Ectopic Mineralization and Conductive Hearing Loss in \textit{Enpp1}^{asj} Mutant Mice, a New Model for Otitis Media and Tympanosclerosis

Cong Tian$^{1,2,*}$, Belinda S. Harris$^1$, Kenneth R. Johnson$^1$

1 The Jackson Laboratory, Bar Harbor, Maine, United States of America, 2 Graduate School of Biomedical Science and Engineering, University of Maine, Orono, Maine, United States of America

Abstract

Otitis media (OM), inflammation of the middle ear, is a common cause of hearing loss in children and in patients with many different syndromic diseases. Studies of the human population and mouse models have revealed that OM is a multifactorial disease with many environmental and genetic contributing factors. Here, we report on otitis media-related hearing loss in \textit{asj} (ages with stiffened joints) mutant mice, which bear a point mutation in the \textit{Enpp1} gene. Auditory-evoked brainstem response (ABR) measurements revealed that around 90\% of the mutant mice (\textit{Enpp1}^{asj/asj}) tested had moderate to severe hearing impairment in at least one ear. The ABR thresholds were variable and generally elevated with age. We found otitis media with effusion (OME) in all of the hearing-impaired \textit{Enpp1}^{asj/asj} mice by anatomic and histological examinations. The volume and inflammatory cell content of the effusion varied among the \textit{asj} mutant mice, but all mutants exhibited a thickened middle ear epithelium with fibrous polyps and more mucin-secreting goblet cells than controls. Other abnormalities observed in the \textit{Enpp1} mutant mice include over-ossification at the round window ridge, thickened and over-calcified stapedial artery, fusion of malleus and incus, and white patches on the inside of tympanic membrane, some of which are typical symptoms of tympanosclerosis. An excessive yellow discharge was detected in the outer ear canal of older \textit{asj} mutant mice, with 100\% penetrance by 5 months of age, and contributes to the progressive nature of the hearing loss. This is the first report of hearing loss and ear pathology associated with an \textit{Enpp1} mutation in mice. The \textit{Enpp1}^{asj} mutant mouse provides a new animal model for studying tympanosclerotic otitis and otitis media with effusion, and also provides a specific model for the hearing loss recently reported to be associated with human \textit{ENPP1} mutations causing generalized arterial calcification of infancy and hypophosphatemic rickets.

Introduction

While many genes have been discovered that underlie Mendelian forms of sensorineural hearing loss (http://hereditaryhearingloss.org/), most cases of conductive hearing loss, often caused
by otitis media and tympanosclerosis, have complex and poorly understood etiologies. Otitis media is an inflammation of the middle ear and is among the most common childhood illnesses. Heritability studies have shown that genetic factors can play an important role in otitis media susceptibility, but few contributing genes have been identified in human populations [1]. In contrast to human studies, a growing number of mouse mutations have been identified that manifest a high incidence of otitis media, including \textit{Eya4}, \textit{Tlr4}, \textit{p73}, \textit{MyD88}, \textit{Fas}, \textit{E2f4}, \textit{Plg}, \textit{Fbxo11}, \textit{Evi1} [1,2], \textit{Sh3pxd2b} [3], \textit{Rpl38} [4], \textit{Isl1} [5], \textit{Chd7} [6], \textit{Lmna1} [7], \textit{Phex} [8], \textit{Oxgr1} [9], \textit{Tgif1} [10], and \textit{Mcph1} [11]. The wide diversity of these genes and their mutant pathologies, including craniofacial abnormalities with Eustachian tube malformations and innate immune response defects, underscores the complex nature of otitis media.

Here we report on the conductive hearing loss associated with a recessive ENU-induced missense mutation of the ectonucleotide pyrophosphatase/phosphodiesterase 1 gene (\textit{Enpp1}) that was discovered at The Jackson Laboratory and named "ages with stiffened joints" (\textit{asj}) because of the progressive ankylosis and osteoarthritis exhibited by mutant mice [12]. The \textit{Enpp1} gene encodes an enzyme (ENPP1) that regulates soft-tissue calcification and bone mineralization by producing inorganic pyrophosphate, a major inhibitor of calcification. A previous analysis of \textit{asj} mutant mice found that enzymatic activity of ENPP1 in the liver and inorganic pyrophosphate (PPi) levels in plasma were markedly reduced [13]. Other mouse mutations of \textit{Enpp1} have been reported, including the naturally occurring "tiptoe walking" (\textit{ttw}) nonsense mutation [14], an ENU-induced C397S missense mutation [15], and a genetically engineered knockout mutation [16]. The \textit{Enpp1} mutant mice in these studies exhibited extensive mineralization defects in a number of tissues, including spinal ligaments, long bones, articular cartilage, heart, aorta, arterial blood vessels, vibrissae, liver, kidneys, and retina. The effects of the \textit{Enpp1} mutations on auditory function and middle and inner ear histology of mice, however, were not examined.

We show here that \textit{Enpp1}\textsuperscript{asj} mutant mice exhibit a conductive hearing loss that is associated with middle and inner ear mineralization abnormalities. These mice provide a new animal model for studies of otitis media and tympanosclerosis and for the hearing loss recently shown to be associated with human \textit{ENPP1} mutations causing generalized arterial calcification of infancy and hypophosphatemic rickets [17,18].

\textbf{Materials and Methods}

\textbf{Mice}

The recessive \textit{asj} mutation was discovered at The Jackson Laboratory in 2004 in the C57BL/6J progeny of an ENU treated C57BL/6J male. The mutant strain name is C57BL/6J-\textit{Enpp1}\textsuperscript{asj}/GrsrJ and is available from The Jackson Laboratory (Stock #012810). Experimental mice were housed in the Research Animal Facility of The Jackson Laboratory, and all procedures involving their use were approved by the Institutional Animal Care and Use Committee. The Jackson Laboratory is accredited by the American Association for the Accreditation of Laboratory Animal Care.

\textbf{Genotyping the \textit{Enpp1}\textsuperscript{asj} mutation}

The \textit{asj} mutation is a single nucleotide substitution of T to A in exon 7 of the \textit{Enpp1} gene resulting in an amino acid substitution from valine to aspartic acid at residue 246 (p.V246D) of protein Reference Sequence NP\_032839. The T to A point mutation (nt 848 of mRNA Reference Sequence NM\_008813) creates a new \textit{TaqI} restriction site (TCGA), which is the basis of the genotyping method described by Harris and colleagues [12] and that was used in this study to distinguish wild-type from mutant alleles.
Assessment of hearing by ABR

Hearing was evaluated in anesthetized (Avertin, 0.4 mg/g mouse mass) wild-type, Enpp1<sup>asj/+</sup>, and Enpp1<sup>asj/asj</sup> mice by measuring auditory brainstem response (ABR). A computer-aided evoked potential system (Intelligent Hearing Systems) was used to measure mouse ABR thresholds as previously described [19].

