ORIGINAL ARTICLE

Contribution of the N-acetyltransferase 2 polymorphism *NAT2*6A* to age-related hearing impairment

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Background: Age-related hearing impairment (ARHI) is the most common sensory impairment in older people, affecting 50% of those aged 80 years. The proportion of older people is increasing in the general population, and as a consequence, the number of people affected with ARHI is growing. ARHI is a complex disorder, with both environmental and genetic factors contributing to the disease. The first studies to elucidate these genetic factors were recently performed, resulting in the identification of the first two susceptibility genes for ARHI, *NAT2* and *KCNQ4*.
Methods: In the present study, the association between ARHI and polymorphisms in genes that contribute to the defence against reactive oxygen species, including *GSTT1*, *GSTM1* and *NAT2*, was tested. Samples originated from seven different countries and were combined into two test population samples, the general European population and the Finnish population. Two distinct phenotypes for ARHI were studied, Z_{low} and Z_{high}, representing hearing in the low and high frequencies, respectively. Statistical analysis was performed for single polymorphisms (*GSTM1*, *GSTT1*, *NAT2*5A*, *NAT2*6A*, and *NAT2*7A*), haplotypes, and gene–environment and gene–gene interactions.
Results: We found an association between ARHI and *GSTT1* and *GSTM1* in the Finnish population sample, and with *NAT2*6A* in the general European population sample. The latter finding replicates previously published data.
Conclusion: As replication is considered the ultimate proof of true associations in the study of complex disorders, this study provides further support for the involvement of *NAT2*6A* in ARHI.

Age-related hearing impairment (ARHI) is the most frequent sensory impairment in older people. It is a complex disorder; both environmental and genetic factors contribute to the disease. The contribution of genetic factors to the development of ARHI has been clearly demonstrated by several heritability studies.[1–3] The number of factors, their individual contribution, and their interaction with each other remain unknown. Much research effort has been put into elucidation of the environmental factors that are involved in ARHI, such as noise exposure,[4–7] ototoxic medication,[8–10] exposure to chemicals,[11 12] medical conditions,[13 14] malnutrition,[15–17] tobacco smoking and alcohol abuse,[14 18–22] although some of these factors remain controversial. In contrast, little is known about the contributory genetic factors.

Two recent genomewide linkage studies and a handful of association studies on candidate genes for ARHI were performed, which aimed to identify some of the genetic factors involved in ARHI. The genomewide linkage studies resulted in a total of seven candidate susceptibility regions for ARHI.[23 24] Association studies on *GSTM* and *GSTT*[25] and *DFNA5*[26] failed to detect an association with ARHI. Two other studies, one studying the involvement of *NAT*[27] and one studying *KCNQ4*,[28] resulted in the identification of the first susceptibility genes for ARHI.

Oxidative stress is considered to participate in the ageing process, and consequently, also in ARHI. It is caused by an imbalance between the production and the removal of reactive oxygen species (ROS).[29] The exact mechanism by which ROS may cause ARHI remains unknown. A series of insults in the inner ear during the lifetime, such as the influence of drugs, exposure to noise, ear diseases and age-dependent degeneration, all against a specific genetic background, might cause increased ROS levels, in turn leading to hair-cell damage.[30 31]

Glutathione (GSH) and glutathione-related antioxidant enzymes, such as glutathione S-transferases (GST), are involved in the detoxification of cytotoxic and carcinogenic molecules, and ROS. GSTs are present in the inner ear,[32 33] which may suggest that they may play a central role in the metabolism and inactivation of ototoxic compounds. Decreased GSH and GST activity levels cause increased susceptibility of cells to noxious insults and cell damage.[31] In the outer hair cells from the basal end of the cochlea, levels of GSH are lower, rendering the basal end of the cochlea more vulnerable to damage by free radicals.[34] Five different GST classes have been identified in humans. Some classes such as *GSTM1* (mu, chromosome 1p13.3), *GSTP1* (pi, chromosome 11q13), and *GSTT1* (theta, chromosome 22q11.2) are polymorphic.[35] The *GSTT1* polymorphism is caused by a deletion of a substantial part of the gene, causing a reduction of *GSTT1* activity in all tissues, whereas in people homozygous for the *GSTM1* deletion, the enzyme is absent.[35]

The N-acetyltransferase (*NAT*) enzymes are responsible for the detoxification of exogenic substrates by N-acetylation or O-acetylation, and are important for the balance of the oxidative

