

BRIEF REPORT

Modification of Human Hearing Loss by Plasma-Membrane Calcium Pump PMCA2

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SUMMARY

Five adult siblings presented with autosomal recessive sensorineural hearing loss: two had high-frequency loss, whereas the other three had severe-to-profound loss affecting all frequencies. Genetic evaluation revealed that a homozygous mutation in *CDH23* (which encodes cadherin 23) caused the hearing loss in all five siblings and that a heterozygous, hypofunctional variant (V586M) in plasma-membrane calcium pump PMCA2, which is encoded by *ATP2B2*, was associated with increased loss in the three severely affected siblings. V586M was detected in two unrelated persons with increased sensorineural hearing loss, in the other caused by a mutation in *MYO6* (which encodes myosin VI) in one and by noise exposure, suggesting that this variant may modify the severity of sensorineural hearing loss caused by a variety of factors.

APPROXIMATELY 1 IN 1000 CHILDREN IS BORN WITH FUNCTIONALLY SIGNIFICANT sensorineural hearing loss, and another 1 in 1000 will have sensorineural hearing loss by nine years of age.¹ At least half these cases have a genetic cause. There are hundreds of genes in which mutations cause sensorineural hearing loss either as the sole clinical feature or in combination with extra-auditory manifestations as part of a syndrome.² Some genes underlie both syndromic and nonsyndromic forms of sensorineural hearing loss: for example, recessive mutations in *CDH23* cause either the Usher syndrome (retinitis pigmentosa and sensorineural hearing loss) or nonsyndromic sensorineural hearing loss.³ *CDH23* encodes cadherin 23, a putative calcium-dependent adhesion molecule required for proper morphogenesis of mechanosensitive hair bundles of the inner-ear neurosensory cells.⁴ There can be clinically significant variation in the severity of sensorineural hearing loss caused by mutations in *CDH23*^{5,6} or other genes⁷ or by exposure to ototoxic factors, such as noise or aminoglycoside antibiotics.^{8,9} Modifier genes, environmental factors, or both most likely account for these individual variations. These same modifiers may also contribute to the pathogenesis of presbycusis, which is increasingly prevalent with advanced age but is thought to arise from complex, lifelong interactions of unknown genetic and nongenetic factors.¹⁰

We evaluated a family in which five siblings were affected by autosomal recessive sensorineural hearing loss. Despite the presumably shared cause of the disorder, there were clinically significant differences among the siblings in the severity of their hearing

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loss. We undertook this study to identify the cause of their sensorineural hearing loss and a potential genetic modifier of its severity. We then sought to determine whether this same modifier might account for variations in the severity of sensorineural hearing loss caused by other factors in unrelated persons.

METHODS

SUBJECTS

This study was approved by the institutional review board of the National Institute of Neurological Disorders and Stroke and the National Institute on Deafness and Other Communication Disorders, National Institutes of Health. All the participants gave written informed consent before participation. The participants were members of the LMG132 family, which is descended from European ancestors. Medical-history interviews, physical examinations, video nystagmography with caloric testing, and pure-tone and speech audiometry were performed. The Usher syndrome was ruled out by funduscopy and electroretinography.

GENETIC ANALYSIS

Genomic DNA was extracted from venous-blood samples (Puregene, Genra Systems). DNA samples were genotyped for short tandem-repeat markers flanking known nonsyndromic recessive deafness loci, and all exons of *CDH23* were sequenced essentially as described.³ Primer sequences and polymerase-chain-reaction amplification and sequencing conditions for *ATP2B2*, which encodes the plasma-membrane calcium pump PMCA2, are provided in Table 1 of the Supplementary Appendix (available with the full text of this article at www.nejm.org). Control DNA samples were obtained from Coriell Cell Repositories and consisted of Human Variation Panels HD01 through HD09, HD027, and HD100CAU (described by the repository as a panel of samples from "self-declared Caucasians"). Our laboratory collected additional normal control DNA samples from 14 unrelated persons with a variety of self-reported European ancestries.

