Evaluating the Risk of Ototoxicity Due to Metals Exposures

By

Krystin Carlson

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Toxicology)
in the University of Michigan
2019

Doctoral Committee:

      Associate Professor Richard Neitzel, Chair
      Associate Professor Niladri Basu
      Professor Bhramar Mukherjee
      Associate Professor Sung Kyun Park
      Professor Emeritus Jochen Schacht

Krystin Carlson

krystin@umich.edu

ORCID iD: 0000-0001-9542-4831

DEDICATION

I dedicate my dissertation to the promotion of all minorities to successful stations in the sciences,

especially to women with children or other family commitments who wish to advance in power.

Diversity in people and situations can make life uncomfortable, challenging, and unfair.

It also can make life full of opportunities for innovation,

replete with time for growth,

and overwhelmingly more rewarding.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS, SYMBOLS, AND ACRONYMS

μg	Micrograms
μg/dL	Micrograms per deciliter (units to measure lead in blood)
μg/L	Micrograms per liter
μM	Micromolar
AABR	Automated auditory brainstem response
ABR	Auditory brainstem response
ACGIH®	American Conference of Governmental Industrial Hygienists
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
As	Arsenic
AST	Aspartate aminotransferase
ATSDR	Agency for Toxic Substances and Disease Registry
BEI®	Biological exposure index
BLL	Blood lead level
BUN	Blood urea nitrogen
C	Celsius (degrees)
Ca	Calcium
CAOHC	Council for Accreditation in Occupational Hearing Conservation
Cd	Cadmium
CDC	US Centers for Disease Control and Prevention
CdCl2	Cadmium chloride
CI	Confidence interval
COHSE	Center for Occupational Health and Safety Engineering
Cr	Chromium
Cu	Copper
dB	Decibel
dBA	A-weighted decibel
dB HL	Decibels hearing level (used in audiometric testing)
DPOAE	Distortion product otoacoustic emissions
EDHI	Early Hearing Detection and Intervention Program
EPA	US Environmental Protection Agency
Fe	Iron
g	Gram
GHS	Ghanaian Cedi
Hg	Mercury
HL	Hearing loss
HLPP	Hearing loss prevention program
Hr	Hour
Hz	Hertz
ICPMS	Inductively-Coupled Plasma-Mass Spectrometry

IRB	Institutional Review Board
ip	Intraperitoneal
K	Potassium
kHz	Kilohertz
L_{EQ}	Equivalent continuous noise level
L_{max}	Maximum noise level
MDHHS	Michigan Department of Health and Human Services
MeHg	Methylmercury
mg/dL	Milligrams per decilitre
mg/kg	Milligrams per kilogram
mg/L	Milligrams per litre
mM	Millimolar
Mn	Manganese
MNBB	Michigan Neonatal Biobank
mV	Millivolt
NDBS	Newborn dried blood spots
NGO	Non-governmental organization
NHANES	National Health and Nutrition Examination Survey
Ni	Nickel
NICU	Neonatal Intensive Care Unit
NIDCD	US National Institute on Deafness and Other Communication Disorders
NIEHS	US National Institute Environmental Health Sciences
NIHL	Noise-induced hearing loss
NIOSH	National Institute for Occupational Health and Safety
NN	Noise notch
OHC	Cochlear outer hair cell
OR	Odds ratio
OSHA	Occupational Safety and Health Administration
Pb	Lead
ppb	Parts per billion
ppm	Parts per million
PTH	Parathyroid hormone
ROS	Reactive oxygen species
SD	Standard deviation
Se	Selenium
SPL	Sound pressure levels
TEOAE	Transient evoked otoacoustic emissions
TLV®	Threshold limit value
TWA	Time weighted average
TXRF	Total reflection x-ray fluorescence
ULAM	Unit for Laboratory Animal Medicine
US	United States
w/v	Weight by volume
WHO	World Health Organization
Zn	Zinc

ABSTRACT

Hearing is vital for speech communication and evaluation of the environment. Damage to this sense harms both physical and social health; poor learning outcomes can also arise. Noise is a well-known exposure that can result in hearing loss (HL), but other environmental exposures in combination with noise may also impact HL. Previous studies have suggested that exposures to toxic metals are associated with HL, while essential elements show protective benefits. This dissertation used three studies to explore the relationship between metal exposures and HL, and accounted for exposure mixtures and protective elements.

First, a controlled laboratory experiment treated mice with lead (Pb) and cadmium (Cd), both alone and in tandem, in drinking water for twelve weeks. Auditory outcomes were measured following treatment with chemical toxicants in addition to the physical agent, noise. Dosing with Pb and Cd reached relevant occupational exposure levels: mean blood levels were 60.1 μg/dL for Pb and 27.2 μg/L for Cd. Animals displayed mean threshold shifts of 42 dB at 32 kHz following noise exposures of 105 dB. However, threshold shifts were not significantly different from controls after treatment with Pb or Cd alone. Combinations of exposures to Pb and noise, Cd and noise, as well as all three toxicants together did not cause threshold shifts significantly different than noise alone. This adult CBA/CaJ mouse model demonstrated a lack of ototoxicity due to Pb and Cd exposure.

Second, a case-control study of newborn infants born between 2003 and 2015 investigated auditory impacts associated with blood levels of Pb and methylmercury (MeHg). Cases with abnormal hearing screenings were matched to controls with normal hearing screenings. Dried blood spots collected after birth were analyzed for MeHg and Pb, in addition to the essential elements calcium, copper, iron, potassium, selenium, and zinc. Conditional logistic regression models of exposure quartiles showed a significant trend (p=0.03) with increasing levels of dried blood MeHg increasing the odds of a hearing screening failure (OR=1.81, 95% CI 1.01-3.24). Models also indicated a significant association with increasing levels of calcium decreasing the odds of hearing

screening failure (OR=0.49, 95% CI 0.34-0.70). A significant interaction between selenium and zinc was also noted in logistic regression models.

Third, a cross-sectional study in a developing nation investigated Pb and other toxicant metals (arsenic, cadmium, manganese, and MeHg), along with essential elements (copper, iron, selenium, and zinc), and noise exposures in a potentially vulnerable community of electronic waste (e-waste) recycling workers. This study recruited 58 e-waste workers, with an average age of 26, from their worksite in Agbogbloshie, Ghana. Sixty percent of participants were observed to have a noise notch, or elevated hearing thresholds at high frequencies. Potentially harmful levels of noise exposure were observed during both occupational and non-occupational tasks. A metric quantifying the variety of work tasks improved multivariate regression models predicting the degree of hearing impairment at high frequencies. Essential elements were not significantly associated with levels of toxicant metals or HL. However, a significant interaction between levels of zinc and noise was observed.

Taken together, these studies do not provide consistent evidence of an ototoxic impact from Pb or Cd exposure; however, MeHg ototoxicity was significant. The three studies also did not find significant evidence of interactions between toxicant metals and noise. Nonetheless, Pb and Cd exposures can damage other tissues relevant to public health.

CHAPTER I: Introduction

Profound impacts on human health and quality of life, as well as a wide variety of adverse social, psychological, occupational, and educational outcomes, can all stem from one disease: hearing loss (HL) [1]. Across the world, HL is the third most disabling disease [2]. This is due in part to an increasingly high prevalence of adult-onset HL in developing nations [3], a large portion of which is due to occupational exposures to potentially harmful conditions [4].

Occupationally, HL is mainly associated with exposures to excessive noise which can cause 'noise-induced hearing loss' (NIHL). Another established link to HL is aging, causing 'age-related hearing impairment'. While HL is indeed most prevalent in the elderly, HL affects those of all ages, especially impairing the quality of life in children, even when cases are mild [5,6]. HL of unknown etiology has been established in a majority of childhood HL cases [7]; the portion of environmental exposures contributing to developmental hearing problems is not established.

However, one cause of HL that is not well understood but is emerging and gaining in prominence is exposure to ototoxic occupational as well as environmental contaminants, in particular metals. There is a need for further research examining the relationship of metals to HL, especially those occurring in mixtures [8]. Exposure to mixtures of metals in the environment are ubiquitous [9]. Pb and Cd occur in occupational and community settings due to their current and past use in gasoline, electronics, plumbing, and household paint [10]. A number of epidemiological studies indicate that exposures to metals, in particular lead (Pb), cadmium (Cd), and mercury (Hg) may be associated with an increased risk of HL [11–15]. Previous experiments on mice have shown that *in utero* exposure to environmental levels of Pb can lead to changes in the nervous system and lowered hearing thresholds [16–18]. While the results of these studies are suggestive, more evidence is required to establish a causal link between metals and HL while accounting for potential confounding effects due to concurrent exposure to noise and nutritional status.

Better understanding of these multi-factorial exposures is essential to enhanced real-world modeling. To address this need, this dissertation comprised three major investigations to study Pb

ototoxicity from both occupational and environmental exposures. Laboratory experiments allow variations of mixtures to be tested in a controlled environment. In the first study, a mouse model was developed to investigate effects on the hearing system through experimental exposures to both environmentally- and occupationally-relevant exposures to Pb and Cd. Epidemiological experiments allow for better understanding of real-world exposure conditions and actual health outcome in humans. A variety of environmental exposures to Pb occur in populations of children living in Michigan [19–21]. Most recently the water crisis in Flint, Michigan has highlighted concerns over heightened exposures to Pb in children [22]. Cd has been also been detected in the environment in Michigan [23,24] and some elevated exposures have been discovered [25]. The second, a case-control study of newborns in Michigan, evaluated relationships between environmental Pb and Cd exposures and abnormal hearing outcomes at birth. Electronic waste workers at Agbogbloshie Market in Accra, Ghana have high occupational exposures to metals [26,27]. The extent to which elevated Pb exposures may play a role in HL outcomes for these workers in Ghana was investigated in a third study using a cross-sectional study design.

To explore more fully the relationship between HL and metals, it is useful to explore several related areas. These include the anatomy and physiology of the hearing system; the association between three key metals (Pb, Cd, and Hg) and HL; the effects of mixed exposures to these metals; potential mechanisms of metal-induced ototoxicity; and relevant research gaps. Each of these areas are described below.

I.A: Hearing system

Hearing is a complex sense that requires intricate bio-machinery working in concert with the nervous system to produce awareness of myriad types of sound. The patterns of sound in a surrounding environment is produced by a wide variety of both natural environmental sources, such as wind rustling trees or waves crashing on a beach, as well as anthropologic environmental sources like trains, jackhammers, and radios. Sounds can be pleasant and soothing when quiet and appealing, or defined as noise when too loud and undesirable. All sounds are the transfer of mechanical energy as molecules in the air vibrate and disrupt adjacent molecules. Transfers of

force occur in three-dimensional waves as variations in pressure are distributed through compression and refraction.

Hearing is the sensory phenomenon of detecting changes in the pressure in the environment as vibrations through the ear. Sound can be described in terms of intensity, pitch, and tone. Intensity, perceived as loudness, is a measure of the amount of energy carried by sound. Highly intense sounds can cause immediate pain and at high enough levels can damage the tympanic membrane and ossicular bones in the middle ear [28]. Intensity is quantified as sound pressure, measured in Pascals, or as sound pressure level, measured in decibels (dB) on a logarithmic scale. High intensity sounds are perceived as being louder. Pitch describes the frequency of sound, measured in hertz (Hz); higher-pitched musical notes, such as those played by a piccolo, have a higher frequency than lower-pitched notes, such as those played by a tuba. Timbre is another variation in the sound quality between musical instruments within the same frequency range, for example a piano and a guitar. This is due to the multiple pitches, overtones, and reverberation produced by energy in a musical instrument or for any type of sound, which can enhance subjective satisfaction with a type of tone for an individual [29].

Conductive HL can occur due to damage to the external and middle ear structures and is not the primary focus of this dissertation. However, describing causes of conductive HL illustrates the anatomy and function of the outer and middle ear. The outer ear structures include the pinna or auricle (the visible part of the ear) as well as the ear canal. The auditory system is open to the environment through both the pinna and ear canal. Deformations of the pinna and obstructions, particularly from occlusion of ear wax, or infections within the ear canal can lead to blockage of the unique external ear structures which conduct and amplify sound [30]. The middle ear structures include the tympanic membrane (ear drum) and the three ossicular bones. The eustachian tube connects the middle ear to the nasopharynx, or nose and mouth, to allow for pressure equalization. Deformation or loss of function in these structures can also cause conductive HL. Problems in the middle ear can include a perforated or deformed tympanic membrane, deformation or fixation (e.g. in otosclerosis) of the malleus, incus, and stapes (the three ossicles), or fluid in the middle ear resulting from an infection [31]. There is special concern following blast trauma or loud bursts of sound, such as those from a gun, which may result in perforation of tympanic membrane, dislocation or fracture of the ossicles, as well as problems in the inner ear, or cochlea [32].

Sensory cells of the inner ear

The stapes transmits vibrations from mechanical motions in the middle ear to fluid motion within the cochlea. Damage to the systems within the cochlea or the connecting nerves producing impairments to hearing are defined as sensorineural and separated from the conductive hearing issues discussed earlier. The key structure inside the cochlea is the organ of Corti which contains the sensory cells responsible for signaling in response to sound stimuli as well as amplifying these sounds. The organ of Corti contains three rows of outer hair cells and one row inner hair cells [33]. In mammalian auditory systems, death of these two types of sensory cells, inner and outer hair cells, is permanent as they do not regenerate [34]. Outer hair cells amplify the movement of the basilar membrane for specific frequencies depending on their location in the cochlea. This amplification allows for better detection of low intensity sounds by the inner hair cells. Inner hair cells perceive vibrations through the bending of stereocilia which opens potassium channels allowing an influx of potassium inside the cell [35]. The increase in potassium triggers an increase in calcium levels as calcium channels open, and this in turn signals for neurotransmitters to be released at the basal end of the cell. The inner hair cells allow for translation of vibration sensations into neuronal signals received by neurons connected to the inner hair cells and contained within the cochlea. Each inner hair cell connects to one afferent Type I neuron or spiral ganglion [36]. Afferent spiral ganglia receive glutamate neurotransmitters sent from the inner hair cells of the ear. They then send action potentials to the auditory cortex of the brain after first passing signals through the brainstem, through the medulla oblongata (cochlear nucleus) and midbrain (inferior colliculus), relayed then through the thalamus (medial geniculate nucleus) [37].

The stria vascularis regulates the levels of ions in the endolymph. Holding a high concentration of potassium in the endolymph allows for an endocochlear potential over 100 mV [38]. This concentration gradient allows for a sudden increase in potassium upon the appropriate channel opening when the inner hair cells, which are bathed in the low potassium levels of the perilymph, are stimulated by vibrations. Damage to the stria vascularis can also result in sensorineural HL, as a secondary effect. These types of impairments tend to be associated with temporary HL and the threshold shift would be flatly elevated [39].

Function of the outer hair cells inside the cochlea can be measured using DPOAEs (distortion product otoacoustic emissions) [40]. DPOAEs are generated by a phenomenon that occurs when

two pure-tones (f_1 and f_2; at frequencies separated by 1.22 ratio of f_1/f_2) enter the cochlea with functional outer hair cells [41]. The amplification function and resonance of the outer hair cells with the basilar membrane produce a third tone (at a lower frequency than the entering sounds by the formula $f_3=2f_1-f_2$) which exits the cochlea and is transmitted in reverse through the hearing system to the outer ear where it can be detected by a microphone [42]. Alterations in DPOAEs are well documented after severe noise exposures and correlated with HL [43]. DPOAEs can be used to localize the location of damage to the outer hair cells by using various low to high frequencies detected in segments ranging from the basal to apex portions of the cochlea. Detecting outer hair cell impairment is important as outer hair cells are more sensitive to damage than inner hair cells, especially by reactive oxygen species (ROS). Outer hair cell damage, whether due to toxicants or noise, results in 30-40 dB HL heightened threshold detection [44].

Neural transmission

Damage within the neural complex of the auditory processing system can also impair hearing. Auditory brainstem response (ABR) detects the threshold sound pressure level which stimulates neuronal pathways in the auditory system allowing for perception of sound. ABR only measures sound-initiated neural activity and not the complete sensation of "hearing," which includes conscious perception of that sound. Action potentials are displayed as waves I-V on an audiogram. Following detection of sounds by spiral ganglia, signals are sent along the eighth cranial nerve (or the vestibulocochlear nerve); this is seen as wave I. As neural signals continue, they are passed to the cochlear nucleus (wave II), the superior olivary complex (wave III), the lateral lemniscus (wave IV), and finally the inferior colliculus (wave V). Action potentials travel further to the medial geniculate body in the auditory thalamus and then the auditory cortex. Auditory processing occurs in other areas of the brain such as the amygdala where auditory inputs can be stored as memory and associated with an emotional response [45]. Abnormal ABR amplitudes (such as the lack of peaks) as well as prolonged or shortened times between waves indicate abnormal neurological processing and sensorineural HL [46].

Pure-tone audiometry is the gold standard for assessing auditory thresholds. It involves an assessment of the full auditory system from outer ear to neural processing and an individual's perception of a sound. Audiometry is best performed in a sound-proof booth and with an individual who is willing and able to report when a pure-tone stimulus is perceived [47]. Despite excellent

diagnostic abilities of audiometry, it has been found to not fully capture all hearing difficulties, specifically those of not hearing speech well in environments with high levels of background sounds [48]. Hearing in background noise is also being explored through neural responses in animals [49]. Audiometry is often recommended in conjunction with other hearing tests such as ABR and DPOAE to fully diagnose hearing disorders.

I.B: Pb and HL

Carlson and Neitzel (2019) recently published an extensive review examining Pb ototoxicity in both animal and human studies [50]. Literature is available in both the fields of toxicology and epidemiology, though results in both areas are suggestive, evidence does not conclusively demonstrate an ototoxic property of Pb. Toxicological studies have all not established a consistent threshold shift or alteration in ABR across different species or different doses of Pb. Epidemiological studies have conflicting results and weaknesses, especially in the areas of establishing the level of exposure to Pb and accounting for confounding noise exposures. The evidence below is compelling, but must be further explored.

Toxicological studies done in various types of animals have noted alterations in the auditory system following Pb exposure. Injections of Pb acetate (PbAc) into chicken embryos resulted in alterations to auditory nuclei neurofilament proteins [51]. Monkeys exposed to PbAc from birth to age 13, had elevated thresholds, especially at higher frequencies compared to non-treated controls [52]. However, within this study, of the six monkeys exposed to Pb, three did not display altered auditory responses. *In vitro* work has shown evidence that transmission of neuronal signlas can be altered after treatment with Pb. Auditory nerve compound action potentials were disrupted due to administration of PbAc in guinea pigs [53]. PbAc decreased outward potassium currents in adult pigmented guinea pigs outer hair cell explants [54]. Pb also impacts the proton gradient of synaptic vesicles and decreases glutamate accumulation inside synaptic vesicles [55]; this may be related to auditory processing as glutamate is the primary neurotransmitter used in ascending auditory neurons [37].

Three studies conducted by Lurie et al. (2008, 2009, and 2010) focused on developmental exposures of Pb throughout gestation in a mouse model. While no changes in auditory thresholds

were observed, neural conduction time was influenced by treatment with Pb in mice during their development to 21 days of age. Pb treatment *in utero* decreased levels of proteins and the neurotransmitter serotonin in addition to altering the superior olivary complex in the brainstems of Pb exposed animals [16–18].

Human studies have also established some evidence of detrimental hearing effects associated with Pb exposure. American adolescents showed a greater likelihood for HL with higher Pb exposures [56]. However, this study accounted for noise exposures using participant reporting of five or more hours a week and did not examine interactions or quantify average noise levels. In a more aged population, x-ray fluorescence has been used to measure life-time accumulated bone Pb levels in participants. Levels of Pb in bone were significantly associated with thresholds increases at higher frequencies and HL increased with Pb exposures, even after accounting for occupational noise exposures [57]. Four occupational studies have noted associations between Pb exposure and HL. In workers, a majority of whom were male and white, an elevated (i.e., worse) hearing threshold at 4000 Hz was associated with blood Pb levels (BLLs) from 1 to 18 µg/dL [58]. Hearing thresholds over 25 dB displayed a significant dose-response relationship to Pb exposures in a case-control study [59]. Both increasing BLL and working-lifetime average BLLs were associated with increasing wave I latency; a cumulative Pb exposure measure, working-lifetime integrated BLLs, accounted for increasing wave V latency and increasing wave I to III intervals [60]. Increasing Pb levels showed an increase in the time between waves I and V due to industrial Pb exposures [61]. Significant findings of Pb ototoxicity have also shown disagreement of occurring at low frequencies [62] or high frequencies [63]. While the literature on higher Pb levels resulting in heightened HL is suggestive, it is also countered by data suggesting that there may be no detrimental effect; though the evidence provided by these six studies may not be generalizable as they were done on children and adults with extremely high BLLs and Pb exposures due to Pb-tile glazing or Pb mining industries in Ecuador [64–69].

Studies on occupational exposures to Pb in combination with high noise levels have suggested an interaction between noise and Pb exposure. This type of potentiation or synergistic interaction has been noted in other ototoxic chemicals like styrene, aminoglycosides, and jet fuel [70–72]. Steel workers in Taiwan with BLLs over 7 µg/dL had higher odds of HL measured by pure-tone audiometry at frequencies of 3-8 kHz [73]. Pb-battery factory workers were analyzed for HL due

to noise exposure and Pb levels; while chronic Pb exposure and loss of hearing were correlated, noise was not and no interaction between Pb and noise was found [74]. In an Egyptian printing facility, workers were exposed to high levels of Pb, but noise exposures were under 50 dB, well below OSHA's time weighted average standard of 90 dB. When pure-tone audiometry hearing thresholds were analyzed and compared with those in a cotton factory with comparable noise levels, those exposed to Pb had significantly higher thresholds [75].

I.C: Cd and HL

DPOAEs and ABR tests on Cd-exposed rats revealed damage to the hearing pathway and cochlea after 30 days of treatment with Cd in the drinking water; the interpeak latency of waves I-III increased significantly along with a shortened latency of wave I [76]. This Cd-related ototoxicity was confirmed in another study on rats examining protective effects of zinc supplementation on Cd ototoxicity; significant shortening in wave I-III and I-V latencies were due to Cd, but zinc treatment had no significant effects [77]. Kim et al. have also used Cd as a model for ototoxicity in rat cell culture explants and in mice [78–80]. However, the does used by Kim et al. are high and may not be relevant for environmental exposures. Human data on Cd ototoxicity is lacking, but US adolescents with the highest levels of Cd in their urine were observed to have three times the rate of low-frequency HL [56]. Choi and Park's study in Korea found that blood levels of Cd were linked to heightened odds of high-frequency HL [81]. These compelling data must be further replicated and explored to confirm the doses and outcomes Cd exposures may have on the auditory system.

I.D: Hg and HL

Animal studies have produced convincing evidence of Hg toxicity. Toxicological studies of Hg in guinea pigs have shown alterations in endocochlear potential latency and heightened potentials following treatment with Hg along with decreased action potential voltage [82]. In developing mice *in utero* doses of Hg chloride and methylmercury, as well as doses administered following weaning, caused delays in ABR wave V latency at seven weeks; effects were largest when animals

received exposures both during gestation and after birth [83]. Low doses (10 mg/kg/day) of Hg in the form of cinnabar caused significant shifts (about 30 dB) in hearing thresholds after ten weeks of treatment in ICR male and female mice [84].

In humans, Hg ototoxicity has been reported, though the specifics of magnitude, frequency, or duration for adverse outcomes have not been determined by extensive research. Ototoxicity of Hg was historically shown following treatments with Hg vapors for lice and documented by Avicenna in the 11th century [85]. The Japan disaster occurring during the 1950s due to Chisso Corporation releasing Hg into Minamata Bay has also shown that Hg exposures can lead to HL [86]. Hg has also been examined in mining populations in Ecuador and Nicaragua where results have been mixed [66,87,88]. These three studies are limited by small sample sizes and cross-sectional study design as well as many confounding factors such as noise.

I.E: Metal toxicity in combination

Pb and Cd, Pb and Hg, and Pb together with Cd and Hg have been explored together as ototoxicants. Studies on other cellular effects of Pb and Cd (i.e. metallothionein expression) have shown that administration of both metals simultaneously evoke a synergistic response [89]. Potentiation of oxidative stress in dual administration of Pb and Cd has also been shown [90]. Pb with both Cd and Hg has shown synergistic toxicity on liver cell viability [91].

Combined exposures to these metals have also been explored in hearing studies. Choi et al. (2012) analyzed National Health and Nutrition Examination Survey (NHANES) data for associations between pure-tone audiometry average hearing thresholds with levels of blood Cd and BLLs; after appropriate age and social adjustments, the highest quintiles of Cd and Pb had significantly higher levels of thresholds, 13.8% and 18.6% higher, respectively [11]. Levels of Pb and Cd in US adults have been found to have additive effects on HL [11]. In adolescents from the US recruited for the National Health and Nutrition Examination Survey, levels of Pb equal to or above 2 µg/dL were found to significantly increase the odds ratios of a having high frequency pure-tone threshold averages (for 3, 4, 6, and 8 kHz) above 15 dB while the highest quartile, with a median level of 0.15 µg urinary Cd /gram creatinine, were only significantly associated with low frequency (0.5,

1, and 2 kHz) pure-tone threshold averages above 15 dB [56]. This same cross-sectional study also examined levels of Hg, which showed no significant associations to overall HL, high frequency HL, or low frequency HL in the 12-19 year-olds [56]. This study did not examine metal interactions.

I.F: Possible mechanisms of toxicity involved with metals and noise

The generation of an imbalance of reactive oxygen species (ROS) may play a key role in metals-induced ototoxicity. Cd and Pb exposure have been linked to increased levels of ROS [92–94]. Hg treatment has also been linked with raising higher lipid peroxidation levels and increased oxidative damage to cellular structures [84]. Animals with Pb exposures have been shown to have increased levels of hydrogen peroxide, a ROS in red blood cells due to Pb inhibition of γ-aminolevulinic acid dehydratase [95]. Inner hair cells from guinea pigs have shown decreased outward potassium currents when exposed to 10 mM of hydrogen peroxide [96]; thus, hydrogen peroxide generation alone may disrupt hearing. When rising ROS levels can no longer be quenched by cellular antioxidant resources, oxidative stress occurs, and continued ROS production may lead to damage. Oxidative stress is associated with HL; specifically, NIHL and ototoxicity of aminoglycoside exposure [97]. The demyelination of the cochlear nerve observed after Pb exposure [98] is thought to be caused by lipid peroxidation due to ROS [99]. The ototoxic effects of Pb, in the forms of PbAc and tetraethyl Pb were attenuated when administration of phenyl-tert-butyl-nitrone, a free-radical scavenger, preceded the Pb injection; suggesting the toxic response works at least partially through ROS [100].

I.G: Research gaps

Mouse models are well established for studying the hearing system and exploring the effects of drugs, age, sex, and noise [101,101,102]. A mouse model has been established to demonstrate HL in mice exposed to Pb *in utero* [16–18], and Cd ototoxicity has been shown using a C57BL/6 adult mouse [78]. There is, however, no literature on use of a mouse model to determine effects of Pb exposures on the adult hearing system.

Whether hearing outcomes seen following *in utero* exposures of Pb to mice [16–18] are likely to appear in humans is not currently known. Information on nutrient and metal levels might further the understanding of nutrient-toxicant interactions and effects on the hearing system; to this date there is no study to explore interactions of nutrients and metal toxicants together in the hearing system. Newborn blood spot analysis offers an efficient approach to confirm whether low levels of Pb may create similar hearing outcomes in human gestational exposures to Pb.

A number of occupational studies have suggested HL in workers exposed to both noise and metals which is greater than expected for the amount of noise exposure alone. However, none of these studies have included detailed information, such as personal noise dosimetry data, on the levels of noise to which workers were exposed. Only one recent study has investigated a link between noise exposure and metals exposures for mining occupations [14] and its findings were inconclusive. In developing nations, both noise and metals exposures are likely to be heightened due to a lack of regulations and enforcements. Studies among vulnerable populations, such as electronic waste recycling workers who have exposures to both noise and metals, are needed to evaluate the effects of these mixed exposures.

I.H: Overarching goals

Each aim of this study sought to address the same overarching questions:

    1) To what extent is Pb ototoxic?

    2) How is this ototoxicity impacted by other modifiable exposures (essential elements, noise, or additional toxic metals) from the environment?

To explore these questions, two research approaches were used: one experimental animal model, and two human epidemiological studies. A mouse model proven to develop HL in a manner similar to that of humans was used in the animal study [103,104]. The mouse model was used to evaluate whether effects occur on the mammalian auditory system due to Pb and Cd, and the extent of these effects with and without noise modification.

The two epidemiological studies in this dissertation were designed to assess associations of metal levels with HL at different age ranges and include co-variates, such as nutrient levels and noise exposures. This approach was intended to elucidate the extent of resilience or vulnerability the hearing system has at two lifecycle stages (newborn or adult) has to metals at different exposure levels. The studies were also intended to assess the interactions of other toxicant metals, nutrient metals, and noise on Pb and Cd ototoxicity.

I.I: Study aims

The three studies were designed to assess the impacts of different toxicant and nutrient mixtures on HL (Figure I-1). More specifically, all three studies explored toxic metals as chemical hazards individually and concomitantly, while two studies explored the additional physical hazard noise, and two studies investigated levels of nutritional metals in blood as protective against HL.

Project 1: Mouse Model

The objective of this study was to experimentally evaluate and quantify, in a well-understood animal model, the relationship between Pb and Cd exposure and damage to the cochlear/vestibular system. The effects of these metals were evaluated singly, in tandem, and also in combination with noise exposure.

Mouse Model, specific aim 1

The dose-response relationships between Pb, Cd, and HL in CBA/CaJ mice were evaluated. Young mice of reproductive age were treated with doses of Pb or Cd in their drinking water for a period of twelve weeks, achieving serum levels commonly found in humans in contaminated areas. Animals' auditory performance at baseline and end of the exposure period were quantified via Auditory Brainstem Response (ABR) and Distortion Product Otoacoustic Emissions (DPOAEs). Cochlear hair cell counts were conducted to enumerate morphological damage, serum levels of Pb and Cd were assessed, and bone Pb levels (in the tibia, femur, and cochlea) were compared as levels of Pb on the cochlea were previously not established. Vestibular damage was also qualitatively evaluated at these same time points through use of a rotarod test. These findings have been published elsewhere [105].

Mouse Model, hypothesis 1

Higher exposures to Pb and Cd will be associated with a greater risk of HL and vestibular damage.

Mouse Model, specific aim 2

The highest dosing treatments in Aim 1 were selected to explore mixtures of exposures with both Pb and Cd. The highest treatments for Pb and Cd were also chosen to evaluate effects of in combination with noise in separate groups of animals. Potentiation, additive, and synergistic effects were evaluated in groups of animals exposed to combinations of Pb and noise; Cd and noise; and all three agents in an experimental setting.

Mouse Model, hypothesis 2

Pb and Cd will act synergistically with noise and mice with exposure to a combination of noise and Pb and/or Cd will have greater HL than those exposed to noise alone.

Project 2: Prenatal Human Exposures

The objective of this study was to evaluate the relationship between newborn blood levels of non-essential metals and newborn hearing outcomes. Hearing test outcomes for a sample of abnormal hearing cases and normal hearing controls matched by birth year-, sex-, race/ethnicity-, and birth city and hospital were obtained from the Michigan Department of Community Health (MDCH). Matched blood spot samples for these newborns were also obtained from the MDCH.

Prenatal Human Exposures, specific aim 1

Blood spot samples were analyzed for two non-essential metals (Pb and Hg). Next, the relationship between blood levels of these metals and hearing screening outcomes was evaluated controlling for age, sex, and birth year, and also accounting for multiple non-essential metals exposures (e.g., toxicant-toxicant interactions).

Prenatal Human Exposures, hypothesis 1

Infants with higher blood levels of non-essential metals Pb and Hg, separately at birth will have greater odds of an abnormal hearing outcome at birth. An interaction between Pb and Hg will cause subject with heightened levels of both Pb and Hg to have synergistically higher odds of hearing screening failure.

Prenatal Human Exposures, specific aim 2

To evaluate the modifying effects by nutritional status, indicators of the observed relationship between newborn blood levels of essential metals were analyzed within infant dried blood spots. Since nutritional status can affect the uptake of metals, potential modifying effects by blood levels of nutritionally vital essential elements were explored in our analyses of the relationship between hearing and non-essential metals. This nutrient-toxicant interaction analysis examined the blood samples for the following essential metals: calcium, copper, potassium, selenium, iron, and zinc.

