Hearing function in heterozygous carriers of a pathogenic *GJB2* gene mutation

D. GROH[1,2], P. SEEMAN[3], M. JILEK[1], J. POPELÁŘ[1], Z. KABELKA[2], J. SYKA[1]

[1]Department of Auditory Neuroscience, Institute of Experimental Medicine AS CR, Vídeňská 1083, 142 20 Prague 4, Czech Republic

[2]Department of ENT, 2nd Faculty of Medicine, Charles University in Prague and University Hospital Motol, V Úvalu 84, 150 06 Prague 5, Czech Republic

[3]Department of Paediatric Neurology, 2nd Faculty of Medicine, Charles University in Prague and University Hospital Motol, V Úvalu 84, 150 06 Prague 5, Czech Republic

***Corresponding author**:*

Daniel Groh, M.D., Ph.D.

Department of ENT, Charles University in Prague, 2nd Faculty of Medicine

V Úvalu 84

150 06 Prague 5

Czech Republic

Phone: (+420) 224432600, (+420) 224432601, Fax: (+420) 224432620

e-mail: daniel.groh@seznam.cz

Short title: Hearing in heterozygotes for GJB2 mutation

Summary

The most frequent hereditary hearing loss is caused by mutations in the *GJB2* gene coding for the gap junction beta 2 protein Connexin 26 (Cx26). In contrast to many studies performed in patients with ~~a~~ bi-allelic mutations, audiometric studies on heterozygotes are sparse and often contradictory. To evaluate hearing function in heterozygous carriers of the *GJB2* c.35delG mutation, audiometry over the extended frequency range and the recording of otoacoustic emissions (OAEs), ie, transient-evoked OAEs (TEOAEs) and distortion product OAEs (DPOAEs), were performed in a group of parents and grandparents of deaf children homozygous for the *GJB2* c.35delG mutation. The comparison of audiograms between control and heterozygous subjects was enabled using audiogram normalization for age and sex. Hearing loss, estimated with this procedure, was found to be significantly larger in *GJB2* c.35delG heterozygous females in comparison with controls for the frequencies of 8-16 kHz; the deterioration of hearing in heterozygous men in comparison with controls was not statisticaly significant. A comparison of TEOAE responses and DPOAE levels between *GJB2* c.35delG heterozygotes and controls did not reveal any significant differences. The results prove the importance of using audiometry over the extended frequency range and audiogram normalization for age and sex to detect minor hearing impairments, even in a relatively small group of subjects of different ages.

Key words: c.35delG mutation; audiogram normalization; high-frequency audiometry; otoacoustic emissions; high-frequency hearing loss

Introduction

Sensorineural hearing loss is a very common inherited sensory defect affecting 1-2 per 1000 newborns. Until now, mutations in more than 39 different genes have been identified as resulting in non-syndromic autosomal recessive deafness (http://hereditaryhearingloss.org). About 50% of these cases are caused by mutations in the *GJB2* gene coding for the gap junction beta 2 protein Connexin 26 (Cx26), resulting in a hereditary hearing loss named DFNB1. Gap junctions in the inner ear have been shown to recycle potassium ions from the hair cells to the endolymph (Holt and Corey 1999). About 3% of the Caucasian population are heterozygous for the c.35delG mutation in

the *GJB2* gene (the deletion of a single guanosine in a sequence of six guanosines extending from position 30 to position 35) (Seeman *et al.* 2004).

Several previous studies have documented moderate to profound hearing loss in homozygotes for the c.35delG mutation (Kelsell *et al.* 1997; Murgia *et al.* 1999). However, audiometric results obtained in *GJB2* heterozygotes are sparse and often contradictory. Engel-Yeger *et al.* (2002) as well as Silva *et al.* (2010) did not find any differences in the audiograms of carriers and non-carriers, but they demonstrated a significantly lower response level of DPOAEs in carriers in comparison with non-carriers. Other studies reported statistically significant differences in hearing thresholds, mainly at high frequencies (Wilcox *et al.* 2000; Franzé *et al.* 2005; Lipan *et al.* 2011), and/or lower DPOAE response levels over a limited frequency range (Silva *et al.* 2010). These studies were performed in small cohorts of subjects, and hearing thresholds were often measured only in the conventional frequency range up to 8 kHz.

