Mitochondrial non-syndromic sensorineural hearing loss: a clinical, audiological and pathological study from Italy, and revision of the literature

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Synopsis
Over the last decade, a number of distinct mutations in the mtDNA (mitochondrial DNA) have been found to be associated with both syndromic and non-syndromic forms of hearing impairment. Their real incidence as a cause of deafness is poorly understood and generally underestimated. Among the known mtDNA mutations, the A1555G mutation in the 12S gene has been identified to be one of the most common genetic cause of deafness, and it has been described to be both associated to non-syndromic progressive SNHL (sensorineural hearing loss) and to aminoglycoside-induced SNHL. In the present study, we have investigated the presence of mtDNA alterations in patients affected by idiopathic non-syndromic SNHL, both familiar and sporadic, in order to evaluate the frequency of mtDNA alterations as a cause of deafness and to describe the audiological manifestations of mitochondrial non-syndromic SNHL. In agreement with previous studies, we found the A1555G mutation to be responsible for a relevant percentage (5.4 %) of cases affected with isolated idiopathic sensorineural hearing impairment.

Key words: cochlea, deafness, mitochondrial DNA (mtDNA), mutation, non-syndromic sensorineural hearing loss

INTRODUCTION
Over the last decade, knowledge of the genetic cause of deafness has considerably increased. Among the genetic factors, mtDNA (mitochondrial DNA) mutations are clearly responsible for several forms of syndromic and non-syndromic deafness, although the role of these mutations is still poorly understood [1].

The cochlea is very sensitive to mitochondrial dysfunctions, because its function is highly energy-dependent or the hair cells do not replicate. The exact mechanism of cochlear damage in mtDNA-associated disorders is still unclear. Normal hearing is dependent upon the function of hair cells and the stria vascularis, which maintain the ionic gradients necessary for sound signal transduction [1,2]. Both the stria vascularis and the hair cells are highly metabolically active and rich in mitochondria, so can be easily compromised by a dysfunction in mitochondrial ATP production as a consequent of mtDNA mutations [1]. Moreover, the hair cells of the cochlea do not replicate and consequently tend to accumulate mutant mtDNA. For this reason progressive SNHL (sensorineural hearing loss), in both its syndromic and non-syndromic forms, has been associated with a large number of mtDNA alterations [2–15]. Similarly to other mitochondrial pathologies, deafness due to mitochondrial dysfunction shows wide clinical variations. This variability is most likely to be related to the complex interaction between mtDNA, nuclear DNA and environmental factors, so that a mtDNA alteration probably causes deafness only in association with other genetic or environmental cofactors [1,16,17].

Non-syndromic progressive SNHL has been shown to be associated with mitochondrial genomic dysfunctions. This is demonstrated by the presence of mtDNA mutations in families with non-syndromic progressive SNHL with a maternal inheritance, as well as in patients affected with sporadic non-syndromic progressive SNHL.

The most commonly reported non-syndromic deafness-causing mtDNA mutations are: A1555G [1,3,4,16,18], C1494T...
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manifestations of mitochondrial non-syndromic SNHL. The mtDNA alterations as a cause of deafness and to describe the audiological idiopathic non-syndromic SNHL, both familiar and sporadic. The previously known mtDNA mutations in patients affected by syndromic bilateral SNHL, with a 2.7 kb mtDNA deletion [32].

SNHL [31]; we have reported a patient affected by sudden non-progressive SNHL and aminoglycoside-induced SNHL. The mtDNA has been described to be associated with both non-syndromic progressive SNHL and diabetes in whom the A3243G point mutation had been excluded; and mtDNA deletions were also analysed.

All patients with a mtDNA mutation underwent a neurological examination, as well as measuring the level of lactic acid in the blood, both at rest and during exercise. Furthermore, after having obtained their informed consent, a muscle biopsy was performed. Routine histological and histochemical studies, as well as biochemical analysis of the respiratory chain enzyme activities, were performed as described previously [34]. The above-mentioned point mutations and mtDNA deletions were also investigated in mtDNA extracted from muscle presence, as described below.

Where possible, the relatives of the patients with mtDNA mutations underwent an audiological evaluation and screening for the mutation in peripheral blood lymphocytes.

