# ARTICLES

# Hearing impairment and neurological dysfunction associated with a mutation in the mitochondrial tRNA$^{Ser(UCN)}$ gene

Kristien Verhoeven[1], Robbert JH Ensink[2], Valeria Tiranti[3], Patrick LM Huygen[2], David F Johnson[4], Isabelle Schatteman[1], Lut Van Laer[1], Margriet Verstreken[1], Paul Van de Heyning[5], Nathan Fischel-Ghodsian[4], Massimo Zeviani[3], Cor WRJ Cremers[2], Patrick J Willems[1] and Guy Van Camp[1]

[1]Department of Medical Genetics, University of Antwerp (UIA), Belgium
[2]Department of Otolaryngology, University Hospital Nijmegen, The Netherlands
[3]Division of Biochemistry and Genetics, National Neurological Institute 'Carlo Besta', Milan, Italy
[4]Medical Genetics Birth Defects Center, Departments of Medicine and Pediatrics, Cedars-Sinai Medical Center and UCLA School of Medicine, Los Angeles, CA, USA
[5]ENT Department, University Hospital of Antwerp, Belgium

We studied a large Dutch family with maternally inherited, progressive, sensorineural hearing loss in 27 patients. Only in a single family member was the hearing loss accompanied by neurological symptoms including ataxia and dysarthria. DNA analysis of the mitochondrial genome revealed the insertion of a C at nucleotide position 7472 in the $tRNA^{Ser(UCN)}$ gene (7472insC mutation). We determined the percentage of mutant DNA (heteroplasmy) in blood from all family members, and found no correlation between hearing loss and leucocyte heteroplasmy. The 7472insC mutation was previously identified in a smaller family from Sicily with sensorineural hearing loss in 9 family members, six of them also presenting neurologically with ataxia and myoclonus. The presence of the 7472insC mutation in two different pedigrees strongly supports its pathogenicity. However, the interfamilial difference in penetrance of the neurologic abnormalities is most likely to be strongly influenced by secondary factors different from the 7472insC mutation, as heteroplasmy or age of the patients were similar in both families. This mutation should therefore be analysed in families with maternally inherited hearing loss, irrespective of whether the hearing loss is non-syndromic or accompanied by neurologic abnormalities.

Keywords: hearing impairment; transfer RNA$^{Ser(UCN)}$; mitochondrial DNA; heteroplasmy; ototoxicity

# Introduction

Several human tissues and organs like the brain, retina, muscle, kidney and endocrine glands are highly dependent on the energy produced by mitochondrial oxidation. As a consequence these tissues are more vulnerable to mitochondrial defects.[1,2] In almost all mitochondrial diseases identified so far one or more of these tissues or organs are involved. In many of these, hearing loss is an accompanying symptom. This is probably caused by a high reliance of the inner ear on mitochondrial energy production.[3]

Until now only three different mutations in the mitochondrial genome have been reported to cause hearing loss as a sole or predominant symptom. The first mutation, an A to G substitution at position 1555 in the *12S rRNA* gene (1555A☐G), was reported in a large Israeli-Arab family with maternally inherited, non-syndromic hearing loss.[3] This mutation was also found in many other pedigrees with non-syndromic deafness,[4–6] and in patients and families with aminoglycoside-induced ototoxic hearing impairment.[4,7–9] Apparently this mutation makes individuals susceptible to hearing impairment after treatment with aminoglycosides at concentrations that normally do not affect hearing. Even without exposure to aminoglycosides many patients with this mutation develop hearing impairment, but it is currently not known which other factors are involved in the etiology.

A second mutation of the mitochondrial genome was described in a Scottish family with maternally-inherited, bilateral, sensorineural hearing loss. The mutation substituted a G for an A at position 7445 (7445A☐G) of the mtDNA[10] and has subsequently been observed in a second pedigree from New Zealand.[11]

The insertion of a C at nucleotide position 7472 in the *tRNA*[Ser(UCN)] gene (7472insC) has been reported in a Sicilian family with maternally-inherited hearing loss as a predominant feature.[12] In six out of nine patients the hearing loss was accompanied by ataxia, dysarthria and focal myoclonus.

