Manganese in toenails is associated with hearing loss at high frequencies in humans

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Additional file accompanies this paper.

Abstract

Purpose: Elevated hearing thresholds from high frequencies are known to be one of the hallmarks of age-related hearing loss. Our recent study showed accumulation of manganese (Mn) in inner ears resulting in acceleration of age-related hearing loss in mice orally exposed to Mn. However, there is no evidence showing an association between Mn in non-invasive biological samples and hearing loss in humans evaluated by pure tone audiometry (PTA). In this study, we evaluated Mn in non-invasive biological samples as a possible biomarker for hearing loss in humans.

Materials and Methods: We determined hearing levels by PTA and Mn levels in toenails, hair and urine with an inductively coupled plasma mass spectrometer (ICP-MS) in 145 healthy subjects in Bangladesh.

Results: Multivariable analyses showed that Mn levels in toenails, but not in hair and urine samples, were significantly associated with hearing loss at 8 kHz and 12 kHz. Moreover, our experimental study showed a significant correlation between Mn levels in inner ears and nails, but not hair, in mice orally exposed to Mn.

Conclusions: The results provide novel evidence that Mn in toenails is a possible biomarker for hearing loss at high frequencies in humans.

Keywords: manganese, hearing loss, toenails, inner ears, ICP-MS.

Clinical significance

Hearing loss at high frequencies was shown to affect the speech perception in humans. However, there is limited information about biomarkers associated with hearing loss at high frequencies in humans. Our recent study showed manganese (Mn)-mediated hearing loss in mice orally exposed to Mn, while there is no direct evidence showing an association between Mn in non-invasive biological samples and hearing loss at high frequencies in humans objectively evaluated by pure tone audiometry (PTA). This study provides novel evidence that Mn in toenails is a possible biomarker associated with hearing loss at high frequencies in humans.

Context

Previous studies have shown that humans exposed to environmental factors such as smoking and arsenic have increased risks of hearing loss at high frequencies including 4, 8 and 12 kHz by pure tone audiometry (PTA) (Ohgami et al., 2011; Li et al., 2017; Das et al., 2018), although it is known that exposure to audible noise affects hearing at 4 kHz in humans (World Health Organization, 2015). Elevated hearing thresholds at high frequencies of 4 and 8 kHz has also been shown to affect speech perception in humans (Helvik et al., 2013) and to be one of the hallmarks of age-related hearing loss in humans (Scholtz et al., 2001) and mice (Ohgami et al., 2012b). These studies suggest the importance of investigating environmental factors affecting hearing levels at high frequencies in humans. However, there is limited information about biomarkers to determine hearing loss at high frequencies in humans.

Manganese (Mn) has been detected in drinking water from tube wells at concentrations ranging from a very low concentration to 34,000 μg/L in Bangladesh (Wasserman et al., 2006; Hafeman et al., 2007) and Malaysia (Kato et al., 2010). In tube wells in the Mekong Delta area in Vietnam, Mn has also been detected at concentrations ranging from 1,000 to 34,000 μg/L (Agusa et al., 2006; Buschmann et al., 2008). In a diet investigation in the US, nuts and legumes including cereal products were also found to contain Mn (3.4-23.2 mg/kg) (ATSDR, 2012). Thus, we are orally exposed to Mn via drinking water and foods in our daily life. Oral exposure of humans to Mn via well water has been shown to be associated with increased evidence of impaired neurological functions (Wasserman et al., 2006; Bouchard et al., 2011). Mn levels in hair and blood have been shown to be associated with attention-deficit/hyperactivity disorder in children (Hong et al., 2014; Shin et al., 2015). There are also risks of exposure to Mn via inhalation in occupational environments. Previous studies showed that excessive exposure of welding workers to Mn via inhalation causes Parkinsonism with neurodegeneration of the substantia nigra (Bowler et al., 2011; Racette et al., 2012).