The aim of the present study was to evaluate hearing function in a group of heterozygous parents and grandparents of 18 congenitally hearing-impaired children that were homozygous for the *GJB2*/c.35delG mutation. Hearing in heterozygous parents and grandparents was evaluated using audiometry over the extended frequency range up to 16 kHz and the recording of OAEs.

Methods

Audiometric testing was performed in a group of 122 healthy control subjects and in a group of 34 parents and grandparents of 18 congenitally hearing-impaired children previously identified for the implantation of a cochlear implant in the University Hospital Motol in Prague. All 18 children were homozygous for the c.35delG mutation in the *GJB2* gene. All subjects had normal results from an otoscopic examination.

The genotyping procedures and audiometric recordings were approved by the Ethics Committee of the University Hospital Motol, Prague, and followed the guidelines of the Declaration of Helsinki.

Genetic testing

The group of 52 parents and grandparents of 18 congenitally hearing-impaired children were examined for the presence or absence of the *GJB2*/c.35delG mutation using a combination of allele-specific polymerase chain reaction (PCR) and fluorescence PCR with subsequent fragment analysis, as described elsewhere (Seeman *et al.* 2004). Deoxyribonucleic acid (DNA) was isolated from the saliva using a commercial kit Oragene. From the total number of 52 relatives, 34 parents and grandparents (65%) (13 males and 21 females) were found to be heterozygous for the c.35delG mutation. The age and gender of all subjects are shown in Table 1.

Pure tone audiometry

Hearing thresholds were obtained bilaterally over an extended frequency range from 125 Hz up to 16 kHz using a clinical audiometer (OB 922, Madsen Orbiter, v.2) equipped with high-frequency headphones (HDA 200, Sennheiser).

Audiogram normalization procedure

Due to age-related hearing loss, the hearing thresholds in individual experimental groups can be compared only between subjects of the same age. For comparing the hearing thresholds of persons of different age and sex, the audiograms were normalized. The age-related threshold shift at frequencies from 125 Hz to 8 kHz is defined in the ISO 7029 standard by a mathematical equation. In this standard, α coefficients for each frequency are given for males and females separately. However, no coefficients are presented for audiometry in the extended frequency range used in this study. In the paper by Jilek and Syka (2008), the ß coefficients for frequencies of 10, 12.5 and 16 kHz were calculated using a linear approximation method. These coefficients were used for audiogram normalization in the extended frequency range.

Recording of otoacoustic emissions

To examine the physiological status of outer hair cells (OHCs) in the cochlea otoacoustic emissions were measured with commercial instrumentation (ILO 292, Otodynamics, Ltd.) using a B-type probe for adults. The signal was processed with an ILO software (Otodynamics, Ltd., v.6).

The acoustical stimuli for transiently evoked otoacoustic emissions (TEOAEs) were conventional broadband clicks (0.5–6.0 kHz, 80 μs duration), with the stimulus gain adjusted to 80 dB peak SPL. Each recording consisted of 260 averagings. Only measurements with a reproducibility of more than 60% were considered.

Stimuli for eliciting $2f_1–f_2$ distortion-product otoacoustic emissions (DPOAEs) (cubic DPOAEs) were two primary tones, f_1 and f_2, with tone levels L1 = L2 = 70 dB SPL, $f_2/ f_1 = 1.22$. Testing was made over the f_2 frequency range from 1-6.3 kHz, at three points/octave. The DPOAE values as a function of f_2 frequency (DP-grams) in individual subjects are presented here.

Statistical tests

Differences between hearing thresholds and DP-grams in control versus heterozygous subjects were tested using one-way ANOVA with the Bonferroni post-hoc test. Average TEOAE responses, measured in control and heterozygous subjects, were compared using a t-test. All statistical calculations were performed using commercial software (GraphPad, v.7, SigmaPlot).

Results

Hearing thresholds

Figure 1 shows the audiograms averaged over decades in controls (Fig. 1 A, B) and heterozygous carriers (Fig. 1 C, D), separately for males (A, C) and females (B, D).

