Original Article

Distribution and phenotype of GJB2 mutations in 102 Sicilian patients with congenital non syndromic sensorineural hearing loss

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Abstract

Objective: To evaluate the frequency of GJB2 mutations and their correlation with phenotype in Sicilian non-syndromic sensorineural hearing loss (NSHL) patients. Design: Sequencing of the coding region, basal promoter, exon 1, and donor splice site of the GJB2 gene; screening for the presence of the two common GJB6 deletions. Study sample: A cohort of 102 Sicilian NSHL patients. Results: Fifteen different mutations in GJB2 and seventeen different genotypes were detected. No GJB6 mutations were found. The hearing impairment was profound in the 64.72% of probands (mean PTA0.25 – 4 kHz of 88.82 ± 26.52 dB HL). A total of 81.37% of patients harboured at least one c.35delG allele; c.167delT and c.-23 +1>G were identified in 10.78% and the 9.8% of patients respectively; c.35delG homozygotes presented more severe hearing impairment (75.59% of profound hearing loss) and a higher mean PTA0.25 – 4 kHz (96.79 ± 21.11 dB HL) with respect to c.35delG/non-c.35delG and c.35delG/Wt patients (P < 0.05). Conclusions: This work underlines the role of c.35delG, c.167delT and c.-23 +1>G as the most frequent causes of NSHL in Sicily. The c.35delG frequency found is similar to those reported in other populations of the Mediterranean area. The analysis of genetic and audiologic data confirmed a variability in the phenotype associated to a single genotype.

Key Words: Sensorineural hearing loss; GJB2; Genotype-Phenotype; Sicily

Approximately 60% of congenital deafness has genetic causes in developed countries (Petit et al, 2001; Piccotti et al, 2009), with a progressive increase of this percentage due to the improvement of public health conditions and consequent reduction of hearing impairment linked to other causes such as infections (Marazita et al, 1993). Genetic sensorineural hearing loss (SNHL) is classified as non-syndromic (70%) and syndromic (30%), with an autosomal recessive mode of inheritance found in about 70–80% of patients. Lower percentages are associated with autosomal dominant (15–20%), X-linked (1%), and mitochondrial (1%) deafness.

Mutations in more than 60 genes were identified and correlated with non-syndromic sensorineural hearing loss (NSHL) (Alford 2011). Variants of GJB2 gene, accounting for up to 50% of NSHL inherited in autosomal recessive manner, clearly play a major role in the onset of this condition (Kenesson et al, 2002). GJB2, expressed in the cochlea, encodes for the gap junction protein Connexin 26 (Cx26), whose function is crucial for intercellular communication, particularly in the recycling of potassium ions (K+) which occurs through a lateral and a medial transcellular pathway. In the lateral route, K+ effluxing from outer hair cells is resorbed by Deiters’ and tectal cells and flows via gap junctions through Hensen’s, Claudius’ and outer sulcus cells to efflux into stroma. K+ subsequently diffuses via gap junctions through type 1 fibrocytes and strial basal and intermediate cells into the intrastrial compartment. In the medial path, the K+ released by the inner hair cells travels through inner phalangeal cells, border cells, inner sulcus cells directly or by way of limbal fibrocytes via gap junctions, similar to the spiral ligament, and is finally released back into the endolymph via the intercellular cells (Spicer & Schulte, 1998).
A decreased synthesis or an altered function of Cx26 may therefore reduce the efficiency of K⁺ circulation leading to impaired hearing sensitivity (Wangemann, 2002). Additionally, as evidenced by the c.250G>C mutation (p.V84L) studied by Beltramello et al. (2005), Cx26 regulates also the permeability to Ca²⁺-mobilizing messenger inositol triphosphate (IP3), allowing a metabolic coupling between connected cells which is necessary to generate an endocochlear potential.