Histological analyses of middle and inner ears

Histological analyses of the middle and inner ears were performed following the methods described previously [20]. Briefly, middle and inner ears from Enpp1<sup>asj/asj</sup> mice and wild-type mice were dissected after transcardial perfusion with Bouin’s fixative. Ear samples were immersed in Bouin’s fixative (7 days for one-month-old mice and 30 days for mice older than 6 months) and embedded in paraffin. Sections (7 mm) were cut and mounted onto Fisher Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) and counterstained in hematoxylin/eosin (H&E). Goblet cells, whose sole function is to secrete mucus, were identified by Mayer’s Mucicarmine staining method following the protocol provided by Electron Microscopy Sciences (Catalog #26320). The stepedial artery was dissected from 6-month-old control and mutant mice, sectioned without decalcification, and stained with Alizarin red following standard procedures [21].

Evaluation of pathology of middle ears

A scoring system of -/+/YYYY/+++ was used to assess the severity of pathology in the middle ears following a previously described method with modifications [22]. Histological analysis of the middle ears of control and mutant mice was performed using an Olympus BX51 microscope. A '-' symbol was assigned when the pathology is absent in the middle ear. A '+' symbol was assigned when pathology was very scarce in the middle ear. A '++' symbol was assigned when pathology was more prevalent, but not to the point of spanning the entire middle ear. A '++++' symbol was assigned when pathology spanned the entire middle ear. The following pathologies were evaluated by this scoring system: middle ear effusion, inflammatory cell infiltration, tissue proliferation (epithelial hyperplasia), abnormal tissue at the Eustachian tube opening in the middle ear cavity, ectopic mineralization, and clusters of goblet cells.

Scanning electron microscopy (SEM)

Middle ears from Enpp1<sup>asj/asj</sup> and wild-type mice were dissected after transcardial perfusion with 4% paraformaldehyde (PFA) and then immersed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH = 7.2) at 4°C overnight. Dissection was performed to expose the middle ear cavities. After three 15-minute washes with cacodylate buffer (pH = 7.2) at room temperature, samples were post-fixed with the osmium-thiocarbohydrazide-osmium-thiocarbohydrazide-osmium (OTOTO) method [23], and dehydrated in increasing concentrations of ethanol at 4°C. Samples were critical point dried with Hexamethyldisilazane (HMDS) and then air dried in fume hood. Samples were then coated with 15nm gold and analyzed in a Hitachi S-3000 scanning electron microscope (Hitachi, Tokyo, Japan) at 20 kV.

Results

Hearing impairment in Enpp1<sup>asj</sup> mutant mice

Hearing in the Enpp1 mutant mice and age-matched controls was evaluated by ABR threshold analysis. Initial ABR measurements of three-month-old mutant mice revealed a moderate to severe hearing loss with threshold increases of 25–35 decibels (dB) compared to the normal
thresholds in the control mice for both click and all pure tone stimuli (8 kHz, 16 kHz, 32 kHz). To further characterize the hearing ability of Enpp1<sup>asj/asj</sup> mice and to determine whether the hearing impairment is progressive with age, recurrent ABR measurements were performed in Enpp1<sup>asj/asj</sup>, Enpp1<sup>asj/+</sup>, and Enpp1<sup>+/+</sup> littermates from 3 weeks to 30 weeks of age (Fig 1, Table 1, Table 2). Enpp1<sup>asj/+</sup> and Enpp1<sup>+/+</sup> mice (grouped as controls) showed normal ABR thresholds even at 30 weeks of age, whereas Enpp1<sup>asj/asj</sup> mice exhibited elevated thresholds by 6 weeks of age. Hearing impairment is not congenital as mutant mice had normal ABR thresholds at weaning age (3 weeks). Hearing loss in mutant mice progressed in two stages: average ABR thresholds increased by about 30–35 dB between 3 and 6 weeks of age, were relatively stable between 6 and 12 weeks, increased another 20–25 dB between 12 and 18 weeks, and remained stable between 18 and 30 weeks. At 12 weeks of age, only one out of 11 Enpp1<sup>asj/asj</sup> mice tested showed normal thresholds in both ears (Table 1), giving a rough estimate of hearing loss penetrance of about 90%. No head bobbing or circling behaviors were observed in the mutant mice at all ages, indicating normal vestibular function.

**Otitis media with effusion in Enpp1<sup>asj/asj</sup> mice**

To assess the causes of hearing impairment in the Enpp1<sup>asj/asj</sup> mice, anatomical analysis of the middle and inner ears was performed after the completion of ABR measurements. We processed the whole bullae for histological studies, which allowed us to observe both middle and inner ear morphology. We did not observe any obvious inner ear defects in the asj mutant mice, such as hair cell and spiral ganglion cell loss, or stria vascularis degeneration. However, we observed major histopathology in the middle and outer ear, with details described below.

**a. Middle ear effusion and epithelial proliferation in Enpp1 mutant mice.** All ears from Enpp1<sup>asj/asj</sup> mice that had elevated ABR thresholds (n = 11) exhibited defects in the middle ear including retracted tympanic membranes and middle ear cavities filled with effusion (Figs 2 and 3, Table 3) and a thickened epithelium with fibrous polyps (Figs 2B, 2D and 3C, Table 3). In addition, older Enpp1<sup>asj/asj</sup> mice exhibited excessive accumulation of discharge in the external ear canal (Fig 2H). All of the ears from the control Enpp1<sup>+/asj</sup> and Enpp1<sup>+/+</sup> mice examined (n = 12) showed a transparent tympanic membrane, a clear middle ear cavity lined with a thin epithelium.

**b. Increased goblet cell density and mucin secretion in Enpp1<sup>asj/asj</sup> mice.** In the epithelium lining the middle ear cavity and the Eustachian tube, mucins secreted by goblet cells build the first line of defense in protecting the host from invading pathogens; movements of the cilia then help to get rid of the pathogens, with mucins serving as a lubricant [24]. However, when chronic inflammation is present, some of the epithelial cells transdifferentiate to mucin-secreting goblet cells [24]. These additional goblet cells secrete excessive mucins, which accumulate in the middle ear cavity and lead to conductive hearing loss (otitis media). We used Mayer’s mucicarmine staining to detect the density of goblet cells in the asj mutant mice. Already at two weeks of age, an excess of goblet cells was observed around the opening of the Eustachian tube and in the Eustachian tube duct of mutant mice (Fig 4B). In the control mice, scattered goblet cells were seen around the opening of the Eustachian tube, but very few were detected along the Eustachian tube duct (Fig 4A). Similar observations were made in two-month-old adult mice: more goblet cells were present in the epithelia lining the middle ear cavity and the Eustachian tube in mutants (Fig 4D) than in controls (Fig 4C).