Hearing threshold normalization for age and sex

To enable the comparison of audiograms between heterozygous carriers and controls, the procedure of audiogram normalization for sex and age was used. The average normalized audiograms of control males and females (Fig. 2A, B, interrupted lines) are almost identical; the differences between the normalized thresholds at individual frequencies did not exceed 1.3 dB, thus providing evidence of the reproducible results achieved by using audiogram normalization. The average normalized audiograms for heterozygous males and females (Fig. 2A, B, full lines) show a pronounced hearing loss at frequencies higher than 2 kHz. Normalized audiograms in control males and heterozygous males are not significantly different. However, the audiograms obtained in heterozygous females are significantly worse at frequencies of 8-16 kHz than those measured in control females ($p<0.0001$).

To analyze more precisely the hearing impairment in heterozygous subjects, the normalized high-frequency (HF) thresholds in the frequency range 8-16 kHz were averaged for each heterozygous male and female and compared with the analogous data obtained in controls. The mean HF thresholds in control males amounted to 0.15±12.2 dB HL and 3.63±16.8 dB HL in heterozygous males ($p<0.222$, t-test). The histograms in Fig. 3A document that 34.9% of controls and 34.6% of heterozygotes had their HF thresholds concentrated at around 0 dB HL. In females, the mean HF thresholds in heterozygous females (10.63±2.302 dB HL) significantly exceeded the mean HF thresholds found in the control females (0.4780±0.733 dB HL) ($p<0.0001$, t- test). Whereas 45.8% of control females had HF thresholds at around 0 dB HL, the largest number of heterozygous females (35.7%) had HF thresholds located at around 10 dB HL (Fig. 3B).

DPOAEs

The average DP grams evaluated in selected age-matched controls and *GJB2* heterozygous carriers are presented in Fig. 4. The differences between the average DP-grams evaluated in control and heterozygous males (Fig. 4A) and control and heterozygous females (Fig. 4B) are not significant at any frequency.

TEOAEs

The TEOAE responses averaged in heterozygous carriers and control males and females are shown in Fig. 5. Similarly to DPOAEs, the average TEOAE responses measured in heterozygous carriers and control males and females are not statistically different.

Discussion

The results of the present study demonstrate that the analysis of TEOAE responses and DPOAE levels did not show significant differences between *GJB2* c.35delG heterozygotes and controls. However, using audiometry in an extended frequency range and the methods of audiogram normalization according to age and sex, a significant hearing loss at frequencies of 8-16 kHz was detected in heterozygote woman in comparison to control woman. The differences between the normalized audiograms in control males and heterozygous men was not statistically significant. These results document the importance of high frequency audiometry and the suitability of audiogram normalization methods for detecting small differences in the audiometric hearing loss of a small number of subjects with large differences in age.

Mutations in the *GJB2* gene represent the most common cause of recessively inherited hearing loss (Petersen and Willems 2006). There have been several publications dealing with the large variability in genetic background and phenotype-genotype deafness. Most of these studies were oriented towards a detailed identification of the bi-allelic *GJB2* mutations causing hearing loss of different severity or even deafness (Cohn and Kelley 1999); only in a few studies have the audiometric manifestations of a heterozygous pattern been systematically studied. Due to the small number of subjects with a *GJB2* gene mutation in the normal population (2-4%) (Seeman *et al.* 2004) and the large variability of hearing loss in these heterozygous subjects (from normal hearing to profound hearing loss), studies examining hearing function in *GJB2* c.35delG heterozygotes have often yielded contradictory results. Engel-Yeger *et al.* (2002) found a significant difference between non-carriers and c.35delG carriers: non-carriers had larger DPOAE responses than

heterozygotes at all frequencies. In contrast to a significant difference in DPOAEs, the hearing thresholds of carriers and non-carriers were found to be similar up to 8 kHz. The large variability of hearing loss in a group of heterozygous carriers of the *GJB2* 35delG mutation was reported in several other studies (Wilcox *et al.* 2000; Franzé *et al.* 2005; Lipan *et al.* 2011). In our study the tested subjects were parents or grandparents of congenitally hearing-impaired children who were diagnosed as having the c.35delG mutation. Such a sample of probands guarantees a high probability of the presence of the c.35delG mutation. A similar strategy, i.e., audiological evaluation of the heterozygous parents of individuals with autosomal recessive hearing loss, was used in the study of Silva *et al.* 2010. These authors found a tendency towards better audiometric thresholds in the control group in comparison with the heterozygous parents, but without statistical significance.