Our previous study showed that oral exposure of mice to Mn at 16.5 mg/L for 4 weeks caused accumulation of Mn in the inner ears, which resulted in hearing loss involving neurodegeneration of spiral ganglion neurons, but not hair cell, mediated by c-Ret tyrosine kinase in inner ears (Ohgami et al., 2016b). Therefore, it would be ideal to determine Mn levels in inner ears in order to evaluate Mn-mediated ototoxicity in humans. However, it is very difficult to non-invasively determine Mn levels in inner ears of humans. Univariate analysis in a previous study showed a significant correlation between distortion product otoacoustic emission (DPOAE) amplitude at 3 kHz and Mn levels in fingernails of humans living in a gold mining community of Nicaragua (Saunders et al., 2013). Therefore, it is possible that nails are suitable non-invasive biological sample to examine hearing loss in humans. However, there is limited information about the utility of biomarkers in determining hearing loss at high frequencies in humans. In this epidemiological study, we therefore examined the association of Mn levels in non-invasive biological samples including toenails with hearing levels at 1, 4, 8 and 12 kHz determined by PTA in humans. We also performed an experimental study with mice as a complement approach in order to determine the correlation between Mn levels in inner ears and nails after oral exposure of mice to Mn at 16.5 mg/L for 4 weeks.

Methods

Ethics approval and consent to participate

This epidemiological study was performed with the approvals by Nagoya University International Bioethics Committee following the regulations of the Japanese government (approval number: 2013-0070) and the Faculty of Biological Science, University of Dhaka (Ref. no. 5509/Bio.Sc). This study got informed consent in written form from all of the subjects. All experiments were performed with the approval by the Institutional Animal Care

and Use Committee in Nagoya University (approval number: 28251) and followed the Japanese Government Regulations for Animal Experiments.

Study subjects

Basic information on the subjects was shown in our previous study (Ohgami et al., 2016a). Briefly, a total of 145 Bangladeshi healthy subjects participated in this study (Fig. 1 and Table 1). All subjects did not have a history of occupational exposure to wielding fumes or ear diseases and a habit of alcohol drinking or using earphones for a portable music player. We obtained information about smoking, age, educational history, weight and height of each participant by a self-reporting questionnaire as described previously (Ohgami et al., 2016a). Body mass index was calculated by using the following formula: weight in kg/height in meters squared.

Hearing test in humans

We performed duplicated measurements by PTA to examine the hearing thresholds at 1, 4, 8 and 12 kHz in a sound-proof room (Ohgami et al., 2011; Ohgami et al., 2016a; Sumit et al., 2015). Hearing level was determined at 12 kHz in this study since hearing level of the extra-high frequency has a high susceptibility to environmental factors including smoking (Ohgami et al., 2011; Ohgami et al., 2016a; Sumit et al., 2015).

Experimental study with mice

We exposed wild-type C57BL/6J mice to manganese chloride ($MnCl_2$) at 16.5 mg/L for 4 weeks via drinking water. The exposure was started at 1 month of age. After exposure, we collected biological samples as described previously (Ohgami et al., 2012a; Ohgami et al., 2016b).

Determination of Mn and arsenic levels in biological samples

We determined Mn and arsenic levels in biological samples by the method previously described (Ohgami et al., 2016a, 2016b). Briefly, the biological samples were incubated in 3 ml of HNO_3 (61%) at 80˚C for 48 hours and then cooled down to room temperature for 1 hour. After adding 3 ml of H_2O_2 (30%) to the samples, the samples were further incubated at 80˚C for 3 hours. After dilution of the samples with an appropriate amount of ultrapure water, the Mn and arsenic level in each sample was determined by using an inductively coupled plasma mass spectrometer (ICP-MS; 7500cx, Agilent Technologies, Inc.) (Ohgami et al., 2015). The limits of detection for Mn in toenails, hair and urine were 0.25 and 0.17 μg/g and 0.12 μg/L, respectively. In urine samples, 60 subjects had Mn levels below the limit, while none of the subjects had Mn levels below the limits in the toenail and hair samples. We assigned the 60 subjects with urinary Mn levels below the limit and the other subjects with urinary Mn levels below the cut-off value to the low group. Values of specific gravity were used to normalize total Mn levels in urinary samples, and the normalized values are shown as μg/L.

Statistical analysis

We performed statistical analyses by the methods previously reported (Ohgami et al., 2016a, 2016b). We determined a significant association between nonparametric variables by Spearman's correlation coefficient, because Mn levels in human biological samples did not show a normal distribution when we performed the Shapiro-Wilks test. We used Pearson's correlation coefficients for animal experiments. We also used the two-tailed Mann-Whitney *U* test for comparison between two groups to determine a significant difference in the auditory thresholds, which are discontinuous variables. We performed Levene's test and Bartlett's test to assess homogeneity of variances. We considered a difference with $p < 0.05$ as significant. We used a logistic regression model with dependent variables of auditory thresholds at 1 kHz

(≥ 10 dB) (Cantley et al., 2015), 4 kHz (≥ 10 dB) (Job et al., 2009), 8 kHz (≥ 25 dB) (Bainbridge et al., 2011; Fabry et al., 2011) and 12 kHz (≥ 40 dB) (Ohgami et al., 2011) and independent variables of Mn levels in hair, toenail and urine samples with adjustment for confounders including sex, smoking and BMI for multivariate analysis as shown previously (Ohgami et al., 2016a). Receiver operating characteristic (ROC) curves and the highest Youden index were used to determine cut-off values for Mn levels in biological samples (Schisterman et al., 2005). We used JMP Pro (version 11.0.0; SAS Institute Inc., Cary, NC, USA) for all statistical analyses.