Screening of mtDNA

Total DNA from patients’ blood or, if available, muscle was extracted using standard protocols [34a]. The mtDNA point mutations were screened by restriction-fragment-length polymorphism PCR analysis. tRNA mutations were detected by single-strand conformational polymorphism analysis. A set of primers that amplifies all the tRNA-coding regions was used. PCR was performed in 25 μl of 10 mM Tris/HC1 (pH 8.9) containing 0.4 μM each of the forward and reverse oligonucleotide, 1.5 mM MgCl2, 0.2 mM each of dATP, dGTP and dTTP, 0.02 mM dCTP, 1 μCi of [α-32P]dCTP and 1.25 units of Taq DNA polymerase (Roche). PCR conditions used were: 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 30 s, and a final extension step at 72 °C for 7 min. Samples were denatured and separated on a 6 % MDE (mutation detection enhancement) polyacrylamide gel (BME) with 5 % glycerol, according to the manufacturer’s instructions. The conformations of the single-stranded DNA were revealed by autoradiography using BIOMAX film (Kodak). Samples with abnormal patterns were sequenced directly using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit and a 310 automatic sequencer (Applied Biosystems). In all cases, the presence of the mutations was confirmed by restriction-fragment-length polymorphism PCR. Southern-blot and long-PCR analysis for the presence of mtDNA deletions were performed as described previously [35].

RESULTS

We found the mtDNA A1555G point mutation in nine of the 167 patients (5.4 %) that were affected by idiopathic non-syndromic

EXPERIMENTAL

Patients and study design

Consecutive patients (167) affected by idiopathic non-syndromic SNHL were studied at the ENT Unit of the University of Pisa, in collaboration with the Neurological Unit, with the aim to investigate the presence of mtDNA alterations as a cause of deafness. The study was composed of 81 males and 86 females, with a mean age of 59 years (ranging from 15 to 76 years). All patients gave their informed consent to participate in the present study. The study had ethical approval.

Any known cause of SNHL had been excluded previously by means of a complete blood and immunological evaluation, using the method described in [33], including sequence analysis of the connexin 26 and 30 genes. HRCT (high-resolution computed tomography) of the petrous bone and HRMR (high-resolution magnetic resonance) of the labyrinth excluded the presence of inner-ear malformations in all of the patients.

The whole sample of patients was subjected to an accurate audiological evaluation, as follows. An accurate clinical history was performed to investigate the presence of clinical signs or symptoms suggestive of mitochondrial dysfunction and the presence of a familial history of hearing impairment or mitochondrial diseases. Otoscopy and otomicroscopy were performed, as well as pure tone audiometry, speech audiometry, tympanometry and stapedial reflex study, brainstem-evoked auditory potentials and oto-acoustic emissions.

All of the patients included in the study were affected by bilateral SNHL. In the 167 patients, 19 (11.3 %) were pre-lingually deafened and in six (3.6 %) the hearing impairment had a sudden onset. In addition, eight of the 167 patients (4.7 %) had been exposed to aminoglycosides during the neonatal period or later before hearing loss onset.

Investigation of homoplasmic and high-rate heteroplasmic mtDNA mutations and deletions in peripheral lymphocytes was performed in all of the patients, as described below. The following mtDNA point mutations were investigated: A1555G, 961T and T1095C of the 12S rRNA gene; A7445G, 7472insC, T7510C and T7511C of the tRNASer(UCN) gene; A3243G of the tRNALeu(UUR) gene; A8926G was investigated only in patients with diabetes in whom the A3243G point mutation had been excluded; and mtDNA deletions were also analysed.

All patients with a mtDNA mutation underwent a neurological examination, as well as measuring the level of lactic acid in the blood, both at rest and during exercise. Furthermore, after having obtained their informed consent, a muscle biopsy was performed. Routine histological and histochemical studies, as well as biochemical analysis of the respiratory chain enzyme activities, were performed as described previously [34]. The above-mentioned point mutations and mtDNA deletions were also investigated in mtDNA extracted from muscle presence, as described below.

Where possible, the relatives of the patients with mtDNA mutations underwent an audiological evaluation and screening for the mutation in peripheral blood lymphocytes.