In the present study we report a second family with the 7472insC mutation. Although neurological abnormalities were found in only a single patient from this large family, no significant difference in heteroplasmy was found between this family and the first family from Sicily, in which seven patients have neurological problems. These results indicate that the strongly reduced penetrance of neurological symptoms in this family is most likely caused by other factors besides the 7472insC mutation.

# Patients and Methods

*Audiometric Studies*

Sixty-four family members including 10 spouses participated in this study (Figure 1). A general otological examination and pure tone audiometry with air and bone conduction was performed. Pure tone average hearing thresholds at 0.25, 0.5, 1, 2, 4 and 8 kHz were determined. Patients with a hearing threshold below the 95th percentile of an age and sex dependent audiometric curve (ISO) were considered to be hearing impaired. Family members with a hearing threshold better than 20 dB or above the 50th percentile were considered to have normal hearing.

Twenty-seven family members were found to be hearing impaired. Their hearing loss was accompanied by tinnitus in the majority of patients, and by dizziness in about one third of patients. Two family members (I:1 and II:16) showed a documented acute drop in their hearing levels after treatment with streptomycin for tuberculous spondylitis and pneumonia, respectively. A third family member from the maternal line (who did not participate in this study) had hetero-anamnestically an acute loss of hearing after 2 weeks of treatment with streptomycin for tuberculous pneumonia. One patient with hearing loss (II:30) had a history of noise exposure and was therefore given an unknown affection status.

In all patients, hearing loss was sensorineural and progressive, mainly affecting the high frequencies in the initial stage and gradually affecting lower frequencies with increasing age. Hearing loss was first noted in the patients between 12 and 45 years of age. Nearly all family members from the maternal line older than 30 were affected, except for four family members, between 38 and 47 years old (II-3, II-6, II-24, II-26).

*Clinical Examination*

All family members were interviewed about their clinical history, and the presence of possible neurological complaints was investigated by questionnaire. Nine different family members of the maternal line were selected for a neurological examination. Eight family members ranging in age from 35 to 55 had no neurological abnormalities, but most of them showed a mild general neurological picture which can best be described as clumsiness. Only the proband (patient I-5) had truncal and limb ataxia, dysarthria and motor and sensory polyneuropathy in the limbs. MRI of the proband's brain showed atrophy of the cerebellar vermis and multiple subcortical white matter lesions. Two deceased brothers of the proband had suffered from a neurological picture similar to that of the proband. However, no clinical data could be recovered from these deceased patients. Ophthalmological examination of four patients was normal with no evidence of tapetoretinal degeneration. Three patients (I-4, I-5, I-13) reported diabetes mellitus type II.