Results

Basic characteristics of study participants

Characteristics of the subjects and variables of confounders are shown in Table 1. The subjects had no clinical problems including diabetes or hypertension. Associations of confounders with hearing levels were shown in our previous study (Ohgami et al., 2016a). In brief, average auditory thresholds showed no significant differences among three groups with BMI based on the WHO categories (underweight, < 18.5; normal range, 18.5–25; overweight, > 25). Average auditory thresholds in the older group (n = 68) were higher compared to those in the younger group (n = 77) at 1 kHz ($p = 0.0098$), 4 kHz ($p < 0.0001$), 8 kHz ($p < 0.0001$) and 12 kHz ($p < 0.0001$). Auditory thresholds in females (n = 76) were higher than those in males (n = 69) at 4 kHz ($p = 0.0257$), 8 kHz ($p = 0.0004$) and 12 kHz ($p = 0.0066$). The smoking group (n = 31) showed higher auditory thresholds compared to those in the non-smoking group (n = 114) at 1 kHz ($p = 0.0057$), 4 kHz ($p < 0.0001$), 8 kHz ($p = 0.0002$) and 12 kHz ($p < 0.0001$). We also considered the educational records of subjects (Helvik et al., 2013) and arsenic (As) levels in biological samples (Li et al., 2017) as confounders.

Univariate analysis of hearing loss in humans with high Mn levels in biological samples

Mn levels (means ± SD) in toenails, hair and urine in all of the subjects were 7.3 ± 7.6 μg/g, 20.3 ± 19.9 μg/g and 0.7 ± 2.1 μg/g, respectively (Table 2). Arsenic levels (means ± SD) in toenails, hair and urine in all of the subjects were 1.4 ± 1.2 μg/g, 0.5 ± 0.5 μg/g and 90.3 ± 103.0 μg/g, respectively (Li et al. unpublished observation). We then classified the subjects into two groups at 1.97 μg/g of Mn in toenails, 4.08 μg/g of Mn in hair and 0.24 μg/L of Mn in urine (Table 3) in order to compare the hearing levels among the two groups (Fig. 2). In this study, we examined hearing levels at 12 kHz in addition to 1, 4 and 8 kHz measured by PTA in humans, since our previous study showed that an auditory threshold at 12 kHz as an extra-high frequency could be a sensitive marker for hearing loss (Ohgami et al., 2016a; Ohgami et al., 2011; Sumit et al., 2015), though hearing thresholds at 1, 4 and 8 kHz are generally measured in the clinical examination of PTA. In toenail and hair samples, the high group showed significantly worse hearing levels at 4, 8 and 12 kHz compared to those in the low group (Fig. 2A, B). There were no significant differences in urine samples between the two groups except for 12 kHz (Fig. 2C).

Multivariable analysis of hearing loss in humans with high Mn levels in biological samples

Multivariable models adjusted for confounders including age, sex, BMI, smoking and educational record (Table 4) showed that Mn levels in toenails in the high group had significant associations with hearing loss at 8 kHz [odds ratio (OR) = 4.19; 95% confidence interval (CI): 1.18, 14.82; $p < 0.05$] and 12 kHz (OR = 3.88; 95% CI: 1.25, 12.06; $p < 0.05$) (Table 4). Mn levels in hair in the high group had significant associations with hearing loss at 4 kHz (OR = 3.82; 95% CI: 1.00, 14.53; $p < 0.05$), while urine samples showed no significant ORs at 1, 4, 8 and 12 kHz in the adjusted models (Table 4). We also performed multivariate analysis adjusted for arsenic levels in biological samples in addition to age, sex, BMI, smoking and educational record. ORs of hearing loss at 8 kHz and 12 kHz remained significant in subjects with high Mn levels in toenails but not in hair (Table 5). Thus, these

results suggest that Mn level in toenails is associated with hearing loss at 8 and 12 kHz in humans. We further verified the models by shifting the cut-off values of the independent variables dividing Mn levels in biological samples. The significance of ORs in toenails remained when the cut-off values in toenails were shifted from 1.05 to 2.08 μg/g [ranging from 53% to 142% of the cut-off value (1.97 μg/g) in toenails], while Mn levels in hair and urine samples did not show significant ORs even when the cut-off values in hair and urine samples were shifted from 2.16 to 5.79 μg/g and from 0.13 to 0.34 μg/L with the same proportions as the toenails, respectively. The shifted cut-off values at the lowest and highest values had the following numbers of subjects in the low/high Mn groups: 24/121 and 55/90 in toenails, 22/123 and 48/97 in hair and 61/84 and 90/55 in urine.