Abbreviations: ARHI, age-related hearing impairment; FDR, false discovery rate; GSH, glutathione; GSR, glutathione reductase; GST, glutathione S-transferase; GSTP1, glutathione S transferase ρ; GXP1, glutathione peroxidase 1; ROS, reactive oxygen species; SOD1, superoxide dismutase 1; SNP, single nucleotide polymorphism

status. Insufficiently acetylated drugs accumulate and may be converted to reactive drug metabolites by oxidative enzymes. It has been suggested that *NAT* reduces the possibility that these reactive metabolites are formed.[36] *NAT1* and *NAT2*, two isoenzymes, are both highly polymorphic.[37] Some of these polymorphisms result in the production of slow acetylating enzymes, while others will form rapid acetylators. Both slow and rapid acetylators have been linked to disease. Within this study, we focused on polymorphisms of *NAT2* as putative causative factors for ARHI. The missense substitutions of the *NAT2* alleles, G191A (*NAT2*14A*), T341C (*NAT2*5A*), G590A (*NAT2*6A*) and/or G857A (*NAT2*7A*), are associated with slow acetylator phenotypes[38] with different functional characteristics. *NAT2*5A* and *NAT2*6A* lead to decreased protein expression, and *NAT2*14A* and *NAT2*7A* result in reduced protein stability.[37] The *NAT2*5* cluster (all different polymorphisms for *NAT2*5*) shows the greatest reduction in acetylation, followed by *NAT2*14*, *NAT2*6* and the *NAT2*7* cluster.[37] In a previously published study, a Turkish Caucasian population was investigated by Ünal *et al*, who found an association of the *NAT2*6A* polymorphism and ARHI.[27]

For the current study, 2111 Caucasian subjects (age range of 53–67 years), from nine different centres and seven different countries, were genotyped. The subjects were divided into two population groups representing the general European population and the Finnish population. The aim of this study was to detect an association between genes related to oxidative stress and ARHI. Single gene polymorphisms (*GSTM1*, *GSTT1*, *NAT2*5A*, *NAT*6A*, and *NAT2*7A*), haplotypes, and gene–environment and gene–gene interactions were analysed.

METHODS

Samples

Caucasian volunteers aged 53–67 years from nine different centres in seven countries were collected from population registries or through clinical consultations. When samples were collected through audiological consultations, the spouses of included subjects were also included. Table 1 gives an overview of the nine different centres and their general collection data. Air conduction was measured at 0.125, 0.25, 0.5, 1, 2, 3, 4, 6 and 8 kHz, and bone conduction at 0.5, 1, 2 and 4 kHz from all participating volunteers. The full inclusion and exclusion criteria have been described previously.[28] Two distinct phenotypes of ARHI, Z_{low} and Z_{high}, were tested within this study. Z-scores were calculated as follows.[39] Based on the ISO 7029 standard,[40] frequency-specific thresholds were converted to Z-scores independent of sex and age,[39] defined as the number of standard deviations that the hearing threshold differs from the median value at a specific frequency. People with better hearing than the age-specific and sex-specific median at a certain frequency have a negative Z-score. For each subject, the better hearing ear was selected by averaging the Z-scores at 0.25, 0.5 and 1 kHz (Z_{low}), and at 0.2, 4 and 8 kHz (Z_{high}). All further calculations were performed using the respective Z-scores of the better hearing ear. After calculating the Z-scores and exclusion of phenotypic outliers for Z_{high}, samples were selected for analysis by selecting the 34% best and 34% worst hearing subjects of both sexes in each separate sample set, based upon Z_{high}. The selection of the samples was performed before data polishing. Analyses for Z_{low} were conducted after excluding the phenotypic outliers for Z_{low} as well.

Data polishing

Data polishing was based on a larger study that analysed 768 single nucleotide polymorphisms (SNPs) derived from 70 candidate susceptibility genes for ARHI (Illumina, San Diego, California; http://www.illumina.com). The first step in the data-polishing process consisted of the removal of all samples that had $\geqslant$10% missing genotypes. After removing these, SNP assays that led to >4% missing genotypes of all genotyped individuals were excluded. In the third step, Hardy-Weinberg equilibrium was tested for every approved SNP on all approved samples by χ^2 test. All the tests for the first three polishing steps were performed using an automated protocol with SAS software (SAS Institute Inc., North Carolina, USA; http://www.SAS.com). The threshold for significance for Hardy-Weinberg equilibrium was set at 0.001. No SNPs were excluded on the basis of Hardy-Weinberg equilibrium.

The fourth step of the polishing process consisted of the detection of genetic outliers, using two programs: CHECKHET[41] and the Graphical Representation of Relationship errors program (GRR; http://bioinformatics.well.ox.ac.uk/GRR).[42] As the homogeneous genetic background of each independent sample set enhances the power of a genetic association study, CHECKHET can be used to detect small numbers of subjects with a different genetic background compared with the genetic background of the majority of the tested sample set. The presence of related individuals in association studies using unrelated samples can lead to misleading conclusions about the significance of statistical tests. GRR detects putative relatives using the fact that related individuals share an excess of alleles identical by state. The cut-off value to exclude samples was 1.75 identical by state. In addition, GRR is also able to detect sample duplications. Table 2 gives an overview of the number of samples that were excluded during the different data-polishing steps. Apart from sample set 9, all centres contained samples with $\geqslant$10% missing genotypes. Only in sample set 3 was an important fraction of the samples lost due to missing genotypes (6.3%). For the other centres, the number of failed samples was negligible (0–1.6%). CHECKHET and GRR were used to detect

