UNIVERSIDADE DE LISBOA
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ANA CLÁUDIA GASPARINHO GONÇALVES

Dissertação
Mestrado em Biologia Molecular e Genética

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List of abbreviations

12S rRNA – 12 Subunit ribosomal Ribonucleic Acid
ºC – degree Celsius
δ – delta
γ – gamma
µL – microliter
µM – micromolar
% - percent sign
A – Adenine
ATP – Adenosine Triphosphate
Ala – Alanine
Arg – Arginine
Asn – Asparagine
Asp – Aspartic acid
BioFIG – Center for Biodiversity, Functional and Integrative Genomics
BLAST – Basic Local Alignment Search Tool
bp – base pair
C – Cytosine
c. – complementary DNA
CFTR - Cystic Fibrosis Conductance Regulator
CHLN – Centro Hospitalar de Lisboa Norte
CHLO – Centro Hospitalar de Lisboa Oriental
cm^2 – square centimetre
COXI – Cytochrome Oxidase I
Cx26 – Connexin-26
Cx30 – Connexin-30
Cys – Cysteine
dB – decibel
DFNB1 – autosomal recessive nonsyndromic deafness
DNA – Deoxyribonucleic Acid
EDTA - Ethylenediamine Tetraacetic Acid
ENT – Ear, Nose and Throat
ER – Endoplasmic Reticulum
G – Guanine
GJB2 – Gap junction Beta-2
GJB6 – Gap junction Beta-6
Gln – Glutamine
Glu – Glutamic acid
Gly – Glycine
GTP – Guanosine triphosphate
H_2O – Water
H_2Od – Distilled water
His – Histidine
HL – Hearing Loss
Hz – Hertz
Ile – Isoleucine

IVS – Intervening Sequence
K^+ - potassium ion
kb – kilobase
kDa – kilo Dalton
Leu – Leucine
LFSNHL – Low Frequency Sensorineural Hearing Loss
Lys – Lysine
m. - mitochondrial
M – Molar
Met – Methionine
mg – milligrams
min – minute
mL – milliliter
mm^2 – square millimeter
mRNA – messenger RNA
mtDNA – mitochondrial DNA
NaCl – Sodium Chloride
NCBI – National Center for Biotechnology Information
p – position on the chromosome's short arm
p. – protein
PCR – Polymerase Chain Reaction
Phe – Phenylalanine
Pro – Proline
PS – Pendred Syndrome
PSDM – PCR-Site Directed Mutagenesis
q – position on the chromosome's long arm
rpm – revolutions per minute
s – second
SDS - Sodium Dodecyl Sulfate
Ser – Serine
SLC26A4 - Solute Carrier Family 26, member 4
STAS - Sulfate Transporter and Antisigma factor antagonist
T – Timine
TBE – Tris/Borate/EDTA
Thr – Threonine
Tm – melting temperature
Tris/HCl – Tris(hydroxymethyl)aminomethane-Hydrochloride
Trp – Tryptophan
tRNA – transfer RNA
Tyr – Tyrosine
U – Unit
Val – Valine
WFS1 – Wolfram Syndrome 1
WS – Wolfram Syndrome

Abstract

About 2/1000 new born present hearing loss (HL) and of these, approximately 50% can be genetically explained.

In this work, 70 Portuguese probands presenting nonsyndromic sensorineural HL were investigated for the presence of mutations in the DFNB1 *locus*. Mutation c.35delG, in the *GJB2* gene, was first screened by restriction analysis. Two individuals were homozygous (2,9%) and five (7,1%) heterozygous for this mutation. The *GJB2* coding exon was then analysed by sequencing in cases found to be negative or heterozygous for the c.35delG mutation. A novel mutation, p.Leu213X, was identified. This mutation wasn't present in a random control sample of 480 individuals from the Portuguese population previously sequenced. Along with the two c.35delG homozygous, the genetic cause for HL due to *GJB2* mutations could be determined for six more individuals, representing a total of 11,4% of the probands. The most frequent *GJB6* deletions were further screened by multiplex PCR in individuals negative or monoallelic for *GJB2* mutations. No mutation was found.

Three common mitochondrial DNA mutations associated to HL were investigated by enzymatic restriction in 143 families, previously screened for DFNB1 and compatible with maternal inheritance. Only the m.1555A>G mutation was found, in one of the analysed families.

Two cases of low-frequency sensorineural HL were investigated regarding the most relevant exons of *WFS1* gene. A novel mutation, p.Asp171Asn, was found in exon 5 of *WFS1* gene. This mutation wasn't present in 100 Portuguese hearing controls later sequenced.

Two cases of Pendred Syndrome were studied. No mutation was found in the relevant exons analysed. So, the genetic cause couldn´t yet be determined.

This study represents a contribution for extending the knowledge on hereditary HL in the Portuguese population, either in the identification of the genetic etiology in affected families, or in the development of more accurate diagnostic protocols.

Keywords: hearing loss, DFNB1, p.Leu213X mutation, p.Asp171Asn mutation.

Resumo

Cerca de 2/1000 recém-nascidos apresentam surdez e, nestes, aproximadamente 50% têm uma causa genética.

No presente trabalho, foram estudados 70 probandos Portugueses com surdez não-sindrómica neurosensorial. A análise iniciou-se com o estudo da mutação c.35delG no gene *GJB2* através de restrição enzimática. Dois indivíduos apresentaram esta mutação em homozigotia (2,9%) e cinco em heterozigotia (7,1%). O exão codificante de *GJB2* foi analisado por sequenciação em todos os casos negativos ou heterozigóticos para c.35delG. Uma nova mutação, p.Leu213X, foi descoberta. Esta mutação não foi encontrada numa amostra aleatória de 480 indivíduos da população Portuguesa. Juntamente com os indivíduos homozigóticos para c.35delG, a causa genética da surdez devida a *GJB2* foi determinada para mais seis indivíduos, representando no conjunto 11,4% dos probandos. As deleções mais frequentes no gene *GJB6* foram investigadas por PCR multiplex em indivíduos monoalélicos ou negativos para *GJB2*. Nenhum dos indivíduos apresentou deleções em *GJB6*.

As três mutações mais comuns associadas a surdez no DNA mitocondrial foram investigadas por restrição enzimática em 143 famílias previamente testadas para *GJB2* e compatíveis com hereditariedade materna. Apenas numa família foi encontrada a mutação m.1555A>G.

Dois casos de surdez neurosensorial com perdas nas baixas frequências foram estudados através da análise dos exões mais relevantes do gene *WFS1*. Uma nova mutação, p.Asp171Asn, foi encontrada no exão 5 deste gene. Esta mutação não foi encontrada em 100 controlos Portugueses ouvintes posteriormente sequenciados.

Dois casos esporádicos de Síndrome de Pendred foram estudados mas nenhuma mutação foi encontrada nos exões analisados, pelo que a causa genética não pôde ser ainda determinada.

Este estudo constituiu uma contribuição para aprofundar o conhecimento sobre a surdez hereditária na população Portuguesa. A importância do estudo genético fica aqui patente, quer na identificação da etiologia genética da surdez em famílias afectadas, quer no desenvolvimento de protocolos de diagnóstico mais eficazes.

Palavras-chave: surdez, DFNB1, mutação p.Leu213X, mutação p.Asp171Asn.

Resumo alargado

A surdez representa a deficiência sensorial mais frequente na população humana, apresentando uma incidência de aproximadamente 2 em cada 1000 nascimentos. A surdez devida exclusivamente a causas genéticas tem tendência a aumentar nos países desenvolvidos, onde já cerca de 50% dos casos são devidos a surdez hereditária, em consequência da melhoria dos cuidados de saúde e diminuição da incidência dos factores ambientais. Dentro destes, em 70% a surdez é o único sintoma clínico (surdez não-sindrómica) e nos restantes 30% a surdez pode estar associada a outras desordens clínicas, sendo designada por surdez sindrómica. As formas de surdez não sindrómica autossómicas recessivas são geneticamente heterogéneas e constituem a forma mais comum da perda auditiva hereditária, contabilizando cerca de 75-85% dos casos. Este número é seguido pela hereditariedade autossómica dominante (12-13%) e pela hereditariedade mitocondrial ou ligada ao cromossoma X, que representa 2-3% dos casos de surdez genética.

Os mais recentes avanços na área da genética molecular permitiram associar a surdez a mais de 400 síndromes, ao mesmo tempo que mais de 140 *loci* relacionados com a surdez não-sindrómica foram mapeados. Até à data, cerca de 60 genes foram já associados com a perda auditiva.

O gene *GJB2* (*Gap Junction β-2*) forma com o gene *GJB6* (*Gap Junction β-6*) o *locus* DFNB1. Estes genes localizam-se no cromossoma 13q11-q12 e codificam para as proteínas conexina-26 (Cx26) e conexina-30 (Cx30), respectivamente. As proteínas conexinas possuem uma localização transmembranar e podem associar-se num hexâmero formando conexões (Martínez, 2009). Os conexões são designados homoméricos, quando são constituídos por um único tipo de conexina ou heteroméricos, quando são formados por diferentes tipos de conexinas. As *gap junctions* resultam da associação entre si de conexões na superfície de células adjacentes e são designadas de homotípicas, quando são formadas por conexões semelhantes ou heterotípicas, quando são formadas por conexões heteroméricos. As células ligadas por *gap junctions* usam este tipo de canais para transferir iões e outras pequenas moléculas entre si. Na cóclea, a Cx26 e a Cx30 encontram-se co-localizadas e co-expressas, contribuindo para a homeostasia coclear. A manutenção desta homeostasia é conseguida através da re-circulação de iões K^+ na endolinfa após estimulação das células ciliadas do ouvido interno.

O gene *GJB2* contém cerca de 5500 pb e um único exão codificante, num total de dois exões. Mais de 100 mutações foram já identificadas neste gene e, dependendo da população,

contribuem para cerca de 10-40% dos casos de surdez hereditária, representando a causa mais frequente de surdez hereditária não-sindrómica autossómica recessiva. A mutação mais comum na população Caucasóide é a c.35delG, que resulta numa terminação precoce da cadeia polipeptídica da Cx26, após uma alteração na grelha de leitura que conduz a um codão STOP prematuro. A frequência alélica para esta mutação foi estimada em 2,5% na população Caucasóide geral, num total de 3% de frequência alélica para todas as mutações em *GJB2*. A mutação c.167delT, por sua vez, possui uma elevada frequência na população de Judeus Ashkenazi (7,5%) e a mutação c.235delC é a mais frequente nas populações Coreanas e Japonesas, com frequências alélicas estimadas em 0,5 e 1%, respectivamente.

O gene *GJB6*, contrariamente ao gene *GJB2*, possui até hoje poucas mutações descritas, apenas seis, que apresentam um padrão de hereditariedade recessiva. Dessas seis mutações, quatro são deleções. As mais frequentes são as deleções Δ(*GJB6*-D13S1830) e Δ(*GJB6*-D13S1854), que, ao mesmo tempo que truncam o gene *GJB6*, inactivam o gene *CRYL1*, eliminando a região entre estes. O gene *CRYL1* codifica para a λ-cristalina e até à data, nenhum caso de indivíduos que possuam quer a deleção Δ(*GJB6*-D13S1830), quer a deleção Δ(*GJB6*-D13S1854), foi reportado juntamente com desordens oculares.

A transmissão do DNA mitocondrial, através de herança materna, também contribui para casos de surdez não-sindrómica quando na presença de determinadas mutações. Uma destas mutações, m.1555A>G, constitui-se como uma causa comum de surdez não-sindrómica familiar. Esta mutação conduz a uma alteração na conformação do gene *12S rRNA*, tornando a sua estrutura semelhante ao gene ribossomal das bactérias. Assim, a exposição a antibióticos aminoglicósidos, como a estreptomicina, torna-se ototóxica, podendo conduzir a um fenótipo de surdez acentuado.

As perdas auditivas nas altas frequências são relativamente comuns, ao contrário de perdas auditivas que afectem predominantemente as frequências abaixo dos 2000Hz. Devido à manutenção da audição nas altas frequências, os indivíduos que sofrem de perdas auditivas nas baixas frequências retêm a capacidade da percepção da fala, pese embora a presbiacúsia ou a exposição a ruídos elevados possam causar perdas auditivas nas altas frequências mais tarde nas suas vidas. A maioria dos casos reportados de perdas auditivas nas baixas frequências é devida a mutações dominantes no gene *WFS1*, que codifica para a wolframina, uma glicoproteína transmembranar localizada no retículo endoplasmático.

Entre os diferentes síndromes em que a surdez se encontra associada a outras anomalias, destaca-se o Síndrome de Pendred. Este síndrome, autossómico recessivo,

representa a forma mais comum de surdez sindrómica e apresenta uma incidência de cerca de 7,5 a 10 casos por cada 100000 indivíduos. Os indivíduos afectados com Síndrome de Pendred possuem mutações bialélicas no gene *SLC26A4* e apresentam, para além de surdez neurosensorial, defeitos na tiróide e malformações no ouvido interno, que podem originar displasia de Mondini.

O principal objectivo deste trabalho foi determinar a causa genética da surdez apresentada por pacientes Portugueses com surdez neurosensorial não-sindrómica. Casos esporádicos de Síndrome de Pendred e de perdas auditivas nas baixas frequências foram também estudados.

Setenta probandos com fenótipo de surdez neurosensorial não-sindrómica foram primeiramente testados para a presença da mutação c.35delG, por restrição enzimática. Esta mutação foi encontrada em homozigotia em dois (2,9%) probandos e em heterozigotia em cinco (7,1%) probandos. Para os 2 indivíduos homozigóticos para a mutação c.35delG estava nesta altura encontrada a causa genética do seu fenótipo de surdez. Para os restantes 68 indivíduos, negativos (n=63) e heterozigóticos (n=5) para a mutação c.35delG, foi feita a sequenciação para o exão codificante do gene *GJB2*. Esta sequenciação revelou a presença de 14 diferentes variantes nos indivíduos considerados. Para seis casos (8,8% 6/68) a causa genética da surdez foi determinada através de sequenciação automática, enquanto para os restantes permaneceu inconclusiva. A causa genética da surdez pôde ser estabelecida para: um probando heterozigótico composto com as mutações p.Met34Thr e p.Iso140Ser; um probando com heterozigotia composta envolvendo as mutações c.333-334delAA e p.Leu213X; um probando homozigótico para a mutação p.Trp24X; um probando com heterozigotia composta envolvendo as mutações p.Met34Thr e p.Arg184Pro; um probando heterozigótico composto c.35delG e p.Trp172X e, um probando apresentando uma heterozigotia composta com as mutações p.Val37Ile e p.Asn206Ser. No conjunto, 11,4% dos probandos (8/70) apresentam surdez associada ao gene *GJB2*. Durante esta etapa, uma nova mutação nunca antes descrita foi reportada, p.Leu213X, com localização no C-terminal da Cx26. Esta mutação não foi encontrada numa amostra aleatória de 480 indivíduos Portugueses que tinha sido previamente sequenciada para o exão codificante do gene *GJB2*.