Screening of mtDNA

Total DNA from patients’ blood or, if available, muscle was extracted using standard protocols [34a]. The mtDNA point mutations were screened by restriction-fragment-length polymorphism PCR analysis. tRNA mutations were detected by single-strand conformational polymorphism analysis. A set of primers that amplifies all the tRNA-coding regions was used. PCR was performed in 25 μl of 10 mM Tris/HC1 (pH 8.9) containing 0.4 μM each of the forward and reverse oligonucleotide, 1.5 mM MgCl2, 0.2 mM each of dATP, dGTP and dTTP, 0.02 mM dCTP, 1 μCi of [α-32P]dCTP and 1.25 units of Taq DNA polymerase (Roche). PCR conditions used were: 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 30 s, and a final extension step at 72 °C for 7 min. Samples were denatured and separated on a 6 % MDE (mutation detection enhancement) polyacrylamide gel (BME) with 5 % glycerol, according to the manufacturer’s instructions. The conformations of the single-stranded DNA were revealed by autoradiography using BIOMAX film (Kodak). Samples with abnormal patterns were sequenced directly using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit and a 310 automatic sequencer (Applied Biosystems). In all cases, the presence of the mutations was confirmed by restriction-fragment-length polymorphism PCR. Southern-blot and long-PCR analysis for the presence of mtDNA deletions were performed as described previously [35].

RESULTS

We found the mtDNA A1555G point mutation in nine of the 167 patients (5.4 %) that were affected by idiopathic non-syndromic
SNHL. We did not find any of the other mutations investigated in peripheral blood lymphocytes.

In all of the patients, the sequence analysis of connexin 26 and 30 genes produced a negative result, as did the radiological study of the inner ear. Table 1 summarizes the main features of the patients with the mtDNA mutations. A brief description of the clinical cases with the mutations is as follows.

### Patient 1

This patient (54 years old, male) was affected by isolated slowly progressive SNHL, which started at the age of 27 years after streptomycin exposure. The audiometric pattern showed a sloping hearing loss (Figure 1A). The homoplasmic A1555G point mutation was detected in blood. The patient’s family history was positive for hearing impairment, with a matrilineal pattern of transmission (Figure 1B). Not all of the hearing-impaired relatives had been exposed to aminoglycosides. We analysed the mtDNA from peripheral lymphocytes of the patient’s brothers, sister and nephews, and we detected the homoplasmic A1555G mutation in all of them (Figure 1B).

The vestibular function examined in the proband was normal. HRCT of the petrous bone and HRMR of the labyrinth were both normal. Neurological examination, as well as lactic acid levels, were both normal. Signs of mitochondrial dysfunction were present at muscular level, as demonstrated by the presence of ragged-red and COX (cytochrome c oxidase)-negative fibres (Figure 1C). The ultrastructural analysis showed mitochondria with focal loss of cristae and crystalline-like bodies.

### Patient 2

This patient (34 years old, female) was affected by isolated slowly progressive SNHL, which started at the age of 15 years. The hearing loss was mild to moderate, and the audiogram shape was flat (Figure 2A). The patient had not been exposed to aminoglycosides. The homoplasmic A1555G point mutation was detected in blood. The patient’s family history was negative for hearing impairment (Figure 2B). The A1555G mutation was also detected in the patient’s mother, sister and daughter, although none of them had any hearing loss (Figure 2B).

The vestibular function examined in the proband was normal. Both HRCT of the petrous bone and HRMR of the labyrinth were normal. Neurological examination, as well as lactic acid levels, were both normal. Ragged-red and COX-negative fibres were also observed in muscle biopsy, and ultrastructural studies showed focal loss of cristae and the presence of crystalline bodies within mitochondria (Figure 2C).

### Patient 3

This patient (66 years old, female) was affected by profound bilateral SNHL, which started at the age of 24 years, after streptomycin consumption and progressively worsened. She has used bilateral hearing aids, without substantial benefits and was a candidate for cochlear implantation. The patient had the homoplasmic A1555G mutation. The proband had three daughters, all positive for the A1555G mutation; two of them were affected by SNHL. One of the two hearing-impaired daughters developed the hearing loss after aminoglycoside exposure. A vestibular function examination showed bilateral labyrinth areflexia. HRCT of the petrous bone and HRMR of the labyrinth were both normal. Neurological examination was normal, whereas lactic acid kinetic study showed slightly abnormal lactic acid levels after exercise. The patient refused to participate in muscular biopsy.

