

# The potential role for use of mitochondrial DNA copy number as predictive biomarker in presbycusis

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**Objectives:** Age-related hearing impairment, or presbycusis, is the most common communication disorder and neurodegenerative disease in the elderly. Its prevalence is expected to increase, due to the trend of growth of the elderly population. The current diagnostic test for detection of presbycusis is implemented after there has been a change in hearing sensitivity. Identification of a pre-diagnostic biomarker would raise the possibility of preserving hearing sensitivity before damage occurs. Mitochondrial dysfunction, including the production of reactive oxygen species and induction of expression of apoptotic genes, participates in the progression of presbycusis. Mitochondrial DNA sequence variation has a critical role in presbycusis. However, the nature of the relationship between mitochondrial DNA copy number, an important biomarker in many other diseases, and presbycusis is undetermined.

**Methods:** Fifty-four subjects with presbycusis and 29 healthy controls were selected after ear, nose, throat examination and pure-tone audiometry. DNA was extracted from peripheral blood samples. The copy number of mitochondrial DNA relative to the nuclear genome was measured by quantitative real-time polymerase chain reaction.

**Results:** Subjects with presbycusis had a lower median mitochondrial DNA copy number than healthy subjects and the difference was statistically significant ( $P=0.007$ ). Mitochondrial DNA copy number was also significantly associated with degree of hearing impairment ( $P=0.025$ ) and audiogram configuration ( $P=0.022$ ).

**Conclusion:** The findings of this study suggest that lower mitochondrial DNA copy number is responsible for presbycusis through alteration of mitochondrial function. Moreover, the significant association of mitochondrial DNA copy number in peripheral blood samples with the degree of hearing impairment and audiogram configuration has potential for use as a standard test for presbycusis, providing the possibility of the development of an easy-to-use biomarker for the early detection of this condition.

**Keywords:** age-related hearing impairment, presbycusis, biomarker, mtDNA

## Introduction

Presbycusis, or age-related hearing impairment, manifests as symmetric, bilateral, sensorineural hearing impairment, occurring slowly over a lifetime.<sup>1</sup> It is a major reason for hearing impairment in adults. Recently, Homans et al conducted a large cohort study that showed that 33% of men and 29% of women aged >65 years suffer from presbycusis.<sup>2</sup> Presbycusis affects communication skills, gradually leading to isolation, depression, and frustration. Thus, the condition greatly impacts the quality of human life and general health.<sup>1,3</sup> Panza et al demonstrated that presbycusis is a risk factor for dementia and cognitive decline.<sup>4</sup> There is, as yet, no treatment for

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presbycusis, which develops over many years and people are usually identified after their hearing sensitivity change.<sup>5</sup> Various research groups are seeking a biomarker for this common impairment;<sup>6–9</sup> development of a sensitive and specific biomarker for detection and prediction of presbycusis could reduce the impact of the condition on the health of society in the future.

Mitochondria are intracellular bodies, and their impairment contributes to presbycusis.<sup>10</sup> These are the only cellular organelles that contain their own genomes, known as mtDNA. According to the mitochondrial theory of aging proposed by Harman,<sup>11</sup> accumulation of reactive oxygen species (ROS) produced by mitochondria causes damage to intracellular macromolecules, particularly mtDNA. A lack of introns and protective histones make mtDNA more susceptible to ROS and other types of damage, which could lead to sequence mutations or copy number alterations.<sup>12</sup> Variation of both quality (mutations)<sup>13,14</sup> and quantity (copy number)<sup>15</sup> of mtDNA has been associated with many human diseases, including neurodegenerative diseases,<sup>16</sup> metabolic diseases, and various types of cancer,<sup>17</sup> as well as in the process of aging.<sup>18</sup> Therefore, the application of mtDNA alteration as a biomarker in aging, age-related diseases, cancers, metabolic diseases, and even forensic science is currently being investigated.<sup>19,20</sup>

In recent years, studies have identified some chromosomal loci and genes associated with presbycusis.<sup>3,21,22</sup> A comparison of the mtDNA from temporal bones of presbycusis patients with that of healthy controls revealed a significant relationship between large-scale deletions in mtDNA and presbycusis.<sup>23–25</sup> In addition to large deletions, there are reports of single nucleotide changes to mitochondrial genomes associated with presbycusis.<sup>26</sup> To determine the role of mtDNA copy number in the development of presbycusis and investigate the possibility of using mtDNA copy number as a biomarker for this impairment, we compared the mtDNA copy number in subjects with presbycusis and healthy controls by quantitative real-time polymerase chain reaction (PCR).