Para os probandos monoalélicos ou sem mutações em *GJB2* procedeu-se à análise por PCR multiplex das deleções mais frequentes no gene *GJB6*. Nenhuma das deleções testadas foi encontrada.

Relativamente à análise das três mutações mais comuns no DNA mitocondrial associadas a surdez, dos 143 indivíduos analisados apenas um probando foi diagnosticado com heteroplasmia para a mutação m.1555A>G. Posteriormente, a análise por restrição enzimática à irmã e à mãe revelou neles também a presença desta mutação.

No âmbito deste trabalho, foram também analisadas duas famílias que apresentavam fenótipo de perdas auditivas nas baixas frequências. Foram sequenciados os exões 4, 5, 6 e 8 do gene *WFS1* onde se localizam, até à data, a maioria das mutações patogénicas descritas. Para uma das famílias este rastreio foi inconclusivo. No que diz respeito ao segundo caso, foi encontrada uma nova mutação, p.Asp171Asn, não descrita até à data. Esta mutação, presente em heterozigotia num indivíduo não foi encontrada numa amostra de 100 indivíduos ouvintes Portugueses que foram sequenciados para o exão 5 do gene *WFS1*.

Dois probandos pertencentes a duas diferentes famílias Portuguesas foram encaminhados para este estudo com a indicação de um fenótipo compatível com Síndrome de Pendred. Para estes indivíduos foi feito o estudo por sequenciação automática dos exões 6 e 10 e das regiões IVS8 e IVS14 do gene *SLC26A4*. Não tendo sido encontrada nenhuma variante, a causa genética do fenótipo apresentado por estes indivíduos não ficou elucidada. No entanto, no estudo da região IVS14, onde parte do exão 15 foi também analisada, houve a suspeita de uma nova mutação, p.Ser552Gly, ter sido encontrada. Este facto causou estranheza, pois esta nova mutação surgira nos dois indivíduos de diferentes famílias em homozigotia. Um novo protocolo de diagnóstico molecular foi desenhado e clarificou-se que se tratou de um artefacto induzido ou na reacção de PCR ou na sequenciação automática, não constituindo uma nova mutação.

O estudo molecular reveste-se de grande importância, quando se considera a probabilidade de uma futura gravidez vir a gerar novamente um descendente com perturbações auditivas. Uma análise cuidada do heredograma familiar, juntamente com a análise genética, constitui-se também de grande utilidade, na medida em que outros membros da família podem vir a beneficiar de um teste semelhante, se a causa genética da surdez for familiar. Um diagnóstico genético atempado pode elucidar a causa e a evolução da surdez (progressividade ou não). Com base nesse resultado, melhores estratégias terapêuticas podem ser equacionadas, contribuindo decisivamente para uma melhor saúde auditiva e uma melhor qualidade de vida dos indivíduos afectados e, também, com menores custos associados.

1. Introduction

1.1. Genetic Hearing Loss

Hearing loss (HL) is the most frequent disability of the human senses (Pollak *et al*, 2007). Clinically relevant sensorineural HL (SNHL) is present in at least 2 per 1000 new born at birth, rising to at least 2,7 per 1000 infants by the age of four (Petersen *et al*, 2012). About 50-70% of hearing impaired children have a monogenic cause for their deafness (Matsunaga, 2009).

Hereditary HL can be subdivided into two types: syndromic or nonsyndromic. The syndromic type is associated with other distinctive clinical features beyond deafness and accounts for 30% of hereditary congenital HL. The nonsyndromic type, in which HL is the only clinical manifestation, represents the other 70% (Matsunaga, 2009).

Regarding nonsyndromic HL, autosomal recessive is the most frequent inheritance pattern, accounting for 75-85% of the cases (Snoeckx *et al*, 2005; Ibrahim *et al*, 2011). It is followed by dominant pattern (12-13%) and X-linked or mitochondrial inheritance that accounts for 2-3% of the cases (Ibrahim *et al*, 2011).

More than 140 genetic *loci* that have been associated to nonsyndromic HL were mapped, being identified to date about 60 genes (Minami *et al*, 2012).

1.2. DFNB1 *locus*

At chromosome 13q11-q12, DFNB1 *locus* comprises *Gap Junction β-2* (*GJB2*) and *Gap Junction β-6* (*GJB6*) genes that encode for connexin 26 (Cx26) and connexin 30 (Cx30), respectively (Rodríguez-Paris *et al*, 2011).

Connexin are transmembrane proteins, being constituted by intracellular amino- and carboxy-termini and four transmembrane domains. Connexons are transmembrane hexameric gap junction hemi-channels composed by six connexin proteins (Martínez *et al*, 2009). Connexons can be classified into homomeric, when they are made up of a single type of connexin or heteromeric, when they are formed by different connexin proteins (Erbe *et al*, 2004; Tang *et al*, 2006). Connexons embedded in the surfaces of adjacent cells can associate and form intercellular channels. Intercellular channels then cluster and form gap junctions. Gap junction channels can be homotypic, when are composed of similiar connexons or heterotypic, when made up of different connexons (Martínez, 2009). Cells connected by gap junctions use the channels to transfer ions and other small molecules across cell membranes (Tang *et al*, 2006).

Cx26 and Cx30 are co-localised and co-expressed in the cochlea, an organ in the inner ear, where they contribute to cochlear homeostasis, since they are thought to provide the recirculation of K^+ ions to the endolymph after hair cell's sound stimulation (fig.1) (Rodríguez-Paris *et al*, 2011; Erbe *et al*, 2004).

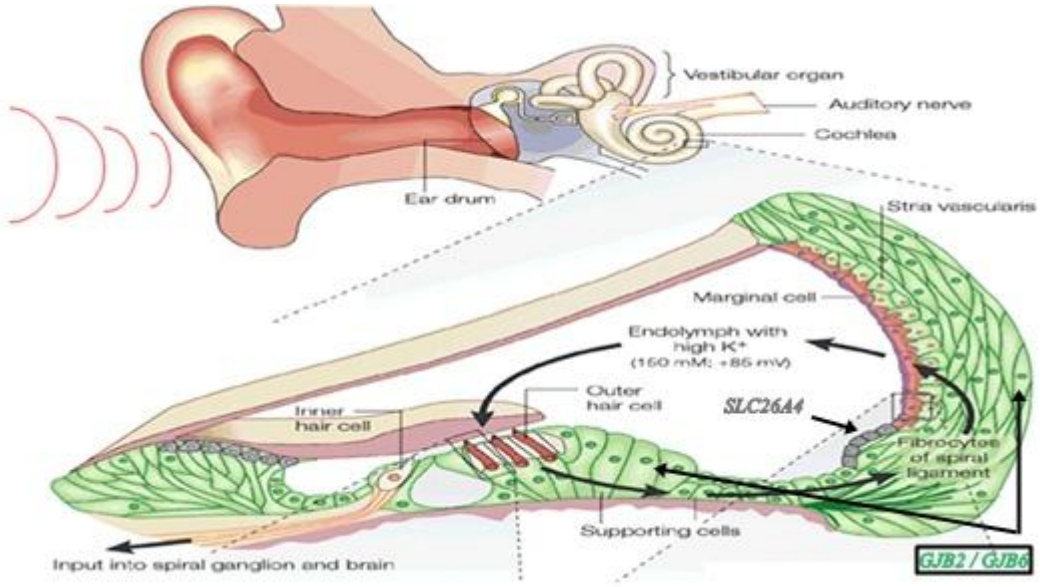

Figure 1 – Schematic representation of the cochlea, showing the location of its different structures. The K^+ recycling pathway is indicated. Adapted from Jentsch, 2000.

1.2.1. *GJB2* gene

GJB2 gene (fig.2) has 5500 bp and a single coding exon, in a total of two exons (Falah *et al*, 2011). More than 200 different pathogenic mutations were identified in this gene that account for 10-40% of congenital HL depending of ethnicity, being the most frequent cause of nonsyndromic autosomal recessive hereditary HL (Tang *et al*, 2006; Falah *et al*, 2011).

The majority of mutated Cx26 alleles among Caucasoids worldwide are due to a deletion of a guanine within a string of six guanines at nucleotide 35 (c.35delG) that results in a premature chain termination (Gasparini *et al*, 2000). Carrier frequency for this allele was estimated to be 2,86% in countries from southern Europe and 1,27% in countries from central and northern Europe (Gasparini *et al*, 2000). In United States Caucasoids c.35delG carrier rate was found to be 2,5% (Green *et al*, 1999). In Portugal, the carrier rate for c.35delG in general population was estimated to be 0,88% (Chora *et al*, 2011). The absence of the c.35delG mutation in other populations, like North American Blacks, Egyptians and Yemenite Jews, for example, could be explained as a consequence of a single origin, somewhere in Europe or in the Middle East (Gasparini *et al*, 2000). The high frequencies observed for c.35delG carriers

in some Caucasoid population suggest either a founder effect or a selective advantage for heterozygotes, or even a combination of both (Gasparini *et al*, 2000). It was hypothesized (Gasparini *et al*, 2000) that since Cx26 is expressed among a variety of tissues, it is conceivable that a putative heterozygote advantage is related to a function of Cx26 in one of these tissues, but clearly not the cochlea. This advantage could be associated to specific functions of gap junctions and could be involved in climate, food, toxic factors and infectious agents, among others, reflecting geographic and cultural different conditions that could influence the frequency of c.35delG allele (Gasparini *et al*, 2000).

GJB2 mutations associated with other specific ethnic groups include c.167delT, with a carrier rate of 7,5% in Ashkenazi Jews and c.235delC, with a carrier rate of 0,5% to 1% in Korean and Japanese populations (Erbe *et al*, 2004).

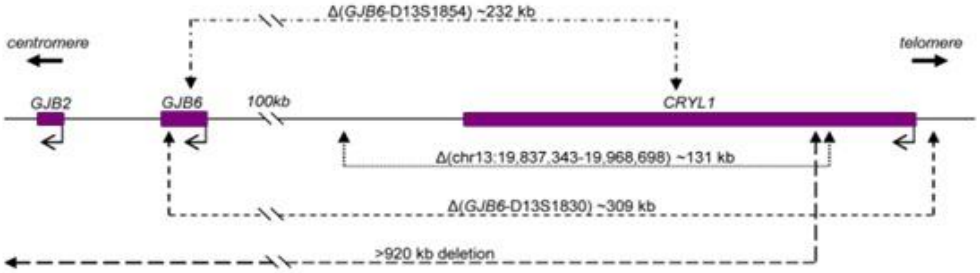

Figure 2 – Map of the region on chromosome 13q11-12 showing *GJB2*, *GJB6* and *CRYL1* genes, relatively to centromere. The regions disrupted by the deletions are indicated. Adapted from Rodríguez-Paris *et al*, 2011.

1.2.2. *GJB6* gene

Whilst many mutations have been described in *GJB2* gene, so far only six mutations with a recessive pattern of inheritance were reported in *GJB6* gene or the region upstream causing deafness (Ballana *et al*, 2012). Four of them are deletions, as shown in figure 2. The most frequent deletions are Δ(*GJB6*-D13S1830) and Δ(*GJB6*-D13S1854), which truncate the *GJB6* gene. Both Δ(*GJB6*-D13S1830) and Δ(*GJB6*-D13S1854) mutations inactivate the *CRYL1* gene and eliminate the sequence between *GJB6* and *CRYL1*, where no additional genes have been reported so far. λ-crystallin is the product of *CRYL1* gene and no contribution of λ-crystallin to DFNB1 HL was found to date. Additionally, no individual carrying either Δ(*GJB6*-D13S1830) or Δ(*GJB6*-D13S1854) was found to present any eye disorder (del Castillo *et al*, 2002; del Castillo *et al*, 2005). The other two deletions affecting *GJB6* gene are private mutations, one of which (>920 kb) deletes both *GJB2* and *GJB6* genes and the other one [(Δ(chr13:19,837,343–19,968,698))] does not affect either gene and is located upstream of *GJB6* (Rodríguez-Paris *et al*, 2011).

1.3. Low Frequency Sensorineural Hearing Loss

Low frequency SNHL (LFSNHL) is an unusual form of HL, in which frequencies at 2000 Hz and below are predominantly affected. Due to maintenance of high frequency hearing, LFSNHL patients retain understanding of speech, although presbycusis or noise exposure may cause high frequency loss later in their life (Bespalova *et al*, 2001). Four *loci*, DFNA1, DFNA6, DFNA14 and DFNA38, are reported as being associated with LFSNHL (Bespalova *et al*, 2001; Young *et al*, 2001).

Most of the families with LFSNHL carry mutations in *WFS1* gene that maps to chromosome 4p16 and has a coding transcript of 2673 bp. *WFS1* gene has 8 exons, of which only the last seven are coding (Gürtler *et al*, 2004; Minami *et al*, 2012). The product of *WFS1* is wolframin, a membrane glycoprotein that is located primarily in the endoplasmic reticulum (ER). Its expression in the human cochlea remains unknown (Gürtler *et al*, 2004). However, its location in the ER suggests a possible role for wolframin in ion homeostasis retained by the canalicular reticulum, a specialized form of ER (Minami *et al*, 2012). Studies of functional analysis suggest that the autosomal dominant pattern of LFSNHL is due to reduced amount of wolframin (Gürtler *et al*, 2004).

Mutations in *WFS1* gene can cause autosomal dominant LFSNHL (DFNA6/14/38) (Bespalova *et al*, 2001; Cryns *et al*, 2003). They are also associated with Wolfram Syndrome (WS), an autosomal recessive syndrome, characterized by insulin-dependent diabetes mellitus and bilateral progressive optic atrophy, usually presenting in childhood or early adult life (Cryns *et al*, 2003; Aloi *et al*, 2012).

1.4. Syndromic Hearing Loss

The syndromic type of hereditary HL includes about 400 syndromes, such as Pendred Syndrome (PS), the most frequent form of syndromic HL. It accounts for 4–10% of the inherited cases (Fraser, 1965; Illum *et al*, 1972; Reardon *et al*, 1997), and is inherited in an autosomal recessive pattern, with an incidence estimated to be as high as 7,5 to 10 in 100000 individuals (Reardon *et al*, 1997; Fraser, 1965).

PS is characterized by SNHL, goiter and a partial defect in iodide organification. These features are generally accompanied by malformations of the inner ear, ranging from enlarged vestibular aqueduct (EVA) to Mondini dysplasia (Pera *et al*, 2008).