Due to the large age range of heterozygotes in our study (from 30 to 70 y), it was not possible to divide the total number of heterozygous subjects into smaller age groups and to compare their audiograms with those of the corresponding age groups of the control subjects. To overcome this limitation, the method of normalizing all of the audiograms for age and sex was used. Audiogram normalization for every frequency in the frequency range 0.125 – 8 kHz was performed using α coefficients according to the ISO 7029 standard, while for frequencies of 10, 12.5 and 16 kHz the ß coefficients were calculated using a linear approximation method (Jilek and Syka, 2008). This procedure enabled us to detect a pronounced hearing threshold elevation at high frequencies that was statistically highly significant in females, but not in males. This result was confirmed by computing the average HF threshold (i.e., the average of the threshold values at frequencies of 8-16 kHz). The distribution histograms of the average HF thresholds demonstrated the differences between the mechanisms of hearing impairment between heterozygous males and females: whereas the distribution histogram of the average HF threshold values in heterozygous males is almost identical with that obtained for control males and only a minority exhibited a relatively large threshold deterioration, the distribution histogram of the average HF threshold values in

heterozygous females was significantly shifted by approximately 10 dB to higher values compared to control values, suggesting the presence of HF hearing deterioration in a large portion of heterozygous females. Hearing impairment of heterozygous females seems be not age-dependent since pronounced hearing loss was detected in several young as well as old heterozygous females. To the best of our knowledge, this paper represents the first study reporting a significantly worse deterioration of hearing function in c.35delG heterozygous females than in heterozygous males.

Impaired potassium recycling in Connexin 26 (Cx26) mutants may cause a fluid and ion imbalance in the cochlea and change the endolymphatic potential, which is necessary for the proper functioning of the OHCs and the inner hair cells (IHCs) (Kikuchi *et al.* 2000). Since OHC impairment due to a *GJB2* mutation may be reflected in impaired OAEs, our original hypothesis was that the OAEs in *GJB2* c.35delG heterozygous carriers could be altered as well. Unfortunately, the method of audiogram normalization is not possible to use in the case of OAE recording, and thus no significant differences were detected in the average TEOAE responses or in the DPOAE amplitudes between heterozygous and control males or females. The reason for this lack of differences might be the fact that the hearing loss in heterozygous subjects was localized at high frequencies (8-16 kHz), whereas OAEs were recorded at frequencies up to 5 kHz. On the other hand, several studies have presented data showing that a high frequency hearing deficit can be reflected in changes of lower-frequency DPOAEs (Arnold *et al.* 1999; Avan *et al.* 1997; Groh *et al.* 2006). However, in our study a hearing impairment was demonstrated only using audiogram normalization, but not with the recording of OAEs.

An interesting result of our study is the finding that the hearing deterioration in c.35delG heterozygous carriers was more profound in females than in males. Several previous papers suggested the possibility that sex differences in the brain and behavior may be mediated by genetic mechanisms that do not involve gonadal hormones (Blecher and Erickson, 2007; Bocklandt and Vilain, 2007). Thus, several mechanisms can be responsible for the observed differences in hearing

loss between heterozygous males and females; however, for a fuller explanation of this fact, further studies are necessary.

In conclusion, the results of the present study document that computing normalized audiograms for age and sex seems to be a suitable method for detecting slight differences in the audiograms of a relatively small number of subjects exhibiting a large variability in their ages. In addition, because the differences appeared at frequencies higher than 8 kHz, these results demonstrate the importance of using audiometry over an extended frequency range as well as the use of new normalization coefficients for 10-16 kHz to detect slight hearing impairments as observed in c.35delG heterozygous females.

The preliminary data were presented at the 45th Inner Ear Biology Workshop, September 21-24, 2008, Ferrara, Italy, and published as an abstract in the Workshop Proceedings.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

Supported by the project (Ministry of Health, Czech Republic) for conceptual development of research organization 00064203 (University Hospital Motol, Prague, Czech Republic) and by grants GACR 304/12/1342 and GACR P304/10/1872.

References:

ARNOLD DJ, LONSBURY-MARTIN BL, MARTIN GK: High-frequency hearing influences lower-frequency distortion-product otoacoustic emissions. *Arch Otolaryngol Head Neck Surg* **125**: 215-222, 1999.