Table 1 Overview of sample collections

Centre number	Country	City	Collection	Age range (years)	No of samples	No selected for analysis
1	Belgium	Antwerp	Population registries	54–66	769	567
2	Belgium	Ghent	Clinic + spouses	55–65	237	203
3	UK	Cardiff	Clinic + spouses	53–67	374	252
4	The Netherlands	Nijmegen	Clinic + spouses	55–65	276	188
5	Germany	Tübingen	Clinic + population registries	53–67	395	276
6	Denmark	Copenhagen	Clinic + spouses	55–65	404	278
7	Italy	Padova	Clinic + spouses	53–67	359	246
8*	Finland	Oulu	Population registries	53–67	504	346
9*	Finland	Tampere	Clinic + spouses	55–65	256	184

For statistical analysis, the samples were grouped into two populations, a general European population containing all samples except those from Finland and the samples from centre 3, and the Finnish population, containing the two sample sets from Finland*.

Table 2 Number of samples used for further analysis after various data-polishing steps

Centre	No of selected samples	No of failed samples	CHECKHET	GRR	Total no of genetic outliers	Total no used for analysis
1	567	4	14	3	17	546
2	203	2	2	1	3	198
3	252	16	5	2	7	229
4	188	1	3	0	3	184
5	276	3	7	1	8	265
6	278	1	7	1	8	269
7	246	4	7	2	9	233
8	346	2	5	4	9	335
9	184	0	5	0	5	179

genetic outliers, which were found in all nine sample sets (1.5–3.7%) (table 2). Samples that were marked by CHECKHET and/or GRR as putative genetic outliers were excluded from further statistical analysis.

Correction for population stratification

Correction for population stratification was performed using the genomic control approach for quantitative traits as proposed by Devlin *et al*.[43] In brief, 70 independent markers (SNPs) that were not associated with the phenotype were selected across the genome and genotyped. For each of these markers, the quantitative phenotype (Z-score) was regressed on the genotype, coded linearly. In each regression, a test statistic was calculated as the squared ratio of the regression coefficient for genotype and its standard error. In the absence of stratification, this statistic has a χ^2 distribution with one degree of freedom. In cases of population substructure, this test statistic is inflated by a factor λ. To estimate λ, the observed median of the test statistic was divided by 0.456, which is the median under the hypothesis of no substructure. Subsequently, all p values were multiplied by this estimate of λ.[44] The λ values were calculated for the general European ($\lambda = 1.29$) and the Finnish population ($\lambda = 1.09$) groups.

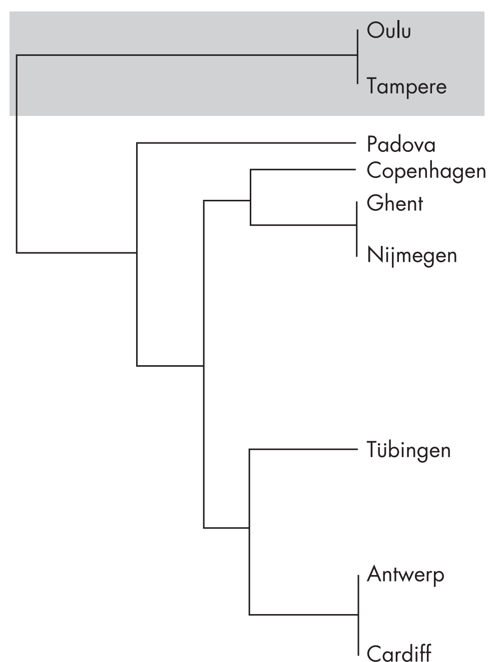

Figure 1 Phylogram based upon pairwise F statistics and the UPGMA method. From the nine original sample sets, the Finnish participants were combined together (Finnish population group, indicated in grey), and the remaining seven sample sets formed a second group (general European population group).

Combining several sample sets

To visualise the relationships of the sample sets, a phylogram of all centres was constructed based upon the SNP markers genotyped by Illumina. For this purpose, we applied the unweighted pair group method with arithmetic mean (UPGMA) clustering method to the distance matrix, which was determined by the pairwise F-statistics between the nine different sample sets.[45] The calculations were performed using Powermarker V.3.0 software (http://statgen.ncsu.edu/powermarker/[46]) and the tree topology was plotted with Treeview V.1.6.6 software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). The resulting phylogram showed that the samples fell into two population groups (figure 1). The general European population group (n = 1695) contained all sample sets, except for the two Finnish sample sets and the samples from centre 3 that were excluded from further analysis because of the low DNA quality. The Finnish population sample (n = 514) contained Finnish participants only. All further analyses were performed on the two population samples.