### Patient 4

This patient (36 years old, female) was affected by bilateral SNHL, which started at the age of 18 years and progressively worsened. From the age of 20, she used bilateral hearing aids. No exposure to aminoglycosides was known. The patient had two severely hearing-impaired twins (aged 3 years) who had been exposed to aminoglycosides in neonatal intensive care unit. The mother and both sons carried the homoplasmic A1555G mutation. The vestibular function in the proband was normal. HRCT of the petrous bone and HRMR of the labyrinth were both normal. Neurological examination was normal, as well as lactic acid levels. The patient refused to participate in muscular biopsy.

### Patient 5

This patient (40 years old, female) was affected by severe bilateral SNHL, which started at the age of 19 years and progressively worsened. She has used a unilateral hearing aid since the age of 26 years. The patient had never been exposed to aminoglycosides. We detected the homoplasmic A1555G mutation in peripheral lymphocyte mtDNA. The patient’s family history was negative for hearing impairment, and she has a normal-hearing daughter. Both mother and daughter have the A1555G mutation.

The vestibular function in the mother was normal. HRCT of the petrous bone and HRMR of the labyrinth were both normal. Neurological examination, as well as lactic acid levels, were both normal. The muscle biopsy was normal.

### Patient 6

This patient (50 years old, female) was affected by bilateral down-sloping SNHL, which started during childhood and progressively worsened (Figure 3A). The patient had not been exposed to aminoglycosides. The family history was positive for hearing impairment. The patient had two hearing-impaired brothers and one normal-hearing sister. One of the brothers, with severe bilateral hearing loss, had been exposed to streptomycin before the onset of the hearing loss. The patient, her brothers and sister had the homoplasmic A1555G mutation. The mutation was also detected in her sons who had no hearing impairment (Figure 3B).

The vestibular function explored in the proband was normal. HRCT of the petrous bone and HRMR of the labyrinth were both normal. Neurological examination, as well as lactic acid levels, were also normal. The muscle biopsy was normal.
Table 1 General and audiological features of the sample of patients with mtDNA mutations
HL, hearing loss; SDH, succinate dehydrogenase.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (years)</th>
<th>mtDNA mutation</th>
<th>SNHL degree</th>
<th>Age at onset (years)</th>
<th>HL progression</th>
<th>Site of lesion</th>
<th>Vestibular function</th>
<th>Aminoglycoside exposure</th>
<th>Family history positive for HL</th>
<th>Lactic acid levels</th>
<th>Muscular biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>54</td>
<td>A1555G</td>
<td>Moderate</td>
<td>Bilateral, down-sloping</td>
<td>Yes</td>
<td>Cochlear</td>
<td>Normal</td>
<td>Yes*</td>
<td>Yes</td>
<td>Yes</td>
<td>Normal</td>
</tr>
<tr>
<td>2</td>
<td>Female</td>
<td>34</td>
<td>A1555G</td>
<td>Mild-to-moderate</td>
<td>Bilateral, flat</td>
<td>15</td>
<td>Yes</td>
<td>Cochlear</td>
<td>Normal</td>
<td>No</td>
<td>No</td>
<td>Normal</td>
</tr>
<tr>
<td>3</td>
<td>Female</td>
<td>66</td>
<td>A1555G</td>
<td>Profound</td>
<td>Bilateral, down-sloping</td>
<td>24</td>
<td>Yes</td>
<td>Cochlear, Bilateral labyrinth areflexia</td>
<td>Yes*</td>
<td>Yes</td>
<td>Abnormal</td>
<td>Not carried out</td>
</tr>
<tr>
<td>4</td>
<td>Female</td>
<td>36</td>
<td>A1555G</td>
<td>Severe</td>
<td>Bilateral, down-sloping</td>
<td>18</td>
<td>Yes</td>
<td>Cochlear</td>
<td>Normal</td>
<td>No</td>
<td>Yes</td>
<td>Normal</td>
</tr>
<tr>
<td>5</td>
<td>Female</td>
<td>40</td>
<td>A1555G</td>
<td>Severe</td>
<td>Bilateral, down-sloping</td>
<td>19</td>
<td>Yes</td>
<td>Cochlear</td>
<td>Normal</td>
<td>No</td>
<td>No</td>
<td>Normal</td>
</tr>
<tr>
<td>6</td>
<td>Female</td>
<td>50</td>
<td>A1555G</td>
<td>Moderate</td>
<td>Bilateral, down-sloping</td>
<td>Childhood</td>
<td>Yes</td>
<td>Cochlear</td>
<td>Normal</td>
<td>No</td>
<td>Yes</td>
<td>Normal</td>
</tr>
<tr>
<td>7</td>
<td>Female</td>
<td>49</td>
<td>A1555G</td>
<td>Severe</td>
<td>Bilateral, down-sloping</td>
<td>6</td>
<td>Yes</td>
<td>Cochlear</td>
<td>Normal</td>
<td>No</td>
<td>Yes</td>
<td>Normal</td>
</tr>
<tr>
<td>8</td>
<td>Male</td>
<td>66</td>
<td>A1555G</td>
<td>Profound</td>
<td>Bilateral, down-sloping</td>
<td>Childhood</td>
<td>Yes</td>
<td>Cochlear</td>
<td>Normal</td>
<td>No</td>
<td>Yes</td>
<td>Negative</td>
</tr>
<tr>
<td>9</td>
<td>Male</td>
<td>71</td>
<td>A1555G</td>
<td>Profound</td>
<td>Bilateral, down-sloping</td>
<td>Childhood</td>
<td>Yes</td>
<td>Cochlear</td>
<td>Normal, Yes†</td>
<td>Yes†</td>
<td>Yes†</td>
<td>Normal</td>
</tr>
</tbody>
</table>