*MtDNA Mutation Analysis*

Blood samples were taken after informed consent. Total DNA was extracted from blood samples by standard techniques. To detect large mitochondrial rearrangements, Southern blot analysis was performed. The presence of the 7445A☐G and 1555A☐G mutations associated with hearing loss, was investigated.[3,10] Analysis of the 7472insC mutation in
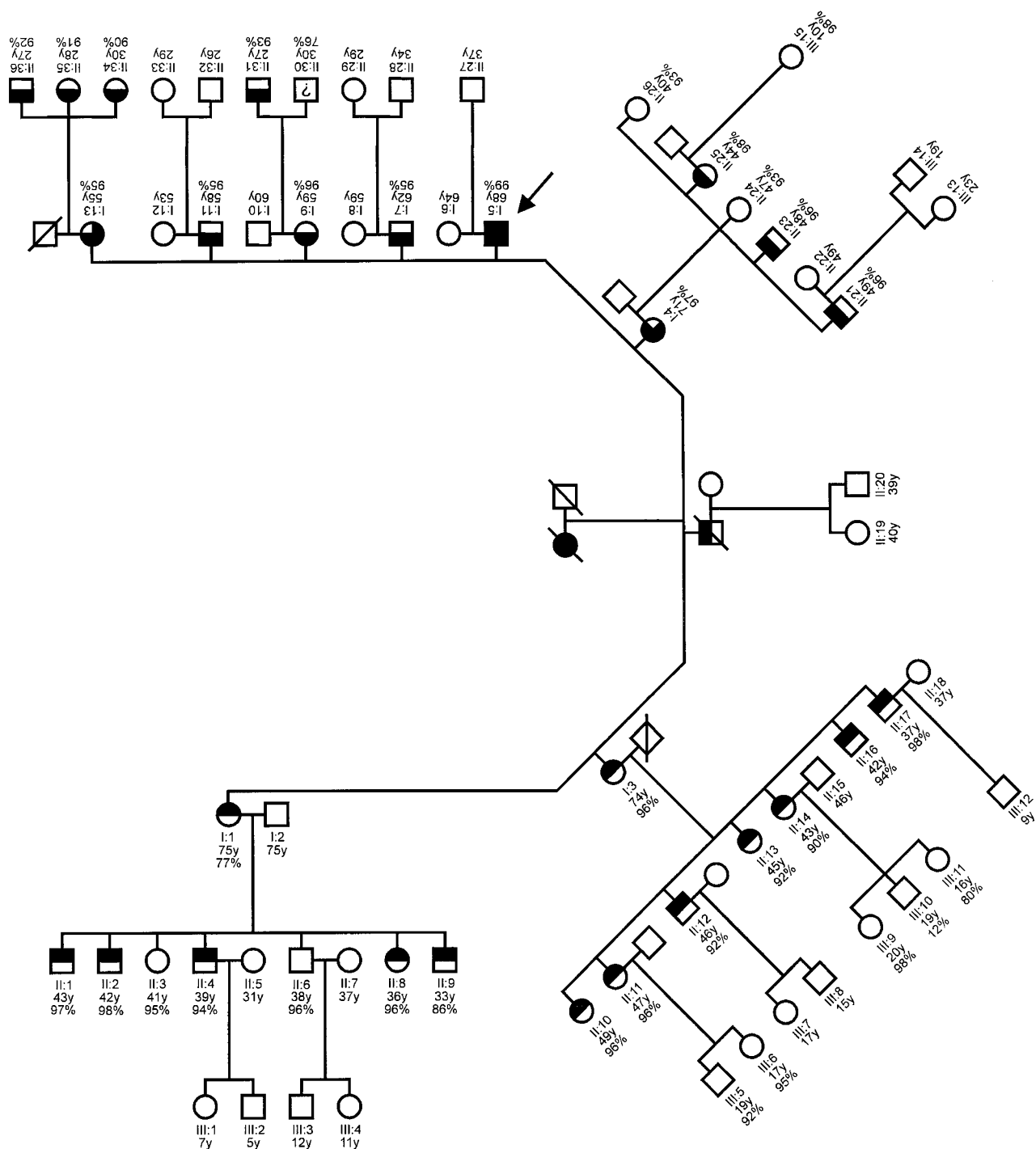
**Figure 1** *Pedigree of the Dutch family members who participated in this study. Only the family members from whom a DNA sample was available are numbered. In all family members of the maternal line the presence of the 7472insC mutation was demonstrated. Patients with hearing loss only are indicated by a half-filled symbol (◐,◨). Patients with hearing loss and diabetes mellitus type II are indicated by a three-quarter filled symbol (◕,◧). The patient with hearing loss, diabetes mellitus and neurological symptoms is indicated by a solid symbol (■). Unaffected family members are indicated with open symbols (□,○). Individual II-30 had hearing loss probably caused by noise exposure, and was therefore given an unknown affection status (?). The ages of the living family members and the percentage of mutant mitochondrial DNA are indicated below the symbols.*

the *tRNA*<sup>Ser(UCN)</sup> gene was done by modified PCR amplification followed by digestion with XcmI, as previously described.[12]

In order to determine the percentage of mutant mtDNA, solid phase mini sequencing (SPMS) analysis was carried out as described previously.[12] DNA sequencing of the PCR fragment containing the 7472insC mutation was performed on a Perkin-Elmer automated DNA sequencer using dye-primer cycle sequencing kits (PE).