Association between Mn levels in inner ear and nails in mice after oral exposure to Mn

We determined the correlation between Mn levels in inner ears and nails by ICP-MS after oral exposure of mice to Mn at 16.5 mg/L for 4 weeks (Fig. 3). There was a significant correlation between Mn levels in inner ears and nails ($r = 0.7030$, $p = 0.0035$) (Fig. 3A), whereas there was no correlation between Mn levels in inner ears and hair ($r = 0.1512$, $p = 0.5906$) (Fig. 3B).

Discussion

In this pilot study, Mn in toenails was associated with hearing loss in humans. We demonstrated significant correlations between duration of drinking well water and Mn levels in toenails (Fig. S1E) and between duration of drinking well water and hearing levels at 4, 8 and 12 kHz in subjects with no occupational record of inhalation exposure to Mn (Fig. S1B-D). Thus, the results indicate the possibility that oral exposure to Mn via drinking water causes hearing loss at high frequencies in humans, although there is a possibility that oral exposure to Mn via food is also associated with hearing loss.

Our results are partly consistent with the results of our previous study showing acceleration of age-related hearing loss in mice orally exposed to $MnCl_2$ (Ohgami et al., 2016b). Thus, it is possible that oral exposure to Mn via drinking water is associated with age-related hearing loss in humans, although the chemical form of Mn in drinking water in this study remains unknown. In this study, ORs of hearing loss at 8 and 12 kHz remained significant in humans with high Mn levels in toenails in our multivariate analysis adjusted for arsenic in addition to other confounders. Thus, the results suggest that Mn level in toenails is independently associated with hearing loss at high frequencies of 8 and 12 kHz in humans. Hearing loss at higher frequencies has been shown to affect speech perception in humans (Aydelott et al., 2010), although speech perception in humans was not determined in this study. Further study is needed to determine the associations among Mn levels in nails, hearing loss at higher frequencies and speech perception with a larger number of subjects.

Our results showed a significant association of hearing loss with Mn levels in toenails but not with Mn levels in hair. These results partly correspond to the results of our experimental study showing that Mn levels in inner ears significantly correlated with those in nails but not in hair, though the reason for the different correlations of Mn levels in inner ears with those in nails and hair is not clear. The expression level of a metal transporter in a neural tissue has been shown to correlate with the accumulation of Mn in the same tissue in rats (Thompson et al. 2007). A previous study showed that the metal transporters DMT1, Zip8 and Zip14, which have been shown to mediate cellular uptake of Mn, are expressed in inner ears in mice (Ma et al., 2008), although the tissue distributions of these transporters in the nail matrix and hair follicle, which have been shown to produce nails (Kitahara and Ogawa. 1997) and hair (Rogers. 2004), respectively, are not clear. It would be interesting to investigate whether these transporters are expressed in the nail matrix and hair follicle. The level of an element in toenails has been shown to be a reliable biomarker reflecting the status of chronic exposure (Garland et al., 1993; Longnecker et al., 1993), while the levels of an

element in urine and blood are usually measured for acute exposure. A previous study showed no association between Mn levels in blood samples and hearing levels in factory workers (Chuang et al., 2007). Thus, our results suggest that chronic exposure to Mn causes hearing loss in humans.

Conclusions

In this pilot study, we epidemiologically demonstrated that Mn in toenails is significantly associated with hearing loss at high frequencies in humans evaluated by PTA. In the experimental study, wild-type mice that were orally exposed to Mn correspondingly showed a significant correlation between Mn levels in inner ears and nails. These results combined with the results of our epidemiological study indicate that Mn in toenails is a possible biomarker for hearing loss at high frequencies in humans.

Acknowledgements

We thank members of the Department of Occupational and Environmental Health, Nagoya University Graduate School of Medicine for helpful discussions and technical assistances.