PMCA2 FUNCTIONAL ASSAY

Expression vectors were constructed for the PMCA2a splice isoform of PMCA2, since the former is the predominant PMCA isoform expressed in hair bundles in bullfrogs and inner-ear neurosensory cells in rats.¹¹ Full-length human complementary DNA

fragments encoding wild-type PMCA2a or PMCA2a with the V586M variant (in which methionine replaces valine at amino acid position 586) were sequenced in their entirety and subcloned into baculovirus expression vector pVL1392 (Invitrogen). Details of the cloning procedures are provided in the Methods section of the Supplementary Appendix. Preparation and amplification of recombinant baculovirus, expression of PMCA2a in Sf9 cells, preparation of microsomes, and measurement of ATPase activity were performed as described elsewhere.¹² Equivalent amounts of the expressed wild-type and mutant proteins were used in their respective reactions. The free calcium concentration was calculated with the Maxchelator program (www.stanford.edu/~cpatton/maxc.html).

CALCULATION OF *ATP2B2*^{V586M} FREQUENCIES

Some of the samples used to calculate *ATP2B2*^{V586M} frequencies were derived from members of the same families. To avoid duplicative counting of alleles that were identical by descent among members of the same family, we examined each pedigree to deduce the numbers of independent wild-type and *ATP2B2*^{V586M} alleles. If we could not determine whether two sampled alleles were identical or not identical by descent, we defined minimum and maximum possible values, respectively, which were used to calculate high and low composite estimates of the frequency of *ATP2B2*^{V586M} in the entire group of samples. Since the frequency of the *ATP2B2*^{V586M} allele was low and no homozygotes were detected, carrier frequency was approximated by doubling the allele frequency.

CASE REPORT

Five affected offspring (42 to 55 years of age) of a consanguineous union in Family LMG132 had autosomal recessive, nonsyndromic sensorineural hearing loss, with normal vestibular and retinal function (Fig. 1A). All five siblings had severe-to-profound high-frequency sensorineural hearing loss that had begun, according to their recollection, in the first decade of life, after the initial development of speech and language, and that had steadily progressed to current levels during the subsequent decade. However, there were two different phenotypes among the siblings: Subjects II-4 and II-6 had normal low-tone hearing, whereas Subjects II-1, II-9, and II-10 had severe-to-profound low-frequency loss that had begun in the first or second decade