*HL started after aminoglycoside exposure.
†HL worsened after aminoglycoside exposure.
‡Family history was not clearly suggestive of a matrilinear transmission.
Mitochondrial non-syndromic sensorineural hearing loss

Figure 1  Data for Patient 1

(A) Audiogram showing down-sloping hearing loss [BC = AC, bone conduction = air conduction; circle, right air conduction; cross, left air conduction; x-axis, frequency (Hz); y-axis, hearing level (dB)]; (B) matrilinear pattern of transmission of hearing impairment in the family; (C) muscle biopsy, with immunohistochemical staining for COX (asterisk indicates COX-negative fibres). Magnification, × 20.

Patient 7
This patient (49 years old, female) was affected by bilateral severe down-sloping SNHL, which started at the age of 6 years and progressively worsened. The patient also suffered from Type 2 diabetes and hypertension. The patient had not been exposed to aminoglycosides. The patient’s mother was hearing-impaired. The patient had two sons, both with normal hearing. We detected the homoplasmic A1555G mutation in the proband. The other relatives were not analysed.

The vestibular function was normal. HRCT of the petrous bone and HRMR of the labyrinth were both normal. Neurological examination, as well as lactic acid levels, were both normal. The muscle biopsy was normal.

Patient 8
This patient (66 years old, male) was affected by bilateral profound SNHL, which started during childhood and progressively worsened. At the age of 25 years, when the hearing loss became severe bilaterally, the patient started using hearing aids, without substantial benefits. The patient had not been exposed to aminoglycosides. The patient also suffered from Type 2 diabetes. The patient had four brothers, all of them hearing-impaired; in particular, three of them were affected by severe bilateral hearing loss and were hearing-aid users. The patient’s daughter had normal hearing. We detected the homoplasmic A1555G mutation in the patient and in his brothers.

The vestibular function in the proband was normal. HRCT of the petrous bone and HRMR of the labyrinth were both normal. Neurological examination as well as lactic acid levels were both normal. The muscle biopsy was normal.