Funding

This study was supported in part by Grants-in-Aid for Scientific Research on Innovative Areas (24108001 and 16H01639), Scientific Research (A) (15H01743 and 15H02588), (B) (16H02962, 17KT0033) and (C) (16K08343) and Grant-in-Aid for Challenging Exploratory Research (26670525) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), the Japan Health Foundation, the Mitsui & Co., Ltd. Environment Fund, Foundation from Center for Advanced Medical and Clinical Research Nagoya University Hospital, the Mitsubishi Foundation, AEON Environmental Foundation, Nagono Medical Foundation, The Salt Science Research Foundation and Grant for Environmental

Research Projects from the Sumitomo Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflict of interest statement

All authors declare to have no actual or potential conflicts of interest.

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Figure legends

Fig. 1. Diagram of the study protocol.

Fig. 2. Hearing loss in humans with high Mn levels in biological samples. (**A-C**) Auditory thresholds (means ± SD) from frequencies of 1 kHz to 12 kHz in the high Mn level group (closed circles) and low Mn level group (open squares) in toenails (**A**), hair (**B**) and urine (**C**) are displayed. Cut-off values of Mn and the number of subjects in each group in each biological sample are shown in Table 3. Significant differences (**$p < 0.01$, ***$p < 0.001$) compared to the low Mn level group were analyzed by the Mann-Whitney *U* test.

Fig. 3. Wild-type mice orally exposed to Mn via drinking water showed a correlation between Mn levels in inner ears and nails. (**A, B**) Scatter plots of Mn levels (ng/g) in inner ears on the Y-axis and in nails on the X-axis (**A**) and hair on the X-axis (**B**) from wild-type mice with oral exposure to Mn at 16.5 mg/L via drinking water for 4 weeks (n = 8) and with no exposure (n = 7) are presented. Pearson's correlation coefficients are also shown.

Table 1. Basic characteristics of study participants (n=145)

	Mean ± SD	Max	Min	Variables	Participants (%)
Age	29.6 ± 11.0	55	12		
Sex				Male	47.6
				Female	52.4
BMI	22.0 ± 3.4	31.9	13.8	< 18.5	15.9
				18.5-25	64.8
				> 25	19.3
Smoking				Yes	21.4
				No	78.6
Educational record				Primary school	31.7
				Junior high school	35.2
				High school	22.1
				University	11.0

Table 2. Manganese levels (μg/g) in biological samples (n=145)

	Mean ± SD	Max	Min
Toenails	7.3 ± 7.6	49.6	0.3
Hair	20.3 ± 19.9	85.4	0.4
Urine*	0.7 ± 2.1	21.3	0.0

*Manganese concentration in urine was corrected by specific gravity (μg/L).

Table 3. Classification according to manganese levels in biological samples

Manganese (μg/g)		Participants	Percentage (%)
Toenails	Low (< 1.97)	44	30.3
	High (≥ 1.97)	101	69.7
Hair	Low (< 4.08)	40	27.6
	High (≥ 4.08)	105	72.4
Urine*	Low (< 0.24)	79	54.5
	High (≥ 0.24)	66	45.5

*Manganese concentration in urine was corrected by specific gravity (μg/L).

Table 4. Adjusted odds ratios (95% CI) for hearing loss and Mn levels in biological samples (n=145)[a].