**c. Impaired Eustachian tube function due to epithelia proliferation.** Out of 10 ear preparations (8–30 weeks) that allowed us to observe the opening of the Eustachian tube in the middle ear cavity, 7 had amorphous tissues that could potentially block the Eustachian tube (Table 3) thereby disrupting middle ear pressure regulation and ciliary clearance of secretions.
d. Impaired middle ear epithelial clearance function due to excess mucin and loss of microciliary function. Using scanning electron microscopy (Fig 5), we assessed the integrity of the mucociliary epithelium in 1-month-old and 6-month-old wild-type and Enpp1<sup>asj/asj</sup> mice (n = 3 each genotype). The epithelium of one-month-old mutant mice had scattered

![Fig 1. Progressive hearing loss in Enpp1<sup>asj/asj</sup> mice. ABR threshold means are shown for Enpp1<sup>asj/asj</sup> mice and littermate control mice, tested at the ages of 3 (n = 6 mutant ears/8 control ears), 6 (n = 10/10), 8 (n = 6/6), 12 (n = 22/16), 18 (n = 8/6), 26 (n = 10/8), and 30 (n = 12/14) weeks. Starting from 6 weeks of age, the mutant mice exhibit significantly higher mean ABR threshold values at all the stimulus frequencies tested (click, 8 kHz, 16 kHz, 32 kHz) compared to those of the littermate controls. Thresholds of mutant mice continue to increase with age and by 18 weeks most of the Enpp1<sup>asj/asj</sup> mice are profoundly hearing impaired. The increase in 32 kHz ABR thresholds of the control mice is due to the B6 background, a strain known to exhibit age-related hearing loss starting at high frequencies. Error bars indicate standard errors of the mean.](doi:10.1371/journal.pone.0168159.g001)
areas with high densities of goblet cells (Fig 5B), which was confirmed by Mayer’s mucincarmin staining. Goblet cell density in 6-month-old mutants could not be determined because of the obscuring layer of mucin (Fig 5D). These results indicate that excessive mucin secreted by increased numbers of goblet cells and the hindering effect of the mucin layer on ciliary function in the asj mutant mice are contributing factors to the occurrence of otitis media.

Table 1. ABR thresholds of asj/asj mutant mice

| Mouse ID | Group | Test Age | Sex | Click 8 kHz | 16 kHz | 32 kHz | Left Ear thresholds (dB SPL) |
|----------|-------|----------|-----|-------------|--------|--------|----------------------------|
| 5729     | 3 wk  | 23       | F   | 30          | 30     | 10     | 35                         | 40          | 45 | 15 | 35 |
| 5055     | 3 wk  | 22       | M   | 30          | 40     | 10     | 40                         | 35          | 40 | 15 | 40 |
| 5738     | 3 wk  | 25       | M   | 30          | 40     | 10     | 40                         | 30          | 30 | 10 | 35 |
| 4195     | 6 wk  | 38       | M   | 35          | 70     | 50     | 80                         | 75          | 80 | 60 | 70 |
| 5001     | 6 wk  | 39       | F   | 70          | 80     | 40     | 70                         | 45          | 50 | 30 | 60 |
| 5003     | 6 wk  | 39       | M   | 60          | 80     | 40     | 80                         | 60          | 90 | 50 | 70 |
| 4094     | 6 wk  | 41       | F   | 70          | 80     | 70     | 70                         | 75          | 80 | 55 | 70 |
| 4098     | 6 wk  | 41       | M   | 35          | 70     | 50     | 60                         | 70          | 80 | 60 | 70 |
| 3915     | 8 wk  | 50       | F   | 75          | 80     | 50     | 85                         | 70          | 75 | 40 | 80 |
| 3891     | 8 wk  | 55       | F   | 35          | 60     | 35     | 45                         | 75          | 75 | 55 | 75 |
| 3893     | 8 wk  | 55       | M   | 75          | 85     | 75     | 70                         | 75          | 85 | 70 | 75 |
| 3902     | 12 wk | 75       | F   | 45          | 50     | 30     | 40                         | 40          | 50 | 30 | 40 |
| 3904     | 12 wk | 75       | M   | 65          | 75     | 40     | 70                         | 40          | 40 | 40 | 50 |
| 3905     | 12 wk | 75       | M   | 30          | 50     | 20     | 40                         | 80          | 85 | 60 | 90 |
| 3899     | 12 wk | 75       | F   | 60          | 65     | 50     | 70                         | 70          | 85 | 50 | 65 |
| 4038     | 12 wk | 89       | F   | 100         | 90     | 60     | 90                         | 80          | 80 | 50 | 70 |
| 4039     | 12 wk | 89       | F   | 65          | 80     | 40     | 70                         | 80          | 80 | 50 | 80 |
| 4040     | 12 wk | 89       | M   | 70          | 80     | 60     | 90                         | 70          | 85 | 50 | 90 |
| 4030     | 12 wk | 101      | F   | 90          | 90     | 80     | 90                         | 90          | 90 | 80 | 90 |
| 4033     | 12 wk | 101      | M   | 60          | 80     | 70     | 70                         | 40          | 60 | 60 | 70 |
| 4034     | 12 wk | 101      | M   | 40          | 70     | 50     | 70                         | 70          | 80 | 50 | 80 |
| 4035     | 12 wk | 101      | M   | 70          | 80     | 50     | 70                         | 40          | 50 | 50 | 60 |
| 5005     | 18 wk | 123      | F   | 95          | 100    | 95     | 100                        | 100         | 100 | 95 | 100 |
| 5007     | 18 wk | 125      | F   | 90          | 100    | 70     | 90                         | 60          | 90 | 50 | 90 |
| 5010     | 18 wk | 136      | F   | 90          | 100    | 70     | 85                         | 80          | 90 | 80 | 75 |
| 5008     | 18 wk | 144      | F   | 80          | 90     | 70     | 90                         | 90          | 90 | 80 | 90 |
| 3884     | 26 wk | 172      | M   | 100         | 100    | 90     | 90                         | 75          | 100 | 60 | 100 |
| 3885     | 26 wk | 172      | F   | 90          | 100    | 70     | 80                         | 100         | 100 | 100 | 100 |
| 3886     | 26 wk | 172      | M   | 100         | 100    | 90     | 90                         | 70          | 85 | 70 | 60 |
| 3906     | 26 wk | 188      | F   | 100         | 100    | 80     | 100                        | 95          | 90 | 70 | 100 |
| 3913     | 26 wk | 188      | M   | 60          | 70     | 40     | 90                         | 60          | 60 | 30 | 70 |
| 3106     | 30 wk | 202      | F   | 100         | 100    | 100    | 100                        | 90          | 90 | 70 | 100 |
| 3107     | 30 wk | 202      | F   | 100         | 100    | 90     | 100                        | 100         | 100 | 90 | 100 |
| 3283     | 30 wk | 204      | F   | 100         | 100    | 90     | 100                        | 100         | 100 | 90 | 100 |
| 3098     | 30 wk | 206      | F   | 80          | 90     | 80     | 90                         | 50          | 80 | 40 | 70 |
| 3102     | 30 wk | 206      | M   | 70          | 80     | 70     | 90                         | 90          | 90 | 60 | 90 |
| 3286     | 30 wk | 223      | M   | 80          | 90     | 50     | 100                        | 50          | 80 | 50 | 100 |
| 3290     | 30 wk | 223      | M   | 100         | 100    | 90     | 100                        | 80          | 80 | 65 | 90 |