> Prenatal Human Exposures, hypothesis 2
>
> Newborn babies with poor nutritional status (low levels of essential metals) will be more susceptible to nonessential metal exposure-related abnormal hearing outcomes at birth.

Project 3: Occupational Human Exposures

The levels of both nutritional and non-essential metals together taken at the same time in vulnerable workers are not clearly established; the extent to which these levels may influence actions of ototoxic metals and noise on audiometric thresholds is also unknown. This study evaluated these factors among informal e-waste recycling workers at the Agbogbloshie dump in Accra, Ghana. Noise levels may be higher in developing nations [4], however to date, no studies have investigated the occupational exposures to noise related to electronic waste processing, recycling, and disposal. After levels of exposures were established, hearing abilities of workers with relatively heightened exposures to toxicant metals and heightened levels of noise exposure were compared to hearing abilities of workers with lower levels of metals and lower levels of noise to determine possible interactions of physical and chemical hazards.

Occupational Human Exposures, specific aim 1

This study investigated the extent to which various levels of non-essential metal exposures (Pb, Hg, Cd, manganese and arsenic) and noise were associated with hearing damage e-waste workers.

> Occupational Human Exposures, hypothesis 1
>
> Participants with higher thresholds will have higher levels of BLL on average. Higher levels of toxicant metals in blood will be associated with greater auditory thresholds. Each toxicant metal will be a significant predictor of increased thresholds within the high frequency of human hearing (4, 6, and 8 kHz).

Occupational Human Exposures, specific aim 2

This aim investigated the extent to which detrimental effects on hearing due to cumulative or combinations of toxicant metal (Pb, Hg, Cd, Mn and As) exposures were additive, synergistic, or resulted in potentiation. Nutritional intakes in Ghana are exceptionally different than in the United States [106], so we assessed essential metal (copper, zinc, selenium and iron) levels in the blood of workers and their corresponding associations with levels of non-essential metals, along with possible impacts on HL. This aim also addressed whether associations of metal exposures and HL were modified by nutritional factors (blood levels of calcium, iron, copper, and zinc) and noise exposures in Ghanaian waste site workers.

Occupational Human Exposures, hypothesis 2

Participants with both higher noise and higher metals exposures will have more HL than those with higher noise exposures but lower metals levels; a synergistic relationship between metal and noise ototoxicity is expected to be observed.

Occupational Human Exposures, hypothesis 3

Higher levels of essential metals calcium, iron, copper, and zinc will be associated with better threshold levels and abate detrimental effects due to metals and noise exposures in combination.

I.J: Dissertation overview

This dissertation is organized into five chapters. Chapter II provides details of the experimental Mouse Model study. Chapter III describes features of the Prenatal Human Exposures study, combining blood and hearing screening data from Michigander newborns. Chapter IV describes the Occupational Human Exposures study which explored data on Ghanaian electronic waste workers from surveys, biological exposure measures and real-time dosimetry. Conclusions integrating and combining the results of these three novel studies are provided in Chapter V. Chapter V also addresses the combined strengths and limitations for this set of analyses, along with recommendations for future directions and possibilities for improvements to the field of mixtures research in toxicology and epidemiology.

I.K: Figures

Figure I-1. Venn diagram showing hazardous factors and protective factors investigated by this study as predictors of HL
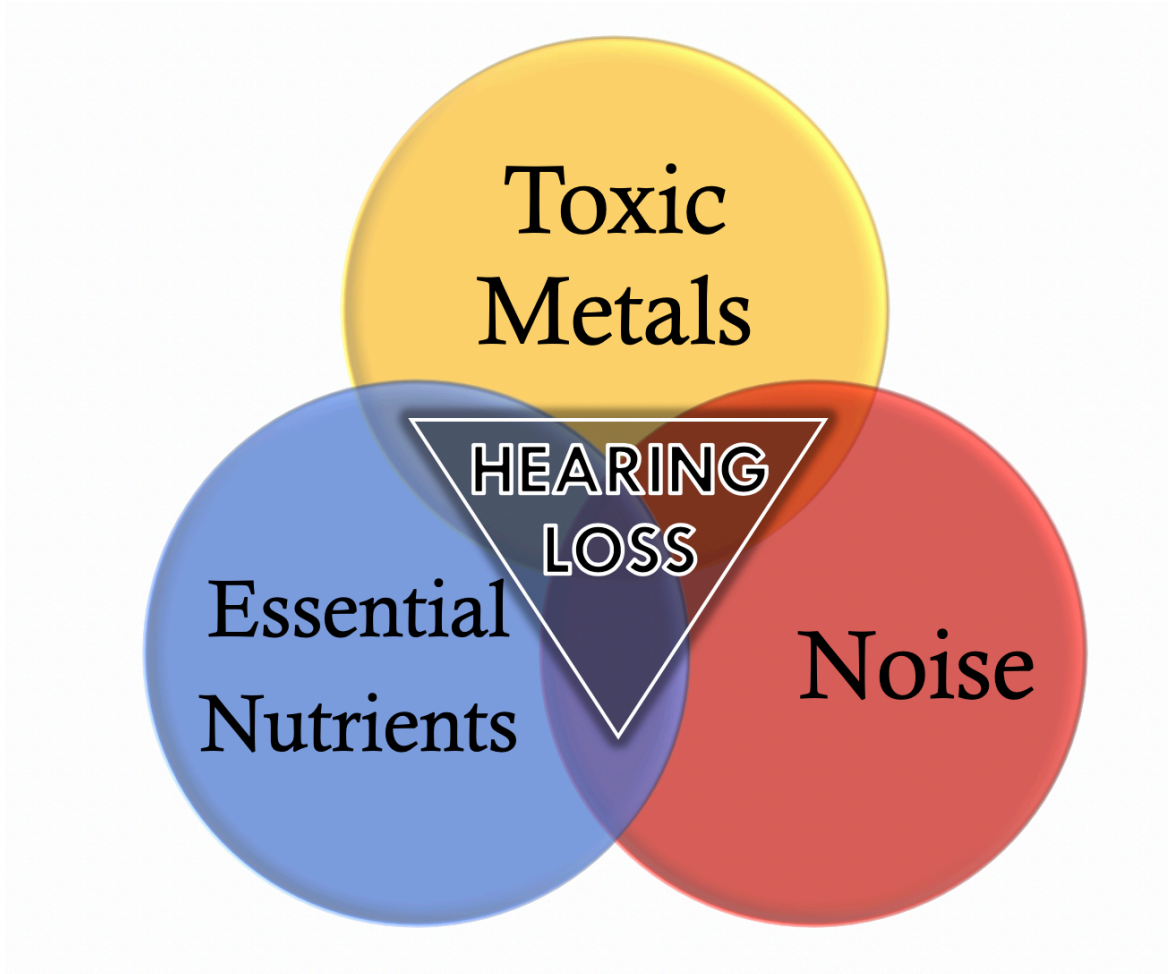
CHAPTER II: Assessing ototoxicity due to chronic lead and cadmium intake with and without noise exposure in the mature mouse

Note: This chapter has already been published in a peer-reviewed journal [107].

II.A: Abstract

Exposure to metals may lead to hearing impairment. However, experimental studies have not explored this issue with and without noise exposure in mature animals with environmentally relevant doses. The aim of this study was to investigate ototoxicity produced by lead (Pb) and cadmium (Cd) and noise, singly and in combination, in the adult CBA/CaJ mouse. Metals were delivered via drinking water (0.03 mM, 1 mM, and 3 mM Pb; or 30, 100, and 300 μM Cd) for 12 weeks, resulting in environmentally- and occupationally-relevant mean (± standard deviations) blood levels of Pb (2.89 ± 0.44, 38.5 ± 4.9, and 60.1 ± 6.6 μg/dL, respectively) and Cd (1.3 ± 0.23, 6.37 ± 0.87, 27.2 ± 4.1 μg/L, respectively). Metal treatment was also combined with a noise exposure consisting of a 105 dB broadband (2-20 kHz) stimulus for 2 hours or a sham exposure. Auditory performance was determined by comparing auditory brainstem responses (ABR) and otoacoustic emissions (DPOAE) at baseline and after 11 weeks of metal treatment. Metal-exposed animals did not develop significant auditory deficits and did not exhibit morphological damage to cochlear hair cells. In contrast, noise-exposed animals, including those exposed to combinations of metals and noise, demonstrated significant hair cell loss, reduced DPOAE amplitudes, and ABR threshold shifts of 42.2 ± 13 dB at 32 kHz (105 dB noise alone). No significant potentiation or synergistic effects were found in groups exposed to multiple agents. This study established a highly reproducible adult mouse model that may be used to evaluate a variety of environmental exposure mixtures.

II.B: Introduction

Worldwide, over 466 million people are estimated to suffer from hearing loss (HL) [108], and non-age-related HL ranks as the fifth cause of years lived with disability globally [3]. HL in almost any

form exerts detrimental impact at all stages of life. Even mild losses in children are associated with poorer speech perception and significantly lowered performance on basic skills tests [6,109]. In adults, HL may impair a wide variety of adverse social, psychological, educational, clinical and occupational outcomes [110].

Exposures to high levels of noise produce HL [111], and other environmental agents may also cause or contribute to HL [112,113]. Auditory toxicity (ototoxicity) resulting from exposures to certain clinically relevant drugs [114] is well established. Research from the past and present identified another potential source of HL: exposure to non-essential metals, including lead (Pb) and cadmium (Cd) [115,116], which are common environmental and occupational contaminants in industrialized communities [117,118]. Pb is present in paint in US homes built before 1977 and exposure may occur through the water supply in older homes, soil and household dust [119], children's toys [120], and even through some food products [121]. Cd exposure may occur through contact with contaminated soils and dusts, tobacco smoke [122], and through dietary intake [123].

Contradictory information exists on the consequences of prolonged low-level metal exposures in adults. Several reports suggested that Pb may be ototoxic in human adults [13,124] but not all studies agree [125]. The potential ototoxicity of Cd in human adults has been addressed in one study [11]. Epidemiological evidence also suggests that HL associated with Pb and Cd exposures in humans might be synergistically or additively affected by concurrent noise exposure [74,126].

Pb has also been implicated as a potential ototoxicant in developing animals, including mice [127–129] and monkeys [130] and acute Pb exposures may generate auditory dysfunction in guinea pigs [100]. Similarly, Cd in drinking water at 5, 15, or 150 ppm produced ototoxic effects in 30-day exposures in two rat and one mouse study [76–78]. However, as with Pb, not all studies of Cd demonstrated ototoxicity [131]. These discrepancies largely arise from the lack of an established animal model to guide a consistent approach to potential ototoxic exposures by metals.

While detrimental neurological effects of metals have been established during development in both children [132] and animals [16], though this is controversial as some studies show no consistent effects [64,133]. Adult animal models using higher occupational exposures to Pb have been exploited to a lesser extent. Therefore, adult animal models are necessary to address ambiguities in the epidemiological findings and explore potential causal connections between exposure to Pb

and/or Cd and HL. In order to close these knowledge gaps, the objective of this study was to evaluate and quantify, in a well-controlled animal model, the relationship between Pb and Cd exposure and ototoxicity as measured by auditory threshold shifts and cochlear hair cell loss. Based upon environmental and occupational exposure parameters, the effects of the metals were evaluated singly, in tandem, and also in combination with noise.

II.C: Methods

Animals

The University of Michigan's University Committee on the Use and Care of Animals approved all animal protocols for this work. Routine care for animals was provided by the University of Michigan's Unit for Laboratory Animal Medicine (ULAM). Mice were housed in a containment facility with unlimited access to both food and treatment water. Treated water bottles were changed twice a week. The facility maintained a 12-hr light-dark cycle. In-cage 72-hr noise exposure measurements were taken monthly using a personal noise dosimeter (Spark 706RC, Larson Davis, Depew, NY). Average ambient sound pressure levels (SPL) were consistently at or below 60 dBA, a level sufficient to eliminate the risk of noise-induced HL in mice [134,135].

CBA/CaJ mice arrived from the Jackson Laboratory (Bar Harbor, ME) at 4 weeks of age weighing 23 g and were allowed one week for acclimation. All mice were housed 5 to a cage if possible. Baseline auditory brainstem response (ABR) measurements at age 5 weeks determined hearing thresholds before chemical treatments began at 6 weeks of age (Figure II-1). A treatment schedule beginning no earlier than age 5 weeks was selected to coincide with mouse reproductive maturity and comparable to the age of young workers in the US. Treatments continued for 11 weeks. Sacrifice by anesthesia and decapitation followed by blood, bone, liver, kidney, and cochlear tissue harvest occurred at 17 weeks of age. Noise exposures were administered during the sixth week of treatment (11 weeks of age). Final hearing thresholds were measured during week 11 of treatment (16 weeks of age).

All animals were visually inspected daily and weighed weekly to verify consistent growth. No cases of overt toxicity from Pb or Cd were observed. Animals were sacrificed after final hearing

assessments for pathological analysis, cochlear assessment, and tissue Pb and Cd measurements. One control mouse died during our experiment due to acute obstructive uropathy unrelated to the experimental protocols. All other mice completed the full experimental treatment.

Treatments

Mouse treatment groups and total group size throughout this study are shown in Table II-1. This study was run in three stages. The first stage used various levels of Pb, Cd, and noise treatments to determine the level of treatment that caused damage. During the second stage, doses of 3 mM Pb, 300 μM Cd, and 105 dB noise were selected to investigate effects of these treatments under concurrent dosing regimens. The third stage exclusively investigated the DPOAE outcome. Controls were included in each period thus larger numbers of control animals are reflected in Table II-1. Each treatment group included at least 6 mice to allow for health outcomes to show through individual variability. Only male mice were used due to documented estrus-related hearing fluctuations in female mice [136].

Pb exposure

An aqueous 2% w/v lead acetate solution (Fisher Scientific, Waltham, MA; #429132) was diluted into highly purified and filtered water (Merck Millipore, Billerica, MA Milli-Q System). Final concentrations of Pb in drinking water were 0.03 mM, 1 mM, and 3 mM; concentrations were selected to obtain serum levels representing those seen in US workers from average environmental, high environmental and legal limits of occupational exposures, respectively.

Cd exposure

Cd in the aqueous form of $CdCl_2$ (VWR, Radnor, PA; # 101443-260) was diluted into Milli-Q water to final concentrations of 30 μM, 100 μM, or 300 μM, again selected to achieve serum levels to represent US exposures ranging from environmental to occupational.

Combined exposure to Pb and Cd consisted of treated water containing the highest doses of Pb (3 mM) and Cd (300 μM). Random samples from each concentration were taken from all water bottles at monthly intervals to verify consistency in concentrations over the study.

Noise exposure

A single two-hour noise exposure was administered in week 5 of the chemical treatment period. Using a method previously described [137], mice were housed in individual wire cages in a ventilated chamber with a loudspeaker mounted at the top of the chamber. Two or three awake animals were simultaneously exposed to 2 hours of broadband white noise (2 to 20 kHz) at intensities of 102, 105, or 108 dB SPL. Noise levels were confirmed within the wire cage with sound level meters before and during exposure (B&K sound level meter model 2231, with type 4155 1/2" microphone).

Pathophysiology

Auditory brainstem response (ABR)

For ABR testing, animals were anesthetized with intraperitoneal injections of ketamine (65 mg/kg), xylazine (7 mg/kg), and acepromazine (2 mg/kg) to insure relaxation and immobilization as described in previous literature [138]. Additional injections of ketamine and xylazine were administered as necessary to maintain anesthesia for the duration of the examination; an injection of glycopyrrolate (0.2 mg/kg) was administered to aid recovery. The ear canals and tympanic membranes of all animals were evaluated for signs of obstruction or infection prior to hearing assessments. No obstructions or infections were observed in any of the mice.

Needle electrodes were inserted subdermally at the vertex of each mouse's skull equidistant to each external auditory meatus, a reference electrode was inserted below the pinna of the left ear, and a ground electrode was inserted contralaterally [138,139]. Sound stimuli were carried in a closed acoustic system to the left external auditory meatus and then transmitted through an ear bar connected to a Beyer DT-48 transducer (Beyer Dynamic, Farmingdale, NY, USA). The test output was transmitted to an amplifier, viewed via oscilloscope, and recorded using SigGen software (Tucker-Davis Technologies, Gainsville, FL USA). Thresholds were determined at low, mid-range, and high frequencies (8, 16, and 32 kHz, respectively) by progressive reductions in sound intensity by 10 dB SPL steps initially, and 5 dB SPL steps near threshold. Thresholds were defined as the lowest stimulus at which a positive waveform was seen [140] and threshold shifts were calculated for individual animals as the difference between measurement at baseline and the

21

conclusion of the experiment (chemical treatment week 11). Pilot work included preliminary analyses for groups suspected to show the largest changes of final ABR peak I through V amplitude and latency data as well as inter-peak latencies (I-II, II-III, III-IV, IV-V, and I-V) were calculated using MATLAB (Mathworks, Natick, MA) at 80 dB for frequencies of 8, 16, and 32 kHz for control mice, and those in the highest Pb and Cd treatment groups. Due to inconsistencies in interpreting ABR waveforms of noise-exposed animals, we excluded any animal with noise treatment from these analyses.

Distortion product otoacoustic emissions (DPOAEs)

DPAOEs were collected at the same time points as ABR (i.e., baseline and eleventh week of treatment). DPOAE tests were run as described previously [140,141] at 32 kHz following administration of anesthetics as described above for ABR. The ratio of the intensity of the primary tones f1 and f2, remained constant at f2/f1 = 1.2. F1 intensity was adjusted in 5–10 dB SPL increments, while f2 intensity was held 10 dB SPL below f1. Tucker-Davis Technologies System II (Gainsville, FL) hardware and SigGen/BioSig software captured responses and presented stimuli tones. Amplitude shifts were calculated by subtracting the final amplitude from the initial.

General pathology

Immediately following euthanasia via deep anesthesia with ketamine (100 mg/kg) all animals were exsanguinated via cardiac puncture to collect up to 1 ml blood. Upon exsanguination, 0.5 ml blood was placed in a trace metals analysis tube (Becton Dickinson, Franklin Lakes, NJ #368381). Any remaining blood was placed in to a serum separator tube, spun for two min and frozen in preparation for blood chemistry analysis. Blood serum was analyzed for markers of kidney and liver function: creatinine, aspartate amino transferase (AST), albumin, alanine amino transferase (ALT), total bilirubin, blood urea nitrogen (BUN), and alkaline phosphatase (ALP). Mice were necropsied and gross visual inspection was made for systemic damage. Liver and kidneys were removed during necropsy on all mice and placed into formalin fixative for at least 24 hours before they were trimmed and stained with eosin and hemotoxylin. Right femur and right tibia bones were collected, scraped of extraneous tissues with a ceramic blade, weighed and stored under

refrigeration. Cochleae were collected and placed on ice in phosphate buffered saline (PBS) for dissection.

Tissue analysis

A University of Michigan Unit for Laboratory Animal Medicine (ULAM) veterinary pathologist blinded to treatment status evaluated liver and kidney slides for organ-specific lesions. ULAM also completed blood chemistry panel analyses. Blood samples were analyzed for Cd and Pb levels using inductively coupled plasma mass spectrometry (ICP-MS) at the Michigan Department of Health (Lansing, MI). Cochlear bone Pb and Cd levels were determined from the entire cochlear-vestibular apparatus along with a portion of the temporal bone. Right and left cochlea were weighed together and digested in OPTIMA grade nitric acid. Tibia and femur bones were similarly digested Pb and Cd content was determined using ICP-MS by the Michigan Department of Health. Detection limits were 0.05 mg/kg Pb and 0.05 mg/kg Cd for the bone and cochlear metal analyses.

Hair cell counts

Surface preparations of cochleae were examined to determine numbers of all cochlear inner and outer hair cells. Both left and right auditory systems were examined using a light microscope for abnormalities and signs of infection. The round window, oval window, and apex were opened to perfuse the entire cochlea with 4% paraformaldehyde in 10 mM phosphate-buffered saline (PBS) at pH 7.4. Cochleae were then decalcified with 4% EDTA in 10 mM PBS, stained with rhodamine phalloidin to label actin and hair cells were counted along the entire length of the cochlea [139,142]. Average outer hair cell losses were tallied in the apex, middle section, and base.

Statistics

Data were compiled, and descriptive statistics computed, in Excel (Microsoft, Redmond WA). Further analyses were run using R 3.3.1 (Vienna, Austria) and figures were generated in GraphPad Prism 6 (San Diego, CA). Counts of mice with ABR threshold shifts >20 dB, a level that has been used as a threshold for human hearing impairment [143], were also computed. Means across exposure groups were compared using Student's t-test and one-way ANOVA using a Bonferroni

correction for multiple comparisons; distributional comparisons were conducted via χ^2. Results were considered significant at α = 0.05. Blood serum above or below established normal values [144] were counted were pooled from similar treatment groups using Pearson's Chi-squared test with Yates' continuity.

II.D: Results

Animals

Pilot work in CBA/J male mice revealed a number of ear infections not identifiable during visual inspection before performing ABR and observed after cochlear dissections. The CBA/CaJ strain of mouse was therefore selected for this investigation due to their low incidence of ear infections compared to other mouse strains [145] and stable hearing thresholds into advanced age (12-18 months) [135,138]. Our study noted only one mouse with an ear infection on the right (non-ABR) side out of a total 150 mice undergoing ABR and cochlear dissection. Body weight measures were maintained throughout the study and no overt changes of health were noted. As reported in pathology results, hepatotoxicity and systemic toxicity were not found at this dose.

Treatments

All control water samples tested below the limit of detection (0.2 mg/L) for both Pb and Cd. All Pb treatment water tested below limits of detection (LOD) for Cd and all Cd water tested below LOD for Pb. Water samples showed that mean treatment concentrations of Pb and Cd were within 25% error below the intended concentrations, and no individual samples tested above the intended concentrations of Pb or Cd. Mean water sample concentrations (all results reported to two significant figures) of Pb were 0.023, 0.91, and 2.7 mM (4.8, 190, and 550 mg/L) for the 0.03, 1, and 3 mM treatment groups, respectively. Mean Cd water sample concentrations were 27, 93, and 240 μM (3.0, 11, and 27 mg/L) for the 30, 100, and 300 μM treatment groups, respectively. Mean water sample concentrations for the combined Pb and Cd treatment groups were 2.4 mM (510 mg/L) and 280 μM (31 mg/L) for Pb and Cd, respectively.

Noise

Pilot study results indicated that noise exposures of 102 dB SPL produced no hearing damage, while 105 dB or 108 dB SPL exposures averaged similar threshold shifts (approximately 35-40 dB). Therefore, the 105 dB SPL exposure, which produced a robust threshold shift without a full elimination of auditory responses, was selected for evaluation of exposures in combination with metals in order to optimize our ability to assess possible potentiation effects of Pb and Cd on hearing thresholds.

Pathophysiology

ABR baseline levels

Baseline thresholds decreased with increasing test frequency, as is typical for CBA/CaJ mice. Variability in baseline thresholds was low for the 147 mice that underwent ABR. The highest threshold mean ($\pm$ SD) of 26 ± 3 dB was observed at 8 kHz, compared to 17 ± 3 dB at 16 kHz and 17 ± 3 dB at 32 kHz.

ABR threshold shifts

No significant changes from baseline thresholds were observed in the control animals at 8 and 32 kHz (Table II-2 also shown in Figures II-2, II-3, and II-4). Threshold shift variability in the metal treatment groups was similar at all frequencies to that of the control group. No Pb or Cd treatment groups (including single and combination metal exposures) displayed threshold shifts at any frequency that differed significantly from control, though changes from baseline thresholds among the Pb and Cd single treatment groups were higher on average at 8 and 32 kHz than those of controls. Only 1 of 47 mice (2.1%) in a combination of the control, all Cd single treatment, and all Pb single treatment groups experienced a 20-dB or greater threshold shift at 32 kHz, and only 2 of 42 (4.7%) experienced such a shift at 8 kHz.

Noise-exposed mice showed threshold shifts much larger and more consistent than those from Cd, Pb, or Cd+Pb treatments, and which differed significantly from control (Table II-2; results for controls, 105 dB noise exposures, and highest Pb and Cd treatments singly or in combination are shown in Figures II-2, II-3, and II-4). Mice exposed to 102 dB only exhibited alterations from

baseline thresholds 10-11 dB greater than controls at 8 and 16 kHz; however, these differences were not significant. Threshold shifts in the noise-only treatment groups of 105 dB and 108 dB were not significantly different at any frequency. Average threshold shifts were significantly different from controls at 8, 16, and 32 kHz in mice treated with 105 dB noise alone or 105 dB noise in combination with Pb, Cd, or Pb and Cd (data not shown). Threshold shifts in the 105 dB noise plus Pb and/or Cd exposure groups did not differ significantly from the 105 dB noise-only treatment group.

The Cd+Pb+Noise group unexpectedly showed significantly lower changes from baseline thresholds than the Cd+Noise treatment at 16 kHz. Changes remained lower, though not significant at 8 kHz and 32 kHz; differences ranged from 7.3 dB at 8 kHz to 19.8 dB at 16 kHz. The fractions of animals experiencing substantial threshold shifts (greater or equal to 20 dB) were similar among mice treated with mixtures of noise, Pb, and Cd, compared to mice exposed singly to 105 dB noise.

ABR peak and latency
ABR waves I, II, III, IV, and V amplitudes and latencies were analyzed as well as inter-peak I-II, I-III, I-IV, and I-V. Preliminary findings did not show significance of any wave peaks or latencies in the highest treatment groups compared with controls, so we did not pursue further deeper analysis. No significant differences from the control group were found among mice treated with 3 mM Pb or 300 μM Cd.

DPOAE thresholds
DPOAE amplitude shifts at 32 kHz among the Pb, Cd, 105 dB, Pb+Noise, and Cd+Noise groups were similar to those seen in ABR at 32 kHz (Table II-3). DPOAE shifts among Pb and Cd treatment groups were not significantly different from zero, and mean differences of amplitude shifts among noise-exposed mice were not significantly different. Mice exposed to noise displayed significantly higher threshold shifts than mice treated with Cd and Pb. One mouse in the noise-only exposure group did not experience a DPOAE threshold shift; this created a larger SD in this group compared to other treatment groups. DPOAE amplitude shift averages for Pb+Noise [28 ± 9.5 dB SPL] were similar to Cd+Noise [26 ± 11.7 dB SPL] and not significantly different.

Tissue assessment

Blood levels of metals

Pb blood levels at sacrifice were not altered by addition of noise or Cd treatment. No mice from control or Cd treatment groups had detectable levels of Pb while all mice treated with Pb had detectable levels of Pb in their blood. Blood Pb levels were significantly different among the three Pb-only treatment groups (Table II-4). The mean blood Pb levels across the combination exposure groups (using 3 mM Pb) were not significantly different from each other or the single treatment of 3 mM Pb. All mice treated with Cd had detectable levels of blood Cd. Three mice not in a Cd treatment group (one control, two exposed to Pb only) had a detectable level of blood Cd. Blood Cd levels differed significantly among the exposure groups (Table II-4 and Figure II-5).

In combination treatment, Pb levels were unchanged in mice concurrently exposed to Cd (Figure II-6). In contrast, mean levels of blood Cd in mice were altered by addition of Pb treatment, though not among noise-exposed animals (Figure II-5). Blood Cd levels for the 300 μM treatment were significantly decreased when combined with Pb exposures. Both the Cd+Pb groups and the Cd+Pb+Noise group exhibited significantly lower blood Cd levels than Cd treatments alone.

Bones and cochleae

All mice had detectable levels of Pb in their tibia and femur, though levels were 1,000-fold higher among the Pb treatment groups than non-Pb-groups. Only mice treated with Cd displayed detectable levels of Cd (Table II-5). Among Pb treatment groups, Pb bone levels in the femur were higher than those in the tibia. Mean femur Pb was significantly higher in the Pb-only group than the Cd+Pb group; however, this was not the case for tibia Pb. Bone Cd levels were not significantly different between Cd treatment groups.

As with bones, detectable levels of Pb were found in all cochlear tissue and bone samples, while detectable levels of Cd were observed only in the cochlear tissue and bone of Cd treatment groups (Table II-6). Levels of cochlear Pb were not significantly different among non-Pb-treatment groups, and levels of cochlear Cd were not significantly different among non-Cd-treatment groups.

Cochlear assessments

Significant cochlear hair cell losses were only seen in noise-exposed groups (Table II-7). Numerous outer hair cells were missing in the basal area of the cochlea among all noise exposure groups, while no outer hair cells were missing in the apex and mid ranges of all treatment groups. There was a dose-dependent rise in mean loss of basal hair cells with increasing noise exposure; mean levels were 3 ± 2, 6 ± 4, and 14 ± 17 hair cells missing for the 102, 105, and 108 dB exposure groups, respectively. Among combination noise and metal treatment groups, mean basal hair cell loss was highest in the Cd+Noise group [12 ± 10] and lowest in the Pb+Noise group [3 ± 2]; the Cd+Pb+Noise group fell between these [7 ± 5]. Inner hair cells were noted to be intact under all treatment conditions.

General pathology

Histology

Only mice treated with Pb showed signs of kidney distress, but even the levels noted histologically were not indicative of major systemic health problems. Karyomegaly in the S3 tubular epithelium, which is known to be a characteristic lesion of Pb exposure in mice, was present in 91% of mice treated with 3 mM Pb alone, 86% of mice in the Cd+Pb+Noise group, and in 100% of mice in the Pb+Noise and Cd+Pb groups. Intranuclear inclusions were also present in the highest Pb treatment groups with similar proportions to karyomegaly. One mouse in the 108 dB Noise treatment group displayed both these lesions; that was the only mouse without Pb treatment found to exhibit these lesions. Karyomegaly was absent in the 0.03 mM Pb group and rare (13% of mice) in the 1 mM Pb group. No other lesions were characteristic of any treatment group. Mild (affecting under 10% of tubules in the tissue) tubular hyperplasia occurred in over 50% of animals in all but one treatment group and lesions observed displayed no regeneration or fibrosis. Mild hepatic lesions also presented in a wide range of treatment groups at low levels. Two control animals presented with both centrilobular or random degeneration and necrosis multifocal neutrophilic with mononuclear inflammation. All other groups had 0 to 2 mice with similar lesions; no dose-response patterns were noted. Histology data is shown in Table II-8.

Blood serum results

Serum ALP levels at sacrifice tested at biologically relevant low levels in Cd treated mice at higher treatments (4 of 9, or 44 %, in the 300 μM Cd group, 3 of 9, or 33 %, in the Cd+Noise group, and 1 of 12, or 8.3 %, among controls) though these levels were not significantly different from controls.

Most mice showed total bilirubin counts below normal values of 0.12 to 0.58 mg/dL. At sacrifice, blood serum levels of creatinine, AST, ALT, and BUN were not always within the normal reference ranges but were not significantly different between treatment groups and did not show dose-response changes with increasing treatment levels.

II.E: Discussion

Previous studies exploring the ototoxic interaction of chemicals with noise have used rats [146]. An alternative animal model such as the mouse is increasingly important in modern research making up about 90% of species used today in animal studies and was thus selected. Molecular tools as well as transgenic strains render this model more versatile than any other species for research. In addition, exploration of interactions between genetic and environmental factors in mice offers a much more flexible and yet highly controlled experimental approach than does epidemiological modelling [147], and the relevance of this research can be increased even further through the use of transgenic mice mimicking human disorders [148].

In the CBA/CaJ adult mouse model presented here blood levels of Pb and Cd relevant to occupational situations in the US were achieved and simulated environmental exposures that occur in certain areas internationally. Although levels of Pb in the general environment decline in the US, higher levels may still be experienced in certain occupations and remain important to public health. Further, recent research suggests that exposure to noise (both occupational and recreational) may have more severe consequences than previously assumed [109]. Even low-level exposure that only initiates temporary threshold shifts in young but mature hearing systems may produce earlier and greater age-related HLs [149]. Globally, adults in low- and middle-income nations have high levels of noise exposures as well as high Pb and Cd exposures in both occupational and community settings [4,150].

With regards to occupational exposures to Pb, the current OSHA Permissible Exposure Limit for Pb exposures among workers is 50 μg/dl; in our study, the exposures achieved in the highest Pb-exposed group was equivalent to 60 μg/dl. The levels of Cd in our highest-exposed mice (27 μg/L) were double the OSHA occupational limit (10 μg/L), and Cd exposures in combined treatment of Pb and Cd still resulted in levels above the same Cd OSHA limit. When environmental exposures are considered, levels in treated mice were above the updated CDC Community Action Limit for Pb (5 μg/dl) [151]. Even exposures below this CDC limit are coming under increasing scrutiny as research continues to demonstrate the importance of cumulative effects [152,153].

