

II. Nagy AL, Csáki R, Klem J, Rovó L, Tóth F, Tálosi G, Jóri J, Kovács K, Kiss JG.: Minimally invasive genetic screen for GJB2 related deafness using dried blood spots. Int J Pediatr Otorhinolaryngol. 2010 Jan;74(1):75-81.

III. A GJB2 gén 35delG mutációjának vizsgálata a Szegeden cochlearisan implantáltak és rokonaik populációjában allél specifikus polimeráz láncreakcióval.Nagy L. Attila, Dr. Csáki Róbert, Dr. Tálosi Gyula, Klem József, Dr. Jóri József, Dr. Kovács Kornél, Dr. Kiss József Géza (accepted for publication)

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List of Abbreviations

AA	Amino Acid
AD	Autosomal Dominant
AR	Autosomal Recessive
ARNSHL	Autosomal Recessive NonSyndromic Hearing Loss
AS-PCR	Allele-Specific PCR
dB	deci Bel (measure of loudness)
DBS	Dried Blood Spot (usually on a piece of Guthrie paper)
DFN	DeaFNess; abbreviation for X-linked NSHL's
DFNA	DeaFNess; abbreviation for AD NSHL's
DFNB	DeaFNess, abbreviation for AR NSHL's
dHPLC	Denaturing High Performance Liquid Chromatography
DNA	DeoxyriboNucleic Acid
EDTA	EthyleneDiamide-TetraAcetate
GJB	Gap Junction Beta – genes (with a numbered suffix) that code various subunits of the Gap Junction Beta family of proteins
HL	Hearing Loss
Hz	Hertz (measure of frequency)
IHC	Inner Hair Cell
kb or kbp	kilo base or kilo base pairs
mRNA	messenger RNA
NSHL	NonSyndromic Hearing Loss
OHC	Outer Hair Cell
PCR	Polymerase Chain Reaction
RNA	RiboNucleic Acid
rRNA	ribosomal RNA
SNP	Single Nucleotid Polymorphism
TEAA	TriEthylAmmonium-Amine

1 Introduction

1.1 Hearing disorders in general

Hearing disorders are amongst the most frequent sensory organ deficits in humans. Its causes are unknown in around 40% of all the investigated cases. In 30%, the etiology can be genetic, where hearing losses caused by various syndrome-causing genetic defects take about 3%, and cases with nonsyndromic origin take between 25-29%. Prenatal causes (rubella, CMV, alcohol, measles, bone malformations, etc), account for 11-12%, perinatal etiology (asphyxia, prematurity, drugs, etc) account for 9%. Postnatal etiology (meningitis, trauma, chemotherapy, for example) accounts for about 6-8% [1]. Probably some of the cases that were previously described as unknown are of genetic origin as well.

1.2 A short overview of the hearing process

The human hearing system is divided into three parts: the external ear, the middle ear, and the inner ear.

The external ear consists of the auricle, the external auditory canal and the eardrum (tympanum). The middle ear consists of the hearing ossicles (malleus, incus, stapes- hammer, anvil, stirrup), situated in the tympanic cavity. The Eustachian tube joins the tympanic cavity with the outer air, and hence equalizes pressure through the nasopharynx. In the inner ear, the cochlea and the semicircular canals can be found.

The energy of the sound, collected by the auricles, passes through the external auditory canal, then the movements of the air are displaced by the eardrum onto the chain of heaing ossicles, and finally into the fluid-filled space of the inner ear, the cochlea, by the stapes through the oval window. If this energy would simply pass from the surrounding air into some fluid, most of it would be lost, that is, reflected back from the surface of the fluid. The eardrum acts as an amplifier, it's surface is around 55 mm^2 on the outside, and the surface of the malleus (hammer) is about 3.2 mm^2 on the inside, that joins the eardrum from the direction of the middle ear. This alone makes a 17 times amplification possible, which takes 24,5dB gain. If the pressure would not be equalized by the Eustachian tube, the eardrum could become too tense, and that would mean to lose this amplification. The chain of the hearing ossicles provides some more gain, around 1,3-1,5 times [2].

Inside the cochlea, the movement of the fluid is transformed into electrical signals by the organ of Corti (the process of the so-called mechanoelectrical transduction), and then this information arrives into the central nervous system through the VIIIth cranial nerve. (Figure 1.)

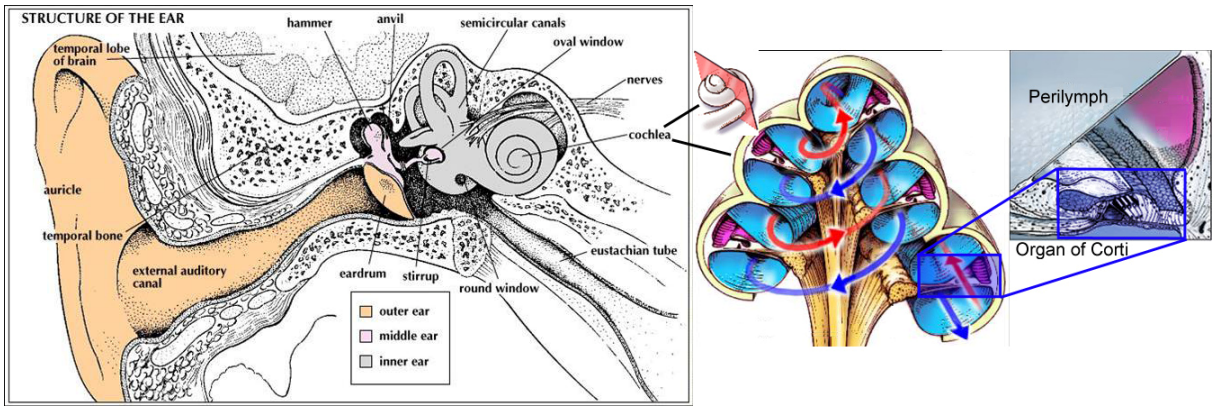

Figure 1.
The schematic drawing of the human hearing system
To the left the three parts are shown, the outer-, middle-, and the inner ear. The cochlea is cut into half, and is shown in detail. The organ of Corti – with its complex structures – is shown in the insert to the right

1.3 Genetics of hearing

To date, approximately 100-150 genes are estimated to be involved in the physiological processes related to hearing. 146 disease-causing alleles of 42 genes have been identified so far (57 dominant, 77 recessive, 8 X-linked, 1 Y-linked, 2 modifiers, 1 auditory neuropathy) [3]. The large number of genes and loci complicate the genetic analysis of nonsyndromic hearing losses.

1.3.1 Nomenclature of nonsyndromic hearing losses

The three letters “DFN” (DeaFNess), without a suffix, are only used in case of nonsyndromic genetic disorders that are X-linked.

“DFNA” denotes autosomal dominant deafness that is passed directly through generations.

“DFNB” denotes autosomal recessive nonsyndromic hearing losses that require the allele to be present on both homologus chromosomes. All three abbreviations are used with a numbered suffix that corresponds to the order they were described and published. These names do not necessarily correspond to different genes; they rather denote loci. In fact, there

are more genes that have more DFN's, there are genes that's mutation can cause both dominant and recessive forms of nonsyndromic hearing losses.

1.3.2 The studied genes

Gene						Site of expression	DFN locus
Family	Name	Chromosome localization	Size(bp)/ number of coding exon(s)/ size of coding exon (bp)	Product			
				Name	Length (AA)/molecular weight (kDa)		
Connexins	**GJB2**	13q12-13 [4]	5510/1/ 681	Connexin26/Gap Junction Beta-2 subunit	226/26,215	inner ear, skin	DFNB1 [5], DFNA3 [6]
	GJB3	1p35.1	5178/1/ 813	Connexin 31/Gap Junction Beta 3 subunit	270/30,818	cochlea, VIIIth cranial nerve [7]	DFNA2B [8]
	GJB6	13q12	10356/1/ 783	Connexin 31/Gap Junction Beta 6 subunit	261/30,387	trachea, thyroid, brain, cochlea[9] [10]	DFNA3 [10]
	GJA1	6q21-q23.2	14129/1/?	Connexin 43/Gap Junction Alfa 1 subunit	382/43,008	Heart [11], liver, autonomic and sensory neurons [12]	DFNA3 [13]
Unconventional myosins	**MYO6**	6q13	170346/ 32/? [14]	Myosine VI	1294/149, 691	Inner hair cells [15][16]	DFNA22 [17], DFNB37 [18]
Transcription factors	**POU3F4**	Xq21.1	1491bp/?/ 1083	Brain-specific homeobox/POU domain protein 4	361/39,427	Brain, neural tube, otic vesicle (mouse) [19]	DFN3 [20]
Ion channels	**SLC26A4**	7q31	57175/?/?	Pendrin	780/85,723	inner ear, thyroid, kidneys [21]	DFNB4 [22]
	KCNQ4	1p34	54677/?/ 695	Potassium voltage-gated channel subfamily KQT member 4	695/77,092	Outer hair cells, basal lamina [23]	DFNA2A [24]
	COCH	14q12-q13	16058/?/?	Cochlin precursor/Coagulation factor C homology	550/59,483	Cochlea, vestibulum [25]	DFNA9 [26]
Mitochondrial ribosomal RNA	**12s rRNA/ MTRNR1**	19q13.33 [27]	954/1/954	12s rRNA	-	Mitochondria in every human cell	DFNA4 [27][28] [29]

Table 1.
A table showing the genes, some of their properties, and the DFN locus that was mapped to them. The numbers in square brackets correspond to their respective numbers in the chapter "References"

1.3.2.1 Connexins

Connexins are a family proteins that form the so-called gap junctions between cells. There are 24 known connexins until today. A gap junction consists of two connexons, which are

composed of six connexins (Figure 2.) Connexins can form heterohexamers, so they can substitute each other, in these cases, their function can be partially restored. Connexins are only expressed in vertebrates. In humans, connexins are mostly expressed in the connective tissue underlying the skin, in the central and peripheral nervous system, in the kidneys, in the liver, and in the thyroid for example. They enable the flux of some smaller molecules or hydrated ions. The pore, or channel, that is formed by the hexamers is 10-15 Å in diameter, and can let pass particles through in the size range between 400Da and 1kDa, depending on the type of the actual connexin [30]. In the cochlea [31][32] connexins facilitate the flux, and the recycling of K^+ ions from the intracellular space to the endolympha [33].

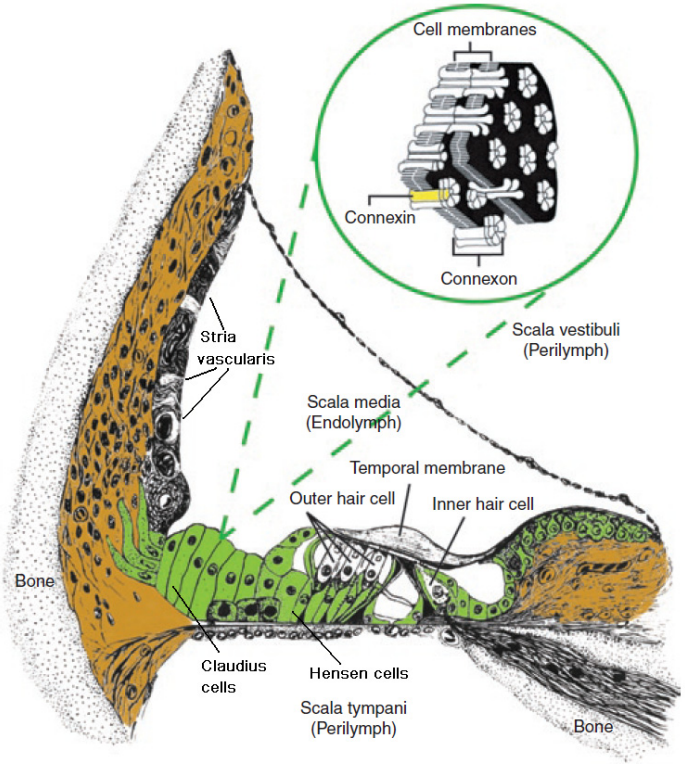

Figure 2.
Organization of connexins and their approximate site of expression in the inner ear.
The insert shows the units of the gap junction ("Connexin", painted yellow), the "Connexon", which is formed by six connexins, and the whole gap junction, which is formed by two connexons. In the inner ear, K^+ is transported from the hair cells through the Hensen and Claudius cells to the stria vascularis and from there they are transported back to the endolympha.
Reproduced from [34]

1.3.2.1.1 GJB2 (Connexin 26)

DFNB1 was the first identified locus; its autosomal recessive mutation causes nonsyndromic hearing loss [5]. Using co-segregation analysis, this locus was mapped to 13q12-13[4].

In 1994, Chaib et al. described the first dominant nonsyndromic hearing loss of genetic origin to 13q12-13.[6] Mutation in the coding region of Gap Junction protein Beta 2 (GJB2) - also called Connexin26 (Cx26) - was the first to be linked to nonsyndromic hearing loss of genetic origin.[35] Connexin 26 is a protein, which belongs to the family of connexins. The length of the functional, protein coding exon of GJB2 is 681 bp, and it codes a 226 amino acid polypeptide. 35delG causes a frameshift mutation, the deletion of one guanine residue in a stretch of six guanines in the coding region of the gene at position 35, resulting in a nonsense mutation at the 13th codon. 35delG mutation of the GJB2 gene accounts for 7-15% of nonsyndromic hearing losses of genetic origin in the European population [36][37][38], and regarding this mutation, about 1-5% of the Caucasian population is a carrier [39]

1.3.2.1.2 GJB3 (Connexin 31)

This protein was first cloned in 1998 [40]. Xia et al. [8] mapped DFNA2B to this gene in 1998. It has been shown also, that mutations in the coding region of this protein can lead to ARNSHL as well [41]. This protein is mostly expressed in the cochlea, and in the VIIIth cranial nerve [7]. Liu et al. showed that in the mouse cochlea CX31 is co-expressed with Cx26 [42]. Its chromosome localization is 1p35.

1.3.2.1.3 GJB6 (Connexin 30)

This protein is mostly expressed in the skin, in the trachea, thyroid, brain, and in the cochlea [9][10]. DFNA3 was mapped to this locus [10]. Part of a digenic GJB2/GJB6 deafness was also mapped to this gene [43]

1.3.2.1.4 GJA1 (Connexin 43)

The gene map locus for GJA1 is 6q21-q23.2. It has been shown by Liu et al. that mutations in this gene can lead to ARNSHL [13], although this connexin is mostly expressed in the human heart [11], the liver, and in certain autonomic and sensory neurons [12].

1.3.2.2 MYO 6 (Myosin VI)

The gene map locus for MYO6 is 6q13. This gene has 32 exons, its length is 70 kb [14]. Two loci, DFNA22 [17] and DFNB37 [18] were both mapped to this gene. The protein product of MYO6 plays an important role in the intracellular vesicle and organelle transport and acts as

an anchor protein as well [44]. There are reports that MYO6 may be necessary for the maturation of IHCs [15] [16]

1.3.2.3 Transcription factors

Transcription factors are proteins that bind to the DNA and regulate the production of mRNA, and hence gene expression. They play an important role in the development of cells and the organism.

1.3.2.3.1 POU3F4

Gene map locus is Xq21.1. The rat homologue of this gene, called RHS2 is expressed in the inner ear during the embryonic development [18]. DFN3 has been shown to map here [20]. There may be inner ear malformations connected to mutations in this locus described as early as 1967 [45], that later have been confirmed [46].

1.3.2.4 Transporter proteins

These proteins are involved in the transport of certain types of ions or molecules across membranes. Sometimes they work against the chemical concentration gradient, and in these cases, they use ATP as the source of energy.

As the processes of hearing, more specifically the mechanoelectric transduction in the organ of Corti needs very precisely controlled ionic composition of fluids in the cochlea [2] (and see also Figure 1. and Figure 2.), these type of proteins play a crucial role in the maintenance of homeostasis in the inner ear, and thus in the process of hearing.

1.3.2.4.1 SLC26A4 (Pendrin, PDS gene)

Its chromosome localization is 7q31. The gene's transcript is around 5 kb, and it is coding a 780 AA long protein. It is expressed in the thyroid, the inner ear and in the kidneys. SLC26A4 is an anion transporter; it transports chloride, iodide [47] and carbonate [21]. DFNB4 is mapped here [22], as well as the mutation that causes the Pendred syndrome [48], and the Enlarged Vestibular Aqueduct syndrome [49].

1.3.2.4.2 KCNQ4

Its chromosome localization is 1p34. This is a Potassium channel, which regulates the ionic composition of the endolympha in the inner ear, and is also involved in the electrical signal transduction. It was first cloned in 1999 [24]. KCNQ4 is only expressed in the OHCs, in their

basal membrane [23]. In the vestibular system, KCNQ4 is only expressed in the type I hair cells and the afferent nerve endings ensheathing these sensory cells. It is also expressed in certain nuclei of the central auditory pathway, and is absent from most other regions of the central nervous system. It is present, amongst others, in the cochlear nuclei, the nuclei of the lateral lemniscus, and the inferior colliculus. This is the first ion channel shown to be specifically expressed in a sensory pathway [23]. DFNA2 is known to map here [24].

1.3.2.5 COCH (Cochlin)

Its chromosome localization is 14q12-q13. DFNA9 has been mapped to this gene [25]. The mutation in the COCH gene prevents the deposition of cochlin into the extracellular matrix as suggested by Grabski et al. [25].

1.3.2.6 12s rRNA (MTRNR1)

It is located on 19q13.33. The gene codes the mitochondrial 12S rRNA. It is found to be the site of a mutation that has been identified as a basis for aminoglycoside-induced deafness and familial progressive sensorineural deafness. Because DFNA4 maps to 19q13 [27] and because of a relationship of the ribosomal protein gene to the ribosomal RNA, the gene encoding mitochondrial ribosomal protein S12 may be the site of mutations causing DFNA4 [28][29]. This hypothesis is further supported by the facts that the mitochondrial ribosome is very similar to the bacterial chromosome, and bacterial ribosomes are the main targets of aminoglycoside antibiotics [50].

1.4 The use of Guthrie papers

A Guthrie paper is a specially manufactured absorbent filter paper. Blood is drawn to it after 2-4 days of birth, from the finger, heel, or toe. The blood saturates the paper, and is dried for several hours, in room air, or in exsiccator.

Since the 1950's [51] and 1960's [52] national neonatal screening programs begun to operate in the advanced countries, they mostly perform screening for metabolic diseases [53][54]. These programs generated an enormous amount of DBS'es on Guthrie cards. These cards have the advantage of easy transportation, and their storage conditions are much cheaper than any other way of storing blood samples.

1.5 dHPLC

During the dHPLC measurements the DNA, under specialized conditions, forms hetero-, and homoduplexes (wild type-wild type, SNP-SNP, SNP-wild type, and wild type-SNP), that can be separated, and detected. This method makes it possible to discover unknown genetic variants, the presence of mutations, without having to perform an actual sequencing analysis.
During these experiments, an SNP containing part of the DNA is amplified with PCR. After the PCR reaction the amplicon is gradually cooled from 95 °C to 65 °C so that four types of duplexes from: heteroduplexes, that contain one copy of the "faulty" region, and one copy of the error-free region, and two types of homoduplexes, that contain either only the error-free parts or only the SNPs. Because of their sequence differences these duplexes form distant 3D structures, that are eluted at different speeds, thus at different time frames from the HPLC column. By graphing the resistance of the solution - that is eluted from the column - as a function of time, we get a so-called "chromatogram". Based on this chromatogram, it is possible to tell how many, and what type of duplexes there are. If there is more than one peak on the graph, than there is likely an SNP in the amplified DNA region. [61]
By utilizing this method the costs can be reduced, since only those samples have to be sequenced that are actually found to contain some mutation. By using this technique it is possible to perform around 200-300 screens daily. The actual number depends on many factors, including, but not limited, to the volume of sample injected onto the column, and the volume of the buffers that has to be supplied during the measurements. Because of this relatively high number of possible screens per day, this is a so-called "high throughput" method.