* Highlighted ID numbers indicate ears were examined for pathology

doi:10.1371/journal.pone.0168159.t001
Over-calcification of middle ear structures (tympanosclerosis)

6-month old control mice have transparent tympanic membrane (Fig 6A), mutant mice at the same age have retracted tympanic membrane due to the pressure of excessive discharge and white patch were observed at pars tensa of tympanic membrane in the mutant mice (Fig 6B).

We dissected middle ear ossicles of 6-month-old Enpp1 asj/asj mice with littermate controls. Control mice have normal morphology of ossicles (Fig 6C). We found that stapes in the mutant mice have normal morphology and are freely removable from the round window (Fig 6D). Although malleus an incus has relative normal morphology, these two bones are fused (Fig 6D). The wall of the stapedial artery, which passes through the ring of the stapes, is thicker.

Table 2. ABR thresholds of +/+ or +/asj control mice

| Mouse | Age | Test Age | Right Ear thresholds (dB SPL) | Left Ear thresholds (dB SPL) |
|-------|-----|----------|-------------------------------|-----------------------------|
|       |     |          | Click 8 kHz | 16 kHz | 32 kHz | Click 8 kHz | 16 kHz | 32 kHz |
| ID *  | Group | Days | Sex | Click | 30 | 20 | 40 | Click | 30 | 20 | 40 |
| 5044  | 3 wk | 21   | F   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 5045  | 3 wk | 21   | M   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 5054  | 3 wk | 22   | F   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 5056  | 3 wk | 22   | M   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 4193 *| 6 wk | 38   | M   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 5002  | 6 wk | 39   | F   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 5004  | 6 wk | 39   | M   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 4095 *| 6 wk | 41   | F   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 4097  | 6 wk | 41   | M   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 3914  | 8 wk | 50   | F   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 3888  | 8 wk | 55   | F   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 3892 *| 8 wk | 55   | M   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 3900 *| 12 wk | 75   | F   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 3901 | 12 wk | 75   | M   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 3903 | 12 wk | 75   | F   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 4036  | 12 wk | 89   | F   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 4037 | 12 wk | 89   | M   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 4032  | 12 wk | 101  | M   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 4029  | 12 wk | 101  | M   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 4031  | 12 wk | 101  | F   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 5006  | 18 wk | 123  | M   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 5011  | 18 wk | 136  | F   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 5009  | 18 wk | 144  | F   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 3883 *| 26 wk | 172  | F   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 3887 *| 26 wk | 172  | M   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 3910  | 26 wk | 188  | M   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 3911  | 26 wk | 188  | F   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 3110  | 30 wk | 202  | F   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 3112  | 30 wk | 202  | M   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 3286  | 30 wk | 204  | M   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 3101  | 30 wk | 206  | M   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 3104  | 30 wk | 206  | M   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 5014  | 30 wk | 231  | F   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |
| 5012  | 30 wk | 234  | F   | 35   | 40 | 10 | 30 | 30   | 10 | 30 |

* Highlighted ID numbers indicate ears were examined for pathology

doi:10.1371/journal.pone.0168159.t002
in *Enpp1*mutant mice (Fig 2F) than controls (Fig 2E). Alarin red staining indicates excessive calcium deposition in the artery wall of the mutant mice (Fig 6F). Therefore, enlarged and stiffened stapedial artery could potentially impede the movement of the stapes and lead to impaired sound transmission to the inner ear. We observed overossification of round window ridge in the mutant mice (Fig 2B, Table 3), which may indirectly contribute to otitis media and hearing loss. We observed that the round window membrane in *Enpp1*mutant mice (Fig 3H) was thicker than in control mice (Fig 3G).
Fig 3. Development of otitis media in *Enpp1*^{asj/asj} mice. A-D: 6–12 week time course of middle ear pathology of *Enpp1*^{asj/asj} mutant mice (B, C, D) compared with a 12-week-old control (A). At 6 weeks of age (B), an aqueous effusion (black asterisk) and a slightly thickened epithelium (arrow) are observed in the middle ear cavity of the
Bacterial infection does not play a role in the development of otitis media in Enpp1\textsuperscript{asj/asj} mice

The effusion was confined within the middle ear cavity and did not appear to extend through the round window into the inner ear. To detect if otitis media in the \textit{asj} mutant mice was caused by bacterial infection, we performed gram staining in the freshly prepared middle ear sections. We failed to detect any pathogens from the middle ear cavities of the \textit{asj} mutant mice (data not shown), suggesting a non-infection origin of the otitis media in the \textit{asj} mutant mice.

Discussion

Otitis media-related ear pathology of \textit{Enpp1}\textsuperscript{asj/asj} mutant mice

Beginning at 6 weeks of age, an effusion starts to appear in the middle ears of \textit{asj} mutant mice, and moderate epithelial proliferation is observed lining the middle ear cavity. The degree of middle ear effusion and epithelial thickening in \textit{asj} mutant mice correlates with ABR thresholds. At 12 weeks of age the content of the effusion changes from serous to suppurative, with a corresponding increase in ABR thresholds. These effusions in the middle ear cavity may interfere with the normal vibration of the tympanic membrane and movement of the ossicle chain; with suppurative effusion having a much stronger effect. We observed discharge in the outer ear cavity starting around 4 months of age. All of the ears examined from seven 6~7-month-old \textit{asj} mutant mice showed complete blockage of the outer ear canal by discharge, which could explain the secondary increase in ABR thresholds that occurs in \textit{asj} mutant mice between 12 and 18 weeks of age.