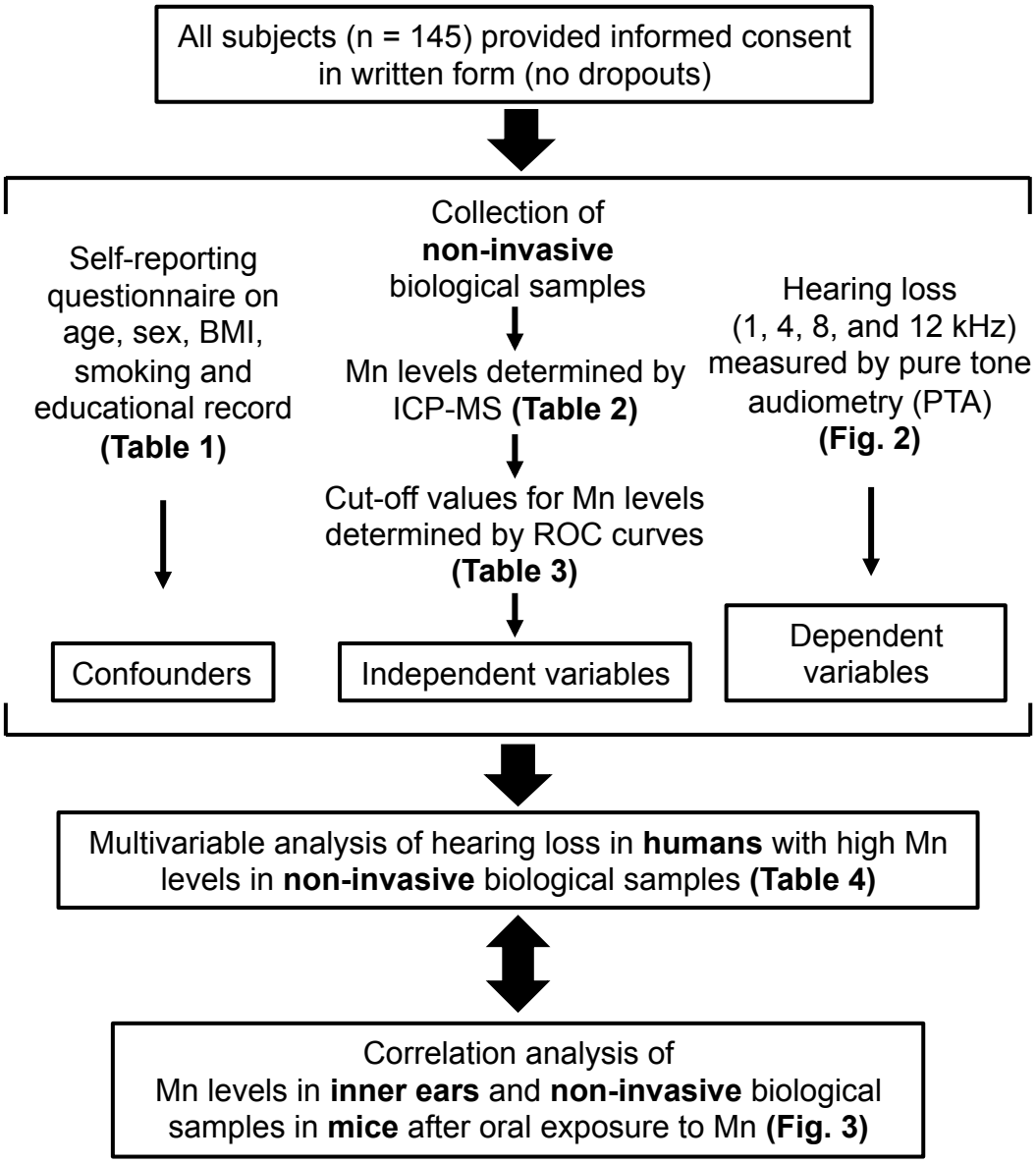
	1 kHz (≥10 dB)	4 kHz (≥10 dB)	8 kHz (≥25 dB)	12 kHz (≥40 dB)
Mn in toenails				
Low	Reference	Reference	Reference	Reference
High	1.09 (0.32, 3.79)	1.36 (0.42, 4.34)	4.19* (1.18, 14.82)	3.88* (1.25, 12.06)
Mn in hair				
Low	Reference	Reference	Reference	Reference
High	1.03 (0.27, 3.98)	3.82* (1.00, 14.53)	3.25 (0.83, 12.67)	1.64 (0.46, 5.94)
Mn in urine				
Low	Reference	Reference	Reference	Reference
High	1.39 (0.58, 3.35)	1.26 (0.53, 2.99)	1.15 (0.47, 2.84)	1.39 (0.59, 3.28)

Abbreviation: CI, confidence interval. [a]Adjusted for age, sex, BMI, smoking and educational record. *$p < 0.05$.