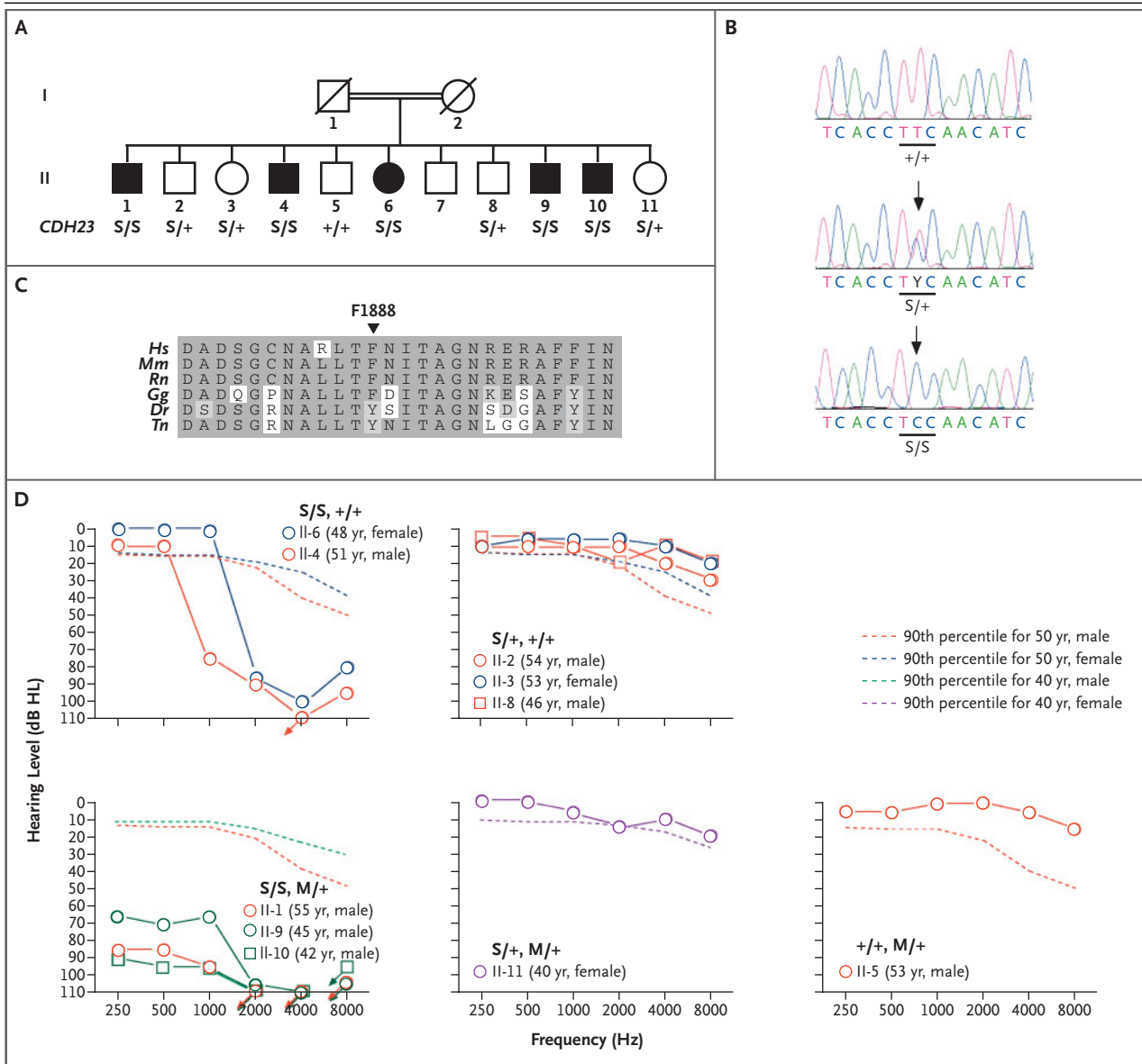


Figure 1. CDH23 Genotypes and Phenotypes of Members of Family LMG132.

Panel A shows the pedigree of five affected siblings, the offspring of a consanguineous union (double line), who were homozygous for the F1888S mutation (S) of *CDH23*. The 25-year-old child of a first cousin of the siblings had nonsyndromic, congenital, severe-to-profound sensorineural hearing loss affecting all frequencies (not shown). She was a compound heterozygote for F1888S and a novel frame-shift mutation (8882_8883insT) in *CDH23*. Solid symbols indicate persons with nonsyndromic sensorineural hearing loss, and symbols with a slash indicate deceased family members. Panel B shows electropherograms of wild-type (Subject II-5), heterozygous (Subject II-2), and homozygous (Subject II-10) genomic nucleotide sequences with respect to the missense mutation F1888S in exon 42 (arrows). Panel C shows the alignment of cadherin 23 amino acid sequences including and flanking F1888 (arrowhead) from *Homo sapiens* (Hs), *Mus musculus* (Mm), *Rattus norvegicus* (Rn), *Gallus gallus* (Gg), *Danio rerio* (Dr), and *Tetraodon nigroviridis* (Tn) (GenBank accession numbers AAG27034, AAG52817, NP_446096, XP_421595, NP_999974, and CAG04741, respectively). The alignment program ClustalW was used. Identical residues are indicated by dark shading and conservatively substituted residues by light shading. Amino acids are denoted by their single-letter codes. Panel D shows pure-tone air-conduction thresholds for the better-hearing ear of Subjects II-1 through II-11. Bone-conduction thresholds were consistent with the presence of sensorineural hearing loss (data not shown). Arrows indicate that there was no response to a stimulus at the highest tested level. Normative 90th percentile pure-tone thresholds are from International Organization for Standardization publication ISO 7029.²³ dB HL denotes decibels hearing level. Audiometric profiles are grouped according to *CDH23* and *ATP2B2* genotypes, where S denotes the F1888S mutation and M the V586M variant.

of life and that had been followed by steady progression to current levels during the subsequent few decades (Fig. 1D). The latter group relied on sign language, lip-reading, hearing aids, or a cochlear implant for communication, in contrast to the two siblings whose intact low-frequency and mid-frequency hearing permitted oral and auditory communication without hearing aids. There was no history of exposure to aminoglycoside antibiotics, ototoxic noise levels, head trauma, or systemic or otic infections that could account for the sensorineural hearing loss in the five affected siblings.

RESULTS AND DISCUSSION

CDH23 DEAFNESS IN FAMILY LMG132

All five affected siblings were homozygous for short tandem-repeat markers linked to *CDH23* on chromosome 10q22.1 (data not shown). Genomic nucleotide-sequence analysis of *CDH23* exons in the affected siblings revealed homozygosity for a point mutation (5663T→C; GenBank accession number, AY010111) in exon 42, predicted to result in the substitution of serine for phenylalanine at amino acid position 1888 (F1888S; GenBank accession number, AAG27034) in the extracellular domain of cadherin 23 (Fig. 1B). This phenylalanine residue is conserved in mouse, rat, and chicken cadherin 23 (Fig. 1C) but is not located within the motifs involved in calcium-mediated intermolecular associations among cadherins.¹⁴ The *CDH23*^{F1888S/F1888S} genotype cosegregated with sensorineural hearing loss in Family LMG132, and the *CDH23*^{F1888S} mutation was not detected in 108 European (“Caucasian”) control samples.