Our data appears to be the first study to test for Pb and Cd levels in the mouse cochlea. It was found that levels were detectable and similar to bone levels in the tibia and femur, though these were more similar for Cd than Pb. Mean Pb levels in the cochlea were 36% less than in the femur and 17% less than Pb in tibia. Detectable levels of Pb in non-Pb-treated mice were identified. This Pb exposure may originate from their feed, which contained detectable levels of Pb in our tests. Cd concentrations in bone and cochlea were not detectable in mice that were not treated with Cd. Most animals not exposed to Pb or Cd did not show detectable levels in their blood. However, three mice exposed to the control, or Pb alone showed detectable levels of Cd in their blood. All bone samples, regardless of Pb treatment status or not, showed detectable levels of Pb. Contamination in animal food has been previously shown as is a likely contributor to the few mice which showed detectable levels of Cd and an ongoing exposure to low levels of Pb [154,155]. For mice treated with a combination of Pb and Cd lowered concentrations of both Pb and Cd were observed in the femur and tibia; this is likely due to competing metal uptake. Cochleae from these mice were not tested for metals.

Mixture-based research such as this is increasingly recognized as a critical area for epidemiological research. Therefore, our study included a thorough analysis of the ototoxicity of Pb and Cd in combination with noise. In agreement with previous investigations [156,157], noise exposures produced significant detriments to thresholds in nearly all mice, validating the model. In contrast, our study did not identify any significant differences in HL risk resulting from metal exposure, thus exposing a potential difference between developing and mature animals. It is also possible that Pb or Cd, are more ototoxic in humans, or that longer-term exposures are required in order to produce hearing deficits in older animals.

It is noteworthy that our model explored cochlear histology but did not investigate peripheral or central neurological damage that may disrupt auditory processing. Fortune and Lurie (2009) noted alterations to the superior olivary complex and normal monoaminergic expression albeit in developing mice [17]. These are exciting areas for future studies to examine as Pb treatment may well distort auditory temporal processes. Further, subthreshold damage at a young age might manifest as increased HL as the animals age [158]. A long-term follow-up would be an important contribution to our understanding of metal toxicity.

The results of our study appear to disagree with two previously published studies that showed ototoxicity due to Pb exposure. The first used 6 monkeys, treated from birth to age 13, and pure tone thresholds to establish ototoxicity [130]. Results of this study demonstrated normal thresholds in three monkeys and lowered thresholds at high frequencies in three monkeys [130]. The second study investigated HL in Wistar rats exposed to 4 mg/kg Pb acetate by gavage for 30 days [159]. HL was demonstrated as an increased latency in the ABR peaks from I to V [159]. This was not found in analysis of ABR peaks within our study. Assuming the daily amount of water consumed by one 30 g mouse is about 4 ml [160], the Pb dose mice in our study received was about 82.67 mg/kg, a higher level than given to the rats above. However, differences in absorption due to developmental exposure times, species differences, digestion times, and stress levels due to mode of administration may also alter this comparison.

In contrast to Pb, Cd is not as well-studied. Kim et al. (2008) demonstrated ototoxic properties attributed to Cd. *In vitro*, over half of hair cells were apoptotic, detected through the use of TUNEL staining, following 24 hours direct exposure of organ of Corti explants from rats to Cd (10 μM) [161]. This study further explored the ABR threshold shifts in mice following 150 mg/L (1334 μM) Cd in drinking water for 30 days. Thresholds of control mice at 32 kHz were near 30 dB and averaged near 55 dB following 30 days of Cd treatment. A likely reason for the discrepancy to our study is the dosing by Kim et al. (2008) which is over 4-fold higher than the levels administered in our study and should have resulted in comparatively higher serum levels [161]. Increases of ALP in mouse blood due to Cd treatment noted in our highest Cd treatment group were also observed previously [162].

Conclusions

This comprehensive study design with both positive (noise) and negative (water) controls confirms the findings of many previous studies regarding the severity and consistency of noise-related damage to auditory function [163,164]. For combination treatment, a similar strategy to studies on the interactions of styrene and noise was used [146,165]. To the best of our knowledge, our study is the first of its kind to use mice and had the longest treatment period of experiments of this type identified in a literature search. Therefore, this investigation offers a novel model for examining interactions within the auditory and vestibular [105] systems.

It is significant that a CBA/CaJ adult mouse model was developed with blood levels of Pb and Cd relevant to human health in real-world mixture exposure settings. Interestingly, these mice did not demonstrate significant ototoxic effects of Pb or Cd singly or in combination. It is also important that throughout the chronic exposure all animals maintained good general health and only showed subtle pathological changes in renal parameters parallel to those seen in rodents [166] and comparable to observed renal outcomes in humans [167]. The use of this robust adult model might advance toxicology knowledge and research methods on mixed exposures.

Future studies investigating ototoxicity of metals and possible interactions of chemical agents with noise need to further explore the complex pathways of signals and cell bodies in auditory neurons and ascending pathways for a more complete understanding of health outcomes due to ototoxicant interactions. Metals are known neurotoxicants [168,169]; it is not unreasonable that changes in auditory perception may be due to auditory synaptopathy, which may be aggravated by noise exposures [170], or central effects. One example of a chemical producing adverse effects in the brain stem following noise exposure is 1,3-dinitrobenzene [171]. Finally, it is possible that the CBA/CaJ mouse may not be sufficiently vulnerable to demonstrate effects potentially seen in more sensitive human populations. Species and strain differences are important and well documented variables in research and deserve further exploration.

II.F: Tables

Table II-1. Description of treatment groups

Group	N
Total	150
Control (no Pb, no Cd, sham noise)	16
Pb (no Cd, sham noise)	
3 mM	16
1mM	8
0.03 mM	8
Cd (no Pb, sham noise)	
300 μM	16
100 μM	7
30 μM	6
Noise (no Pb, no Cd)	
108 dB	7
105 dB	15
102 dB	7
300 μM Cd + 3 mM Pb (sham noise)	9
3 mM Pb + 105 dB Noise (no Cd)	14
300 μM Cd + 105 dB Noise (no Pb)	14
300 μM Cd + 3 mM Pb + 105 dB Noise	7

Table II-2. Threshold shift averages (dB) and counts of shifts equal to or over 20 dB by treatment groups

Treatment Group	32 kHz				8 kHz			
	N	Avg Shift	SD	N Shifts ≥ 20 dB	N	Avg Shift	SD	N Shifts ≥ 20 dB
Control	15	3.9	4.7	0	15	2.6	4.1	0
Pb								
3 mM	16	3.9	4.3	0	11	5.2	4.1	0
1 mM	8	6.6	5	0	8	12.75	5.6	2
0.03 mM	8	6.3	8.9	1	8	8.0	4.9	0
Cd								
300 µM	16	4.5	5.3	0	11	4.6	3.5	0
100 µM	6	5.5	3.3	0	5	9.4	5.6	0
30 µM	6	7.8	4.4	0	6	10.7	6.7	0
Noise								
108 dB	6	41.0	18.8	5	6	25.2	16.5	3
105 dB	15	42.2	13.5	14	10	21.4	11.3	5
102 dB	7	15.3	6.5	1	7	13	3.3	0
Mixtures								
300 µM Cd + 3 mM Pb	9	0.8	3.5	0	9	3.1	3.4	0
3 mM Pb + 105 dB Noise	14	38.9	12.3	13	9	16.6	8.2	2
300 µM Cd + 105 dB Noise	14	39.3	8	14	9	23	11.5	6
300 µM Cd + 3 mM Pb + 105 dB Noise	7	29.6	18	5	7	15.7	7.8	2

Table II-3. DPOAE average amplitude shifts (dB) and matched ABR threshold shifts (dB) at 32 kHz

		Threshold Shifts		Amplitude Shifts	
Group	N	ABR	SD	DPOAE	SD
Pb	5	2	3.1	-1.1	2.6
Cd	4	0.5	2.5	0.6	0.9
Noise	5	42.2	21.0	-21.9	15.7
Pb+Noise	5	45.8	4.3	-27.7	9.5
Cd+Noise	5	37.8	7.3	-26.1	11.7

Table II-4. Concentrations of metals in blood by treatment group

		Whole Blood Metals			
		Pb (µg/dL)^		Cd (µg/L)^^	
Treatment Group	N	Avg	SD	Avg	SD
Control	16	ND	-	ND*	-
Pb					
3 mM	11	60.1	6.6	ND	-
1 mM	8	38.5	4.9	ND**	-
0.03 mM	8	2.89	0.44	ND***	-
Cd					
300 µM	12	ND	-	27.2	4.1
100 µM	7	ND	-	6.37	0.87
30 µM	6	ND	-	1.3	0.23
Noise					
108 dB	7	ND	-	ND	-
105 dB	10	ND	-	ND	-
102 dB	6	ND	-	ND	-
Mixtures					
300 µM Cd + 3 mM Pb	9	57.8	2.2	16.2	1.2
3 mM Pb + 105 dB Noise	9	57.0	3.9	ND	-
300 µM Cd + 105 dB Noise	9	ND	-	24	2.9
300 µM Cd + 3 mM Pb + 105 dB Noise	7	58.9	3.9	16.7	1.8

^Detection limit is 0.5

^^Detection limit is 0.25

*One sample above DL: 0.26

**One samples above DL: 0.32

***One sample above DL: 0.27

Table II-5. Concentrations of metals (mg/kg) in bone

| | | Femur | | | | Tibia | | | |
| | | Pb | | Cd | | Pb | | Cd | |
Treatment Group	N	Avg	SD	Avg	SD	Avg	SD	Avg	SD
Control	5	0.16*	0.14	ND**	-	0.16	0.06	ND	-
3 mM Pb	12	287	45	ND^	-	216	51	ND	-
300 µM Cd	11	0.13^^	0.01	0.27	0.03	0.18	0.04	0.23	0.03
300 µM Cd + 3 mM Pb	9	236	24	0.26	0.02	207	23	0.21	0.02

*All five samples run in control groups showed levels of Pb were detectable and above the limit of detection of 0.05 mg/kg

**All five samples in control groups showed levels of Cd were below the limit of detection of 0.05 mg/kg.

^Two femur and tibia samples run in Pb treatment groups showed levels of Cd were below the limit of detection of 0.05 mg/kg. Results were similar to control levels.

^^Two femur and tibia samples run in Cd treatment groups showed levels of Pb were detectable and above the limit of detection of 0.05 mg/kg. Results were similar to control levels.

Table II-6. Concentrations of metals (mg/kg) in cochlea and adjoining tissue

| | | Cochlea | | | |
| | | Pb | | Cd | |
Treatment Group	N	Avg	SD	Avg	SD
105 dB Noise	5	0.250	0.153	ND*	-
3 mM Pb	5	185.0	34.9	ND*	-
300 µM Cd	4	0.228	0.04	0.287	0.066

*All Pb treatments and Noise treatment groups had levels of Cd that were not detectable at the limit of detection of 0.05 ppm or mg/kg.

Table II-7. Cochlea cytogram missing outer hair cell counts

Treatment Group	N	Apex Avg	Apex SD	Middle Avg	Middle SD	Base Avg	Base SD
Control	15	0.2	0.2	0.2	0.3	0.3	0.4
3 mM Pb*	11	0.2	0.2	0.3	0.2	0.1	0.1
300 µM Cd*	12	0.2	0.3	0.2	0.2	0.2	0.2
102 dB Noise	7	0.2	0.2	0.3	0.3	3.1	2.3
105 dB Noise*	10	0.2	0.2	0.4	0.2	5.8	4.4
108 dB Noise	8	0.3	0.1	1.6	2.6	13.8	16.7
Cd+Pb	9	0.1	0.3	0.2	0.2	0.7	1.2
Pb+Noise	9	0.3	0.3	0.3	0.3	2.9	2.3
Cd+Noise	9	0.2	0.1	0.5	0.4	11.6	9.6
Cd+Pb+Noise	6	0.1	0.1	0.3	0.3	7.3	5.0

*Group treatment levels used for mixtures (3 mM Pb, 300 µM Cd, & 105 dB Noise)

Table II-8. Histology data in counts of mice with observed lesions

Treatment	N	Renal Lesions						ANY RENAL LESION	Hepatic Lesions		ANY HEPATIC LESION
		THP	KM	TD	MII	FM	INI		DGN	MFI	
Control	16	9	0	4	0	0	0	9	2	2	2
102 dB Noise	7	5	0	2	1	0	0	6	1	2	2
105 dB Noise*	10	9	0	5	1	0	0	9	1	1	1
108 dB Noise	7	3	1	4	1	0	1	4	0	0	0
30 µM Cd	6	4	0	2	0	0	0	4	0	0	0
100 µM Cd	7	6	0	4	1	0	0	6	1	1	2
300 µM Cd*	12	9	0	6	1	0	0	9	1	1	1
Cd+Noise	9	5	0	0	0	0	0	5	0	0	0
0.03 mM Pb	8	8	0	3	0	0	0	8	1	1	1
1 mM Pb	8	7	1	4	0	0	1	7	2	2	2
3 mM Pb*	11	9	10	6	0	0	10	11	0	0	0
Pb+Noise	9	8	9	1	0	4	8	9	0	1	1
Cd+Pb	9	7	9	1	0	0	9	9	0	1	1
Cd+Pb+Noise	7	6	6	0	0	2	6	7	0	0	0

*Group treatment levels used for mixtures (3 mM Pb, 300 µM Cd, & 105 dB Noise)

DGN - degeneration and necrosis, centrilobular and random

FM - focal mineralization

INI - intranuclear inclusions

KM - karyomegaly, tubular epithelium, S3

MFI - multifocal neutrophilic and mononuclear inflammation

MII - mononuclear interstitial inflammation

TD - tubular degeneration

THP - simple tubular hyperplasia

II.G: Figures

Figure II-1. Timeline of exposures and treatments along with corresponding age of mice
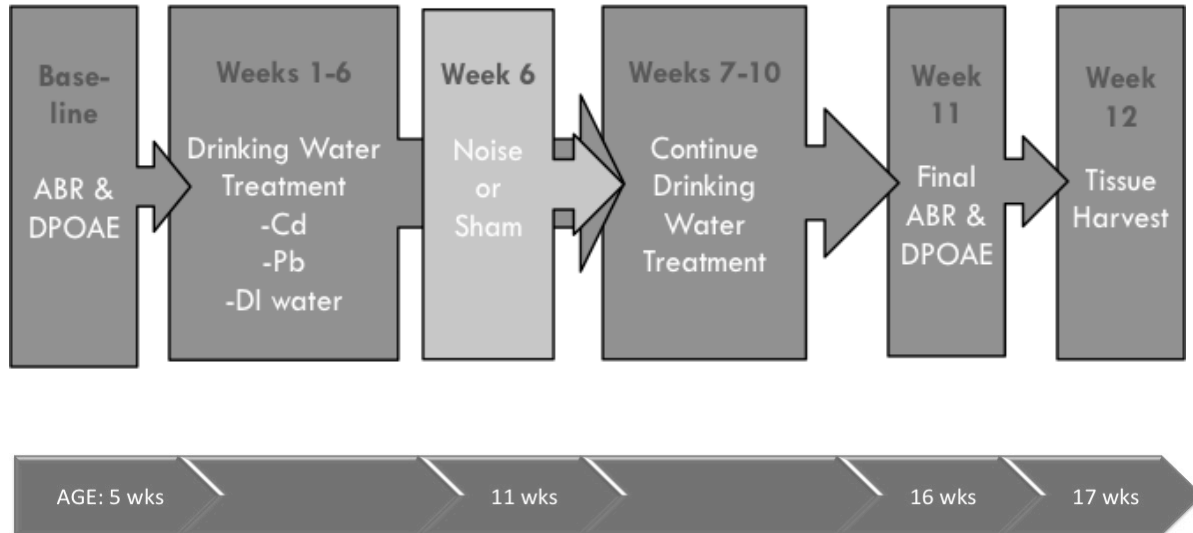
Figure II-2. Mean and standard deviations for ABR threshold shifts at 8 kHz for single treatment and mixture groups with 3 mM Pb, 300 μM Cd, and 105 dB
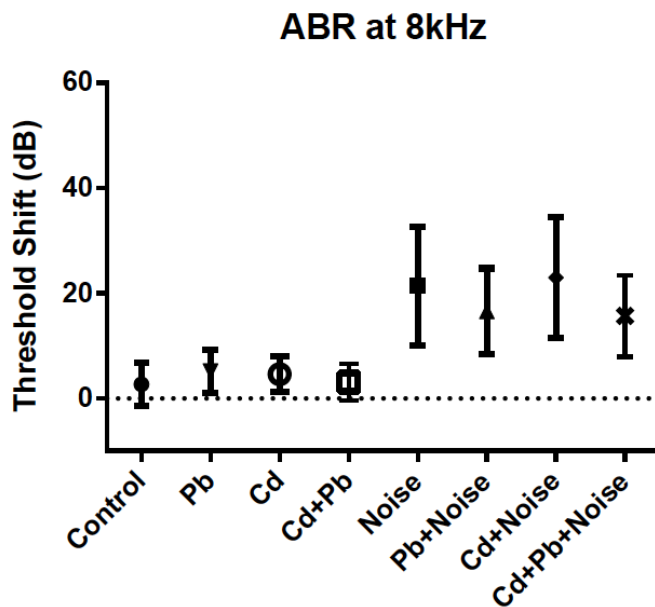
Figure II-3. Mean and standard deviations for ABR Threshold shifts at 16 kHz for single treatment and mixture groups with 3 mM Pb, 300 μM Cd, and 105 dB
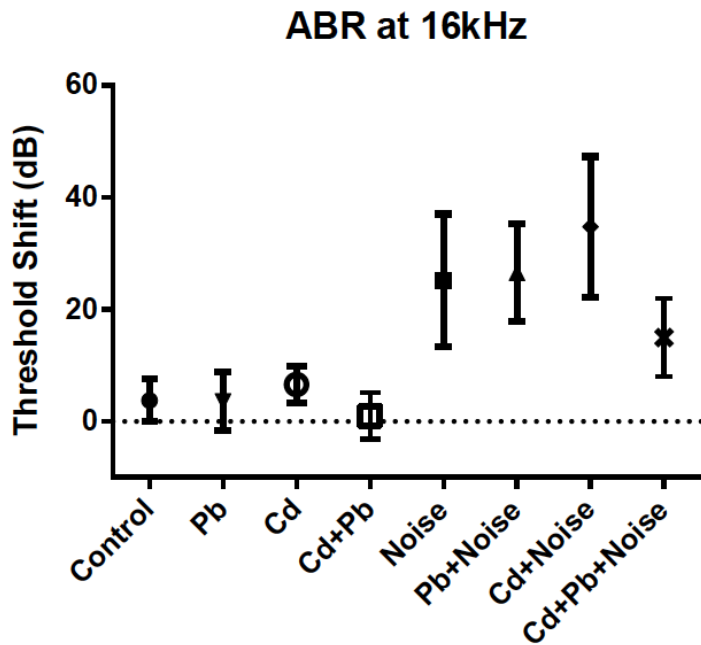
Figure II-4. Mean and standard deviations for ABR Threshold shifts at 32 kHz for single treatment and mixture groups with 3 mM Pb, 300 μM Cd, and 105 dB
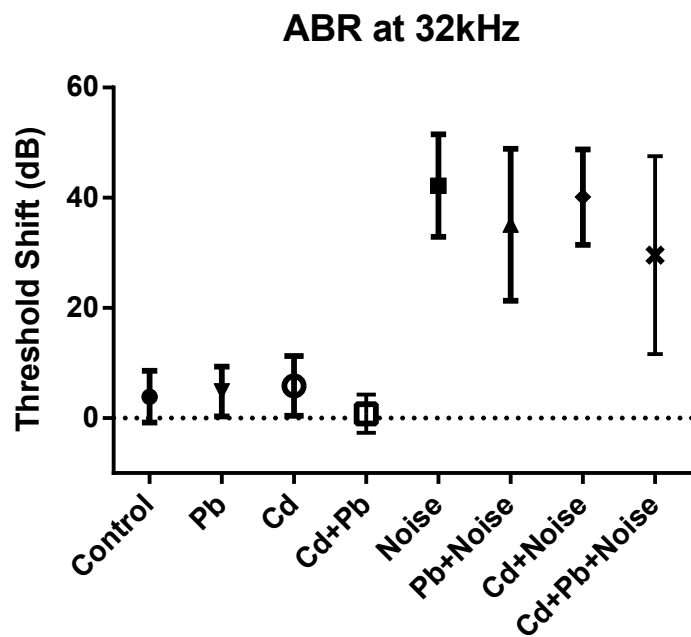
Figure II-5. Blood Cd levels (μg/L) by treatment groups for single treatment and mixture groups with 3 mM Pb, 300 μM Cd, and 105 dB

Blood Cd Levels

* p-value < 0.0001

Detection limit
0.25 μg/L
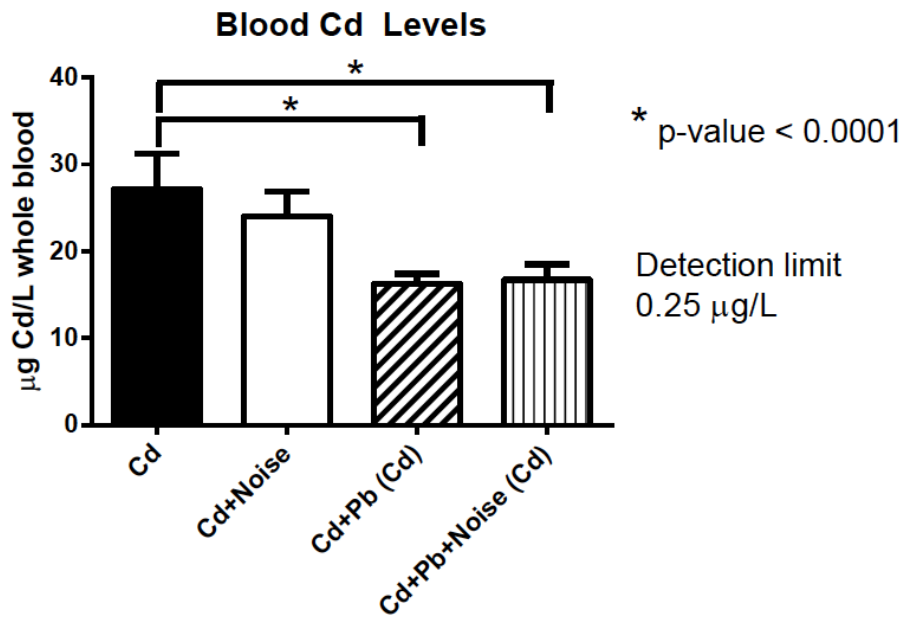
Figure II-6. Blood Pb levels (μg/dL) by treatment groups for single treatment and mixture groups with 3 mM Pb, 300 μM Cd, and 105 dB

Blood Pb Levels

No significant
differences
p-value 0.486

Detection limit
0.5 μg/dL
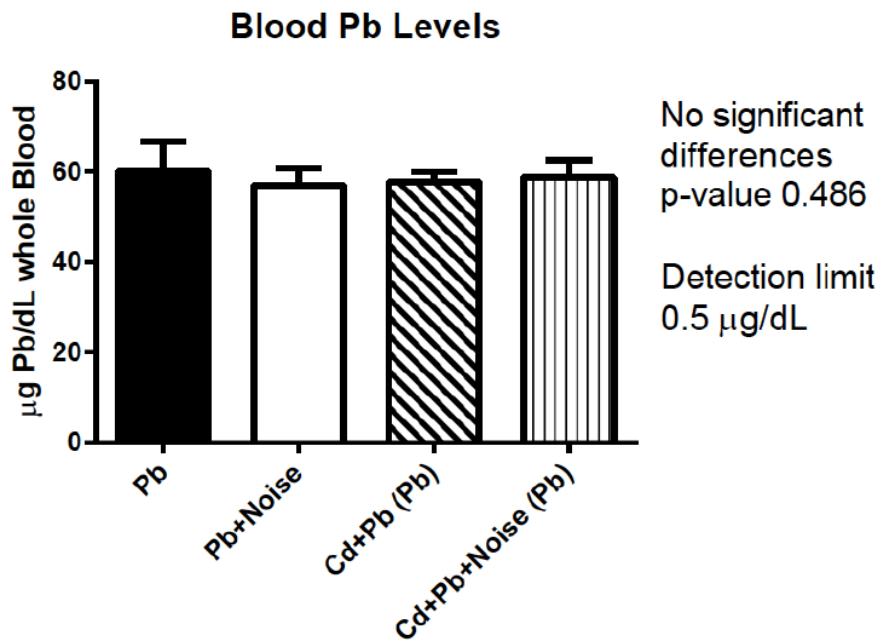
CHAPTER III: Toxic and essential elements from newborn dried blood spots associated with abnormal hearing screening outcomes: a case-control study

III.A: Abstract

Although toxicant metals have been associated with hearing loss in children and adults, little is known about the role of prenatal exposure to metals in hearing health in neonates. This case-control study explored the relationship between newborn hearing test outcomes and neonatal blood levels of two non-essential metals, lead and methylmercury (MeHg), along with six essential elements (calcium, copper, iron, potassium, selenium, and zinc). Data were obtained from Michigan State surveillance programs for 338 infants with an abnormal hearing screening result and 338 infants with a normal hearing screening result, matched by birth year, sex, and race. Elemental levels in the newborn dried blood spots were determined by Total Reflection X-Ray Fluorescence and cold vapor atomic fluorescence spectrometry. Conditional logistic regression models were used to estimate odds ratios (ORs) and 95% confidence intervals (CI) for the risk of abnormal hearing screening result. Borderline significance was shown for MeHg as a continuous variable (OR=1.23, 95% CI: 0.99-1.53). A significant trend (p=0.03) was observed for quartile analysis. The odds of a hearing screening failure were significantly higher in the highest quartile of MeHg compared to the lowest quartile (OR=1.81 95% CI: 1.01-3.24). A statistically significant association was found between blood spot calcium levels and decreased odds of hearing screening failure (OR=0.49, 95% CI: 0.34-0.70). Lead and other essential elements were not significantly associated with odds of hearing screening failure. Potassium and iron alone were not associated with hearing screening failures, whereas when introduced in a model with calcium both potassium and Iron became significant predictors, though increasing levels of both potassium and Iron were associated with higher odds of hearing screening failure (potassium: OR=1.47, 95% CI: 1.11-1.93; Iron: OR=1.43, 95% CI: 1.10-1.86). These results remained unchanged in sensitivity analyses of using only cases with bilateral hearing loss screening failures. A significant multiplicative interaction was found between selenium and zinc (selenium: OR=0.51, 95% CI: 0.27-0.93; zinc: OR=0.38, 95% CI: 0.19-0.75; selenium × zinc: OR=1.32, 95% CI: 1.08-1.61). The best model included MeHg, calcium,

iron, selenium, zinc and a selenium × zinc interaction term (likelihood ratio test=45, p=5e-8). These findings, though they must be interpreted with caution, suggest that neonatal blood calcium, potassium, and iron levels may be important determinants of hearing health, though further studies are warranted to understand the physiological mechanisms. The study demonstrated that MeHg appears to be a risk factor for hearing in the developing auditory system, and that newborn blood spots represent a novel metric for assessing infant environmental exposures.

III.B: Introduction

Globally, in 2016, all types of hearing loss (HL) collectively were the third top cause of years lived with disability, contributing to estimates as high as 51 million years lived with disability [172]. Loss of hearing has a profound impact on health and quality of life, and is associated with a wide variety of adverse social, psychological, educational, and occupational outcomes [110]. HL in children and young adults is of great concern due to impairment of intellectual development. Even mild losses in children are associated with poorer speech perception and significantly lowered performance on basic skills tests [173]. Children with HL are also likely to have additional health and social disparities [174].

A number of exposures to biological, chemical, and physical hazards are known to cause HL, including noise [175] and certain pharmaceuticals [114]. Cytomegalovirus is also a cause of HL cases prenatally [176,177]. However, recent research has identified a new potential source of HL: environmental and occupational exposure to non-essential metals, including lead and mercury [116]. The mechanism of this toxicity has not been elucidated, but lead has been implicated as ototoxic in animals [52,53], as well as in human adults [57,73] and adolescents [56]. Mercury may also have ototoxic effects in animals [178] and human adults [179,180].

Essential elements may also play a role in hearing health, either by enhancing auditory cellular processes or by counteracting adverse effects of toxic metals. Higher intakes of calcium, iron, and zinc have been associated with lower lead absorption by infants [181]. Selenium is an essential element that is known to mitigate mercury toxicity [182]. Healthy diets and essential metal nutrient intake have also been linked to hearing health [183,184]. Deficiency from dietary zinc, an essential

element, has been shown to raise hearing thresholds at high frequencies in mice [185]. Iron deficiencies have also been shown in human adults to be associated with sudden sensorineural HL [186]. However, to our knowledge, the role of prenatal exposure to toxic metals and essential minerals and their potential interactions in the developing hearing system has never been explored. This is likely due to the difficulty of assessing exposures during this vulnerable developmental stage.

As part of neonatal health screening programs, newborn dried blood spots (NDBS) are collected to help reduce mortality or morbidity in newborns or children having, or being at risk for, heritable disorders [187]. Over the past decade there has been increasing interest in using residual NDBS to gauge early life exposures to various agents, such as toxic metals and essential elements [188]. In addition to NDBS-based screening, many jurisdictions also perform hearing tests on newborns, thus offering a great opportunity to link datasets for exploratory research into prenatal health impacts.

NDBS samples and hearing screening tests are required of all newborn infants born in Michigan before they leave the hospital. Between 2004 and 2008 98% infants born in Michigan completed an initial hearing screening. Failure of the screening test leads to a referral for further hearing testing. From 2004-2008, there was a prevalence of 1.3 per 1,000 births for any permanent HL; for bilateral HL, the rate was 1 per 1,000 births. These rates are similar to national averages from states responding to the National CDC early hearing detection and intervention (Michigan Early Hearing Detection and Intervention and Michigan Department of Community Health, 2011; Michigan Early Hearing Detection and Intervention and Michigan Department of Community Health, 2013).

The State of Michigan has a long history of concern over exposures to toxic metals, and especially lead and mercury. The geographic focus of this study, Southeast Michigan, includes numerous sources of environmental metals contamination and legacy and ongoing industrial pollution and automobile and rail traffic, and has historically shown high levels of blood lead among monitored children [191]. For example, elevated blood lead levels have been documented in children in Detroit and throughout Michigan [20,192], and the water crisis in Flint has renewed and escalated these concerns [22]. Methylmercury pollution is of on-going concern in the Great Lakes region

44

with the major source to human populations being the consumption of contaminated fish [193]. In the 2011 National Listing of Fish Advisories, the entire State of Michigan had an advisory, mainly due to methylmercury contamination [194].

While newborns across Michigan may be exposed to elevated levels of lead and methylmercury, and studies suggest links between these elements and adverse hearing outcomes, direct evaluation of these potential associations is challenging. Due to the opportunities presented by elevated lead exposures and Michigan's historical data in NDBS, we have been able to explore the effects of these exposures in a novel fashion. This paper describes a case-control study to assess the associations of essential and non-essential metals in NDBS with failures of infant hearing screenings.

III.C: Methods

Prior to the start of our research, we obtained Institutional Review Board approval from four institutions: The University of Michigan, the Michigan BioTrust for Health Scientific Advisory Board, the Early Hearing Detection and Intervention (EHDI) Program, and the Michigan Department of Health and Human Services (MDHHS, formerly called the Michigan Department of Community Health).

All NDBS samples, collected within 48 hours after birth, are maintained by the Michigan Neonatal Biobank (MNBB) and represent a measure of prenatal exposure to environmental contaminants. The EHDI program within the MDHHS tracks newborn hearing screening tests, administered to all infants born in Michigan since 2003 [189]. These tests, also collected shortly after birth (usually within 24 hours), provide a snapshot of hearing ability separately in both the left and right ears prior to any substantial postnatal noise exposure. The hearing test data are collected using multiple methods, depending on the preference of the hospital. These include auditory brainstem response (ABR), automated ABR, distortion product otoacoustic emissions, and rarely transient evoked otoacoustic emissions. These tests, when used in a hearing screening context, do not provide quantitative measurements, but instead indicate a normal or abnormal hearing test outcome. Information available from the newborn screening cards linked to the MNBB and EHDI data include: birth year, hour of birth, birth weight, gestational age, single or multiple birth, Neonatal

Intensive Care Unit (NICU) or Special Care nursery, transfusions, race/ethnicity, as well as mother's city of residence.