1.6 AS-PCR

AS-PCR exploits the use of primers that overlap with the mutation site. Two forward primers are designed in a way, that one of them has a perfect match with the SNP in question, whereas the other corresponds to the wild genotype. Both the wild type sequence and the SNP must be known for which the primers are designed.
AS-PCR is used to discriminate between the presence of the wild genotype and the mutant allele. Because base mismatch will not enable replication, PCR amplicons will be produced

only in case of perfect base pairing. This involves two reactions, one with each primer. (Figure 3.).

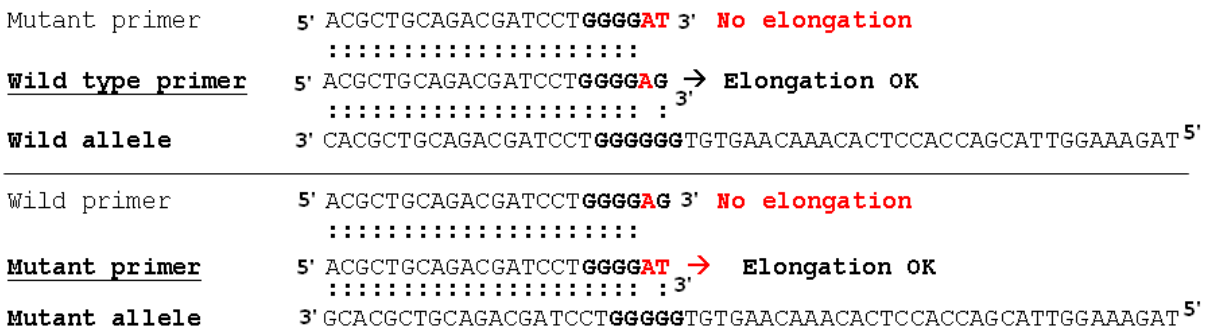

Figure 3.
Theory of the AS-PCR experiments
Two primers are designed so that at the 3'end one primer contains the SNP ("mutant primer"), while the other does not ("wild primer"). If there is that SNP than the "mutant primer" elongates, while - because of the mismatch - the other cannot. In case of the wild type allele, the "mutant primer" cannot elongate, while the "wild primer" can. In case of heterozygosis, both primers can elongate and both their products can be observed with gel electrophoresis, in about equal quantities.

This way all three possible genotypes are indicated with one combination of the amplicons, so homozygous wild, homozygous mutant and heterozygous alleles can all be detected (Figure 4.).

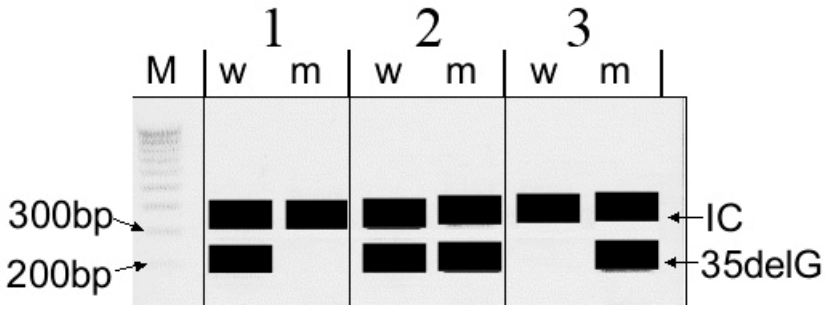

Figure 4.
This is a hypothetical picture of three patients with three genotypes.
1 denotes the homozygous wild, 2 denotes the heterozygous, and 3 denotes the homozygous recessive („mutant") genotype, respectively. M: molecular weight calibration, IC: internal control, 35delG: 35delG allele. w: amplicon of the wild type sequence matching forward primer, m: amplicon of the 35delG-matching forward primer.
In case both primers can be seen the patient is heterozygous (case 2),. In case of homozygosity (cases 1 and 3), only one of the primers can elongate and can its product be detected.

2 Aims

Our aim was to improve the genetic testing of hearing loss by optimizing, or determining the following parameters:

I. Evaluation of three different DNA extraction methods from dried blood spots for genetic testing.
II. Assessment of the quality and quantity of the DNA extracted from DBS'es as a function of time: how does the length of storage influence these parameters?
III. Determination of the number of PCR experiments that can be carried out from a genomic DNA solution extracted from a dried blood spot.

Our aims were also to determine the following parameters in our cohorts of patients:

IV. Determination of the frequency of the 35delG mutation of the GJB2 gene in our group of patients.
V. Description of other mutations, that occur in our groups of patients, in the GJB2 gene.
VI. Determination of the abundance of mutations in other (non-GJB2) genes in our population.
VII. Explore how mutations in other genes affect the patient's hearing?
VIII. Determination of the correlation of the genetic profile with the audiological profile of our patients.
IX. Potential benefits for cochlear implantation from these experiments - is it possible to deduce the audiological profile based on the genetic findings of a person?

3 Materials and methods

3.1 Sample and patient selection

All participants involved in the trials were informed according to the Guidelines of University's Ethical Committee, and all have signed a written consent.

3.1.1 Guthrie cards

Double blind tests were performed on the spot blood samples taken for the routine, population wide metabolic screening tests of neonates. The samples were taken on Guthrie cards at the 3^{rd}-4^{th} life day of newborns at any neonatal ward where the neonates were cared in the eastern part of Hungary and sent via conventional mail. The test cards were stored at room air at the Department of Pediatrics, University of Szeged. The Guthrie cards were selected as follows: 48 pieces from the years 1996, and 1997, and further 96 pieces from the years 1999, 2000, 2001, 2004, 2005 respectively. The total number of DBS'es was n=576. As these cards were selected randomly, they could be used as a "generic population sample".

3.1.2 Patients

The other population we examined consists of 318 of patients. These patients were Cochlear Implant (CI) users, their relatives, CI candidates, their relatives, and a few individual patients from the ENT Clinic. CI users and CI candidates were selected based on the following criteria: if there was a family history of hearing losses, and there were no organic abnormalities (anatomical variations, or developmental problems) or other diseases that are known to cause hearing loss or deafness in the patient's history. In case of the CI users (n=20+32=52) and CI candidates (n=56) the average hearing threshold level was bellow 70dB and speech recognition performance was under 25%. Those patients and CI users were excluded - and hence left out of the trial -, whose patient history contained some form of disease that can cause deafness, or who suffered a head trauma, or head injury, that can account for their hearing problems. The CI users' and CI candidates' family members (n=163) have various levels of hearing loss, from no hearing loss at all to severe to moderate levels. There were some individuals who wanted to participate in our study with various levels but

with unknown origin of hearing loss (n=47). 20 CI users (out of n=52) were individuals with no screened relatives. Our group of control persons consisted of people with hearing threshold levels at 5dB or better on both ears (n=20) and no family history of hearing losses.

3.2 Molecular biology testing

3.2.1 Selection of the genomic regions

Genomic regions were selected that contained known and published mutations at the time of the planning of the experiments. The decisions were based on the table found at http://hearing.harvard.edu/db/genelist.htm. As the planning stage of the experiments took place in the spring of 2004, and until then the table contained relatively fresh data, we used this as our starting point. See the slightly edited version of the table (Table 9.) in "Appendix A– Primers and sequences", 9.1 "A concise table on the involved genes, regions, and their related publications", on page 59.

3.2.2 DNA extraction

3.2.2.1 Preparation of DNA from DBS for AS-PCR

4 mm diameter pieces from the bloodspot test cards were punched out. Three methods were examined to prepare genomic DNA from the Guthrie papers.

The first method (method "a") was as follows: the paper discs were put into PCR tubes along with 200 µl 1x PCR buffer (Eppendorf HotMaster taq). The DNA was extracted from the DBS with the following procedure: 10 minutes at 96 °C then 1 minute at 25 °C and 10 minutes at 96 °C again, in a thermal cycler. The samples were then centrifuged at 16 000 g for 2 minutes. The supernatant was then transferred into a sterile microcentrifuge tube, and stored at 4 °C until utilization [55][56].

In the second series of experiments (method "b") the paper discs were put into 200 µl 1x PCR buffer (Eppendorf HotMaster taq), and then the PCR tubes into 55°C water bath for 10 minutes. The samples then were centrifuged at 16 000 g for 2 minutes, and the supernatant was then used for PCR tests.

The third method (method “c”) involved the same preparation steps as method two, but the samples were sonicated for 10 minutes in the water bath at 55°C. The samples were then centrifuged at 16 000 g and the supernatant was then used.

3.2.2.2 Preparation of DNA from venous blood for AS-PCR

3 ml blood was collected from patients having Cochlear Implant, their relatives, CI candidates, their relatives, and a few people with hearing loss of unknown origin (see 3.1.2 “Patients” on page 11 for details). Blood anti-coagulant was EDTA. Genomic DNA (gDNA) was purified from 400 µl of blood using Versagene Blood Kit (Gentra) according to the manufacturer’s instructions. The concentration of the DNA was measured with spectrophotometer and was calculated by the adsorption at 260 and 360 nm.

3.2.3 Polymerase Chain Reactions

3.2.3.1 DNA integrity test reactions

These reactions were carried out using HotMaster Taq DNA Polymerase (Eppendorf). 30 µl final volume of the reaction mix contained 3 µl (10x) HotMaster Taq buffer (Eppendorf), 2.5 µl dNTP (2.5 mM), 0.5-0.5 µl (15 pM) DF2F-DF2R primer pair (Table 1); 1 U HotMaster Taq (Eppendorf); 6 µl gDNA template and 16.5 µl water.

PCR program was as follows: 2 minutes at 96°C for 35 cycles, (96°C 30 seconds – 61°C 30 seconds - 68°C 35 seconds), and after these 35 cycles 96°C for 5 minutes. Negative control experiment was also done with paper discs originating from the “blood-free” parts of the Guthrie papers. PCR fragments were analyzed by agarose gel electrophoresis on 1.5 % agarose gel (AbGen) with 1X TBE buffer. The internal control used in all the AS-PCR experiments were the primer pair ICF and ICR. They amplificate a part of the serine proteinase inhibitor gene (see Table 2. in “Properties of DNA purified from Guthrie cards and from EDTA-anticoagulated blood” on page 17)

3.2.3.2 AS-PCR reactions

30 µl final volume of the reaction mix contained: 3 µl (10x) HotMaster Taq buffer (Eppendorf), 2.5 µl dNTP (2.5 mM), 0.5-0.5 µl primers GJC-GJW pair for wild allele detection and GJC-GJM for 35delG mutant allele detection (Table 2.). (15 pM), 0.4-0.4 µl

(15 pM) internal control primer pair (ICF-ICR); 1 U HotMaster Taq (Eppendorf); 6 µl gDNA template and 16,5 µl water.

The PCR program was as follows: first, denaturation step at 95 °C for 5 minutes, then 35 cycles at 96 °C for 30 seconds, at 65 °C for 35 seconds, and at 68 °C for 38 seconds , and as the last step, 10 minutes incubation time at 68 °C. PCR fragments were analyzed by agarose gel electrophoresis on 1.5 % agarose gel (AbGen) with 1X TBE buffer.

3.2.4 DNA sequencing and sequence analysis

PCR fragments were generated as described in section 3.2.3.2. PCR products were desalted on Microcon columns (Millipore). The purified PCR products were eluted in 30 µl of water. The DNA sequences were determined by automated sequencing at Macrogen Inc. (Korea) on both strands. The sequences were aligned to the wild type reference sequence with the CLUSTALW program [58].

3.2.5 dHPLC experiments

We used the Varian Inc. Helix System and Varian Star Workstation at the Department of 2nd Internal Medicine and Cardiological Centre. For the table of dHPLC programs that were used with our primers during our tests please see Table 10. on page 80 in 11 “Appendix C– dHPLC parameters”.

The composition of the buffers used with the device (“Buffer A” and “Buffer B”, respectively) are as follows:

Buffer A: ≤ 2% TEAA in 3000ml aqueous solution. [60]

Buffer B: ≤25% acetonitrile, ≤1% triethylamine, ≤0.6% acetic acid, ≤0.01% EDTA sodium salt in 3000ml aqueous solution [61]

3.3 Audiology testing

During our audiological examinations subjective audiological tests were performed. By using audiograms, it was possible to assess the hearing of our patients according to the routine evaluation (clinical) procedure.

Most of the subjective audiology measurements were made with GSI 16 audiometers at the audiology station of the department. Some audiograms were taken at other institutions in the country.

The standard procedure involved measuring the pure tone hearing threshold levels at the following frequencies: 125 Hz, 250 Hz, 500 Hz, 1000 Hz, 2000 Hz, 4000 Hz, 8000 Hz, according to the international standards.

4 Results

4.1 Preparation of DNA from DBS for AS-PCR

Three methods were examined in order to extract genomic DNA from dried blood spots. Regions of the GJB2 gene from the gDNA were amplified with PCR, and then the samples were analyzed with gel electrophoresis.

During our work, the first method (method "a", with boiling at 98°) was used, as described in 3.2.2.1 "Preparation of DNA from DBS for AS-PCR" on page 12. By gel electrophoresis of the PCR products, it was found that the method with water bath and ultrasound sonication gave somewhat worse results. (Figure 5.) As also can be seen on the figure, methods "a" and "b" gave similar results, but as method "a" was technically simpler to carry out, it was used later on.

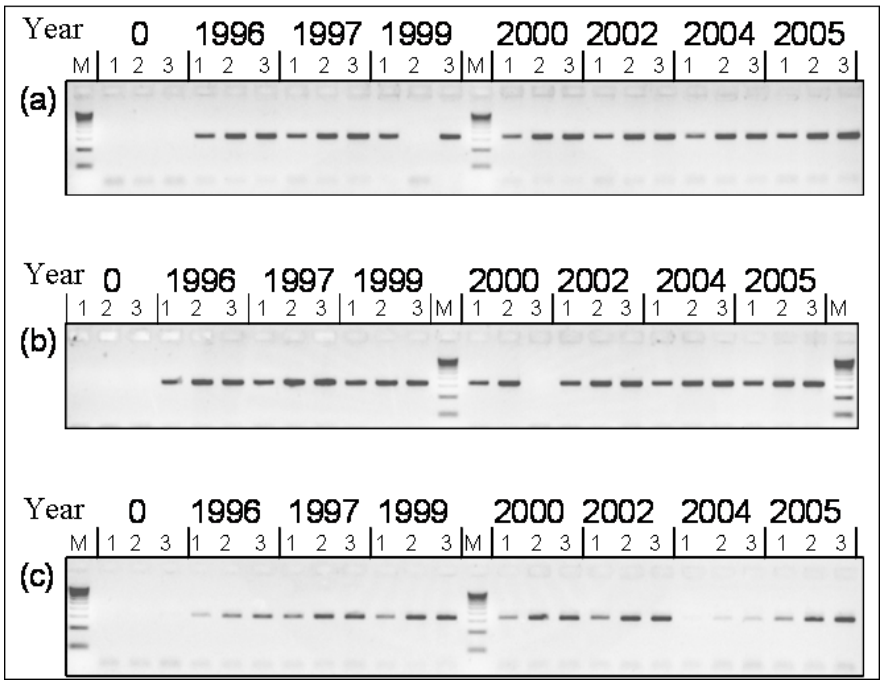

Figure 5.
Comparison of the three methods for DNA extraction from blood drops on Guthrie cards.
1 = 0.5µl, 2 = 2.5µl, 3 = 10 µl genomic DNA template solution
(a): 10 min @96°C 1 min @25°C 10 min @96°C minutes in thermal cycler
(b): 10 min @55°C in water bath
(c): 10 min @55°C in water bath with ultrasound
Samples 1, 2, 3 are always from the same blood spot, on all three ((a), (b). (c)) respective series in the same years. Note, that while series (a) and (b) yielded similar results, there are missing bands in series (c), it's quality is noticeably inferior.

4.2 Properties of DNA purified from Guthrie cards and from EDTA-anticoagulated blood

200 µl of DNA solution were obtained from one tube of EDTA anticoagulated blood and 200 µl DNA solution from one 4 mm diameter piece of the bloodspot test cards.

The amount and the integrity of the purified DNA samples was tested by PCR with the primers DF1F and DF2F (see Table 2.) which amplify parts of GJB2's coding exon. The length of the PCR products was 420 and 324 base pairs. They covered the whole exon. The experiments were performed with 6 µl of genomic DNA (gDNA) template. The samples were analyzed by gel electrophoresis. 1x, 5x, 50x dilutions were made from blood spots and tested by PCR. The 200 µl gDNA solution, when diluted 50 times, is still acceptable for PCR testing, and that volume (200 µl x 50 = 10 000 µl) is sufficient theoretically for more than a thousand PCR reactions per purified sample, or 4mm paper disc (Figure 6.) As a negative control, a blood-free paper disc was used from a Guthrie card.

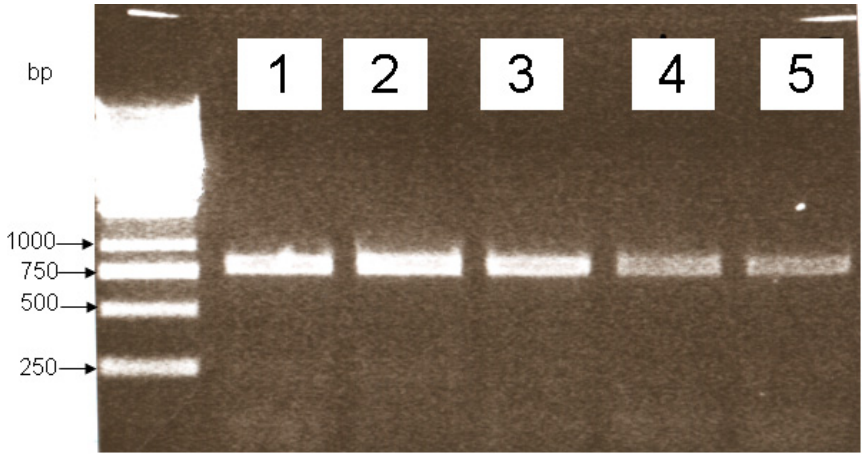

Figure 6.
Approximate amount of PCR products when DNA is extracted from a 4mm paper disc from a Guthrie card
Numbers: 1. no dilution, 2. diluted 5x times, 3. 50x 4. 500x, 5. 1000x dilution, respectively
"bp": length calibration
The picture shows the PCR product of the whole coding exon of the GJB2 gene, the primer used was DF2F. The length of the PCR product is 809 bp. The intensity of the bands lowers, as the applied gDNA solution is diluted.

As Figure 7. shows, even the DNA extracted from a twelve years old blood spot gave good results.

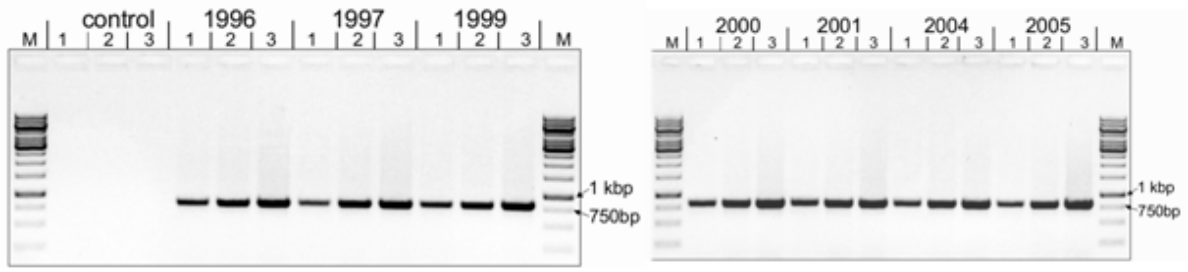

Figure 7.
PCR experiments showing the impact of age of the Guthrie cards the DNA was extracted from, on he amount of PCR products.
The primer DF2F was used. 1 kbp and 750 kbp are the molecular weight calibrations ("M").
As the picture shows, there were no aspecific PCR products when this primer was used, with the (a) method. Here 1, 2, and 3 all denote different samples. Three samples were tested from every year.