The clinical features observed in PS are consequence of biallelic mutations in the *SLC26A4* gene. This gene, containing 21 exons, is located on chromosome 7 (7q22.3-q31.1) and codes for the multifunctional anion exchanger pendrin (fig.3) (Pera *et al*, 2008; Bizhanova, 2010). Pendrin is a 73 kDa membrane protein that belongs to the solute carrier family 26A. It is comprised of 780 amino acids and is predicted to have 12 putative transmembrane domains, being both the amino- and carboxi-termini located on the cytosol (Royaux *et al*, 2000; Gillam *et al*, 2004). Pendrin has a sulfate transporter domain and a sulfate transporter and antisigma factor antagonist (STAS) domain. The last one has been suggested to play a role in nucleotide binding or to interact with other proteins, such as cystic fibrosis conductance regulator (CFTR) (Bizhanova *et al*, 2010).

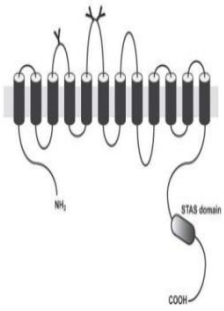

Figure 3 – Schematic representation of pendrin protein.
Figure adapted from Bizhanova, 2010.

1.5. Mitochondrial Hearing Loss

Mitochondria are organelles involved in oxidative metabolism, ion homeostasis, signal transduction and apoptosis. These cellular organelles contribute to pathogenicity by a variety of mechanisms involving maternally inherited diseases due to mutations in mitochondrial (mt) DNA, as well as Mendelian-inherited diseases resulting from mutations in nuclear genes required for mitochondrial function (Raimundo *et al*, 2012). The mtDNA is a double-stranded circular genome composed of 16569 bp that codes for 13 subunits of respiratory complexes (Angulo *et al*, 2011). Normal cochlear function requires a very high rate of ATP production and mtDNA mutations have often been found to cause hearing deficiencies either in syndromic and nonsyndromic forms of HL (Guan *et al*, 2008). Up to now, eight mutations in mtDNA have been clearly associated to HL phenotype (Mitomap, 2012). One of these, the m.1555A>G mutation in *12S rRNA* gene (fig.4) is a common cause of familiar nonsyndromic post-lingual SNHL. The HL phenotype due to this mutation is significantly exacerbated after exposure to aminoglycosides, in particular streptomycin, because this mutation alters the *12S rRNA* gene conformation, making it similar to the bacterial ribosomal gene, thus enhancing aminoglycoside binding and its toxic effects in the ear (Angulo *et al*, 2011).

The m.7445A>G mutation in the *tRNA*$^{Ser(UCN)}$ gene, also called *COX*I (*Cytochrome oxidase* I) gene, is another mtDNA mutation associated with HL (Tekin *et al*, 2003). It occurs in the immediately adjacent nucleotide to the 3'-end of *tRNA*$^{Ser(UCN)}$ and previous studies indicate that it influences the normal processing of the light strand polycistronic mRNA, being the primary defect a significant decrease in serine tRNA level and protein synthesis rate in mitochondria (Guan *et al*, 2008). The m.7511T>C mutation also occurs in *tRNA*$^{Ser(UCN)}$ gene and was associated to nonsyndromic deafness in several families from different ethnic groups (Zheng *et al*, 2012). This mutation is responsible for the substitution of a highly conserved A-U to a G-U base pairing on the 5' side of the acceptor stem of the tRNA$^{Ser(UCN)}$ (Zheng *et al*, 2012). Mutations in the *tRNA*$^{Ser(UCN)}$ gene often occur at high degrees of heteroplasmy or in homoplasmy, indicating a high threshold for pathogenicity (Zheng *et al*, 2012). In figure 5 it is shown a schematic representation of the human mtDNA.

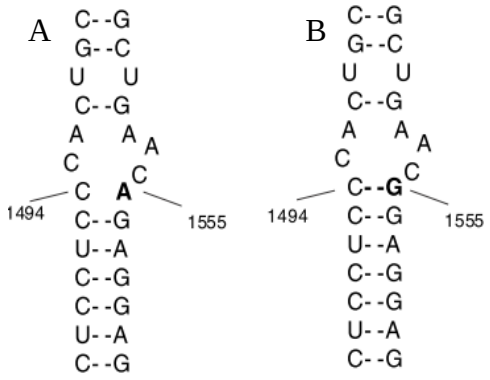

Figure 4 – The coding region of the *12S rRNA* human mtDNA gene. A – wild-type and B – m.1555A>G mutation. Adapted from Böttger, 2010.

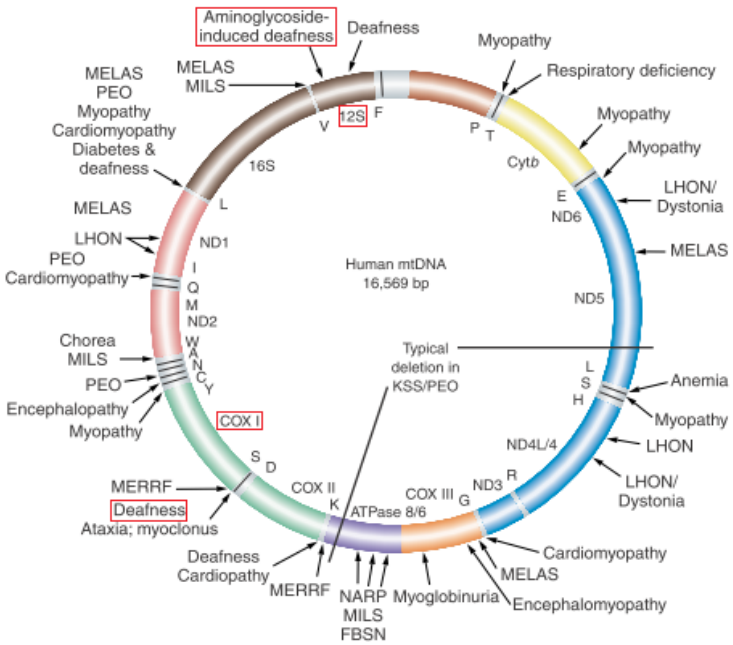

Figure 5 – Schematic representation of human mtDNA showing the *loci* involving in mtDNA associated diseases. The genes *12S* and *COX I* are emphasized with red boxes. Adapted from Griffiths, 2004.

2. Objectives

The main objective of this work was to investigate the genetic etiology of the hearing--impaired phenotype presented by Portuguese patients with SNHL.

The specific objectives were:

- To analyse the coding exon of the *GJB2* gene;
- To perform the screening of the most common deletions in *GJB6* gene;
- To analyse the most frequent mutations in mtDNA associated with deafness;
- To screen the *WFS1* relevant exons in LFSNHL cases;
- To screen the *SLC26A4* relevant exons in PS cases.

3. Materials and Methods

3.1. Sample characterization

A total of 152 Portuguese hearing impaired individuals were selected for this study: 70 patients referred by the ENT Department, CHLO - Hospital Egas Moniz; 81 cochlear implanted individuals referred by the ENT Department, CHC, EPE - Hospital dos Covões, Coimbra, previously analysed for the *GJB2* coding region and identified with negative results (Chora *et al*, 2010) and one individual from ENT Department, CHLN - Hospital Santa Maria. The majority of these patients presented bilateral nonsyndromic SNHL. Two individuals presenting LFSNHL and two cases of syndromic HL were also selected and included in this study. Blood samples were always codified with a blind code, composed of letters (family code) plus numbers (individual code), prior to the DNA extraction and sample manipulation. The personal information of each individual was carefully stored with restricted access. A detailed clinical history of each proband was taken to ensure that the HL was not a result of infection, acoustic trauma, ototoxic drugs or premature birth. For some patients, however, family histories weren't possible to establish. Written informed consent was obtained from all the participants in this study.

3.2. Audiologic examination

The probands and some members of their families underwent otoscopic and audiometric examinations by using age-appropriate methods. Pure tone audiometry was obtained by the clinician's teams of the considered Hospitals listed in 3.1 section in a sound proof room according to current clinical standards. The level of HL was classified following the European Working Group on Genetics of Hearing Impairment as slight (21–40 dB), moderate (41–70 dB), severe (71–95 dB), or profound (>95dB), from an average at 500, 1000, 2000 and 4000 Hz in the better ear.

3.3. Extraction of genomic DNA from blood samples

The protocols for the extraction of genomic DNA from blood samples are presented in section 1 of the supplementary data.

3.4. Amplification by Polymerase Chain Reaction (PCR)

PCR was performed in order to amplify specific studied DNA regions described below. In each PCR reaction a negative control, in which DNA wasn't added, was used to confirm the absence of contaminants in the reagents. All primers used are listed in table 5 of

the supplementary data. PCR reactions were made in Biometra T1 Thermocycler and PCR products were always run in a 1% agarose gel in TBE 0,5x by electrophoresis, as described in section 2 of the supplementary data.

3.5. Screening of c.35delG mutation

PCR-mediated Site Directed Mutagenesis was the method chosen to test for the c.35delG mutation in the *GJB2* gene. It consisted in originating another restriction site for the restriction enzyme *Bsl*I (New England Biolabs) in the presence of the mutation, after amplification with a modified specific primer. The PCR reaction was performed for the test fragment (called δ) and for an internal control of the restriction (called γ). PCR reaction for δ fragment used 22BF 80µM and 22BR 20µM primer pair and is described in detail in table 1 of the supplementary data. The γ fragment was amplified using FP 10µM and RP 10µM primer pair following the conditions listed in detail in table 1 of the supplementary data. PCR programme for the amplification of δ and γ fragments is shown in table 2 of the supplementary data.

3.5.1. Restriction with *Bsl*I

After PCR amplification, the screening of c.35delG mutation was made with the restriction enzyme *Bsl*I as referred. The γ fragment, which also has a recognition site for *Bsl*I, was used as an internal control of the restriction. To maintain DNA concentration in the reaction mix, 8µL of δ product + 8µL of γ product were concentrated during 7 min at 45ºC on DNA SpeedVac. The total volume of each sample restriction and the incubation conditions are indicated in table 4 of the supplementary data.

The δ fragment is not digested in the absence of c.35delG mutation but, in the presence of the mutation, this fragment of 207 bp is digested into 181 + 26 bp. The control γ fragment has 2 overlapping cutting sites. So, when the enzyme cuts one of them, the other disappears, and the 153 bp control fragment can be digested into 97 + 56 bp or 99 + 54 bp.

3.6. Amplification of *GJB2* coding exon

PCR amplification of the coding exon of the *GJB2* gene is listed in detail in table 1 of the supplementary data. Standard PCR programme was used following the conditions shown in table 2 of the supplementary data. The amplified PCR product had 928 bp. *GJB2* PCR products from the probands heterozygous or negative for c.35delG mutation and, when necessary from parents and siblings, were automatically sequenced after purification with

JETQUICK PCR Product Purification Spin Kit (Genomed) according to manufacturer's instructions.

3.7. Screening for *GJB6* deletions

The *GJB6* deletions del(*GJB6*-D13S1830) and del(*GJB6*-D13S1854) can be detected by multiplex PCR (del Castillo, 2005). Three different primer pairs were used, which amplified according to their proximity, depending on the presence of the deletions. The Cx30Ex1A and Cx30Ex1B primer pair amplifies a 333 bp fragment in the presence of a wild--type allele. The Del BK1 and Del BK2 primer pair amplifies a 564 bp fragment in the presence of a mutated allele with del(*GJB6*-D13S1854) deletion. The GJB6-1R and BKR-1 primer pair amplifies a 460 bp fragment in presence of del(*GJB6*-D13S1830). PCR amplification of the *GJB6* screened deletions followed the conditions listed in detail in table 1 of the supplementary data. All primers were used at 10µM. The PCR program included a touchdown step, as described in table 3 of the supplementary data.

3.8. Mitochondrial DNA analysis

Screening of three known mutations: m.1555A>G, m.7445A>G and m.7511T>C was performed for all those cases compatible with maternal inheritance and which cause of deafness remained to be elucidated.

3.8.1. Detection of m.1555A>G mutation

This mutation can be detected by PCR with specific primers that amplify the *12S rRNA* gene, followed by digestion with the restriction enzyme *Hae*III (Promega). PCR mix is described in detail in table 1 of the supplementary data. The standard PCR program is listed in table 2 of the supplementary data. The PCR product had 339 bp.

The restriction enzyme *Hae*III always recognizes a cleavage site in the mentioned amplified PCR area and cuts originating two fragments of 218 bp + 121 bp. The m.1555A>G mutation creates one other cleavage site and thus the enzyme cuts twice, originating three fragments of 218 bp + 91 bp + 30 bp. The restriction mix and the incubation conditions are indicated in table 4 of the supplementary data.

3.8.2. Detection of m.7445A>G and m.7511T>C mutations

The m.7445A>G and m.7511T>C mutations can be detected by PCR with specific primers that amplify part of the the $tRNA^{Ser(UCN)}$ gene, followed by digestion with the restriction enzyme *Xba*I (Promega) and *Mbo*II (New England Biolabs), respectively. PCR mix

is described in detail in table 1 of the supplementary data. The standard PCR program is also listed in table 2 of the supplementary data. The PCR product had 215 bp.

The m.7445A>G mutation is recognized by the loss of a cleavage site for the *Xba*I restriction enzyme, as compared to the wild-type. So, in the wild-type, *Xba*I always has a recognition site and cleaves, originating two fragments of 166 bp + 49 bp. The restriction mix and the incubation conditions are shown in table 4 of the supplementary data.

*Mbo*II restriction enzyme recognizes a cleavage site only in the wild-type. This enzyme cuts in the absence of the mutation m.7511T>C, originating two fragments of 175 bp + 40 bp. The restriction mix and the incubation conditions are indicated in table 4 of the supplementary data.

3.9. Screening of *WFS1* exons 4, 5, 6 and 8

For those individuals presenting LFSNHL, automatic sequencing of exons 4, 5, 6 and 8 of *WFS1* gene was performed. PCR amplification mix is listed in table 1 of the supplementary data. The standard PCR programme is also shown in table 2 of the supplementary data. Exon 8 was amplified in two PCR reactions (designed in this study by 8a and 8b) because its large size is not compatible with good quality of automatic sequencing. *WFS1* PCR products of exons 4 (222 bp), 5 (225 bp), 8a (872 bp) and 8b (1096 bp) were automatically sequenced after purification with JETQUICK PCR Product Purification Spin Kit (Genomed) according to manufacturer's instructions. PCR products of exon 6 (186 bp) were automatically sequenced after purification with Zymoclean™ Gel DNA Recovery Kit (Zymoresearch) following manufacturer's instructions.