Patient 9
This patient (71 years old, male) was affected by bilateral profound SNHL, which started during childhood and progressively worsened after streptomycin consumption at the age of 28 years. At 28 years, when the hearing loss became severe bilaterally, the patient started using hearing aids, without substantial benefits. The patient was also affected by a mild form of Type 2 diabetes.
Figure 2 Data for Patient 2
(A) Audiogram (flat-shape) [see Figure 1(A) for an explanation of the symbols etc.]. (B) Muscle fibres with accumulation of mitochondria with variable shapes and dimensions, within intermyofibrillar and subsarcolemmal sites. Mitochondria present focal loss of cristae and crystalline-like bodies. Magnification, $\times 25\,000$. (C) Pedigree.

○§ subjects harbouring the A1555G point mutation
○* exposure to aminoglycosides
○ not exposed to aminoglycosides

○ Woman normal hearing
● Woman with hearing loss
□ Man normal hearing
■ Man with hearing loss

Figure 3 Data for Patient 6
(A) Audiogram showing down-sloping SNHL [see Figure 1(A) for an explanation of the symbols etc.]; (B) pedigree.

○§ subject harbouring the A1555G point mutation
○* exposure to aminoglycosides
○ not exposed to aminoglycosides

○ Woman normal hearing
● Woman with hearing loss
□ Man normal hearing
■ Man with hearing loss
The patient had two brothers and one of them was hearing-impaired and a hearing-aid user. The patient’s daughter was also hearing-impaired. We have detected the homoplasmic A1555G mutation in the father, but not in the daughter.

The vestibular function in the proband was normal. HRCT of the petrous bone and HRMR of the labyrinth were both normal. Neurological examination, lactic acid levels and muscle biopsy were normal.

**DISCUSSION**

Genetic deafness accounts for approx. 60% of SNHL; mtDNA alterations play an important role in genetic causes of deafness. In this regard, over the last decade, a number of distinct mutations in the mtDNA have been found to be associated with both syndromic and non-syndromic forms of hearing impairment [1,16,36], although their real incidence is poorly understood and generally underestimated. The reported frequency of mitochondrial deafness is quite variable, ranging from 3 to 27%, depending on the variability of the studies of the literature [23,37–42]; in fact, most of the studies are difficult to interpret, due to both the differences in the patients' selection criteria (sporadic or familial cases) and the variability in the mutations investigated, and this lack of uniformity leads to quite variable and not completely comparable results [23,37–42]. In this regard the reported frequency of the most frequent deafness-related mtDNA mutations, A1555G and A3243G, range from 3 to 6.9% in sporadic cases [11,38,42], whereas a higher rate is generally reported in familial cases, ranging from 16 to 27% [5,8,17].

In agreement with previous work, our present study demonstrates that mitochondrial dysfunctions are a relevant cause of sensorineural deafness: 5.4% (9/167) of our patients affected with idiopathic SNHL have the A1555G mutation, thus confirming that this point mutation is the most frequent among the known mtDNA mutations related to sensorineural deafness and is globally one of the most relevant causes of genetic deafness. In our patients, we did not observe any other mtDNA mutations. In a previous study [43], we detected the A3243G point mutation at a very low level (3%) in muscle and urine, but not in peripheral blood, in a patient affected with profound SNHL and submitted to cochlear implant. The A3243G mutation is one of the most common mtDNA mutations related to deafness, and it is considered to cause mainly syndromic deafness and rarely non-syndromic hearing loss [1,2]. Oshima et al. [38] described three patients with the A3243G mutation affected with non-syndromic SNHL, reporting a wide variability in the audiological features, as well as in the evidence of a clear matrilineal kind of transmission. Chinnery et al. [2] reported two A3243G cases with non-syndromic SNHL, and, to our knowledge, this is the first case observed in Italy of non-syndromic deafness with a very low level of the A3243G mutation. These data reinforce the finding that this mutation can be responsible for both syndromic and non-syndromic deafness, similarly to other mtDNA mutations such as A7445G [1]. In our patient, the A3243G mutation was present at very low level in muscle (3%) and in urine (1%), whereas it was not present at in peripheral blood and it is difficult to explain how such low levels of mutant mtDNA cause mitochondrial dysfunction and clinical symptoms [43]. We can speculate that a mutation with high pathogenic potential such as A3243G may be clinically limited to deafness if present at very low levels. Furthermore, this case confirms that mitochondrial deafness is suitable for treatment by cochlear implantation, as reported previously [44–47].