## Materials and methods

All 50- to 90-year-old volunteers participating in the molecular investigation of presbycusis at the ENT and Head and Neck Research Center and Department, Iran University of Medical Sciences completed a questionnaire, based on that developed by the European presbycusis consortium, which included questions to determine a full medical history and details of exposure to environmental factors that affect

hearing.<sup>21,27</sup> Participants with confounding factors (any known reason for hearing loss, exposure to ototoxic drugs, and other pathologies correlated with hearing loss, such as cardiovascular disorder, liver cirrhosis, renal failure, and head and neck tumor), neurodegenerative diseases, and cancers were excluded. Otoloscopic examination and pure-tone audiometry were performed on all volunteers. Air conduction was measured at octave frequencies of 250–8,000 Hz and bone conduction at 250–4,000 Hz using an Amplaid 319 audiometer (Milan, Italy). Calibration was performed according to the American National Standards Institute (ANSI, 2004a). Subjects with any kind of middle ear pathology affecting hearing sensitivity, conductive hearing loss (mean air-bone differences at 500, 1,000, and 2,000 Hz of  $\geq 15$  dB), and extensive noise exposure (self-reported) were excluded.

Presbycusis patients (n=54) with symmetric, bilateral hearing impairment with the pure-tone average of mean frequencies 1, 2, 4, and 8 kHz of  $\geq 30$  dB HL were selected. Healthy subjects (n=29) with normal ear examination and the pure-tone average of mean frequencies  $\leq 25$  dB HL were selected. The mean age of patients was  $66.87 \pm 8.9$  years and that of healthy subjects was  $63 \pm 5.3$  years. The gender ratio was 54.2% male and 45.8% female, and there were no statistically significant differences in age and gender between presbycusis and healthy subjects ( $P > 0.05$ ). This study was approved by the Ethics Committee of Iran University of Medical Sciences, and all subjects provided signed informed consent.

Genomic DNA was extracted from peripheral blood samples using the QIAamp DNA Mini Kit (Cat No 51304; Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Relative mtDNA copy number was determined through simultaneous assessment of the nuclear gene, *TMIE* (NC\_000003.12), and the mitochondrial gene, *MT-RNR1* (NC\_012920.1), using quantitative PCR (qPCR). Primers were as follows: *MT-RNR1-F*, 5'-AGTAG AGTGCTTAGTTGAGC-3'; *MT-RNR1-R*, 5'-AG TACACTTCCATGTTACG-3'; *TMIEF*, 5'-CCATTC CTTGGGTCTCTGAA-3'; and *TMIER*, 5'-AGCAGA GGAACAGGGTGAC-3'. qPCR was performed using a Rotor-gene 6000 instrument (Corbett Life Science, Sydney, Australia) and QuantiFast SYBR Green PCR master mix (Cat No 204054, Qiagen). All reactions were performed in duplicate with total reaction volumes of 10  $\mu$ L, containing 5  $\mu$ L of 1X Quanti Fast SYBR Green PCR master mix, 0.12  $\mu$ L of each primer (10 pmol), 0.7  $\mu$ L of DNA, and 4  $\mu$ L of RNase-free water. After initial heating for 1 min at 95°C as an activation step, 40 cycles of denaturation at 95°C for

10 s, followed by annealing and extension at 60°C for 45 s, were performed.

The threshold cycle (Ct) of each sample was determined for *MT-RNR1* and *TMIE*. Relative mtDNA copy number was determined using the following equation:  $2^{-\Delta Ct}$  ( $\Delta Ct = Ct_{MT-RNR1} - Ct_{TMIE}$ ).