Also, mutations in GJB6, a gene encoding for Connexin 30 (Cx30), a gap junction protein co-localized in the inner ear with Cx26, can be responsible for NSHL. Particularly, two large deletions in GJB6 have been studied and have often been identified in trans with single GJB2 recessive mutations, suggesting a possible recessive digenic inheritance (Del Castillo et al., 2002, 2005). GJB6 deletions are also linked with hearing impairment in patients who carry one GJB2 recessive mutation in trans, as a result of the abolition of an as-yet-undefined cis-regulatory element responsible for the expression of the GJB2 gene in the inner ear. Common et al. (2005), analysing immunohistochemical data of the sweat glands of an individual compound heterozygous for c.35delG in GJB2 and del(GJB6-D13S1830), demonstrated that the mutated GJB2 allele in trans with del(GJB6-D13S1830) was expressed, whereas the GJB2 allele located in cis with the deletion was not expressed at all. Also del(GJB6-D13S1854), similar to del(GJB6-D13S1830), disrupts human GJB2 expression at the transcriptional level, but, differently from del(GJB6-D13S1830), it is associated with low residual GJB2 expression (Rodriguez-Paris et al., 2011). The c.35delG mutation is by far the most common pathogenic GJB2 allele in Caucasians, (Denoyelle et al., 1997) with a carrier frequency among European hearing individuals from 1% to 4% (Kempen et al., 2002). A South-North gradient in c.35delG carrier frequency was observed by Gasparini et al. (2000), with an average carrier frequency of 2.8% in Southern Europe, higher than Central and Northern Europe (1.3%). Particularly the Mediterranean region presents the highest c.35delG frequency with Greece representing, as suggested by Luquette (2007), the ‘geographical center’ of this mutation; in Sicily, which was one of the regions of Ancient Greek colonization, Niceta et al. (2007), studying 1040 newborns who underwent GJB2 gene mutational screening, estimated a c.35delG carrier rate of 2.9%.

The main objective of this work was to study the frequency of GJB2 mutations among Sicilian NSHL patients, to try and correlate the genotype with the severity of hearing impairment. In particular, considering the high prevalence of c.35delG found, we analysed the variability of the degree of deafness between c.35delG homozygotes and c.35delG heterozygotes. We compared also the genetic profile of our sample with those of other populations of the Mediterranean area to evidence the main differences or similarities.

### Materials and Methods

#### Study sample

We performed a retrospective study of audiological and genetic data that we collected from 102 patients with a variable degree of hearing impairment over recent years at the Audiology Department of the University of Palermo. All the included subjects, aged between 6 and 65 years, were affected by bilateral NSHL and carried at least one GJB2 mutation. Individuals with syndromic, unilateral or acquired hearing impairment were excluded from this study. A detailed history was taken to rule out the presence of environmental causes associated with SNHL (Joint Committee on Infant Hearing, 2007). After Ethical Committee approval, an informed consent was obtained from all participants and from parents of patients younger than 18 years old.

#### Clinical and audiometric evaluation

All patients underwent a physical examination of the ears through micro-otoscopy; audiometric tests and tympanograms were performed in all subjects studied. Air conduction pure-tone average thresholds at frequencies 0.5–1–2–4 kHz (PTA0.5–4kHz) were calculated for each ear and were used to classify hearing loss severity. Average thresholds ranging between 21 and 40 dB HL were defined as mild hearing loss, 41–70 dB HL as moderate, 71–95 as severe hearing loss, and >95 dB HL as profound hearing loss, as suggested by Liu et al. (2005). SNHL was classified in ‘symmetrical’ when in presence of inter-aural threshold differences <30 dB, and ‘asymmetrical’, when inter-aural threshold difference was ≥30 dB.

#### Mutation detection

After extracting DNA samples from peripheral blood, all subjects were analysed regarding the basal promoter, exon 1 and the coding region of GJB2. Genomic DNA was extracted classically from peripheral blood samples with the salting-out method. Molecular analysis of GJB2 gene was performed with PCR and direct sequencing. Exon 1 was amplified with the primers Cx1 forward (5′-TCAAAAGAACTAGGATCCG-3′) and Cx1 reverse (5′-CAAGGACGTGTGTTGTCGAC-3′). Exon 2 was amplified with the primers GAPI forward (5′-CCTATGACAAAC-TAAGTTGGTC-3′) and CONN reverse (5′-GACAGCTGACGACGGTTGCGTC-3′).