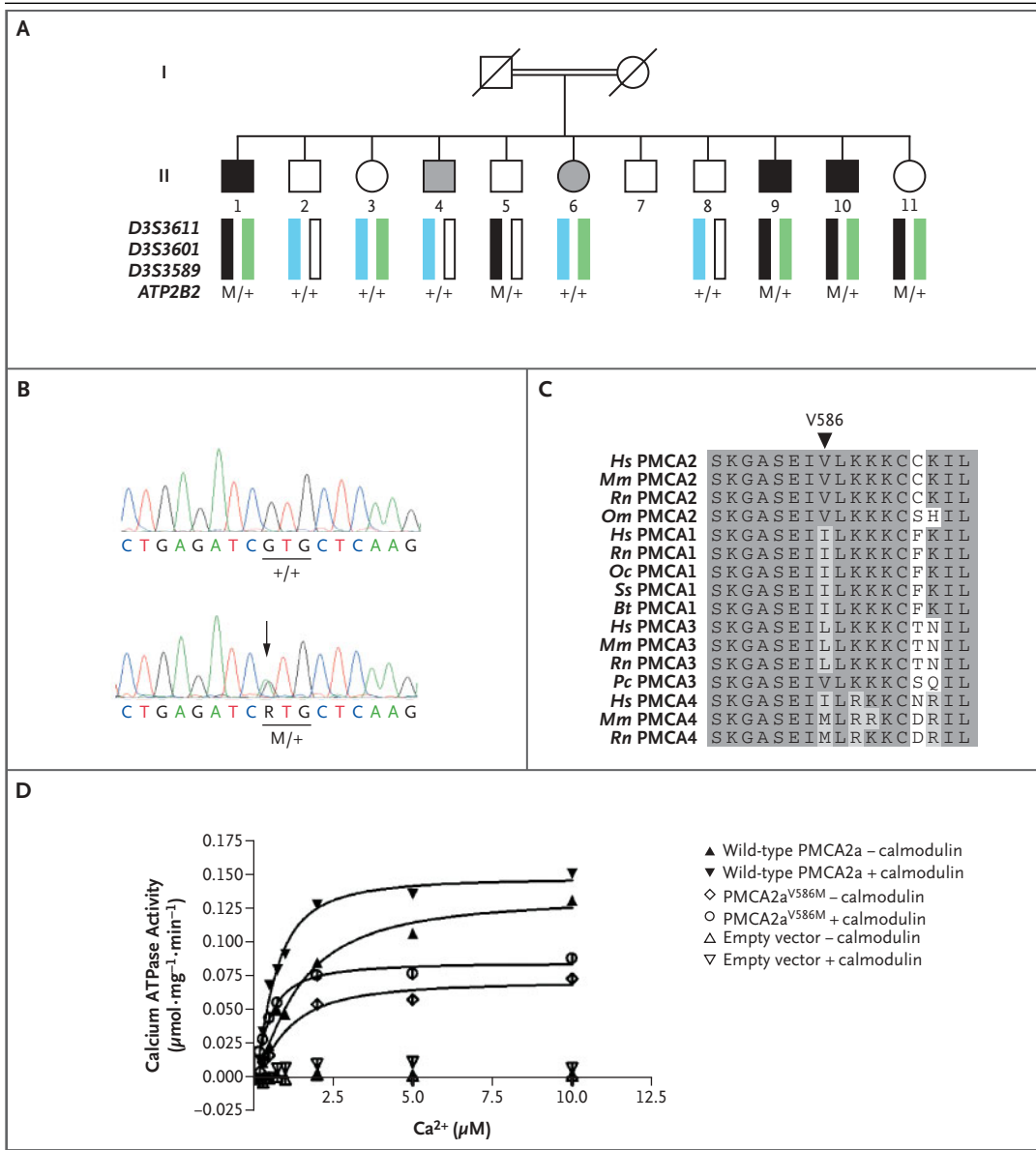
ATP2B2 AS A MODIFIER OF CDH23 DEAFNESS IN FAMILY LMG132

A variety of recessive mutations of *Cdh23* cause profound deafness and vestibular dysfunction in homozygous waltzer mice,¹⁵ whereas another allele of *Cdh23*, called *ahl*, underlies less severe, age-related hearing loss in many inbred mouse strains.¹⁶ The severity of this age-related hearing loss is significantly increased by heterozygosity for the *dfw2j* deaf-waddler allele of *Atp2b2*,¹⁶ which encodes PMCA2, the predominant PMCA of hair bundles. This interaction has been attributed to a reduction in PMCA2 activity that results in a decrease in extracellular calcium concentrations around hair bundles, where calcium-dependent, cadherin-mediated adhesion is thought to occur.^{17,18}

Figure 2 (facing page). V586M Allele of ATP2B2.

Panel A shows *ATP2B2*-linked short tandem-repeat marker (D3S3611, D3S3601, and D3S3589) haplotypes and *ATP2B2* genotypes of each member of generation II of Family LMG132. Solid symbols indicate persons with severe-to-profound sensorineural hearing loss across all frequencies, and shaded symbols persons with normal low-tone hearing thresholds and severe-to-profound sensorineural hearing loss at high frequencies. Subjects II-1, II-9, and II-10, all of whom had severe-to-profound hearing loss affecting all frequencies, were heterozygous for the haplotype that cosegregates with V586M (M) (black bars). The green, blue, and open bars represent different haplotypes. Panel B shows electropherograms of wild-type (Subject II-4) and heterozygous (Subject II-10) genomic nucleotide sequences with respect to the missense substitution V586M in exon 12 of *ATP2B2* (arrow). Panel C shows the alignment of amino acid sequences including and flanking V586 (arrowhead) of *ATP2B2* orthologues and paralogues: *Homo sapiens* (*Hs*) PMCA2, *Mus musculus* (*Mm*) PMCA2, *Rattus norvegicus* (*Rn*) PMCA2, *Oreochromis mossambicus* (*Om*) PMCA2, *H. sapiens* PMCA1, *R. norvegicus* PMCA1, *Oryzotolagus cuniculus* (*Oc*) PMCA1, *Sus scrofa* (*Ss*) PMCA1, *Bos taurus* (*Bt*) PMCA1, *H. sapiens* PMCA3, *M. musculus* PMCA3, *R. norvegicus* PMCA3, *Procambarus clarkii* (*Pc*) PMCA3, *H. sapiens* PMCA4, *M. musculus* PMCA4, and *R. norvegicus* PMCA4 (GenBank accession numbers NP_001674, NP_033853, P11506, P58165, P20020, P11505, Q00804, NP_999517, NP_777121, Q16720, NP_796210, XP_343840, AAR28532, NP_001001396, NP_998781, and NP_001005871, respectively). The alignment program ClustalW was used. Identical residues are indicated by dark shading, and conservatively substituted residues by light shading. Amino acids are denoted by their single-letter codes. Panel D shows the calcium ATPase activity of wild-type PMCA2a and PMCA2a^{V586M} in the absence or presence of 300 nM calmodulin. Three ATPase experiments were performed for each expressed protein from three microsomal preparations of two different expressed-protein preparations. Representative data are shown from one of the three experiments. The slope and standard error of the determination of activity were calculated by linear regression.

We hypothesized that one or more alleles of *ATP2B2* modify the severity of sensorineural hearing loss caused by *CDH23*^{F1888S/F1888S}. DNA samples from Subjects II-1, II-4, II-6, II-9, and II-10 were genotyped for short tandem-repeat markers linked to *ATP2B2*. The resulting haplotypes were consistent with a model in which a dominant allele of *ATP2B2* (Fig. 2A, black haplotype bar) exacerbates sensorineural hearing loss in a manner analogous to the interaction between the *dfw2j* allele of *Atp2b2* and the *ahl* allele of *Cdh23* in mice. Nucleotide-sequence analysis of *ATP2B2* exons in *CDH23*^{F1888S}



homozygotes revealed that heterozygosity for a point mutation in exon 12 (2075G→A; GenBank accession number, NM_001683) was linked to this haplotype. The 2075G→A mutation is predicted to result in the substitution of methionine for valine at amino acid position 586 (V586M; GenBank accession number, NP_001674) in the T4-T5 intracellular catalytic loop of PMCA2 (Fig. 2B). Molecular modeling of V586M based on the three-dimensional structure of the closely related sarcoplasmic reticulum calcium pump predicts that substitution with the sterically larger methionine side chain distorts packing underneath the ATP-binding interface or

increases its projection from the external solvent-exposed surface of the nucleotide-binding domain (data not shown).