Standard curves were prepared for both nuclear and mitochondrial PCR.  $R^2$  correlation for each standard curve was  $\geq 0.99$ . Melting curve analysis of qPCR and running of PCR products on agarose gels (1.5%) were performed to confirm amplification of a specific product without primer dimer or unwanted PCR product.

Statistical analyses were performed using SPSS 22 for Windows (IBM, Chicago, IL, USA). All numerical variables were checked for normal distribution using the one-sample Kolmogorov–Smirnov test. The association between the mtDNA copy number in presbycusis and healthy subjects was evaluated using the Mann–Whitney *U*-test. The associations between mtDNA copy number and degree of hearing impairment and audiogram configuration were evaluated using Kruskal–Wallis H-test. Statistical significance was defined as  $P < 0.05$ .

## Results

The degree of hearing impairment of subjects with presbycusis was classified into five categories according to the American Speech–Language–Hearing Association: mild, moderate, moderately severe, severe, and profound.<sup>28</sup> Patients in this study suffered from mild, moderate, and moderately severe hearing impairment (Figure 1A).

Based on audiogram configuration described previously, patients were divided into five groups: flat, high-frequency

gently sloping (HFGS), high-frequency steeply sloping (HFSS), low-frequency ascending, and mid-frequency U-shape.<sup>29</sup> Presbycusis subjects in this study exhibited flat, HFGS, and HFSS shapes (Figure 1B).

The mean audiograms of presbycusis patients and healthy subjects are presented in Figure 2.

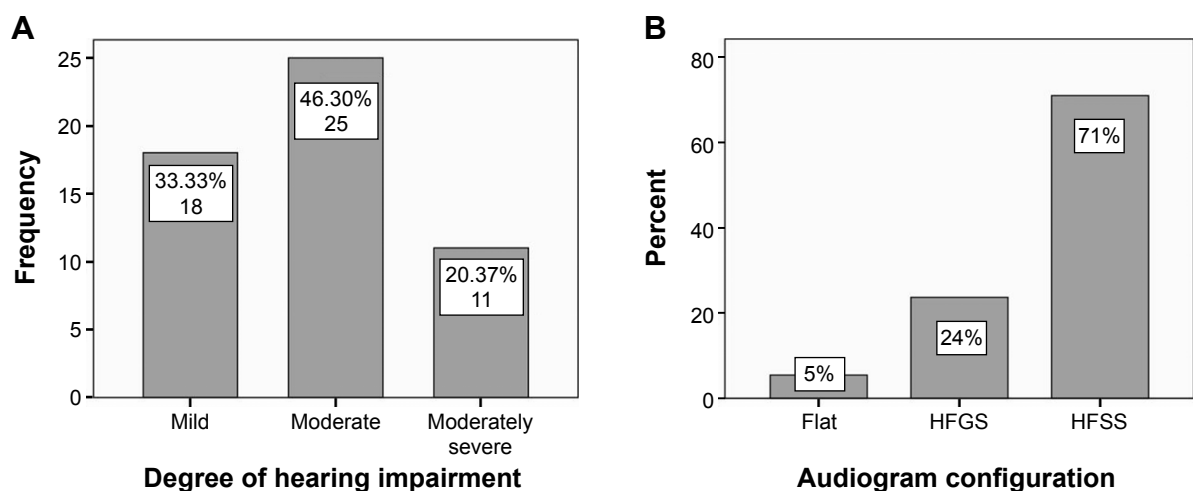
The mitochondrial copy number of study participants was evaluated by measuring the proportion of mtDNA relative to nuclear DNA using the level of *MT-RNR1* as an evaluation index of the mitochondrial genome and that of *TMIE* for nuclear DNA. Median mtDNA copy number for subjects with presbycusis was 0.76, whereas that of healthy subjects was 1.45, indicating that the mitochondrial copy number of subjects with presbycusis was almost halved compared with the healthy controls (Figure 3). The difference in mtDNA copy number between the two groups was statistically significant ( $P=0.007$ ).