Genotyping

GSTM1 and GSTT1

All PCR reactions were conducted using 40 ng of DNA in 16 mmol/l $(NH_4)_2SO_4$, 67 mmol/l Tris-HCl (pH8.8 at 25℃), 0.1% Tween-20 and 1.5 mmol/l $MgCl_2$ (Eurogentec, Seraing, Belgium). Primer sequences are listed in table 3. To detect the homozygous null and the homozygous wild-type genotype of *GSTM1*, the PCR reaction was performed with *GSTM1* primers (0.1 μmol/l; length of PCR product 1030 bp)[47] and *HBB1* (haemoglobin β b1 subunit) primers (0.1 μmol/l; length of PCR product 1566 bp) (both Invitrogen, Carlsbad, California, USA), 250 μmol/l dNTPs (Amresco, Ohio, USA) and 0.04 U/μl Silverstar Taq polymerase (Eurogentec, Seraing, Belgium). The PCR reaction was initiated by a denaturation step at 94℃ for 5 min. The amplification reaction consisted of 35 cycles of denaturation at 94℃ for 45 s, annealing at 57.4℃ for 45 s and extension at 72℃ for 90 s. A final extension was performed at 72℃ for 10 min. The *GSTT1* genotyping assay consisted of a multiplex PCR reaction (0.5 μmol/l of each primer; length of PCR products 466 bp and 1460 bp) (Invitrogen),[48] with 200 μmol/l dNTPs (Amresco) and 0.0072 U/μl Silverstar Taq polymerase (Eurogentec). After a denaturation step of 94℃ for 5 min, the amplification reaction comprised 35 cycles of denaturation at 94℃ for 1 min, annealing at 68℃ for 1 min and extension at 72℃ for 1 min. A final extension was performed at 72℃ for 10 min.

Table 3 Primer and probe sequences for GSTM1, GSTT1 and NAT2 analysis

	Sequence
Primers	
GSTM1 forward	5′-AGACAGAAGAGGAGAAGATTC-3′
GSTM1 reverse	5′-TCCAAGTACTTTGGCTTCAGT-3′
HBB1 forward	5′-GACACAACTGTGTTCACTAGC-3′
HBB1 reverse	5′-CAGAATCCAGATGCTCAAGG-3′
GSTT1 (1) forward	5′-CAGTTGTGAGCCACCGTACCC-3′
GSTT1 (1) reverse	5′-CGATAGTTGCTGGCCCCCTC-3′
GSTT1 (2) forward	5′-CCAGCTCACCGGATCATGGCCAG-3′
GSTT1 (2) reverse	5′-CCTTCCTTACTGGTCCTCACATCTC-3′
NAT2*5A*6A*7A_forward	TGCATTTTCTGCTTGACA
NAT2*5A*6A*7A_reverse	GTTGGGTGATACATACACAA
NAT2*14A_forward	CATTGTGGGCAAGCCA
NAT2*14A_reverse	GTTGGGTGATACATACACAA
Probes	
NAT2*5A_FL	GCATTTTCTGCTTGACAGAAGAGAGAGGA-FL
NAT2*5A_LC	LC-TCTGGTACCTGGACCAAATCAGGA-PH
NAT2*6A_FL	GACGTCTGCAGGTATGTATTCATAGACTCAAAAT-FL
NAT2*6A_LC	LC-TCAATTGTTCGAGGTTCAAGCGT-PH
NAT2*7A_FL	TTCCTTGGGGAGAAATCTCGTGC-FL
NAT2*7A_LC	LC-CAAACCTGGTGATGGATCCCT-PH
NAT2*14A_FL	CACCCACCCCGGTTTCTTC-FL
NAT2*14A_LC	LC-TACAATGTGATCAAAAATAGCCTCTAAGCCC-PH

FL, fluorescein label with a detection wave length of 533 nm; LC, LightCycler red label with a detection wavelength of 640 nm; PH, phosphate.

NAT2

Genotyping of the NAT2 polymorphisms (*NAT2*5A*, *NAT2*6A*, *NAT2*7A* and *NAT2*14A*) was performed using primers and probes (TIB MOLBIOL, Berlin, Germany) designed for genotyping analysis on a real-time PCR system (LightCycler 480; Roche Diagnostics, Basel, Switzerland) (table 3). To perform the reaction, the system kit (LightCycler Genotyping Master Kit; Roche Diagnostics) was used in a final volume of 5 µl in 384 well plates with 20 ng of DNA. Final concentrations of PCR primers and probes were 0.5 µmol/l and 0.2 µmol/l, respectively. Protocols for genotyping of the four polymorphisms are given in table 4.

Statistical analysis

Genotype frequencies of *GSTs* and *NAT2* in the investigated sample sets

The frequencies of the investigated polymorphisms (*GSTM1*, *GSTT1*, *NAT2*5A*, *NAT2*6A* and *NAT2*7A*) in the population samples were calculated using SPSS V.12.0 for Windows (SPSS Inc., Chicago, Illinois, USA). The differences in frequencies between the general European and the Finnish population samples were calculated using Fisher's exact test with R Console V.2.0.1 software (http://www.r-project.org).