One hundred normal hearing Portuguese controls were sequenced for the exon 5 of *WFS1* gene after purification with JETQUICK PCR Product Purification Spin Kit (Genomed).

3.10. Screening of *SLC26A4* exon 6, exon 10, IVS8 and IVS14 regions

For those individuals presenting medical diagnostic of PS, automatic sequencing of exons 6 and exon 10, as well as IVS8 and IVS14 regions of *SLC26A4* gene, was performed. PCR amplification of each region of *SLC26A4* gene is shown in detail in table 1 of the supplementary data. In table 2 of the supplementary data is listed the standard PCR programme used. *SLC26A4* PCR products of exons 6 (459 bp), exon 10 (606 bp), IVS8 (499 bp) region and IVS14 (185 bp) region were automatically sequenced after purification with JETQUICK PCR Product Purification Spin Kit (Genomed) according to manufacturer's instructions.

Results and Discussion

4.1. *GJB2* gene analysis

GJB2 analysis was the first step performed to determine the genetic cause of HL in probands from 70 families recently referred to the Deafness Group of the BioFIG. This analysis started by screening c.35delG mutation in probands, since it represents the most frequent mutation in Cx26 gene in Caucasoid population (Zelante *et al*, 1997). Its detection was done for all the probands prior to the analysis of the *GJB2* coding exon, performed for the patients shown to present only one or no c.35delG mutation.

4.2. Screening of the c.35delG mutation

After restriction analysis for c.35delG mutation, seven of the 70 probands (10%) were found to be either heterozygous (7,1%) or homozygous (2,9%) for this mutation. An image of a c.35delG restriction analysis agarose gel is shown in figure 6.

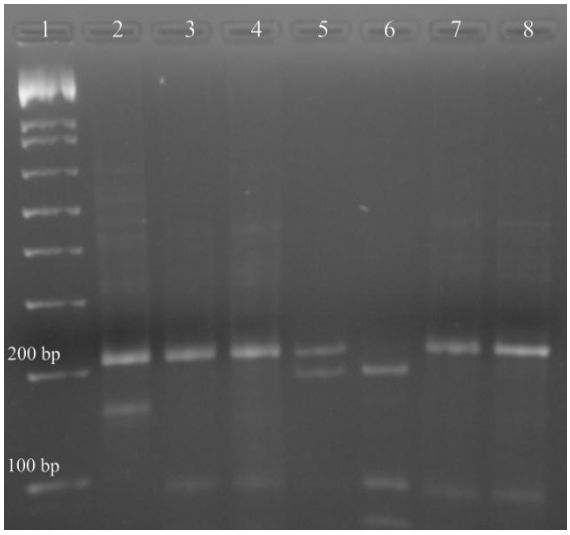

Figure 6 – Agarose gel from a c.35delG restriction analysis. Lanes: 1 – 1kb DNA plus ladder (Invitrogen); 2 – non-digested control; 3-4 and 7-8 – wild-type samples for c.35delG; 5 – heterozygous sample for c.35delG; 6 – homozygous sample for c.35delG.

One of the heterozygous proband was later shown, after sequencing of *GJB2* coding exon (section 4.3.11), to have the c.35delG mutation in compound heterozygosity with another *GJB2* mutation, p.Trp172X.

So, in the present study c.35delG mutation explains the HL in three of the 70 patients (4,3%): two homozygous and one in compound heterozygosity with p.Trp172X mutation. The mutation c.35delG has been reported as the most common mutated allele found among Mediterranean HL families (Zelante *et al*, 1997). The results of this study agree with this finding, since c.35delG was the most common allele found among the studied probands, representing 9 alleles in a total of 140 (6,4%).

4.3. Automatic sequencing of the *GJB2* coding exon

Automatic sequencing of coding exon of *GJB2* gene was performed for the probands from the remaining 68 families, heterozygous and negative for c.35delG mutation. When a mutation/variant was found, the parents and siblings DNA, if available, was also sequenced.

Fifty-six of the 68 sequenced probands (82,3%) were shown to be wild-type for *GJB2* alleles. In 12 probands (17,6%, 12/68), Cx26 variants were found and in 6 families (8,8%, 6/68) the diagnosis for HL could be establish. The relevant results are described below, for each family concerned.

4.3.1. Case KQ

A single individual presenting SNHL was available for study. His *GJB2* genotype was found to be [=] + [p.Lys224Gln]. The p.Lys224Gln (fig.1, supplementary data) mutation occurs at the intracellular C-terminal domain, which is involved in pH gating of Cx26 channel (Kelley *et al*, 1998). Due to the fact that this is a recessive mutation, no conclusion could be drawn as regards the etiology of the HL in this individual.

4.3.2. Family MV

This family is composed of two siblings presenting moderate SNHL and the normal hearing parents. The *GJB2* genotype was found to be [p.Met34Thr] + [p.Val95Met] for one of the siblings (fig.7, II:2) and [=] + [p.Val95Met] for the other one (fig.7, II:1). The p.Val95Met variant (fig.2, supplementary data), occurring in intracellular loop (Martinez *et al*, 2009), was inherited from the mother who presents the genotype [=] + [p.Val95Met]. The father was found to be a p.Met34Thr (fig.3, supplementary data) carrier.

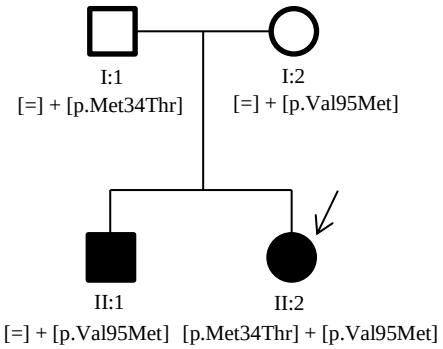

Figure 7 – Heredogram of Family MV.

The p.Val95Met is a controversial variant. Studies by Wang *et al* (2003) and Zhang *et al* (2005), shows that the ionic permeability of p.Val95Met-Cx26 channels is not affected. However, the same study by Zhang, and Beltramello *et al* (2005) conclude that the permeability to large molecules is impaired. The p.Val95Met was found in compound heterozygosity with other Cx26 mutations (c.35delG, p.Met34Thr and p.Leu90Pro) in several HL patients (Cryns *et al*, 2004; Snoeckx *et al*, 2005).

The p.Met34Thr variant, occuring in transmembranar domain 1 of Cx26, is a controversial variant, described as a recessive mutation by some authors (Snoeckx *et al*, 2005) and as a benign polymorphism by other authors (Feldmann *et al*, 2004). Data in literature are quite contradictory for p.Met34Thr variant, with some studies concluding that p.Met34Thr does not interfere with efficient formation of stable connexons (Oshima, 2003) and other studies concluding that the defect observed in the function of mutant protein is due to a shift in the gating response of the channel, resulting in a low conductance and permeability (Skerrett *et al*, 2004).

Due to the fact of both mutations being controversial and the affected son having the same genotype as his hearing mother, we cannot assume that the HL in this family is associated to the *GJB2* genotypes observed.

4.3.3. Family NI

This family is composed of three normal hearing siblings (fig.8 II:1, II:2 and II:3) and their parents (fig.8 I:1 and I:2), having other relatives with deafness. The siblings and their mother presented the [=] + [p.Arg127His] *GJB2* genotype. The variation p.Arg127His (fig.4, supplementary data) is localised to the intracellular loop 2 of Cx26 (Martinez *et al*, 2009).

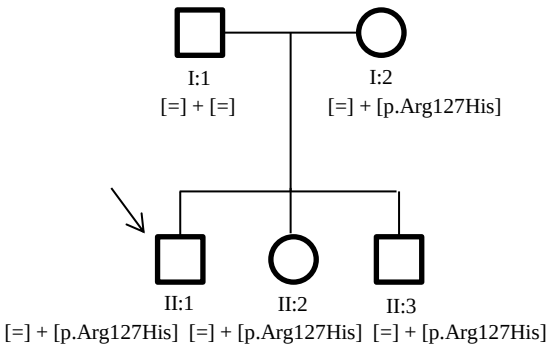

Figure 8 – Heredogram of Family NI.

The p.Arg127His variant was found to greatly impair the ability of Cx26 protein to form functional gap junctions, by reducing the channel permeability (Wang *et al*, 2003). However, other study reported homozygous individuals for p.Arg127His that harboured normal hearing condition (Roux *et al*, 2004). Moreover, additional data show that frequency of p.Arg127His between normal hearing individuals and HL patients is not statistically different, demonstrate that p.Arg127His is effectively a polymorphism (RamShankar *et al*, 2003).

Mutation p.Arg127His is still controversial, but it is possible that it exerts a pathogenic effect depending on the environment and genetic background (Matos *et al*, 2010). In this family all the affected individuals carry the p.Arg127His. They may be coincidental carriers, but it is also possible that a noncoding *GJB2*/*GJB6* mutation *in trans* with p.Arg127His might be present in these family members, thus accounting for the HL.

4.3.4. Case NP

A single individual presenting severe bilateral SNHL was available for study. His *GJB2* genotype was found to be [=] + [p.Gly160Ser]. The p.Gly160Ser mutation (fig.5, supplementary data) localizes to the extracellular loop 2 of Cx26 and is described as a polymorphism (Tang *et al*, 2006). These data are not sufficient for the establishment of the HL genetic cause in this individual.

4.3.5. Case NT

A single individual presenting familiar cases of deafness was available for study. The +785 A>T and +792 C>T variations were detected on *GJB2* gene in heterozygosity. Both variations were first reported by Tang and his collaborators (Tang *et al*, 2006), and were found at high frequence among the african-american population. This results aren't strange considering the similarity of the genetic background of Portuguese population in relation with the African population. No genetic cause was yet determined for the HL in this individual.

4.3.6. Case NY

One individual presenting bilateral severe nonsyndromic SNHL was available for study. His *GJB2* genotype was found to be compound heterozygous for the variants [p.Met34Thr]+[p.Ile140Ser] (Gonçalves *et al*, 2012, A). This individual has a sister with HL, of unknown genotype since she was not available for study. Mutation p.Ile140Ser (fig.6, supplementary data), localised to transmembrane domain 3 of Cx26, was first reported in 2005 (Snoeckx *et al*, 2005) as being recessive, and is not enough to explain the HL of this individual (Martinez *et al*, 2009; Snoeckx *et al*, 2005). Taking into account the role of p.Met34Thr variant described in 4.3.2 section, the conjugation of p.Ile140Ser with p.Met34Thr variant may justify the HL phenotype observed.

4.3.7. Family OB

This family is composed of two hearing-impaired siblings (fig.9) with moderate and severe nonsyndromic SNHL (fig.10), respectively aged 11 (fig.9 II:2, fig.10D) and 15 (fig.9

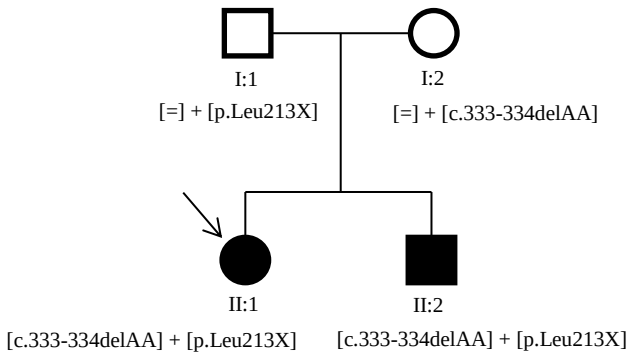

Figure 9 – Heredogram of Family OB.

II:1, fig.10C) (proband) years and their normal hearing parents (fig.9 I:1 and I:2 and fig.10 A and B, respectively). Both siblings shared the same *GJB2* genotype [c.333-334delAA] + [p.Leu213X], being the deletion c.333-334delAA inherited from the mother. The p.Leu213X is a **novel variant**, identified for the first time in this study and is present in both siblings (Gonçalves *et al*, 2012, *accepted paper*, B). This mutation was later found in heterozygosity in their father.

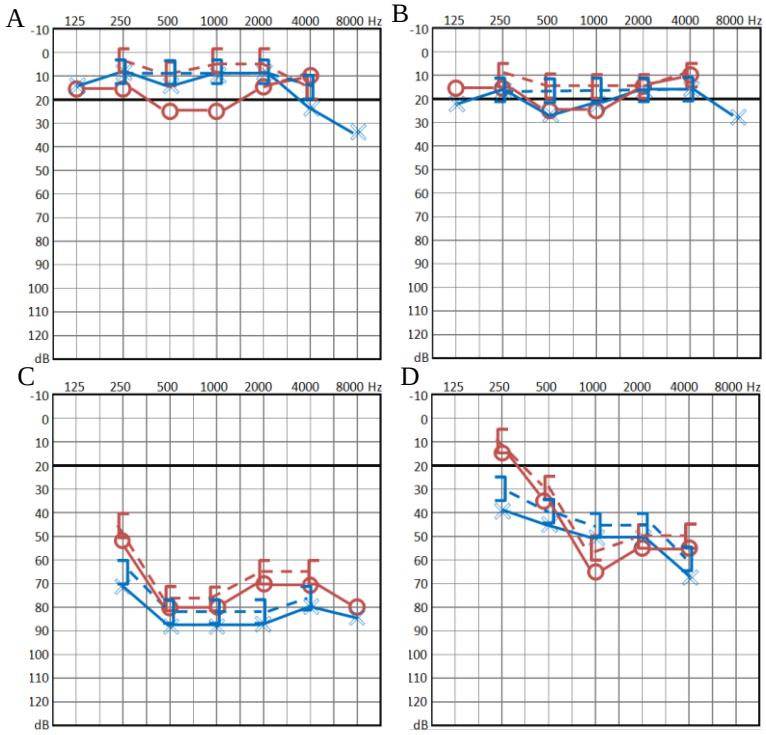

Figure 10 – Representative audiograms of Family OB. Individual I:1 (A), individual I:2 (B), individual II:1 (C) and individual II:2 (D) showing pure-tone audiometry results for air conduction bilaterally. Circles in blue represent the right ear; crosses in red represent the left ear.

The recessive deletion c.333-334delAA (fig.11,B) (Kelley *et al*, 1998), localised to the intracellular loop of Cx26, causes a *frameshift* which results in chain termination after an additional novel amino acid, truncating about half of the protein. It is the first time that this mutation is reported in Portuguese HL patients.

The novel recessive mutation p.Leu213X (fig.11,D) creates a premature STOP codon by changing the codon 213 (TTG) which codes for a leucine, to a STOP codon (TAG). This mutation leads to the deletion of the last 14 amino acids of the protein. The Leu213 amino acid residue is localised to the C-terminus domain of the Cx26 protein. The residues in the intracellular loop region and C-terminus are very different among different connexins and are hence thought to be responsible for regulation, thus imparting unique properties to the various connexin molecules (Mani *et al*, 2009). The p.Leu213X mutation wasn't present in 480

Portuguese individuals from a random control sample that were previously sequenced for *GJB2* gene (Chora *et al*, 2011). Future functional studies are necessary to characterize this novel mutation p.Leu213X.