A Kruskal–Wallis H-test showed that there was a statistically significant difference in mtDNA copy number between the different hearing degrees,  $\chi^2(3) = 9.33$ ,  $P=0.025$ , with a mean rank mtDNA copy number of 50.79 for normal, 29.72 for mild, 38.20 for moderate, and 44.64 for moderately severe.

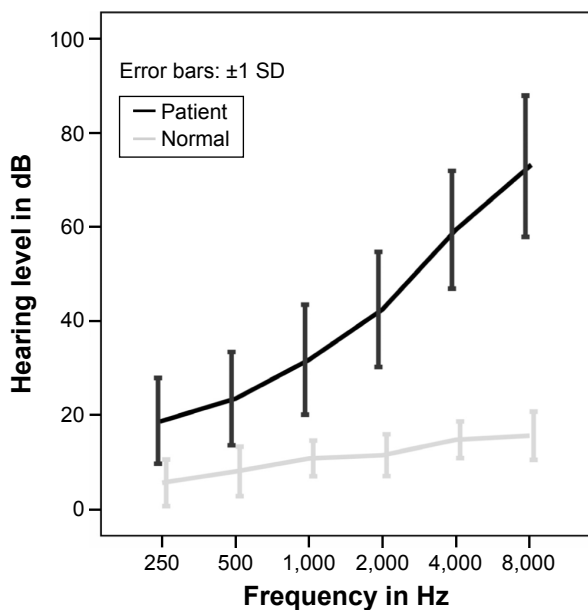
A Kruskal–Wallis H-test showed that there was a statistically significant difference in mtDNA copy number between the different audiogram configurations,  $\chi^2(2) = 7.62$ ,  $P=0.022$ , with a mean rank mtDNA copy number of 51.23 for flat shape, 32.96 for HFGS shape, and 37.68 for HFSS shape.

## Discussion

Presbycusis is categorized as a complex or multifactorial disorder.<sup>30,31</sup> Factors affecting its development include genetic background, environmental factors, ototoxic drugs,



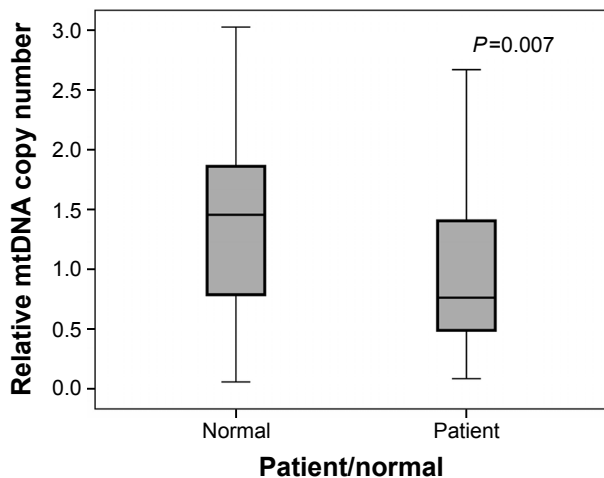
**Figure 1** (A) Classification of presbycusis patients by degree of hearing impairment (n=54). (B) Classification of patients by shape of audiogram. **Abbreviations:** HFGS, high-frequency gently sloping; HFSS, high-frequency steeply sloping.



**Figure 2** Mean audiograms of patients with presbycusis and healthy subjects. Error bars represent SDs.

**Abbreviation:** SD, standard deviation.

medical conditions, and hormonal factors.<sup>32–34</sup> Presbycusis occurs as a result of degeneration of both the inner ear structure and the central auditory system of the brain.<sup>35–37</sup> Mitochondria play a key role in cellular damage and death in the aging process of hearing.<sup>38,39</sup> Mitochondrial dysfunction is related to the development of certain neurodegenerative, age-related diseases, and cancer.<sup>14,40</sup> Mutations in the *MT-RNR1* and *MT-TF* genes can cause hearing loss.<sup>26,41</sup> Investigation