Association testing

All data were analysed using SPSS V.12.0 for Windows (SPSS Inc.). We tested for association between the Z-scores for both high and low frequencies and the polymorphisms of *GSTT1*, *GSTM1*, and *NAT2* respectively. A two-way analysis of variance (ANOVA) was used to account for sex differences.[49] Two-way ANOVA models were constructed in a stepwise backward fashion, with a saturated model including main effects for sex and genotype, as well as the interaction term sex × genotype. If no significant interaction was found, the interaction term was omitted from the analysis and a new model, consisting solely of the main effects for sex and genotype, was used. The normality of the residuals was tested for each genotype of each tested marker. We did not detect major differences in variance between the genotype groups, which is in agreement with the assumptions of ANOVA. If the interaction term was significant, one-way ANOVA was used to test men and women separately. The interaction between *GSTM1* and *GSTT1* and the haplotypes of *NAT2* were also analysed. To test the interaction between *GSTM1* and *GSTT1*, a similar two-way ANOVA model was used, replacing the sex interaction term by the *GSTM1* × *GSTT1* interaction term. Haplotypes were built using FAMHAP (http://iuni-bonn.de/~umt7oe/becker.htm[50]) and were analysed in the same way as the single SNPs. Significance was set at 0.05.

Environment–gene interaction

All study subjects were asked to fill in a questionnaire regarding their medical history, smoking habits, exposure to noise and solvents, and some general features such as body height, body weight, and eye colour (questionnaire available on request). Some of the answers on the questionnaire (see supplementary table S1, available online at http://jmg.bmj.com/supplemental)

Table 4 LightCycler protocols for *NAT2*5A*, *NAT2*6A*, *NAT2*7A* and *NAT2*14A*

Programme	Cycles	Analysis mode	Temperature (℃)	Hold	Ramp (℃/s)	Acquisition
Denaturation	1	None	95	10 min	4.8	None
Amplification	45	Quantification	95	5 s	4.8	None
			52/**55**	10 s	2.5	Single
			72	20/**35** s	4.8	None
Melting curve	1	Melting curve	95	1 min	4.8	None
			50	1 min	2	None
			75	1 s	–	Continuous
Cooling	1	None	40	30 s	2	None

Numbers in bold are the target temperature and incubation time for *NAT2*14A*.

had to be processed before statistical analysis could be initiated. For whiplash and diabetes, subjects were dichotomised into affected and non-affected groups. The latter group also contained subjects who did not recall a history of whiplash or did not know whether they had diabetes. The body mass index for each subject was calculated by dividing weight (kg) by height square (m^2). For the analysis of smoking habits, subjects were dichotomised into smokers and never smokers. Next, the number of pack years was estimated by multiplying the number of years a subject had been smoking, weighting for daily consumption of tobacco (weighting of 0.5 for <10 cigarettes/day, 1 for 10–20 cigarettes/day, 1.5 for >20 cigarettes/day). For non-smokers the number of pack years was set to zero. Noise exposure was not analysed in this study because the information obtained on hearing protection and noise exposure from our questionnaire was limited.

We tested interactions between a single *GST* or *NAT2* SNP and an environmental factor for both phenotypes (high and low frequency hearing loss) using SPSS V.12.0 for Windows (SPSS Inc). Because Z_{high} shows sex differences, sex was included in the statistical model as a main effect to correct for these differences. Z_{low} does not show such sex differences. Hence, sex was not taken into account for the statistical analysis of Z_{low}.

A two-way ANOVA was used to analyse the interaction of the *GST* or the *NAT2* genotype and categorical environmental factors. To test for interactions for Z_{high}, a saturated model was constructed including sex, environmental factor, and genotype as main effects, and an environmental × genotype interaction term. For Z_{low}, sex was omitted from the model. In the case of a continuous environmental factor, or if the size of the smallest test group for a categorical environmental factor was <10 (which violates the assumptions necessary to perform a two-way ANOVA) linear regression was performed. In addition, linear regression was performed if an interaction was found using two-way ANOVA on a categorical environmental factor. For linear regression, a two-model based approach was pursued. The first model contained sex (only for Z_{high}), genotype and environmental factor as main effects. In the second model, the main effect was the genotype × environment interaction term. If the interaction term remained significant, a one-way ANOVA was used to test the effect of the particular environmental factor.