The valine residue at position 586 is completely conserved among mouse, rat, and fish PMCA2 orthologues, and either valine or a conservatively substituted residue (isoleucine) is present at this position in all known PMCA1 and PMCA3 amino acid sequences (Fig. 2C). Mouse and rat PMCA4 has methionine at this residue, but PMCA2 has a faster calcium-activation time than PMCA4.¹⁹ Since up-regulation and relocation of PMCA1 and PMCA4 to stereocilia do not rescue auditory function in *dfw2J*

deaf-waddler mice, the faster calcium-activation time of PMCA2 may be required for normal hearing.¹⁷ When expressed as a recombinant baculovirus protein in Sf9 cells, human PMCA2a^{V586M} has approximately 50 percent of the calcium ATPase activity of wild-type PMCA2a (Fig. 2D).

Studies of heterozygous deaf-waddler mice demonstrate that partial loss of PMCA2 activity is expected for an allele that modifies, but is not itself sufficient to cause, hearing loss. The *dfw* allele product contains a pathogenic amino acid substitution in the T2–T3 cytoplasmic loop; this product retains approximately 30 percent of wild-type PMCA2 activity.²⁰ *Atp2b2^{dfw/+}* mice have normal hearing thresholds, whereas mice that are heterozygous for loss-of-function alleles (*dfw^{2J}* and *dfw^{3J}*) of *Atp2b2* have functionally significant sensorineural hearing loss on the same genetic background.¹⁸ Analogous to *Atp2b2^{dfw}*, *ATP2B2^{V586M}* is not itself a dominant deafness-causing allele, since two siblings with normal hearing in Family LMG132 (Subjects II-5 and II-11) were *ATP2B2^{V586M}* heterozygotes (Fig. 1D and 2A).

ATP2B2^{V586M} AS A MODIFIER OF OTHER FORMS OF HEARING LOSS

To explore the phenotypic consequences of the *ATP2B2^{V586M}* allele further, we screened 57 affected members of unrelated families with various progressive hearing-loss phenotypes and identified *ATP2B2^{V586M}* in 1 subject. The affected *ATP2B2^{V586M}* heterozygote (Subject IV-6 described by Mohiddin et al.²¹) had age-related hearing loss associated with a dominant missense substitution of *MYO6*. She had low-frequency (0.25-, 0.5-, and 1-kHz) hearing loss that was more severe than would be predicted by linear regression estimates of sensorineural hearing loss as a function of age in her affected relatives (Fig. 3A). We also identified three *ATP2B2^{V586M}* heterozygotes, all of European ancestry, among 128 (119 self-reported European) unaffected members of families with a variety of other phenotypes. Two of these *ATP2B2^{V586M}* carriers had normal hearing (Fig. 3B and 3C), but the third had a history of occupational and recreational noise exposure and high-frequency sensorineural hearing loss that was highly characteristic of noise-induced ototoxicity (Fig. 3D). Four of the 125 subjects who did not carry the *ATP2B2^{V586M}* allele also had audiometric phenotypes consistent with noise-induced sensorineural hearing loss (data not shown). All four *ATP2B2^{V586M}* carriers with normal hearing (Subjects II-5 and II-11 of Family LMG132 [Fig. 1D] and

the two carriers described above [Fig. 3B and 3C]) reported that they had no history of noise exposure. Although we cannot conclusively correlate the sensorineural hearing loss in the carrier with noise exposure (Fig. 3D) with the *ATP2B2^{V586M/+}* genotype, it has been reported that heterozygosity for a null allele of *Atp2b2* predisposes mice to noise-induced sensorineural hearing loss.⁸