*Statistical Analysis*
We wished to find out whether there was a significant correlation between the percentage of mutant mtDNA and the hearing loss. Because the percentage of mutant mtDNA was clearly not normally distributed, we used nonparametric statistics. Spearman's rank correlation test was used to evaluate the correlation and the Mann-Whitney test was used for comparing differences in heteroplasmy between groups of patients with different symptoms and signs. The level of significance used was $P = 0.05$ in all tests.
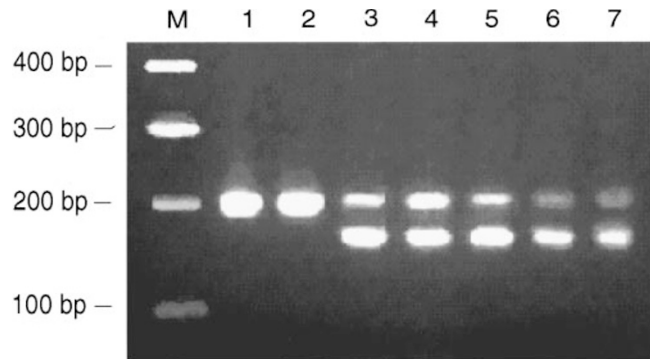


**Figure 2** *PCR amplification and XcmI digestion of a mitochondrial DNA fragment (position 7432–7640). In patients II-9, I-1, I-3, II-12 and I-13 with the 7472insC mutation (lanes 3 to 7), the 208 bp PCR product is cut into two fragments of 168 bp and 40 bp. The 40 bp fragment is not visible on the gel. Due to heteroplasmy, the undigested fragment remains visible. Lanes 1 and 2 show controls in which the 208 bp product remains undigested. The left lane (M) contains a 100 bp ladder as size standard.*

## Results

*MtDNA Mutation Analysis*
As the hearing loss was inherited maternally, a mitochondrial inheritance was assumed and mutation analysis of the mtDNA was performed. Southern blot analysis of five patients revealed no large mtDNA rearrangements.

Three different mitochondrial point mutations have been reported in association with hearing loss as a sole or predominant symptom, including the 1555A□G mutation,[3] the 7445A□G mutation[10] and the 7472insC mutation.[12] PCR amplification from lymphocyte DNA followed by digestion with a specific restriction enzyme, was performed in ten family members from the maternal line in order to examine the presence or absence of these three point mutations. The 1555A□G and 7445A□G mutations were not present in any of the patients. However, the 7472insC mutation was present in all ten family members. The 208 bp fragment is cut into two fragments measuring 168 bp and 40 bp by XcmI in the patients with the mutation (Figure 2). In controls, the PCR fragment remains uncut. To prove the presence of the 7472insC mutation in this family by sequence analysis, we PCR-amplified and cloned an mtDNA fragment containing the 7472insC mutation in the *tRNA*<sup>Ser(UCN)</sup> gene from two patients (II-21, I-7). Sequence analysis of the cloned material of the two patients revealed the insertion of a seventh cytosine in a stretch of six cytosines (7467–7472) that are part of the *tRNA*<sup>Ser(UCN)</sup> gene. Subsequently, all family members of the maternal lineage, including those without hearing loss, were tested by PCR and restriction digest with XcmI. They all showed the 7472insC mutation (Figure 1). One hundred unrelated normal controls were also tested, but we did not detect the 7472insC mutation.