Data set and demographics

All data were integrated and subsequently de-identified at the MNBB before being delivered to us for analysis. Two datasets of NDBS punches from newborns born in Michigan were analyzed separately and together; the two datasets resulted from the two grants, obtained at different times, that supported this research. The first dataset consisted of 100 matched pairs of cases and controls (N=200 newborns); cases and controls were matched by birth year (2004-2007), sex, race, mother's city of residence, and birth hospital. The second data set consisted of 238 matched pairs of cases (defined in the same way as set 1) and controls (N=476 newborns). In the second set, matching was performed using birth year in two-year intervals (2003-2004, 2005-2006, 2007-2008, 2009-2010, 2011-2012, 2013-2014 and 2015), sex, and race (white, black or other). The matching criteria for this second set were reduced in order to increase the available sample of newborn HL cases without creating potentially identifiable subjects.

Both sample sets were drawn from the Detroit-Warren-Flint Consolidated Statistical Area, which accounts for 54% of state population [195]. Infants who spent time in a NICU or special care nursery were excluded from our sample, as these infants may have incurred HL after time spent in an incubator prior to their hearing screening test [196]. The EHDI surveillance program confirmed abnormal hearing screening cases for all data, either as a failure at the initial screening with a subsequent abnormal hearing assessment, or a failure at screening with no subsequent information provided (failures with no follow up were treated as confirmed). EHDI provided us with a de-identified demographic information dataset.

Metal levels in NDBS

Laboratory NDBS analyses required 80 mm^2 of dried blood per subject, or nine 1/8th inch diameter blood spot punches per subject (with each 1/8th inch punch being roughly 8 mm^2). These samples were prepared and de-identified by MDHHS. They were shipped and stored in polypropylene bags.

Novel laboratory methods involving total reflection x-ray fluorescence (TXRF) with a S2 PICOFOX (Bruker AXS Microanalysis GmbH, Berlin) were developed [197] to evaluate the essential and non-essential elements in the NDBS punches. NDBS were handled in a Class100 ISO clean hood. For elemental analyses of lead, calcium, copper, iron, selenium, and zinc via TXRF, a 1/8" punch was taken from a NDBS and placed into an acid-washed microcentrifuge tube to which 15 μL of concentrated HCl and 5 mmol EDTA was added. The sample was vortexed and then digested for 1.5 hours at 55 C. Following the digestion, the sample was centrifuged for 15 minutes at 25 C and 12,000 rpm. An 8 μL portion of the extraction fluid was removed into a second microcentrifuge vial, to which was added 4 μL of a solution containing gallium (internal standard), lead (spike), and polyvinyl alcohol. This solution was mixed, and an aliquot was placed onto an RBS™-conditioned quartz disc and allowed to dry before TXRF analyses. Samples were read for 2,500 seconds, after which the results were analyzed using the instrument's software. The TXRF approach used here had an analytical accuracy of 77-116% and precision of 12-25% for the analytes lead, calcium, copper, iron, selenium, and zinc by testing against seven different blood standard reference materials from which NDBS were created. The resulting data overlapped nearly completely with the expected reference range values. Analyte digests were also analyzed for methylmercury via a GC-CVAFS unit (Tekran 2700, Tekran Instruments Corporation, Toronto) [198]. To standardize the data, and act as a negative control, a nearby punch unspotted with blood was taken from the sample card.

Statistical analyses

The demographic and metals datasets were merged and imported into R for statistical analysis (version 3.5.1; The R Foundation for Statistical Computing). Descriptive statistics were computed to evaluate the demographics and NDBS datasets and evaluate potential outliers. For paired cases and controls, when metals data was missing for one of the pairs, it was removed for the other member of the pair. One control (and its paired case) were removed from the dataset due to consistently having the highest levels (values ranged from 2-23 times higher than the next highest sample) of all metals analyzed by TXRF. One sample was lost during laboratory analysis. Visualization tools (e.g., histograms and boxplots) and correlation coefficients were used to assess data distributions and bivariate relationships. Non-normally distributed variables were log-

transformed and reported in terms of geometric mean and geometric standard deviation. Negative values were used in analyses based on quartiles. Quartiles were created by dividing the range of values for each element in the set of control data into four equal segments. Conditional logistic regression modeling was used to estimate the odds ratios (OR) and 95% confidence intervals (CI) for newborn hearing screening failure associated with blood levels of contaminants (lead and methylmercury) as well as nutrient levels (calcium, copper, iron, potassium, selenium, and zinc). All models were conditioned on matching factors (birth year, sex, and race). Conditional logistic regression was run with the survival package in R. Other subject-specific variables (i.e., gestational age, single or multiple birth, and transfusions) were included in subsequent models as covariates.

In addition, to investigate potential effect modification, interaction effects were evaluated between lead or methylmercury and essential elements, as well as two or more essential metals, on the odds of having a newborn hearing screening failure. Sensitivity analyses were conducted on the subset of cases with bilateral HL, as exposures to these metals would not be expected to affect just one ear selectively during development *in utero*. Models with logarithmic transformation of Pb and Hg were explored. Factor analysis and principle component analysis was also used to analyze significant predictor groups.

III.D: Results

Demographics

The majority of newborns in the overall sample were white (69%), male (55%), non-Hispanic (65%), and full-term (91%) (Table III-1). The majority (74% for cases and controls combined) of all final hearing screening tests were conducted using automated auditory brainstem response (AABR). Cases were more likely to be screened with distortion product otoacoustic emissions (DPOAE) (28%) than controls (15%). The majority (64%) of cases of hearing screening failures were bilateral (i.e., failure in both right and left ears).

In addition to the overall sample, demographic characteristics are shown by dataset in Table III-1. Dataset 1 was obtained first and represents older data, with the majority of the sample born during 2005-2006 (43%). Over half of set 2 was born during 2007-2010 (51%), and these years represent

45% of our data set in total. The majority of newborns were white (68% for Set 1 and 69% for Set 2) and male (51% and 56%, respectively) in both data sets. Babies who were black were more heavily represented in set 1 (23%) over set 2 (16%), and other racial groups were represented more heavily in set 2 (15%) over set 1 (9%). Mothers reported smoking in 15% of cases and 12% of controls overall, with similar fractions in set 1 (14% for cases and 8% for control) and set 2 (16% and 14%, respectively). However, set 2 had a much larger percentage of unknown smoking status overall for the mother (21%) compared to set 1 (2%).

The geographical distribution of newborns in sets 1 and 2 is provided in Table III-2. For set 1, data were available regarding mother's residence city and birth hospital and for set 2, only residential county information was available. The most common cities of maternal residence for newborns in set 1 were Detroit (Wayne county) and Ann Arbor (Washtenaw county), and the most common counties of residence for set 2 were Wayne and Oakland.

Metal levels in NDBS

Geometric mean and standard deviation concentrations of the eight measured NDBS analytes are shown in Table III-3, both for the overall dataset (III-3A) and stratified by datasets 1 (III-3B) and 2 (III-3C). Concentrations of lead were much higher in set 1 than in set 2, likely reflecting the higher fraction of blood lead concentrations measured after 2008 in set 2 compared to set 1 and the generally downward trend in mean blood lead concentrations over time (Figure III-1). NDBS concentrations of nearly all analytes were higher in set 1 than in set 2, with the exception of blood methylmercury. These results did not differ substantially when the analysis was restricted to bilateral cases of HL only (data not shown). In both datasets, cases had higher, though not significantly higher, levels of lead, copper, iron, potassium, and selenium. Controls had higher levels of calcium and zinc across both data sets. Levels of methylmercury were higher in controls for set1 and in cases for set 2. Concentrations of copper were significantly ($p = 0.04$) higher in cases compared to controls in set 1, but not in set 2 or overall. Concentrations of calcium were significantly higher in cases than controls in set 2 ($p = 0.04$) and overall ($p = 0.03$). No other significant differences were noted between cases and controls.

Odds of hearing screening failure

Toxicant metals: Lead and methylmercury

ORs for newborn hearing screening failure across both toxic metals, as well as the essential elements for univariate analysis, are shown in Table III-4. Methylmercury was found to be significant risk factor for cases with unilateral HL and has borderline significance as a risk factor in the overall dataset. Lead had a large confidence interval and borderline significance as a risk factor for unilateral HL. No subsets of the data by gender or race indicated blood lead levels as a risk factor for newborn hearing screening failure.

Table III-5 shows the results of conditional logistic regression modeling of the OR of a newborn hearing screening failure associated with lead and methylmercury exposure independently, both adjusted and unadjusted for a number of covariates. The results for the lead models (Table III-5A, Models 1-5) consistently indicate elevated but non-significant odds of a newborn HL for newborns in the highest quartile of lead exposure. The results of the methylmercury models (Table III-5B, Models 6-10) all show significant or borderline significant dose–response trends across increasing quartiles of exposure. Models 6 and 8 show methylmercury blood levels as a significant risk factor, with increased odds ratios for the highest quartile compared to the lowest. For methylmercury, protective effects were seen for all second quartile levels of methylmercury, and the third quartile for levels of methylmercury adjusted for blood levels of calcium (Model 7). The results presented in Table 4 did not differ substantially when the analysis was restricted from all cases of HL to bilateral cases only (data not shown).

Effect modification was assessed by including elements into models with toxic metals to examine the impacts on OR and significance. Methylmercury's effect increased when it was considered with four other essential elements were added to the model: calcium, copper, selenium, and zinc. The methylmercury ORs were only significant when considered with calcium (Table III-5B, Model 7: OR=1.31 95% CI: 1.03-1.65). Calcium also remained a significant protective factor in this model (OR=0.47, 95% CI: 0.33-0.68). The same essential elements increased the effect of lead as a continuous variable in additive models, but lead never reached statistical significance. Lead became a borderline significant risk factor when copper and zinc were included in the model. Multiplicative interactions assessed by adding an interaction term for either lead or methylmercury

and the essential element tested to additive models revealed no significant interactions. Models with logarithmic transformation of lead and methylmercury did not reveal significant findings for lead or methylmercury. Factor analysis and principle component analysis did not reveal any significant predictors for combinations of toxic metals or essential elements (data not shown).

Essential elements: Calcium, copper, iron, potassium, selenium, and zinc
Figure III-2 displays scatterplots (with least squares fitted lines) and correlations (with p-values) for three essential elements: calcium, iron, and potassium. Normal distributions were observed for iron and potassium, whereas the distribution for calcium was skewed right. Correlations ranged from weak (calcium and iron) to strong (iron and potassium), but all were highly significant.

ORs for a newborn hearing screening failure associated with individual essential elements by increase in interquartile range are shown in Table III-4B. Calcium consistently showed a protective effect for both data sets, both sexes, white race, and both unilateral and bilateral screening failures. Higher levels of iron were associated with an increased risk of newborn HL across all examined subsets. Iron showed increased ORs for newborn HL significant among males, but not females. The OR for iron levels in black newborns approached significance, as did the OR for iron levels for all data. Increasing levels of potassium showed non-significant protective effects for females and the white racial group; however, all other subsets of the data showed non-significantly increased risk for HL with increased potassium levels. No other metals analyzed showed significant OR across any data subsets except copper, which did show increasing levels of copper associated with increasing likelihood of HL for data set 1 (OR=1.70, 95% CI: 1.05-2.78).

The best-fitting conditional logistic regression models with essential elements and additive interaction effects are shown in Table III-6 (Models 11-17) with ORs for continuous variables as well as for quartiles with the lowest quartile for reference. These models included calcium alone, iron alone, potassium alone, calcium and iron together (Ca+Fe) and calcium and potassium together (Ca+K) (Models 11-15 respectively). Models 16 and 17 include a model with calcium, iron, and potassium as well as mother's smoking status. Calcium consistently showed a significant protective effect against newborn hearing screening failure and was most protective in Model 15 (Ca+K). Iron and potassium were significant when considered with calcium in Models 14 and 15 (Ca+Fe and Ca+K, respectively), and insignificant when calcium was not included (Models 12 and

13, respectively). Interaction terms for calcium, iron, and potassium when one or more predictors were included in these models were not found to be significant, though the model with an interaction term for calcium and iron showed a slightly higher protective effect against newborn HL (OR=0.34, 95% CI: 0.14-0.82) for calcium (data not shown). The detrimental effects of iron at higher levels were reduced to non-significance in this model.

When the analysis was restricted to bilateral HL cases, the number of available cases and their matched controls was reduced from 672 to 428. The models described above were re-run in this restricted dataset and are shown in Table III-7 (models 18-24). Calcium remained protective and significant in both Model 11 and 18 and increasing levels of iron remained a risk factor in Model 12 and 19. Iron was borderline significant in Model 21 (Ca+Fe). Therefore, model Ca+Fe, shown in models 14 and 21, was selected as the best model from our analysis, as this model showed consistency across the entire data set and the bilateral case subset (likelihood ratio test = 18 for bilateral failures and 29 for all types of failures).

Potential effect modifications were assessed for calcium. All other elements (both essential and non-essential), except for zinc, made calcium a protective when included in bivariate models (data not shown). Interactions were also assessed for essential elements and three significant multiplicative interactions were discovered: selenium-zinc, copper-zinc, and calcium-copper. Including both of these pairs of elements in additive models did not show significance until the multiplicative factor was introduced. All predictors in the zinc-selenium models were significant (N=650, selenium-zinc: OR=1.32 95% CI: 1.08-1.61). These predictors remained significant when this model was explored in only bilateral cases (N=410, selenium-zinc: OR=1.49 95% CI: 1.12-1.97). Interaction terms OR and CIs were borderline significant for copper-zinc (OR=1.09 95% CI: 0.99-1.20) and calcium-copper (OR=1.12 95% CI: 1.00-1.26). These two models lost significance when restricted to bilateral HL cases only.

One final set of models (Table III-8) was run combining the significant predictors of calcium and iron along with the significant selenium-zinc interaction term. These models were run with and without methylmercury (Table III-8). All terms were significant predictors for this model and each essential element was protective except iron. When data were restricted to bilateral cases, iron was no longer significant, though all other terms in the model remained significant and protective.

When methylmercury was included in this model, it was significant in all types of hearing failures, however it fell out of significance when included in the model with only bilateral cases.

III.E: Discussion

Our case-control study is the first study in infants to investigate levels of lead and methylmercury associated with abnormal hearing screenings. To do so, we created a connection between existing NDBS samples and existing hearing screening data. Previous studies have shown both lead and methylmercury to be developmental toxicants [199]. In agreement with these previous findings, we observed a significant increase in the odds of hearing screening failure for newborns in the highest quartile of methylmercury compared to the lowest quartile. A significant trend of increasing odds ratios across methylmercury quartiles was also observed. However, the continuously-modeled exposures alone only reached borderline significance. No associations with hearing screening failures were established for lead. We also found that higher calcium levels were associated with lowered odds of an abnormal hearing screening. This association with calcium was significant in both males and females (males OR=0.52, 95% CI 0.31-0.88; females OR=0.46, 95% CI 0.28-0.77), white infants (OR=0.46, 95% CI 0.30-0.72), and in cases of bilateral HL (OR=0.48, 95% CI: 0.30-0.75). This significant protective effect of higher levels of calcium remained across all logistic regression models.

Toxicant metals and HL

Mercury exposures, usually measured as the biologically more toxic form of methylmercury [200], have previously been documented to lead to HL. Delayed brainstem auditory evoked potentials of 14 year old children from the Faroe Islands were shown to be associated in with higher levels of methylmercury in their umbilical cord blood [201]. In Ecuadorian children exposed to mercury in artisanal gold mining contexts, level of mercury were correlated with hearing thresholds at 3 kHz [87]. Developmental exposures to methylmercury produced lowered thresholds in monkeys that was not evident until adulthood [202]. However, not all studies of human adolescents and adults [56,203] and children [204] have shown ototoxic effects.

Previous studies in mice have shown that *in utero* lead exposures can alter processing pathways in the auditory system in mice [16–18]. Monkeys treated with lead during development have also demonstrated alterations in auditory brainstem response and auditory thresholds [205–207]. In humans, lead levels have been shown to be associated with impaired auditory brainstem response as well as increased thresholds across both high and low frequencies [208]. Blood lead levels have also been shown to be associated with higher thresholds in adolescents in the US [56]. In contrast to these previous findings, this study did not indicate that lead levels in whole blood were significantly associated with failures of infant hearing screenings, though they did consistently show non-significant increases in the highest quartile of lead exposure.

Essential elements and HL

Our finding that low levels of blood calcium in newborns are associated with lowered odds of hearing screening failures does not appear to have been previously reported in the literature. However, hypocalcemia has been observed previously to be associated with poor general health in infants [209,210]. Calcium is absorbed from dietary sources, then absorbed into the blood, where calcium levels are tightly regulated. Calcium concentrations are extremely important in the auditory system, as imbalances of calcium in auditory cells and tissues can lead to temporary or permanent HL [211]. Dysregulation of calcium is also associated with age-related HL [212].

Parathyroid hormone (PTH) disorders may be indicated by hypocalcemia, as PTH is needed to control levels of calcium in the blood. Hypocalcemia is symptomatic of DiGeorge's syndrome (a genetic condition), associated with sensorineural HL, in which the parathyroid gland is completely or partially absent [213,214]. CHARGE syndrome (named for abnormalities in multiple systems: coloboma, heart, atresia choanae, retardation of growth, genitals, and ear) also has both hypocalcemia and HL symptoms [140]. As a protective effect of calcium, or increased blood calcium levels associated with less failure of a hearing screening, was demonstrated by our data, cases likely include individuals with CHARGE syndrome, or other syndromic diseases which decrease levels of calcium as well as the auditory system. Hypoparathyroidism has also been linked with sensorineural hearing disorders [215].

In addition to PTH abnormalities, deficiency of vitamin D can cause lowered absorption of calcium and lowered blood calcium. Low levels of calcium in our sample could be linked to low levels of vitamin D, which has been observed in pregnant mothers in higher latitudes, especially in those with darker skin pigmentation [216,217]. Normal ranges of calcium are also essential to the development of a high functioning hearing system as well as vitamin D [218,219]. Complex neurocognition within the brain is also a necessity for normal auditory function, however, low levels of vitamin D during fetal development are associated with ongoing neurocognitive disorders at age eight [220].Vitamin D and calcium sensing is also essential to neuronal development [218,221,222].

Vitamin D deficiency has previously been shown to be associated with HL [223]. Although not measured directly, vitamin D deficiency is a concern in this particular cohort. Vitamin D deficiency is noted especially at high latitudes similar to those in Michigan [224], in non-white races, and in smokers [225]. Smoking has been shown to lower calcium absorption, lower levels of PTH, and increase risk of vitamin D deficiency; however serum calcium levels of smokers and non-smokers have been found to not be significantly different [226,227]. It is important to note that smoking status was self-reported by mothers and this may have resulted in omission of smoking status. Prevalence for vitamin D deficiency is seen in populations at higher levels [228] than levels the prevalence for newborn HL [229]. Vitamin D has been investigated in NDBS in the past [230,231], however vitamin D quantification may be compromised by similar metabolites present in the blood [232]. This study lends support for further investigations into association between calcium, vitamin D, and auditory function. More vulnerable members of our sample may be showing signs of HL related to vitamin D deficiency to a greater extent than those who are more resilient.

Iron and potassium have been both linked to HL outcomes separately. Anemia, or deficient levels of iron, has been associated with raised thresholds in rats [233] and humans [234]. Potassium has also been linked to problems of sudden sensorineural HL [235]; and it is involved in a treatment for blast trauma which provides osmotic stabilization [236]. Adding potassium and iron to the models with calcium made both iron and calcium significant, for all types of HL. Biologically, the electrolytes calcium, iron, and potassium are linked and critical to not only the nervous system, but also the auditory system. These electrolytes are also linked as calcium is administered to patients with dangerously high potassium levels as it increases the potential of cellular thresholds

[237]. Supplementation with high levels of potassium in pregnant dairy cows has been shown to increase the amount of calcium excreted in urine [238]. Dietary calcium has been shown to inhibit absorption of dietary iron, though this issue is not usually a concern in Western societies [239]. A study using the healthy eating index, a generalized metric of nutritional intake calculated from dietary recall National Health and Nutrition Examination Survey data, rather than specific biomarkers, has also indicated high quality nutritional intake to be associated with better hearing, as measured by pure-tone audiometry [240]. Alcohol consumption is one other confounding factor not measured in our study which has been shown to impact homeostasis, though results varied by trimester of pregnancy, of the essential elements calcium, potassium, and iron [241]. Likewise, fetal alcohol syndrome is also associated with hearing disorders [242].

Literature on the other essential elements studied here is conflicted as far as the implications of hearing. High levels of serum selenium have been shown to be protective against cisplatin ototoxicity [243]. However, in a case-control study of adult factory workers in Taiwan with HLs, blood levels of selenium were not significantly protective in all models or in both ears [59]. In animal models, zinc deficiency has been shown to increase auditory brainstem response thresholds [185], as well as have no effects on them [244]. Our study agreed with the later findings showing no significant protective effect due to blood levels of zinc. However, a significant multiplicative interaction was found between selenium and zinc. In the rat, an interaction between selenium and zinc has been previously observed, in that administration of zinc increased retention of selenium [245].

Limitations

This study had several major strengths, including the large sample size of data combining two separately drawn sets of cases and control from the MNBB, and the confirmation of abnormal hearing screenings among cases. However, the study had two central limitations: the NDBS exposure metric used, and the sample source of predominantly white and male Michigander infants. First, while the blood spot represents a unique method for measuring essential and non-essential metals, this metric has several issues that future studies will need to address. The methods we used were developed for the purpose of the study [197], and have several weaknesses, most

notably an inability to account for the amount of blood present on each blood spot punch, and apparent differential deposition of elements across the area of blood spots [246,247]. Errors in blood spot levels could have resulted in exposure misclassification. Seven newborns (2 cases and 5 controls) had negative values reported for blood lead concentrations. Such challenges are not unexpected with new laboratory methods but introduced minor difficulties into our analysis. The few negative values obtained after analysis of blood lead could be due to several possible causes, including systematic changes in our methods over time that occurred despite stringent quality control procedures; changes in the way in which NDBS were collected (for example, the use of different lots or types of filter papers); and potential effects of differing amounts of time in storage for the NDBS themselves. This is concerning; however, we are confident that the rank order of the lead in the samples is stable, and this is perhaps most important when running epidemiological models. While using NDBS samples has many advantages in the long-term, all components available in fresh blood samples are not available to be quantified in dried blood.

The second central limitation relates to potential limitations in the generalizability of our results due to the specific population of Michigander infants we analyzed. Blood lead levels in the US are on average much lower than in other parts of the world [248]. Consequently, these results may not be applicable worldwide or in areas of extremely high lead or mercury exposures. However, previous studies have shown significant findings related to blood lead levels over 10 μg/dL [208], which overlaps with our sample geometric mean of blood lead (26 μg/dL). So, similar findings should have been observed, though the life stage examined by other studies was not in infants, but adolescents and children who can demonstrate deficits in hearing with more nuance [56,208]. We were forced to sample across multiple due to the small number of children with abnormal hearing screening results in a given year. Blood lead levels in US children have been declining since the 1970s [249], though children parts of the US Midwest did not enjoy such a reduction [250]. Also, the sample is predominately white, so levels of vitamin D deficiency may lead to different results in people of color [251,252].

Additional study limitations include the lack of a thorough neurological processing analysis, assessment of exposures at a single timepoint, and the absence of a full battery of metal levels during pregnancy. The pass/fail nature of hearing screenings was unable to investigate hearing as deeply some previous studies, which analyzed complete waveforms from ABR. Our study also did

not involve the same auditory test for all subjects. The timing of lead exposures and variation over the development of the fetus was not captured by a single measure taken after birth and may not have captured the most concerning time-frame for lead exposures to negatively impact the hearing system. Cochlear development occurs in humans at eight weeks and innervation continues through week twenty-two [253]. While the cochlea may only be sensitive to damage in a narrow time range, studies exploring neurotoxicants show that sensitive points in development continue into early childhood [254,255]. Thus, the appropriate window and exposures during this time frame may indeed not have been completely captured by this study. Hearing impairments may take time to develop and some controls may become cases later. This would represent non-differential misclassification, which would bias results toward the null. The amount of calcium, potassium, and iron in the blood are usually tightly regulated, so these may be best interpreted as signals of homeostasis dysregulation and not indicators nutritional status.

Conclusions

To our knowledge, the novel analysis presented here represents the largest of its kind to date; nevertheless, due to the rarity of newborn HL, we may still have had insufficient statistical power to detect associations. These findings support use of the NDBS as a feasible neonatal exposure metric for toxicant and essential metals. While our analysis is the first of its kind, it is likely that our NDBS exposure estimates contain substantial measurement error that would be expected to attenuate the true associations between lead and methylmercury and newborn HL, and also to reduce the effects of nutritional status on HL. Nevertheless, we have demonstrated significant associations between concentrations above 1.8 μg/L methylmercury in NDBS and adverse hearing outcomes among newborns. We also found significant associations between nutrient blood levels and hearing screening failures that give need for further investigations into neonatal (calcium, iron, potassium, selenium, and zinc) blood levels as well as how these factors may be associated with abnormal newborn hearing.

III.F: Tables

Table III-1. Characteristics of 676 newborns in total and by data set [N (%)]

	Total (n=676)		Set 1 (n=200)[†]		Set 2 (n=476)[‡]	
	Cases (n=338)	Controls (n=338)	Cases (n=100)	Controls (n=100)	Cases (n=238)	Controls (n=238)
Sex						
Male	185 (54.7)	185 (54.7)	51 (51)	51 (51)	134 (56.3)	134 (56.3)
Female	153 (45.3)	153 (45.3)	49 (49)	49 (49)	104 (43.7)	104 (43.7)
Race						
White	232 (68.6)	232 (68.6)	68 (68)	68 (68)	164 (68.9)	164 (68.9)
Black	62 (18.3)	62 (18.3)	23 (23)	23 (23)	39 (16.4)	39 (16.4)
Other	44 (13.0)	44 (13.0)	9 (9)	9 (9)	35 (14.7)	35 (14.7)
Ethnicity						
Non-Hispanic	218 (64.5)	217 (64.2)	64 (64)	57 (57)	154 (64.7)	160 (67.2)
Hispanic	6 (1.8)	8 (2.4)	1 (1)	4 (4)	5 (2.1)	4 (1.7)
Unknown	114 (33.7)	113 (33.4)	35 (35)	39 (39)	79 (33.2)	74 (31.1)
Gestational age						
Preterm	15 (4.4)	12 (3.6)	5 (5)	3 (3)	10 (4.2)	9 (3.8)
Full-term	308 (91.1)	312 (92.3)	92 (92)	89 (89)	216 (90.8)	223 (93.7)
Unknown	15 (4.4)	14 (4.1)	3 (3)	8 (8)	12 (5.0)	6 (2.5)
Cigarette smoking*						
Yes	51 (15.1)	41 (12.1)	14 (14)	8 (8)	37 (15.6)	33 (13.9)
No	247 (73.1)	253 (74.9)	92 (92)	98 (98)	152 (63.9)	155 (65.1)
Unknown	51 (15.1)	52 (15.4)	2 (2)	2 (2)	49 (20.6)	50 (21.0)
Birth year						
2003-2004	33 (9.8)	33 (9.8)	27 (27)	27 (27)	6 (2.5)	6 (2.5)
2005-2006	66 (19.5)	66 (19.5)	43 (43)	43 (43)	23 (9.7)	23 (9.7)
2007-2008	92 (27.2)	92 (27.2)	30 (30)	30 (30)	62 (26.1)	62 (26.1)
2009-2010	60 (17.8)	60 (17.8)	0 (0)	0 (0)	60 (25.2)	60 (25.2)
2011-2012	53 (15.7)	53 (15.7)	0 (0)	0 (0)	53 (22.3)	53 (22.3)
2013-2014	33 (9.8)	33 (9.8)	0 (0)	0 (0)	33 (13.9)	33 (13.9)
2015	1 (0.3)	1 (0.3)	0 (0)	0 (0)	1 (0.4)	1 (0.4)
Type of test						
AABR	221 (65.4)	282 (83.4)	57 (57)	82 (82)	164 (68.9)	200 (84.0)
ABR	22 (6.5)	5 (1.5)	10 (10)	1 (1)	12 (5.0)	4 (1.7)
DPOAE	93 (27.5)	50 (14.8)	32 (32)	17 (17)	61 (25.6)	33 (13.9)
TEOAE	2 (0.6)	1 (0.3)	1 (1)	0 (0)	1 (0.4)	1 (0.4)
Type of hearing loss						
Bilateral	215 (63.6)	--	68 (68)	--	147 (61.8)	--
Unilateral	123 (36.4)	--	32 (32)	--	91 (38.2)	--

†In data set 1, cases and controls were matched on birth year, sex, race, mother's city, and hospital.
‡In data set 2, cases and controls were matched on birth year, sex, and race.
*Summary statistics only for data set 1 – no individual level data available for conditional logistic regression models

Table III-2. Geographical information collected for total sample (N=676 newborns)

Geographical data obtained	Set 1[†] N (%)	Set 2[‡] N (%)
Mother's city (county)		
Ann Arbor (Washtenaw)	14 (7.0)	14 (2.9)
Canton (Wayne)	8 (4.0)	4 (0.8)
Detroit (Wayne)	36 (18.0)	56 (11.8)
Flint (Genesee)	6 (3.0)	16 (3.4)
Rochester Hills (Oakland)	8 (4.0)	4 (0.8)
Sterling Heights (Macomb)	8 (4.0)	14 (2.9)
Ypsilanti (Washtenaw)	8 (4.0)	16 (3.4)
Other	112 (56.0)	356 (74.8)
Birth hospital (county)		
Henry Ford Hospital – Macomb (Macomb)	12 (6.0)	10 (2.1)
Hurley Medical Center (Genesee)	10 (5.0)	24 (5.0)
Huron Valley Hospital – Sinai (Oakland)	10 (5.0)	10 (2.1)
Hutzel Women's Hospital (Wayne)	14 (7.0)	30 (6.3)
St. John's Hospital (Wayne)	16 (8.0)	16 (3.4)
St. Joseph Mercy Hospital (Washtenaw)	34 (17.0)	18 (3.8)
University of Michigan Hospital (Washtenaw)	12 (6.0)	46 (9.7)
William Beaumont Hospital – Royal Oak (Oakland)	22 (11.0)	74 (15.5)
Other	70 (35.0)	248 (52.1)
Residential county		
Genesee	10 (5)	45 (9.5)
Livingston	4 (2)	14 (2.9)
Macomb	39 (20)	90 (18.9)
Monroe	4 (2)	11 (2.3)
Oakland	36 (18)	106 (22.3)
Washtenaw	24 (12)	36 (7.6)
Wayne	73 (37)	146 (30.7)
Other	26 (13)	27 (5.7)
Unknown	0 (0)	1 (0.2)

[†]Data set 1, N=200, no individual level data available for residential county.
[‡]Data set 2, N=476, no individual level data available for birth hospital and mother's city.

Table III-3. Geometric mean and standard deviation (GM and GSD) blood spot element levels in µg/L and results of t-tests comparing cases and controls overall (III-3A) and for datasets 1 (III-3B) and 2 (III-3C)

Element	N	Cases GM (GSD)	Controls GM (GSD)	p
A. OVERALL				
Non-essential				
Lead	336	2.75×10^2 (1.7)	2.63×10^2 (1.9)	0.80
Methylmercury	336	1.28 (1.86)	1.22 (1.72)	0.33
Essential elements				
Calcium	336	1.17×10^5 (1.5)	1.25×10^5 (1.5×10^5)	0.03
Copper	336	1.04×10^3 (1.5)	1.034×10^3 (1.4×10^5)	0.93
Iron[†]	336	6.36×10^5 (1.47×10^5)	6.20×10^5 (1.45×10^5)	0.15
Potassium[†]	336	214×10^6 (5.58×10^5)	2.13×10^6 (5.17×10^5)	0.75
Selenium[†]	325	1.94×10^2 (5.36×10^1)	1.91×10^2 (4.72×10^1)	0.44
Zinc	336	4.14×10^3 (1.4)	4.22×10^3 (1.5)	0.49
B. SET 1				
Non-essential				
Lead	100	5.14×10^2 (1.4)	5.03×10^2 (1.4)	0.69
Methylmercury	100	1.25 (2.28)	1.19 (1.93)	0.60
Essential elements				
Calcium	100	1.42×10^5 (1.5)	1.51×10^5 (1.5)	0.33
Copper	100	1.07×10^3 (1.3)	1.01×10^3 (1.2)	0.04
Iron[†]	100	7.26×10^5 (1.30×10^5)	6.99×10^5 (1.50×10^4)	0.17
Potassium[†]	100	2.31×10^6 (6.40×10^5)	2.28×10^5 (5.40×10^5)	0.74
Selenium[†]	94	2.05×10^2 (5.68×10^1)	1.96×10^2 (4.45×10^1)	0.22
Zinc	100	4.72×10^3 (1.4)	4.94×10^3 (1.6)	0.41
C. SET 2				
Non-essential				
Lead	236	2.08×10^2 (1.6)	1.96×10^2 (1.8)	0.79
Methylmercury	236	1.29 (1.66)	1.23 (1.63)	0.40
Essential elements				
Calcium	236	1.07×10^5 (1.4)	1.15×10^5 (1.5)	0.04
Copper	236	1.02×10^3 (1.5)	1.05×10^3 (1.5)	0.57
Iron[†]	236	5.98×10^5 (1.39×10^5)	5.86×10^5 (1.30×10^5)	0.35
Potassium[†]	236	2.08×10^6 (5.05×10^5)	2.07×10^6 (4.96×10^5)	0.89
Selenium[†]	231	1.90×10^2 (5.16×10^1)	1.89×10^2 (4.82×10^1)	0.90
Zinc	236	3.91×10^3 (1.4)	3.95×10^3 (1.5×10^3)	0.77

[†]Arithmetic mean and standard deviation used due to normal distribution of data.