USB® ExoSAP-IT® PCR Product Cleanup protocol (37°C for 30 minutes and then 95°C for 5 minutes) was performed to remove leftover primers and unincorporated dNTPs (Affymetrix, Inc.). Cleaned up PCR products were sequenced from both ends, using the same primers as used for PCR reactions, with Applied Biosystem (ABI) PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kits and analysed with ABI PRISM 3100 DNA automatic sequencer. Following PCR protocol and using primers described by Del Castillo et al. (2005), the GJB6 gene was also checked to rule out the presence of mutations del(GJB6-D13S1830) and del(GJB6-D13S1854).

#### Statistical analysis

Statistical analysis was performed with Matlab® computer program; χ² test, odds ratio (or) and/or exact test of Fisher were used.
following usual conditions of application. Group data were expressed as percentages and median (95% confidence intervals). Statistical comparisons between two groups were made using Student’s paired t-test. Significance was set at 0.05.

Results

The study sample comprised 102 NSHL patients, 52 males (50.99%) and 50 females (49.01%) (Sex ratio = 1.04). With a mean age of 13.72 ± 9.75 years (median 11.5 years), the majority of participants (75.49%) were within the range of 6–17 years.

Audioligic evaluation revealed that the 95.1% (97/102) of the total population was affected by symmetric SNHL, whereas the others (4.9%) presented an asymmetric hearing loss. The majority of subjects (96.08%, 98/102) had a stable SNHL and only four persons (3.92%) presented a progressive worsening of hearing.

As shown in Table 1, all degrees of hearing loss were observed in our population. The hearing impairment was profound in 66 (3.92%) subjects (96.08%, 98/102) had a stable SNHL and only four persons (3.92%) presented a progressive worsening of hearing.

Of the 102 patients of our cohort, 83 (81.37%) harboured at least one c.35delG mutation, 11 (10.78%) a c.167delT, and 10 (9.8%) a c.-23 + 1G > A; c.35delG, c.167delT, and c.-23 + 1G > A represented respectively the 76.3% (132/173), 6.93% (12/173) and 15.78% (3/19) of cases respectively; eleven probands were affected by profound, four by severe, three by moderate and only one by mild SNHL.

We classified c.35delG carriers in three subgroups: the first (G1), composed by 49 patients who were homozygous for the c.35delG mutation; the second (G2), and the third (G3) respectively constituted by 15 subjects carrying a c.35delG mutation associated with other GJB2 mutations in trans (c.35delG/ non-c.35delG), and 19 patients with a c.35delG/Wt genotype (Table 1).

Table 1. Frequency, hearing loss degree, and hearing threshold of all genotypes identified in the cohort.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>N(%)</th>
<th>Hearing loss degree</th>
<th>Hearing threshold (dB HL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mild</td>
<td>Moderate</td>
</tr>
<tr>
<td>35delG/35delG (G1)</td>
<td>49(48.03)</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>35delG/non-35delG (G2)</td>
<td>15(14.7)</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>35delG/Wt (G3)</td>
<td>19(18.62)</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>35delG / -23 + 1G &gt; A</td>
<td>6(5.88)</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>35delG/167delT</td>
<td>4(3.94)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>35delG/269T&gt;C(L90P)</td>
<td>4(3.94)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>167delT/Wt</td>
<td>3(2.94)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>457G&gt;A/167delT/Wt</td>
<td>3(2.94)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>167delT/139G&gt;T(E47X)</td>
<td>2(1.96)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-23 + 1G &gt; A/Wt</td>
<td>2(1.96)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>101T&gt;C(M34T)/Wt</td>
<td>2(1.96)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>313del14/-23 + 1G &gt; A</td>
<td>2(1.96)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>167delT/167delT</td>
<td>1(0.98)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>35delG/553G&gt;C(D179H)</td>
<td>1(0.98)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>310del14/551G&gt;C(R184P)</td>
<td>1(0.98)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>101T&gt;C(M34T)/269T&gt;C(L90P)</td>
<td>1(0.98)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>380&gt;G&gt;A/R127H/Wt</td>
<td>1(0.98)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>301del5/Wt</td>
<td>1(0.98)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total*</td>
<td>102</td>
<td>3</td>
<td>13</td>
</tr>
</tbody>
</table>