In most family members with the 7472insC mutation the normal band remains visible, suggesting heteroplasmy for the mutation. Therefore, the percentages of wild-type and mutant lymphocyte mtDNA were analysed by SPMS. The percentage of mutant mtDNA observed in each family member of the maternal line is shown in Figure 1. Although the heteroplasmy ranges from 12 to 99% mutant mtDNA, the majority of family members showed values between 90 and 99%. In the proband (I-5) the heteroplasmy was determined in lymphocytes as well as in muscle. No difference between percentage of mutant lymphocyte and muscle mtDNA was found.

*Statistical Analysis*
We statistically tested a possible correlation between the degree of hearing impairment at the low frequencies 0.25, 0.5, 1 kHz and at the high frequencies 2, 4 and 8 kHz and the percentage of heteroplasmy. The Spearman's rank correlation test was not significant ($n = 38$, $r = 0.26$ and 0.12) for a correlation between the degree of hearing impairment and heteroplasmy
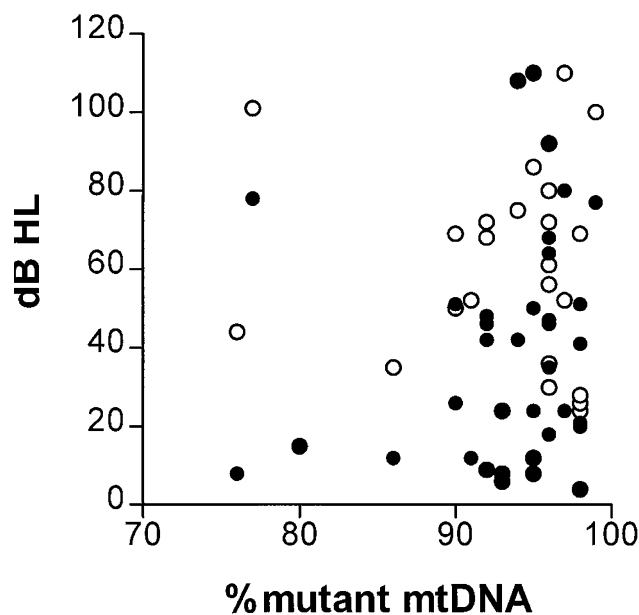
**Figure 3** *Hearing threshold (dB HL) plotted against percentage of mutant mtDNA in 37 members of the family. Average hearing thresholds for the frequencies 0.25, 0.5 and 1 kHz are indicated by a full circle (●); average hearing thresholds at frequencies 2, 4 and 8 kHz are indicated by an open symbol (○). Patient III-10 (12%, 10 dB low, 10 dB high) is not included in the figure for clarity.*

(Figure 3). We also failed to find a significant correlation between age and heteroplasmy (data not shown).

## Analysis of Patients with Aminoglycoside Ototoxicity

As three family members with the 7472insC mutation developed hearing loss after the use of aminoglycosides, we investigated the possibility that this mutation causes susceptibility to aminoglycoside ototoxicity. We analysed the presence of this mutation in 52 patients who suffered from acute hearing loss after the use of aminoglycosides.[13] Forty-three of these patients were unrelated. In seven of them the 1555A☐G mutation was present, but the 7472insC mutation was not detected in any of the patients.

## Discussion

We found the previously reported mitochondrial mutation 7472insC in a Dutch family with maternally inherited sensorineural, bilateral hearing loss. The fact that the mutation is present in two independent families with hearing loss as a predominant symptom[12] is strong evidence for the pathogenic role of the

7472insC mutation. Additional support for the disease-causing nature of this mutation includes:

i) the segregation pattern of the hearing loss in the large Dutch family is clearly mitochondrial,

ii) the mutation was present in all members of the maternal lineage of the Dutch and the Italian family,

iii) the mutation was not present in any of the 100 Dutch control persons in this study, nor in any of the 381 Italian control persons in a previous study,[12]

iv) the mutation changes the conformation of the conserved TΨC loop in the secondary structure of the *tRNA*[Ser(UCN)] gene,

v) using homoplasmic cybrids it was previously shown that the mutation reduces oxidative phosphorylation,[12] and

vi) the mutation in both families is heteroplasmic, which is considered to be an indication of the pathogenicity of a mutation.[14]