Mucins are secreted by goblet cells that lay scattered in the epithelium of digestive, respiratory, urinary, and reproductive tracts either at a basal level or at a high level upon stimulation \cite{25}. Mucins, together with the cilia that line the epithelia, protect the host by cleaning invading pathogens. A similar mechanism is applied by the middle ear mucociliary system to clear middle ear effusions \cite{24}. Disturbed phosphate homeostasis can cause systematic inflammation. Inflammation within the middle ear cavity triggers secretion of mucin into the middle ear cavity and transdifferentiation of more epithelial cells into goblet cells, which lead to excessive effusion accumulation in the middle ear cavity. Continued chronic middle ear inflammation leads to tissue destruction and fibrosis. Thickened middle ear epithelia or fibrosis of epithelia, especially the epithelia around the opening of the Eustachian tube in the middle ear cavity can block the Eustachian tube and facilitate effusion accumulation. Although we don’t have supporting evidence, excessive discharge in the outer ear canal might be caused by inflammation in the external ear. Defective action of the cilia lining the middle ear epithelia is often associated with development of otitis media, as seen in patients with primary ciliary dyskinesia (PCD) \cite{26}. Impaired mucociliary function and increased number of goblet cells in the middle ear cavity of the \textit{asj} mutant mice, confirmed by SEM and Mayer’s mucicarmine staining, indicate that the
Table 3. Histologic assessment of the middle ears of the *Enpp1* mutant and control mice. MEE: Middle Ear Effusion; EH: Epithelial Hyperplasia; IC: Inflammatory Cells; ETO: Eustachian Tube Opening; EM: Ectopic Mineralization; CGC: Cluster of Goblet Cells.

| Mouse ID | Age | Genotype | MEE | EH | IC | ETO | EM | CGC |
|----------|-----|----------|-----|----|----|-----|----|-----|
| 4012L    | 1wk | Control  | -   | +  | -  | N/A | -  | -   |
| 4017     | 1wk | Control  | -   | -  | -  | N/A | -  | -   |
| 4015L    | 1wk | Control  | -   | -  | -  | N/A | -  | -   |
| 4016L    | 1wk | Mutant   | -   | -  | -  | N/A | -  | -   |
| 4016R    | 1wk | Mutant   | -   | -  | -  | N/A | -  | -   |
| 4018L    | 1wk | Mutant   | -   | +  | -  | N/A | -  | +   |
| 4022L    | 2wk | Control  | -   | -  | -  | N/A | -  | -   |
| 4022R    | 2wk | Control  | -   | -  | -  | N/A | -  | -   |
| 4019R    | 2wk | Control  | -   | -  | -  | N/A | -  | -   |
| 4023L    | 2wk | Mutant   | -   | +  | -  | N/A | -  | -   |
| 4023R    | 2wk | Mutant   | -   | +  | -  | N/A | -  | -   |
| 4020L    | 2wk | Mutant   | -   | ++ | +  | N/A | -  | -   |
| 4020R    | 2wk | Mutant   | -   | -  | -  | N/A | -  | -   |
| 4008L    | 2wk | Mutant   | -   | +  | -  | N/A | -  | -   |
| 4008R    | 2wk | Mutant   | -   | +  | -  | N/A | -  | -   |
| 4007L    | 2wk | Mutant   | -   | +  | -  | N/A | -  | +   |
| 4007R    | 2wk | Mutant   | -   | +  | -  | N/A | -  | +   |
| 4002L    | 3wk | Control  | -   | -  | -  | N/A | -  | -   |
| 4002R    | 3wk | Control  | -   | -  | -  | N/A | -  | -   |
| 4001L    | 3wk | Control  | -   | -  | -  | N/A | -  | +   |
| 4001R    | 3wk | Control  | -   | -  | -  | N/A | -  | +   |
| 4004L    | 3wk | Mutant   | -   | -  | -  | N/A | -  | -   |
| 4004R    | 3wk | Mutant   | -   | -  | -  | N/A | -  | -   |
| 4000L    | 3wk | Mutant   | -   | -  | -  | N/A | -  | -   |
| 4000R    | 3wk | Mutant   | -   | -  | -  | N/A | -  | -   |
| 5055R*   | 3wk | Mutant   | -   | +  | -  | N/A | -  | -   |
| 5055L*   | 3wk | Mutant   | -   | +  | -  | N/A | -  | -   |
| 4193R*   | 6wk | Control  | -   | +  | -  | N/A | -  | +   |
| 4195R*   | 6wk | Mutant   | +   | -  | -  | N/A | +  | +   |
| 4094R*   | 6wk | Mutant   | +++ | +++| +  | N/A | +  | +++ |
| 3892L*   | 8wk | Control  | -   | -  | -  | -   | -   | -   |
| 3893R*   | 8wk | Mutant   | +++ | +++| +  | +++ | +  | ++  |
| 3891R*   | 8wk | Mutant   | +   | +  | -  | N/A | +  | -   |
| 3900R*   | 12wk | Control | -   | -  | -  | N/A | +  | -   |
| 3905L*   | 12wk | Mutant | +++ | +++| ++ | N/A | +  | +++ |
| 3905R*   | 12wk | Mutant | +   | +  | +  | +   | +   | -   |
| 3899L*   | 12wk | Mutant | +   | +++| ++ | +++ | +  | ++  |
| 3899R*   | 12wk | Mutant | ++  | +++| ++ | N/A | +  | ++  |
| 3883L*   | 26wk | Control | -   | -  | -  | N/A | -  | -   |
| 3883R*   | 26wk | Control | -   | -  | -  | N/A | -  | -   |
| 3887L*   | 26wk | Control | -   | -  | -  | N/A | -  | +   |
| 3887R*   | 26wk | Control | -   | -  | -  | N/A | -  | -   |
| 3884L*   | 26wk | Mutant | +++ | ++  | ++ | ++ | -  | +++ |
| 3884R*   | 26wk | Mutant | +   | ++ | +  | ++ | +  | +   |
| 3885L*   | 26wk | Mutant | -   | -  | +  | -  | -  | +   |
| 3885R*   | 26wk | Mutant | ++  | +++| ++ | +  | -  | ++  |
| 2094R    | 30wk | Mutant | ++  | ++ | ++ | +  | +  | ++  |

(Continued)
middle ears of the \textit{asj} mutant mice cannot maintain their ability to clear effusion, which therefore leads to conductive hearing loss. Overall, middle ear inflammation with effusion, amorphous tissue mass in the middle ear cavity, excessive discharge in the outer ear canal, and ectopic mineralization contribute to the conductive hearing loss in the \textit{asj} mutant mice.