There were no experiments that produced no results, and this means, that it was possible to screen for 35delG with AS-PCR on all 576 DBS samples. Figure 7. shows randomly selected samples from our tests. As shown on the image, there are no smaller sized fragments in detectable quantity bellow our AS-PCR product on the gel.

The gDNA solution prepared from whole blood could be diluted by 100x and it still gave acceptable results. (Figure 8.) When diluted further (500x, 1000x, 5000x), the PCR experiments were less reliable, the bands disappeared.

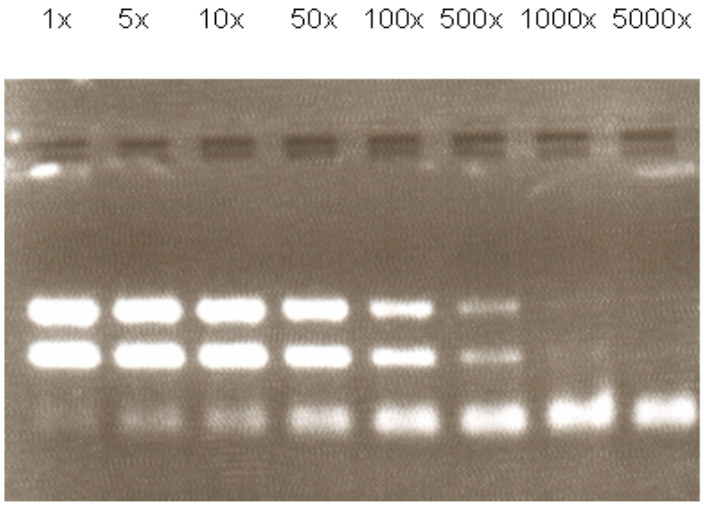

Figure 8.
Genomic DNA extracted from EDTA-anticoagulated blood, and diluted.
The GJW primer was used in this experiment (detects the 35delG wild genotype).
Numbers denote the degree of dilution.
In the last line PCR primer dimers can be seen that hybridize to each other during the PCR runs, yielding double stranded DNA fragments in approximately 50 bps in size. As the amount of PCR products decreases, the amount of such PCR-byproducts grows, hence the increase of intensity of those bands.

The longest PCR product used by us is the 809 bp long product of the primers GJB2F and GJB2R (Table 2.) This PCR product was used to validate the AS-PCR experiments with sequence analysis.

Primer name	Sequence	Description
ICF	CCC ACC TTC CCC TCT CTC CAG GCA AAT GGG	Internal control (serine proteinase inhibitor gene)
ICR	GGG CCT CAG TCC CAA CAT GGC TAA GAG GTG	Internal control (serine proteinase inhibitor gene)
GJC	AGT GAT CGT AGC ACA CGT TCT TGC A	Common reverse primer for GJB2
GJW	GCA CGC TGC AGA CGA TCC TGG GGA G	Primer for 35delG Wild allele detection
GJM	CAC GCT GCA GAC GAT CCT GGG GAT	Primer for 35delG mutant allele detection
DF2F	TCT CCC TGT TCT GTC CTA GC	GJB2 exon and flanking region for sequencing
DF2R	TTT CCC AAG GCC TCT TCC AC	GJB2 exon and flanking region for sequencing

Table 2.
Primers used in the DNA extraction and purification experiments

As can be seen on (Figure 9.) there were no aspecific PCR products in the AS-PCR experiments.

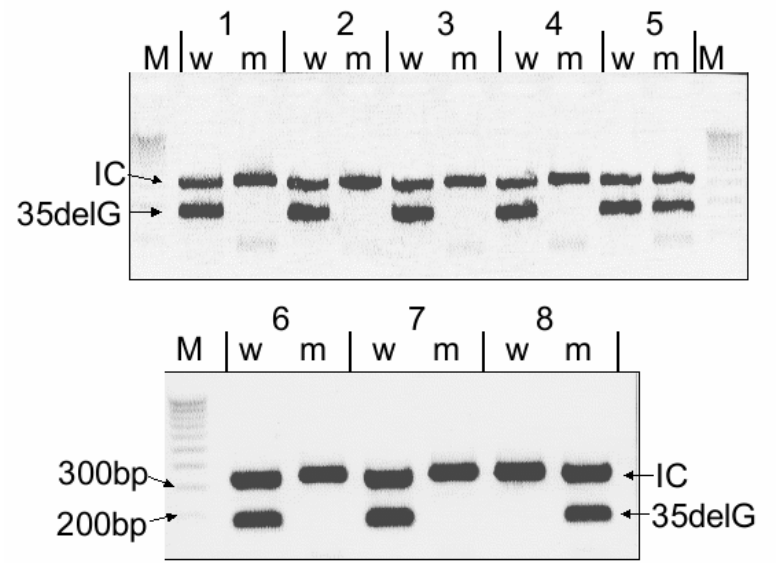

Figure 9.
A picture showing a 35delG AS-PCR experiment.
M: Molecular weight (ladder), w: wild type allele (primer GJW), m: 35delG+ allele (primer GJM) IC: internal control (primers ICF or ICR)
There are no aspecific bands on this gel.
Samples 1, 2, 3, 4, 6, and 7 all represent the homozygous wild genotype. Sample 5 belongs to a patient with a 35delG heterozygous genotype (both PCR primers elongated). Sample 8 shows a 35delG homozygous recessive genotype; as no 35delG wild allele was found, the GJW primer could not elongate during the PCR runs.

Regarding the other primers in this present work, the PCR experiments yielded no aspecific products in detectable quantity. (Figure 10.)

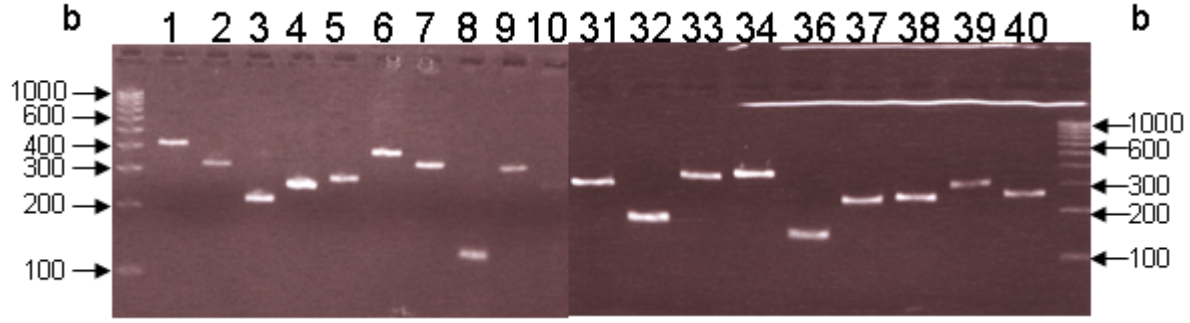

Figure 10.
The numbers represent the primers that can be found in 9.3 - "Sequences of PCR primers used in the PCR experiments" of the following genes:
1, 2, 36: GJB2; 3, 4: 12SrRNA; 5, 6, 7: COCH; 8: GJA1; 9, 10: GJB3; 31, 32, 33, 34: MYO6; 37, 38, 39: GJB6; and 40: POU3F4. As these fragments were optimized for dHPLC experiments, their sizes are in the range 135–420 bp.
Numbers on both sides represent the length of the PCR products, in bases, as a unit.

4.3 Examination of the GJB2 gene

Special attention was paid for the GJB2 gene, and its 35delG mutation. The highest number of nonsyndromic hearing losses is caused by various mutations in this gene. 2-3% of the Caucasian population is a carrier regarding the 35delG mutation, and various other mutations in this gene have been shown to cause ARNSL.

4.3.1 35delG mutation

All the 576 DBS, and 318 samples from peripheral blood samples were evaluated for the 35delG mutation. These two groups were treated separately, as the samples on Guthrie cards can be considered as a randomized population sample, whereas the patients who came to our department because of various hearing problems cannot be treated a randomized group. On Guthrie cards 13 heterozygotes were found regarding the GJB2's 35delG mutation, which means that the carrier frequency is 2.3% in this population (Hungary)[39][62] (Table 3.A). No homozygotes were found, and that can be attributed to the fact that the incidence of the homozygous 35delG mutation is around 1/1000-2/1000 [39]. Three samples were found to belong to the same persons on blood spots and EDTA-anticoagulated blood samples. All three samples gave the same results when sequenced from whole blood and from DBS'es as well,

indicating that blood spots of several years of age may be a good source for GJB2 35delG AS-PCR tests.

From the DNA extracted from peripheral blood, 24 subjects with homozygous recessive genotype, which takes 7,6% of the screened population, and 51 heterozygous patients out of the 318 (16,0%) were found. The total number of patients with homozygous wild genotype was 243 (76,4%) (Table 3.B). 11 cochlear implantees were homozygous for 35delG, and 5 CI users, or CI candidates were heterozygous (Table 3.C). All of the 35delG homozygous recessives' relatives (who could be investigated) were heterozygous. 36 of the implantees, and 78 of all the implantees' relatives were homozygous wild out of the total of 318 patients.

	Total	**35delG +/-**	**35delG -/-**	**35delG +/+**
A - Guthrie papers				
Persons	576	13	0	563
Percent	100	2.3	0	97.7
B - EDTA-anticoagulated blood				
Persons	318	51	24	243
Percent	100	16.0	7.6	76.4
C - CI users (from EDTA-anticoagulated blood)				
Persons	52	5	11	36
Percent	100	9.6	21.2	69.2

Table 3.
Number and percent of 35delG mutations found in the DNA samples from the Guthrie cards (A), from the EDTA-anticoagulated blood (B), and among our CI users (C)

4 subjects with combined alleles, two with 35delG+- /G71A+-, one with 35delG+-/ G139T+-E47STOP +-, and one with 35delG+-/G95A+- were found.

All of the patients with the homozygous recessive 35delG genotype had a pure tone audiometry with 70dB or greater hearing loss. 8 of our CI users have the homozygous recessive 35delG genotype. 5 of the CI users are heterozygous 35delG carriers; one of them has a combined 35delG+- / G95A+- genotype. All their hearing levels were bellow 80 dB.

4 CI users have at least one SNP in the other investigated genes, two of them having a 35delG-- genotype with a MYO6 SNP (in DF33). These two patients' audiograms show even more degraded hearing performance, their levels were bellow 90 dB, while 35delG-- - only patients have slightly higher hearing threshold levels, but still bellow 70dB.

Three out of the four patients who have two mutations in their GJB2 coding exon have no hearing problems. One of them has pure tone audiograms between -55 and -110 dB, but this latter patient has three SNPs in two other genes, in GJB3 (DF9 and DF11), and KCNQ4 (DF17).

For the summary of the found GJB2 35delG alleles and the hearing levels of all subjects, please see 10.1 “GJB2 35delG allele” in “10Appendix B– Summary tables of mutations” on page 70.

4.3.2 Sequencing of the coding exon of the GJB2 gene

Homozygous 35delG, heterozygous, and homozygous wild type samples were sequenced.

All AS-PCR experiments gave consequent and reproducible results when gDNA was purified both from EDTA-anticoagulated blood and from dried blood spots (see Figure 9. on page 19).

Figure 11.
Three examples of a 35delG sequence.
Wild: a sample of a patient with homozygous wild genotype
35delG +/-: a sample of a patient with heterozygous 35delG genotype
35delG -/-: a sample of a patient with homozygous recessive 35delG genotype
Note that the 35delG mutation occurs in the 35th position, which is towards the left on the above pictures, as the sequence is shown backwards.

As the PCR products of GJB2F and GJB2R were 809 bp long, the sequence analysis could cover the full length of the GJB2 gene’s coding exon on both strands (Figure 11.).

The GJB2 gene was sequenced on all the venous blood samples (n=318). With the optimized reaction conditions, no false positive or false negative results were obtained.

All sequencing chromatograms were clear and of good quality and readable up to at least 760 bps (Figure 12.).

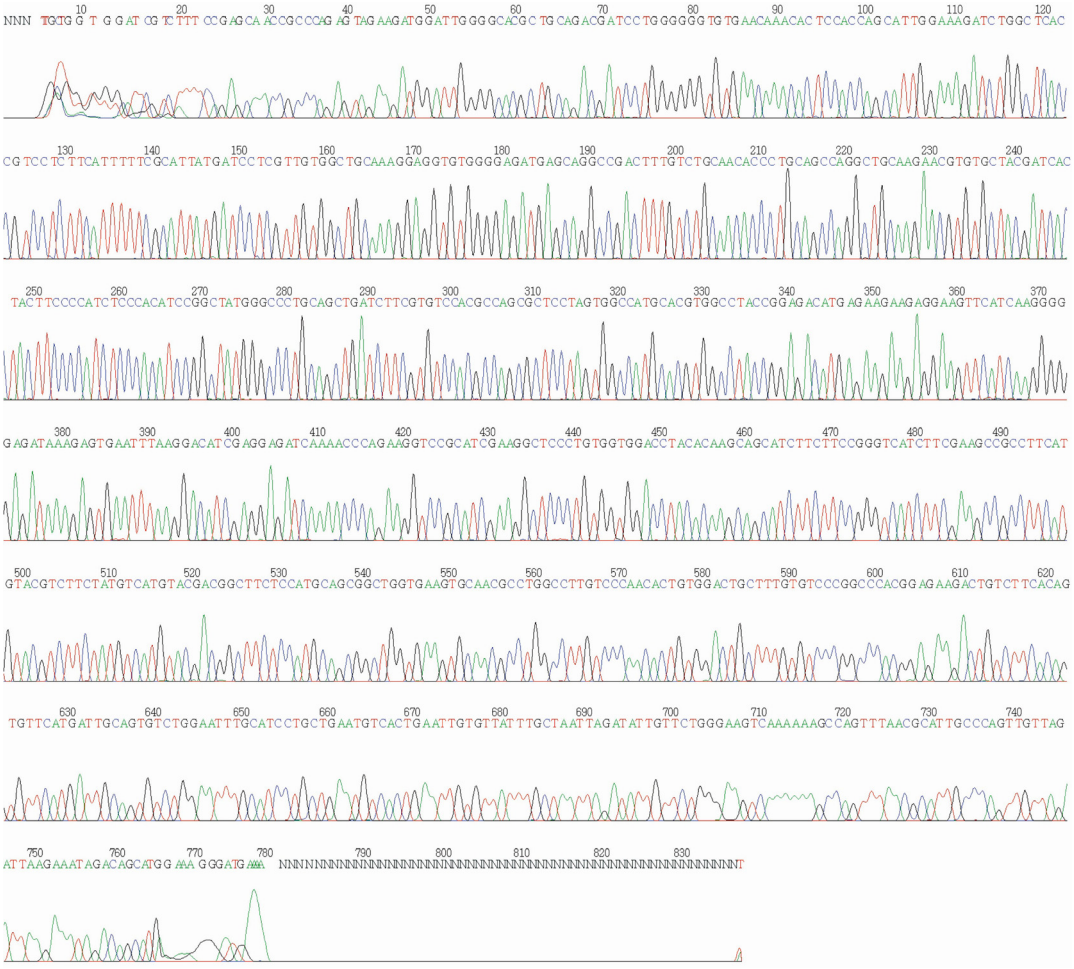

Figure 12.
An example of the whole sequence of the GJB2 coding exon.
The picture above shows a homozygous wild genotype, for clarity reasons: as Figure 9 shows, a heterozygous sample would be hard to evaluate beyond the site of deletion.

25 patients were found with various, but not 35delG-related mutations in the GJB2 gene. 2 G109A +- (V37I+), 1 T269C+- (L90P+-), 2 G95A +- (R32H+-), 1 T101C+- (M34T +-), 2 G380A -/- (R127H--), 7 G380A +- (R127H+-), 3 G71A +- (W24STOP+-), 2 G139T+- (E47STOP+-), 1 C164T+-, 1 G478A+-, 1 176 delG +-, 2 A341G +- (E114G +-) mutations were found.

One G109A+- occurred with an SNP in the GJB3 gene (DF9), with no audiological problems. One occurred without any other detectable genetic alteration, yet the patient has a cochlear implant, possibly the cause of an undetected mutation.

The patient with the T269C/L90P has a CI as well, but no evidence was found of an other SNP in any of the other investigated regions. The patient's hearing threshold level is bellow 100dB. This can probably be attributed to yet another SNP somewhere else in the genome.

One out of the two G95A+- carriers has a 35delG +- genotype too, as well as SNPs in GJB3 and KCNQ4 (DF9, DF11, DF17). This patient has a CI, but since neither of the GJB2 mutations are dominant, the hearing loss can be attributed either to the other SNPs that were found, or to another, undetected mutations in his or her genome. The other subject with G95A+- and 35delG+- has SNPs in GJB3, MYO6, and KCNQ4 (DF9, DF17, DF33), but has no detectable hearing problems.

One patient with the T101C+- mutation has a severe hearing loss (between 60 and 90 dB), and no other detected mutation.

9 patients have G380A/R127H mutation, two of them are homozygous, 6 of them are heterozygous, and one is a G380A -+/56insC compound heterozygote. One G380A-- and one G380A+- patient has developed profound hearing loss, the G380A -- deaf patient has an SNP in the KCNQ4 gene (DF48) too.

One patient was found with a truncating mutation, G71A+-/W24STOP. That patient has hearing levels between 80 and 110 dB.

5 patients are compound heterozygotes, 2 35delG+-/G71A+-, 2 A341G+-/E114G+-, and 1 35delG+-/G139T+- E47STOP+-. The 2 35delG+-/G71A+- compound heterozygotes are siblings. One of them has an SNP in the GJB6 gene (DF12), and the 35delG+-/G139T+-E47STOP+- patient in the 12S rRNA. None of them has developed hearing problems.

One subject has C164T+- as the only mutation, with no hearing problems.

A 176delC+- patient has profound hearing loss, with no other SNP or mutation, and one CI user has a G478A+- genotype, and no other SNP or mutation.

For the summary of the found GJB2 mutations (excluding 35delG) please see the table in 10.2 "Other GJB2 mutations" on page 72.

4.3.3 dHPLC of the GJB2 gene

The GJB2 gene was used as a "calibration test" for the dHPLC experiments that were done on other regions of other genes (DF3-DF48). This validation could be done, because all GJB2 samples were sequenced and measured with dHPLC as well.

No false positive or false negative results were found, all dHPLC chromatograms could be matched to the appropriate GJB2 mutation that was found with sequencing analysis. A few examples are shown in "12 Appendix D - Sample dHPLC chromatograms" - 12.1"GJB2 (35delG) chromatograms" on page 83 on Figure 13, Figure 14, Figure 15, and Figure 16, respectively.

4.4 Other examined genes

All regions, DF1-DF49, (see the Table in 9.2 "Appendix A– Primers and sequences" for details), were analyzed with dHPLC. DF10 and DF15 could not be taken into account, because the data was lost due to computer error. These two primers are marked with red in the Table in 9.2 "Appendix A– Primers and sequences". In this section the data concerning the GJB2 is excluded, as it has been discussed in detail in 4.3."Examination of the GJB2 gene"

131 dHPLC chromatograms show some form of variation, compared to other samples in their appropriate measurement series. In GJA1, and POU3F4 there was no evidence of any mutation, zero differing chromatograms were found in their investigated regions (0 in DF8 in GJA1, and in POU3F4's 7 regions DF24-DF26 and DF40-DF43).