Table III-4. Odds ratios and 95% CI for a newborn hearing failure per each interquartile range increase[†] in element levels of whole blood (N=672 newborns)

A. Toxicant metals

Variable/ category	N	Lead OR	Lead 95% CI	Methylmercury OR	Methylmercury 95% CI
All Data	672	1.04	0.86 – 1.26	1.23	0.99 – 1.53
Set 1	200	1.00	0.81 – 1.23	1.25	0.90 – 1.76
Set 2	472	1.21	0.79 – 1.85	1.22	0.92 – 1.62
Males	368	1.13	0.82 – 1.57	1.16	0.87 – 1.55
Females	304	0.99	0.78 – 1.25	1.32	0.96 – 1.84
White	460	0.99	0.80 – 1.22	1.24	0.95 – 1.63
Black	124	1.29	0.70 – 2.35	1.24	0.67 – 2.29
Other	88	1.27	0.66 – 2.45	1.20	0.76 – 1.89
Bilateral HL	428	0.94	0.75 – 1.17	1.07	0.82 – 1.40
Unilateral HL	244	1.67	0.90 – 3.07	1.55	1.06 – 2.25

B. Essential elements

Variable/ category	N	Calcium OR	Calcium 95% CI	Iron OR	Iron 95% CI	Potassium OR	Potassium 95% CI	Copper OR	Copper 95% CI	Selenium OR	Selenium 95% CI	Zinc OR	Zinc 95% CI
All Data	672*	0.49	0.34 – 0.70	1.27	0.99 – 1.61	1.04	0.84 – 1.30	1.00	0.93 – 1.08	1.11	0.89 – 1.38	0.94	0.85 – 1.04
Set 1	200*	0.72	0.46 – 1.12	1.32	0.90 – 1.95	1.05	0.78 – 1.41	1.70	1.05 – 2.78	1.26	0.86 – 1.86	0.92	0.80 – 1.07
Set 2	472*	0.26	0.13 – 0.51	1.23	0.89 – 1.68	1.04	0.75 – 1.44	0.98	0.91 – 1.07	1.03	0.77 – 1.36	0.97	0.82 – 1.14
Males	368*	0.52	0.31 – 0.88	1.41	1.01 – 1.98	1.30	0.91 – 1.85	1.00	0.91 – 1.10	1.14	0.82 – 1.59	0.88	0.72 – 1.08
Females	304*	0.46	0.28 – 0.77	1.12	0.79 – 1.59	0.91	0.68 – 1.20	1.00	0.88 – 1.13	1.08	0.80 – 1.46	1.00	0.86 – 1.16
White	460*	0.46	0.30 – 0.72	1.16	0.87 – 1.54	0.91	0.73 – 1.23	0.96	0.85 – 1.07	1.18	0.88 – 1.57	0.94	0.84 – 1.04
Black	124*	0.51	0.22 – 1.17	2.01	0.99 – 4.07	1.45	0.78 – 2.68	1.03	0.90 – 1.18	0.93	0.58 – 1.51	0.96	0.65 – 1.43
Other	88*	0.63	0.23 – 1.72	1.30	0.69 – 2.43	1.27	0.68 – 2.37	1.14	0.85 – 1.53	1.11	0.66 – 1.85	1.01	0.60 – 1.69
Bilateral HL	428*	0.48	0.30 – 0.75	1.26	0.94 – 1.69	0.94	0.94 – 1.69	1.01	0.93 – 1.10	1.13	0.87 – 1.48	0.96	0.83 – 1.11
Unilateral HL	244*	0.51	0.27 – 0.94	1.29	0.83 – 2.00	1.24	0.83 – 2.00	0.93	0.76 – 1.15	1.04	0.69 - 1.58	0.92	0.78 - 1.09

[*] N values for selenium differ slightly: 650 for all, 188 set 1, 462 set 2; 361 males, 289 females; 444 whites, 122 blacks, 84 other; 410 bilateral HL, 240 unilateral HL

[†] IQRs (µg/L whole blood): Lead = 224.3; Methylmercury = 0.9235; Calcium = 50587.25; Iron = 174282.1; Potassium = 627897.3; Copper = 50587.25; Selenium = 174282.1; Zinc = 6278

Table III-5. Odds ratios for a newborn hearing failure per each interquartile range increase[†] in levels of whole blood lead or methylmercury levels

A. Lead

		Quartile of lead in whole blood				
		1	**2**	**3**	**4**	
Range (µg/L)		-41 to 186	187 to 250	251 to 410	411 to 3558	
n cases/total		77/162	97/180	75/160	87/170	
Models	**N**	**OR (CI)**	**OR (CI)**	**OR (CI)**	**OR (CI)**	**p (trend)**
1. Pb only	672	Reference	1.32 (0.84 - 2.07)	0.99 (0.60 - 1.63)	1.20 (0.61 - 2.34)	0.75
2. Pb, calcium in whole blood	672	Reference	1.38 (0.88 - 2.18)	1.04 (0.63 - 1.73)	1.37 (0.69 - 2.74)	0.50
3. Pb, mother smoking status*	672	Reference	1.32 (0.84 - 2.09)	0.99 (0.60 - 1.65)	1.20 (0.61 - 2.36)	0.75
4. Pb, preterm birth	643	Reference	1.25 (0.79 - 2.00)	0.88 (0.52 - 1.47)	1.04 (0.52 - 2.11)	0.90
5. Pb, calcium in whole blood, mother smoking status*, preterm birth	643	Reference	1.30 (0.81 - 2.10)	0.94 (0.55 - 1.61)	1.21 (0.58 - 2.53)	0.79

B. Methylmercury

		Quartile of methylmercury in whole blood				
		1	**2**	**3**	**4**	
Range (µg/L)		0.08 to 0.89	1.90 to 1.24	1.25 to 1.76	1.77 to 10	
n cases/total		81/166	67/151	84/168	104/187	
Models	**N**	**OR (CI)**	**OR (CI)**	**OR (CI)**	**OR (CI)**	**p (trend)**
6. MeHg only	672	Reference	0.87 (0.54 - 1.41)	1.27 (0.75 - 2.14)	1.81 (1.01 - 3.24)	0.030
7. MeHg, calcium in whole blood	670	Reference	0.73 (0.36 - 1.45)	0.76 (0.43 - 1.34)	1.00 (0.51 - 1.96)	0.025
8. MeHg, mother smoking status*	672	Reference	0.88 (0.54 - 1.44)	1.3 (0.76 - 2.22)	1.84 (1.02 - 3.32)	0.026
9. MeHg, preterm birth	643	Reference	0.88 (0.53 - 1.45)	1.22 (0.7 - 2.12)	1.65 (0.89 - 3.07)	0.084
10. MeHg, calcium in whole blood, mother smoking status*, preterm birth	641	Reference	0.89 (0.53 - 1.49)	1.22 (0.68 - 2.21)	1.72 (0.89 - 3.32)	0.079

*smoking status was indicated as smoker/non-smoker/unknown. †IQR (µg/L whole blood): Lead = 224.3; Methylmercury = 0.92. Pb=lead; MeHg=methylmercury

Table III-6. Odds ratios for a newborn hearing failure per each interquartile range increase[†] in levels of whole blood elements for all (unilateral and bilateral) hearing loss cases (N=672)

		Quartiles in whole blood			
		1	2	3	4
Calcium	Range (µg/L)	3.1E4 to 9.6E4	9.6E4 to 1.2E5	1.2E5 to 1.5E5	1.5E5 to 4.3E5
	n cases/total	100/184	88/173	80/163	68/152
Iron	Range (µg/L)	3837 to 5.3E5	5.3E5 to 6.2E5	6.2E5 to 7.0E5	7.0E5 to 1.5E6
	n cases/total	72/157	91/175	70/153	103/187
Potassium	Range (µg/L)	2014 to 1.8E6	1.8E6 to 2.1E6	2.1E6 to 2.5E6	2.5E6 to 4.5E6
	n cases/total	74/158	99/183	80/164	83/167

Models	Continuous OR (CI)	OR (CI)	OR (CI)	OR (CI)	OR (CI)	p (trend)
11. Calcium only	0.49 (0.34-0.70)	Reference	0.73 (0.43 - 1.24)	0.58 (0.32 - 1.06)	0.34 (0.16 - 0.72)	0.0053
12. Iron only	1.26 (0.99-1.61)	Reference	1.41 (0.87 - 2.27)	1.14 (0.69 - 1.87)	1.77 (1.03 - 3.06)	0.11
13. Potassium only	1.04 (0.84-1.30)	Reference	1.36 (0.87 - 2.15)	1.11 (0.67 - 1.84)	1.16 (0.67 - 1.98)	0.86
14. Ca+Fe						
Calcium	0.45 (0.31-0.65)	Reference	0.67 (0.39 - 1.15)	0.55 (0.30 - 1.01)	0.30 (0.14 - 0.64)	0.0024
Iron	1.43 (1.10-1.86)	Reference	1.49 (0.92 - 2.44)	1.27 (0.76 - 2.10)	2.07 (1.18 - 3.63)	0.040
15. Ca+K						
Calcium	0.37 (0.24-0.57)	Reference	0.63 (0.36 - 1.10)	0.46 (0.24 - 0.89)	0.26 (0.11 - 0.58)	0.0018
Potassium	1.47 (1.11-1.93)	Reference	1.66 (0.95 - 2.91)	1.48 (0.77 - 2.84)	1.80 (0.79 - 4.08)	0.13
16. Ca+Fe+K						
Calcium	0.39 (0.25 - 0.62)	Reference	0.64 (0.36 - 1.12)	0.51 (0.26 - 1.00)	0.28 (0.12 - 0.65)	0.0050
Iron	1.21 (0.82 - 1.80)	Reference	1.26 (0.73 - 2.18)	1.13 (0.60 - 2.12)	2.00 (0.95 - 4.20)	0.16
Potassium	1.26 (0.83 - 1.91)	Reference	1.52 (0.87 - 2.63)	1.25 (0.63 - 2.50)	1.17 (0.51 - 2.67)	0.90
17. Ca+Fe+K+smoking status						
Calcium	0.39 (0.25 - 0.63)	Reference	0.63 (0.36 - 1.11)	0.50 (0.26 - 0.99)	0.28 (0.12 - 0.65)	0.0048
Iron	1.21 (0.81 - 1.80)	Reference	1.24 (0.72 - 2.16)	1.11 (0.59 - 2.10)	1.97 (0.93 - 4.16)	0.16
Potassium	1.26 (0.83 - 1.91)	Reference	1.52 (0.88 - 2.66)	1.26 (0.63 - 2.53)	1.18 (0.52 - 2.69)	0.88

† IQRs (µg/L whole blood): Calcium = 50587.25; Iron = 174282.1, Potassium = 627897.3. Ca=calcium; Fe=iron; K=potassium

Table III-7. Odds ratios for a newborn hearing failure outcome per each interquartile range increase[†] in levels of whole blood elements for only bilateral cases (N=428)

		Quartiles in whole blood			
		1	**2**	**3**	**4**
Calcium	Range (µg/L)	3.1E4 to 9.9E4	9.9E4 to 1.2E5	1.2E5 to 1.5E5	1.5E5 to 4.3E5
	n cases/total	70/124	52/106	50/103	42/95
Iron	Range (µg/L)	3837 to 5.3E5	5.3E5 to 6.2E5	6.2E5 to 7.1E5	7.1E5 to 1.2E6
	n cases/total	42/97	60/112	49/104	63/115
Potassium	Range (µg/L)	2014 to 1.8E6	1.8E6 to 2.1E6	2.1E6 to 2.5E6	2.5E6 to 3.6E6
	n cases/total	52/106	59/112	57/111	46/99

Models	Continuous OR (CI)	OR (CI)	OR (CI)	OR (CI)	OR (CI)	p (trend)
18. Calcium only	0.48 (0.30-0.75)	Reference	0.61 (0.32 - 1.16)	0.54 (0.27 - 1.06)	0.34 (0.15 - 0.79)	0.013
19. Iron only	1.26 (0.94-1.69)	Reference	1.61 (0.90 - 2.89)	1.30 (0.72 - 2.37)	1.80 (0.95 - 3.40)	0.15
20. Potassium only	0.95 (0.72-1.25)	Reference	1.14 (0.65 - 2.02)	1.05 (0.57 - 1.94)	0.86 (0.44 - 1.70)	0.62
21. Ca+Fe						
Calcium	0.46 (0.29-0.73)	Reference	0.58 (0.30 - 1.13)	0.51 (0.25 - 1.01)	0.31 (0.13 - 0.73)	0.0072
Iron	1.33 (0.98-1.81)	Reference	1.66 (0.91 - 3.01)	1.45 (0.79 - 2.68)	2.06 (1.07 - 3.97)	0.070
22. Ca+K						
Calcium	0.39 (0.23-0.68)	Reference	0.55 (0.28 - 1.10)	0.46 (0.22 - 0.98)	0.30 (0.12 - 0.74)	0.012
Potassium	1.29 (0.92-1.81)	Reference	1.39 (0.76 - 2.54)	1.47 (0.74 - 2.91)	1.37 (0.63 - 2.98)	0.52
23. Ca+Fe+K						
Calcium	0.44 (0.25 - 0.78)	Reference	0.62 (0.31 - 1.25)	0.56 (0.25 - 1.22)	0.36 (0.14 - 0.93)	0.0460
Iron	1.29 (0.81 - 2.04)	Reference	1.62 (0.82 - 3.19)	1.55 (0.70 - 3.44)	2.69 (1.07 - 6.78)	0.054
Potassium	1.04 (0.63 - 1.74)	Reference	1.06 (0.52 - 2.16)	0.93 (0.37 - 2.32)	0.64 (0.21 - 1.94)	0.35
24. Ca+Fe+K+smoking status						
Calcium	0.44 (0.25 - 0.78)	Reference	0.63 (0.31 - 1.26)	0.56 (0.25 - 1.24)	0.36 (0.14 - 0.94)	0.047
Iron	1.29 (0.81 - 2.05)	Reference	1.64 (0.82 - 3.31)	1.57 (0.70 - 3.52)	2.74 (1.07 - 7.03)	0.056
Potassium	1.04 (0.62 - 1.74)	Reference	1.05 (0.51 - 2.16)	0.92 (0.36 - 2.31)	0.62 (0.20 - 1.93)	0.34

[†] IQRs (µg/L whole blood): Calcium = 50587.25; Iron = 174282.1; Potassium = 627897.3. Ca=calcium; Fe=iron; K=potassium

Table III-8. Odds ratios for newborn hearing screening failure per each interquartile range increase† with toxic metals, essential elements, and interaction term

	Model 25	Model 26	Model 27	Model 28
	All cases	**Bivariate HL**	**All cases**	**Bivariate HL**
MeHg	-	-	1.37 (1.07 - 1.77)	1.15 (0.85 - 1.54)
Ca	0.42 (0.27 - 0.65)	0.40 (0.23 - 0.69)	0.41 (0.27 - 0.64)	0.40 (0.23 - 0.69)
Fe	1.49 (1.06 - 2.10)	1.30 (0.86 - 1.98)	1.46 (1.04 - 2.06)	1.30 (0.86 - 1.96)
Se	0.46 (0.23 - 0.92)	0.39 (0.16 - 1.00)	0.44 (0.22 - 0.88)	0.39 (0.15 - 0.98)
Zn	0.40 (0.19 - 0.83)	0.30 (0.11 - 0.83)	0.36 (0.17 - 0.76)	0.29 (0.10 - 0.80)
Se*Zn	1.30 (1.06 - 1.61)	1.42 (1.06 - 1.90)	1.33 (1.08 - 1.65)	1.43 (1.07 - 1.92)

†IQR (μg/L whole blood): Methylmercury = 0.9235; Calcium = 50587.25; Iron = 174282.1, Selenium = 174282.1; Zinc = 6278
MeHg=methylmercury; Ca=calcium; Fe=iron; Se=selenium; Zn=zinc

III.G: Figures

Figure III-1. Lead in blood by two-year* increments for cases and controls
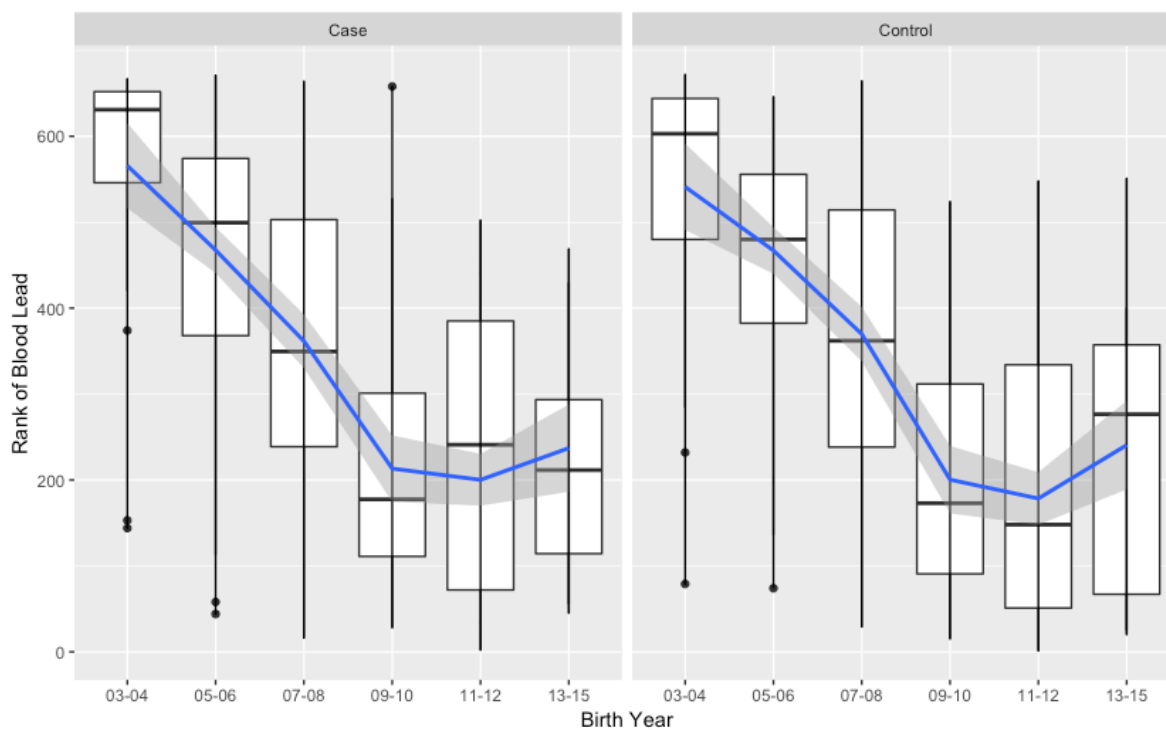
*Two individuals from 2015 were included with the 2013-2014 group

66

Figure III-2. Calcium, Iron and Potassium correlation matrix with scatterplots (and fitted lines), frequency distribution and correlations values (p-values shown)
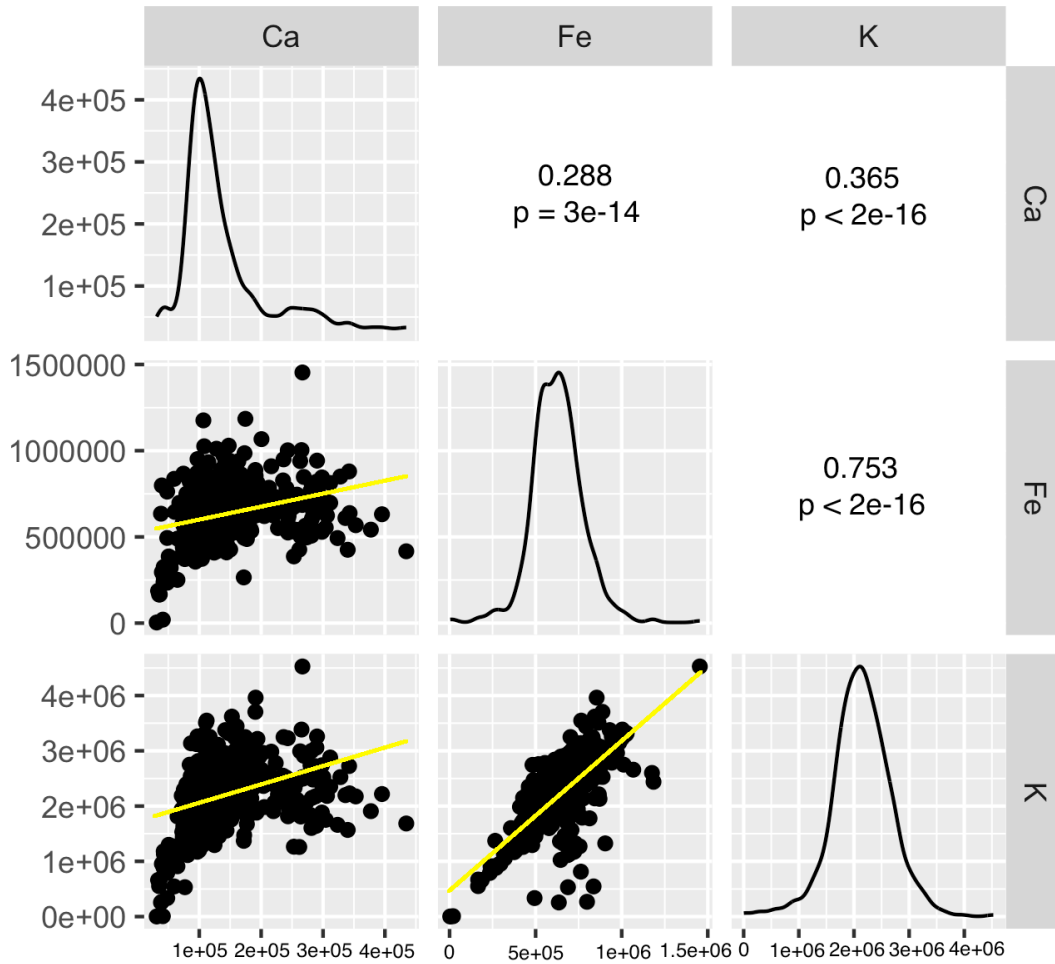
CHAPTER IV: Metal exposures, noise exposures, and audiometery from e-waste workers in Agbogbloshie Ghana

IV.A: Abstract

Metals such as lead, mercury, and cadmium may have ototoxic properties, but are not well understood as causal agents of hearing loss, especially in conjunction with noise exposure. This cross-sectional study investigated hearing health, noise exposures, and metal exposures in a marginalized population of electronic waste (e-waste) recycling workers at Agbogbloshie Market in Accra, Ghana. Workers were recruited on-site and participated in audiometric testing, a survey, blood collection, and personal noise dosimetry. An indicator of work activity diversity was developed to quantify the proportion of possible tasks in which workers engaged. Audiometric results were analyzed by test frequency using several noise-induced hearing loss notch indices. We recruited a total of 58 workers; 60% of whom showed evidence of audiometric notches, most commonly centered at 6 kHz. Most workers (86%) reported high noise exposures occurring "often" or "fairly often" in their work. Daily average noise levels, including both occupational and non-occupational time periods, ranged from 74.4 - 90.0 dBA. Linear regression models indicated that participants who lived at Agbogbloshie for longer periods were significantly associated with worse thresholds at both 4 and 6 kHz. Linear models did not identify blood levels of lead, mercury, or cadmium as significant predictors of worse hearing thresholds or larger noise notches, but increasing levels of selenium were significantly associated with higher thresholds at 6 kHz and a larger noise notch. Workers with higher work diversity had significantly larger noise notches in the final linear model. Final models predicting thresholds at 4 and 6 kHz were improved by including a significant interaction term between the maximum noise exposure and the level of zinc in whole blood. This interaction indicates that zinc may be protective against detrimental hearing outcomes by noise exposures, but no longer protective at heightened noise levels. Further study of the relationships between metals, noise, and HL is needed among informal e-waste recyclers and other high-risk occupations with multiple hazardous exposures as well as health vulnerabilities.

IV.B: Introduction

Worldwide, 466 million people are estimated to suffer from hearing loss (HL) and global prevalence is increasing [256]. The prevalence of adult-onset HL in developing nations, a large portion of which is due to occupational noise [4], is also increasing [3]. HL has profound impacts on human health and quality of life as well as a wide variety of adverse social, psychological, occupational, and educational outcomes [1]. Individuals with HL experience detrimental impacts on educational achievements, employment status as well as challenges within the workplace [257,258].

One type of HL, noise-induced HL (NIHL), is completely preventable but also unfortunately prevalent. In the US, one in four adults has indications of NIHL and about one in three adults working in noise have NIHL [259]. Estimating the exact prevalence of NIHL is challenging as many individuals in the US either do not have access to, or do not give priority to, their hearing health, even when they would benefit from treatment. Only three in ten adults un under age 35 with hearing impairments will obtain a hearing aid in the US [260].

With such high numbers of HL cases, understanding how chemicals in the environment and taken therapeutically may be linked to HL is important. Pharmaceuticals such as aminoglycosides are well known to cause negative outcomes in the auditory system [261], however, the emerging ototoxic effects of metals such as lead (Pb), mercury (Hg), and cadmium (Cd), are less understood. Metals occurring in occupational exposures are an emerging area of ototoxicity research [262,263]. The National Institute for Occupational Safety and Health (NIOSH) has highlighted the need for further research examining the relationship of metals to HL, especially those occurring in mixtures [8].

Elevated exposures to Pb, Hg, and Cd occur in many occupational settings, and environmental contamination with these metals is common in industrial and post-industrial communities. Though the precise mechanisms by which Pb and Cd exposure produces ototoxicity are not yet known, these three metals can induce oxidative stress [264,265], an action that fits well with prevailing hypotheses of noise trauma and drug-induced HL.

A number of epidemiological studies indicate that exposures to metals, in particular Pb and Cd, may be associated with an increased risk of HL [11–14]. American adolescents with higher Pb and Cd exposures have shown a greater likelihood for HL [56]. In a more aged population, x-ray fluorescence has been used to measure life-time accumulated bone Pb levels, and levels of Pb in bone were significantly associated with threshold increases at higher frequencies [57]. Park et al. (2010) also found increases in HL with Pb exposures, even after accounting for occupational noise exposures [57]. While data on Hg and HL are lacking in humans, Hg has also been associated with adult-onset HL in monkeys [202].

Exposures to metals mixtures can evoke a synergistic response [89], and synergism and potentiation interactions have been noted for known non-metal ototoxicants like styrene, aminoglycosides, and jet fuel [70–72]. At least one study has suggested a possible interaction between noise and Pb exposure [74]. A handful of studies have assessed the contributions of Pb and noise to HL [57,73–75], but none have evaluated the effects of combined exposures to Cd and noise, nor have any studies evaluated the potential interactions between noise and metal exposures alongside nutritional essential element levels. Better understanding of multi-factorial exposures is necessary to understand and reduce the risk of HL among workers.

While Pb, Hg, and Cd exposures in high-income countries are relatively well-controlled, the same is not true in low- and middle-income countries. In Ghana and other West African countries, for example, the high demand for electronic and electrical equipment (EEE) at affordable prices has resulted in massive importation of mostly secondhand EEE from developed countries [266,267]. Though Ghana, as of 2003, is a signatory of the Basel Convention [268], which forbids transboundary movement of hazardous wastes, exporters send unusable EEE into Ghana every year under the pretense of donating second-hand EEE [266,269]. These waste electronics are commonly referred to as "e-waste." In 2009, 35% of secondhand EEE imports to Ghana (a total of 280,000 tons) were non-functioning, illegal e-waste [269], typically recycled by informal workers. E-waste flows to Ghana are projected to at least double by 2020 [270].

The informal sector in Ghana largely processes e-waste in two ways: manual dismantling with rudimentary tools and open-air burning [270]. Many of the workers are young, and few use any personal protective equipment [26,270]. Reusable parts and resalable copper, aluminum, gold,

silver, iron, and brass are extracted and sold from electronic and electric products such as televisions, monitors, computers, audio equipment, cameras, printers, telephones, mobile phones, and household appliances [270,271]. However, e-waste also contains metals, including Pb, Cd, and Hg, as well as plastic components, which when burned produce toxic organic compounds [267]. Several studies at Agbogbloshie Market, Ghana's largest e-waste site, have found elevated levels of Cd and Pb in urine of e-waste workers compared with reference sites [26] and elevated concentrations of Pb and Cd in dust at the site and its surroundings [27].

The aims of this study were three-fold. First, we characterized the blood metal levels, occupational habits, and noise exposures experienced by e-waste workers at Agbogbloshie Market. Second, we assessed the associations of essential and toxic metals in whole blood from e-waste workers with pure-tone audiometric thresholds as well as measures of NIHL, or noise notches (NN). Thirdly, we assessed potential interactions between essential and toxic metal levels and noise.

IV.C: Methods

All research procedures were reviewed and approved by the University of Michigan Health Sciences and Behavioral Sciences Institutional Review Board (HUM00084062) and the Noguchi Memorial Institute for Medical Research Institutional Review Board (NMIMR-IRB CPN 070/13-14) prior to collection of data. Participants received a meal and a small financial incentive for participating in the study.

Participant recruitment

Participants were recruited from Agbogbloshie Market with assistance from the local Chairman of the Greater Accra Scrap Dealer's Association (who nominally oversaw the site, though employed no workers directly), as well as from three translators, one of which was a tribal chief in Northern Ghana. The first eight participants were approached directly by the chief, then subsequent workers were recruited by previous participants; a small fraction were recruited directly by research staff. All participants were over the age of 18 and worked at Agbogbloshie for at least the past six

months. Often, workers were recruited in clusters of two to five people working on similar waste materials and in similar locations.

Participants were transported at no cost to the Ghana Post Clinic nearby to Agbogbloshie. At the clinic, participants underwent five study procedures, described in detail below. All study procedures were facilitated by a translator.

Health evaluation

A brief medical examination was conducted for each participant. Information recorded during the exam included: current health insurance, height, weight, waist circumference, hip circumference, numbers and sites of work-related scars or deformities, skin disorders, and any other health complaints.

An outdoor clinic at the e-waste work site was held at the conclusion of data collection to allow participants to meet with the doctor again and go over any health concerns discussed during the survey or not addressed within our study. Medications for common fungal infections and malaria were made available to those needing treatment.

Hearing assessment

An audiometric test was administered using an OtoPod M2 portable audiometer (Otovation, Inc.). We obtained threshold levels from the frequencies 500, 1000, 2000, 3000, 4000, 6000, and 8000 Hz on both ears. Two thresholds on both the right and left side were collected at 1000 Hz for verification of data quality. Background noise from the surrounding environment was attenuated with ear cups and insert headphones, which were estimated to reduce noise exposures by approximately 40 dB across the frequency range of interest. Background noise levels were checked prior to the start of each audiometric test; in the event of high background noise levels (>50 dBA), the test was interrupted until the noise levels declined. All testing was conducted by a technician certified by the Council for Accreditation in Occupational Hearing Conservation (CAOHC).

Survey

A survey was administered to participants to assess various occupational- and hearing-related factors. Surveys were filled out for the participants by the translators who administered them. Survey items included demographic information about sex, age, education, marriage, income, hours worked per day, smoking habits, and living arrangements. Other risk factors for HL (e.g., congenital HL, ear infections, etc.), along with self-reported hearing status, were assessed to control for possible confounding. The items specific to hearing status included: whether participants had problems with their hearing, and, if so, when they started having these problems; whether a doctor had diagnosed any HL; and whether they were taking any medications for this diagnosis. Additional items enquired about participants' perceived occupational and non-occupational noise exposure and annoyance associated with these exposures. The questionnaire collected further information on occupational history and exposures, socioeconomic status, occupational and residential noise exposures, use of personal protective equipment, and dietary intake.