It can be conclude that the etiology of deafness in these siblings is most likely due to the *GJB2* genotype involving the c.333-334delAA deletion and the novel p.Leu213X mutation in compound heterozygosity.

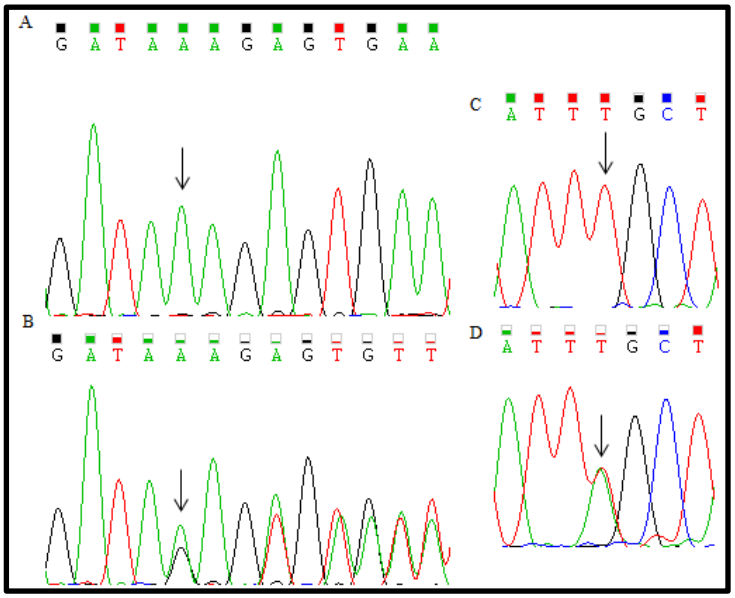

Figure 11 – Electrophoretograms of c.333-334delAA and p.Leu213X mutations, respectively. A and B – wild-type and c.333-334delAA mutation in heterozygosity, respectively. C and D – wild-type and p.Leu213X mutation in heterozygosity, respectively.

4.3.8. Family OF

This gypsy family is composed of five individuals, three siblings and two related individuals. A previously described variation in the nucleotide position -40 (from the ATG initiation codon) was found in all the individuals of this family. This variation, c.-22-18T>A, was found in homozygosity in the proband and his affected siblings. It was also found in heterozygosity in the two relatives. This variation was reported previously (Tang *et al*, 2006), but in heterozygosity. So, to our knowledge, this is the first time that this variation is reported in homozygosity.

One of the two relatives carrying the c.-22-18T>A variation, was also found to be a carrier for the *GJB2* mutation p.Trp24X. The role of this mutation will be described next in 4.3.9 section.

The second relative was found to present, besides the c.-22-18T>A heterozygosity, the p.Phe83Leu polymorphism. The polymorphism p.Phe83Leu localised to the transmembranar

domain 2 of Cx26 and was previously reported as a non-pathogenic variation (Bruzzone *et al*, 2002). The genetic cause of the HL wasn't yet elucidated for this family.

4.3.9. Family OH

This gypsy family is composed of two siblings (fig.12 II:1 and II:2), presenting profound bilateral SNHL phenotype. Their *GJB2* genotype was found to be the same [p.Trp24X] + [p.Trp24X]. The nonsense recessive mutation p.Trp24X (fig.7, supplementary data), localised to the transmembranar domain 1, truncates Cx26 protein, leading to the formation of 24 amino acids, instead of the normal 226 polypeptide.

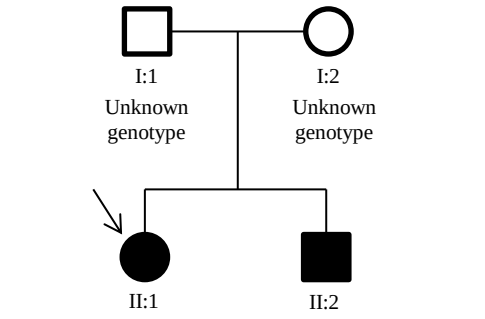

Figure 12 – Heredogram of Family OH.

According to literature (Minárik *et al*, 2003), in individuals homozygous for p.Trp24X, no functional Cx26 channels are present in the cochlea, which has a negative impact on K^+ recycling to the endolymph, thus resulting a week or null physiological response to sound stimuli (Minárik *et al*, 2003). The p.Trp24X mutation is found in high frequency among gypsy populations, namely Spanish Romani, Slovak Romani and Indian, with recent data pointing to the specificity of this mutation to Indian population (Álvarez *et al*, 2005; Minárik *et al*, 2003; Padma *et al*, 2009), since it was found to account for 73% of all pathogenic mutations in *GJB2* gene (Mani *et al*, 2009) in this population. Its higher frequency (2,4%) in Indian population leads to the question if the high carrier frequency of p.Trp24X mutation is also associated with a heterozygote advantage (Mani *et al*, 2009). Because of its premature STOP codon, p.Trp24X is not expected to form a protein, but a study demonstrate an apparently full-length protein but with defective cellular localisation, being retained in cytoplasm (Mani *et al*, 2009). This evidence points for the need of performing functional studies even with mutations leading to premature STOP codon. All this data lead us to conclude that the homozygosity of p.Trp24X in both siblings is certainly the cause of their HL phenotype.

4.3.10. Family OI

This family present two daughters (fig.13 II:1 and II:2) with severe and moderate to severe nonsyndromic bilateral SNHL, respectively. The sisters were found to carry two different coding variants, and thus having the same *GJB2* genotype [p.Met34Thr] +

[p.Arg184Pro], (Gonçalves *et al*, 2012, C). Their hearing father (fig.13 I:1) is a p.Arg184Pro carrier, while their hearing mother (fig.12 I:2) is a p.Met34Thr carrier.

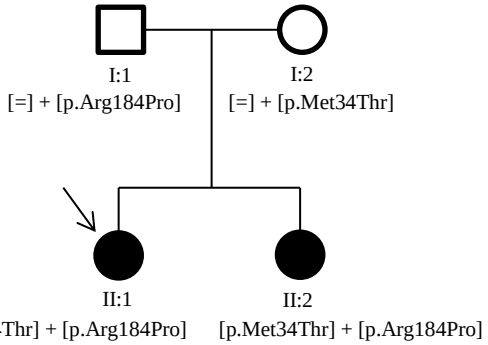

Figure 13 – Heredogram of Family OI.

Mutation p.Arg184Pro (fig.8, supplementary data) was previously reported as pathogenic and with a recessive pattern of transmission (Martinez *et al*, 2009; Bruzzone *et al*, 2002). This mutation occurs at extracellular loop 2 of Cx26 and prevents not only the traffic of the protein to the membrane but also its correct oligomerization (Martinez *et al*, 2009; Bruzzone *et al*, 2002), thus resulting that no gap junction channels are formed.

Because p.Arg184Pro mutation is a recessive one, its presence in only one allele in both sisters does not explain by itself the HL of them. However, its association with p.Met34Thr leads us to assume that the genotype [p.Met34Thr] + [p.Arg184Pro] could be the cause of the deafness observed in both individuals.

The data concerning this family and case NY, above described (section 4.3.6), thus point to a possible pathogenic role of p.Met34Thr variant (discussed in section 4.3.2), either as a recessive allele or as a polymorphism which increases the severity of the phenotype of a recessive monoallelic mutation.

4.3.11. Case OM

This single individual presented profound SNHL was previously found to be heterozygous for c.35delG mutation in section 4.2. Due to this heterozygosity, this proband's DNA was sequenced for *GJB2* gene and his genotype was found to be [c.35delG] + [p.Trp172X]. The p.Trp172X mutation (fig.9, supplementary data) is localised to the extracellular loop 2 of Cx26 protein and leads to a premature STOP codon. This mutation was only reported twice. It was reported for the first time on a Brazilian patient present in an homozygous state (Pfeilsticker *et al*, 2004) and the second time in a multicentre study from Snoeckx and collaborators (Snoeckx *et al*, 2005).

Due to the presence of c.35delG mutation that leads to a *frameshift* causing a premature chain termination and its conjugation with p.Trp172X mutation that causes a premature STOP codon, the genetic cause for the profound HL phenotype presented by this proband can be due to the compound heterozygosity [c.35delG] + [p.Trp172X].

4.3.12. Family PD

This family is composed of an eight year old proband (fig.14 II:1) presenting moderate bilateral SNHL and his normal hearing parents. Proband's *GJB2* genotype was found to be [p.Val37Ile] + [p.Asn206Ser], having inherited from the father the mutation p.Val37Ile and from the mother (fig.14 I:2) the p.Asn206Ser mutation.

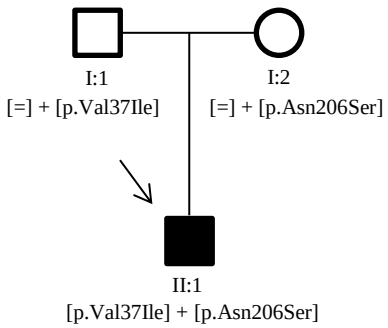

Figure 14 – Heredogram of Family PD.

The p.Val37Ile mutation (fig.10, supplementary data) is localised to the transmembranar domain 1 of Cx26 protein (Martinez, 2009) and its pathogenic role is still controversial. This mutation was first described as being a polymorphism, because of its high carrier frequency in the general population (Kelley *et al*, 1998). However, several studies have proposed that p.Val37Ile is in fact pathogenic with a reduced penetrance pattern because it was found overexpressed among individuals with mild to moderate HL (Snoeckx *et al*, 2005; Pollak *et al*, 2007; Ma *et al*, 2010). Pollak also hypothesised that p.Val37Ile mutation can be related to relatively late onset and progression of HL (Pollak *et al*, 2007).

The p.Asn206Ser mutation (fig.11, supplementary data) is located in transmembranar domain 4 of Cx26 (Martinez *et al*, 2009), and was associated with a higher proportion of moderate or mild HL (Marlin *et al*, 2005). This mutation can lead to the formation of functional channels with levels of conductance similar to those observed in wild-type Cx26 channels, but with small differences in gating or permeability (Martinez *et al*, 2009).

Having into consideration the above data on both mutations, the phenotype presented by this proband may be considered as the result of the genotype [p.Val37Ile] + [p.Asn206Ser] observed.

4.4. Summing up on *GJB2* screening

The results described above, whereupon 25,7% of the studied families (18/70) were found to harbour mutations in *GJB2* gene are according to previous determined ones, in which the DFNB1 was a likely cause to explain 20,8% of the HL phenotype in the Portuguese population (Matos, 2012). In the present study, we identify two families with HL due to c.35delG, and 6 other families where HL is due to other genotypes identified in *GJB2* gene, accounting for a total of 11,4% of elucidated cases. Figure 15 shows the Cx26 variants found

during this work, with special emphasis for the novel mutation, p.Leu213X, reported here for the first time.

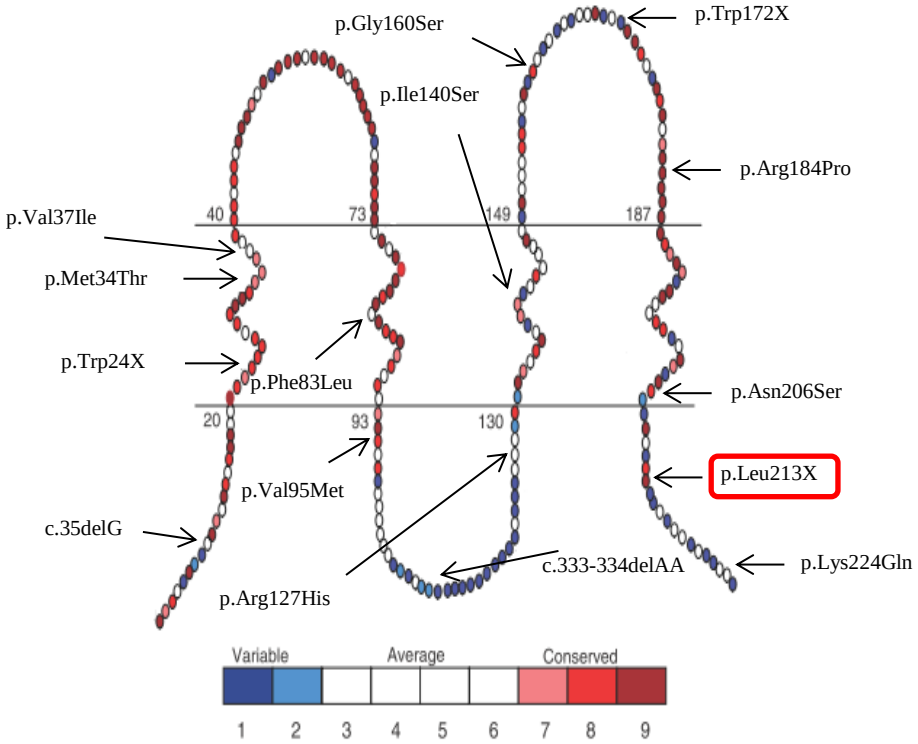

Figure 15 – Schematic representation of Cx26 protein. The arrows indicate the mutations and variants identified in this study and the novel mutation p.Leu213X is shown in red box. The extent of amino acid conservation is colour-coded, with residues shown in blue (1-2) not conserved and rapidly evolving. Residues in white (3-6) show an average degree of conservation and residues in red (7-9) are highly conserved and are slowly evolving. The degree of conservation of the polymorphic residues was analysed using ConSeq, the sequence only variant of Rate4Site, an algorithmic tool for the identification of functional regions in proteins. Adapted from Mani *et al*, 2009.

Sixty genes were mapped until now and more than 100 *loci* were identified as being responsible for nonsyndromic HL cases (Minami *et al*, 2012). So, it can always be considered the possibility that the genetic cause of HL for the remaining families could due to mutations in other genes.

4.5. *GJB6* analysis

None of the 62 analysed individuals presented any of the *GJB6* deletions (fig.16). These data point to a low prevalence of *GJB6* deletions in Portugal, corroborating previous results (Teixeira *et al*, 2006; Chora *et al*, 2010). On the contrary, in Spain, France, United Kingdom, Israel and Brazil the prevalence of *GJB6* deletions is very high, where del(*GJB6*-D13S1830) allele accounts for 5,9-9,7% of all DFNB1 alleles (del Castillo *et al*, 2003). Also the del(*GJB6*-D13S1854) mutation has a frequency of 2,2% in Spain, being part of the five most common DFNB1 alleles in that country (del Castillo *et al*, 2005).