29 SNP's in the 12s rRNA (1 in DF3, and 28 in DF4), 6 in the COCH gene (0 in DF5 and DF7, 1 in DF49 and 5 in DF6), 32 in the GJB3 gene (0 in DF10, 19 in DF9, and 13 in DF11), 5 in the GJB6 gene (0 in DF13, DF14, DF38, DF39, 3 in DF12, and 2 in DF37), 37 in the KCNQ4 gene (0 in DF15, and in DF47, 1 in DF16, 11 in DF17, and 25 in DF48), 2 in the SLC26A4 (0 in DF18, DF20, DF21, DF23, and in DF43-46, 1 in DF19, and 1 in DF22), and 21 in MYO6 (all of them in DF33, we have found none in DF31, DF32, DF34) were found. These results are summarized in Table 4.

Sum	12s rRNA	COCH	GJA1	GJB3	GJB6	KCNQ4	SLC26A4	POU3F4	MYO6
131	29	6	0	32	5	37	2	0	21

Table 4.
The number of SNPs and the genes they were found in with dHPLC

These 131 SNPs were found in 98 patients.

We have found 73 individuals with one SNP in the investigated regions (excluding the GJB2 gene), 17 with two SNPs, and 8 with three SNPs. There were no patients with more than three SNPs in the screened regions. For the detailed summary, please see 10.3 thru 10.9 on pages 73 to 77 in 10 “Appendix B– Summary tables of mutations”.

Example dHPLC chromatograms of these experiments with audiograms of the respective patients are shown in 12.2 “Examples of dHPLC chromatograms from other genes, with audiograms”, from page 85. Figure 17. shows an SNP in the KCNQ4 gene, Figure 18. shows a chromatogram of an SNP in the 12s rRNA gene, in the DF4 region, Figure 19. shows an SNP in the cochlin gene in the DF6 region, and Figure 20. shows the chromatogram of an SNP in the GJB3 gene, in the DF9 region.

22 of all the 98 subjects have at least one mutation in their GJB2 gene, 4 of them have the homozygous recessive 35delG genotype, and 18 are 35delG carriers (heterozygous). 10 of them have some other mutation in the GJB2 gene, 3 out of this ten are 35delG carriers as well. 21 CI users have SNP is other genes than GJB2. Two of these 21 are 35delG homozygous recessive, one of them is a 35delG carrier, and one of them has a 35delG+- + G95A+- combined genotype. All the other CI users (n=17) have at least one SNP in the other examined genes. 7 of them have an SNP in the MYO 6 gene (DF 33), but there are patients who have SNP only their DF33 as well, have no hearing problem, but are close relatives of CI users. In case of 17 out of the 52 CI users, no mutation could be found, neither in the GJB2 gene, nor any SNP in any of the other genes. Their hearing threshold levels (pre-implantation) show strong variation, from around -70dB to no hearing at all.

In two cases out of the ten 35delG-- CI users, one SNP was found in the MYO6 gene (DF33). In six cases, no other mutation or SNP could be found but the 35delG homozygous recessive allele. Their (pre-implantation) pure tone audiometry hearing threshold levels vary between 70dB to 120dB (no hearing at all).

17 CI users have no GJB2-related mutation, but do have some other SNPs in some of their other genes. 14 of them have 1 SNP. 6 in MYO6 (DF33), 3 in the KCNQ4 (2 in DF17, 1 in DF48), 1 in the COCH gene (DF6), 1 in the GJB3 (DF9), 3 in 12s rRNA (DF4), and 1 in GJB6 (DF12). Their audiograms show some variation, between 70-90dB and 100-120dB.

4.5 Genetic findings of cochlear implant users

In 19 cases no evidence of genetic alteration could be found in this group. In twelve cases out of the 52, the only mutation that was found in the patient's genome is in the coding exon of GJB2 gene (Table 5.)

Mutation	# of patients
35delG --only	6
35delG-+ only	2
T269C+- L90P	1
G109A +- V37I	1
G380A +- R127H	1
G 478 A+-	1

Table 5.
This table shows the number of CI user patients and the mutations in the coding exon of the GJB2 gene

In 21 cases a broad diversity of SNP were found. In four cases with some SNP in the GJB2 gene, in one case even with two SNPs in the exon. 17 patients have only one SNP in one gene that's no GJB2, 2 patients have two SNPs in two different genes, and one patient has 3 SNP in three genes. For details see Table 6.

Patient nr.	**1**	**2**	**3**	**4**	**5**	**6**	**7**
GJB2 Mutation	35 delG --				35 delG --		
dHPLC primer	DF33	DF33	DF9,DF11,DF33	DF9,DF33	DF33	DF33	DF9,DF17
Gene	MYO6	MYO6	12srRNA, GJB3, MYO6	12srRNA, MYO6	MYO6	MYO6	12srRNA, KCNQ4
Patient nr.	**8**	**9**	**10**	**11**	**12**	**13**	**14**
GJB2 Mutation				35 delG+-			
dHPLC primer	DF17	DF17	DF6	DF9	DF33	DF9	DF48
Gene	KCNQ4	KCNQ4	COCH	GJB3	MYO6	GJB3	KCNQ4
Patient nr.	**15**	**16**	**17**	**18**	**19**	**20**	**21**
GJB2 Mutation			35 delG+-/ G95A-				
dHPLC primer	DF33	DF33	DF9, DF11, DF17	DF4	DF4	DF12	DF4
Gene	MYO6	MYO6	KCNQ4, GJB3, KCNQ4	12srRNA	12srRNA	GJB6	12srRNA

Table 6.
Detailed table showing the localization of the SNPs found in CI users, where other gene was, or other genes were involved than the GJB2 gene.

Note, that a more detailed table can be found with the above data on page 78 in 10.10 "Cochlear implant users"

4.6 Audiograms

538 audiograms from 119 patients were collected during our work. No audiogram was taken from relatives, who stated that they did not have hearing problems. There were a few relatives who refused to participate in audiology testing, but they all stated that they do not have hearing problems.

52 cochlear implant users were in our group, all of them had hearing threshold levels bellow 80 dB (which is one criterion for the cochlear implantation also). All the GJB2 35delG homozygous recessive patients had hearing levels bellow 70dB, with the exception of one subject. This exception contradicts our knowledge of the 35delG mutation, and may represent an error, as that mutation in its homozygous form is known to cause severe hearing loss, and no other mutation (that could possibly restore the function of Cx26) has been found in this case.

Audiograms from 67 other people were collected. 11 of these patients have hearing threshold levels between 0 and 20 dB. 4 of them have hearing threshold levels between 20-30 dB, 14 of them between 30-70 dB, 15 of them between 70-90 dB. Finally, 21 of them had hearing threshold levels bellow 90dB, or no hearing at all. See Table 7. for the summary of these data.

Hearing threshold level (dB)	>20	20-30	30-70	70-90	90<
Number of patients	11	4	14	15	23

Table 7.
Summary of the hearing threshold levels in our group of patients
-10-20 dB: normal hearing range
20-30 dB: mild hearing loss
30-70 dB: moderate hearing loss
70-90 dB: severe hearing loss
90< dB: profound hearing loss

Out of this 119, 40 subjects have only 1 audiogram, 63 have between 2 and 10, 10 have between 10 and 20, and 6 people have more than 20 audiograms. Those who had the most audiograms were CI users, whose CI device was regularly fitted, and checked with pure tone audiometry.

Because of the relatively high number of variations among the audiograms, it was not possible to calculate averages or significance.

Follow-up could not be done, because of the high variability of mutations, and the low number of patients who had more audiograms.

5 Discussion

5.1 Properties of DNA purified from Guthrie cards

Different blood storage methods were tested if they can provide appropriate DNA samples for AS-PCR tests and for sequencing of the GJB2 gene. DNA was obtained from EDTA-anticoagulated venous blood, and from dried blood spots. The possibility of the use of dried blood spots on Guthrie cards as a source of DNA for genetic testing after an extended period of storage under suboptimal conditions was evaluated.

According to our experiments, both DNA sources gave satisfactory results. The usability of the PCR products from either template is equal when used in AS-PCR experiments, or in sequencing. Previous works have shown that extracting DNA or RNA is possible from DBS'es [55][56], but none have evaluated the effect of storage conditions.

AS-PCR primers were validated on samples that contain the 35delG mutation in the GJB2 gene's coding exon. All the DBS samples with the 35delG allele were sequenced and all sequences certified the AS-PCR results. Whole GJB2 gene sequences of samples resulted in wild type signals with AS-PCR proved that the AS-PCR experiments did not give false negative results.

Three samples were found on blood spots in the randomized group that belonged to our patients from whom we drew blood in EDTA-anticoagulated tubes. The samples were analyzed both with AS-PCR and with sequencing, and these two methods showed the same results, not only on these three, but also on all that were compared using the two approaches. All six experiments, however (AS-PCR and sequencing on all three corresponding DBS and whole-blood samples), showed the respective, matching results as well. Although more experiments using the same patient's whole blood and DBS would have been more desirable, mainly due to financial and organizational reasons, this could not be achieved.

These results support that our AS-PCR test is suitable for large-scale screening of dried blood spots as well as for simple and cost-effective detection of selected – and not only, or necessarily GJB2-related – point mutations on individuals or in family samples.

Because of the reliability of these methods, it is possible to screen for larger genes, or to screen for multiple SNPs in the same, or even in more genes simultaneously. By multiplexing the PCR primers, more point mutations can be addressed in one, quick and cheap experiment.
The costs of traveling this amount of people to hospitals, or university hospitals, just to draw blood are enormous. With DBS'es, the costs can be cut down. Blood can be drawn by their physician, and blood transport do not need to take place in a controlled manner, the temperature for transportation and the time it takes to transport the anticoagulated blood to the screening centers is of no consideration anymore.
On the other hand, if the need arises to carry out further genetic testing, the DNA, which can be extracted from a DBS, is enough to carry out hundreds or even more than a thousand PCR experiments. In fact very little amount of genomic DNA solution is needed to carry out a successful PCR. Whole blood cannot be stored long until it noticeably degrades, and the costs of storing the gDNA solution from peripheral blood (buying, or maintaining a refrigerator for example), and the laboratory room consumed by the needed equipment cannot be compared to the storage demands of the dried blood spots. With the methods presented, dried blood spots can not only be used to test for metabolic diseases but to carry out genetic experiments as well.
As calculated by our lab we can get enough gDNA for a few hundreds of PCR runs from about 2/3 of the money if working with DBS, than needed to do the same number of experiments with EDTA-anticoagulated whole blood, and this is only the financial calculation that concerns the acquisition and the maintenance of the equipment. In addition, it requires less time, as the purification of 96 samples takes an hour with the previously described method from DBS, but almost a whole day with whole blood, even using a good kit. Significantly more work can be done in a given time frame when gDNA is extracted from dried blood spots. Purifying gDNA from DBS'es is more simple, requires less work and lab equipment, and the gDNA's quality – based on the PCR experiments described in this work – is on par with the gDNA solution that can be extracted from the EDTA-anticoagulated blood. Beyond all these advantages, AS-PCR is a cost effective, precise, and quick tool that can help us to screen newborns for specific alleles.
It was estimated, that there are around 800-1000 people in a Hungary-sized country per year who may need cochlear implantation. The number of severe or profound hearing losses is

even higher, around 1-2% in the European population. Ideally about 100 000 subjects should be screened for the background of their hearing losses. Considering this high number of potential hearing screens, the DBS method has an enormous advantage over any other previous method used, both in the technical and in the financial sense. It also has the advantage that a lot of samples are already available in the Guthrie paper banks at selected institutions in the country, so population-wide screening is – at least theoretically – possible.

5.2 Examination of the GJB2 gene

5.2.1 35delG mutation

Carrier rates of mutations that cause nonsyndromic deafness show strong variation according to the literature. The frequency of some of these mutations is not even known, as they are only analyzed on one or two families [63]. Some of these mutations are researched in more detail, and their carrier rates are known [63] According to some researchers the 35delG mutation is the single most responsible mutation for nonsyndromic hearing losses in the European population [64]. Still, if not the single cause for most of the nonsyndromic hearing losses, this is one of the leading causes for autosomal recessive nonsyndromic hearing losses (ARNSLs) [63]. Our findings indicate that in the Hungarian population the carrier frequency of the 35delG mutation is around 2.3%, as we have found 13 heterozygotes on Guthrie cards. Our results are similar to that of Tóth et al., they carried out their work in a population in Northern Hungary [62]. It seems that geographic (and hence minor ethnic) differences do not play an important role in the distribution of the 35delG alleles in Hungary, because our randomized samples came mostly from the Southern-, and Southeastern parts of the country. The incidence of the homozygous 35delG mutation is roughly 1/1000 to 1/2000, the carrier rates are in the range of 1-3% [36][39][65] and, as expected, no 35delG homozygous patients were found in the randomized group.

According to the literature, far more 35delG alleles are found amongst CI users than in the normal population, but the genetic background of hearing loss does not seem to make any difference in the success of the later rehabilitation, according to Coletti et al. and Fischer et al. [66][67]. Green et al. – on the contrary – found that cochlear implant recipients with GJB2-related deafness have greater improvement with cochlear implant, than subjects with

congenital deafness with other root cause and non-cochlear implant recipients [69]. Speech development, however, can be normal, or close to normal, when the child is fitted with cochlear implant in the early ages. As a consequence, the procedure for the selection of a Cochlear implantation must be carried out in the very early years of life – ideally between 1,5-3 years, or even earlier if possible [68].

In the cochlear implanted population, 8 patients were found with homozygous 35delG genotype. Five cochlear implantees were heterozygous for 35delG. All of the 11 homozygous recessives' relatives (who could be investigated) were heterozygous. 28 of the implantees, and 78 of all the implantees' relatives were homozygous wild out of the total of 318 patients.

Only 12 of all the 35delG allele carriers have CI, and even less, 8 of them have homozygous recessive 35delG genotype. These numbers indicate that screening for only the 35delG mutation is not always sufficient.

5.2.2 Sequencing of the coding exon of the GJB2 gene

Numerous GJB2 mutations have been described in the literature until now, but because of their very low abundance, only a few could be studied in detail. Their inheritance and the hearing loss they may cause are not known in all cases.

The non-35delG mutations found in the coding exon of the GJB2 gene in our cohort of patients are summarized in Table 8.

Number of GJB2 mutations	**Percent of GJB2 mutations**	**G109A +- V37I**	**T269C+- L90P**	**G95A-+ R32H +-**	**T101C-+ M34T +-**	**G380A -- R127H**	**G380A +- R127H**
15		2	1	2	1	2	7
	5.66	0,63	0,31	0,63	0,31	0,63	2,20
		G71A +- W24STOP	**G139T+- E47STOP +-**	**C164T+-**	**G478 A+-**	**176delG +-**	**A341G+- E114G+-**
10		3	2	1	1	1	2
	3.14	0,94	0,63	0,31	0,31	0,31	0,63
25	7.86						
Total = 318	100						

Table 8
This table summarizes the non-35delG mutations found in the GJB2 gene
Please note that due to space constraints the table's first row is continued in the fourth row, the second in the fifth, and the third in the sixth, respectively

By sequencing the complete coding exon of the GJB2 gene, 12 other mutations have been found in this group, four of them together with a 35delG allele.

Two of them were homozygous, and even that one – the G380A – is a non-truncating mutation. It has been shown, however, that this mutation can render the Connexin 26 unsuitable for forming functional gap junctions between the cells [70]. This may make us cautious about this mutation. On the other hand, Thönissen et al. [71] and Roux et al. [72] concluded that this mutation might not have any impact on the hearing. Dahl et al. found a proband that had a homozygous recessive form G380A, and had no hearing loss; they concluded too, that the G380A in its homozygous form does not cause nonsyndromic hearing loss [10][73]. Interestingly, our data show that these two homozygotes are in a parent-child relationship, and one sibling, and one parent is heterozygous, yet they have normal hearing.

Apart from this family, 5 other patients have the G380A +- genotype, one of them has a cochlear implant device. The CI user has no other mutation in the GJB2 gene, nor was found any other SNP in any other examined regions. The other four R127H patients have not developed hearing loss.

Only sporadic occurrences have been found of other non-35delG GJB2 mutations. None of them were in their homozygous forms.

Three of these carriers, 1 T269C +- (L90P+-), 1 G109A+- (V37I+-), and 1 G478A+-, have cochlear implants, but this can also be a consequence of other, undetected mutations.

The L90P transition (leucine to proline at the 90^{th} position) was first described by Murgia, et al. as a polymorphism in 1999 [73]. According to the opinion of Loffer et al. from 2001 [75], this mutation is a recessive mutation, that only causes hearing loss in its homozygous form. Janecke et al., suggested that L90P, with some other recessive GJB2 mutation, causes only mild to moderate hearing loss [76]. In our case, the hearing loss is more severe, and this may indicate more things. Firstly, descriptions so far may have been wrong. In our case, no other mutation either in the GJB2 gene or in any other examined region could be found. Secondly, the genetic background of the hearing remains hidden in other genomic regions that fell outside of the scope of this work.

V37I has been described as a polymorphism from a control group by Kelley et al. in 1998 [77]. Bason et al. described the first homozygous V37I allele in GJB2 [78]. Dahl et al. [73] and Huculak et al. [79] both found that V7I in its homozygous form causes only slight to mild degree of hearing loss. All these findings indicate that our patients may have yet an other genetic background for their profound hearing loss.

For the G478A (G160S) change there is only one report to date. Guo et al. described this change as a polymorphism in a large Chinese population [80]. No other reports could be found about this alteration.

Two G95A (R32H) +- heterozygous patients were found. The first report of this mutation comes from Mustapha et al. [81]. Only a few reports are available concerning this SNP, and none of them describes the phenomenology, or the change in hearing it may cause. See the reports of Mahdieh [82] and Feldmann [83] as examples. Santos et al. identified this mutation as a possibly damaging mutation due to the change of polarity in the transmembrane region of the connexin 26 protein [84].

T101C (M34T) was described by Kelsell et al. as a possible autosomal dominant hearing loss causing allele [35]. They described a family where this was the only mutation in the GJB2 gene. The reported family has dermatological problems. Scott et al. found, however, that in fact this may not be true [85]. They described a family, where they found this allele, but none of the family members developed hearing loss. In our single case, nobody in this family has dermatological problems, and there was not found any other mutation neither in the coding exon of the GJB2 gene, nor in any of the other genes or regions that were examined during our experiments. Our patient has a severe hearing loss with hearing levels between 65 and 90 dB. The cause of the hearing remains loss is unclear in this case.

3 G71A +- (W24STOP) mutations were found. Two individuals are 35delG+- / G71A compound heterozygotes, with no hearing loss. One patient has hearing levels between 40 and 70 dB on one ear, and between 80 and 110 dB on the other ear. This latter patient has an SNP in KCNQ4 as well (in DF48). These data may indicate that the hearing loss is not caused by the W24STOP mutation. Although it is a truncating mutation, it seemingly does not cause problems in its heterozygous form. Unfortunately, no homozygous form of this mutation could be found. This base change was first described by Kelsell et al. in 1997 [35]. W24STOP is the predominating DFNB1 allele in India [86][87]. Interestingly, the only W24STOP patient found is a gipsy and it has been reported several times that this amino acid change is more frequent amongst romanis. Both Minárik et al. in Slovakian a population in 2003 [88], and in 2005 Alvarez et al. in a Spanish population [89] reported this. They both concluded that this high frequency in those populations might be a consequence of a founder effect.

G139T+- or E47STOP has been first described by Denoyelle et al. in 1997 [90]. As this is a rare allele, it has only been described a few times. (see [91] and [92] and [93] as examples). Ben Arab et al. described this mutation in Tunisia but they did not provide audiological details [94]. Samanich et al. found a homozygous E47STOP proband, and they concluded that in this form this truncating mutation causes autosomal recessive hearing loss [95]. One of our patients is a 35delG+- / G139T+- compound heterozygote with an SNP in the 12s rRNA gene (DF4), the other one has this mutation only. None of them has any hearing problems.

