

## Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Vandebona H, Mitchell P, Manwaring N, et al. Prevalence of mitochondrial 1555A→G mutation in adults of European descent. *N Engl J Med* 2009;360:642-4.

## **Methods**

### ***Patient recruitment***

In 1991, we identified 4443 eligible non-institutionalized permanent residents aged 49 years or older in a door-to-door census of two suburban postcode areas, west of Sydney. Of this target population, 3654 persons (82.4%) participated in the Blue Mountains Eye baseline survey (1992-4, BMES I). During 1997-9, 2335 of the 3111 survivors (75.1%) participated in 5-year follow-up examinations (BMES IIA). Participants of BMES IIA were invited to participate in the BMHS during 1997-2000. In 1999, a repeat door-to-door census was conducted in the same area; 1174 of the 1378 newly eligible residents who had moved into the study area or entered into the study age group (85.2%) participated in BMES IIB during 1999-2000. Of these 3509 participants, 2956 (84.2%) also took part in the BMHS. All participants gave written, informed consent and the University of Sydney and local Human Research Ethics Committees approved the study in accordance with the tenets of the Declaration of Helsinki. We obtained written informed consent from all participants.

An interview was conducted face to face to collect a comprehensive battery of medical information. A history of hearing and life-style factors was obtained from all participants. An audiologist-administered questionnaire was given to collect demographic and socioeconomic data, a history of any self-perceived hearing problem, information regarding its severity, onset and duration, in addition to whether primary care practitioners or other professionals had been consulted and if a hearing aid had been provided. The medical history enquired about information

regarding hearing loss risk factors, medications used, exercise, smoking and caffeine or alcohol consumption. We also collected information regarding family history of hearing loss, past medical or surgical treatment of otologic conditions, diseases associated with hearing loss and risk factors for ear disease. Other questions addressed exposure to noise at work, during military service or leisure activities, and past use of ototoxic drugs. The duration of the noise exposure was also categorized in years. Participants also self-reported ancestral background by completing a multiple choice questionnaire with the following categories: White, Black, Asian, Indian, Hispanic, Oceanian, and Aboriginal (indigenous). 97% of the population was self-reported White.

### ***Hearing assessment***

Pure-tone audiometry was performed by qualified audiologists. Audiometric thresholds for air-conduction stimuli (in both ears) were established for frequencies at 250, 500, 1000, 2000, 4000, 6000, and 8000 Hz, with 3000 Hz added if a 20 decibel (dB) difference existed between the 2000 & 4000 Hz thresholds. Bone conduction was evaluated whenever air conduction thresholds were greater than 15 decibels hearing level (dB HL) for frequencies of 500, 1000, 2000, and 4000 Hz. For the purposes of this analysis, we defined hearing impairment as the pure-tone average of audiometric hearing thresholds at 500, 1000, 2000 and 4000 Hz,  $>25$ ,  $>40$  or  $>60$  dB HL, in the better of the two ears. Hearing thresholds  $>25$  dB HL but  $\leq 40$  dB were defined to indicate 'mild' hearing loss; thresholds  $>40$  dB HL but  $\leq 60$  dB indicated 'moderate' hearing loss and

thresholds >60 dB HL indicated 'marked' hearing loss.

### ***MtDNA analysis***

We collected blood and hair follicles samples for DNA extraction from 2856 of the 2956 BMHS participants (96.6%). Ninety participants, classified with either childhood onset hearing loss (n=14), conductive hearing loss (n=58) or otosclerosis (n=18) were excluded, as well as one participant without complete audiological data, and ten patients whose DNA could not be amplified, resulting in 2856 participants with both DNA and complete relevant audiological data available for analysis. We extracted DNA from hair follicles (see Sue et al J Neurol Sci 1998;161:36-9) and blood using standard techniques. We detected the m.1555A>G mutation using PCR/RFLP techniques. MtDNA 1555 nucleotide region was amplified by PCR using 20-base-pair forward and reverse primers from mtDNA nt1492 and nt1803 regions respectively. The PCR product was digested with *BsmA1* restriction enzyme (New England Biolabs, USA). The resulting restriction fragments were resolved in a 5% polyacrylamide gel and scanned by gelscan 2000 (Corbett Research, Sydney, Australia). Wild type products with A nucleotide at 1555 position of mitochondrial DNA resolved two DNA fragments in the gel (64 bp and 248 bp), whereas the m.1555A>G mutation causes a loss of restriction site, and thus only a single band (312 bp) is detected in the presence of a homoplasmic m.1555A>G mutation. When detected by PCR/RFLP analysis, the presence of the m.1555A>G mutation was confirmed by direct sequencing of a repeat sample on an ABI PRISM 3100

Genetic Analyser (Applied Biosystems). MtDNA haplogroup analysis was performed using standard techniques (see Manwaring et al., Intern Med J 2006;36:530-3).

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**Table 1: Clinical features of individuals with m.1555A>G mutation**

Hearing loss data from the Blue Mountains Hearing Study (baseline)													
ID #	Age at test (yrs)	Gender	Haplogroup	Better ear †	Worse ear †	Duration of HL (yrs)	AAO	Maternal FH of HL	Ototoxic drug exposure	Noise exposure	Diabetes	Current smoking	Hearing aid
0201	78	F	J	40-60	>60	> 20	58	Yes	Quinine; Frusemide	No	Yes	No	Yes
0356	73	F	unassigned	25-40	>60	10-11	61	Yes	NR	No	No	No	Yes
2320	63	F	U	25-40	25-40	1-5	58	No	No	No	No	Yes	No
3126	77	M	U	25-40	25-40	1-5	70	Yes	No	Yes	No	No	No
3187	72	F	H	<25	25-40	N/A*	N/A*	No	NR	No	No	No	No
3628	74	F	J	25-40	25-40	6-10	66	No	No	No	Yes	No	No

HL = hearing loss; N/A = not applicable. \*This participant did not complain of hearing loss so duration and age of onset of hearing loss were not reported. AAO = Age at onset of hearing loss. Maternal FH of HL = Maternal family history of hearing loss (defined as at least 1 relative such as mother, siblings or children (if a female carrier), having hearing loss). NR= not reported.

†Hearing loss defined as the pure-tone average of audiometric hearing thresholds at 500, 1000, 2000 and 4000 Hz (dB HL).

Table 2: Summary of audiogram data in individuals with m.1555A>G mutation

Patient ID	250Hz R/L	500Hz R/L	1000Hz R/L	2000Hz R/L	4000Hz R/L	6000Hz R/L	8000Hz R/L
0201	30/ <b>40</b>	35/ <b>40</b>	40/ <b>60</b>	60/ <b>70</b>	75/ <b>80</b>	<b>110</b> /95	888/888
0356	<b>85</b> /60	<b>80</b> /30	<b>60</b> /35	<b>60</b> /30	55/ <b>60</b>	<b>70</b> /65	<b>75</b> /65
2320	5/ <b>20</b>	10/ <b>20</b>	15/15	30/30	70/ <b>75</b>	90/ <b>95</b>	85/ <b>100</b>
3126	5/ <b>10</b>	<b>10</b> /5	15/15	15/ <b>25</b>	<b>65</b> /60	60/ <b>75</b>	65/ <b>75</b>
3187	15/15	20/20	<b>25</b> /5	<b>30</b> /20	<b>45</b> /30	<b>55</b> /35	<b>65</b> /45
3628	20/ <b>30</b>	20/20	10/ <b>20</b>	<b>20</b> /15	<b>65</b> /60	75/ <b>95</b>	65/ <b>70</b>

R= right ear; L=left ear. Worst ear readings are in bolded text.