This mutation was previously reported in a Sicilian family. Of the thirteen members of the Sicilian family with the mutation, four were unaffected, two had only hearing loss, and seven had neurological symptoms, four being severely neurologically affected.[12] In the Dutch family, 27 of the 38 family members with the mutation had hearing loss as an isolated symptom. All family members above the age of 30 with the mutation had hearing loss, except for four persons. The fact that these four family members have normal hearing might be due to a highly variable age of onset or to reduced penetrance of the hearing impairment. In the Dutch family only the index patient (I-5) has ataxia of both limbs and dysartheria in addition to hearing loss. So the Dutch family has a lower penetrance of neurological symptoms than the Sicilian family.

We analysed the percentages of heteroplasmy in the previously described Sicilian family and the Dutch family in relation to the presence or absence of hearing impairment or neurological impairment in patients aged 25 years and over. There was a significant difference in the percentage of heteroplasmy between the family members with and without hearing/neurological impairment in the Sicilian family (Mann-Whitney test, $P < 0.05$). In the Sicilian family, a threshold of approximately 95% was suggested for the manifestation of all

neurological symptoms in addition to hearing loss.[12] However, in the Dutch family, 20 family members with 95% heteroplasmy or more, had no additional neurological symptoms. Only the proband, who had the highest percentage of heteroplasmy (99%), had a clear neurological picture.

However, it is very possible that a higher threshold (99%) for heteroplasmy is necessary for the manifestation of the complete neurological phenotype in the Dutch family. This different threshold must be explained by the influence of a second factor, environmental or genetic. This factor might have a 'protective' influence in the Dutch family or alternatively a 'pathogenic' influence in the Sicilian family. Also for other mitochondrial mutations, additional factors are known to be involved, but have remained unidentified.[15,16] Such a factor might be another mtDNA variation, as suggested for the 7445A⎕G mutation. However, sequencing of the complete mitochondrial genome would be needed to investigate this possibility in our family.

On the other hand, nuclear genes may also influence the penetrance and expression of mitochondrial mutations. The recent finding that mice with a mutation in the *myosin VIIA* gene are protected from aminoglycoside ototoxicity[17] suggests that the *myosin VIIA* gene may be a nuclear factor influencing mitochondrial mutations that predispose to aminoglycoside induced deafness.

We investigated possible statistical correlations between hearing impairment and heteroplasmy or between age and heteroplasmy. Both were not significant. The absence of a correlation between hearing thresholds and heteroplasmy is remarkable, as the causative role of the mutation is beyond doubt. However, heteroplasmy was determined in blood, and a significant correlation might be found in other tissues. Up to now, heteroplasmy levels in muscle have only been determined in a single patient of the Dutch family and in two patients of the Sicilian family. Although identical values for blood and muscle were obtained in these three cases, more patients need to be analysed to produce statistics.

It is noteworthy that three Dutch family members of the maternal line reported acute hearing loss after the use of streptomycin. This suggests the involvement of the 7472insC mutation in susceptibility to ototoxic medication. However, the 7472insC mutation was not found in a set of 45 additional patients with aminoglycoside-induced hearing loss without the 1555A⎕G

mutation. This could indicate that the presence of three patients with aminoglycoside-induced deafness in the Dutch family is purely coincidental. Alternatively, if the 7472insC mutation does influence the sensitivity to aminoglycosides, it is probably a rare cause of aminoglycoside-induced hearing loss.

In this study we report a family in which the 7472insC mutation causes non-syndromic hearing loss in most patients, and syndromic hearing loss in a single patient. It is therefore obvious that, in addition to the 1555A⎕G and the 7445A⎕G mutations, the 7472insC mutation is an important mutation to be analysed in families with maternally-inherited non-syndromic hearing loss.

## Acknowledgements

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