As shown by the above-mentioned case, the heteroplasmic mtDNA mutations can be missed if only peripheral blood lymphocytes are investigated, and not the muscle. Therefore we can speculate that the incidence of mtDNA mutations as a cause of deafness could be more frequent than reported in the literature.

In addition to being one of the most common causes of nonsyndromic SNHL, the homoplasmic A1555G point mutation in the mitochondrial 12S rRNA gene has been described in association to aminoglycoside-induced deafness. This mutation was first discovered by Prezant et al. [12] in a large Arab–Israeli family, with a striking pattern of maternal inheritance and a quite variable clinical expression. Later it was detected in various ethnic groups from Europe [4,5,8,18,39], Asia [23,40–42] and Africa [37], both in familial and in sporadic cases [4,18,23,29,48–50]. The reported prevalence varies from 0.5 to approx. 5% in Europe and 3.45% in Japan [4,49,51], although higher percentages have been found in Spain [5,8,17]. This reported variability is probably due to the heterogeneity of the samples of the various studies and to the different use of aminoglycoside antibiotics among the different countries.

The phenotype varies considerably among matrilineal relatives within families or among different families, ranging from severe deafness to moderate progressive hearing loss, or even completely normal hearing [1,3,4,16,18]. Ballana et al. [16] found that 63% of the subjects with the A1555G mutation presented SNHL, whereas the remaining 37% were asymptomatic. In several cases, a bilateral down-sloping SNHL has been described, whereas flat or rising audiograms have been reported in a minority of cases. In the patients described in the present study, the hearing threshold shape was down-sloping in all, with the exception of one patient who showed a flat curve. The age of onset is also very variable. It is more common in adult or young adult age, although congenital or childhood-onset cases have been reported [3,48]. In the present study, the hearing-loss onset was found to be in childhood or adulthood. The site of lesion is generally reported to be the cochlea, as demonstrated by oto-acoustic emissions, stapedia reflex study and brainstem-evoked auditory potential results [3,4,7,8,11,52,53]. Usually, the vestibular function is normal, which is also the case with aminoglycoside exposure [1,3,54]. The relationship between A1555G and aminoglycoside exposure is also quite variable, and aminoglycosides seem to play a role only in approx. 20% of the cases. Today, in developed countries, aminoglycosides are used rarely and this could affect the data [1,4,8,55,56]. Aminoglycosides seem to enhance the susceptibility to develop hearing loss in patients with this mutation. However, even in the absence of aminoglycoside...
exposure, the mutation could be itself responsible of hearing impairment [1,4,8,5,56]. Other studies have reported that patients carrying the A1555G mutation exposed to aminoglycosides develop a more severe deafness with an earlier onset [3,4,7,8,18,20,57]. However, other studies did not confirm that hypothesis [58]. Two out of the three patients in our present study with profound SNHL had been exposed to aminoglycosides.

The expression of the deafness phenotype in carriers of the A1555G mutation probably requires the contribution of additional environmental and/or genetic factors. The factors that could modulate the expressivity of this mutation in hearing impairment have not yet been resolved and some hypotheses have been proposed. Aminoglycosides are probably only one of the factors interacting with the mutation in determining the deafness phenotype. Mitochondrial haplotypes may explain some of the differences between families and ethnic groups [59,60], and also the nuclear background may modulate the phenotypic expression of the mutation [61,62]. Studies by Bykhovskaya et al. [48,63,64] have revealed that nuclear-modifying factors are likely to be numerous and, although a region in chromosome 8p23 has been proposed as a putative localization for a modifier locus, the gene has not been identified. The connexin 26 gene has also been proposed to be a modifier gene, but, according to Lopez-Bigas et al. [52], mutations in the connexin 26 gene do not seem to modify the deafness phenotype arising from the A1555G mutation. In the present study, connexin 26 and 30 genes mutation were not present in any of the subjects with the A1555G mutation.

In 2003, Del Castillo et al. [5] found some individuals (5.7 % of the subjects) with a variable degree of heteroplasmy (from 3.75 to 96.6 %). The authors [5] attempted to correlate the rate of heteroplasmy to the severity of the phenotype and found that most of the patients with a low rate of heteroplasmy (below 20 %) presented normal hearing or a mild hearing impairment, whereas subjects with a high rate of heteroplasmy (above 52 %) were affected by moderate-to-severe SNHL [5].