*With the exclusion of c.35delG/non-c.35delG genotype.
Severe

Moderate

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statistical significant difference between G1, G2, and G3 (P

hearing loss than G1 (P

percentage (46.66%, 7/15) of patients affected by mild to moderate

ated with a higher variability in the distribution of data and a greater

dB HL) and G3 subjects (mean IQR 0.25-4 kHz

additionally, concerning 0.25 and 0.5 kHz frequencies, G2 is associ-

of hearing loss

a prevalence of 79.59% (39/49), 40% (6/15), and 57.89% (11/19)

one by mild SNHL, with a mean PTA 0.25-4 kHz of 67.2

were affected by profound, four by severe, three by moderate, and

c.-23

and distributed in three genotypes (c.-23

ence (P

non c.35delG /c.35delG carriers but without any signifi  cant differ-

logical

GJB2

mutations c.35delG accounts

for up to 75% of mutated alleles in populations with European

ancestry (Gasparini, 2000; Oguchi et al, 2005). In the Mediterra-

nean area, with a great diffusion in the general population (carrier

frequency of ~1/30), c.35delG represents the most common patho-

logical GJB2 allele associated with NSHL (Lucotte et al, 2005). We

evidenced in fact a c.35delG frequency of 76.3% among the total

mutated GJB2 alleles (132/173), in line with data relative to Tunisia

(85.4%), Spain (82%) Algeria (76%), Turkey (76%), France (69%),

Portugal (85%), Morocco (90.8%), and Greece (95.2%) (Rabionet


It is important to underline that GJB6 mutations, which were not

found in our cohort, represent only a small percentage of DFNB1

mutations in our country; in fact, as demonstrated by many studies

published previously, GJB6 mutations were not or sporadically rec-

ognized in Italian NSHL patients, with an evident difference between

Northern and Southern Italy (Del Castillo et al, 2003; Gualandi et al, 2010; Rihai et al, 2013).

Discussion

Among more than 90 different GJB2 mutations c.35delG accounts

to G3 group for all frequencies studied (P < 0.01) and to G1 limited

to 1–2–4 kHz frequencies (P ≤ 0.05).

The study of a box plot (Figure 2) for each group revealed a

lower variability in the degree of SNHL among G1 patients (mean

IQR0.25-4 kHz = 26 dB HL) than among G2 (mean IQR0.25-4 kHz = 41.5

dB HL) and G3 subjects (mean IQR0.25-4 kHz = 40 dB HL). With

a prevalence of 79.59% (39/49), 40% (6/15), and 57.89% (11/19)
of hearing loss ≥ 90 dB HL for 2–4 kHz frequencies, it resulted a

statistical significant difference between G1, G2 and G3 (P = 0.012);

additionally, concerning 0.25 and 0.5 kHz frequencies, G3 is associ-

ated with a higher variability in the distribution of data and a greater

percentage (46.66%, 7/15) of patients affected by mild to moderate

hearing loss than G1 (P = 0.032).

The c.167delT mutation was found in 11 probands (10.78%),

associated with four different genotypes: c.167delT homozygous, c.167delT/c.35delG, c.167delT/c.139G, and c.167delT/Wt. From

the study of the audiologic data of all c.167delT carriers it resulted in

a mean PTA0.25-4 kHz of 90.18 ± 26.49 dB HL and a profound

SNHL in the majority of cases (72.72%, 8/11). Particularly, the fi ve

subjects with a c.167delT/c.35delG genotype presented a variable

degree of hearing loss (one moderate, one severe, and three pro-

found) and a mean PTA0.25-4 kHz of 86.6 ± 17.76 dB HL, lower than

non c.35delG /c.35delG carriers but without any signifi cant differ-

ence (P = 0.067).