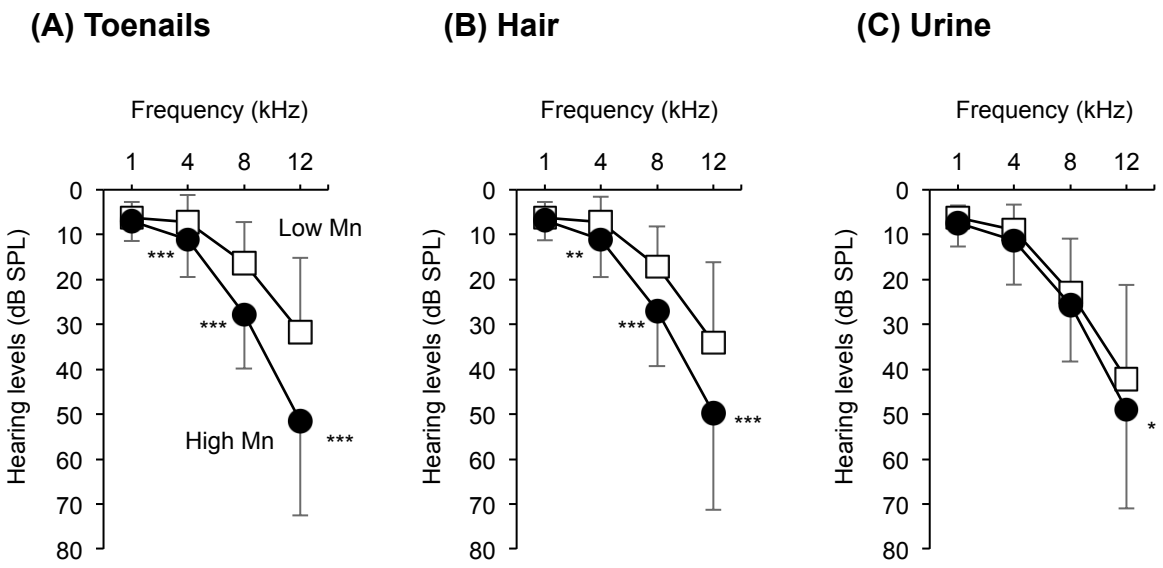
Table 5. Adjusted odds ratios (95% CI) for hearing loss and Mn levels in biological samples (n=145) after adding the confounder factor of arsenic[a].

	1 kHz (≥10 dB)	4 kHz (≥10 dB)	8 kHz (≥25 dB)	12 kHz (≥40 dB)
Mn in toenails				
Low	Reference	Reference	Reference	Reference
High	1.14 (0.32, 4.15)	1.65 (0.48, 5.62)	7.66** (1.76, 33.45)	5.07* (1.45, 17.69)
Mn in hair				
Low	Reference	Reference	Reference	Reference
High	0.91 (0.22, 3.68)	3.70 (0.96, 14.25)	3.23 (0.82, 12.64)	1.56 (0.43, 5.75)
Mn in urine				
Low	Reference	Reference	Reference	Reference
High	1.36 (0.56, 3.32)	1.29 (0.54, 3.08)	1.15 (0.47, 2.84)	1.38 (0.59, 3.27)

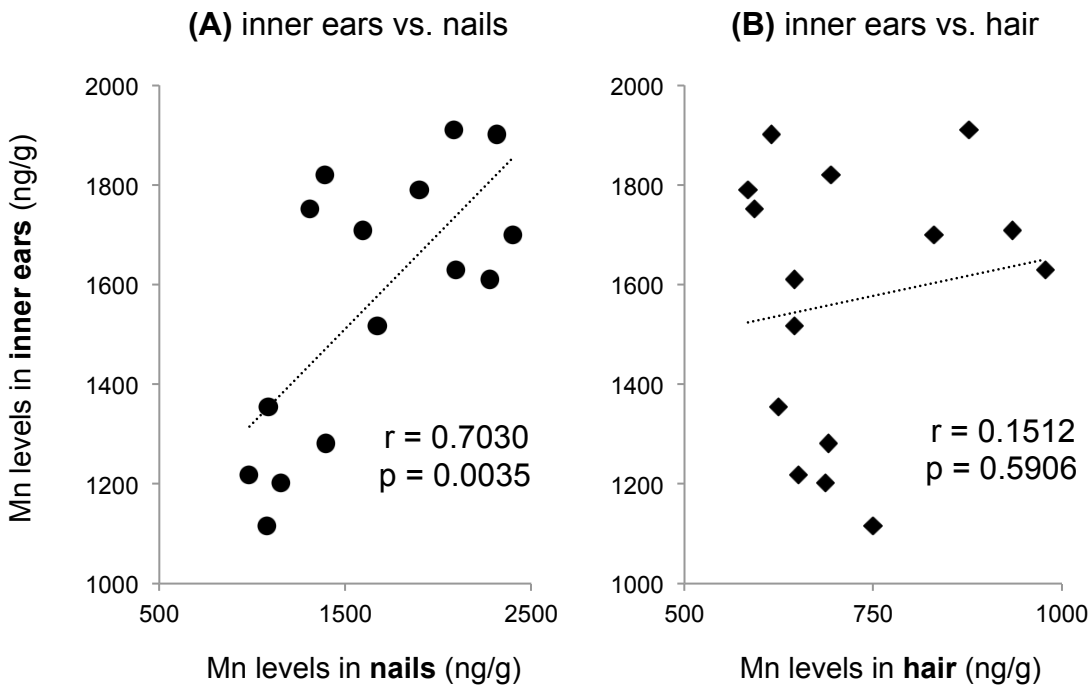
Abbreviation: CI, confidence interval. [a]Adjusted for age, sex, BMI, smoking, educational record and arsenic. *$p < 0.05$, **$p < 0.01$.