The allele frequency of *ATP2B2^{V586M}* was cumulatively estimated in the European members of Family LMG132 and these cohorts. The lowest and highest estimates of allele frequency for the entire group were 4 of 258 (1.6 percent; 95 percent confidence interval, 0.6 to 3.9 percent) and 5 of 218 (2.3 percent; 95 percent confidence interval, 1.0 to 5.2 percent), respectively. The differing estimates arose from ambiguities in the segregation, and thus the independence, of alleles within some pedigrees. The corresponding low and high heterozygous carrier frequencies were deduced to be 4 of 129 (3.1 percent; 95 percent confidence interval, 1.3 to 7.7 percent) and 5 of 109 (4.6 percent; 95 percent confidence interval, 2.0 to 10.3 percent), respectively. In agreement with these findings, we also detected *ATP2B2^{V586M}* in 4 of 87 normal “Caucasian” control samples from an independent source (Coriell Cell Repositories) (4.6 percent; 95 percent confidence interval, 1.9 to 11.2 percent). We did not detect *ATP2B2^{V586M}* in 87 normal Pakistani control samples, but we did detect *ATP2B2^{V586M}* in 3 of 84 DNA samples from a Human Diversity Panel (Coriell Cell Repositories) representing 10 ethnic backgrounds. All three *ATP2B2^{V586M/+}* samples were from a subgroup of five Pima Indian samples in this panel, although we could find no literature on hearing loss in Pima Indians. These carrier frequencies are consistent with a potential role for *ATP2B2^{V586M}* or other alleles of *ATP2B2* in the etiology of presbycusis.

Although the interaction of a heterozygous *dfw* allele with a hypomorphic *Cdh23* allele in *ahl* strains of mice suggests that *ATP2B2^{V586M}* could act as a dominant modifier allele in humans, our results do not formally rule out a model in which it is a recessive modifier allele that, in combination with another haplotype in Family LMG132 (Fig. 2A, green haplotype bars), exacerbates sensorineural hearing loss. It is possible that important sequence variants within noncoding regions of this allele were missed by our genomic sequencing protocol. Nonetheless, our study indicates that *ATP2B2^{V586M}* or other alleles of *ATP2B2* may be general modifiers of a variety of human hearing-loss phenotypes that are due