Gene–gene interactions

Analysis for gene–gene interactions was conducted with R software (http://www.r-project.org). To test genetic interactions with *GST* or *NAT2*, we used a hypothesis-driven based approach, selecting only SNPs from other candidate ARHI susceptibility genes that were related to oxidative stress and genotyped by Illumina. In total, 22 SNPs in the *superoxide dismutase 1* (*SOD1*), *glutathione peroxidase 1* (*GXP1*), *CATALASE*, *glutathione reductase* (*GSR*) and *glutathione S transferase ρ 1* (*GSTP1*) genes were tested for interactions. For this analysis, phenotypic outliers for Z_{low} were also excluded in the Z_{high} analysis. A two-way ANOVA was performed to detect interactions. Because of sex differences in hearing, sex was introduced into the statistical analysis as a main effect (only for Z_{high}), correcting for differences between men and women. Interaction was fitted by introducing sex (only for Z_{high}), genotypes, and the genotype × genotype interaction term as main effects. Further strategies were identical to those for the single SNP and haplotype analyses.

RESULTS

Genotype frequencies of GSTs and NAT2 in the investigated sample sets

Table 5 shows the frequencies for the null deletions of *GSTM1* and *GSTT1*, and the slow acetylating genotypes of *NAT2*5A*, *NAT2*6A* and *NAT2*7A* in the eight different centres that we included in the final investigations. Because *NAT2*14A* was not polymorphic within our study sample, the genotyping analysis of *NAT2*14A* was limited to a subset of 920 samples. For this same reason, *NAT2*14A* was omitted from further analysis, and was not included in this table. We noticed significant differences in frequencies of the *GSTM1* ($p = 0.0005$) and *GSTT1* ($p = 0.004$) deletions and of *NAT2*6A* ($p = 0.028$) between the general European and the Finnish population samples, but the difference between the frequencies of the general European and the Finnish population groups for *NAT2*7A* was not significant ($p = 0.15$).

Statistical analysis of individual polymorphisms and haplotypes

No significant associations for *GSTM1*, *GSTT1* and ARHI could be detected in the general European population sample for Z_{low} and Z_{high}. However, analysis of the Finnish population sample resulted in a nominally significant p value for *GSTT1* in women only (Z_{high}) ($p = 0.035$) (table 6). Women homozygous for the *GSTT1* deletion had significantly worse hearing at high frequencies (figure 2A). Analysis for *GSTM1* in the Finnish population sample resulted in a significant p value (0.027) for Z_{high} (table 6). Subjects homozygous for the *GSTM1* deletion had significantly better hearing in the high frequencies than those who were not homozygous for the deletion (figure 2B). Interactions of both deletions were tested, but no effect on hearing could be demonstrated (data not shown).

Analysis for Z_{low} and Z_{high} in the general European population sample resulted in significant p values for *NAT2*6A* (Z_{high}, $p = 0.013$) (table 6). Subjects with the AA genotype for *NAT2*6A* had worse hearing compared with heterozygous or homozygous GG subjects, indicating that in the general European population sample, an increased risk of developing ARHI existed for subjects with the AA genotype of *NAT2*6A* (figure 3). No significant p values were found for the *NAT2* polymorphisms in the Finnish population sample.

For both the European and the Finnish population samples, six different haplotypes were constructed with three *NAT2* polymorphisms (*NAT2*5A*, *NAT2*6A* and *NAT2*7A*). For each population, three haplotypes were considered to be 'rare' and were grouped together. Subsequent statistical haplotype analysis did not result in any significant p values (data not shown).

Gene–environment interaction

For every tested subject, data on 20 environmental factors were available. Statistical analysis for gene–environment interactions in the general European and the Finnish population samples resulted in several significant p values for the *GST* deletions and *NAT2* in both samples. A false discovery rate (FDR) correction for multiple testing was calculated independently for GST deletions and *NAT2* in combination with the other SNPs following the Benjamini and Hochberg method described by Sabatti *et al*.[51] None of the significant p values survived this correction for multiple testing. All data on gene–environment interactions are available on request.

Gene–gene interaction

Gene–gene interactions were performed between *GSTM1*, *GSTT1* and *NAT2* polymorphisms and 22 additional SNPs originating from five candidate susceptibility genes for oxidative stress in ARHI. Statistical analysis resulted in many significant p values for the *GSTT1*, *GSTM1* and *NAT2* polymorphisms (range $p = 0.05$ to 0.0001). FDR was calculated using the method of Benjamini and Hochberg described by Sabatti *et al*.[51] None of the interaction p values remained significant after correction for multiple testing, except for two interactions with *NAT2*6A* and

Table 5 Frequencies (%) of GST null polymorphisms and NAT2 slow acetylating genotypes in different European countries

Centre	Country	*GSTM1*	*GSTT1*	*NAT2*5A*	*NAT2*6A*	*NAT2*7A*
Antwerp	Belgium	50	17	17	9	0
Gent	Belgium	60	11	20	6	0
Copenhagen	Denmark	50	13	21	10	0.4
Tübingen	Germany	48	12	17	8	0
Padova	Italy	46	14	19	9	0
Nijmegen	The Netherlands	51	15	17	10	0
Oulu*	Finland	42	10	16	7	0.3
Tampere*	Finland	38	6	19	4	0.5
General European population		50	15	18	9	0.06
Finnish population		40	9	17	6	0.4

*Finnish population.