One C164T allele was found, that has not been described so far. This is a novel polymorphism. This patient has no hearing problems, and has no other mutations in the other examined regions. No other family member has this mutation.

One patient with 176 delG +- has been found with significant hearing loss (between 65 and 110 dB) on all frequencies. This mutation has not been described so far. C176G was described by Heathcote et al. in 2000 that causes palmoplantar keratoderma [96]. They concluded that the transition would disrupt one of the extracellular loops in the Gap Junction Beta 2 protein, and hence dysfunctional connexins form. By deleting one G from the "GGC" triplet, the reading frame is shifted and it becomes "GCU", and glycine becomes alanine. Considering the chemical structures of the two amino acids, this mutation, even in its homozygous form, may be a function-preserving mutation. It is not sure, however, that this mutation in its heterozygous form causes the hearing problem of our patient.

Neither of the two A341G+- (E114G +-) patients has hearing loss. One of them has an SNP in the GJB6 gene, in the DF12 region. Pandya et al. in 2001 described a G79A + A341G (valine at 27 into isoleucyne and glutamine at 114 into glicyne) compound heterozygote. Fuse et al. found more patients with both homozygous and heterozygous forms of this mutation, with and without hearing problems [97]. Kudo et al. described the allele frequency of this mutation in a Japanese population, but gave no data on the mutation's impact on hearing [98]. Park and co-workers found numerous homo- and heterozygotes with A341G mutation, their hearing loss varied from profound hearing loss to no hearing loss at all. They found this mutation in the control group as well. They concluded that although it is possible that this mutation causes anomalies in the function of the GJB2 protein - because this base transition substitutes glycine for glutamate in the cytoplasmic loop area of the polypeptide -, their data do not support this relationship clearly [99]. Choung et al., however, stated that wild type and E114G mutant

transfected cells show the same Cx26 immunohistochemical properties, and that might imply normal GJB2 protein function [100]. Our data is not sufficient to support either hypotheses.

No insertions in size bigger than one base, or deletions in size bigger than one base have been found in our population.

5.2.3 dHPLC of the GJB2 gene

Lin et al. in 2001 reported 100% sensitivity and specificity with this method in 154 patients [101]. Although the method itself is reliable and it is possible to screen with the help of it several hundred samples per day, it has not been widely adopted by the genetic hearing loss research community.

5.3 Mutation detection in other examined genes

No SNPs in the GJA1 gene (primer DF8), and in the POU3F4 transcription factor (primers DF24, DF25, DF26, DF40, DF41, DF42, DF43) were found. This may indicate that mutations in these genes are very rare in the Hungarian population.

5.3.1 12S rRNA

A relatively high number of SNPs were found in various regions of this gene. While there was only one SNP in DF3, DF4 contained 28 SNPs.

This gene – also known as MTRNR1 - is responsible for the coding of the 12s rRNA in the nucleus of the cell. It has been described in 1993 that its A1555G mutation makes an individual highly sensitive to aminoglycoside type of antibiotics, and hence related deafness [102]. This number of SNPs in this gene should make us cautious about utilizing possibly ototoxic drugs. There are only 2 patients where there were no other genetic alterations found, yet they both have cochlear implants. It is possible that there are ototoxic reasons in the background of their hearing loss. Administering antibiotics in relatively low doses can lead to hearing problems in case of these individuals. Although the problem may be prominent, only little is known about the amount of antibiotics that cause deafness in genetically sensitivised patients. Guan et al. investigated the effects of different concentrations of paromomycin on lymphoblastoid cells derived from the members of a family with A1555G-mutation and

deafness, and compared them with normal cells [103]. Their work, however, is hard to apply in clinical circumstances, as they carried out in vitro experiments.

5.3.2 COCH

5 SNPs were found in the COCH gene, 4 in DF6, and 1 in DF49.

In 2 cases, the only detectable genetic alteration was in the cochlin gene, and only one of the two patients had profound hearing loss. This patient also has a cochlear implant. The other patient with the detected SNP does not have any hearing problem, but is the mother of the male cochlear implanted child. No mutation could be detected in case of the father or the sibling of the mentioned CI user. According to the literature, mutations found so far in the COCH gene are mostly autosomal dominant (DFNA9). The first description of these is that of Manolis et al. from 1996 [104].

Our finding, however, may indicate a presence of an X-linked mutation.

A GJB2 G380A+- / COCH compound heterozygote was also found, without detectable hearing loss. According to our findings in 5.2.2 “Sequencing of the coding exon of the GJB2 gene”, the G380A mutation is an autosomal recessive mutation, and if the SNP in this case in the COCH is “silent” then this compound heterozygote may not develop hearing loss.

An other patient with GJB2 35delG+- / COCH / GJB6 triple mutation was also found with severe (around 50 dB) hearing loss. In this case, it is nearly impossible to tell which of the found genetic alterations causes the hearing loss. 35delG is an autosomal recessive mutation, COCH [104] had been described to cause autosomal dominant nonsyndromic hearing losses, but the role of GJB6 is not clear; DFNB1 [105] and DFNA3 [10] both have been mapped to this gene. DFNB1 was mapped to Connexin 26 as well [35].

There is a female, who has SNPs in the SLC26A4, 12S rRNA and in the COCH gene as well, with mild-to severe hearing loss, an audiogram between -25 and -60 dB. Her brother has hearing problems too, with SNPs in the GJB3 and 12S rRNA, but with more severe hearing loss (between 50 and 80dB). It is known from their family history that they were not administered ototoxic drugs, although the only common genetic variance seems to support this idea. Multiple mutation sites have been described in the MTRNR1 gene, but all, except the A1555G transition are outside of our primer DF3. Our findings might indicate the

possibility that there may be aminoglycoside-deafness sensitizing “hot spots” in other regions of the gene as well.

5.3.3 GJB3

In 7 out of the 27 cases, an SNP in the GJB3 gene was the only genetic alteration found. 9 subjects have point mutations in their GJB2 gene, 6 35delG+-, 1 35delG+-/G95A+-, 1 G95A, and 1 G109A have been found. None of them developed hearing problems.

12 of the 27 patients have some degree of hearing loss, and all of their hearing levels are bellow 40 dB. Four of them have mutation only in the GJB3 gene, all the others have SNPs in one of the COCH, KCNQ4, 12s rRNA, or in the MYO6 genes.

Mutations in GJB3 were described to cause dermal diseases [106], or – in a few cases – hearing loss was associated with tinnitus [8], and had an impact on the high frequencies [8]. More marked decrease in the hearing level of the subjects was found by Lopez-Bigas et al. [7], where they assumed nerve myelination problems.

5.3.4 GJB6

5 patients have SNPs in the GJB6 gene, 2 in DF37 and 3 in DF12. One of them is a CI user and this, the SNP is his or her DF12, is the only genetic alteration that could be found.

Two subjects have mutations in their GJB2, one 35delG+-, and one A341G+-, but neither them, nor the other two patients have hearing loss.

Because of the controversial data on GJB6 (see for example [10][35][105]), and the relatively low number of SNPs in this gene amongst our patients, it is hard to tell what impact these SNPs may have on the hearing of our subjects.

5.3.5 KCNQ4

DFNA2 is known to map here [10][24][73]. According to current knowledge, no autosomal recessive hearing loss causing allele has been described in gene.

Out of the 35 patients who have at least one SNP in this gene, ten have developed severe to profound hearing loss. Five of them are CI users, and only one of them has a 35delG+- genotype besides the SNP in KCNQ4. Three of these hard-of-hearing personnel have a GJB2

mutation that can account for their hearing problem, one with 35delG--, one with G71A (W24STOP, a truncating mutation as well), and one with G380A-- (R127H).

The data for this latter patient seems to conflict with the opinion of Thönissen et al. [71] and Roux et al. [72] as they have identified this mutation as a "silent mutation", or a polymorphism. This issue has been discussed in more detail in 5.2.2 "Sequencing of the coding exon of the GJB2 gene".

Five patients have hearing levels bellow 70 dB, and four of them have an SNP in the GJB3 gene too. Taking into account that mutations so far described in both GJB3 and KCNQ4 are mostly dominant [3], their hearing problems may be related to mutations in both genes.

5.3.6 SLC26A4

Only two SNPs were found in two patients in this gene. One of them has no hearing related problems at all, and has this SNP as the only genetic alteration in the studied genes and regions.

The other patient has SNPs in the 12s rRNA and the COCH gene as well, and has mild-to-severe hearing loss on both ears. The patient's hearing problem may be related to the SNP in the 12s rRNA, as her sibling has some severe-to-profound hearing loss on both ears, and three SNPs, in which only the 12s rRNA is common.

5.3.7 MYO6

Myosin VI is one of the unconventional myosins, and plays a role in the intracellular vesicle and organelle transport [107], and in the maturation of the IHCs [15][16]. Both autosomal dominant [17] and autosomal recessive [18] nonsyndromic hearing loss-causing loci have been identified in this gene.

21 SNPs were found in 21 patients. In 8 cases out of the of the 21, MYO6 contained the only genetic alteration in those parts of the genome that was studied. 5 of them have a cochlear implant, and these patients do not have any other mutation in any other studied genetic region. One child's mother has an SNP in MYO6 as well, but she does not have hearing problems. These findings may indicate the presence of a nonsyndromic dominant hearing loss causing mutation in these patients.

Two CI users are homozygous 35delG recessives, and have an SNP in the MYO6 gene, but this SNP is most likely "silent" as their hearing threshold levels are around the same as that of other CI users'.

Four of these patients are 35delG carriers, and have an SNP in MYO6, but they all have normal hearing.

Two of the CI users in this group have SNPs in both GJB3, and MYO6. Neither of them has any other mutation in the studied regions. No simultaneous occurrences of mutations in these genes were described before, so this issue might need some further investigation. One of them had only residual hearing, and one of them had a pure tone threshold level bellow 100 dB before the cochlear implantation.

A patient with severe hearing loss (hearing threshold levels between 55 and 90dB, with lower thresholds on the middle frequencies) has SNPs in three genes, in 12srRNA, GJB3, MYO6. Again, this combination is not studied in enough detail to able to judge the cause of the hearing loss.

5.4 Cochlear implant users

19 out of the 52 cochlear implant users have no mutation in neither of the genes and regions studied in our present work. This is a relatively high number, 36.5% of all the CI users amongst our patients. On the other hand, our cohort was selected so, that their cause of hearing loss was unknown. By systematic checking, some genetic background could be found in almost two-third of these cases.

In 17 cases SNPs were found in one gene, and more genes in four cases out of this 17, that is not GJB2-related.

In 12 cases, there was a mutation in the GJB2 gene only.

These 52 CI users took approximately one-fourth of our CI users at the time the work was in progress. The above numbers suggest that genetic tests should be made during the examinations that forego the implantation procedure, as a large percent of hearing-related problems may be caused by genetic defects. In selected patients (and possibly their families) who have some kind of serious hearing problem (so that cochlear implantation comes into question), the likelihood of such genetic defects is much higher than in the normal population. The 35delG allele was only found in several cases in the "general" Hungarian population, the

carrier rate is approximately 2.2%, but this allele showed a much higher frequency in the other group, 99/318, around ~31%. If similar or even lower ratios in cases of other genes are assumed, then the amount of possible mutations is still very high.

In Hungary, there is a Cochlear Implantation program ongoing. As the Hungarian social insurance finances only a limited amount of cochlear implants per year per institute, the requisites for getting a device must be set very strictly. As quite a few of our cochlear implant users and aspirants have a hearing problem of unknown origin (85 out of 204 as of December 2008), genetic testing can be a tool that can help in making a more thorough decision. Because of some financial constraints - the device is expensive - there exists a waiting list as well. If there is a genetic background in case of a CI candidate, the odds are better to get sooner on that list, as autosomal recessive nonsyndromic hearing loss is an indicative factor for an implant. The child can be implanted earlier, and so its chances are better to learn, to hear, to talk, and to communicate.

Speech development can be normal, or close to normal, when the child is fitted with cochlear implant in the early ages. As a consequence, the procedure of the selection for a cochlear implantation must be carried out in the very early years of life – ideally between 1,5-3 years, or even earlier if possible [68]. As the objective audiological measurements are very hard to carry out properly at this age, genetic testing plays an even more important role. The use of dried blood spots (DBS) makes this task much easier, because if the DBS bank is correctly maintained there will almost surely be a sample from the given patient. In this case, the genetic testing can be carried out without having to bring a child to the hospital, and draw blood from her or him, which is a painful and inconvenient or uneasy procedure, sometimes even for the doctor too.

About $1/4^{th}$ of our country belongs to our department and that takes nearly 2 million people. According to statistics, there are around 800-1000 people per 10 million inhabitants per year who need cochlear implantation, and the number of severe or profound hearing losses is higher, between 1-2% in the European population. That takes 100 000 - 200 000 subjects who ideally should be screened for the background of their hearing losses, just based on the severity, because this could make them a potential CI receiver. These are estimates based on our daily work, and our own experiences with patients. Others suggested somewhat lower numbers [57].

The costs of traveling this amount of people to hospitals, or university hospitals, just to draw blood are enormous. With DBS'es, the costs are considerably lower. Blood can be drawn by their physician, and blood transport do not need to take place in a controlled manner i.e. temperature and the time it takes to transport the anticoagulated blood to the screening centers is of no consideration anymore.

6 Summary

I. We examined three methods to extract DNA from dried blood spots, and tested the obtained DNA. We showed that with a relatively simple procedure (by boiling the DBS'es in water) it is possible to extract DNA that is suitable for genetic testing. It is possible to extract DNA from 96 tubes (this number depends on the capacity of the thermal cycler) in around half an hour. In contrast, this costs significantly more with the use of DNA purifying kits (from EDTA-anticoagulated blood), and takes about 5-6 hours of lab work. [II]

II. During our examinations, we showed that DNA remains usable for both SNP detection (AS-PCR), and sequencing for at least ten years, even when stored under rogue conditions. We could sequence the whole coding exon of the GJB2 gene from DNA that was purified with the methods we applied. [II]

III. We have shown that from one piece of Guthrie paper (3-4 drops of blood) we can get DNA solution that is enough for hundreds, or even thousands of PCR experiments. Taking this into account, even some larger genes can be sequenced if needed. [II]

IV. It was shown that in the population we examined, that consists of samples from South- and Southeastern Hungary, the 35delG mutation in the GJB2 gene is found in about the same percent as others have described in the Caucasian population. Amongst patients whose origin of hearing loss was unknown the 35delG is found much more frequently, the risk of having such a mutation is almost 15 times higher than in the normal hearing population, 2.2% versus ~31 %. [III, I]

V. We have found numerous other mutations in the GJB2 gene in our population of patients. C164T and 176delG have not been described so far. 176delG may even be a novel autosomal dominant nonsyndromic hearing loss causing mutation. C164T is most likely a polymorphism. None of the other mutations hasve been studied in more detail

together with the regions of other genes in the Hungarian population until this work. [submitted]

VI. 29 patients out of the 318 who have some level of hearing loss have possibly other causes for their problems than a mutation in the GJB2 gene. This is almost 11% of this population.

97 SNPs were found in the studied genes and regions of these 318 people. 17 with SNPs in two regions, in two cases in the same gene, and in 8 cases with three SNPs, in 5 cases two of the three SNPs were found in different primers within the same gene. [submitted]

VII. Numerous SNPs were found, but we lack family data to be able to precisely map the affected gene and locus, and to be able to precisely follow the audiological traits between the relatives. Most genetic examinations are done on families, where extended kinships exist, mostly out of cultural conventions. In Hungary (in the European/Western countries in general), it is hard to achieve this. On the other hand, after having reviewed the literature, certain mutations are typical to different human races and geographic regions, and thus genetic data originating from different parts of the world may not be appropriate in certain populations. [submitted]

VIII. Based on our data it is only possible to reliably predict the possible level of hearing loss, in case of a few, well studied, and standalone (or few-gene, or few-locus) nonsyndromic hearing loss-causing mutations. As the frequency of these mutations is very low, we would need a nation-wide screening program to achieve this goal. [submitted]

IX. The pre-selection examinations for cochlear implantation should include genetic testing, and not even on one gene. Although at present state it is not possible to deduce the level of hearing loss based only on the genetic background of the patient, the relatively high number of mutations we found indicates that, that in fact more hearing losses may be attributed to various genetic backgrounds in Hungary than we thought earlier. [submitted]

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9 Appendix A– Primers and sequences

9.1 A concise table on the involved genes, regions, and their related publications

GENE	LOCUS or SYNDROME	AMINO ACID CHANGE	NUCLEOTIDE CHANGE	EXON	ETHNICITY (Number of families)	REFERENCES
COCH	**DFNA9**	P51S	207C>T	exon 4	Belgian (1), Dutch (2)	Fransen 1999
		V66G	253T>G	exon 4	American (1)	Robertson 1998
		G88E	319G>A	exon 5	American (1)	Robertson 1998
		I109N	382T>A	exon 5	Australian (1)	Kamarinos 2001
		W117R	405T>C	exon 5	American (1)	Robertson 1998
GJA1	Recessive Hearing Loss	L11F	30C>T	exon	African American (3)	Liu 2001
		V24A	71T>C	exon	African American (1)	Liu 2001
	Heart Malformations	T326A			N/A (1)	Britz-Cunningham 1995
		F335Q			N/A (1)	Britz-Cunningham 1995
		S373G	A>G		N/A (1)	Britz-Cunningham 1995
		E352G	A>G		N/A (2)	Britz-Cunningham 1995
		S364P	T>C		N/A (5)	Britz-Cunningham 1995
		S365N	G>A		N/A (1)	Britz-Cunningham 1995
GJB3	**DFNA2**	R180X	538C>T		Chinese (1)	Xia 1998
		E183K	547G>A		Chinese (1)	Xia 1998
	Recessive Deafness	141delI	423delATT		Chinese (2)	Liu 2000
		I141V	423A>G		Chinese (2)	Liu 2000
	Neuropathy and Hearing Impairment	R32W	1227C>T	exon 2	Spanish (1)	Lopez-Bigas 2001*
			*Mutation was also found in some controls			
			1610G>A	exon 2	Spanish (1)	Lopez-Bigas 2001
			1700C>T	exon 2	Spanish (1)	Lopez-Bigas 2001
		V200I	1731G>A	exon 2	Spanish (1)	Lopez-Bigas 2001
	Erythrokeratodermia Variabilis	G12R	34G>C		Swiss (1)	Richard 1998
		G12D	35G>A		European (1)	Richard 1998
		R32W	1227C>T		N/A (1)	Kelsell 2000
		L34P	101T>C		Israeli (1)	Gottfried 2002
		R42P	125G>C		N/A (1); Italian (1)	Wilgoss 1999; Richard 2000
		C86S	256T>A		Euroepan (2)	Richard 1998
		F137L	409T>C		British (1)	Richard 2000