**Mineralization disorders and conductive hearing loss in \textit{Enpp1}^{asj/asj} mice**

Mineralization disorders due to abnormal phosphate levels have been associated with inflammation in several different diseases, including chronic kidney disease \cite{27,28} and

| Mouse ID | Age | Genotype | MEE | EH | IC | ETO | EM | CGC |
|----------|-----|----------|-----|----|----|-----|----|-----|
| 2096R    | 30wk| Mutant   | +++ | +++| +++| ++  | +  | ++  |
| 2098R    | 30wk| Mutant   | +++ | +++| ++  | +++ | +  | ++  |

* Indicate ears with ABR data.

doi:10.1371/journal.pone.0168159.t003

---

**Fig 4. Increased density of goblet cells in \textit{Enpp1}^{asj/asj} mice.** Mayer's mucincarmine method was used to visualize goblet cells (stained red) in the epithelia lining the Eustachian tube (A, B) and middle ear cavity (C, D) of mutant and control mice. A, B: Few goblet cells are seen in the epithelia lining the Eustachian tube of littermate control mice (A, empty arrow points to Eustachian tube, insert shows higher magnification of the Eustachian tube epithelia). By contrast, goblet cells are present at high density in the epithelia lining the Eustachian tube of \textit{Enpp1}^{asj/asj} mutant mice (B, empty arrow points to Eustachian tube, magnified inset shows goblet cells, marked by arrows, in the Eustachian tube epithelia). C, D: More goblet cells are present in the epithelium in the middle ear cavity (MEC) of the asj mutant mouse (arrows in D) than in the control (C). Scale bars: A, B = 200 \( \mu m \), C, D = 50 \( \mu m \), A and B inserts = 20 \( \mu m \).

doi:10.1371/journal.pone.0168159.g004
cardiovascular disease [29]. Enpp1<sup>asj</sup> adds to a growing list of mouse mutations causing phosphate homeostasis disorders that have associated conductive hearing loss, including mutations of the Ank, Phex, Rpl38, and Fgf23 genes. Ank (progressive ankylosis) encodes a multiple-pass transmembrane protein that regulates pyrophosphate levels, and Ank mutant mice were reported to have middle ear ossicle fusions and associated conductive hearing loss [30]. ANK mutations in human patients also have been reported with associated with conductive hearing loss [31]. PHEX (X-linked phosphate regulating endopeptidase) is an enzyme that is involved in regulating the balance of phosphate in the body. PHEX mutations are associated with X-linked hypophosphatemic rickets in human patients, with hearing loss as one of the symptoms [32–34]. Mice with Phex mutations exhibit hypophosphatemia-related abnormalities and hearing impairment [35], which recently was shown to be associated with middle ear effusion and ciliary defects [8]. Rpl38 (ribosomal protein L38) is not known to be directly involved in phosphate regulation; however, elevated organic phosphate levels in Rpl38<sup>Ts</sup> mutant mice [4] suggest a potential but unknown function of Rpl38 in phosphate homeostasis. Rpl38<sup>Ts</sup> mutant mice have conductive hearing loss caused by ectopic ossification and cholesterol crystal deposition in the middle ear cavity, enlarged Eustachian tube, and chronic inflammation with effusion [4]. FGF23 (fibroblast growth factor 23) is a circulating hormone that controls phosphate and calcium homeostasis and bone mineralization. Abnormal serum levels of FGF23 lead to systemic pathologies in humans, including renal phosphate wasting diseases and hyperphosphatemia. FGF23-deficient mice show a mixed hearing loss and middle ear malformations [36], and changes in circulating FGF23 have been observed in humans and mice with ENPP1 and PHEX deficiencies [16,37].

ENPP1 produces inorganic pyrophosphate (PPI), an inhibitor of mineralization. Because PPI levels are markedly reduced in Enpp1<sup>asj/asj</sup> mice [13], it is not surprising to see ectopic mineralization and calcification of middle ear tissues, as was observed in other soft tissues of these mutant mice [13]. Calcification of soft tissues within the middle ear cavity could contribute in various ways to the conductive hearing loss of Enpp1<sup>asj/asj</sup> mice. The stapedial artery is an embryonic artery that disappears at the early embryo stage in humans but is conserved in mice through adult ages [38]. We observed a thickened stapedial artery wall in Enpp1<sup>asj</sup> mutant...
Fig 6. Calcification of middle ear structures (or tympanosclerosis) in Enpp1<sup>as/as</sup> mice. In the control mice (A), the outer ear canal is clear without any discharge. The tympanic membrane appears to be transparent and malleus (blue asterisk) is clearly visible. In the age matched mutant mice (B), surrounding bone must be removed to expose the tympanic membrane, most of which is completely covered by discharge. White patches (inside black dashed lines) were observed on the tympanic membrane of the mutant mice (B). The ossicle bones of the age matched control and mutant mice appear to have similar morphology (C-D), but malleus and incus are fused in the mutant mice (D, red arrow). M: Malleus; I: Incus; S: Stapes. Alizarin red staining reveals extensive mineralization in the stapedial artery wall (green asterisk) in Enpp1<sup>as/as</sup> mice (F), but not in the control mice (E).

doi:10.1371/journal.pone.0168159.g006
mice, which is likely due to calcification as has been observed in other soft tissues. The thicker arterial wall is not likely to affect transport function, but because the artery passes through the ring of the stapes, its thickened wall could potentially impede the vibration of the stapes and contribute to the conductive hearing loss of mutant mice.

Abnormal mineralization can also cause otosclerosis and tympanosclerosis, two conditions that commonly lead to hearing loss in human patients. Disruption of bone homeostasis of the otic capsule can lead to otosclerosis [39], and the most common feature of otosclerosis is stapes fixation. Although we observed ectopic mineralization and bone deposition in the otic capsule of asj mutant mice starting from around 6 weeks of age, stapes fixation is absent from Enpp1 mutant mice. Instead, we observed white patch on the tympanic membrane, malleus and incus fusion, which are typical symptoms of tympanosclerosis. We observed a thickened round window membrane in Enpp1<sup>asj/asj</sup> mice at 8 weeks of age, which may increase the rigidity of the membrane and impede proper cochlear fluid movement and hair cell stimulation [40]. Therefore, Enpp1<sup>asj</sup> mice can serve as a model for studying tympanosclerosis.