GSR for both Z_{low} (6.5×10^{-6}) and Z_{high} (0.00061) in the Finnish population sample. The other gene–gene interaction results are not described within this paper. All data are available on request.

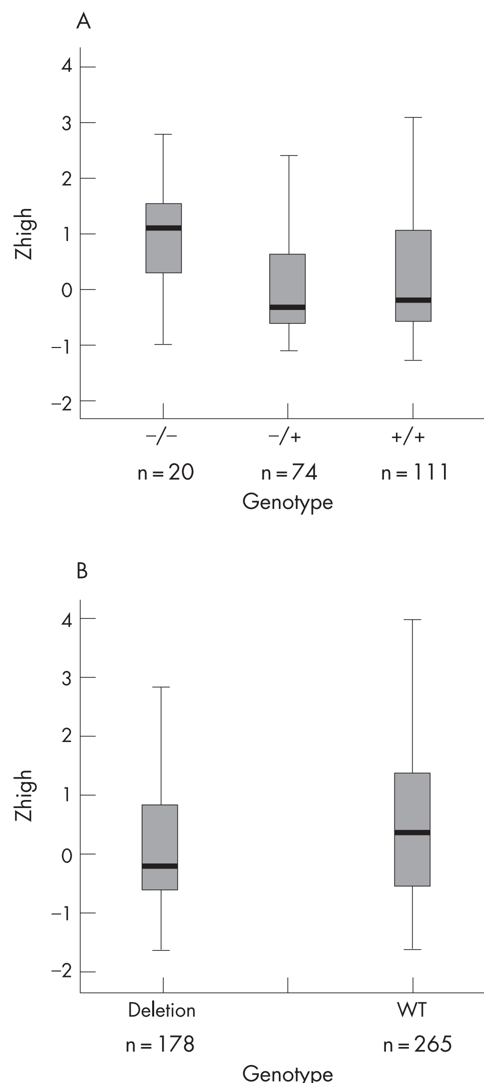

Figure 2 Box plots of significantly associated *GSTT1* and *GSTM1* polymorphisms in the Finnish population: (A) *GSTT1* for Z_{high} in women; (B) *GSTM1* for Z_{high} in the overall Finnish population. The sample size for each genotype is indicated below each box. The upper flag represents the 90th percentile (P90), the upper border of the box P75, the bold line P50, the lower border of the box P25 and the lower flag P10.

DISCUSSION

To date, only a handful of association studies on candidate susceptibility genes for ARHI have been published. A few of these studies concentrated on genes involved in oxidative stress.[25 27] Ates *et al* studied the contribution of variations in GSTs (*GSTM1*, *GSTP1*, *GSTT1*) to ARHI.[25] Individuals homozygous for the deletion of *GSTM1* or *GSTT1* cannot conjugate metabolites or toxins specific for these enzymes.[31 35] This might cause an increased susceptibility to ototoxic compounds and oxidative stress, with cell damage in the inner ear as a consequence. Starting from this working hypothesis, Ates *et al* performed a case–control study with 68 subjects and 69 healthy controls, but could not detect an association. The relatively small sample size and the consequent low power of this study might have been responsible for this negative result. Within the current study, a large set of 2111 independent samples was divided into two population groups, a general European and a Finnish population samples. The null mutation frequencies that we found for *GSTM1* and *GSTT1* in our study population are in agreement with previously published findings.[25 52] We found a significant difference in the frequencies of the *GSTM1* and *GSTT1* deletions between the general European and the Finnish population samples, perhaps because the Finnish population is genetically distinct from the general European population.[53]

In the general European population sample, we could not detect an association between *GSTM1* or *GSTT1* and ARHI.

Table 6 Two way analysis of variance (ANOVA) analysis for *GSTM1*, *GSTT1* and *NAT2* polymorphisms

	Z_{low}	Z_{high}
General European population		
GSTM1	1.00	1.00
GSTT1	0.37	0.80
NAT2*5A	1.00	0.68
NAT2*6A	0.21	**0.013**
NAT2*7A	0.72	1.00
Finnish population		
GSTM1	0.78	**0.027**
GSTT1	0.33	F = **0.035**, M = 0.32 (0.040*)
NAT2*5A	0.73	0.54
NAT2*6A	0.64	0.74
NAT2*7A	0.57	0.79

All p values were multiplied by λ to correct for population stratification (λ_{Europe} = 1.29; $\lambda_{Finland}$ = 1.09). If p was >1, these values were rounded down to 1.00.
Significant p values (p<0.05) are in bold.
Results of NAT2*14A are not included in this table because it was not polymorphic within our study populations.
*Two-way ANOVA sex × genotype interaction.