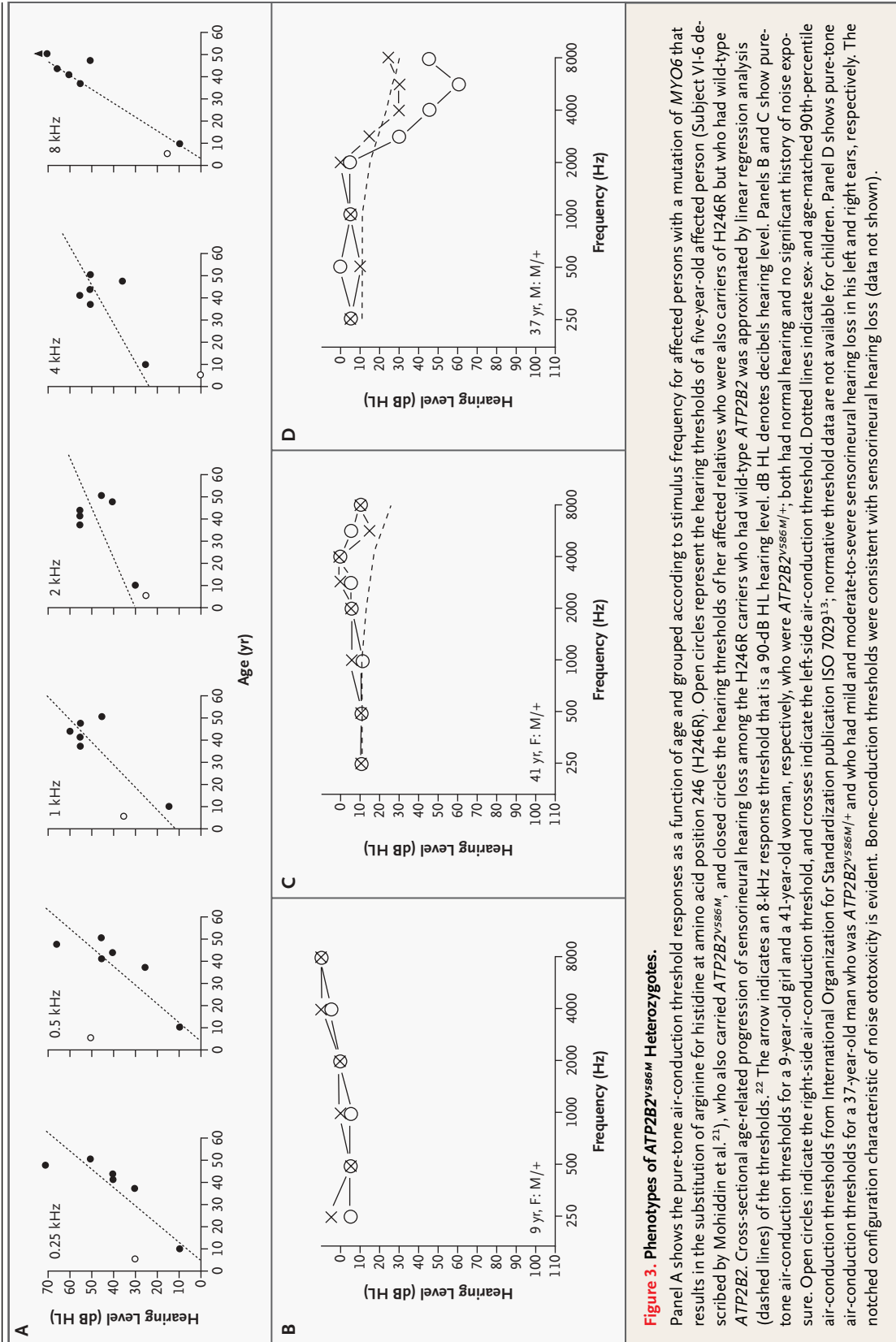


Figure 3. Phenotypes of ATP2B2^{V586M} Heterozygotes.

Panel A shows the pure-tone air-conduction threshold responses as a function of age and grouped according to stimulus frequency for affected persons with a mutation of MYO6 that results in the substitution of arginine for histidine at amino acid position 246 (H246R). Open circles represent the hearing thresholds of a five-year-old affected person (Subject VI-6 described by Mohiddin et al.²¹), who also carried ATP2B2^{V586M}, and closed circles the hearing thresholds of her affected relatives who were also carriers of H246R but who had wild-type ATP2B2. Cross-sectional age-related progression of sensorineural hearing loss among the H246R carriers who had wild-type ATP2B2 was approximated by linear regression analysis (dashed lines) of the thresholds.²² The arrow indicates an 8-kHz response threshold that is a 90-dB HL hearing level. dB HL denotes decibels hearing level. Panels B and C show pure-tone air-conduction thresholds for a 9-year-old girl and a 41-year-old woman, respectively, who were ATP2B2^{V586M/+}; both had normal hearing and no significant history of noise exposure. Open circles indicate the right-side air-conduction threshold, and crosses indicate the left-side air-conduction threshold. Dotted lines indicate sex- and age-matched 90th-percentile air-conduction thresholds from International Organization for Standardization publication ISO 7029²³; normative threshold data are not available for children. Panel D shows pure-tone air-conduction thresholds for a 37-year-old man who was ATP2B2^{V586M/+} and who had mild and moderate-to-severe sensorineural hearing loss in his left and right ears, respectively. The notched configuration characteristic of noise ototoxicity is evident. Bone-conduction thresholds were consistent with sensorineural hearing loss (data not shown).

to genetic determinants, environmental factors, or combinations of these influences. Since *CDH23* and *MYO6* mutations and ototoxic noise directly affect sensory hair cells of the inner ear,^{2,4,23} the effects of *ATP2B2*^{V586M} may be confined to sensorineural hearing loss characterized by pathologic processes affecting primarily the hair cell. Although audiometric differences in *ATP2B2*^{V586M} carriers are most obvious with respect to low-frequency hearing in Family LMG132 (Fig. 1D) and in the family with a *MYO6* mutation (Fig. 3A), the lack of detectable high-frequency hearing in *ATP2B2*^{V586M} carriers in Family LMG132 (Subjects II-1, II-9, and II-10) and the sensorineural hearing loss in the *ATP2B2*^{V586M} carrier with noise exposure (Fig. 3D) raise the possibility that *ATP2B2*^{V586M} can modify hearing loss at all frequencies. Additional studies

are needed to address these questions and to provide accurate genetic, prognostic, lifestyle, and occupational (i.e., noise avoidance) counseling as well as communication-rehabilitation counseling based on *ATP2B2* genotype results.

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CORRECTION

**Modification of Human Hearing Loss by
Plasma-Membrane Calcium Pump PMCA2**

Modification of Human Hearing Loss by Plasma-Membrane Calcium Pump PMCA2 . On page 1557, lines 7 through 9 of the Summary should have read, "V586M was detected in two unrelated persons with increased sensorineural hearing loss, caused by a mutation in *MYO6* (which encodes myosin VI) in one and by noise exposure in the other . . .," rather than "V586M was detected in two unrelated persons with increased sensorineural hearing loss, in the other caused by a mutation in *MYO6* (which encodes myosin VI) in one and by noise exposure . . .," as printed. We regret the error.