AVAN P, ELBEZ M, BONFILS P: Click-evoked otoacoustic emissions and the influence of high-frequency hearing losses in humans. *J Acoust Soc Am* **101**: 2771–2777, 1997.

BLECHER SR, ERICKSON RP: Genetics of sexual development: a new paradigm. *Am J Med Genet A* **143**: 3054-3068, 2007.

BOCKLANDT S, VILAIN E: Sex differences in brain and behavior: hormones versus genes. *Adv Genet* **59**: 245-266, 2007.

COHN ES, KELLEY PM: Clinical phenotype and mutations in connexin 26 (DFNB1/*GJB2*), the most common cause of childhood hearing loss. *Am J Med Genet* **89**: 130-136, 1999.

ENGEL-YEGER B, ZAAROURA S, ZLOTOGORA J, SHALEV S, HUJEIRAT Y, CARRASQUILLO M, SALEH B, PRATT H: The effects of a Connexin 26 mutation--35delG - on oto-acoustic emissions and brainstem evoked potentials: homozygotes and carriers. *Hear Res* **163**: 93-100, 2002.

FRANZÉ A, CARAVELLI A, DI LEVA F, MARCIANO E, AULETTA G, D'AULOS F, SAULINO C, ESPOSITO L, CARELLA M, GASPARINI P: Audiometric evaluation of carriers of the connexin 26 mutation 35delG. *Eur Arch Otorhinolaryngol* **262**: 921-924, 2005.

GROH D, PELANOVA J, JILEK M, POPELAR J, KABELKA Z, SYKA J: Changes in otoacoustic emissions and high-frequency hearing thresholds in children and adolescents. *Hear Res* **212**: 90-98, 2006.

HOLT JR, COREY DP: Ion channel defects in hereditary hearing loss. *Neuron* **22**: 217–219, 1999.

ISO 7029 2000. Acoustics – Statistical distribution of hearing thresholds as a function of age. Geneva: International Organization for Standardization.

JILEK M, SYKA J. Age related hearing thresholds in an extended frequency range. The XIV International Symposium in Audiological Medicine, IAPA 2008, Ferrara, Italy, September 18-21, 2008, Abstract Proceedings p.55, 2008.

KELSELL DP, DUNLOP J, STEVENS HP, LENCH NJ, LIANG JN, PARRY G, MUELLER RF, LEIGH IM: Connexin 26 mutations in hereditary non-syndromic sensorineural deafness. *Nature* **387**: 80-83, 1997.

KIKUCHI T, ADAMS JC, MIYABE Y, SO E, KOBAYASHI T: Potassium ion recycling pathway via gap junction systems in the mammalian cochlea and its interruption in hereditary nonsyndromic deafness. *Med Electron Microsc* **33**: 51-56, 2000.

LIPAN M, OUYANG X, YAN D, ANGELI S, DU LL, LIU XZ: Clinical comparison of hearing-impaired patients with DFNB1 against heterozygote carriers of connexin 26 mutations. *Laryngoscope* **121**: 811-814, 2011.

MURGIA A, ORZAN E, POLLI R, MARTELLA M, VINANZI C, LEONARDI E, ARSLAN E, ZACCHELLO F: Cx26 deafness: mutation analysis and clinical variability. *J Med Genet* **36**: 829-832, 1999.

PETERSEN MB, WILLEMS PJ: Non-syndromic, autosomal-recessive deafness. *Clin Genet* **69**: 371–392, 2006.

SEEMAN P, MALÍKOVÁ M, RASKOVÁ D, BENDOVÁ O, GROH D, KUBÁLKOVÁ M, SAKMARYOVÁ I, SEEMANOVÁ E, KABELKA Z: Spectrum and frequencies of mutations in the *GJB2* (Cx26) gene among 156 Czech patients with pre-lingual deafness. *Clin Genet* **66**: 152-157, 2004.

SILVA LS, NETTO RC, SANCHES SG, CARVALLO RM: Auditory measurements in parents of individuals with autosomal recessive hearing loss. *Pro Fono* **22**: 403-408, 2010.

WILCOX SA, SAUNDERS K, OSBORN AH, ARNOLD A, WUNDERLICH J, KELLY T, COLLINS V, WILCOX LJ, MCKINLAY GARDNER RJ, KAMARINOS M, CONE-WESSON B, WILLIAMSON R, DAHL HH: High frequency hearing loss correlated with mutations in the *GJB2* gene. *Hum Genet* **106**: 399-405, 2000.