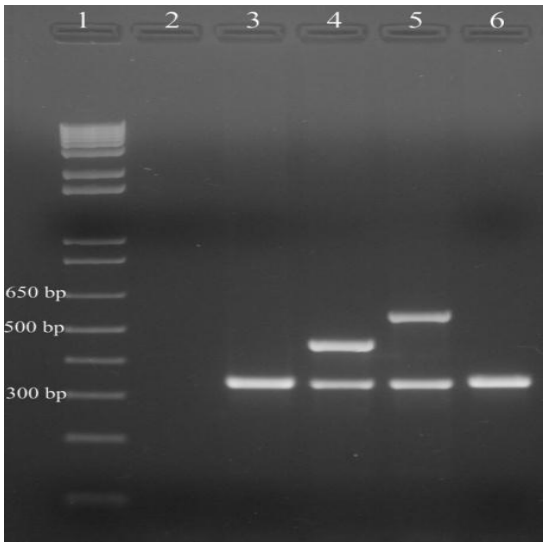

Figure 16 – Agarose gel from a *GJB6* multiplex PCR. Lanes: 1– 1 kb DNA plus ladder (Invitrogen); 2– PCR's negative control; 3 and 6– negative samples for both *GJB6* deletions; 4– heterozygous control for del(*GJB6*-D13S1830); 5– heterozygous control for del(*GJB6*-D13S1854).

4.6. Mitochondrial DNA analysis

Three mtDNA mutations were screened by enzymatic restriction: m.1555A>G, m.7445A>G and m.7511T>C. In addition to the 62 families screened in the previous steps described, more 81 individuals also negative for *GJB2*/*GJB6* were screened for mtDNA mutations.

Only one family (family NL) presented the mtDNA mutation m.1555A>G (fig.17). The mutation was first detected in the proband and his sister and was later confirmed in their mother (fig.18).

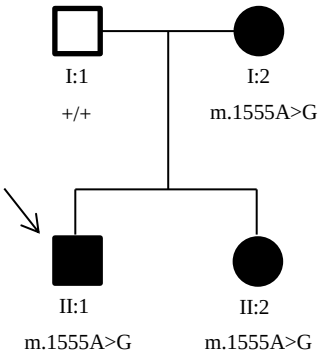

Figure 17 – Heredogram of Family NL.

The HL phenotype caused by m.1555A>G is irreversible, leading to death of critical cells in the inner ear, by a pathogenic cell-death pathway yet unknown (Prezant *et al*, 1993; Raimundo *et al*, 2012). The pathology is caused by oxidative phosporilation due to alterations in mitochondrial ribosome function and translation, triggered by m.1555A>G mutation (Guan *et al*, 1996; Raimundo *et al*, 2012). Raimundo and his colleagues concluded that individuals harbouring the m.1555A>G mutation would be more prone to eventual loss of irreplaceble

inner ear cells, due to mitochondrial stress pathway to proapoptotic nuclear transcription factor E2F1, thus explain the irreversible HL in a spontaneous way or as a function of age, noise or aminoglycosides (Raimundo *et al*, 2012). The presence of m.1555A>G mutation in heteroplasmy in these individuals was found to be the cause of their HL phenotype.

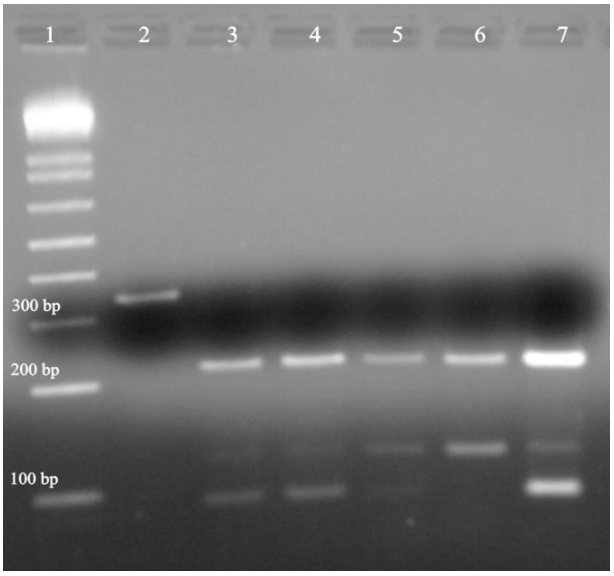

Figure 18 – Agarose gel from the restriction analysis of m.1555A>G mutation in mtDNA of Family NL. Lanes: 1 - 1 kb DNA plus ladder (Invitrogen); 2 – non-digested control; 3-4 – restriction analysis for individual II:1 and II:2, respectively; 5 – heteroplasmic control for m.1555A>G mutation; 6-7 – restriction analysis for individual I:1 and I:2, respectively.

No individual was found carrying m.7445A>G or m.7511T>C mutations in mtDNA. Images of electrophoresis for restriction analysis of both m.7445A>G and m.7511T>C mutations are shown on figure 12, A and B, respectively, of the supplementary data.

4.7. *WFS1* analysis

WFS1 gene analysis was performed for individuals of two families, both presented LFSNHL. Exons 4, 5, 6 and 8 were chosen for analysis by automatic sequencing since these are the *WFS1* regions where most pathogenic mutations were found to date (Cryns *et al*, 2002; Gürtler *et al*, 2005; Fukuoka *et al*, 2007).

4.7.1. Case BK

One single individual presenting LFSHNL phenotype was available for study (fig.19A). A **novel mutation,** p.Asp171Asn (Gonçalves *et al*, 2012, D), was found in heterozygosity in exon 5 of *WFS1* gene (fig.19 C). This mutation alters the 511 residue of wolframin (fig.21), by changing an aspartic acid to an asparagine. The aspartic acid is an acid polar negatively charged amino acid frequently involved in protein active or binding sites. Its substitution for a neutral polar amino acid as asparagine may cause some defect in the interaction with, for example, positively-charged non-protein atoms (Barnes *et al*, 2003). The p.Asp171Asn mutation is located close to the N-terminus domain of the protein, situated in the extracytoplasmic region (Komatsu *et al*, 2002).

One hundred normal hearing Portuguese controls were sequenced for the exon 5 of *WFS1* gene and in none allele the p.Asp171Asn mutation was found. In this specific gene many known pathogenic mutations generally occur in heterozygous dominant pattern, conferring HL (Bespalova *et al*, 2001). However, it cannot be established if this mutation is recessive or dominant, since members of other generations of this family weren't available to be tested and no information on their phenotype is known.

One intronic variant, IVS4 – 9 A>G, previously reported (Van Den Ouweland *et al*, 2003), was identified in homozygosity in this patient (fig.13, supplementary data). In addition, polymorphism p.Arg228Arg was also found in exon 6, in homozygous state (fig.14, supplementary data).

The auditory phenotype of this patient might probably be due to the novel mutation p.Asp171Asn. Its functional characterization should be performed in order to assess its effect on the protein and to clarify in which way the introduced change in the residue 171 leads to low frequency HL.

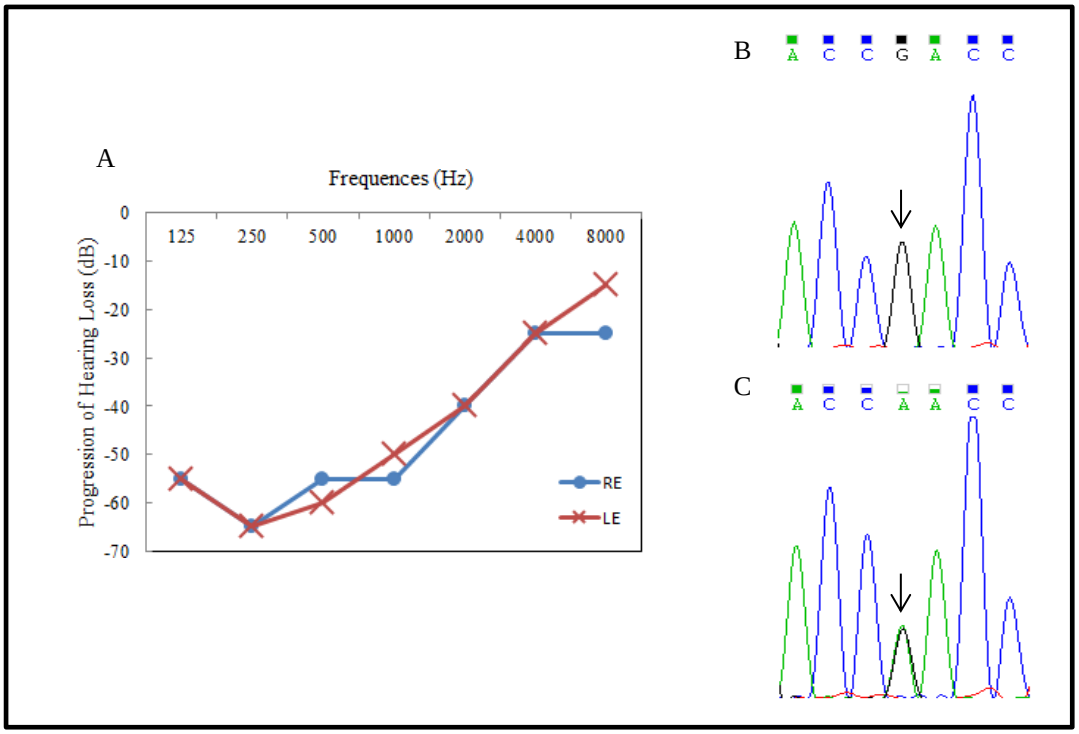

Figure 19 – Pure-tone audiogram of individual BK1 and electrophoretogram of p.Asp171Asn mutation. A – Pure-tone audiogram with circles in blue represent the right ear (RE) and crosses in red represent the left ear (LE). Electrophoretogram showing: B – wild-type ; C – p.Asp171Asn mutation in heterozygosity.

4.7.2. Family PF

This family was composed of mother (fig.20 I:2) and daughter (fig.20 II:1). No information was available regarding the father. Both mother and daughter were affected only in low frequencies with an indication of a dominant pattern of inheritance. The mother was found to exhibit a common polymorphism in exon 6, p.Arg228Arg, in heterozygosity (fig.15, supplementary data), which doesn't explain her HL phenotype (Fujikawa *et al*, 2009). The mother also presented in exon 8 the p.Ala602Val mutation (fig.16, supplementary data) in heterozygosity. Previous studies indicate that this mutation is more frequent in patients with mental disorders than in controls, being present, for example, in schizophrenic patients (Torres *et al*, 2001). However, results from other study do not support the pathophysiological significance of wolframin in bipolar disorders (Kato *et al*, 2003). Polymorphism p.Val333Ile (fig.17, supplementary data), present in exon 8, was found in heterozygosity in mother and daughter but doesn't explain their HL. So, the genetic cause for the LFSNHL phenotype observed in this family couldn't be determined in this study.

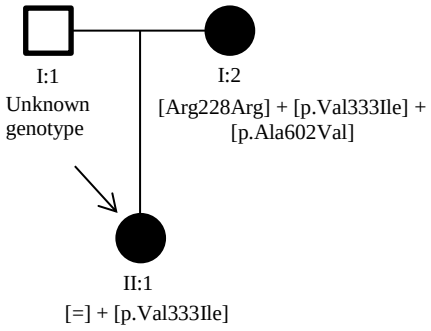

Figure 20 – Heredogram of Family PF.

The variations found in this study are shown in a schematic representation of wolframin, in figure 21, with special emphasis in the novel mutation, p.Asp171Asn, reported for the first time in this study.

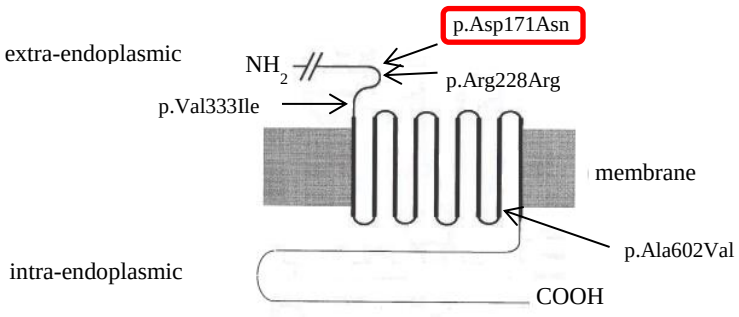

Figure 21 – Schematic representation of wolframin protein. Arrows indicate mutations and variants found in this study and the novel mutation p.Asp171Asn is shown in red box. Figure adapted from Komatsu, 2002.

4.8. *SLC26A4* analysis

SLC26A4 gene analysis was made for individuals belonging to two unrelated Portuguese families diagnosed with PS. Screening of exons 6 and 10, and region IVS8 was performed because these are the regions presenting higher frequency of pathogenic mutations in the Caucasoid population (Tsukamoto *et al*, 2003). IVS14 region was also tested, since in a previous study a novel mutation (IVS14-2A>G) was identified in Portuguese Pendred patients (Simões-Teixeira *et al*, 2010). No mutation was found in the studied regions for the considered individuals. The genetic cause for PS wasn't yet determined for any of the analysed families.

4.9. Pitfall

The first sequencing of the IVS14/exon 15 region of *SLC26A4* gene revealed an alteration in the nucleotide +1654 of this gene, with an adenine being substituted by a guanine. This alteration appeared in a homozygous state (fig.22 A) in each proband of the tested families, NR3 and OQ1, and was found to be a novel mutation, p.Ser552Gly, not reported until now. This alteration could only be detected with the forward primer (IVS14+2F.P , fig.22C) because it was located in the area compatible with the annealing of the reverse primer (IVS14+2R.P , fig.22C), not being detected in the sequencing with the reverse primer. Due to the fact that a new mutation, never reported before, was observed in two individuals belonging to different families, the available members of the NR family and three normal hearing Portuguese controls were also sequenced in order to check the presence of the new alteration. The variation was observed, also in homozygosity in the sequencing with the forward primer. This situation originated more strangeness and a doubt about the viability of both the PCR product and the automatic sequencing procedure could be considered.

The presence of an unreported mutation in homozigosity in affected and hearing individuals would be improbable. In order to clarify this situation, a new reverse primer was design (IVS14+2R.P2 , fig.22C), to amplify a larger region. The same forward primer was used with the new reverse primer in the PCR reaction and in automatic sequencing. When sequencing these new PCR products, the variation was no longer observed (fig.22B) either in the probands of the considered families neither in the normal hearing Portuguese controls used. It was then assumed that the occurrence that was happened was no more than an artefact made by the PCR or by the automatic sequencing procedure. This situation acts as an alert for

the importance of confirmation by sequencing in both directions, for instance, and validation of new results as for the critical and precautious spirit that the molecular analysis requires.