Decreased PPi levels in Enpp1<sup>asj/asj</sup> mice also lead to otitis media, perhaps the most important factor contributing to the hearing loss, but the underlying mechanism of pathology is uncertain. ENPP1 deficiency is known to cause elevated serum levels of FGF23 in Enpp1 mutant mice [16], and excess FGF23 secreted in the middle ear may trigger mucoperiosteum proliferation, which may contribute to the development of otitis media. In support of this possibility, mucoperiosteum proliferation in Enpp1<sup>asj/asj</sup> mice is remarkably enhanced around the regions of the otic capsule that exhibit ectopic mineralization. Enpp1<sup>asj</sup> mice provide a tool to unravel the underlying mechanism of the development of otitis media that is associated with abnormal phosphate homeostasis.

This is the first report of hearing loss and ear pathology associated with a mutation of the mouse Enpp1 gene. The conductive hearing loss of Enpp1<sup>asj</sup> mutant mice provides a new animal model for studying otitis media and tympanosclerosis related to mineralization defects. It also provides a specific model for understanding the hearing loss recently reported to be a clinical feature associated with human ENPP1 mutations [17,18].

**Acknowledgments**

We thank Sandra Gray for mouse husbandry. We thank Chantal M. Longo-Guess for initial hearing assessment. This work was supported by National Institutes of Health Grants R01 DC004301 (KRJ), P40 OD010972 (The Jackson Laboratory Mouse Mutant Resource), and P30 CA034196 (The Jackson Laboratory Shared Services).

**Author Contributions**

Conceptualization: CT KRJ.

Data curation: CT.

Formal analysis: CT KRJ.

Funding acquisition: KRJ.

Investigation: CT.

Methodology: CT.

Project administration: CT KRJ.

Resources: CT KRJ BSH.
References

1. Rye MS, Bhutta MF, Cheseman MT, Burgner D, Blackwell JM, et al. (2011) Unraveling the genetics of otitis media: from mouse to human and back again. Mamm Genome 22: 86–92. doi: 10.1007/s00335-010-9295-1 PMID: 21107580

2. Zheng QY, Hardisty-Hughes R, Brown SD (2006) Mouse models as a tool to unravel the genetic basis for human otitis media. Brain Res 1091: 9–15. PMID: 16917982

3. Yang B, Tian C, Zhang ZG, Han FC, Azem R, et al. (2011) Sh3pxd2b mice are a model for craniofacial dysmorphology and otitis media. PLoS One 6: e22622. doi: 10.1371/journal.pone.0022622 PMID: 21818352

4. Noben-Trauth K, Latoche JR (2011) Ectopic mineralization in the middle ear and chronic otitis media with effusion caused by RPL38 deficiency in the Tail-short (Ts) mouse. J Biol Chem 286: 3079–3093. doi: 10.1074/jbc.M110.184598 PMID: 21062742

5. Hilton JM, Lewis MA, Grati M, Ingham N, Pearson S, et al. (2011) Exome sequencing identifies a missense mutation in Iis1 associated with low penetrance otitis media in daisrie mice. Genome Biol 12: R90. doi: 10.1186/gb-2011-12-9-r90 PMID: 21939904

6. Tian C, Yu H, Yang B, Han F, Zheng Y, et al. (2012) Otitis media in a new mouse model for CHARGE syndrome with a deletion in the Chd7 gene. PLOS One 7: e34944. doi: 10.1371/journal.pone.0034944 PMID: 22539851

7. Zhang Y, Yu H, Xu M, Han F, Tian C, et al. (2012) Pathological features in the LmnaDhe/+ mutant mouse provide a novel model of human otitis media and laminoopathies. Am J Pathol 181: 761–774. doi: 10.1016/j.ajpath.2012.05.031 PMID: 22819531

8. Han F, Yu H, Li P, Zhang J, Tian C, et al. (2012) Mutation in Phex gene predisposes BALB/c-Phex(Hyp-Duk)/Y mice to otitis media. PLoS One 7: e30310. doi: 10.1371/journal.pone.0030310 PMID: 23028440

9. Kerschneer JE, Hong W, Taylor SR, Kerschneer JA, Khampang P, et al. (2013) A novel model of spontaneous otitis media with effusion (OME) in the Oxgr1 knock-out mouse. Int J Pediatr Otorhinolaryngol 77: 79–84. doi: 10.1016/j.ijporl.2012.09.037 PMID: 23200873

10. Tateossian H, Moraes S, Parker A, Mburu P, Warn N, et al. (2013) Otitis media in the Tgfβ knockout mouse implicates TGFbeta signalling in chronic middle ear inflammatory disease. Hum Mol Genet 22: 2553–2565. doi: 10.1093/hmg/ddt103 PMID: 23459932

11. Chen J, Ingham N, Clare S, Raisen C, Vancollie VE, et al. (2013) Mcph1-deficient mice reveal a role for MCPH1 in otitis media. PLoS One 8: e58156. doi: 10.1371/journal.pone.0058156 PMID: 23564444

12. Harris BS, Ward-Bailey PF, Yu H, Bergstrom DE, Bronson RT, Donahue LR (2012) Ages with stiffened joints, A new mutation in Enpp1. http://mousemutantjax.org/articles/mmrmutants#html.

13. Li Q, Guo H, Chou DW, Berndt A, Sundberg JP, et al. (2013) Mutant Enpp1asj mice as a model for generalized arterial calcification of infancy. Dis Model Mech 6: 1227–1235. doi: 10.1242/dmm.012765 PMID: 23798568

14. Okawa A, Nakamura I, Goto S, Moriya H, Nakamura Y, et al. (1998) Mutation in NppS in a mouse model of ossification of the posterior longitudinal ligament of the spine. Nat Genet 19: 271–273. doi: 10.1038/956 PMID: 9662402

15. Babij P, Roudier M, Graves T, Han CY, Chhoa M, et al. (2009) New variants in the Enpp1 and Ptpn6 genes cause low BMD, crystal-related arthropathy, and vascular calcification. J Bone Miner Res 24: 1552–1564. doi: 10.1359/jbmr.0906417 PMID: 19419305

16. Mackenzie NC, Zhu D, Milne EM, van ’t Hof R, Martin A, et al. (2012) Altered bone development and an increase in FGF-23 expression in Enpp1(-/-) mice. PLoS One 7: e32177. doi: 10.1371/journal.pone.0032177 PMID: 22359666