The c.-23 + 1G> A mutation was identified in 10 patients (9.8%)

and distributed in three genotypes (c.-23 + 1G> A /c.35delG, c.-23 + 1G> A /c.313del14, c.-23 + 1G> A /Wt); two probands

were affected by profound, four by severe, three by moderate, and

one by mild SNHL, with a mean PTA0.25-4 kHz of 67.2 ± 24.8 dB HL,

lower than that of c.167delT carriers (P = 0.006).

Figure 1. Relative frequencies of the degree of hearing loss in

c.35delG/c.35delG, c.35delG/non-c.35delG, and c.35delG/Wt

genotypes. Mild SNHLs were not reported.

Figure 2. Box plot. Distributions of PTA 0.25–4 kHz of patients

with c.35delG/c.35delG, c.35delG/non-c.35delG, and c.35delG/Wt

genotypes.
129 hearing-impaired infants from the Campania region, did not evidence any \(GJB6\) mutations; Primignani et al (2009) and Berto et al (2009) instead found \(GJB6\) mutations only in three (0.4%) and five patients (1.3%) of two cohorts from Northern Italy composed respectively by 734 and 385 patients. On the contrary, in other countries of Western Europe such as Portugal and France, higher percentages of these mutations were observed (7.4% and 8.2% respectively) and also in Spain, where the \(GJB6\) mutations represent the second most frequent cause of prelingual deafness (Del Castillo et al, 2003; Del Castillo et al (2002) described previously this peculiar geographical distribution of \(GJB6\) mutations, especially of del(GJB6-D13S1830), for which they suggested a 'common founder' to justify the greater prevalence in certain populations.

In the 48.04% (49/102) of c.35delG homozygotes in our cohort, 95.92% (47/49) had a severe to profound degree of hearing loss and a median PTA\(_{0.5-2\,kHz}\) of 100 dB HL, similar to data reported by Hismi (90.9% of severe to profound HL and a median PTA\(_{0.5-2\,kHz}\) of 104 dB HL) and Cryns (91.56% of severe to profound HL and a median PTA\(_{0.5-2\,kHz}\) of 102 dB HL) (Cryns et al, 2004; Hismi et al, 2006). Additionally, also from the comparison of our results with those of Iliadou (100% of severe to profound HL and a mean PTA\(_{0.5-2\,kHz}\) of 99.78 dB HL) and Santos (100% of severe to profound HL and a mean PTA\(_{0.5-2\,kHz}\) of 101.5 dB HL), no significant difference was evidenced with respect to our data (Iliadou et al, 2004; Santos et al, 2005). However, even if c.35delG/c.35delG was the most represented genotype among the studies cited, a certain variability in its prevalence emerged. Hismi et al (2006) and Cryns et al (2004), for example, found higher frequencies of this genotype (69.8% and 60% respectively), differently from our work (48.03%, 49/102) and from Iliadou et al (2004) and Santos et al (2005) (25.2% and 47.36% respectively). This discrepancy could be explained considering that the first two authors cited included in their works only subjects carrying biallelic \(GJB2\) mutations. In fact, by excluding patients carrying only one \(GJB2\) mutation, the c.35delG/c.35delG percentage found in our cohort increases to 69.01% (49/71). Furthermore, it is clear that there is a correlation between this genotype and an audiologic phenotype characterized by severe to profound hearing loss, even if, cases of mild to moderate SNHL were previously reported (Wilcox et al, 2000; Snoeckx et al, 2005; Hismi et al, 2006) and were also observed in our sample (2/49 of c.35delG/c.35delG carriers). The presence of different degree of hearing impairment in patients carrying the same \(GJB2\) mutations is not clearly understood, but many authors underlined the role of environmental factors or modifier genes as potential causes of this variability (Hismi et al, 2006; Hilgert et al, 2009; Picciotti et al, 2009).