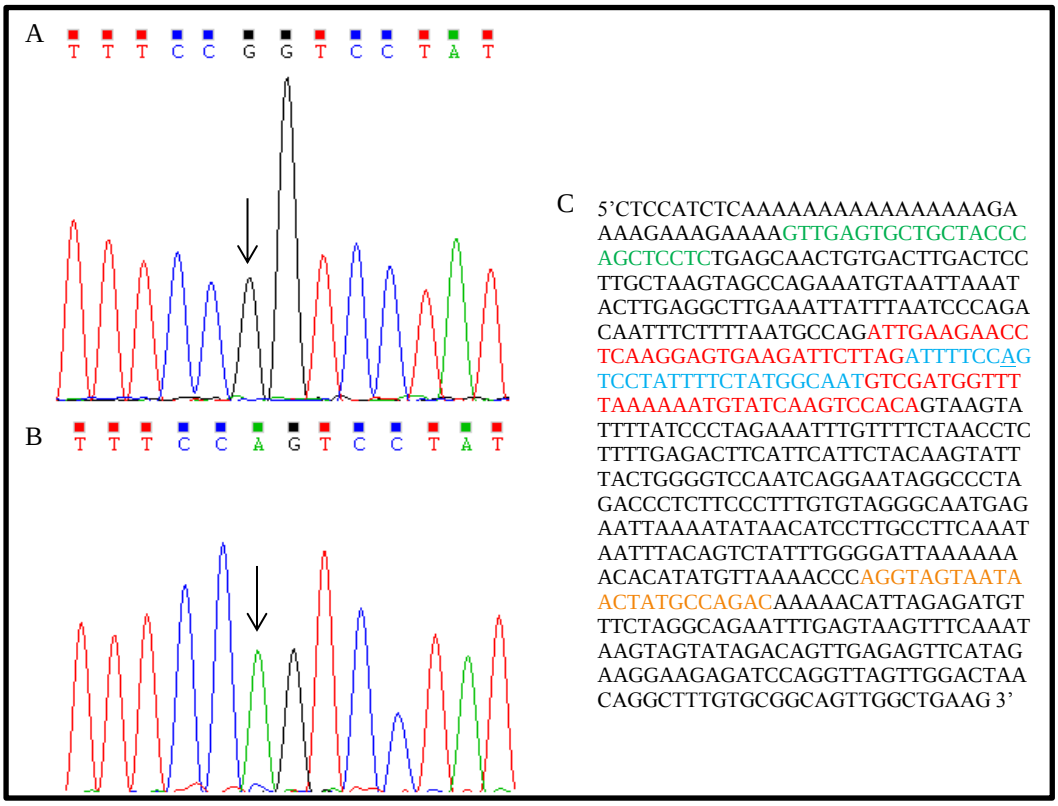

Figure 22 – Pitfall in *SLC26A4* gene analysis. Electrophoretogram showing: A - guanine homozygosity in the nt +1654 of *SLC26A4* gene. B – adenine homozygosity in the nt +1654 of *SLC26A4* gene. C - Section of the SLC26A4 gene. In green is indicated the sequence of the forward primer IVS14+2F.P. In red is indicated the exon 15. In blue is indicated the reverse primer IVS14+2R.P. In orange is indicated the second designed reverse primer IVS14+2R.P2.

5. Conclusion

In this study, 70 families presenting HL were analysed as regards *GJB2*/*GJB6* genes (DFNB1 *locus*). About 1:4 families (25,7%) harboured mutations in this locus being the cause of HL estimated for 11,4% (8/70) of the families. Fourteen different variants previously reported and one **novel mutation**, p.Leu213X, were identified in *GJB2* gene. As to *GJB6* gene, no deletion was found. This data are according to the low frequency of these deletions previously observed in Portugal.

Considering the fact that it only was analysed the *GJB2* coding region, in families with monoallelic recessive mutations in Cx26 it would be recommended to perform screening for non-coding regions, since some mutations have already been identified in these regions (Matos *et al* 2012).

Concerning mtDNA, only one proband from the 143 analysed, presented an m.1555A>G mutation in mtDNA *12S rRNA* gene. Mutations m.7445A>G and m.7511T>C were also screened but none was found in this study. The m.1555A>G is associated with aminoglycosides exposure and also with a progressive form of deafness. Its identification in a family is extreme relevant when the probands are females considering the maternal inheritance of mtDNA.

Two families presenting LFSNHL were also included in this project. An analysis of the regions of *WFS1* gene, where most of the disease-associated mutations are located, was performed and a **novel mutation**, p.Asp171Asn, localised to the N-terminus of wolframin protein was found in heterozygosity in one of the families. Some polymorphisms and one intronic variation previously reported were also identified during the study of the *WFS1* gene.

Two families presenting PS were studied in order to elucidate the genetic cause of the syndrome. Sequencing of relevant regions of *SLC26A4* gene was performed. No mutations were found and the genetic cause of the PS remained to be elucidated.

As molecular tests are minimally invasive nowadays, the genetic study became more widely available and accepted. With the identification of the genetic cause of HL it can be predicted the chance of recurrence in a future pregnancy and the expected evolution of HL determining if it is progressive or stable. A careful analysis of the heredogram of the family under consideration can enable genetic counselling of other family members who may potentially benefit in the future of the same test.

Communications resulted from the present work:

Articles

Gonçalves AC, Chora J, Matos TD, O'Neill A, Escada P, Fialho G, Caria H. 2012. A Novel p.Leu213X Mutation in *GJB2* Gene in a Portuguese Family. Accepted. *International Journal of Pediatric Otorhinolaryngology*.

Matos TD, Simões-Teixeira H, Caria H, Gonçalves AC, Chora J, Rosa H, Monteiro L, *et al*. 2012. Contribution of *GJB2* mutations to nonsyndromic sensorineural hearing loss in Portugal. (Submitted).

Posters

Gonçalves AC, Santos R, Cavilhas P, Madeira da Silva, O'Neill A, Fialho G, Caria H. 2012. Case report on a Portuguese family with deafness of genetic etiology. *XXII International Symposium on Morphological Sciences*, São Paulo, Brasil.

Gonçalves AC, Santos R, Cavilhas P, Madeira da Silva, O'Neill A, Fialho G, Caria H. 2012. Patogenicidade da mutação controversa M34T no gene *GJB2* numa família portuguesa com surdez neurosensorial bilateral. *59º Congresso Nacional da Sociedade Portuguesa de Otorrinolaringologia e Cirurgia Cérvico-Facial. XIV Congresso Luso-Espanhol de ORL*.

Gonçalves AC, Santos R, Cavilhas P, Madeira da Silva, O'Neill A, Fialho G, Caria H. 2012. The Controversial p.M34T Mutation in *GJB2*: Report on Three Portuguese patients with NSSHL. *XXXVII Jornadas Portuguesas de Genética*.

Gonçalves AC, Matos TD, Santos R, Cavilhas P, Madeira da Silva, O'Neill A, Fialho G, Caria H. 2012. A Novel Wolframin Mutation, p.D171N, in a Nonsyndromic Sensorineural Low-Frequency Hearing Loss Case. *Inner Ear Biology Workshop*.

Gonçalves AC, Chora J, Santos R, Cavilhas P, Madeira da Silva, O'Neill A, Fialho G, Caria H. 2012. The Controversial p.M34T Mutation in *GJB2*: Report on Three Portuguese patients with NSSHL. *Inner Ear Biology Workshop*.

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7. Supplementary data

7.1. Extraction of genomic DNA from blood samples

Blood samples for DNA extraction were collected in two different forms: extraction of about 5 mL of peripheral blood for tubes containing EDTA as an anticoagulant agent, which were stored at -20ºC; and in FTA or Guthrie Cards, stored at room temperature.

The extraction of genomic DNA from liquid blood samples in tubes was made using the JetQuick Blood and Cell Culture Kit (GENOMED), according to the manufacturer's instructions.

For the blood in FTA or Guthrie Cards, genomic DNA was extracted following the next steps: 1cm^2 of card was cut in 2mm^2 pieces. 500µL of DLB buffer (1mL Tris/HCl 1M pH=7,4; 0,2mL NaCl 5M; 2mL EDTA 0,5M pH=8,0; 96,8mL H_2Od), 50µL of SDS (10%) and 5µL of proteinase K (20mg/mL) were added. The mixture was incubated at 56ºC overnight and was gently shaking. Then, 20µL of NaCl (5M) and 575µL of Phenol**:**Chloroform**:**Isoamyl Alcohol (25:24:1) were added. After mix by inversion, the solution was separated in two phases through a 12000rpm centrifugation during 3 minutes. The upper layer was recovered and then were added 575µL of Chloroform**:**Isoamyl Alcohol (24:1). Another centrifugation at 12000rpm during 3 minutes was made. The upper layer was recovered again and 1mL of ethanol 96% stored at -20ºC was added. The solution was incubated during 1 hour at -20ºC. After a centrifugation of 15 minutes at 4ºC and 12000rpm the ethanol was discard and the precipitate was dried at room temperature. After this process, 100µL of ultra-pure H_2O was added to rehydrate DNA and the solution was incubated at 56ºC overnight.

To confirm the extraction, DNA samples were always run in a 1% agarose gel in TBE 0,5x, by electrophoresis, with EtBr staining under UV light. 1Kb Plus Ladder (Invitrogen) was used to compare the sizes of the DNA fragments. Photographs were taken using microDOC System from Cleaver Scientific Ltd.

7.2. Electrophoresis of PCR products

All the PCR products were run in a 1% agarose gel in TBE 0,5x, by electrophoresis, with EtBr staining under UV light. 1Kb Plus Ladder (Invitrogen) was used to compare the sizes of the PCR fragments. Photographs were taken using microDOC System from Cleaver Scientific Ltd.

Table 1 - Reaction of PCR mix per sample.

Specific PCR / Reagents	Standard and γ c.35delG	*GJB6* multiplex	δ c.35delG and mtDNA regions
	Volume (µL)		
miliQ ultra-pure H2O	29,7	3,85	14,75
5x MyTaq Buffer	10	3	5
Primer Forward	2	1	1
Primer Reverse	2	1	1
MyTaq DNA polymerase	0,3	0,15	0,25
DNA template	6	2	3
Total	50	15	25

Table 2 – Standard PCR programme.

Time	Temperature	Cycles
1m	94ºC	
1m	94ºC	x 30 cycles
1m	Specific Tm(ºC)	
1m	72ºC	
7m	72ºC	
∞	4ºC	

Table 3 - *GJB6* multiplex PCR programme.

Time	Temperature	Cycles
5m	95ºC	
40s	94ºC	
40s	60ºC	Turns to #2 5*
40s	94ºC	x 24 cycles
40s	55ºC	
7m	72ºC	
∞	4ºC	

* = Touchdown step in which 1 degree is reduced in the annealing temperature in each cycle. The touchdown step is initiated with 5ºC above the ideal annealing temperature for the considered primers. 5 cycles of denaturing are repeated at 94ºC, following the decrease of 1 degree in the annealing temperature per cycle, which allows an highly specific amplification in the first steps of PCR reaction.

Table 4 - Restriction analysis mix per sample.

c.35delG	**m.1555A>G**	**m.7445A>G**	**m.7511T>C**
H_2O miliQ – 9,8µL	H_2O miliQ – 9,5µL	H_2O miliQ - 14µL	H_2O miliQ – 14,96 µL
10x NEBuffer 3 – 2µL	Buffer C - 2µL	Buffer D - 2µL	Buffer 4 1x - 2µL
*Bsl*I – 2U / µL	*Hae*III – 5U / µL	BSA 1mg/mL - 2µL	BSA 1mg/mL - 2µL
PCR product - 8µL	PCR product - 8µL	*Xba*I – 10U	*Mbo*II – 0,4U
Total – 20µL	Total - 20µL	PCR product - 1µL	PCR product - 1µL
		Total - 20µL	Total - 20µL

A non-digested control that contained all the mix reagents, but without the specific restriction enzyme, was used in each restriction reaction.

- For c.35delG restriction analysis the samples were incubated at 55ºC during 3h;
- For m.1555A>G restriction analysis the samples were incubated at 37ºC overnight;
- For m.7445A>G restriction analysis the samples were incubated at 37ºC during 3h;
- For m.7511T>C restriction analysis the samples were incubated at 37ºC during 1h.

All the restriction samples were run in a 2% agarose gel in TBE 0,5x, by electrophoresis, with EtBr staining under UV light. 1Kb Plus Ladder (Invitrogen) was used to compare the sizes of the DNA fragments. Photographs were taken using microDOC System from Cleaver Scientific Ltd.

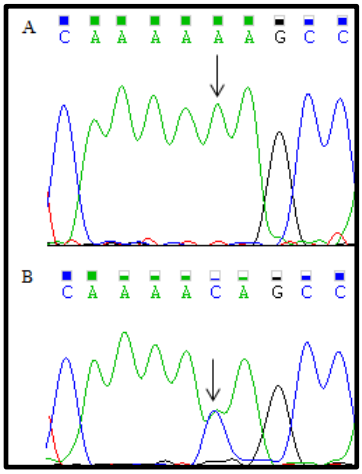

Figure 1 – Electrophoretogram showing: A – wild-type; B - p.Lys224Gln mutation in heterozygosity.

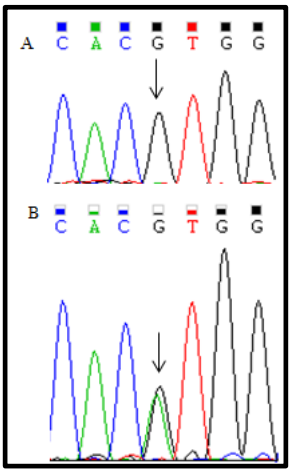

Figure 2 – Electrophoretogram showing: A – wild-type; B - p.Val95Met variant in heterozygosity.

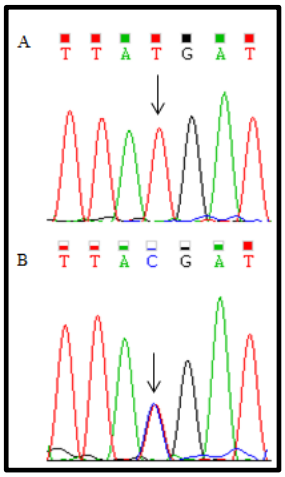

Figure 3 – Electrophoretogram showing: A – wild-type; B - p.Met34Thr variant in heterozygosity.

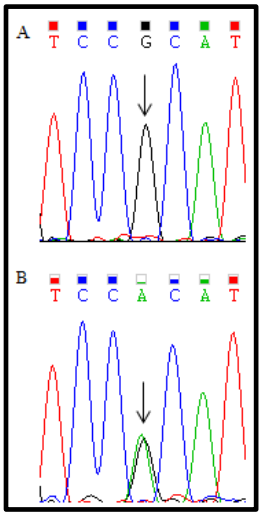

Figure 4 – Electrophoretogram showing: A – wild-type; B - p.Arg127His variant in heterozygosity.