17. Nitschke Y, Baujat G, Botschen U, Wittkampf T, du Moulin M, et al. (2012) Generalized arterial calcification of infancy and pseudoxanthoma elasticum can be caused by mutations in either ENPP1 or ABCG6. Am J Hum Genet 90: 25–39. doi: 10.1016/j.ajhg.2011.11.029 PMID: 22209248
18. Brachet C, Mansbach AL, Clerckx A, Deltenre P, Heinrichs C (2014) Hearing loss is part of the clinical picture of ENPP1 loss of function mutation. Horm Res Paediatr 81: 63–66. doi: 10.1159/000354661 PMID: 24216977

19. Zheng QY, Johnson KR, Erway LC (1999) Assessment of hearing in 80 inbred strains of mice by ABR threshold analyses. Hear Res 130: 94–107. PMID: 10320101

20. Johnson KR, Gagnon LH, Webb LS, Peters LL, Hawes NL, et al. (2003) Mouse models of Usher1C and DFNB18: phenotypic and molecular analyses of two new spontaneous mutations of the Ush1c gene. Hum Mol Genet 12: 3075–3086. doi: 10.1093/hmg/ddg332 PMID: 14519688

21. Li Q, Pratt CH, Dionne LA, Fairfield H, Karst SY, et al. (2014) Spontaneous asj-2J mutant mouse as a model for generalized arterial calcification of infancy: a large deletion/insertion mutation in the Enpp1 gene. PLoS One 9: e113542. doi: 10.1371/journal.pone.0113542 PMID: 25479107

22. Han F, Yu H, Zhang J, Tian C, Schmidt C, et al. (2009) Otitis media in a mouse model for Down syndrome. Int J Exp Pathol 90: 480–488. doi: 10.1111/j.1365-2613.2009.00677.x PMID: 19765102

23. Tian C, Liu XZ, Han F, Yu H, Longo-Guess C, et al. (2010) Ush1c gene expression levels in the ear and eye suggest different roles for Ush1c in neurosensory organs in a new Ush1c knockout mouse. Brain Res 1328: 57–70. doi: 10.1016/j.brainres.2010.02.079 PMID: 20211154

24. Lin J, Caye-Thomasen P, Tono T, Zhang QA, Nakamura Y, et al. (2012) Mucin production and mucous cell metaplasia in otitis media. Int J Otalaryngol 2012: 745325. doi: 10.1155/2012/745325 PMID: 22685463

25. Linden SK, Sutton P, Karlsson NG, Korolik V, McGuckin MA (2008) Mucins in the mucosal barrier to infection. Mucosal Immunol 1: 183–197. doi: 10.1038/mi.2008.5 PMID: 19079178

26. Mata M, Milian L, Armengot M, Carda C (2014) Gene mutations in primary ciliary dyskinesia related to otitis media. J Clin Endocrinol Metab 96: E189–198. doi: 10.1210/jc.2010-1539 PMID: 20943778

27. Terkeltaub RA (2001) Inorganic pyrophosphate generation and disposition in pathophysiology. Am J Physiol Cell Physiol 281: C1–C11. PMID: 11401820

28. Yamada S, Tokumoto M, Tatsu moto N, Taniguchi M, Noguchi H, et al. (2014) Phosphate overload directly induces systemic inflammation and malnutrition as well as vascular calcification in uremia. Am J Physiol Renal Physiol 306: F1418–1428. doi: 10.1152/ajpregu.00633.2013 PMID: 24808541

29. Hofmann Bowman MA, McNally EM (2012) Genetic pathways of vascular calcification. Trends Cardiovasc Med 22: 93–98. doi: 10.1016/j.tcm.2012.07.002 PMID: 23040839

30. Gurley KA, Reimer RJ, Kingsley DM (2006) Biochemical and genetic analysis of ANK in arthritis and bone disease. Am J Hum Genet 79: 1017–1029. doi: 10.1086/509881 PMID: 17186460

31. Morava E, Kuhnisch J, Drijvers JM, Robben JH, Cremers C, et al. (2011) Autosomal recessive mental retardation, deafness, ankylosis, and mild hypophosphatemia associated with a novel ANKH mutation in a consanguineous family. J Clin Endocrinol Metab 96: E189–198. doi: 10.1210/jc.2010-1539 PMID: 20943778

32. Davies M, Kane R, Valentine J (1984) Impaired hearing in X-linked hypophosphataemic (vitamin-D-resistant) osteomalacia. Ann Intern Med 100: 230–232. PMID: 6691666

33. O’Malley SP, Adams JE, Davies M, Ramsden RT (1988) The petrous temporal bone and deafness in X-linked hypophosphataemic osteomalacia. Clin Radiol 39: 528–530. PMID: 3180671

34. Weir N (1977) Sensorineural deafness associated with recessive hypophosphataemic rickets. J Laryngol Otol 91: 717–722. PMID: 894124

35. Lorenz-Depiereux B, Guido VE, Johnson KR, Zheng QY, Gagnon LH, et al. (2004) New intragenic deletions in the Phex gene clarify X-linked hypophosphatemia-related abnormalities in mice. Mamm Genome 15: 151–161. PMID: 15029877

36. Lysaght AC, Yuan Q, Fan Y, Kalwani N, Caruso P, et al. (2014) FGF23 Deficiency Leads to Mixed Hearing Loss and Middle Ear Malformation in Mice. PLoS One 9: e107681. doi: 10.1371/journal.pone.0107681 PMID: 25243481

37. Lorenz-Depiereux B, Schnabel D, Tiosano D, Hausler G, Strom TM (2010) Loss-of-function ENPP1 mutations cause both generalized arterial calcification of infancy and autosomal-recessive hypophosphatemic rickets. Am J Hum Genet 86: 267–272. doi: 10.1016/j.ajhg.2010.01.006 PMID: 20137773

38. Diamond MK (1989) Coarctation of the stapedial artery: an unusual adaptive response to competing functional demands in the middle ear of some eutherians. J Morphol 200: 71–86. doi: 10.1002/jmor.1052000109 PMID: 2716063

39. Thys M, Van Camp G (2009) Genetics of otosclerosis. Otol Neurotol 30: 1021–1032. doi: 10.1097/MAO.0b013e3181a86509 PMID: 19546831

40. Goycoolea MV, Lundman L (1997) Round window membrane. Structure function and permeability: a review. Microsc Res Tech 36: 201–211. doi: 10.1002/(SICI)1097-0029(19970201)36:3<201::AID-JEMT8>3.0.CO;2-R PMID: 9080410