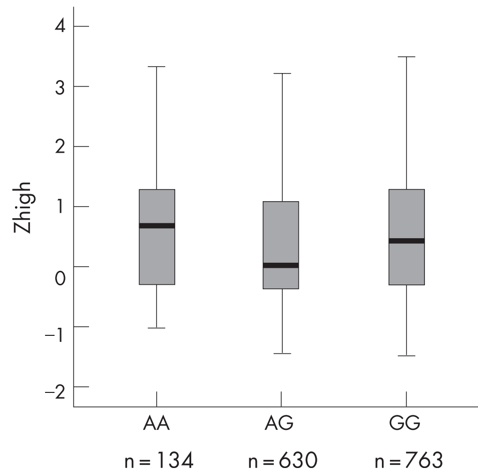

Figure 3 Box plots of the significantly associated NAT2*6A polymorphism in the general European population for Z_{high}. The sample size for each genotype is indicated below each box. The upper flag represents the 90th percentile (P90), the upper border of the box P75, the bold line P50, the lower border of the box P25 and the lower flag P10.

However, in the Finnish population sample, we found significant associations for both genes. In addition to the genetic distinctness observed in the Finnish population sample,[53] it has been demonstrated that the distribution of the *GSTM1* and *GSTT1* deletions differ between ethnic groups, and even between subpopulations of the same ethnicity,[54 55] thus it is very plausible that there is an increased risk for ARHI due to *GSTM1* and *GSTT1* polymorphisms in Finnish inhabitants only. This might also partly explain why Ates *et al* did not detect an association, as they were studying a Turkish population.[25]

A striking, but unexpected finding was that Finnish subjects homozygous for the *GSTM1* deletion had better hearing abilities than those without the deletion. This might indicate that this finding represents a false positive result. Bekris *et al*, however, had suggested that deletions of GSTs might have a protective effect on the development of diabetes.[56] In addition, an unexplainable protective effect against coronary artery disease and acute myocardial infarction of the *GSTM1* null genotype has been found.[57]

The fact that subjects lacking *GSTT1* had worse hearing is in agreement with our working hypothesis. The fact that this could only be found in women requires explanation. In another study, GST enzyme activity was found to be higher in women than in men.[58] If we extend this to our study, this might suggest that women are more vulnerable to develop ARHI due to oxidative stress and *GSTT1* deletions.

Ünal *et al* investigated the effect of *NAT2* polymorphisms on ARHI,[27] and found a significant association with *NAT2*6A*. In the current study we were able to replicate this finding, as we observed significant associations between ARHI and *NAT2*6A* for Z_{high} in the general European population sample. This is a strong indication of the contribution of *NAT2*6A* to the development of hearing impairment in older people. The *NAT2*6A* AA genotype encodes a slow acetylator, which slows down the detoxification mechanisms.[36] This might lead to a higher concentration of xenobiotics in the inner ear, which in turn might increase the number of acquired mitochondrial mutations, eventually leading to cell damage and hearing loss. Ünal *et al* observed a prevalence of 1–7% for slow acetylators in the controls of their tested population samples. Within our study, 9% slow acetylating genotypes (AA) were present for *NAT2*6A* in the general European population sample, and 6% in the Finnish population sample. This is in agreement with Ünal's findings. In addition, all slow acetylating genotype frequencies of *NAT2*5A*, *NAT2*6A* and *NAT2*7A* in the eight different sample sets were similar to previously published data.[27 52]

In addition to single SNP analysis, a gene–gene interaction analysis between *NAT2* and the *GSTM* and *GSTT* genes and five other genes that are involved in oxidative stress was performed. Two of these interactions, between *NAT2*6A* and the *GSR* gene, survived the FDR correction. This may indicate that some genes of the oxidative stress defence mechanism, specifically *GSR*, do contribute with *NAT2* to the development of ARHI.

Performing association studies for common disorders with many contributing factors with small effects will probably result in only marginally significant p values,[59] and there is always a question as to whether these are true findings or false positives. Usually, a correction for multiple testing is suggested. However, exactly how to correct remains an unresolved issue among genetic epidemiologists. The Bonferroni correction is usually considered too strict, and might lead to false negative results. Other, less conservative methods, such as FDR, have been suggested;[51] however, if we would perform an FDR correction for the single SNP analysis, none of the significant p values would survive. For single gene analysis, some authors have expressed doubt as to whether a correction for multiple testing is necessary, and stress the importance of replication rather than detecting very low p values.[60] For gene–environment and gene–gene interactions, we opted to rely on FDR to correct for multiple testing, as we performed many tests for these analyses.

In conclusion, the current study provides evidence that GST genes may be involved in ARHI, buth additional research is required to prove our recent findings. In addition, we present here the first replication of a previously described association, providing further support for the hypothesis that *NAT2* is one of the genes involved in the development of ARHI.

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The supplementary table 1 can be viewed on the JMG website at http://jmg.bmj.com/supplemental

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