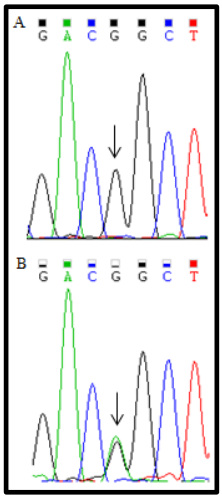

Figure 5 – Electrophoretogram showing: A – wild-type; B - p.Gly160Ser mutation in heterozygosity.

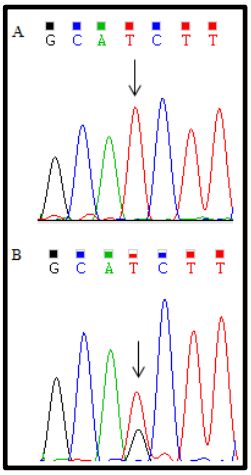

Figure 6 – Electrophoretogram showing: A – wild-type; B - p.Ile140Ser mutation in heterozygosity.

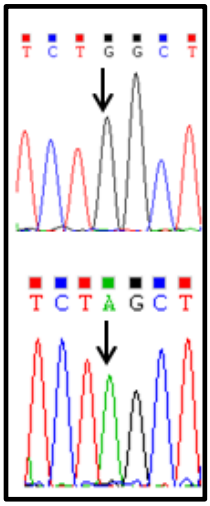

Figure 7 – Electrophoretogram showing: A – wild-type; B - p.Trp24X mutation in homozygosity.

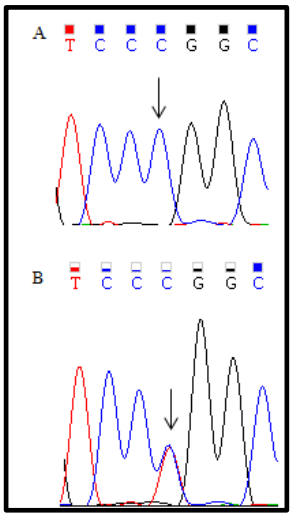

Figure 8 – Electrophoretogram showing: A – wild-type; B - p.Arg184Pro mutation in heterozygosity.

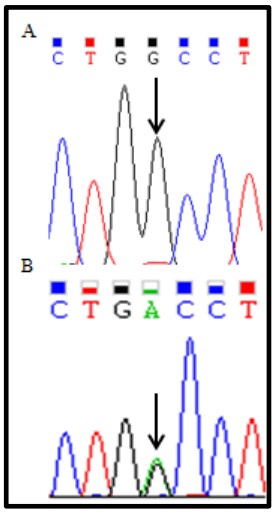

Figure 9 – Electrophoretogram showing: A – wild-type; B - p.Trp172X mutation in heterozygosity.

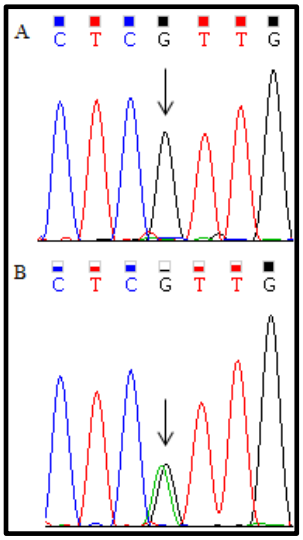

Figure 10 – Electrophoretogram showing: A – wild-type; B - p.Val37Ile mutation in heterozygosity.

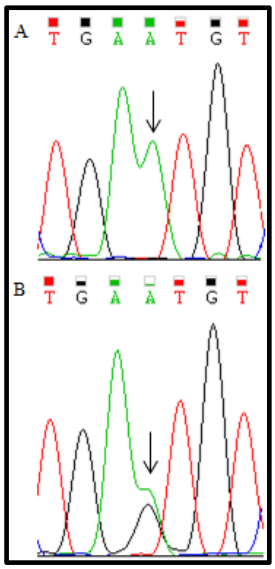

Figure 11 – Electrophoretogram showing: A – wild-type; B - p.Asn206Ser mutation in heterozygosity.

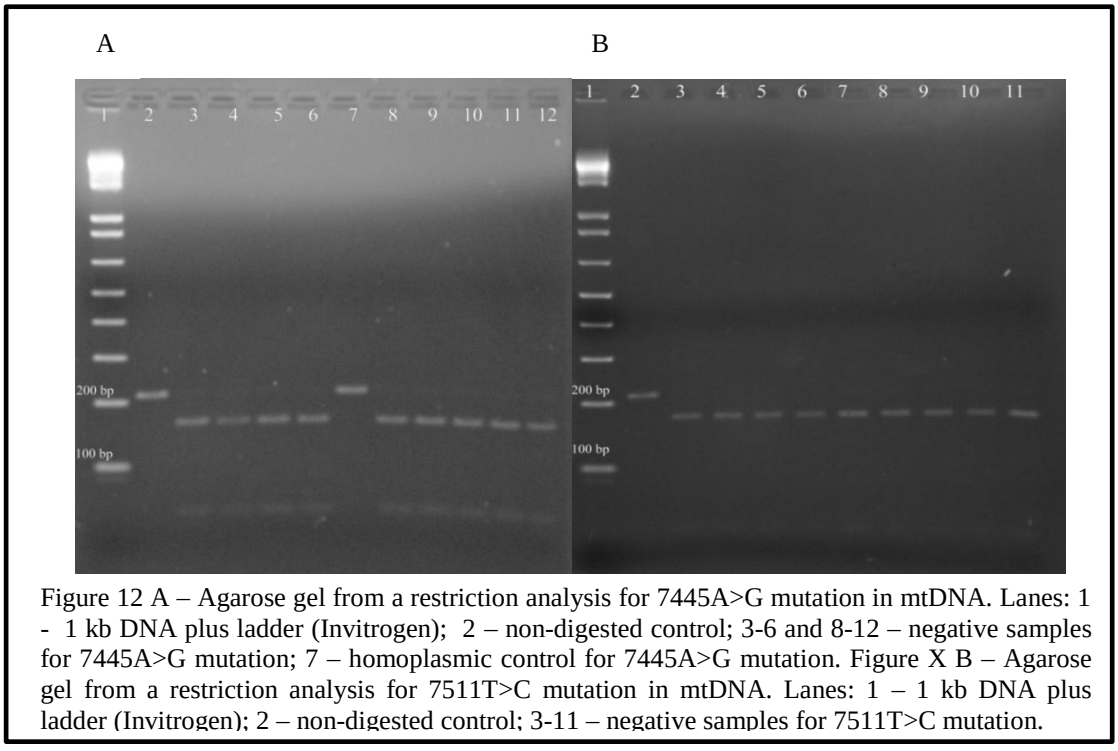

Figure 12 A – Agarose gel from a restriction analysis for 7445A>G mutation in mtDNA. Lanes: 1 - 1 kb DNA plus ladder (Invitrogen); 2 – non-digested control; 3-6 and 8-12 – negative samples for 7445A>G mutation; 7 – homoplasmic control for 7445A>G mutation. Figure X B – Agarose gel from a restriction analysis for 7511T>C mutation in mtDNA. Lanes: 1 – 1 kb DNA plus ladder (Invitrogen); 2 – non-digested control; 3-11 – negative samples for 7511T>C mutation.

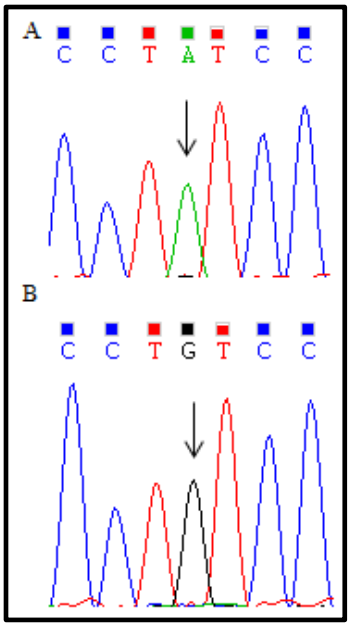

Figure 13 – Electrophoretogram showing: A – wild-type; B – IVS4 – 9 A>G intronic variant in homozygosity.

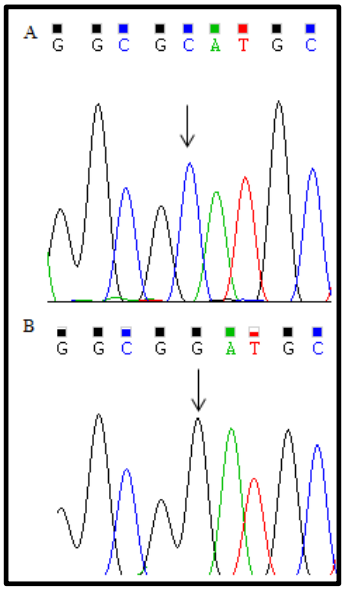

Figure 14 – Electrophoretogram showing: A – wild-type; B - p.Arg228Arg polymorphism in homozygosity.

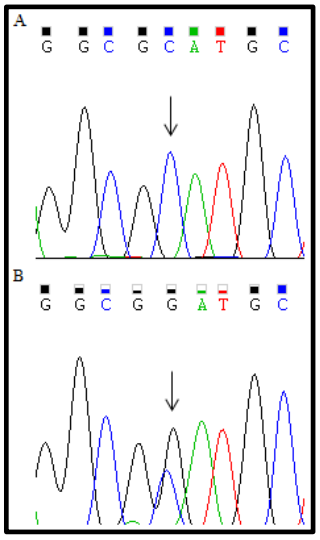

Figure 15 – Electrophoretogram showing: A – wild-type; B - p.Arg228Arg polymorphism in heterozygosity.

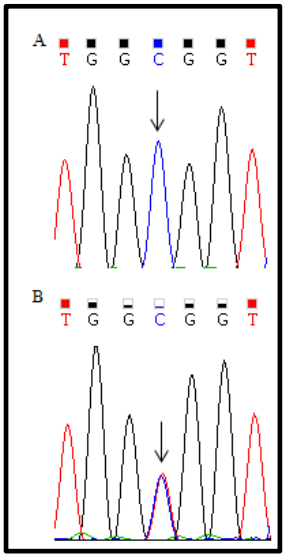

Figure 16 – Electrophoretogram showing: A – wild-type; B - p.Ala602Val mutation in heterozygosity.

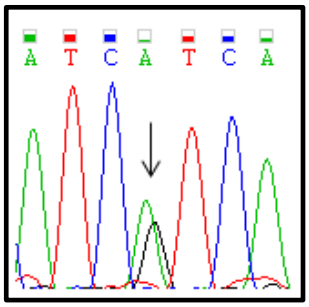

Figure 17 – Electrophoretogram showing: A – wild-type; B - p.Val333Ile variant in heterozygosity.

All the electrophoretograms obtained during this study were evaluated by visual inspection and pairwise alignment to reference sequences using NCBI's BLAST. Chromas and BioEdit softwares were used to analyse the sequences obtained after bidirectional sequencing.

Table 5 – Primers used in PCR's reactions.

Gene	Primer name	Sequence	Tm (ºC)
GJB2	22BF	(5'-GGTGAGGTTGTGTAAGAGTTGG-3')	55
	22BR	(5'-CTGGTGGAGTGTTTGTTCCCAC-3')	
	FP	(5'-GGGAGATGAGCAGCCGACT-3')	55
	RP	(5'-ACGTGCATGGCCACTAGGAGC-3')	
	2AF	(5'-AAGTCTCCCTGTTCTGTCCT-3')	60,7
	2BR	(5'-GGCATCTGGAGTTTCACC-3')	
GJB6	Cx30Ex1A	(5'-CGTCTTTGGGGGTGTTGCTT-3')	60
	Cx30Ex1B	(5'-CATGAAGAGGGCGTACAAGTTAGAA-3')	
	Del BK1	(5'-TCATAGTGAAGAACTCGATGCTGTTT-3')	
	Del BK2	(5'-CAGCGGCTACCCTAGTTGTGGT-3')	
	GJB6-1R	(5'-TTTAGGGCATGATTGGGGTGATTT-3')	
	BKR-1	(5'-CACCATGCGTAGCCTTAACCATTTT-3')	
12S rRNA	rRNA12SF	(5'-GCTCAGCCTATATACCGCCATCTTTCAGCAA-3')	63,9
	rRNA12SR	(5'-TTTCCAGTACACTTACCATGTTACGACTTG-3')	
*COX*I	7392F	(5'-GGATGCCCCCCACCCTACC-3')	60
	7588R	(5'-TACTTGCGCTGCATGTGCC-3')	
WFS1	ex4F.W	(5'-CGGAGAATCTGGAGGCTGAC-3')	64
	ex4R.W	(5'-CAACCCTCCAGAGGCTGTTC-3')	
	ex5F.W	(5'-ACAAGGCCTTTGACCACATC-3')	62
	ex5R.W	(5'-GTGCCCAGGGTGAATCCTC-3')	
	ex6F.W	(5'-CTGTTAATCCACCCTGTCCC-3')	68
	ex6R.W	(5'-GAGTCGCACAGGAAGGAGAG-3')	
	ex8aF.W	(5'-TTCCCACGTACCATCTTTCC-3')	58,7
	ex8aR.W	(5'-GGGCAAAGAGGAAGAGGAAG-3')	
	ex8bF.W	(5'-GTGAGCTCTCCGTGGTCATC-3')	55,8
	ex8bR.W	(5'-CCTCATGGCAACATGCAC-3')	
SLC26 A4	ex6F.P	(5'-ATTTTTGTGCTATAGGCAGG -3')	58
	ex6R.P	(5'-ATGAGGTCTCACGTCTCAAA-3')	
	ex10F.P	(5'-TTATCGAGAGCAATGAGACC-3')	60
	ex10R.P	(5'-TCAGTTGTTATTGACCACAGC -3')	
	IVS8F	(5'- GTGTGCGTGTAGCAGCAGG -3')	64
	IVS8R	(5'- GGACTATTGAAGGAGTATCAGTG -3')	
	IVS14+2F.P	(5'-GTTGAGTGCTGCTACCCAGCTCCTC-3')	64
	IVS14+2R.P	(5'-ATTGCCATAGAAAATAGGACCGGAAAAT-3')	
	IVS14+2R.P2*	(5'- AGGTAGTAATAACTATGCCAGAC-3')	

The C base is altered and substitutes an A in the wild-type sequence to creating a recognition site for *Bsl*I in the presence of c.35delG mutation.

*= new designed reverse primer.