**Figure 3** Distribution of peripheral blood mtDNA copy number in Iranian patients with presbycusis and healthy controls. The relative mtDNA copy number was evaluated by measuring the proportion of mtDNA relative to nuclear DNA. The difference between mtDNA copy number in the presbycusis and healthy subject groups was statistically significant (Mann–Whitney *U*-test,  $P=0.007$ ). The box plots show the median (solid line across the box), inter-quartile range (shaded boxes), and range (whiskers).

of the temporal bone of presbycusis patients showed an increase in deletions and point mutations in mtDNA. Bai et al detected a 4,977 bp deletion, known as the common deletion, in the mtDNA of human cochlear tissue of presbycusis patients.<sup>23,24</sup> Markaryan demonstrated that the expression of the *MT-CO3* gene is decreased in spiral ganglion cells from individuals with presbycusis, suggesting that deficits in the electron transport chain of spiral ganglion cells contribute to presbycusis.<sup>25</sup>

The results of this study indicate a statistically significant decrease in mtDNA copy number in presbycusis subjects. Our study is the first to investigate the relationship between mtDNA copy number and presbycusis. Previously, Santos et al proposed a decrease in mtDNA copy number as an important marker for oocyte quality, having shown that such a decrease is due to insufficient mitochondrial biogenesis or cytoplasmic maturation, causing fertilization failure.<sup>42</sup> Studies of the relationship between neurodegenerative disease and mtDNA content have produced similar results to those of our study. Podlesniy et al suggested low content of mtDNA in cerebrospinal fluid as a novel biomarker for the early detection of preclinical Alzheimer's disease.<sup>43</sup> Petersen et al demonstrated reduced mtDNA copy number in blood leukocytes from patients with Huntington's disease.<sup>16</sup>

Our results are consistent with previous studies demonstrating that presbycusis is commonly a high-frequency hearing impairment, as HFSS was the most common configuration in patients in this study (70.9%), followed by HFGS (23.64%) (Figure 1B).<sup>1,44</sup>

According to previous studies, a decrease in the number of mitochondrial genomes can cause impaired or reduced mitochondrial activity. A study using HeLa cells indicated that depletion of the mitochondrial genome by decreasing catalase expression enhances oxidative damage to nuclear DNA and influences nuclear genome stability.<sup>45</sup> Oxidative stress is associated with excessive ROS production in the mitochondrial matrix; ROS increases death of cellular structures of the cochlear, leading to an increase in mutations in the mitochondrial genome.<sup>40</sup> In transgenic mice, accumulation of mtDNA mutations by mitochondrial dysfunction influences energy metabolism. The consequent reduction in energy production induces apoptosis, leading to a loss of critical cochlear cells and resulting in age-related hearing impairment.<sup>46</sup>

The results of this study, together with those of previous reports demonstrating the relationship between mtDNA sequence variation and presbycusis,<sup>23,26,47</sup> highlight the role of mtDNA in presbycusis progression. A significant reduction



in the number of mitochondrial genomes in presbycusis subjects, due to mitochondrial dysfunction, may be one of the molecular mechanisms involved in the development of this impairment. In addition, quantification of mtDNA in peripheral blood samples may constitute an easily assessed biomarker of presbycusis, since the high-sensitivity qPCR-based assay can detect even minute variations in mtDNA copy number. This blood biomarker could easily be incorporated into regular physical examinations as it employs a simple and cheap screening method. Moreover, the results of this study demonstrate that mtDNA copy number is statistically significantly associated with the degree of hearing ( $P=0.025$ ) and audiogram configuration ( $P=0.022$ ). Clearly, these results require further confirmation in a larger study population to determine whether the method is appropriate for clinical application. In addition, more research is required to understand the relationship between the decrease in mtDNA copy number and the onset of presbycusis.

Age-related hearing impairment is the most common sensory disease in the elderly population.<sup>10</sup> With the growth of the aging population, the prevalence of age-related disorders such as presbycusis is increasing.<sup>1</sup> The World Health Organization estimates that in 2025, more than 500 million individuals will suffer from presbycusis.<sup>48</sup> Presbycusis begins slowly, without clinical manifestation. Further human studies are required to elucidate methods for early diagnosis and prevention.

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## Disclosure

The authors report no conflicts of interest in this work.

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