Age group (years)	Controls			Heterozygotes		
	males	females	all	males	females	all
26 – 34	13	12	25	3	6	9
35 – 44	10	11	21	3	2	5
45 – 54	7	15	22	2	2	4
55 – 64	16	15	31	3	9	12
> 65	10	13	23	2	2	4
total	**56**	**66**	**122**	**13**	**21**	**34**

Table 1: Distribution of control and heterozygous males and females by age (in decades).

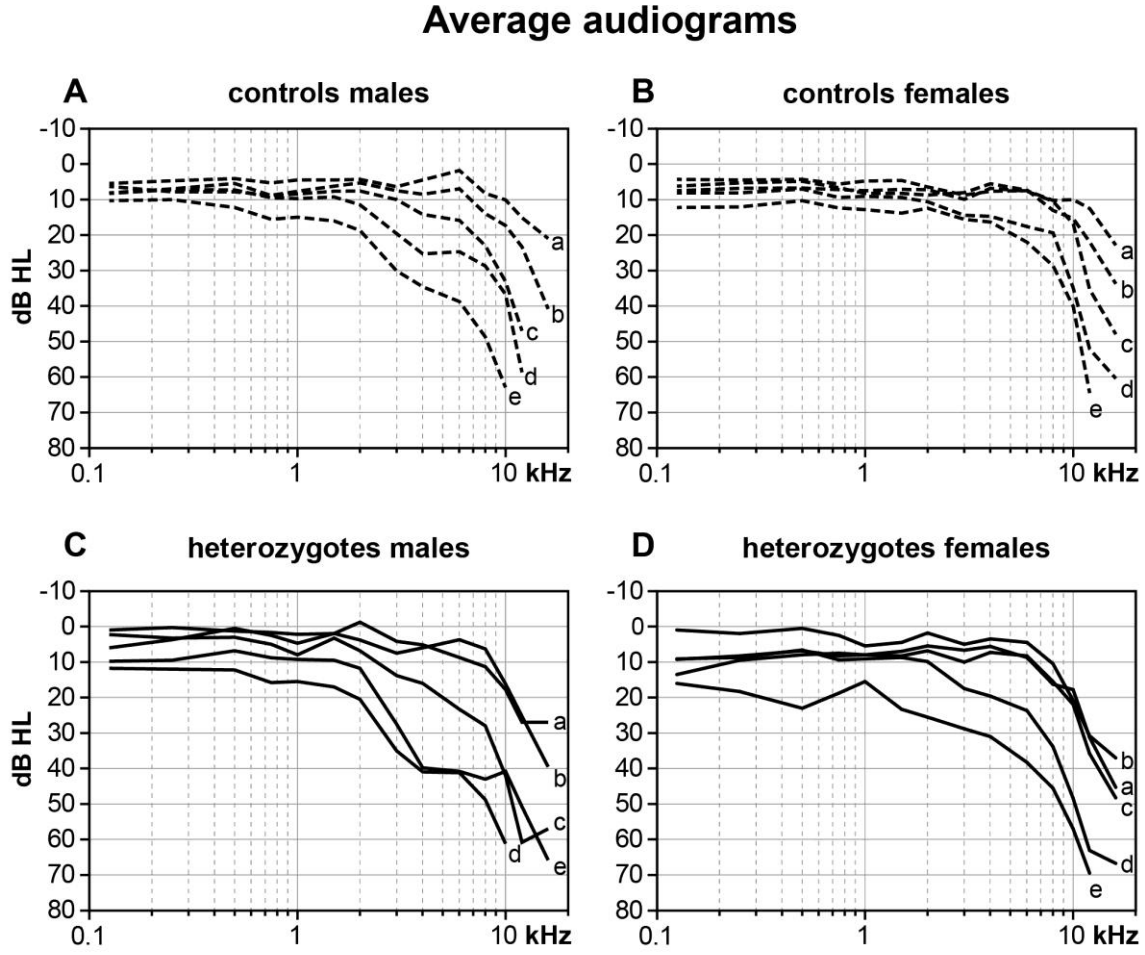

Fig. 1. Audiograms of controls and heterozygotes averaged in age decades for males (A,C) and females (B,D). Age decades: a=25-34 y, b=35-44 y, c=45-54 y, d=55-64 y, e= over 65 y.