Blood biomarkers

A trained phlebotomist collected a blood sample from each participant using standard laboratory procedures. Blood was drawn in a Vacutainer tube and refrigerated. Samples were analyzed at McGill University using Inductively-Coupled Plasma-Mass Spectrometry (ICPMS, Varian 720, Agilent, Santa Clara, CA USA) for levels of Pb and Cd and a direct mercury analyzer (DMA) for methylmercury (MeHg). We also measured a number of essential and toxic elements, including calcium (Ca), manganese (Mn), copper (Cu), iron (Fe), and zinc (Zn), and arsenic (As), again using ICPMS.

Personal noise measurements

Noise exposures were assessed over a nominally 24-hour period on each subject. Measurements were collected using a personal dosimeter (Etymotic Research ER-200D) that datalogged the equivalent continuous average exposure (L_{EQ}) and maximum (L_{max}) noise levels every 3.75 min

over the 24-hour period; this datalogging interval was the only one available on the device. The measurement range of the dosimeter was 70-130 dBA, and the dosimeter was configured according to the guidelines of the World Health Organization [272]. The monitored period was intended to include work and non-work activities such as prayers, exercise, and sleep. Activity times and durations were collected following the measurement period using a separate researcher-administered activity log to allow for determination of noise levels during specific activities, as well as over the entire 24-hour period. L_{EQ} and L_{max} data was used in linear and logistic modeling for differing time periods of overall, occupational, and non-occupational. Occupational noise exposures were converted to 8-hour Time-Weighted Average (TWA) levels to allow for comparison to occupational exposure limits using equation 1:

$$TWA = 10 x log_{10}[\ 1/128 \int_{i=1}^{N} 1 x 10^{L_{AEQ}/10}]$$ (Equation 1)

where L_{AEQ} is a 3.75-minute average equivalent continuous noise level, N is the total number of 3.75-minute intervals i in the measured shift, and 128 is the number of 3.75-minute intervals in a 480-minute (i.e., 8-hour) period.

Analysis

Statistical analyses were performed in R (version 3.5.1, R Foundation for Statistical Computing: www.r-project.org) using RStudio (version 1.1.456, RStudio Inc.). Descriptive statistics were computed for all variables. Significance was determined by p-values below 0.05 and borderline significance was determined by p-values less than 0.10.

Both L_{max} and L_{EQ} levels were explored in bivariate and regression analyses. Numerous 3.75 minute dosimeter intervals had values of 0 dBA; these indicated measured noise levels that were below the dosimeter's threshold of 70 dBA for the entire 3.75 minute interval. To account for this limit of detection issue, we replaced these 0 dBA values with a value of 50 dBA.

In addition to analysis of survey items on primary jobs and e-waste activity involvement, a variable to describe work activity diversity, WorkD, was created by dividing the number of different e-waste activities workers said they had performed in the past three months by the total number of activities evaluated. This variable was intended to summarize the diversity of a worker's job activities as a surrogate for the variability in their exposure to various metals in e-waste. A few workers were not asked about all activities on the survey; in these cases, the denominator used was the number of activities for which responses were recorded. One participant who did not report performing any of the activities had a WorkD=0 and was removed from further analyses based on this statistic.

The outcome of interest, audiometric hearing threshold levels in dB HL, was explored in several ways. We analyzed hearing thresholds at each audiometric test frequency; for these analyses, larger positive numbers indicate a greater degree of HL. Since NIHL is characterized by the appearance of worse hearing thresholds at 4 and/or 6 kHz (the so-called 4 kHz noise notch, NN), we also computed several NN indices to evaluate the presence and area of audiometric notches. These indices calculate NN as a negative number, with larger negative numbers indicating a worse notch. All NN calculations consisted of a background average threshold compared to a threshold at a frequency or average across multiple frequencies at the expected notch location(s). The notch criteria considered are shown below in equations 2-5 [273]:

$$NN_A = (\text{average of 1 and 8 kHz}) - (\text{average of 3, 4, and 6 kHz}) \qquad \text{(Equation 2)}$$

$$NN_B = (\text{average of 2 and 8 kHz}) - (\text{average of 3, 4, and 6 kHz}) \qquad \text{(Equation 3)}$$

$$NN_C = (\text{average of 3 and 8 kHz}) - (\text{average of 4 and 6 kHz}) \qquad \text{(Equation 4)}$$

$$NN_D = (\text{average of 3 and 8 kHz}) - (6 \text{ kHz}) \qquad \text{(Equation 5)}$$

Bivariate relationships between all variables (e.g., survey responses, work activities, measured noise exposures, blood metals levels, and hearing threshold levels) were evaluated using non-parametric Spearman correlation coefficients and scatterplots. Variables showing moderate or

greater correlations with hearing threshold levels or the various NN values were further explored in a series of univariate linear regressions.

Finally, a series of multivariate linear regression models was developed using a forward stepwise selection process utilizing variables which showed significance and borderline significance to assess the effects of various predictors identified *a priori* as important exposure determinants or confounders. *A priori* predictors included: age; years working in noisy environments; years working in scrap; years living at Agbogbloshie; overall averages for levels of noise exposure; maximums for noise exposures; averages for occupational noise exposures; levels of toxic metals Pb, Hg, Cd, As, and Mn (adjusted for interquartile range [IQR]); levels of essential elements Cu, Fe, Se, Zn (adjusted for IQR); level of income; number of times eating meat in a week (as an indicator of nutritional status). Occupational task-based analyses of hearing and noise exposure to determine the extent to which certain tasks may influence hearing over others included worker's primary task (limited to one of four: dealing, dismantling, collecting, or other), as well as analysis of recently performed tasks (including all possible tasks reported on the survey: Pb battery recycling, smelting, repair, collection, sorting, removing wires, dismantling, burning, and sorting through ash).

Final models were identified using forward stepwise regression techniques with variables which showed significance or borderline significance in univariate models and then subsequently adding more *a priori* variables to improve the significance of the terms. Three final models were selected for three hearing outcomes (frequency-specific thresholds at 4 and 6 kHz and NN_D, using equation 5) based on model performance, as measured by the highest coefficient of determination, adjusted R^2. Principle component and factor analysis approaches in modeling groups of metals and job tasks were also utilized in regression modeling.

IV.D: Results

Data collection occurred over seven days in April 2014. A total of 58 e-waste workers were recruited, with the majority (n=32) recruited over three consecutive days in the middle of the data collection period. At least 20 workers refused to participate in the study; refusals were primarily due to schedule conflicts and disruptions in the Agbogbloshie community due to the death of a

prominent local leader shortly before data collection began. A few workers denied interest in participation due to a low-level recruitment fee in comparison to their wages dismantling waste.

Table IV-1 shows basic demographic characteristics of the sample. Though we had obtained IRB approval to recruit participants as young as 16, the youngest participant was 18. Participants were generally young adults (mean age of 26), low income (38% had daily income <10 GHS, or about $3 US based on the 12/31/2014 exchange rate of $1 to 3.22 GHS), and Muslim (97%), and had worked in e-waste activities and lived near Agbogbloshie for approximately 6 years on average. Most workers (78%) moved to Agbogbloshie from the places like Tamale, Bolgatanga, Kumbungu, and Gushiegu in the Northern region of Ghana. The majority of participants were single (38%) or married and living with their spouse (38%), and had no formal education (53%). The vast majority of workers recruited (71%) lived and slept at the site itself; fifteen workers (26%) lived near the site. Only two participants (3%) lived far from the site. The workers and the site were transient with no permanent structures or formal industries. Given this temporary nature and lower social status of workers, health was an overall concern; surveys showed that 46 percent of the workers felt their health was poor or fair (data not shown).

Noise

Participants worked an average of 6.1±4.2 years in loud noise and 6.2±4.2 working on scrap or e-waste (Table IV-1). For their current work in e-waste, the vast majority of participants reported being in loud noise very often (85%, Table IV-2). Summary statistics for noise measurement data are given in Table IV-2. Roughly half of the 1090.63 hours of noise data collected were below the dosimeters' 70 dBA limit of detection (data not shown). L_{EQ} exposures measured over a large fraction of a day (19.1±4.1 hours on average) had a mean of 82.5±3.8 dBA, with a range of 74.4-90.0 dBA (Table IV-2). Participants worked 10.6±2.9 hours/day on average. The mean 8-hour TWA was 84.6 dBA, just below the 85-dBA NIOSH Recommended Exposure Limit, and 397 of the 3.75 minute intervals (>24.8 hours) exceeded 85 dBA. Splined individual noise exposures over the research period showed a diurnal trend in noise levels (Figure IV-1), with nighttime being the quietest time period, but many high (above the WHO recommended 70 dBA daily noise exposure limit) exposures were documented during daytime hours. The maximum noise level for each

individual was 97.0 dBA on average, with a range of 87.5-110.0 dBA, indicating a potential for brief exposures to high levels of noise in e-waste work. Occupational periods showed a higher mean L_{EQ} (85.6 dBA compared to 81.6 dBA), though the L_{max} was higher on average during non-occupational times (96.0 dBA compared to 94.3 dBA). Seventy-nine percent of participants reported that exposures to loud noise were also common during non-occupational periods. Noise exposures on site were dominated by impact sounds from hammers and chisels used to dismantle electric and electronic items. No workers reported, or were ever observed to be, wearing hearing protection devices. Detailed reporting indicating occupational activities and durations was collected from only 16 (29%) of our participants; the remaining participants failed to complete and/or submit activity logs. Researchers observed many workers actively engaging in social activities on the work site and in proximity to work-related activities including impact noise exposures. Muslims practiced prayer and washing rituals a few feet away from occupational activities.

Occupational characteristics

Details of participants' occupational activities are shown in Table IV-3. A total of nine e-waste activities were reported by participants. The vast majority of workers participated in dismantling (86%) and sorting (84%) activities during the six months prior to the study, while relatively few workers had engaged in the more specialized activities of smelting (38%) and e-waste repair (35%). Nearly half of workers (28, or 48%) reported dismantling as their primary e-waste task for the past three months.

Blood metals

Means of blood Pb in participants, 97.2 ppb, or 9.72 µg/dL (Table IV-4), were observed to be six times the US geometric mean of 15.2 ppb for adults over 20 [274]. Average blood Cd in our sample was 2.98 ppb, eight times higher than the geometric mean of 0.378 ppb among US adults over 20 years of age [274]. Few occupational standards or recommendations exist with which these blood metal levels can be compared. The US Occupational Safety and Health Administration (OSHA)

78

requires temporary medical removal from a job if a worker's blood Pb level (BLL) is above 60 µg/dL CFR § 1910.1025(k)(1)(i)(A). None of our participants exceeded this level. However, the maximum BLL of 32.6 µg/L exceeded the Biological Exposure Index (BEI) of 30 µg/100 ml set by the American Conference of Governmental Industrial Hygienists (ACGIH) [275]. The blood Cd levels among these workers varied greatly from 0.9 µg/L to 22.7 µg/L. Both OSHA and ACGIH set biological monitoring standards for Cd at 5 µg/L; the subject with the maximum blood Cd level exceeded these US standards.

Neither OSHA nor BEI limits are not currently available for Mn or As. The average blood Mn level was 12.6±4.2 µg/L (range 6.5-24.6 µg/L), and the blood Hg mean (1.8±1.4 µg/L) and maximum (6.4 µg/L) levels among these workers were in the normal range (under 10 µg/L, well below the toxic threshold of over 50µg/L) (Agency for Toxic Substances and Disease Registry [ATSDR] 1999). Levels of measured essential elements Cu, Fe, Se, and Zn were 1.1±0.7 mg/L, 440.1±628.2 mg/L, 163.6±48.5 µg/L, and 5.5±1.9 mg/L, respectively.

Hearing outcomes

Fifteen participants (25.9%) reported having trouble with their hearing, and five (8.9%) reported having had trouble since childhood or adolescence (data not shown). The majority of participants reported ringing in their ears after exposures to loud noise (67%), being bothered by occupational noise (76%), and being bothered by nighttime non-occupational noise (70%, data not shown). Hearing threshold levels (HTLs) were worse in the right ear for all frequencies except 6 kHz (Table IV-5). Mean HTLs in both ears were below 20 dB at all frequencies except 4 kHz and 6 kHz. Excluding low frequency hearing at 500 Hz (which may have been artificially increased due to background noise levels), HTLs were worst at 6 kHz and followed by 4 kHz. The pattern of the mean HTLs across frequencies was consistent with a noise notch or noise-induced hearing damage (Figure IV-2), with damage worst at 4-6 kHz and recovery at 3 and 8 kHz. Variability in HTLs was greatest at 4 and 6 kHz, but quite high across all test frequencies, as shown by large standard deviation bars.

Noise notch analysis

More than half of participants were found to have a noise notch (Table IV-6). Of those having notches, the majority of participants had bilateral notches. In both the right and left ears, notches were most frequently present at 6 kHz, followed by 4 kHz and then 3 kHz. When the four notch indices were compared, NN_D (which focused on 6 kHz) was found to have an average notch depth that was nearly twice that of the other indices. NN_D and left thresholds at 6 kHZ were found to be significantly and negatively correlated (r=-0.66, p=0.00000003), i.e., higher audiometric thresholds were associated with a more negative NN_D. This fact, when combined with the high correlations between indices NN_A through NN_C, and the majority of notches at 6 kHz, led to an exclusive focus on left-ear 6 kHz audiometric frequency and NN_D for subsequent analyses. The left ear was chosen for analysis due to possible learning effects in the right ear, which was the first ear tested for all participants, none of whom had previously ever had an audiometric test.

Bivariate associations between noise and metals impacts on HL

Blood Pb, blood Cd, and blood Hg were not found to have meaningful bivariate relationships with any of the measures of HL (e.g., hearing threshold levels or notch indices). Perceived noise exposure showed no relation to any of the HL measures. Negligible correlations were found between daily L_{EQ} and left threshold levels at 6 kHz or NN_D (Spearman's rho = 0.09, p=0.51; -0.05, p=0.71; respectively). L_{max} correlations with left threshold levels at 6 kHz and NN_D showed similar non-correlated and non-significant results. Cu was somewhat linearly associated with L_{EQ} (data not shown). Blood Cu was also correlated with blood Cd, Pb, and Se (Spearman's rho = 0.51, p=0.0001; 0.58, p=0.00001; and 0.40, p=0.0003; respectively).

Zn was the only significant predictor of NN_D, while Se, As, and L_{max} all reached borderline significance (p=0.08, p=0.08, and p=0.09, respectively). By comparison, for NN_A, only Mn (p=0.06) reached even borderline significance. Thresholds at 6 kHz were significantly predicted by Se, years working in scrap, and years working at Agbogbloshie. Since years working in scrap and years working at Agbogbloshie were highly colinear, only years working at Agbogbloshie was considered for further analysis.

Multivariate regression analyses

The results of our multivariate analyses for three hearing outcomes, both 4 and 6 kHz audiometric test frequency (Models 1 and 2, respectively) and NN_D (Model 3), are shown in Table IV-7. We started with the significant metals found for each outcome above in bivariate analyses and then forced several variables into both models based on our *a priori* assumptions: years living near Agbogbloshie, smoking status, number of times meat was eaten in a week, work diversity, and noise exposure. Note that we did not force age, a known risk factor for HL, into the model due to the young age of our sample. Rather, we relied on the variable years lived in Agbogbloshie to account for duration of probable exposure to noise and metals. Toxic metals, essential elements, noise levels, and work tasks were added to the models sequentially to explore their impacts. Final models were selected based on adjusted R^2, which ranged from 0.24 (Model 1) to 0.31 (Model 3).

For the 4 kHz model (Model 1), all included coefficients (Cd, Mn, number of times meat is eaten in a week, and years of life at Agbogbloshie) reached statistical significance. Increased levels of Mn, amount of meat eaten in a week, and length of time living at Agbogbloshie were associated with small but significant decrements in hearing ability, on the order of 6 dB HL per µg/L of these metals in blood. Cd was unexpectedly associated with a slight improvement in hearing at 4 kHz. In the 6 kHz model (Model 2), a greater number of years living near Agbogbloshie, Se, and work diversity were each associated with significant decrements in hearing. Work diversity, age, Zn, Se, highest L_{max}, daily L_{EQ}, and age were all significantly associated with worse noise notches in the NN_D model (Model 3), while As was unexpectedly protective. High As levels are found in diets rich with seafood [277], so it is possible that higher As indicates better nutritional status.

Interactions between metals and essential elements were explored. Significant interactions for NN_D included Pb*Zn (p=0.02), Cd*Zn (p=0.01), and Cu*Zn (p=0.01). Mn*Zn, Fe*Zn, and As*Zn showed borderline significance. Among predictors of thresholds at 6 kHz, only Pb*Mn (p=0.03) showed significance. Borderline significant was observed for Hg*As, Mn*Zn, Fe*Zn, As*Fe, As*Zn. Significant interactions as predictors of thresholds at 4 kHz were Mn*Zn (p=0.03), Cu*Se (p=0.03), Fe*Zn (p=0.05), As*Zn (p=0.04), and Fe*Se (p=0.02); borderline significance was

shown by Se*Zn and Hg*Zn. Given the consistency of Fe*Zn in these models, this interaction term was explored in final models, but did not improve them.

One interaction did improve the adjusted R^2 of two (Model 1 and 2) of the final models. There was a highly significant and consistent interaction between Zn and noise levels. This was across all hearing outcome metrics and multiple noise metrics. Thus, an interaction term for L_{max}*Zn was included in the final models (Table IV-7). Se, Fe, and As showed significant interactions with certain noise metrics as well, however these were not consistent (data not shown). Interactions between noise levels and Pb were borderline significant at 6 kHz thresholds for L_{max} (p=0.07) and NN_D for maximum occupational exposures (p=0.06). These interactions became significant only for thresholds at 6 kHz for average non-occupational noise (p=0.04), maximum non-occupational noise (p=0.02). Due to the number of interactions explored, these p-values no longer show statistical significance when multiple comparisons are taken into consideration.

IV.E: Discussion

We successfully recruited and assessed noise- and metal-related exposures and health outcomes for nearly 60 workers at the Agbogbloshie e-waste recycling site. Our study indicated that these workers had unexpectedly high noise exposures during both occupational and non-occupational periods. These elevated noise exposures could help explain the decrements in hearing threshold levels observed among workers through audiometric testing – average right ear thresholds at 4 and 6 kHZ were over 20 dB, which, in a group of workers as young as those assessed (mean age just under 26 years), is much worse than would be expected in a similar group of noise-free workers [278]. The high noise levels observed – the average occupational noise exposure was 85.6 dBA – combined with indications of NIHL (mean left noise notch depth of 9 dB HL) in many workers, suggest that HL prevention efforts are warranted among this group. Additionally, subject report and researcher observations indicated that these workers never use hearing protection devices, though use of other protective equipment (e.g., boots, gloves, and thick aprons for activities with Pb-acid batteries) was reported and observed. Overall, there was interest in many workers for future studies as they understood the hazardous nature of their work.

At Agbogbloshie, e-waste recycling work is conducted outdoors, and workers never wear hearing protection. We observed that the workspace for e-waste recycling workers is often communal, and many non-work-related activities are integrated with work, including social activities. This poses the challenge of effectively separating occupational and non-occupational noise exposures and reinforces the notion that exposures assessment for these workers cannot simply focus on traditional occupational activities. Our noise exposure assessment on these workers, which spanned a period of up to 24-hours, suggests that a holistic approach that integrates exposures from occupational and non-occupational activities is critical to understand the risk of NIHL among these workers, as exposures during non-occupational periods were similar to, and in some cases exceeded, occupational exposures.

Community noise control for Accra, Ghana should be a priority given these findings. The Agbogbloshie market contained traffic, loud music, as well as worker-related noise. The Ghana Environmental Protection Agency (EPA) has been taking action regarding noise pollution by holding a workshop [279] and setting day and night standards by types of activities taking place in an area [280]. Ghana also has noise standards for energy sector employers requiring use of hearing protection over 85 dB [281]. However, given the informal nature of work at Agbogbloshie Market, the diffuse sources of noise in the community, and other pressing public health needs such as sanitation, implementing noise control will be challenging.

While the lack of formal employment arrangements eliminates the potential for an organized, employer-sponsored HL prevention program (HLPP), it is still possible for governmental or non-governmental organizations (NGOs) to provide support to protect workers' hearing. The most basic and evident need for workers is training on occupational health hazards, including noise and metals, as well as their associated potential health impacts. Inexpensive hearing protection devices could also be distributed to the workers, though it should be noted that the likelihood of sustainable provision of hearing protectors to the workforce is extremely small due to economic and logistic challenges, and that such an effort would be largely useless in the absence of concurrent training. Support could also take the form of expert guidance on methods for e-waste recycling that create the least noise possible; this approach may have the added benefit of reducing exposures to metals, dust, and other occupational hazards. Finally, a concerted effort could be made to enroll workers

in the Ghanaian National Health Insurance scheme; this could provide workers with improved access to medical care, including otolaryngological care.

In addition to noise exposure and HL, we have documented exposures to a number of toxic metals, along with several essential metals, among the studied e-waste workers. Average levels of blood Pb in participants (mean = 9.72 µg/dL Pb) were observed to be eight times the US adult average. The average levels of blood Pb in participants were below the OSHA criterion for occupational medical removal at 40µg/dL. However, two participants (3%) had blood Pb levels higher than the more protective ACGIH BEI (30 µg/dL). Average blood Cd was over seven times higher in our sample than the average in US nonsmoking adults (mean = 2.98 ppb Cd). The highest blood Cd levels measured in participants were four times greater than that OSHA criterion of 5 µg/L. The two participants with the highest Cd levels lived far from Agbogbloshie. Both participants were married and lived with their spouse in a rented room, and both had levels of Cd which five to ten times higher than all other participants. The cause of these elevated exposures is unknown, but it is possible they reflect additional, unrecognized local sources of Cd contamination in Accra.

The average blood Mn level in our participants (12.6µg/L, SD=4.2) was higher than the reported average manganese concentration in blood in healthy adults in the US (9µg/L, range from 4-15µg/L) [282]. South African manganese smelter workers with a mean blood manganese level of 12.5 µg/L (SD 5.6) showed significantly worse performance in neurobehavioral tests than workers with lower exposures [283]. Blood Mn alone does not constitute an adequate indicator of overexposure to Mn. Nevertheless, our participants may be at risk of health effects as a result of high blood Mn levels.

The average concentration of blood Se in our participants was 163.6 µg/L (range 72.6-319.3 µg/L), which is higher than the average level for all ages and both sexes of US population (124.8 µg/L) [284]. This level is also comparable to the average levels observed in other populations in Canada, China, Greece, Italy, Finland, New Zealand and Germany, ranging from 59 µg/L to 206 µg/L (SD=6~37) [285–294]. However, the blood Se concentrations in our participants had higher maximum and lower minimum levels [289–291,295] than those observed in other populations, which may reflect dietary variability or insecurity. Both Se overexposure and deficiency are associated with adverse health effects [296,297].

Arsenic (As) is cleared rapidly from blood, and As blood levels therefore indicate only very recent exposures. The average blood arsenic level in our participants was higher than typical values observed in non-exposed individuals (<1µg/L) [298–300] indicating that dietary or drinking water exposures are likely. High levels of As in water and food have been observed in Ghana in the past [301–303]. Conversely, their blood MeHg levels were within a normal range of healthy adults (<10 µg/L, toxic threshold >50µg/L). [276].

The average levels for essential metals Cu, Zn, and Fe were similar to the reported average levels in non-exposed individuals [304–306]. However, individuals with higher levels of these metals may be at risk of some health effects [304]. Iron deficiency has been linked with HL in adults [234]; however the effect of high levels of iron on hearing outcomes is not established.

Our finding of a significant interaction related to noise exposures and blood zinc levels has not previously been shown in the literature. Zinc levels in blood are related to immediate dietary intake [307,308] and deficits have been shown to be associated with hearing problems [309]. Multivariate models using IQR-adjusted and centered data in this study showed that higher levels of zinc was protective when predicting auditory thresholds at 4 kHz and 6 kHz; however the interaction between zinc and noise was positive and significant indicating that zinc may be protective against noise, but at high noise levels zinc is no longer able to protect against auditory threshold decline. In models predicting the noise notch depth, increasing levels of zinc were associated with deeper noise notches, or worse hearing and the interaction term was no longer significant. While findings were somewhat contradictory, the number of models showing significance for zinc and maximum noise exposures demonstrate that this relationship warrants further explorations in future study.

While we have demonstrated the potential for high noise exposures among e-waste workers and have documented exposures to metals in these workers, it is possible that there are additional, unmeasured metals or other chemicals that may have contributed to these findings through confounding or effect modification. It is also possible that there was an interaction between noise exposures and concentrations of some metals, or that there was a relationship between income or some work activities and these exposures that we could not account for. While we explored our dataset to look for such relationships, we were unable to identify any. Blood levels of metals were not as high among these workers as initially expected, and it may be that ototoxic effects of metals

are not present at the exposure levels encountered. Further research is needed to further explore the unexpected results of our study and possible unexpected negative effects of nutrient metals on the hearing system.

Given the paucity of studies on this vulnerable occupational group, it is difficult to compare our findings to those of other researchers. Previous literature has examined surface dust samples and determined extremely high hazard quotients at the Agbogbloshie waste recycling site for Pb and Cu, with levels of great concern to child health [27]. Cd and Zn also displayed concerning hazard quotients while Fe, Mn, chromium, and nickel were not of concern [27]. Our study found high blood values of Pb, Cd, and Cu in some participating adults supporting this study's assertion of high levels of health risks due to Pb and Cd, especially concerning where children may be present on site. High levels of Zn and Cu were found in our study participants as well, highlighting the need to further explore these metals as contaminant concerns.

While recent literature has not explored metals in the blood of e-waste workers, one study examined levels of numerous trace elements in their urine [26]. This study found high levels of As which was not found to be due to drinking water, but likely fish and shellfish sources [26]. Cd was not found to be elevated in worker urine, however Fe, antimony, and Pb were after adjusting for age [26]. While our study did not examine antimony, we also found evidence to support concerning elevated Fe and Pb in blood samples. Asante et al. did not examine how the levels found may influence health outcomes of workers as we have done [26].

Limitations

Our study had a number of limitations. The first and primary limitation was the small sample size, which may limit the generalizability of our results. However, given the relatively homogeneity observed among e-waste recycling activities across Agbogbloshie, and the fact that this site is one of, if not the, largest e-waste recycling sites in the world, even in the absence of generalizability our study results still have great utility. A second limitation pertains to the audiometric test results that form the primary outcome of the study. Conditions at the Ghana Post Clinic were not optimal for audiometric testing, which ideally would have been conducted in a sound-dampened audiometric test booth. As a result, it is possible that some of the hearing thresholds measured

among our workers were artificially elevated by masking due to background noise, particularly in the low frequencies (e.g., <1000 Hz). Our use of insert-type earplugs and attenuating audiometric earcups reduced background noise at participants' ears, but we cannot be certain that the attenuation achieved was sufficient to overcome masking effects. A third limitation resulted from insufficient detail collected from a number of workers at the completion of their noise dosimetry. This limited our ability to conduct task-specific noise exposure assessment, which might have provided useful information regarding activities that generated the highest noise levels. Our survey design, along with the delivery of the survey to the participants, represents a fourth limitation. We relied on self-reported information by participants and given their strict religious practices, there may have been some misreporting resulting in misclassifications. Translation provided by the translators hired for this study may have been inconsistent, resulting in misclassification and errors in participants' survey results. Also, some survey items were consistently omitted by one translator, reducing the amount of valid data obtained from participants he interviewed.

Our final limitation relates to the site itself. Agbogbloshie Market is one of the most polluted areas in the world [310], and there are many potential but unmeasured environmental contaminants other than metals and noise (e.g., organic and inorganic chemical compounds, bacteria and viruses, etc.) that may contribute to or even dominate the risk of certain otological conditions (e.g., otitis media, mumps, etc.) that could have impacted our measured health outcome even in the absence of exposures to metals and noise. Our participant selection may have biased our sample towards those with health concerns, as access to the local clinic and consultation with a doctor would not otherwise have been possible for most or all of our participants. When asked of concerns not addressed in the survey, 22 participants listed individual health concerns. This indicates that nearly half of our sample was interested in gaining access to free health care. To avoid coercion, our participant incentive was not higher than an average daily wage on the site.

Conclusions

Our study has demonstrated that e-waste recycling workers at Agbogbloshie face substantial occupational health issues, including high exposures to noise and metals. These exposures appear to be associated with an increased risk of HL among these workers and indicate that HL prevention

and metal exposure reduction efforts are needed among these workers as part of an overall effort to decrease occupational hazards and improve health outcomes among e-waste recycling workers. The relationship between metals and HL is still unclear and further study is needed. Further occupational hazard assessments are warranted among vulnerable e-waste workers in low-income countries such as Ghana, as exposures among these workers are likely to grow worse over time with the expected increase in e-waste generation. However, research is also needed on e-waste recycling workers in high-income countries like the US, where business organizations are quite different and best practices may be developed and evaluated for potential subsequent transfer to lower-income countries. Only by understanding the exposures associated with this important work in multiple contexts can we improve occupational health conditions for e-waste workers.

IV.F: Tables

Table IV-1. Demographic information (N=58 e-waste recycling workers)

Variable	Mean	SD
Age (years)	25.9	7.9
Estimated age (16/58 = 28%)	23.1	4.8
BMI (kg/m²)	22.8	2.3
Years living near Agbogbloshie	6	4.2
Years worked on e-waste*	6.2	4.2
Years worked in loud noise	6.1	4.2
Typical hours worked per day*	10.7	2.9

Variable/Category	n	%
Estimated (rather than known) age	16	28
Self-reported hearing difficulties	15	26
Sleep at Agbogbloshie	41	71
Muslim	56	97
Daily income (US dollar estimation^)		
≤ GHS 10 (~US $3)	22	38
GHS 11-20 (~US $3-6)	18	31
GHS 21-30 (~US $6-9)	7	12
GHS 31-40 (~US $9-12)	4	7
> GHS 40 (~US $12)	7	12
Marital status		
Separated or divorced	3	5
Married, living with spouse	22	38
Married, not living together	11	19
Single	22	38
Highest level of education		
None	31	53
Primary school	11	19
Middle school	12	21
Secondary school	4	7
Moved to Agbogbloshie from**		
Northern Ghana	42	78
Southern Ghana	9	17
Other	2	4

*N=55; **N=54; ^exchange rate based on 12/31/2014 US $1=3.22 GHS

Table IV-2. Daily noise measurement data (N=56 e-waste recycling workers)*

Period/Variable	N	Mean	SD	Minimum	Maximum
Overall					
Duration (hrs)	56	19.1	4.87	4.1	23.5
L_{EQ} (dBA)	56	82.5	3.84	74.4	90.0
L_{max} (dBA)	56	97.0	4.15	87.5	110.0
Occupational period					
Duration (hrs)	17	1.3	2.61	0.5	13.4
L_{EQ} (dBA)	17	85.6	3.52	78.6	90.2
L_{max} (dBA)	17	94.3	4.53	85.7	102.5
Non-occupational period					
Duration (hrs)	56	18.6	5.98	4.1	23.5
L_{EQ} (dBA)	56	81.6	4.35	70.3	90.0
L_{max} (dBA)	56	96.0	4.98	80.0	110.0

	Very often		Fairly often		Sometimes		Never & almost never	
	N	%	N	%	N	%	N	%
Self-reported frequency of noise exposure at work (N=58)	49	84.5	1	1.7	6	10.3	2	3.4

*2 of 58 total participants did not complete a noise dosimetry measurement

Table IV-3. Occupational characteristics (N=58 e-waste recycling workers)

Variable/category	N	Yes n	Yes %
Activities performed in past six months*			
Ash	57	32	56
Burn	55	40	73
Collect	55	41	75
Dismantle	57	49	86
Lead Batteries	57	27	47
Sort	57	48	84
Smelt	56	21	38
Repair	57	20	35
Remove wire (not by burning)	57	43	75
Primary e-waste activity (N=58)			
Collecting		10	17
Dealing		12	21
Dismantling		28	48
Other		8	14

*N=57; one participant was not asked any of these questions

Table IV-4. Element levels (ppb or μg/L) in whole blood (N=58 e-waste recycling workers)

Metal	Mean	SD	IQR	Minimum	Maximum
Toxic Metals					
Arsenic (As)	4.63	2.73	4.05	0.75	12.08
Cadmium (Cd)	2.97	3.03	1.11	0.92	22.74
Lead (Pb)	97.2	57.8	59.2	29.9	326.1
Manganese (Mn)	12.6	4.2	4.5	6.5	24.6
Methylmercury (MeHg)	1.8	1.4	1.4	0.5	6.4
Essential Elements					
Copper (Cu)	1061	716	201	566	6164
Iron (Fe)*	440161	62824	66710	253078	573369
Selenium (Se)	163.6	48.5	55.2	72.6	319.3
Zinc (Zn)	5539	1965	1193	2392	16255

*N=57; one sample Fe level not reported by lab

Table IV-5. Audiometric hearing thresholds (N=57 e-waste recycling workers)*

Ear/frequency	Hearing threshold level (dB HL)			
	Mean	SD	Minimum	Maximum
Right				
500	25	11	10	65
1000**	19	10	0	55
2000	18	12	0	55
3000	19	13	0	60
4000	23	16	0	90
6000	26	20	5	90
8000	22	17	0	90
Left				
500	23	13	5	60
1000**	16	9	0	50
2000	15	11	0	60
3000	17	15	0	75
4000	19	19	0	90
6000	27	18	5	90
8000	20	18	0	75
Worst threshold	40	18	20	90
Right side	35	17	15	90
Left side	34	18	10	90

*One participant did not complete audiometric testing due to an inability to wear the testing headphones
**Two measurements were taken, however only the second reading is reported here

Table IV-6. Noise notch results for individual frequencies and notch indices (N=55 e-waste recycling workers*)

VARIABLE/category	n	%
Presence of noise notch		
None	20	40
Both right and left ears	16	32
Right ear only	10	20
Left ear only	9	18
Right ear notch		
At 3 kHz	7	13
At 4 kHz	10	18
At 6 kHz	15	27
Left ear notch		
At 3 kHz	7	13
At 4 kHz	11	20
At 6 kHz	17	31
Notch index	Mean (dB)	SD (dB)
NN_A	-3.3	9.9
NN_B	-3.8	9.6
NN_C	-4.9	9.1
NN_D	-9	10.3

*Noise notch analysis was not run on 2 of the 57 participants with complete threshold data. One of the two eliminated participants was due to age, the other due to profound deafness on the right side.