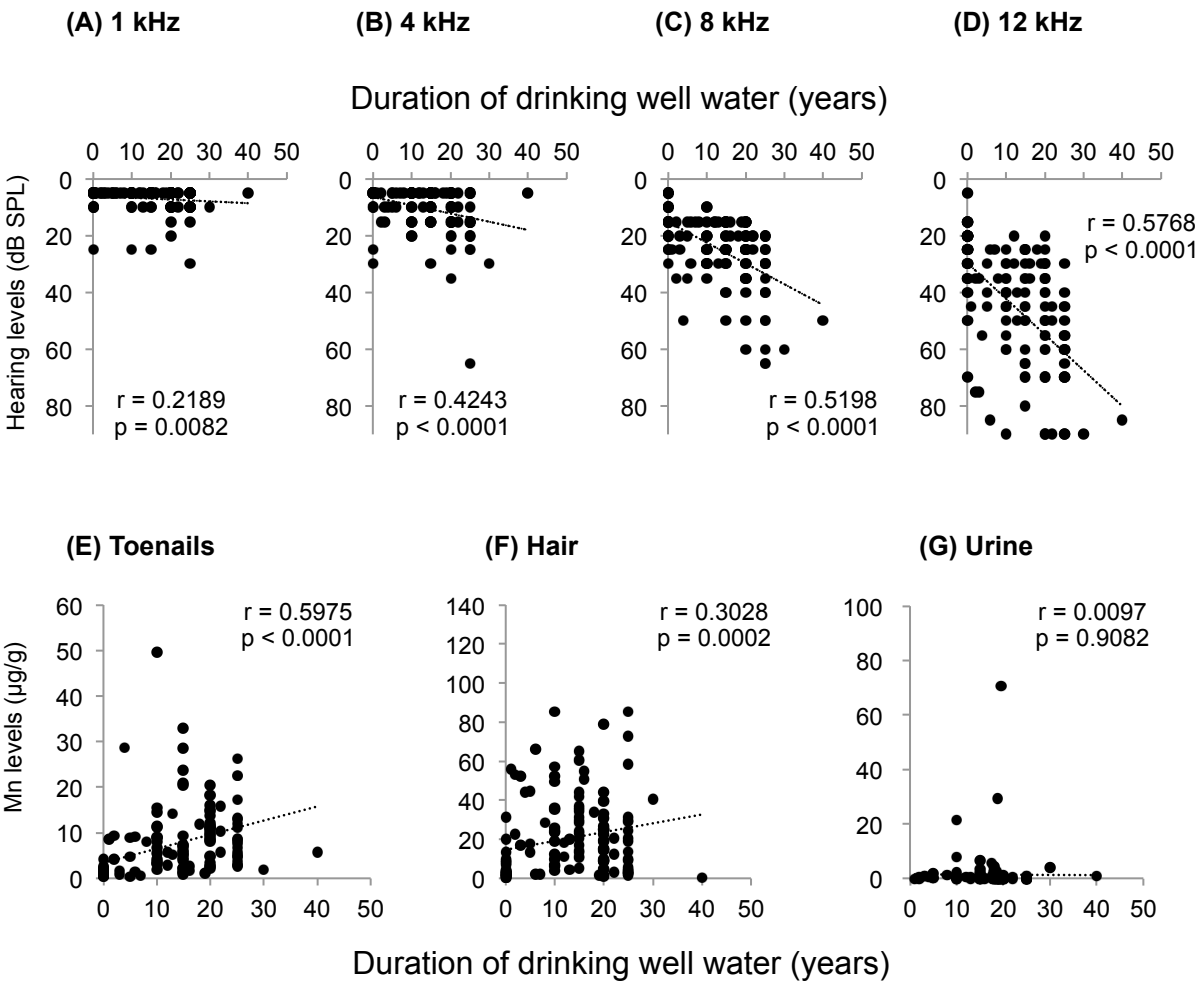
Ohgami et al., Figure 1



Ohgami et al., Figure 2



Ohgami et al., Figure 3



Ohgami et al., Figure S1