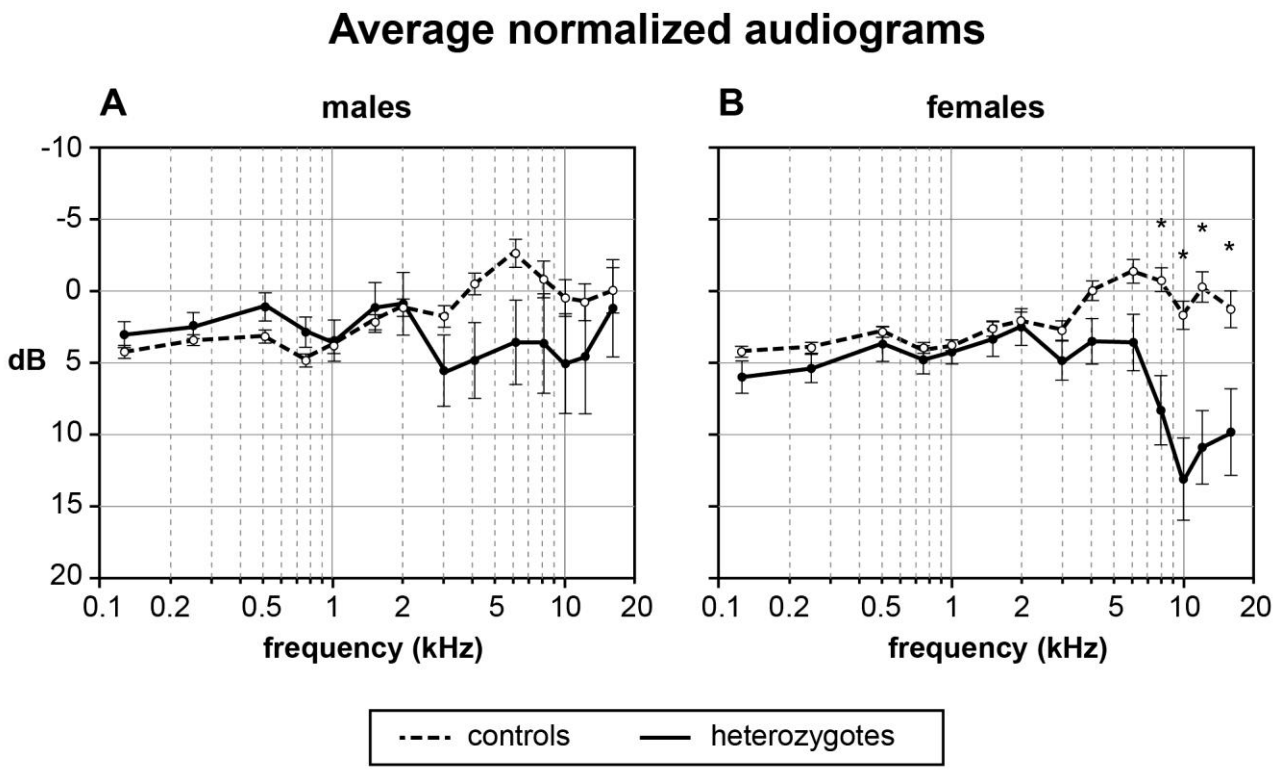

Fig. 2: Averaged normalized audiograms of heterozygous (n=13) and control (n=56) males (A) and heterozygous (n=21) and control (n=66) females (B). (* $p<0.0001$). *Error bars represent* ± *standard error of the mean.*