Table IV-7. Regression models predicting hearing outcomes in e-waste recycling workers (N=55)

Model/Variable	Model Before Interaction Term					Model Including Interaction Term				
	Adj. R^2	p-value	Coef.	SE	p-value	Adj. R^2	p-value	Coef.	SE	p-value
*Model 1: Outcome of 4 kHz hearing threshold level**	0.28	6E-4				0.39	1E-4			
Intercept			17.34	2.23	5E-10			15.64	2.15	5E-9
Cadmium (Cd)[†^] (µg/L)			-3.79	1.66	0.03			-2.63	1.64	0.1
Manganese (Mn)[†^] (µg/L)			6.15	2.49	0.02			4.85	2.46	0.05
Number of times eat meat[^] (per week)			0.93	0.30	0.003			0.75	0.29	0.01
Years living at Agbogbloshie[^] (years)			1.37	0.52	0.01			1.63	0.49	0.002
L_{max}[^] (dBA)			-	-	-			-0.64	0.62	0.3
Zinc (Zn)[†^] (µg/L)			-	-	-			-2.80	1.70	0.1
Interaction term: L_{max}*Zn[†^]			-	-	-			0.87	0.26	0.001
*Model 2: Outcome of 6 kHz hearing threshold level**	0.24	0.002				0.39	1E-4			
Intercept			24.55	2.01	0.1			22.98	1.89	1E-15
Copper (Cu)[†^] (µg/L)			2.14	2.11	0.3			4.04	2.04	0.05
Selenium (Se)[†^] (µg/L)			6.43	2.60	0.02			6.46	2.43	0.01
Years living at Agbogbloshie[^] (years)			1.14	0.47	0.02			1.49	0.44	0.002
Work activity diversity[^] (range from 0 to 1)			13.62	10.89	0.22			7.51	10.13	0.5
L_{max}[^] (dBA)			-	-	-			-0.71	0.56	0.2
Zn[†^] (µg/L)			-	-	-			-2.91	1.64	0.08
Interaction term: L_{max}*Zn[†^]			-	-	-			0.90	0.24	6E-4
*Model 3: Outcome of notch index NN_D***	0.17	0.03				0.21	0.02			
Intercept			24.97	2.12	3E-15			24.14	2.10	1E-14
Zn[†^] (µg/L)			0.30	1.66	0.86			-1.61	1.90	0.4
Arsenic (As)[†^] (µg/L)			-3.05	3.15	0.3			-1.26	3.20	0.7
Se[†^] (µg/L)			7.73	2.55	0.004			7.83	2.48	0.003
L_{max}* (dBA)			1.09	0.92	0.2			-0.04	1.07	0.9
Overall L_{EQ}[^] (dBA)			-0.92	0.95	0.3			-0.10	1.02	0.9
Work activity diversity[^] (range from 0 to 1)			21.61	11.94	0.08			16.26	11.93	0.2
Age[^]			0.65	0.34	0.06			0.62	0.33	0.07
Interaction term: L_{max}*Zn[†^]			-	-	-			0.55	0.29	0.06

* Positive coefficients indicate worse hearing; ** Negative coefficients indicate worse hearing; † IQR adjusted – As: 4.0 µg/L; Cd: 1.0 µg/L; Cu: 206 µg/L; Mn: 4.4 µg/L; Se: 55 µg/L; Zn: 982 µg/L; ^Variables are mean-centered after adjusting for IQR.

IV.G: Figures

Figure IV-1. Individual noise exposures (N=56) with splined average shown in black for e-waste recycling workers
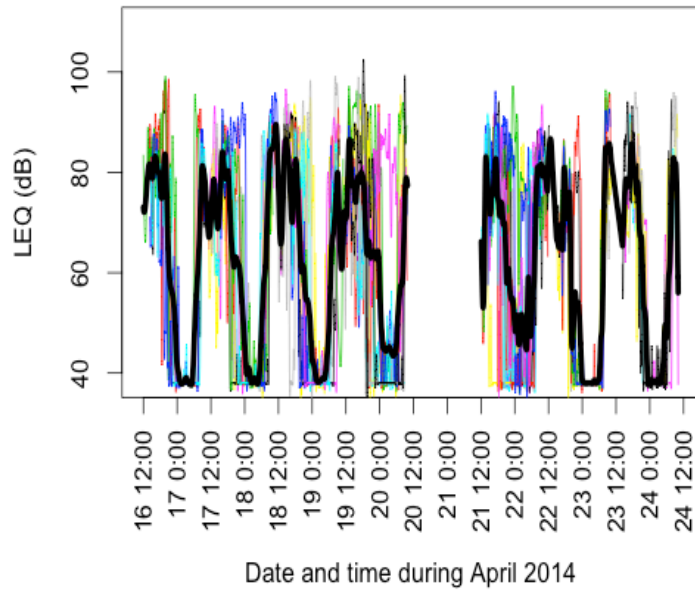
Figure IV-2. Audiometric hearing thresholds for e-waste recycling workers (N=57)
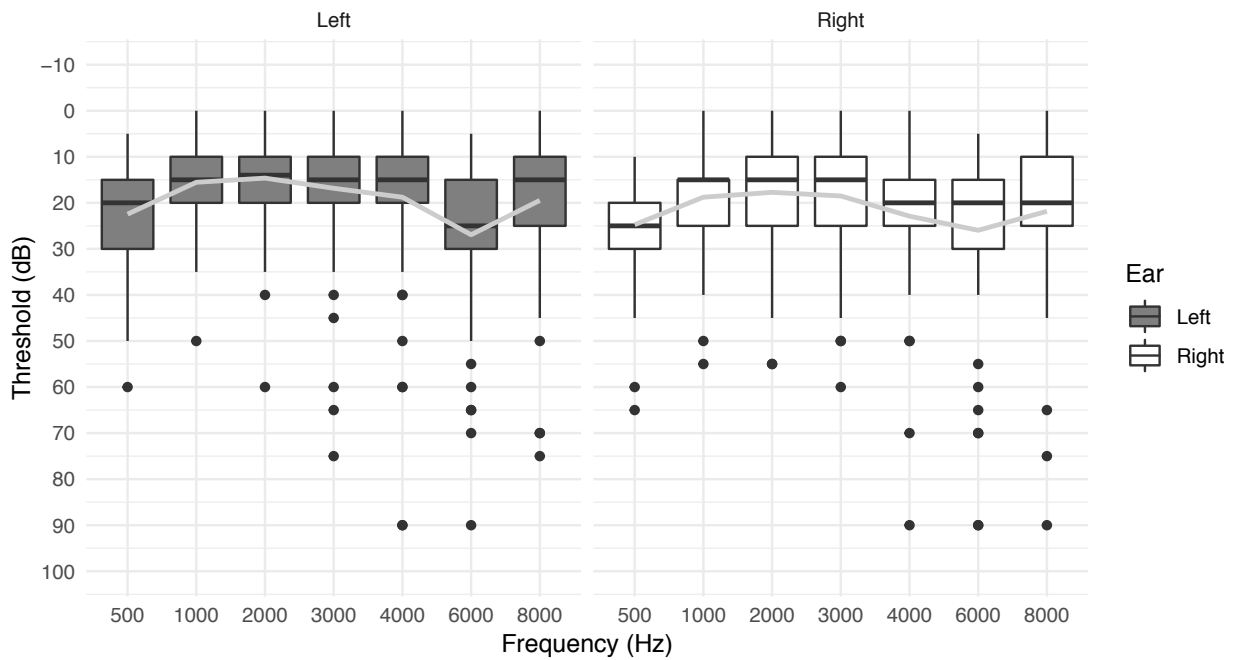
CHAPTER V: Conclusions

V.A: Introduction

In both environmental as well as occupational exposure settings, the likelihood of being exposed to toxic metals is high. Whether these metals are lead (Pb), mercury (Hg), or cadmium (Cd), the potential for nervous system damage is unambiguous. What is unclear is how to measure damage before overt toxicity is irreversible. The potential for metals to impact the auditory system creates a unique and sensitive measure of nervous system damage. Hearing losses (HLs) caused by metals could be a useful indicator of hazardous environments and monitoring auditory health would not only improve future health of populations, but also could provide early flags demonstrating areas where exposures to metals is necessary to improve overall health when consistently measured across the lifetime.

Exploration of HL caused by metals is challenging for many reasons, including the need to determine an appropriate exposure metal biometric (blood or bone) in addition to establishing a suitable auditory outcome. Understanding the specific life-stages of vulnerability and dose ranges resulting in negative impacts is critical. Conducting toxicological and epidemiological investigations to demonstrate ototoxicity in both laboratory and real-world settings strengthens the field of ototoxicity research, as each type of study informs and complements the other. This dissertation investigated the ototoxicity of Pb and other metals, the hazard of noise exposure, and the potential otoprotection offered by essential elements. It also directly addressed real-world, developmental exposure to toxicant-toxicant mixtures and nutrient-toxicant mixtures, both of which are important to public health. The three studies comprise work that was done in investigation of Pb ototoxicity in various life stages, exposure settings, and both humans and animals, as shown in Figure V-1. Methodologically, the Mouse Model provided a controlled setting where low and high doses of metals could be experimentally explored in mice, and allowed for evaluation of causality. The Michigander Newborn study and the E-waste Worker study each provide critical information about real-life exposure levels to metals associated with detrimental

auditory outcomes occurring among humans. The Michigander Newborn study focused on a period of exposure (neonatal) where there has been great vulnerability to various developing systems in the fetus, while the E-waste Worker study assessed a vulnerable adult population in a developing nation.

Worldwide, Pb exposures pose a health risk to an estimated 22 million people [311]. Hg exposures are similarly prevalent, with 19 million people potentially at risk [311]. Cd is not as well-researched globally, but is of concern at levels, which are low, but seem to be increasing in agricultural settings [312]. Pb, Hg, and Cd are toxic metals which are all used in batteries and consumer electronics. Production, recycling, and disposal of these items have potential to release the toxic metals into the environment. While localized pollution results from environmental releases, atmospheric deposition distributes these hazardous metals making them pervasive globally [24,313]. These three non-essential metals are persistent pollutants which remain in the environment; when these metals are taken up by plants and animals, they bioaccumulate [314]. Given the wide use and reach of these toxic metals, their detrimental health effects on hearing must be better understood.

V.B: Impact of HL

Loss of hearing has a profound impact on human health and quality of life, and is associated with a wide variety of adverse social, psychological, educational, and occupational outcomes [1]. HL is the leading work-related injury in the United States [315]. While blame is typically placed on noise exposure, other sources contribute [316], potentially including metals. To develop the best HL prevention strategies, we must explore the relationship between metals exposure and HL as well as how factors such as nutrition, noise, and exposure mixtures may exacerbate detrimental hearing effects. HL due to these factors should be largely preventable, especially in controllable occupational settings.

The preservation of auditory health is important for people of all ages. In workers, normal hearing is associated with improved response to emergency alerts, productivity, and less social isolation [317,318]. In children, HL can produce additional struggles with education and employment [257]. Auditory health is often ignored until the damage is severe and irreversible [260,319]. Auditory

rehabilitation with hearing aids can be complicated, expensive, and may not restore normal hearing if patients wait too long to address their hearing problems. Many patients with HL become socially isolated and have depression and cognitive impairment [320]. However, those successfully using hearing aids show a normal decline in cognition [321]. Not only can the individual with HL suffer negative outcomes, but they are often not aware of the impact on co-workers and family members as a result of communication challenges. While even mild HL is of concern, HL in children is particularly concerning given their vulnerability and the impacts of HL on intellectual development. Even mild losses in children are associated with poorer speech perception and significantly lowered means on basic skills tests [173]. Other groups are also vulnerable, including people in developing nations. For example, in Africa, over 37 million people have moderate to complete HL, and typically have no access to behavioral rehabilitation and technological aids [2]. Even more concerning is the estimation from the World Health Organization that 50% of current HL cases were originally preventable [108]. This implies that modifications in local environments, such as lowering noise levels, can have a profound effect on millions of people.

The specific aims, key findings, strengths, and limitations of each of the three studies are summarized separately below in V.C, V.D, and V.E. Conclusions following the findings of these three studies taken together are discussed in V.F. Directions for future studies are summarized in V.G. A final section discusses the importance of preventing HL and policy directions in V.H.

V.C: Project 1: Mouse Model

Specific aims

Specific Aims for the Mouse Model included 1) evaluation of the dose-response relationships for Pb and Cd individually on the outcomes of threshold shift and outer hair cell loss and 2) evaluation of toxicant-toxicant interactions, exploring possible potentiation on hearing outcomes through combinations of exposures to Pb and Cd; Pb and noise; Cd and noise; and Pb, Cd, and noise.

Key findings

Dose-response relationships between Pb, Cd, and HL were assessed in the CBA/CaJ young adult mouse over a twelve-week treatment period. No effective dose was found as no significant threshold shifts in mice exposed to Pb or Cd were noted, so the highest dosing group for each metal, most resembling occupational exposures in humans, was selected for combination treatments. Noise was found to significantly increase mouse Auditory Brainstem Response (ABR) thresholds at the mid and high treatment groups. In order to avoid threshold shifts that might be too large and prevent visible differences from being observable in combinations of treatments, the mid-range dose of noise was selected for combination treatments. Threshold shifts following noise exposures were not significantly different in groups with combinations of exposures compared to the group exposed to noise alone. Thus, no potentiation was observed between Pb and noise or Cd and noise. Pb and Cd in combination did not show ABR threshold differences from the control group, indicating no metal-metal toxic interactions affecting the auditory system. Distortion Product Otoacoustic Emissions (DPOAE) showed similar results to ABR in combination groups. Cochlear outer hair cell counts did not show significantly different numbers of dead cells than control for animals treated with Pb or Cd. This indicated that either cochlear outer hair cells are not the cellular target most effected by Pb or Cd treatment, or that the dose administered was not at sufficient to produce a toxic response.

Comparisons to past studies

The hypothesized synergism between Pb and noise, similar to that established for styrene and noise [322], was not observed. A study conducted very recently in young adult mice of a different strain, C57BL/6, found evidence of potentiation between Pb and noise [323].This study used only six mice and dosed a level of Pb that was slightly lower (2 mM instead of 3 mM) than my work. Previous studies on Pb damaging hearing had only been done in monkeys, guinea pigs, and rats [53,159,324]. However, these studies reflect different dosing regimens and species. No previous studies investigated Pb levels in the cochlea, though previous studies have documented Pb in fish and eel otoliths [325,326]. Cd effects on ABR were also not in agreement with a previous study, though this study used higher doses of Cd over a shorter time and a different strain of mouse [78].

Strengths

This study offered controlled laboratory experimental conditions where dosing and all outside exposures were highly controlled. Dosing of Pb and Cd reached relevant levels comparable to human occupational exposures above the US OSHA permissible exposure limits. Noise exposures were a key positive control in the study design confirming that the model produced damage to hearing visible as auditory brainstem response threshold shifts, DPOAE shifts, and cochlear basal outer hair cell loss. Blood serum levels, pathology, and histology documented in this experiment confirmed that the levels of Pb and Cd did not cause systemic toxicity to either the liver or the kidney. Exposure levels were well documented in multiple organ tissues: blood, tibia, femur, and cochlear bone with adjacent tissues. This was the first study to demonstrate increased Pb levels in cochlear tissue in a mammalian model.

Limitations

Of the three studies in this dissertation, the mouse model provided the strongest evidence against Pb ototoxicity; however, the findings of this study require restraint in application. Firstly, through utilization of a mouse strain that was robustly resistant to ear infections, this model may have used a strain that does not represent a more vulnerable population's susceptibility to Pb ototoxicity. Pigmented animals have been found to be more robust in their hearing after noise exposure [327]. The CBA/CaJ as an inbred mouse strain can lead to difficulties in mirroring the genetic variability of humans, especially for sensitive individuals [328]. Secondly, the timeframe of this study utilized a reproductively mature mouse, and this may not be the lifecycle timepoint of highest vulnerability. Age-related HL exacerbations were not examined and neither were developmental effects. Thirdly, further dosing or exposures to all types of toxicants (Pb, Cd, and noise) may be required to display ototoxic characteristics that would be biologically and statistically significant in this model. Budget and time constraints prevented this model from being expanded for ototoxicity exploration for this dissertation.

Knowledge added

The Mouse Model created within this dissertation generated knowledge that in this specific exposure scenario and in this strain of mouse, CBA/CaJ at the timepoint from 5 weeks to 17 weeks, there is no evidence that Pb exposure through drinking water caused lowered average auditory thresholds or cochlear hair cells loss. When combined with noise exposures, cadmium exposures, and both noise and cadmium exposures there was no indication of an effect that was significantly different from noise. Cadmium doses were significantly decreased when included with Pb exposures, however no negatives outcomes in thresholds or ABR were observed. Noise exposures given in this exposure setting were found to significantly decrease thresholds and controls maintained their hearing threshold levels across the twelve-week experiment.

Needs identified

Further studies in industrial mixture toxicology are needed to elucidate the possible outcomes of HL in relation to combinations of chemical and noise exposures. These settings that are ubiquitous in occupational settings for human workers are neglected in toxicology work. Development of combinations of physical hazards in combinations with chemical hazards leaves many opportunities. There also needs to be further study on hearing health of mice and specific strains as inbred strains may not fully capture the full variability of human hearing health. Laboratory toxicology studies on mixtures in mice have many strengths that can clarify exposures and outcomes where they are cloudy in epidemiological studies. However, work done in animals must also show relevance to the settings of everyday human life.

V.D: Project 2: Prenatal Human Exposures

Specific aims

Specific Aims for the Prenatal Human Exposure study included 1) evaluation of the impact Pb and MeHg levels had individually on the odds of a newborn hearing screening failure as well as toxicant-toxicant interactions effects from both Pb and MeHg together and 2) investigation on the

impact of essential elements and interactions with the toxic metals (Pb and MeHg) on hearing screening failures in newborns.

Key findings

Dried blood spots from infants collected within 48 hours of birth showed levels of MeHg with a significant hazardous effect on hearing screening outcomes. MeHg levels produced a significant trend across quartiles as well as a significant increased odds of hearing screening failure in the highest quartile compared to the lowest quartile in the prenatal human exposure study. Quantitative logistic models produced with MeHg levels alone showed borderline-significant results. However, this study did not produce evidence of Pb ototoxicity. The odds ratios of those with higher-ranked Pb levels were not significantly different across any quartiles or in any quantitative logistic regression models. Toxicant-toxicant interactions between Pb and MeHg were also not significant. While the original hypothesis was not confirmed, this study did yield an interesting side finding related to levels of calcium in newborns. Increasing levels of blood calcium were singly, and in all models including the combined dataset from both Set 1, with 100 cases and controls from 2003-2008, and Set 2, from 2003-2015 with 238 cases and controls, significantly protective against a hearing screening failure. This observation was seen in both bilateral and unilateral hearing screening failures, both males and females, in only white races (not black or other), and in Set 2 (not Set 1). Logistic regression models for potassium and iron became significant with calcium included in the model; in these models, however, higher levels of iron or potassium increases the odds of a hearing screening failure. When these logistic models were reduced to include only bilateral HLs, both potassium and iron no longer were significant predictors. A significant interaction between zinc and selenium was also documented on odds of hearing screening outcome.

Comparisons to past studies

Previous studies have shown methylmercury to have ototoxicant properties in developing mice [83]. However, this was not shown by a study investigating hearing in human children at eight to

ten years of age with Hg values taken from umbilical cord blood [204]. Blood Pb level (BLL) effects on the developing auditory system have never been investigated before in humans, though adolescents have shown associations with worse high-frequency hearing and with BLLs over 2 µg/dL [56]. Also, exposures to Pb throughout gestations have proven to alter auditory processing in mice [17,18]. No previous study investigated simultaneously levels of toxicant metals alongside essential elements as they influence hearing in adults or newborn infants. Zinc and selenium interactions have been observed [245], though not in a relationship to auditory function.

Strengths

This study included an extremely large sample size of almost 700 infants obtained by combining data from two sets of similar data. This data set was not only large, but also contained cases of hearing screening failures who went on for further audiometric testing and were confirmed to have abnormal hearing. There is no known reason for Pb or MeHg to lead to a hearing problem only on one side of the body, so this study followed the results of the bilateral hearing participants as those hypothesized to possibly demonstrate hearing difficulties brought on by toxicant metal(s) evenly distributed across coronal and sagittal planes. Initial and subsequent hearing tests indicated failures of individual ears, so the analyses were able to stratify across bilateral and unilateral losses to assess sensitivity of analyses.

Limitations

The premise of this study utilizes a single metric, or bloodspot, taken shortly after birth, along with a hearing screening, that may or may not have included follow up auditory testing. These two data points may not fully capture variabilities in exposures over pregnancy or the windows of heightened vulnerability in auditory performance or development of the complete hearing system.

There were limitations in the ability of this study to address multiple causes of HL. One of the major causes of HL in young children is infection with cytomegalovirus. This communicable disease seems to lead to unilateral HL following initial infection [329]. The leading causes of HL for infants and children are genetic and this study was unable to account for this specific origin of

HL. While we were able to exclude infants who spent time in the NICU, we were not able to control for other possible damaging noise exposures; for example, certain hospital locations have been found to have potentially harmful levels of noise [330].

While this study applied new methodologies for using dried blood spots in assessing levels of MeHg [197], it was unable to develop sufficiently accurate metrics of the levels of blood Pb to substantiate useful models. Utilizing Pb levels in quartiles for these spots was helpful but determining accurate, individual-level quantitative measures reflective of those known to occur in populations could have produced stronger and more easily interpretable statistical results. As levels of blood Pb are dropping in the US, the trend for technological advance in dried blood spot analysis may reach a point where the level of blood Pb is nearing the level of detection for average observable levels in the US.

Knowledge added

The Prenatal Human Exposure study generated knowledge on utility of newborn bloodspots as exposure metrics in early exposure life-cycle studies. Not only were toxicant metal levels explored and quantified, but also essential metal levels were observed to be well within detectable limits. This study design provides a unique approach to investigating mixture epidemiology. It also captured a unique cohort in a case-control study design that can be useful in predicting other health outcomes examined in newborns or young children. It is possible that screening for essential elements in the blood, such as calcium, could be used as early indicators of health issues.

Needs identified

Levels of essential elements in normal newborn blood are not well-established and need further study and documentation. Assessing levels of essential elements in blood of patients with syndromic hearing disorders could assist future epidemiologic studies in the possibility of excluding these types of hearing disorders from environmental causes of HL. Further study in early human life to establish toxicant exposures in a representative sample across all stages of pregnancy is also warranted to establish whether assuming the level of MeHg and Pb from newborn blood

spots is a reasonable representation of exposures during earlier stages of fetal development. Establishing developmental exposures to toxicant metals during the most vulnerable stages of cochlear development is necessary before mechanisms of action upon the auditory system can be fully understood, and before it can be determined if toxicant metal exposures are damaging to the cochlea, specific components of the nervous system processing auditory stimuli, or general neuronal development. Specifying an optimal timeframe (or timeframes) for identifying auditory problems would also be of assistance in identifying best practices for epidemiological investigations into childhood environmental exposures and HL, as subclinical abnormalities in hearing may not be detected until much later in childhood.

V.E: Project 3: Occupational Human Exposures

Specific aims

Specific Aims for the Occupational Human Exposure study included: 1) evaluation of the impact of Pb, Cd, Mn, As and MeHg levels, as well as noise exposures, on the audiometric status of e-waste workers, and: 2) investigation of the impacts of combinations of toxic metals, essential elements, as well as noise exposures on hearing outcomes, considering possible synergism, additivity, potentiation, and antagonism.

Key findings

The Occupational Human Exposure study is important as it has documented exposures and health outcomes in an understudied population of Ghanaian e-waste workers. This study selected participants from an occupation with a wide variety of exposures and quantified many of them through biomarkers, personal exposure monitors, and extensive logs and surveys of participant history and activities. The major finding of the analyses is that in this population of young (average age of 26) e-waste worker some degree of noise notch, indicating noise-induced HL, was found in 60% of participants. This high prevalence of injury to the hearing system is alarming in this population. Personal dosimetry of this population confirms that average noise exposures both during occupational and non-occupational time periods were above the WHO 70 dBA L_{EQ} or

continuous average exposure level. Noise exposure levels averaged over 3.75 minutes and recorded on the dosimeters exceeded over 110 dBA in some cases, which is alarming for participants that are without hearing protection in such extremely loud environments. Ten of fifty-five maximum readings were over 100 dB. For reference, NIOSH recommends exposures at 100 dB not to exceed 15 minutes, exposures of 97 dB not to exceed 30 minutes, and exposures of 94 dB not exceed 60 minutes (due to the logarithmic scale of dB, a doubling of sound intensity occurs with every increase of 3 dB). The average occupational exposure exceeded the NIOSH 85 dBA Recommended Exposure Limit for 8-hours, as well, indicating substantial occupational exposures at the work-shift level. Many occupationally-related noise exposures onsite were generated by impacts, such as hammering and chiseling, and the hearing hazard presented by such exposures may not be accurately captured by average exposure levels.

Models did not indicate significance of blood Pb, Hg, or Cd levels as predictors of lowered thresholds at 6 kHz frequencies or depth of a noise notch. However, models did consistently indicate levels of higher selenium were significantly and adversely associated with worse hearing outcomes. The higher the work diversity score metric for participants, or the more tasks that they reported working on in the past three months, the more likely they were to have HL. This was significant in the final model indicating the size of the noise notch, but while it improved the significance of the overall final model predicting thresholds at 6 kHz, the individual predictor did not reach significance. The metric of years lived at Agbogbloshie was a significant predictor of high frequency HL and years working in high noise was a significant predictor of increased size of noise notches. A significant correlation was discovered between average noise exposure levels and the amount of copper in whole blood. This association may be indicative that workers who are more exposed to metals (particularly copper) may also be more exposed to noise.

Comparisons to past studies

Comparisons to previous studies are challenging for this novel work on occupational health done in an emerging setting. Noise in markets of Accra has been documented as concerning before this study alongside documented noise-induced HL [331]. Noise exposures for artisanal gold miners in Ghana has also been shown to be concerning [332]. Quarry workers in Ghana are also subject

to hearing problems due to damaging levels of noise [333]. Metals exposures have been documented as high in e-waste recycling sites [27,334]. These effects on hearing and interactions with essential elements on noise-induced HL have not previously been investigated in this type of work setting.

Strengths

The strengths of this study include the multiple variables that were documented in this population of transient workers living and working in a landfill in Accra, Ghana. This vulnerable minority population is difficult to collect data from as they are doing work that is illegal by the standards of the nation of Ghana. Gaining access to this community was only done through community involvement from the University of Ghana, translators, and immense planning and commitment from data collectors [335].

Participants completed a detailed survey regarding their health, injuries, stress, work, and demographics. Biomarkers for metals were collected, a hearing screening was conducted, a medical examination took place, and personal noise exposures were measured for 24 hours following the clinic visit. This comprises a broad range of health data on individuals rarely participating in health studies. This study found that many workers dismantling e-waste do work on a wide number of tasks. By developing the work diversity score, the metric of the number of tasks the have performed in the past three months out of nine known tasks, this study was able to quantify the diversity of their work activities. Our documentation of the structure of the workers' occupational lives and understanding how the tasks of their work are performed can inform future studies on e-waste work and this study was the first to discuss how occupational structures at Agbogbloshie are extremely different from those traditionally studied in occupational epidemiology [335].

Limitations

While many measures of hazardous as well as essential exposures—predominately metals—were collected by this investigational work, there were many other hazardous aspects involved in the

waste processing that our study did not take into account: brominated flame retardants, polyvinyl chloride, phthalates, as well as particulate matter and other air pollutants [336]. Other toxic metals were not included in this analysis, but occur in concerning levels in electronic products including, but not limited to: hexavalent chromium, beryllium, cobalt, indium, and lithium [336]. Due to constraints on time and resources this study was designed as a cross-sectional study collecting health metrics and exposure data at the same time. Bone metrics of Pb are more indicative of life-time exposures, but technology is still in development to collect this type of data in the field. Blood Pb biomarkers show more recent Pb exposures, which are most powerful in regression models only if exposures are consistent over time. While it can be assumed that these workers are exposed to varying levels of Pb based on the items they are processing, in the absence of data on variability in exposures, these analyses assumed that their exposures were consistent.

The sample size of participants who were recruited was near capacity for the time-frame of our data collection. However, given the large numbers of variables for this data set, having a smaller number of individuals limits the power of this analysis. This study was also limited by our narrow range of participant recruitment which occurred during a holiday, Easter, for ease of using the clinic space for our study. This could be problematic, particularly for biomarkers and noise dosimetry, if during this holiday period workers were not engaging in normal tasks, or normal amounts of tasks as they usually practice. Indeed, the city of Accra experienced many celebrations occurring from Friday through Monday in observance of Easter. These celebrations may have heightened noise levels in the city, however our population was predominantly Muslim and did not observe Easter. One other problematic finding is that many of the metals correlate significantly with each other. Therefore, identifying which heightened metal might be centrally responsible, if indeed that is the case, was not possible.

Knowledge added

The Occupational Human Exposure study generated knowledge on work-life exposures among electronic waste workers in Agbogbloshie, Ghana. This study also established biological doses for the metals they are exposed to via diet, environment, and occupationally. The levels of noise in the market area of Agbogbloshie and on the waste site were documented to be concerningly high.

There were health concerns within our cohort as many had not been seen by a physician within their lifetime. These health concerns were magnified by the finding of a majority showing signs of noise-induced HL. This study design has shown the possibilities of exploring hearing health in a transient and at-risk population alongside appropriate collaborations and respect for community members. These workers showed a high potential for occupational mixtures to hazards of many types, but certainly physical and chemical. An interaction between noise levels and zinc demonstrated that while zinc may offer some protection against noise on hearing, eventually at heightened noise levels, zinc is no longer protective.

Needs identified

This study established levels of metal exposures not explored before in an open work setting where industrial exposures may be altered because of a lack of a built-environment or ventilation systems. Further study in human occupational environments is warranted, not only in electronic waste workers, but also international populations of factory workers with simultaneous metals and noise exposures over a significant period of their lives. Further prolonged studies need to monitor worker health over an extended amount of time to assess associations between these types of exposure mixtures and age-related HL as well as noise-induced HL. While immediate hearing effects may not occur, hazardous exposure mixtures may accelerate vulnerabilities in a more aged population not represented by these data.