GENE	LOCUS or SYNDROME	AMINO ACID CHANGE	NUCLEOTIDE CHANGE	EXON	ETHNICITY (Number of families)	REFERENCES
GJB6	**DFNA3**	T5M	C>T		Italian (1)	Grifa 1999
			del >140kb		Ashkenazi Jewish (4)	Lerer 2001*
			*found in compound hets with GJB2 (DFNB1)			
			del 342kb		Spanish (22)	Castillo 2002*
			*found in compound hets with GJB2 (DFNB1)			
			del >150kb		N/A (5)	Pallares-Ruiz 2002*
			*homozygous or compound het with GJB2 (DFNB1)			
	Hidrotic Ectodermal Dysplasia (Clouston Syndrome)	G11R	31G>A		French (2), Scottish-Irish (1), African (1), Spanish (1), French Canadian (3)	Lamartine 2000
		V37E	110T>A		Scottish (1)	Smith 2002
		A88V	263C>T		Indian (1), Malaysian (1), Welsh (1)	Lamartine 2000
KCNQ4	**DFNA2**	FS71	211del13	exon 1	Belgian (1)	Coucke 1999
		L274H	821T>A	exon 5	Dutch (1)	Van Hauwe 2000
		W276S	827G>C	exon 5	Dutch (1); Japanese (1); Dutch (1), Japanese (1)	Coucke 1999; Akita 2001; Van Camp 2002
		L281S	842T>C	exon 6	American (1)	Talebizadeh 1999
		G285C	853G>T	exon 6	American (1)	Coucke 1999
		G285S	G>A	exon 6	French (1)	Kubisch 1999
		G321S	961G>A	exon 7	Dutch (1)	Coucke 1999
MYO6	**DFNA22**	C442Y	1325G>A	exon 12	Italian (1)	Melchionda 2001
POU3F4	**DFN3**		del1200kb		N/A (1)	Arellano 2000
			del	entire gene	N/A (1)	de Kok 1996
		Rearrangement		upstream	N/A (1)	de Kok 1995b
			del 120kb	upstream	N/A (1)	de Kok 1996
			del 200kb	upstream	N/A (1)	de Kok 1996
			del 220kb	upstream	N/A (1)	de Kok 1996
			del 30kb	upstream	N/A (1)	de Kok 1996
			del 8kb	upstream	N/A (1)	de Kok 1996
			del 2.6kb, 6.5kb, 7kb, 4.4kb upstream		French (1)	de Kok 1996
		201-202delFK	601-606del6bp	POU specific	Japanese (1)	Hagiwara 1998
		K202X	603del4	POU specific	N/A (1)	de Kok 1995a
		D215X	648delG	POU specific	N/A (1)	de Kok 1995a

GENE	LOCUS or SYNDROME	AMINO ACID CHANGE	NUCLEOTIDE CHANGE	EXON	ETHNICITY (Number of families)	REFERENCES
		T230I	689C>T	POU specific	N/A (1)	Friedman 1997
		FS	862del4	POU homeo	Finnish (1)	Bitner-Glindzicz 1995
		L298X	895delA	POU homeo	N/A (1)	de Kok 1995a
		A312V	935C>T	POU homeo	British (1)	Bitner-Glindzicz 1995
		L317W	950T>G	POU homeo	N/A (1)	de Kok 1995a
		R323G (mosaic)	967C>G	POU homeo	N/A (1)	de Kok 1997
		R329G	985C>G	POU homeo	N/A (1)	Friedman 1997
		R330S	990A>T	POU homeo	N/A (1)	de Kok 1997
		K334E	1000A>G	POU homeo	N/A (1)	de Kok 1995a
12S rRNA	Associated with ototoxicity		961delTinsC		Chinese (1); Italian (1)	Bacino 1995; Casano 1999
	Associated with ototoxicity		1095T>C		N/A (1)	Tessa 2001
	Associated with ototoxicity		1555A>G		Arab-Israeli (1),Chinese (3); Japanese (2), N/A (4); Zairean (12); Japanese (5); Mongolian (2); Spanish (2); Greek (1), English/Irish (1), Italian (1), Mexican (1), Puerto Rican (1), Chinese/Japanese/Caucasian (1), Vietnamese (1); S. African (1); Korean (1) Spanish (19); Italian (2); Spanish/Cuban (8); Japanese (1) Spanish (23); Filipino American (1); Japanese (4)	Prezant 1993; Hutchin 1993a; Matthijs 1996; Usami 1997; Pandya 1997; El-Schahawi 1997; Fischel-Ghodsian 1997; Gardner 1997; Chang 1997 Estivil 1998; Casano 1998; Sarduy 1998; Tono 1998 Castilo 2000; Nye 2000*; Oshima 2001
(SLC26A4)	**DFNB4**		IVS2-2A>G		Italian (1)	Lopez-Bigas 2001
		T132I	395C>T		Italian (1)	Lopez-Bigas 2001
		G209V	626G>T	exon 6	Caucasian (1)	Usami 1999
			Found in Pendred by Van-Huawe 1998			
		L236P	707T>C	exon 6	American (1)	Scott 2000
		X308/wt	917delT	exon 7	Japanese (1)	Usami 1999
		K369E	1105A>G	exon 9	Japanese (1)	Usami 1999
		A372V	1115C>T	exon 9	Japanese (1)	Usami 1999
		T410M	1229C>T	exon 10	Italian (1)	Lopez-Bigas 2001
			Found in Pendred by Coyle 1998			
		T416P/wt	recessive?		American (1)	Scott 2000
			Found in Pendred by Van-Huawe 1998			

GENE	LOCUS or SYNDROME	AMINO ACID CHANGE	NUCLEOTIDE CHANGE	EXON	ETHNICITY (Number of families)	REFERENCES
		V480D	1440T>A	exon 13	American (1)	Scott 2000
			Found in Pendred by Van Hauwe 1998			
		I490L	1468A>C	exon 13	Indian (1)	Li 1998
		G497S	1489G>A	exon 13	Indian (1)	Li 1998
		V653A/wt	recessive?		American (1)	Scott 2000
			Found with CX26 35delG heterozygous			
			Found in Pendred by Campbell 2001			
		T721M	2162C>T	exon 19	Japanese (1)	Usami 1999
			Found in Pendred by Lopez-Bigas 2001			
		X722	2111insGCTGC	exon 19	Japanese (1)	Usami 1999
		H723R	2168A>G	exon 19	Japanese (3); Japanese (1)	Usami 1999; Ishinaga 2002
			Found in Pendred by Van-Huawe 1998			
	Pendred Syndrome	E29Q	85G>C	exon 2	N/A (1)	Campbell 2001
		X96	279delT	exon 3	Brazilian (1)	Kopp 1999
		Y105C	314A>G	exon 4	N/A (1)	Campbell 2001
		A106D	317C>A	exon 4	N/A (1)	Campbell 2001
		X180	336-337insT	exon 4	N/A (1)	Coyle 1998
		FS135	406del5	exon 4	Spanish (1)	Lopez-Bigas 2001
		V138F	412G>T	exon 4	Belgian (1); N/A (1); N/A (1)	Van-Huawe 1998; Coyle 1998; Cambpell 2001
		X141	IVS4+7A>G	exon 4	Spanish (1)	Lopez-Bigas 1999
		G139A	416G>C	exon 5	Dutch (1)	Van-Huawe 1998
		T193I	580C>T	exon 5	Middle Eastern (1)	Adato 2000
		G209V	626G>T	exon 6	Belgian (1); N/A (2)	Van-Huawe 1998; Campbell 2001
		L236P	707T>C	exon 6	Dutch (4), American (1), Danish (1), Belgian (1); N/A (10); N/A (1)	Van-Huawe 1998; Coyle 1998; Campbell 2001
			Found in DFNB4 by Scott 2000			
		X286	753delCTCT	exon 6	N/A (1)	Coyle 1998
		X286	783-784insT	exon 7	N/A (1)	Campbell 2001
		D271H	811G>C	exon 7	Turkish (1)	Van-Huawe 1998
		918+1G>A	IVS7+1G>A	intron 7	Indian (1)	Van-Huawe 1998

GENE	LOCUS or SYNDROME	AMINO ACID CHANGE	NUCLEOTIDE CHANGE	EXON	ETHNICITY (Number of families)	REFERENCES
			IVS7-2A>G	intron 7	Turkish (1)	Couke 1999
		1001+1G>A	IVS8+1G>A	intron 8	N/A (9); Italian (1); N/A (7)	Coyle 1998; Bogazzi 2000; Campbell 2001
		F335L	1003T>C	exon 9	N/A (1)	Campbell 2001
		FS383	1149delC	exon 9	Dutch (1)	Van-Huawe 1998
		E384G	1151A>G	exon 10	N/A (6)	Coyle 1998
		FS400, X431	1197delT	exon 10	Arabic (1); Lebanon (1) Italian (1); Spanish (1)	Everett 1997; Van-Hauwe 1998, Fugazzola 2000; Lopez-Bigas 2001
		R409H	1226G>A	exon 10	Turkish (1); N/A (1)	Van-Huawe 1998, Coyle 1998
		T410M	1229C>T	exon 10	N/A (1)	Coyle 1998
			Found in DFNB4 by Lopez-Bigas 2001			
		T416P	1246A>C	exon 10	Dutch (4), Danish (1); N/A (7); N/A (5)	Van-Huawe 1998; Coyle 1998; Campbell 2001
			Found in DFNB4 by Scott 2000			
		A429del	1284delTGC	exon 11	N/A (1)	Coyle 1998
		L445W	1334T>G	exon 11	Dutch (1); Turkish (1); Southern Tunisian (2); Italian (1)	Van-Huawe 1998; Couke 1999; Masmoudi 2000; Lopez-Bigas 2001
		X454	1341delG	exon 12	Arabic (1)	Everett 1997
		X467	1334insAGTC	exon 12	N/A (1)	Coyle 1998
		V480D	1440T>A	exon 13	N/A (1)	Campbell 2001
		T508N	1523C>A	exon 13	Italian (1)	Bogazzi 2000
		X524	1536delAG	exon 13	N/A (1)	Coyle 1998
		Y530H	1588T>C	exon 14	N/A (1); N/A (2)	Coyle 1998; Campbell 2001
		Y556C	1667A>G	exon 15	N/A (1)	Coyle 1998
		Y556H	1666T>C	exon 15	Italian (1)	Lopez-Bigas 2001
		C565Y	1918G>A	exon 15	American (1)	Van-Huawe 1998
		L597S	1790T>C	exon 16	N/A (3)	Campbell 2001
		S610X			Japanese (1)	Kiyomizu 2002
		FS634	1898del A	exon 17	Belgian (1)	Van-Huawe 1998
		V653A	1958T>C	exon 17	N/A (1)	Campbell 2001
			Found in DFNB4 by Scott 2000			
		S657N		exon 17	Japanese (1)	Kiyomizu 2002
		F667C	2000T>G	exon 17	Arabic (1)	Everett 1997
		G672E	2015G>T	exon 17	N/A (1); N/A (2)	Coyle 1998; Campbell 2001
		X719	2127delT	exon 19	N/A (1)	Coyle 1998

GENE	LOCUS or SYNDROME	AMINO ACID CHANGE	NUCLEOTIDE CHANGE	EXON	ETHNICITY (Number of families)	REFERENCES
		T721M	2162C>T	exon 19	Italian (2)	Lopez-Bigas 2001
			Homozygote had hypothyroidsim, but not goiter			
			Found in DFNB4 by Usami 1999			
		H723R	2168A>G	exon 19	Dutch (1); Japanese (1)	Van-Huawe 1998; Ishinaga 2002
			Found in DFNB4 by Usami 1999			
		Y728X	2182insG	exon 19	Italian (1)	Fugazzola 2000
		X781W	2343A>G		Italian (1)	Lopez-Bigas 2001

Table 9.
This table is an edited and slightly modified version of the table found at http://hearing.harvard.edu/db/genelist.htm. The GJB2 gene has been left out for convenience.

9.2 The regions we studied, and amplificates of the regions

Gene	Exon				Amplificate			
	#	start	end	length	start	end	length	Name
GJB2	1	1430	2110	681	1385	1804	420	**DF1f**
					1798	2121	324	**DF2f**
12SrRNA	1	650	1603	954	1400	1620	221	**DF3f**
	TRNS1-TRND	7446	7586	141	7336	7600	265	**DF4f**
COCH	4	4235	4386	152	4192	4477	286	**DF5f**
	5	4865	4927	63	4733	5125	393	**DF6f**
	6 and 7	5894	6176	283	5854	6196	343	**DF7f**
GJA1	1	11207	12355	1149	11201	11335	135	**DF8f**
GJB3	1	1674	2486	813	1636	1979	344	**DF9f**
					1961	2240	280	**DF10f**
					2239	2492	254	**DF11f**
GJB6	1	734	1519	786	724	1082	359	**DF12f**
					1077	1340	264	**DF13f**
					1272	1583	312	**DF14f**
KCNQ4	1	83	396	314	8	415	408	**DF15f**
	5	35336	35461	126	35281	35477	197	**DF16f**
	6 and 7	35864	36249	386	35861	36262	402	**DF17f**
SLC26A4	4	13530	13714	185	13463	13829	367	**DF18f**
	6 and 7	22568	22903	336	22524	22920	397	**DF19f**
	10 and 11	33769	34082	314	33764	34143	380	**DF20f**
	13	37408	37477	70	37358	37555	198	**DF21f**
	17	43697	43751	55	43648	43799	152	**DF22f**
	19	51905	51988	84	51858	52086	229	**DF23f**
POU3F4	1	33	1119	1087	15	405	391	**DF24f**

Gene	Exon				Amplificate			
	#	start	end	length	start	end	length	Name
					401	783	383	**DF25f**
					777	1132	356	**DF26f**
MYO6	2	68301	68464	164	68259	68570	312	**DF31f**
		91363	91504	142	91332	91518	187	**DF32f**
		92003	92199	197	91872	92216	345	**DF33f**
	31	164846	165097	252	164830	165193	364	**DF34f**
GJB2	1	1430	2110	681	1798	2124	327	**DF35f**
					1651	1804	154	**DF36f**
GJB6	1	734	1519	786	734	981	248	**DF37f**
					978	1233	256	**DF38f**
					1226	1531	306	**DF39f**
POU3F4	1	33	1119	1087	20	277	258	**DF40f**
					259	571	313	**DF41f**
					454	809	356	**DF42f**
					803	1134	332	**DF43f**
SLC26A4	4	13530	13714	185	13523	13733	211	**DF44f**
	6 and 7	22568	22903	336	22558	22837	280	**DF45f**
					22690	23042	353	**DF46f**
KCNQ4	6 and 7	35864	36249	386	35864	36152	289	**DF47f**
					36059	36263	205	**DF48f**
COCH	5	4865	4927	63	4733	4959	227	**DF49f**
GJB2	1	1430	2110	681			809	

9.3 Sequences of PCR primers used in the PCR experiments

9.3.1 Forward primers

Name	Length	Forward primer	Tm
DF1f	23	CCCTCTCATGCTGTCTATTTCTT	51,3
DF2f	22	TCCTCTTCTTCTCATGTCTCCG	52,6
DF3f	23	TATGAAACTTAAGGGTCGAAGGT	51
DF4f	22	TCGAAGCGAAAAGTCCTAATAG	50,4
DF5f	20	AGTCAGTGGGATGCCCTGAA	54,2
DF6f	23	GCAGCAGGATGTTTGTAACTACA	51,5
DF7f	22	GTCTTCCTTTTGTTAATGCCAA	50,7
DF8f	20	GGCAACATGGGTGACTGGAG	55
DF9f	20	ATTGGCAGCCCGCATGTTGC	62,5
DF10f	20	AGTCCACGATGTTGGGGCAG	57
DF11f	20	AAGATGAGCTGCAGGGCCCA	59,2
DF12f	22	CTTGAAATGTTTAGCTTGGGAA	50,9
DF13f	21	GCTTCAAAGATGATTCGGAAA	51,3
DF14f	20	AAGATCAGCTGGAGGGCCCA	58,2
DF15f	20	CGTCTCTGAGCGCCCCGAGC	63,9
DF16f	20	ACATCTCCCAGGCAGGCACA	57,6
DF17f	23	CAGATTACATTGACAACCATCGG	53,6
DF18f	23	CCTATGCAGACACATTGAACATT	51,1
DF19f	20	GCGTGTAGCAGCAGGAAGTA	50,7
DF20f	20	TCCAGGTTGCTGGCATCATC	55,6
DF21f	20	CAAAATACGGCTGTTCCAAA	50,5
DF22f	22	GGGCAGATAAGGTTGTTAATTG	50,2
DF23f	23	GGTGGGTTGATGCTATTCTATTT	51,1
DF24f	20	AGGGGATCCTCACCGACCAT	56,7
DF25f	20	TATCACGTCAAGCGGCCAAC	55,9
DF26f	20	GAAGCCCCTGCTGAACAAGT	53,3
DF31f	22	TCAAAACTGATTCATGTTGCTG	50,5
DF32f	22	TTGTAATGTTCCGTCATGCTAA	50,3
DF33f	22	GTTTTTTCCCCTTTATTTGGTG	51,6
DF34f	23	TGCTGGTATAACTTTCCTTGTTC	50
DF35f	22	TCCTCTTCTTCTCATGTCTCCG	52,6
DF36f	20	AGACGTACATGAAGGCGGCT	53,8
DF37f	21	TTAGCTTGGGAAACCTGTGAT	50,7
DF38f	20	AAGCAGTCAACAAGGTTGGG	51,6
DF39f	20	GTAGTAGGCCACATGCATGG	50,4
DF40f	20	ATCCTCACCGACCATGGCCA	59,6
DF41f	20	TGGACCAGCAGGACGTGAAG	55,8

Name	Length	Forward primer	Tm
DF42f	20	TGAGCGGCATGCTGGAACAC	59,2
DF43f	20	AGGAGGCGGATTCGTCCACA	59
DF44f	20	TCCCCAGGACCTTTTCCAGT	54,7
DF45f	23	CTTTTTATAGACGCTGGTTGAGA	50,4
DF46f	23	CCCAGTCCCTATTCCTATAGAAG	50,2
DF47f	21	ATTACATTGACAACCATCGGC	51,5
DF48f	22	GGTACCTCAGAGGGGCAAGGAT	57,2
DF49f	23	GCAGCAGGATGTTTGTAACTACA	51,5
GJB2seq4F	23	GGCCTACAGGGGTTTCAAATGGT	

Tm: melting temperature

9.3.2 Reverse primers

Name	Length	Reverse primer	Tm
DF1r	21	AAGAGGAAGTTCATCAAGGGG	51,5
DF2r	22	GCCCAGAGTAGAAGATGGATTG	52,1
DF3r	23	TGTTAAGCTACACTCTGGTTCGT	50,4
DF4r	23	GCTGCATGTGCCATTAAGATATA	51,6
DF5r	21	CCATCAAGGTTAAAGAGGCTG	50,9
DF6r	23	CAACAGAGTGAGACCCTGTCTTT	51,7
DF7r	23	CATTTTAACAAGGTTGGTACCTG	50,3
DF8r	20	TGACTCAACCGCTGTCCCCA	58,5
DF9r	20	TGCCCCAACATCGTGGACTG	58,8
DF10r	20	TTCGTCACATGCCCCTCGCT	60
DF11r	20	AGGCGCCATGGACTGGAAGA	59,1
DF12r	23	TGAAGCAGCCTTTATGTATGTGT	51,1
DF13r	23	CAAAAATGTGTGCTATGACCACT	51,2
DF14r	20	CCCAAGGCCTCTTCCACTAA	53,3
DF15r	20	CGCGGGGTCGCAAACTCACA	63,6
DF16r	20	AAAGACCCTCACGCACCGTC	56,3
DF17r	21	GGGCATCTTGTACCTGGATGA	53,8
DF18r	23	GGGTTCCAGGAAATTACTTTGTT	52,5
DF19r	20	AGGAACACCACACTCACCCC	53,3
DF20r	22	GGCAGGAAGCATATAAGAACCA	52,8
DF21r	22	CCTTGTACGTAAAATGGAGCTG	51,2
DF22r	22	GGCTTACGGGAAAGTCTTACAG	51,8
DF23r	23	TTCCCTGACAGTTCTTAATCAGA	50,1
DF24r	20	TGATAGACGGGTTCGGTGCC	57
DF25r	21	GGGCTTCAGCTTGCACATATT	53,6
DF26r	20	TCGCTTCCTCCAGTCAGAGA	52,3
DF31r	23	GCTTTCCCAAATATCTACCTCAT	50,5
DF32r	22	CCCAAAAATATCATTCCAAAGC	52,3
DF33r	22	GCACCTGGCTATATGAAATTTC	50,2
DF34r	23	CCCTCAACCCTGAAATGTAATAA	51,9
DF35r	22	GCCCAGAGTAGAAGATGGATTG	52,1
DF36r	21	AAGAGGAAGTTCATCAAGGGG	51,5
DF37r	23	GCTTTATTTCTAGGCCAACAGAG	53,5
DF38r	22	CCTACTACAGGCACGAAACCAC	53,1
DF39r	20	TAAACCAGCGCAATGGATTG	53,7
DF40r	20	TTCACGTCCTGCTGGTCCAG	55,8
DF41r	20	CAATGGTGCGAGCCCAGTTC	57,7
DF42r	20	GCCTCCTCCAGCCACTTGTT	55,5