The variability of the phenotype related to mtDNA mutations is confirmed also by our present study. The audiological characteristics of our patients with the A1555G mutation are quite variable. The degree of hearing impairment was mild-to-moderate in one case, moderate in two cases, severe in three patients and profound in the remaining three. The audiometric threshold shape was also variable, showing a down-slope in eight cases and flat in one patient. The hearing loss was progressive in all of the patients, started in childhood in four patients and in young adulthood in the remaining five. With regards to aminoglycoside exposure, only two out of the nine patients carrying the A1555G mutation developed hearing loss after exposure to those antibiotics, whereas in one patient there was a progression of the hearing impairment after streptomycin exposure. A matrilinear transmission was clearly evident in six out of nine patients, whereas in one patient the family history was positive for hearing loss, but not suggestive of a matrilinear inheritance. The site of lesion was the cochlea in all of the patients, whereas the vestibular function was normal in every A1555G patient, but one, who presented a bilateral labyrinth areflexia and who had been exposed to aminoglycosides (Table 1).

Some variability was also present between members of the same family, regarding both the degree of hearing loss and the association between hearing-loss onset and aminoglycoside exposure.

In six of the nine patients with the A1555G mutation we have carried out a muscle biopsy, and, interestingly, in two of the patients we found muscular abnormalities that were consistent with mitochondrial dysfunction, such as red-ragged and COX-negative fibres, and mitochondrial ultrastructural abnormalities. To our knowledge, only one study has been published in which a muscular study was performed in patients with non-syndromic deafness with the A1555G point mutation [65]. These authors [65] found muscular alterations in the two patients subjected to muscular biopsy. These findings, demonstrating muscular involvement in some patients affected by isolated deafness and harbouring the A1555G mutation, are extremely interesting, and future research may explain whether muscular involvement in these cases plays a role in the variability of the phenotypic expression of the mutation.

It is worth noting that three of the nine patients carrying the A1555G mutation were affected by Type 2 diabetes. To our knowledge, this is the first time that diabetes has been reported in patients with the A1555G mutation and deafness, and further studies are necessary to establish whether this association is incidental or related to the mutation. In the literature, there are only sporadic cases reported of A1555G patients affected with hearing impairment associated with other clinical manifestations, such as Parkinson’s disease, spinal and pigmentary disturbances [66] and restrictive cardiomyopathy [1,13].

**Conclusions**

Mitochondrial-related hearing loss can present in a variety of clinical forms, both syndromic and non-syndromic, with variable degrees of severity. mtDNA mutations are a relevant cause of non-syndromic hearing loss, and the A1555G point mutation is one of the most frequent causes of genetic deafness.

In agreement with previous studies, we found the A1555G mtDNA mutation to be responsible for a relevant percentage (5.4 %) of cases affected with isolated idiopathic sensorineural hearing impairment. The age of onset and the audiological features (severity of hearing loss, the hearing threshold shape, and relationship to aminoglycoside exposure) were quite variable, both between different families and within the same families.

Interestingly, in two patients with the A1555G mutation, we found muscular abnormalities. Further research could explain whether this mutation, rather than being an organ-specific mutation, might cause a systemic disorder or whether the muscular involvement in these cases plays a role in the variability of the phenotypic expression of the mutation. Additionally, in three out of the nine A1555G patients, Type 2 diabetes was associated with deafness. Further studies are needed to establish whether the association is incidental or related to the A1555G mutation phenotype.

Finally, we want to highlight that, as mtDNA mutations represent one of the most frequent causes of deafness, they should be routinely screened for, particularly as their identification has
quite different genetic counselling implications compared with nuclear gene mutations. Moreover, because of the susceptibility of individuals carrying the A1555G mutation and their maternal relatives to aminoglycoside exposure, at the very least genetic screening for this mutation among deaf patients should be considered. The early detection of the mutation would enable prevention of the onset and/or progression of hearing loss (such as avoiding exposure to aminoglycosides).

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