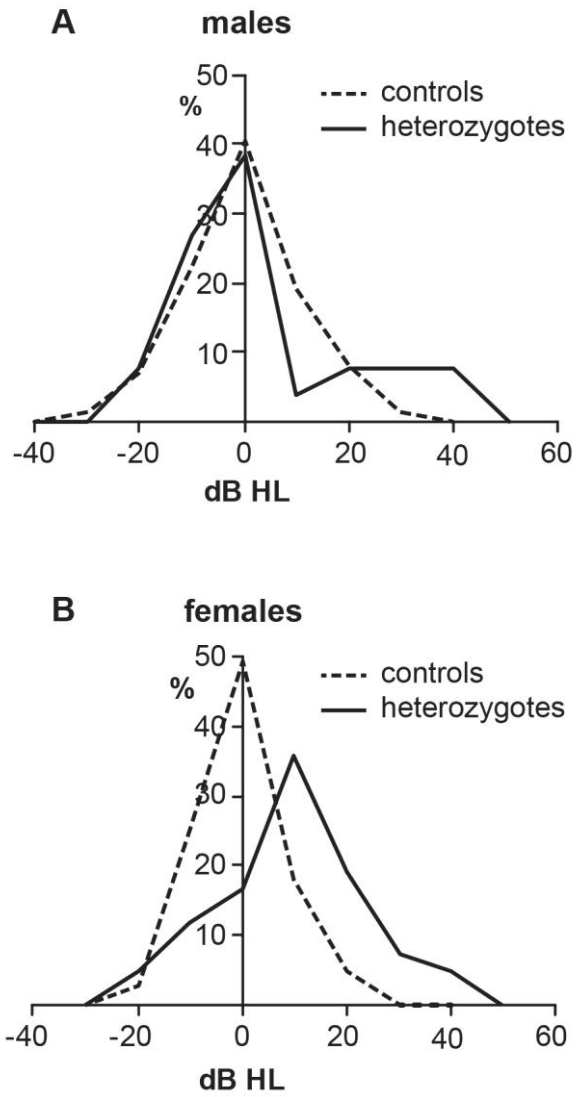

Fig. 3: Histograms of the average normalized HF thresholds (8-16 kHz) in control and heterozygous males (A) and females (B).

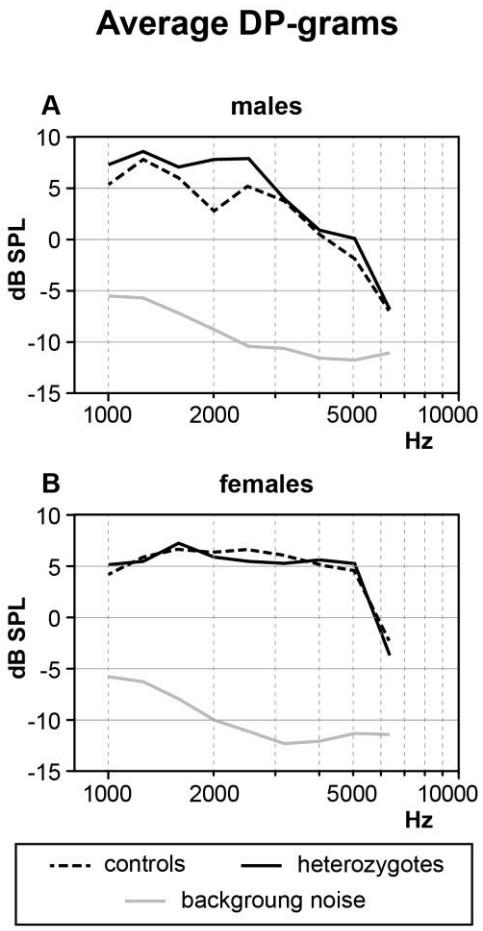

Fig.4: Average DP-grams for control and heterozygous males (A) and females (B).

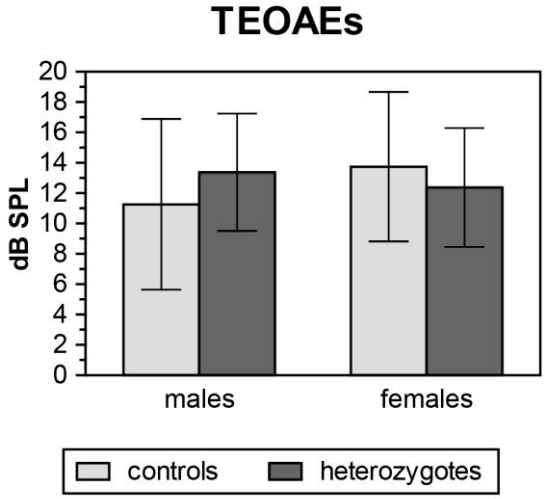

Fig.5: Average TEOAE responses for control and heterozygous males and females. Error bars represent ± standard deviation.