Name	Length	Reverse primer	Tm
DF43r	20	CCTCGCTTCCTCCAGTCAGA	54,2
DF44r	21	GCACCTGACCTAAAACAACGT	51
DF45r	23	GCAGTAGCAATTATCGTCTGAAA	51
DF46r	23	GTTTCTTCCAGATCACACACAAA	51
DF47r	20	TAGAGGGATAGGGCATGGTT	50,3
DF48r	20	CGGGCATCTTGTACCTGGAT	53,8
DF49r	23	GGCAACTAGGAGATAGGTTTCAT	50,5
GJB21R	20	TCATCCCTCTCATGCTGTCT	

Tm: melting temperature

10 Appendix B– Summary tables of mutations

10.1 GJB2 35delG allele

CI	GJB2 Mutation	# of audiograms	Type of audiogram (dB)	dHPLC/Other SNP in primer	Gene
CI	35 delG--	2	90-100	DF33	MYO6
CI	35 delG--	7	50-70		
CI	35 delG--	9	80-120 one side only	DF33	MYO6
CI	35 delG--	24	100		
CI	35 delG--				
CI	35 delG--				
CI	35 delG--	7	no hearing		
CI	35 delG--	3	60		
	35 delG--	8	70-90		
	35 delG--				
	35 delG--				
	35 delG--	1	80-90		
	35 delG--	1	OK		
	35 delG--	1	30-75		
	35 delG--	1	residual hearing		
	35 delG--			DF48	KCNQ4
	35 delG--				
	35 delG--	1	90-120	DF48	KCNQ4
	35 delG--	1	0.25kHz-1kHz 80-110, >2kHz 0		
	35 delG--				
	35 delG--	3	75-110		
	35 delG--	4	70-100		
	35 delG--				
	35 delG--				
	35 delG+-				
	35 delG+-	1	norm		
	35 delG+-	1	norm		
	35 delG+-				
CI	35 delG+-	8	70-90	DF9	GJB3
CI	35 delG+-	5	70-80 on 2 freq. Only		
CI	35 delG+-				
	35 delG+-				
	35 delG+-				

CI	GJB2 Mutation	# of audiograms	Type of audiogram (dB)	dHPLC/Other SNP in primer	Gene
	35 delG+-				
	35 delG+-			DF33	MYO6
	35 delG+-	1	OK	DF9	GJB3
	35 delG+-			DF33	MYO6
	35 delG+-			DF11,DF33	GJB3, MYO6
	35 delG+-			DF37	GJB6
	35 delG+-				
	35 delG+-			DF 33	MYO6
	35 delG+-			DF11, DF17, DF48	GJB3, KCNQ4, KCNA4
	35 delG+-			DF9, DF11	GJB3, GJB3
	35 delG+-	1	50-60	DF6, DF11	COCH, GJB3
	35 delG+-				
	35 delG+-	1	5-35		
	35 delG+-				
	35 delG+-				
	35 delG+-				
	35 delG+-				
	35 delG+-				
	35 delG+-				
	35 delG+-				
	35 delG+-				
	35 delG+-			DF48	KCNQ4
	35 delG+-	1	~80		
	35 delG+-				
	35 delG+-			DF4	12srRNA
	35 delG+-			DF48	KCNQ4
	35 delG+-			DF4	12srRNA
	35 delG+-	1	no hearing		
	35 delG+-	2	no hearing		
	35 delG+-				
	35 delG+-			DF4	12srRNA
	35 delG+-				
	35 delG+-			DF4	12srRNA
	35 delG+-				
	35 delG+-			DF4	12srRNA
	35 delG+-				
	35 delG+-	2	100-110	DF4	12srRNA
	35 delG+-				
	35 delG+- / G139T+-			DF4	12srRNA

CI	GJB2 Mutation	# of audiograms	Type of audiogram (dB)	dHPLC/Other SNP in primer	Gene
	35 delG+- / G71A+-				
	35 delG+- / G71A+-				
CI	35 delG+- G95A-R32H +-	4	55-110	DF9, DF11, DF17	GJB3, GJB3, KCNQ4

10.2 Other GJB2 mutations

CI	GJB2 Mutation	# of audiograms	Type of audiogram (dB)	dHPLC primer	Gene
	176 delG +-	16	65-110		
	35 delG+- / G139T+-			DF4	12srRNA
	35 delG+- / G71A+-				
	35 delG+- / G71A+-				
CI	35 delG+- G95A-R32H +-	4	55-110	DF9, DF11, DF17	GJB3, GJB3, KCNQ4
	A 341 G+- / E114G+-			DF12	GJB6
	C 164 T+-				
	G 380 A+-				
CI	G 478 A+-	18	80-115		
	G109A +- V37I			DF9	GJB3
CI	G109A +- V37I	5	residual hearing		
	G139T+-				
	G380A --	1	-50-90 with hearing aid	DF48	KCNQ4
	G380A --				
	G380A +- / 56insC				
CI	G380A +- R127H				
	G380A +- R127H			DF6	COCH
	G380A +-			DF48	KCNQ4

CI	GJB2 Mutation	# of audiograms	Type of audiogram (dB)	dHPLC primer	Gene
	R127H				
	G380A +- R127H	1	90-120		
	G380A +- R127H				
	G71A +- W24STOP +-	1	left: 40-70; right: 80-110	DF48	KCNQ4
	G95A-R32H +-			DF9, DF17, DF33	GJB3, KCNQ4, MYO6
	T101C-M34T +-	7	65-90		
CI	T269C+- L90P	2	100		

10.3 12s rRNA

CI	GJB2 Mutation	# of audiograms	Type of audiogram (dB)	dHPLC primer	Gene
		2	25-60	DF4, DF19, DF49	12srRNA, SLC26A4, COCH
		2	50-70; 30-80	DF4, DF9	12srRNA, GJB3
				DF4, DF17	12srRNA, KCNQ4
		12	50-90	DF4, DF11, DF33	12srRNA, GJB3, MYO6
control				DF4	12srRNA
control				DF4, DF48	12srRNA, KCNQ4
control				DF4, DF16, DF48	12srRNA, KCNQ4, KCNQ4
CI				DF4	12srRNA
				DF4	12srRNA
	35 delG+-			DF4	12srRNA
	35 delG+-			DF4	12srRNA
				DF4	12srRNA
				DF4	12srRNA
	35 delG+-			DF4	12srRNA
	35 delG+- / G139T+-			DF4	12srRNA
CI				DF4	12srRNA
				DF4	12srRNA
				DF4	12srRNA
				DF4	12srRNA

CI	GJB2 Mutation	# of audiograms	Type of audiogram (dB)	dHPLC primer	Gene
				DF4	12srRNA
		2	no hearing	DF4	12srRNA
CI		20	90-100	DF4	12srRNA
				DF4	12srRNA
				DF4	12srRNA
	35 delG+-			DF4	12srRNA
				DF4	12srRNA
	35 delG+-			DF4	12srRNA
	35 delG+-	2	100-110	DF4	12srRNA

10.4 COCH

CI	GJB2 Mutation	# of audiograms	Type of audiogram (dB)	dHPLC primer	Gene
		2	25-60	DF4, DF19, DF49	12srRNA, SLC26A4, COCH
				DF6	COCH
CI		4	55-65 (HK!)	DF6	COCH
	35 delG+-	1	50-60	DFf6, DF11	COCH, GJB3
	G380A +- R127H			DF6	COCH

HK: measured with hearing aid

10.5 GJB3

CI	GJB2 Mutation	# of audiograms	Type of audiogram (dB)	dHPLC primer	Gene
				DF9,DF48	GJB3,KCNQ4
	35 delG+-	1	OK	DF9	GJB3
				DF9,DF48	GJB3,KCNQ4
				DF9,	GJB3,
				DF9,DF33	GJB3,MYO6
CI		2	20-40	DF9,DF11,DF33	GJB3,GJB3,MYO6
CI		8	no hearing	DF9,DF33	GJB3,MYO6
	35 delG+-			DF11,DF33	GJB3,MYO6
CI		19	110-120	DF9,DF17	GJB3,KCNQ4
				DF9,DF17	GJB3,KCNQ4
		2	50-70;30-80	DF4, DF9	12srRNA, GJB3
	G109A +- V37I			DF9	GJB3

CI	GJB2 Mutation	# of audiograms	Type of audiogram (dB)	dHPLC primer	Gene
CI	35 delG+-	8	70-90	DF9	GJB3
CI		6	100	DF9	GJB3
		1	30-100	DF11	GJB3
		12	55-90	DF4, DF11, DF33	12srRNA, GJB3, MYO6
	35 delG+-			DF11, DF17, DF48	GJB3, KCNQ4, KCNQ4
CI	35 delG+- G95A-R32H +-	4	55-110	DF9, DF11, DF17	GJB3, GJB3, KCNQ4
	35 delG+-			DF9, DF11	GJB3, GJB3
	G95A-R32H +-			DF9, DF17, DF33	GJB3, KCNQ4, MYO6
		3	10-20	DF9, DF11	GJB3, GJB3
				DF11	GJB3
				DF9, DF11, DF48	GJB3, GJB3, KCNQ4
		9	100	DF11, DF48	GJB3, KCNQ4
				DF11	GJB3
	35 delG+-	1	50-60	DFf6, DF11	COCH, GJB3
control				DF9, DF17	GJB3, KCNQ4

10.6 GJB6

CI	GJB2 Mutation	# of audiograms	Type of audiogram (dB)	dHPLC primer	Gene
				DF37	GJB6
	35 delG+-			DF37	GJB6
	A 341 G+- / E114G+-			DF12	GJB6
CI		1	no hearing	DF12	GJB6
				DF12	GJB6

10.7 KCNQ4

CI	GJB2 Mutation	# of audiograms	Type of audiogram (dB)	dHPLC primer	Gene
				DF9,DF48	GJB3,KCNQ4
				DF9,DF48	GJB3,KCNQ4
				DF48	KCNQ4
				DF17	KCNQ4
				DF33,DF48	MYO6,KCNQ4

CI	GJB2 Mutation	# of audiograms	Type of audiogram (dB)	dHPLC primer	Gene
CI		19	100-120	DF9,DF17	GJB3,KCNQ4
		1	OK	DF9,DF17	GJB3,KCNQ4
CI		7	70-80	DF17	KCNQ4
				DF17	KCNQ4
				DF17, DF33	KCNQ4, MYO6
CI		4	60-100	DF17	KCNQ4
				DF4, DF17	12srRNA, KCNQ4
CI		1	55-85	DF48	KCNQ4
	35 delG+-			DF11, DF17, DF48	GJB3, KCNQ4, KCNQ4
CI	35 delG+- G95A-R32H +-	4	55-110	DF9, DF11, DF17	GJB3, GJB3, KCNQ4
	G95A-R32H +-			DF9, DF17, DF33	GJB3, KCNQ4, MYO6
				DF9, DF11, DF48	GJB3, GJB3, KCNQ4
		9	100-110	DF11, DF48	GJB3, KCNQ4
control				DF9, DF17	GJB3, KCNQ4
control				DF4, DF48	12srRNA, KCNQ4
control				DF4, DF16, DF48	12srRNA, KCNQ4, KCNQ4
		17	55-90	DF48	KCNQ4
	35 delG --			DF48	KCNQ4
				DF48	KCNQ4
	35 delG+-			DF48	KCNQ4
	G71A +- W24STOP +-	1	40-70;80-110	DF48	KCNQ4
	G380A +- R127H			DF48	KCNQ4
	G380A --	1	100-120	DF48	KCNQ4
				DF48	KCNQ4
	35 delG --	1	90-120	DF48	KCNQ4
	35 delG+-			DF48	KCNQ4
				DF48	KCNQ4
				DF48	KCNQ4
				DF48	KCNQ4
				DF48	KCNQ4

10.8 SLC26A4

CI	GJB2 Mutation	# of audiograms	Type of audiogram (dB)	dHPLC primer	Gene
		2	25-90	DF4, DF19, DF49	12srRNA, SLC26A4, COCH
				DF22	SLC26A4

10.9 MYO6

CI	GJB2 Mutation	# of audiograms	Type of audiogram (dB)	dHPLC primer	Gene
CI	35 delG --	2	90-00	DF33	MYO6
	35 delG+-			DF33	MYO6
				DF33	MYO6
CI		9	80-120	DF33	MYO6
				DF9,DF33	GJB3,MYO6
				DF33	MYO6
CI		2	20-40	DF9,DF11,DF33	GJB3,GJB3,MYO6
CI		8	no hearing	DF9,DF33	GJB3,MYO6
				DF33	MYO6
	35 delG+-			DF33	MYO6
	35 delG+-			DF11,DF33	GJB3,MYO6
CI	35 delG --	9	80-120 on one side	DF33	MYO6
				DF33,DF48	MYO6,KCNQ4
CI		14	80-120	DF33	MYO6
				DF17, DF33	KCNQ4, MYO6
CI		1	10	DF33	MYO6
CI		1	no hearing	DF33	MYO6
CI				DF33	MYO6
		12	55-90	DF4, DF11, DF33	12srRNA, GJB3, MYO6
	35 delG+-			DF 33	MYO6
	G95A-R32H +-			DF9, DF17, DF33	GJB3, KCNQ4, MYO6

10.10 Cochlear implant users

CI	GJB2 Mutation	# of audiograms	Type of audiogram (dB)	dHPLC primer	Gene
CI	35 delG --	2	90-100	DF33	MYO6
CI		3	80-110; 70-90		
CI		5	60		
CI	35 delG --	7	50-70		
CI		5	40-80		
CI		8	80-120		
CI		9	80-120	DF33	MYO6
CI		2	20-40	DF9,DF11,DF33	12srRNA, GJB3, MYO6
CI		8	no hearing	DF9,DF33	12srRNA, MYO6
CI		6	20-30 ->10		
CI	35 delG --	9	80-120 on one ear	DF33	MYO6
CI		14	80-120	DF33	MYO6
CI		19	110-120	DF9,DF17	12srRNA, KCNQ4
CI		12	120		
CI		7	70-80	DF17	KCNQ4
CI		4	60-100	DF17	KCNQ4
CI		4	55-65	DF6	COCH
CI		12	70-100		
CI	35 delG+-	8	70-90	DF9	GJB3
CI		1	10	DF33	MYO6
CI	35 delG+-	5	80 on 2 freqs only		
CI					
CI	T269C+- L90P	2	100		
CI		1	20-30		
CI		6	100	DF9	GJB3
CI		1	55-85	DF48	KCNQ4
CI		1	noe hearing	DF33	MYO6
CI				DF33	MYO6
CI	35 delG+- G95A-R32H +-	4	55-110	DF9, DF11, DF17	KCNQ4, GJB3, KCNQ4
CI	35 delG+-				
CI	G109A +- V37I	5	residual hearing		
CI		5	100		
CI	35 delG --	24	100		

CI	GJB2 Mutation	# of audiograms	Type of audiogram (dB)	dHPLC primer	Gene
CI		4	no hearing		
CI	G380A +- R127H				
CI					
CI				DF4	12srRNA
CI					
CI					
CI					
CI					
CI	35 delG --				
CI	35 delG --				
CI		1			
CI		9	100		
CI	35 delG --	7	no hearing		
CI				DF4	12srRNA
CI		6	65-90		
CI		1	no hearing	DF12	GJB6
CI		20	90-100	DF4	12srRNA
CI	G 478 A+-	18	80-115		
CI	35delG --	3	60		

Appendix C

11 Appendix C– dHPLC parameters

Primer name	Program	Tempreature °C
DF1f	Medium	58
DF2f	Medium	63
DF3f	Medium	55
DF4f	Medium	56, 57
DF5f	Long	59
DF6f	Long	56
DF7f	Long	55, 57
DF8f	Short	61,8
DF9f	Long	62
DF10f	-	-
DF11f	Long, Medium	64, 60
DF12f	Long	59
DF13f	Long	61
DF14f	Long	59
DF15f	-	-
DF16f	Long	63
DF17f	Long	60
DF18f	Medium	54
DF19f	Medium	54
DF20f	Long	58
DF21f	Medium	56
DF22f	Short	53
DF23f	Medium	55
DF24f	Long	63
DF25f	Long	63
DF26f	Long	62
DF31f	Long	56
DF32f	Long. Short	53, 59
DF33f	Medium	56
DF34f	Long, Medium	50, 58
DF35f	Long	60
DF36f	Medium	60
DF37f	Long	56
DF38f	Long	58
DF39f	Long	62
DF40f	Long	60

Primer name	Program	Tempreature °C
DF41f	Long	62
DF42f	Long	63
DF43f	Long	63
DF44f	Long	57
DF45f	Long	55
DF46f	Long	55
DF47f	Long	58
DF48f	Medium	58, 64
DF49f	Medium	57

Table 10.
The primers, and the programs and temperatures they were measured at

Plate type and size	96 Well Low		**Syringe Speed**	Normal
Syringe volume	250 µl		**Speed factor**	1.0
Sample Loop Volume	200 µl		**Needle height**	4 mm
Needle Tubing Volume	30 µl		**Flush volume**	30 µl
First transport vial	T/R Vial 1		**Plate Cooling Setpoint**	12 °C
Last Tranposrt Vial	T/R Vial 4		**End Time**	1:00 min
plate cooling enabled				

Table 11.
Configuration options for the dHPLC syringe and plates.
These were common for all experiments

Long (@65 °C)					
Pump and CÍM program	**Time (min:sec)**	**%A**	**%B**	**Flow (ml/min)**	**Data acquisition**
	0:00	48	52	0.5	begin
	0:01	43	57		
	3:00	34	66		
	3:30	34	66		
	3:31	48	52		
	4:30	48	52	0.5	end
Shutdown program	0:00	100	0	0	
	1:00	100	0	0	
Oven Control	65 °C				

Table 12.
Example configuration options for the Long method @65 °C. This table shows the ratios of „A" and „B" buffers over the course of one experiment. The temperature is shown in the last line.

Medium (@ 60 °C)					
Pump and CÍM program	**Time (min:sec)**	**%A**	**%B**	**Flow (ml/min)**	**Data acquisition**
	0:00	54	46	0.5	begin
	0:01	49	51		
	3:00	40	60		
	3:30	40	60		
	3:31	54	46		
	4:30	54	46	0.5	end
Shutdown program	0:00	100	0	0	
	1:00	100	0	0	
Oven Control	60 °C				

Table 13.
Example configuration options for the Medium method @60 °C. This table shows the ratios of „A" and „B" buffers over the course of one experiment. The temperature is shown at the last line.

Short (@ 52 °C)					
Pump and CÍM program	**Time (min:sec)**	**%A**	**%B**	**Flow (ml/min)**	**Data acquisition**
	0:00	59	41	0.5	begin
	0:01	54	46		
	3:00	45	55		
	3:30	45	55		
	3:31	59	41		
	4:30	59	41	0.5	end
Shutdown program	0:00	100	0	0	
	1:00	100	0	0	
Oven Control	52 °C				

Table 14.
Example configuration options for theShort method @52 °C. This table shows the ratios of „A" and „B" buffers over the course of one experiment. The temperature is shown at the last line.