We compared the audiological phenotypes of patients homozygous for c.35delG (\(G_1\)), c.35delG/non-c.35delG (\(G_2\)), and c.35delG/Wt carriers (\(G_3\)). From this analysis a more severe hearing impairment in \(G_1\) subgroup (\(P = 0.012\)) emerged, with a statistical significant difference with respect to \(G_3\) group for all frequencies studied (\(P < 0.01\)) and to \(G_2\) limited to 1–2–4 kHz frequencies (\(P < 0.05\)); \(G_3\) instead was characterized by a higher percentage (46.66%, 7/15) of patients affected by mild to moderate hearing loss with respect to \(G_1\) patients (\(P = 0.032\)). The study of distribution of PTA\(_{0.25–4\,kHz}\) hearing thresholds showed in Figure 2 clearly reveals a close hearing loss spectrum for \(G_1\) patients, with an accumulation of values around 100–120 dB HL, especially for high frequencies. Concerning c.167delET, the second most frequent mutation recognized in our cohort (10.78%, 11/102), it was observed associated with c.35delG in the 45.45% (5/11) of cases and was characterized by a moderate to profound hearing loss (mean PTA\(_{0.25–4\,kHz}\) of 90.18 ± 26.49 dB HL); a statistically significant difference emerged between the degree of hearing impairment of c.167delET/c.35delG and c.35delG/ c.-23 + 1G/ A compound heterozygotes (\(P = 0.03\)). The c.167delET/c.35delG genotype was carried by 4.9% (5/102) of our patients, with a median PTA\(_{0.5-2\,kHz}\) = 90 dB HL; our data are comparable to Cryns et al (2005), Marlin et al (2005), Snoeckx et al (2005), and Hismi et al (2006) who reported lower frequencies (<4%) of c.167delET/c.35delG carriers but with similar degree of hearing impairment (mainly severe to profound).

We found the c.-23 + 1G/ A mutation in the 9.8% (10/102) of our cohort with a mild to profound spectrum of hearing loss; lower percentages were evidenced by Cryns et al (2.89%), Snoeckx et al (<1%), and Berto et al (1.7%) studies (Cryns et al 2005; Snoeckx et al, 2005; Berto et al, 2009). These studies identified this mutation mainly in the heterozygous state and associated with c.35delG. From the comparison of the audiological profile of our c.-23 + 1G/ A /c.35delG patients (median PTA\(_{0.5-5\,kHz}\) = 70 dB HL) with literature data, the median hearing threshold seems to be superimposable; in fact Cryns et al (2005) and Snoeckx et al (2005) evidenced respectively median values of 62 dB HL and 64 dB HL among 7 and 16 patients with c.35delG/ c.-23 + 1G/ A genotype. On the contrary Santos et al (2005) described two cases of profound SNHL associated with c.35delG/ c.-23 + 1G/ A (mean PTA\(_{0.5-2\,kHz}\) = 97.9 dB HL); in our cohort one patient was identified with profound SNHL. All these data seems to be contradictory, reflecting a variability in the phenotype associated to a single genotype and are not explainable on the basis of functional studies; in fact, D’andrea et al (2002) and Shanin et al (2002) demonstrated, for c.35delG and c.-23 + 1G/ A, do not yield detectable Cx26 protein and mRNA respectively. This is inconsistent with the observation made by Snoeckx et al (2005) and supported by our data, that c.35delG/c.-23 + 1G/ A compound heterozygotes had significantly less severe hearing impairment compared to c.35delG homozygotes (\(P < 0.0001\)).

In conclusion, the present study, analysing the genetic profile of NSHL Sicilian patients, highlights the role of certain \(GJB2\) mutations (c.35delG, c.167delET, c.-23 + 1G/ A) as the most frequent causes of congenital deafness in Sicily. Also from the study of our data, it emerged a real difficulty to identify precise genotype-phenotype correlations, even in cases of certain genotypes (c.35delG homozygotes) which are mainly associated to a determinate degree of hearing impairment. Future research is necessary to provide additional data that could improve the knowledge about the effects of modifier genes and environmental factors in the variability of audiologic phenotype.

**Acknowledgements**

This work was supported by the Audiology Department of the University of Palermo (Di.Bi.Me.F). We thank all the individuals who participated in this study for their cooperation and support.

**Declaration of interest:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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