12 Appendix D - Sample dHPLC chromatograms

12.1 GJB2 (35delG) chromatograms

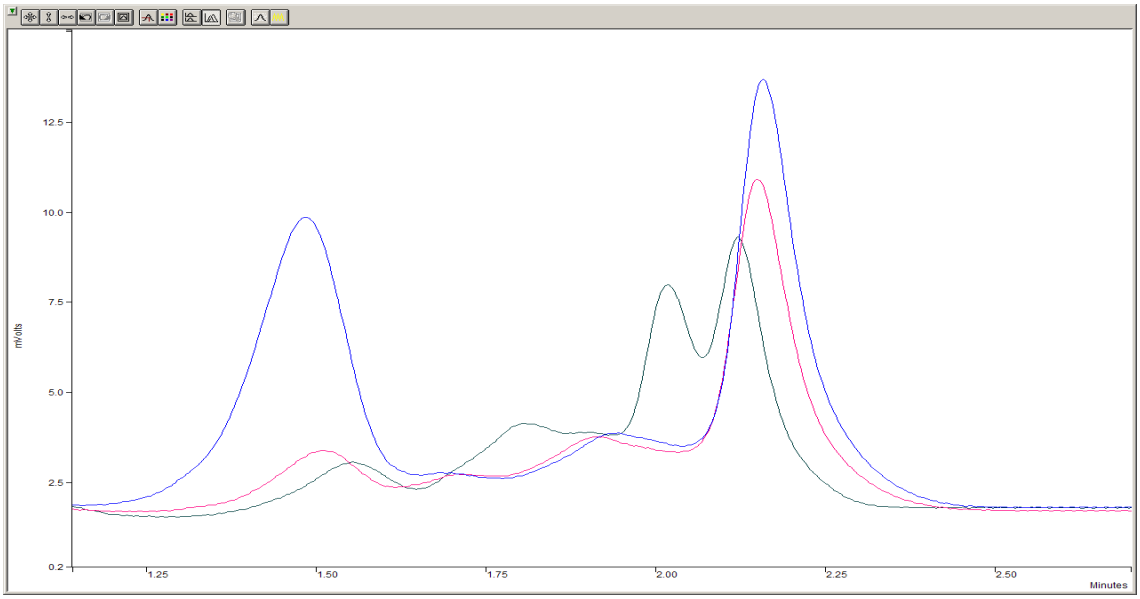

Figure 13.
A sample dHPLC chromatogram showing a homozygous wild type (pink line), a heterozygpous (green line), and a homozygous 35delG (blue line) sample

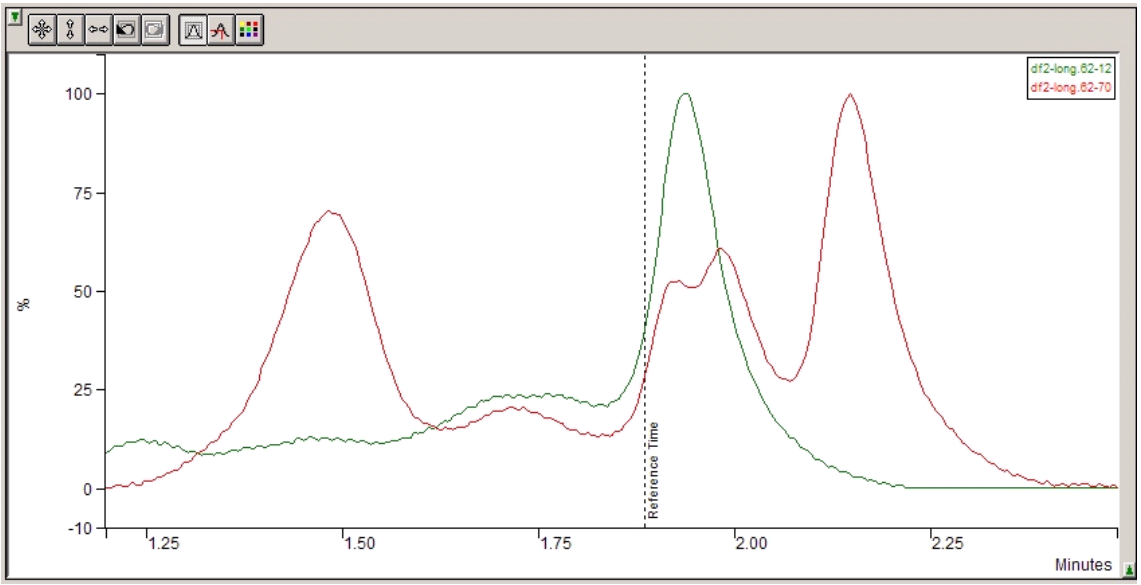

Figure 14.
Sample dHPLC chromatograms showing an M34T (red line), and a wild type allele (green line)

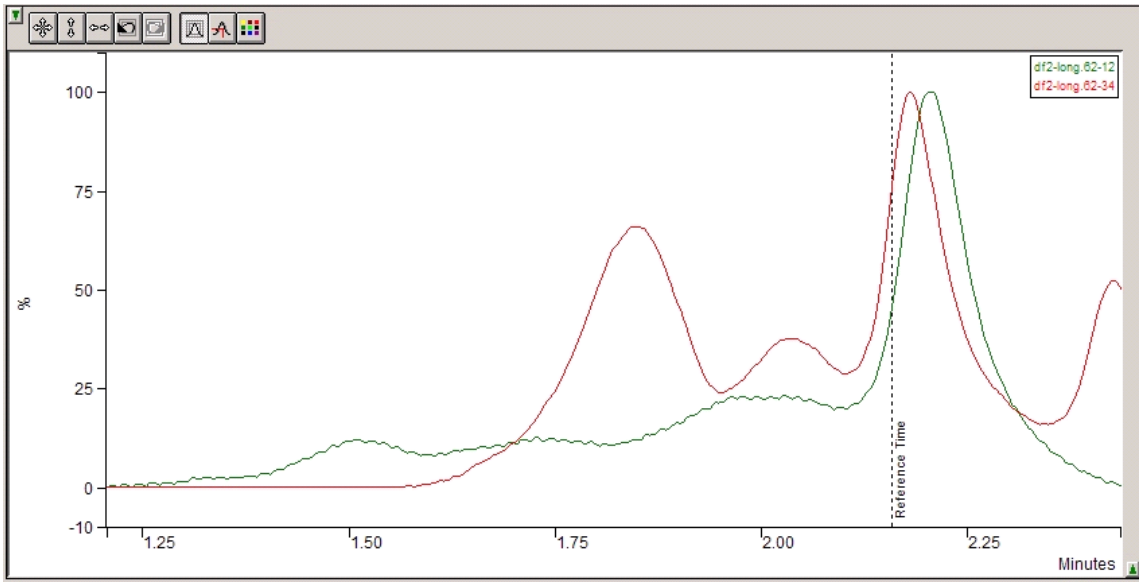

Figure 15.
Sample dHPLC chromatograms showing an R32H heterozygote (red line), and a wild allele (green line)

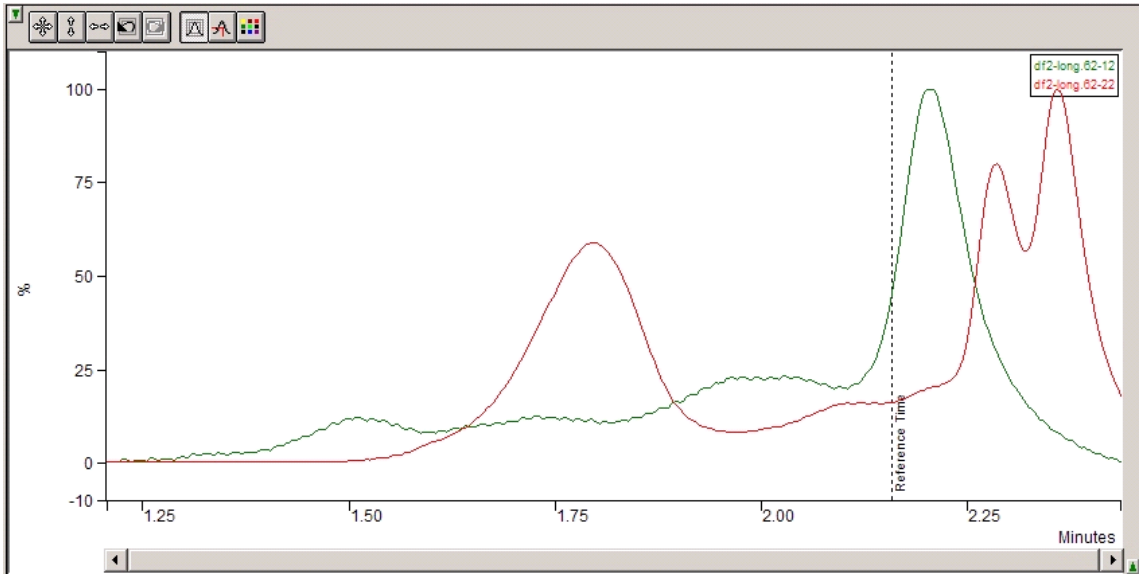

Figure 16.
Sample chromatograms showing a 35delG+- + R32H compound heterozygote (red line), and a wild genotype sample (green line)

12.2 Examples of dHPLC chromatograms from other genes, with audiograms

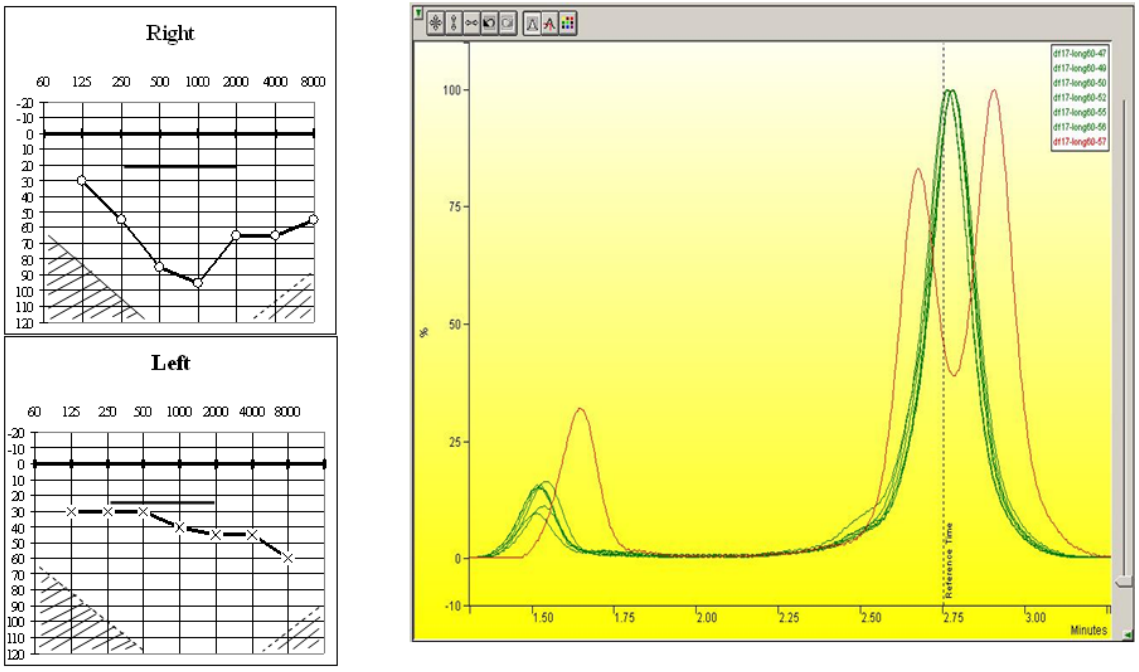

Figure 17.
Sample chromatograms from the KCNQ4 gene, showing an SNP (red line), with the "average" chromatograms (various, non-red colors), and the audiogram of the patient.

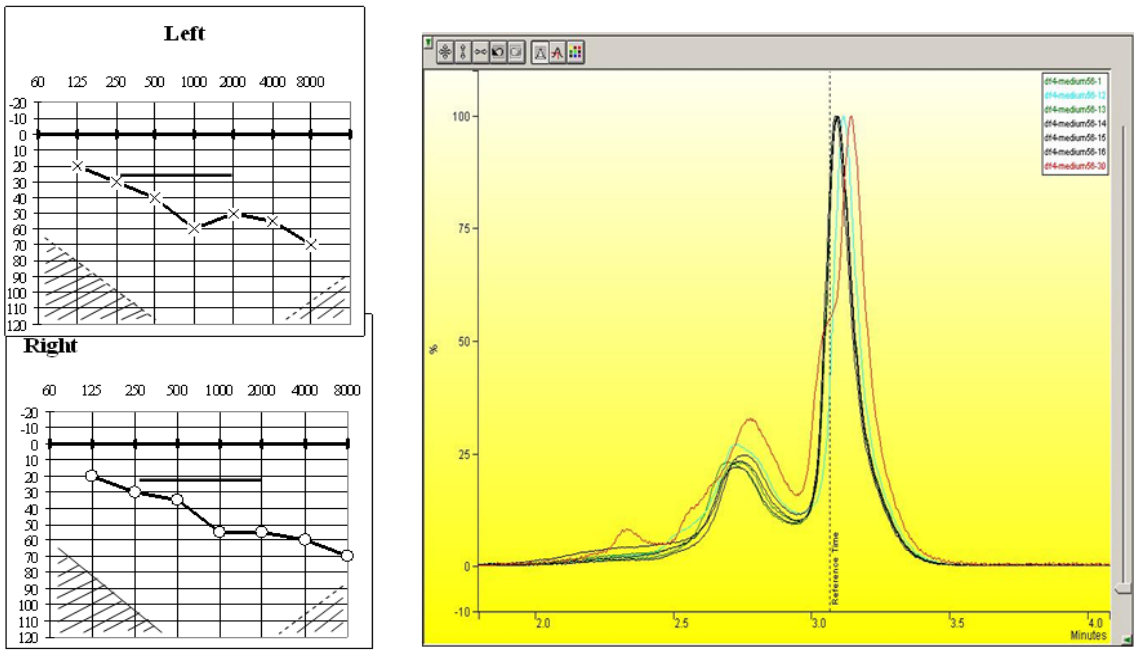

Figure 18.
Sample chromatograms from the 12s rRNA gene (DF4 region), showing an SNP (red line), and the "average" chromatograms. The audiogram of the patient is shown to the left. This particular patient has SNPs in SLC26A4 and COCH, but they are not shown for clarity.

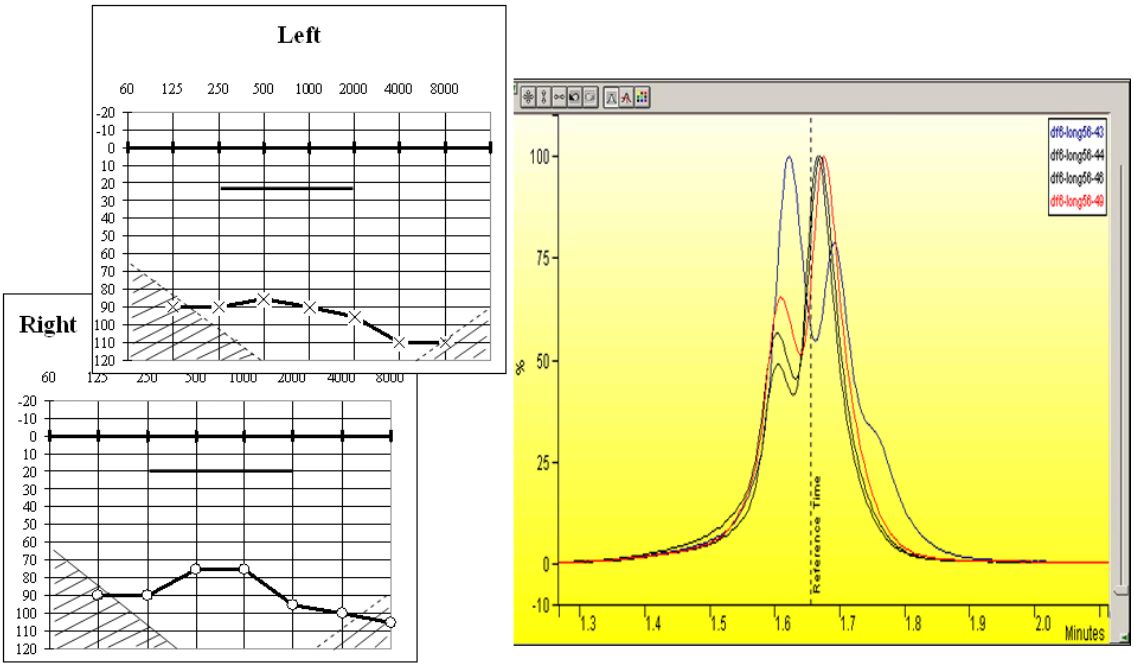

Figure 19.
The figure shows COCH, DF6 region with an SNP (blue line), and the "average samples" with an audiogram.

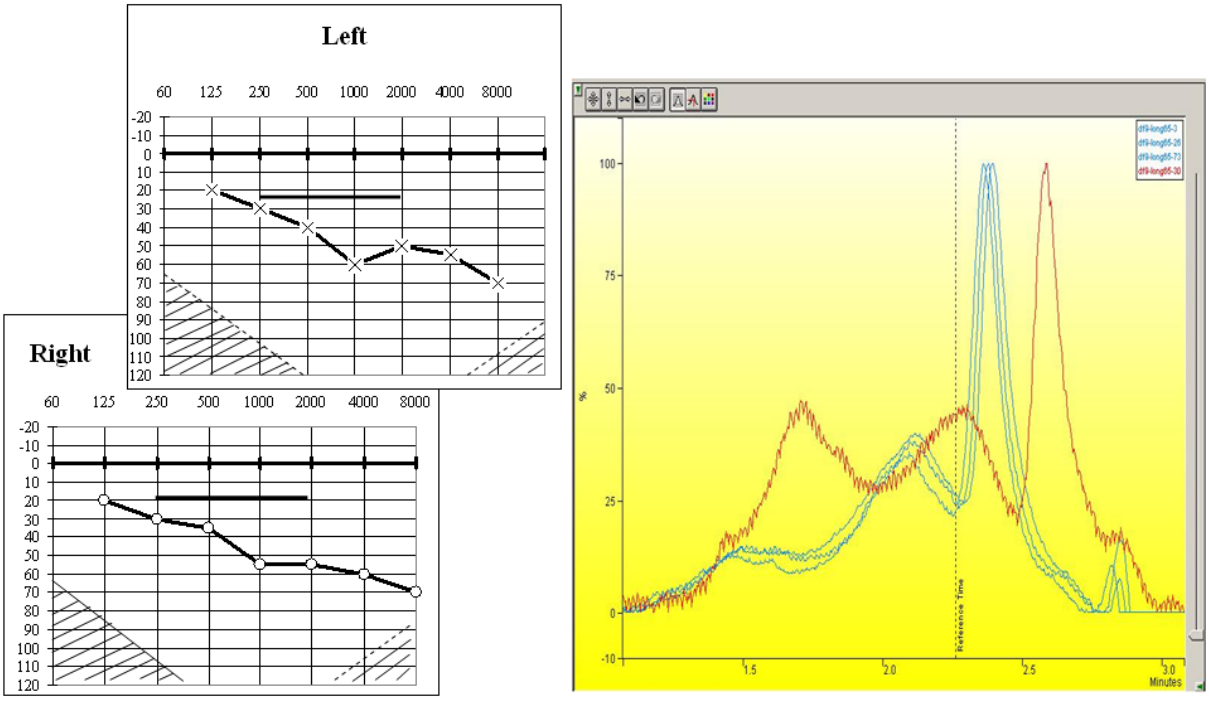

Figure 20.
The figure shows the chromatogram of an SNP in GJB3 in the DF9 region (red line), with an audiogram

I.

II.

III.