More extensive noise monitoring at a personal level is necessary to characterize both occupational and non-occupational exposures. One day of noise measurements was not enough to characterize differences related to e-waste tasks or individual work patterns within this small cohort. Much more research is justified to explore the hazardous conditions related to e-waste occupations. Though, the hazards are real. These workers have already proven living situations that deserve better protection of their health without further data needing to be supplied to provide intervention.

V.F: Overarching ototoxicity observations: From toxicology to epidemiology

This original and ambitious dissertation combines multiple approaches to examine the questions of metal exposure association and influence on auditory performance in a variety of settings including both environmental and occupational exposures. Not only were single exposures investigated, but the combinations and interactions between these elements were also modeled to explore the possibility of antagonism and synergism. The dissertation considered results from a toxicological animal study, an epidemiological state-wide neonatal case-control study, and a cross-sectional occupational exposure assessment. Compelling findings for these three chapters are shown together in Figure V-2. The aspects of ototoxicity, otoprotection, and hazard interaction as addressed by these three studies are summarized below.

Pb and HL

While these three studies have produced clear evidence that Pb ototoxicity may not be a major concern at the levels seen in US occupational workers, in young men in Ghana, or infants in Michigan, the results do not eliminate the possibility that Pb may damage the auditory system in ways not explored by these studies.

The Mouse Model described the strongest results against Pb ototoxicity. No evidence suggesting the ability for exposures in an adult male mouse exposed to Pb in drinking water over twelve weeks was found. This experimental setting controlled all variables in the experiment, manipulating only noise exposures, Pb exposures, and Cd exposures. While these results are robust, the group sizes of 6-16 mice may not have been of appropriate size to detect very low risk levels. Also, the CBA/CaJ strain of mice is resistant to age-related HL and robust against ear infections, so this strain may not be representative of more vulnerable population sub-types most vulnerable to damage related to Pb in the auditory system as. However, when taken together with results from the Prenatal Human Exposures study and Occupational Human Exposures study where ototoxic effects due to Pb exposures were not identified as significantly associated with negative auditory outcomes. In combination, these findings provide convincing evidence that Pb doses observed in current populations may not independently be contributing significantly to hearing impairment.

Cd and HL

The Mouse Model also established a lack of evidence for Cd exposures leading to threshold increases or cochlear hair cell loss. However, these analyses of Cd ototoxicity were confounded by high levels of noise, and our statistical power was limited due to the small sample size. Taken together, these results provide evidence that Cd exposures occurring in occupational environments for young male workers do not independently contribute to HL.

The Prenatal Human exposures study originally was designed to included levels of Cd within the dried blood spot analysis, however levels were not detectable in the biometric, so this analysis was unable to be completed.

Hg and HL

The Prenatal Human Exposures study indicated that heightened levels of MeHg naturally or anthropogenically occurring currently in the state of Michigan may be hazardous to the auditory system of newborn humans. These significant findings were displayed in the highest quartile of MeHg exposures. When broken down into quartiles, MeHg also displayed a significant association with HL.

While MeHg was not explored in the Mouse Model, it was explored in the Occupational Human Exposures study, where there were no significant findings associating blood levels of MeHg with auditory thresholds at 4 kHz, 6 kHz, or noise notched. Taken together, these findings may signal that MeHg is most potent as an ototoxicant during development.

Metals in combination and HL

The combination of Pb and Cd exposures in the Mouse Model did not indicate any evidence of potentiation between the combination of Pb and Cd on auditory threshold shifts. Mice treated with

both Pb and Cd concomitantly displayed hearing thresholds in the normal range and no different than control mice.

The Prenatal Human Exposures study investigated Pb and MeHg levels for interaction associated with hearing screening failures. No interactions between these two neurotoxic metals were observed in this cohort. However, this study design established the possibility of pursuing further toxicant metal interactions on health outcomes from newborn dried blood spots.

A wide variety of toxicant metals were examined in the Occupational Human Exposures study. Combinations of arsenic, Pb, Cd, manganese, and MeHg were explored in multivariate regression models examining both noise notch and hearing threshold outcomes across frequencies from 0.5 kHz to 8 kHz. No significant interactions between these metals were observed.

Metals combined with noise and HL

In the Occupational Human Exposures study, interactions between BLL and noise (both maximum non-occupational and maximum overall) exposures were found to have a significant impact on hearing thresholds at 6 kHz, though this significance must not be overstated due to the large numbers of interactions explored. This finding was not corroborated by results from the mouse study as mice exposed to both Pb and noise did not show significantly different thresholds than mice exposed to noise alone.

The Mouse Model also did not establish any potentiation following the combination of Cd and noise exposures. All three toxicants, Pb, Cd, and noise simultaneously also did not show any suggestion of potentiation. This finding agreed with data from the Occupational Health study where no toxicant metals in any type of interaction were found to significantly influence either the depth of noise notch or the level of auditory thresholds at 4 kHz or 6 kHz.

Essential elements and HL

The unique study design of exploring levels of essential elements in blood and these associations with auditory outcomes has never been explored before in newborns or e-waste workers. This

study feature identified significant findings in both Michigander newborns and Ghanaian young adult men.

Newborns with higher levels of calcium were found to have significantly lower odds of hearing screening failure. This finding may signify that infants born with lower levels of calcium in their blood have systemic disorders that are not properly regulating their calcium homeostasis. This finding associated with auditory dysfunction is curious and deserves future exploration and study.

The Prenatal Human Exposures study also found a significant interaction between zinc and selenium. In multivariate models zinc and selenium were each significantly protective; however, the significant interaction between zinc and selenium showed heightened levels of zinc and selenium together increased risk for a hearing screening failure. This may be indicating toxic outcomes due to hormesis, or it may be indicating that extremely high levels of these essential elements are together indicators of syndromes linked to hearing disorders.

Another significant interaction discovered was between whole blood levels of zinc and noise in the Human Occupational Exposures study. This interaction was significant across a variety of auditory outcomes, such as thresholds at 4 kHz and 6 kHz. Interpreting this interaction shows that zinc had a protective effect against lower-level noise exposures. However, at heightened maximum noise levels, zinc was no longer protective. Since this cohort was not able to take auditory testing after a break from their work, their auditory thresholds may indicate temporary, rather than permanent, threshold shifts. This finding deserves further exploration and research in other occupational populations at risk for both temporary and permanent thresholds.

V.G: Future study directions

These three studies illustrate how important consideration of mixed exposures (or co-exposures) – both chemical-chemical and chemical-physical – is for environmental health research. There is a need for further research exploring our understanding of mechanisms by which damage related to exposure mixtures (as they occur in the real world) [337]. Multipollutant scenarios with multiple sources of health hazards are representative of typical real-world exposures. Environment-wide association studies are emerging as tools for identifying environmental components of complex

diseases and this dissertation captures this idea focused on metals, essential elements, and noise [338,339]. Future work can expand this concept into other environmental factors associated with hearing health in a larger dataset such as NHANES.

In the hearing system, hidden HL (e.g., cochlear synaptopathy, or subclinical damage that may impact peoples' hearing health trajectory) is an important and recently-identified aspect of hearing outcomes [340]. Additional work evaluating such losses can be done in humans and in animals, as was the case here, and will involve different measures of HL than were employed in the current studies.

Toxicologically, further experiments using the mouse model developed and established in the Mouse Model study are needed to work with different levels of metals (e.g., Hg or As). Developmental exposures followed by a lifetime of noise exposure may simulate human exposures that occur in Michigan or the families of e-waste workers living at Agbogbloshie, and identifying thresholds above which harm is likely to occur. Protective interactions on the hearing system, such as those discovered between selenium and zinc the Prenatal Human Exposures study can also be explored with more control in animals than in humans.

The Prenatal Human Exposures study in this dissertation appears to be the first to report results of developmental MeHg exposures on the auditory system. This study can be replicated in other populations where newborn bloodspots are well curated, for example California. MeHg in hair of the mother is also a potential metric to establish exposure levels at different vulnerable timepoints in fetal development, as the second trimester has been shown to be a high risk time for the fetus [341]. Further methodological development of Pb exposure assessment is needed to maximize the utility of blood spots as exposure metrics. Increased use of dried blood spots to measure exposures can allow assessment in populations without means to safely collect or store blood via phlebotomy. Metrics exploring nutritional status are needed to create an understanding of maternal, child, and adult health. This project explored using bloodspot-derived levels of iron, potassium, calcium, zinc, and selenium to indicate the dietary intake of these nutrients as predictors of hearing health; however calcium and iron are kept in tight homeostatic levels, and better nutritional biomarkers are needed. Perhaps a combination of several nutritional markers in blood could be quantified by one metric to indicate dietary vulnerability. It is possible that the early-stage exposures we assessed

may not be clinically relevant until later life; further follow-up on this cohort is warranted. The degree to which genetic disorders and hearing issues were correlated with high calcium levels in infants also warrants further exploration. Additionally, eliminating genetic-related cases of HL from this sample, which we were unable to do, should be considered in future work.

The Occupational Human Exposures study described an opportunistic epidemiological study which is not repeatable. This study explored occupational exposures in a vulnerable population within a developing nation and highlighted the need for better access to hearing health care and screening for other risk factors related to HL in Ghana. There also is a strong need there for community as well as occupational noise limits, protection, and monitoring. Due to many challenges involved in researching this type of community, there is a need for alternative exposure metrics and use of real-world pollution media in laboratory experiments. Examples of this type of research are the converted semi-truck trailer which collects air from Detroit to expose mice for experimental outcomes [342] and soil sample collection for exposure to mice exploring toxicity for residents of Love Canal [343]. Additional research is needed on characterizing occupational exposures to informal workers, as well. The work activity diversity, or WorkD, metric developed in the Human Occupational Exposures study signified multiple jobs done by workers and was associated with HL. Work tasks could be monitored much more thoroughly to produce a better investigation on the levels of noise associated specifically with each task involved in this work.

Another important issue to acknowledge with regards to the Human Occupational Exposures study is the difficulty of the situation for the informal e-waste workers evaluated, which cannot be overstated. These workers can be mistrustful of researchers based on their participation in past studies that have documented a range of concerns but resulted in no improvements to their conditions. In April 2014 when the data for the Human Occupational Exposures study were collected, workers at the site were collaborating with the GreenCross (part of the Blacksmith Institute, now called PureEarth) on projects to minimize harm to e-waste workers. The Blacksmith Institute celebrated a wire stripper installation in October 2014 and a shedding machine was planned for installation in July 2015 [344]. However, these efforts, too, failed to improve conditions at the site, as the wire stripper was often inoperable due to lack of electricity, and was not liked by workers, as it was viewed as slower and less efficient than burning wires. The extreme vulnerability of the workers at Agbogbloshie was highlighted when in the summer of 2015 the

population was removed from their dwellings and their workspaces and other structures on the site demolished by bulldozers hired by a quasi-governmental organization in Accra, demonstrating the impermanence and lack of voice that this population suffers [345,346].

V.H: Prevention of HL

Preventing HL through exposure reduction interventions for metals in the environment and workplace, either through behavioral modifications or environmental remediation, requires a full understanding of ototoxic effects and interacting factors. The lack of a demonstrated causal pathway between exposure to Pb, Hg, and/or Cd and an individual's degree of HL, as well as the absence of characterized dose-response relationships, limit our ability to estimate the impact of exposure to these metals on hearing health. More research is required to elucidate the impacts of metal exposures on the auditory system, and to explore potential interventions to improve hearing health among exposed individuals, particularly in vulnerable populations.

Collectively, these findings indicate that Hg, and potentially Pb and Cd, may contribute to HL under certain circumstances. Further research is needed to assess prenatal and occupational exposures and age-related changes in hearing to understand the role that Pb and other metals play in protection and injury to auditory function. The results of this and future studies may help guide future policies such as the US Centers for Disease Control and Prevention BLL guideline or the regulation of workplace noise by the Occupational Safety and Health Administration for workers exposed to noise, Pb, and/or Cd.

Workers may be subjected to high levels of occupational metal exposures and concomitantly experience high noise levels that can result or exacerbate HL. Regulation of exposures to Pb and other toxic metals, combined with the control and supervision of occupationally-related noise exposures, can improve occupational health. Pregnant families and children are another group with vulnerabilities that would benefit from policies that reduce and warn of sources for hazardous levels of exposures to Pb and other metals. During the analysis and writing of this dissertation, the level of concerning BLLs was lowered by the US Centers for Disease Control and Prevention from 10 μg/dL to 5 μg/dL [151]; however, no safe exposure level to Pb has been established as

acceptable. Public health experts can go further with better research to protect communities and improve health policies.

Elucidating negative health outcomes related to one toxicant in a laboratory setting is complicated enough. However, within the real world and its challenging jumbles of exposures, prioritizing and categorizing hazards is vastly more difficult. These three studies have attempted to capture some of the complexities of the real world, including exposures to toxicants, essential elements, and the physical hazard, noise. While reproducing real-world environmental health impacts in their entirety may be unattainable, innovative methodological approaches can replicate the crucial components, focusing efforts to improve quality of life. This dissertation has demonstrated that research on ototoxicity can push scientific frontiers in the areas of mixture toxicology and exposure assessment while evaluating impacts of environmental hazards on hearing.

V.I: Figures

Figure V-1. Diagram showing methodological variables explored by the three studies of this dissertation
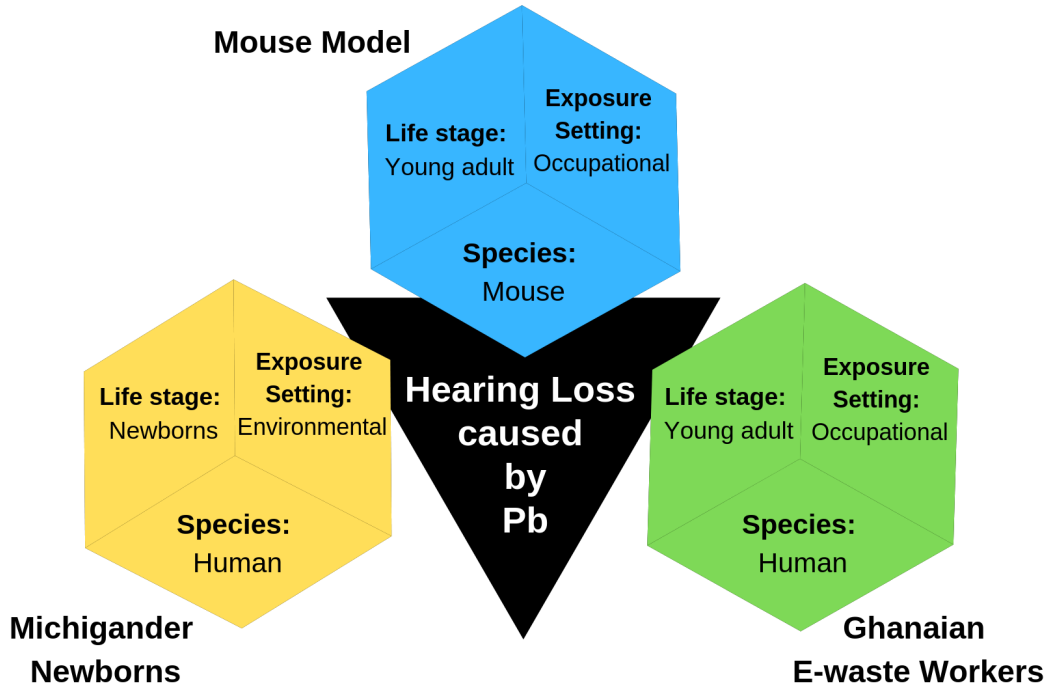
Figure V-2. Diagram summarizing positive findings from this dissertation

Significant finding:
MeHg in Newborns

Significant finding:
Ca in Newborns

Significant interaction:
Zn and Se in Newborns

Toxic
Metals

HEARING
LOSS

Essential
Nutrients

Noise

Significant finding:
Work diversity in Ghana
(worker vulnerability)

Significant interaction:
Zn and Noise in Ghana
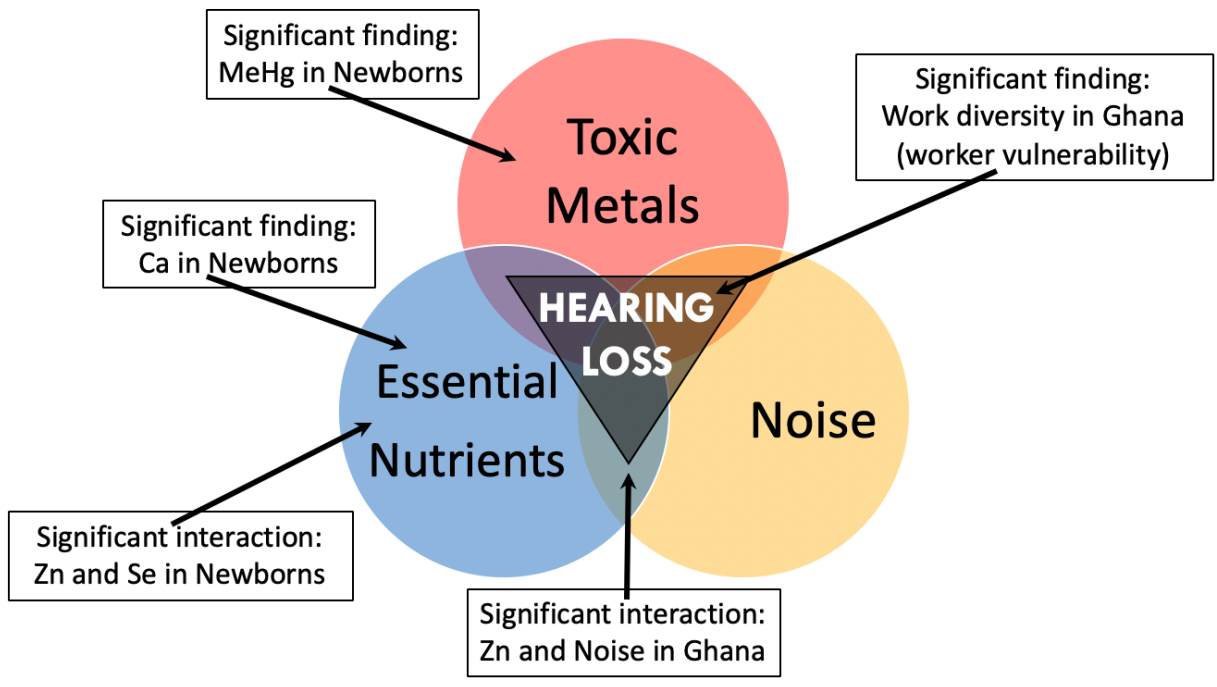
REFERENCES

[1]   Seidman MD, Standring RT. Noise and quality of life. *Int J Environ Res Public Health* 2010;7:3730–8.

[2]   World Health Organization (WHO). The global burden of disease: 2004 update. Geneva, Switzerland: World Health Organization; 2008. doi:10.1038/npp.2011.85.

[3]   Stevens G, Flaxman S, Brunskill E, Mascarenhas M, Mathers CD, Finucane M. Global and regional hearing impairment prevalence: An analysis of 42 studies in 29 countries. *Eur J Public Health* 2013;23:146–52. doi:10.1093/eurpub/ckr176.

[4]   Nelson DI, Nelson RY, Concha-Barrientos M, Fingerhut M. The global burden of occupational noise-induced hearing loss. *Am J Ind Med* 2005;48:446–58. doi:10.1002/ajim.20223.

[5]   Wake M, Hughes EK, Poulakis Z, Collins C, Rickards FW. Outcomes of children with mild-profound congenital hearing loss at 7 to 8 years: a population study. *Ear Hear* 2004;25:1–8. doi:10.1097/01.AUD.0000111262.12219.2F.

[6]   Tharpe AM, Sladen DP, Dodd-Murphy J, Boney SJ. Minimal hearing loss in children: Minimal but not inconsequential. *Semin Hear* 2009;30:80–93. doi:10.1055/s-0029-1215437.

[7]   Dedhia K, Kitsko D, Sabo D, Chi DH. Children with sensorineural hearing loss after passing the newborn hearing screen. *JAMA Otolaryngol - Head Neck Surg* 2013;139:119–23. doi:10.1001/jamaoto.2013.1229.

[8]   Morata TC. Chemical exposure as a risk factor for hearing loss. *J Occup Environ Med* 2003;45:676–82. doi:10.1097/01.jom.0000071507.96740.70.

[9] Wu X, Cobbina SJ, Mao G, Xu H, Zhang Z, Yang L. A review of toxicity and mechanisms of individual and mixtures of heavy metals in the environment. *Environ Sci Pollut Res* 2016;23:8244–59. doi:10.1007/s11356-016-6333-x.

[10] Gloag D. Sources of lead pollution. *Br Med J (Clin Res Ed)* 1981;282:41–4.

[11] Choi Y-H, Hu H, Mukherjee B, Miller J, Park SK. Environmental Cadmium and Lead Exposures and Hearing Loss in US Adults: the National Health and Nutrition Examination Survey, 1999 to 2004. *Environ Health Perspect* 2012.

[12] Shargorodsky J, Curhan SG, Henderson E, Eavey R, Curhan GC. Heavy metals exposure and hearing loss in US adolescents. *Arch Otolaryngol Head Neck Surg* 2011;137:1183–9.

[13] Park SK, Elmarsafawy S, Mukherjee B, Spiro A, Vokonas PS, Nie H, et al. Cumulative lead exposure and age-related hearing loss: the VA Normative Aging Study. *Hear Res* 2010;269:48–55.

[14] Saunders JE, Jastrzembski BG, Buckey JC, Enriquez D, Mackenzie T a, Karagas MR. Hearing loss and heavy metal toxicity in a nicaraguan mining community: audiological results and case reports. *Audiol Neurootol* 2013;18:101–13. doi:10.1159/000345470.

[15] Discalzi G, Fabbro D, Meliga F, Mocellini A, Capellaro F. Effects of occupational exposure to mercury and lead on brainstem auditory evoked potentials. *Int J Psychophysiol* 1993;14:21–5.

[16] Jones LG, Prins J, Park S, Walton JP, Luebke AE, Lurie DI. Lead exposure during development results in increased neurofilament phosphorylation, neuritic beading, and temporal processing deficits within the murine auditory brainstem. *J Comp Neurol* 2008;506:1003–17. doi:10.1002/cne.21563.

[17] Fortune T, Lurie DI. Chronic low-level lead exposure affects the monoaminergic system in the mouse superior olivary complex. *J Comp Neurol* 2009;513:542–58. doi:10.1002/cne.21978.

[18] Prins JM, Brooks DM, Thompson CM, Lurie DI. Chronic low-level Pb exposure during

development decreases the expression of the voltage-dependent anion channel in auditory neurons of the brainstem. *Neurotoxicology* 2010;31:662–73. doi:10.1016/j.neuro.2010.08.004.

[19] Pugh Smith P, Nriagu JO. Lead poisoning and asthma among low-income and African American children in Saginaw, Michigan. *Environ Res* 2011;111:81–6. doi:10.1016/j.envres.2010.11.007.

[20] Nriagu J, Senthamarai-Kannan R, Jamil H, Fakhori M, Korponic S. Lead poisoning among Arab American and African American children in the Detroit metropolitan area, Michigan. *Bull Environ Contam Toxicol* 2011;87:238–44. doi:10.1007/s00128-011-0346-4.

[21] Zahran S, Laidlaw MAS, McElmurry SP, Filippelli GM, Taylor M. Linking Source and Effect: Resuspended Soil Lead, Air Lead, and Children's Blood Lead Levels in Detroit, Michigan. *Environ Sci Technol* 2013. doi:10.1021/es303854c.

[22] Hanna-Attisha M, LaChance J, Sadler RC, Champney Schnepp A. Elevated Blood Lead Levels in Children Associated With the Flint Drinking Water Crisis: A Spatial Analysis of Risk and Public Health Response. *Am J Public Health* 2016;106:283–90. doi:10.2105/AJPH.2015.303003.

[23] Pancras JP, Landis MS, Norris GA, Vedantham R, Dvonch JT. Source apportionment of ambient fine particulate matter in Dearborn, Michigan, using hourly resolved PM chemical composition data. *Sci Total Environ* 2013;448:2–13. doi:10.1016/j.scitotenv.2012.11.083.

[24] Atasi KZ, Fujita G, Le Platte G, Hufnagel C, Keeler G, Graney J, et al. Impact of atmospheric deposition on the headworks of a wastewater treatment plant--a case study. *Water Sci Technol* 2001;43:223–9.

[25] Michigan Department of Community Health. Heavy Metals Surveillance in Michigan. 2010.

[26] Asante KA, Agusa T, Biney CA, Agyekum WA, Bello M, Otsuka M, et al. Multi-trace element levels and arsenic speciation in urine of e-waste recycling workers from Agbogbloshie, Accra in Ghana. *Sci Total Environ* 2012;424:63–73.

doi:10.1016/j.scitotenv.2012.02.072.

[27]   Atiemo SSM, Ofosu FGF, Aboh IJK. Assessing the Heavy Metals Contamination of Surface Dust from Waste Electrical and Electronic Equipment (E-waste) Recycling Site in Accra, Ghana. *Res J Environ Earth Sci* 2012;4:605–11.

[28]   Cho S, Gao SS, Xia A, Wang R, Salles FT, Raphael PD, et al. Mechanisms of Hearing Loss after Blast Injury to the Ear 2013;8. doi:10.1371/journal.pone.0067618.

[29]   Swart DJ, Bekker A, Bienert J. The subjective dimensions of sound quality of standard production electric vehicles. *Appl Acoust* 2018;129:354–64. doi:10.1016/J.APACOUST.2017.08.012.

[30]   Isaacson JE, Vora NM. Differential diagnosis and treatment of hearing loss. *Am Fam Physician* 2003;68:1125–32.

[31]   Webster DB, Packer DJ, Webster M. Functional anatomy of the external and middle ear. *Ear Nose Throat J* 1985;64:8–18. doi:10.1093/jpids/pix105/4823046.

[32]   Choi C-H. Mechanisms and treatment of blast induced hearing loss. *Korean J Audiol* 2012;16:103–7. doi:10.7874/kja.2012.16.3.103.

[33]   Moser T, Starr A. Auditory neuropathy-neural and synaptic mechanisms. *Nat Rev Neurol* 2016;12:135–49. doi:10.1038/nrneurol.2016.10.

[34]   Dallos P. Cochlear amplification, outer hair cells and prestin. *Curr Opin Neurobiol* 2008;18:370–6. doi:10.1016/j.conb.2008.08.016.

[35]   Stöver T, Diensthuber M. Molecular biology of hearing. *Laryngorhinootologie* 2011;90:S22–34. doi:10.1055/s-0030-1270444.

[36]   Carricondo F, Romero-Gómez B. The Cochlear Spiral Ganglion Neurons: The Auditory Portion of the VIII Nerve. *Anat Rec* 2019;302:463–71. doi:10.1002/ar.23815.

[37]   Raphael Y, Altschuler RA. Structure and innervation of the cochlea. *Brain Res Bull* 2003;60:397–422. doi:10.1016/S0361-9230(03)00047-9.

[38] Melichar I, Syka J. Electrophysiological measurements of the stria vascularis potentials in vivo. *Hear Res* 1987;25:35–43. doi:10.1016/0378-5955(87)90077-3.

[39] Kimura RS. Animal models of inner ear vascular disturbances. *Am J Otolaryngol* 1986;7:130–9.

[40] Gorga MP, Dierking DM, Johnson TA, Beauchaine KL, Garner CA, Neely ST. A validation and potential clinical application of multivariate analyses of distortion-product otoacoustic emission data. *Ear Hear* 2005;26:593–607.

[41] Kemp DT. Evidence of mechanical nonlinearity and frequency selective wave amplification in the cochlea. *Arch Otorhinolaryngol* 1979;224:37–45.

[42] Popelka GR, Osterhammel PA, Nielsen LH, Rasmussen AN. Growth of distortion product otoacoustic emissions with primary-tone level in humans. *Hear Res* 1993;71:12–22. doi:10.1016/0378-5955(93)90016-T.

[43] Avan P, Bonfils P, Mom T. Correlations among Distortion Product Otoacoustic Emissions, Thresholds and Sensory Cell Impairments. *Noise Health* 2001;3:1–18.

[44] Abdala C, Visser-Dumont L. Distortion Product Otoacoustic Emissions: A Tool for Hearing Assessment and Scientific Study. *Volta Rev* 2001;103:281–302.

[45] Kraus KS, Canlon B. Neuronal connectivity and interactions between the auditory and limbic systems. Effects of noise and tinnitus. *Hear Res* 2012;288:34–46. doi:10.1016/j.heares.2012.02.009.

[46] Steinhoff HJ, Böhnke F, Janssen T. Click ABR intensity-latency characteristics in diagnosing conductive and cochlear hearing losses. *Arch Otorhinolaryngol* 1988;245:259–65.

[47] Musiek FE, Shinn J, Chermak GD, Bamiou D-E. Perspectives on the Pure-Tone Audiogram. *J Am Acad Audiol* 2017;28:655–71. doi:10.3766/jaaa.16061.

[48] Tremblay KL, Pinto A, Fischer ME, Klein BEK, Klein R, Levy S, et al. Self-reported

hearing difficulties among adults with normal audiograms: The Beaver Dam Offspring Study. *Ear Hear* 2015;36:e290–9. doi:10.1097/AUD.0000000000000195.

[49]   Hershenhoren I, Nelken I. Detection of Tones Masked by Fluctuating Noise in Rat Auditory Cortex. *Cereb Cortex* 2016;27:5130–43. doi:10.1093/cercor/bhw295.

[50]   Carlson K, Neitzel RL. (In press) Hearing loss, lead (Pb) exposure, and noise : A sound approach to ototoxicity exploration. *J Toxicol Environ Heal - Part B Crit Rev* 2019. doi:10.1080/10889868.2013.786018.

[51]   Lurie DI, Brooks DM, Gray LC. The effect of lead on the avian auditory brainstem. *Neurotoxicology* 2006;27:108–17. doi:10.1016/j.neuro.2005.07.005.

[52]   Rice DC. Effects of lifetime lead exposure in monkeys on detection of pure tones. *Toxicol Sci* 1997;36:112–8. doi:10.1093/toxsci/36.2.112.

[53]   Tuncel U, Clerici WJ, Jones RO. Differential ototoxicities induced by lead acetate and tetraethyl lead. *Hear Res* 2002;166:113–23.

[54]   Liang GH, Järlebark L, Ulfendahl M, Bian JT, Moore EJ. Lead (Pb2+) modulation of potassium currents of guinea pig outer hair cells. *Neurotoxicol Teratol* 2004;26:253–60. doi:10.1016/j.ntt.2003.12.002.

[55]   Borisova T, Krisanova N, Sivko R, Kasatkina L, Borysov A, Griffin S, et al. Presynaptic malfunction: The neurotoxic effects of cadmium and lead on the proton gradient of synaptic vesicles and glutamate transport. *Neurochem Int* 2011;59:272–9. doi:10.1016/j.neuint.2011.05.014.

[56]   Shargorodsky J, Curhan SG, Henderson E, Eavey R, Curhan GC. Heavy metals exposure and hearing loss in US adolescents. *Arch Otolaryngol - Head Neck Surg* 2011;137:1183–9. doi:10.1001/archoto.2011.202.

[57]   Park SK, Elmarsafawy S, Mukherjee B, Spiro A, Vokonas PS, Nie H, et al. Cumulative lead exposure and age-related hearing loss: The VA Normative Aging Study. *Hear Res* 2010;269:48–55. doi:10.1016/j.heares.2010.07.004.

[58]   Forst LS, Freels S, Persky V. Occupational lead exposure and hearing loss. *J Occup Environ Med* 1997;39:658–60.

[59]   Chuang H-Y, Kuo C-H, Chiu Y-W, Ho C-K, Chen C-J, Wu T-N. A case-control study on the relationship of hearing function and blood concentrations of lead, manganese, arsenic, and selenium. *Sci Total Environ* 2007;387:79–85. doi:10.1016/j.scitotenv.2007.07.032.

[60]   Bleecker ML, Ford DP, Lindgren KN, Scheetz K, Tiburzi MJ. Association of chronic and current measures of lead exposure with different components of brainstem auditory evoked potentials. *Neurotoxicology* 2003;24:625–31. doi:10.1016/S0161-813X(03)00045-7.

[61]   Discalzi G, Fabbro D, Meliga F, Mocellini A, Capellaro F. Effects of occupational exposure to mercury and lead on brainstem auditory evoked potentials. *Int J Psychophysiol* 1993;14:21–5. doi:10.1016/0167-8760(93)90080-9.

[62]   Forst LS, Freels S, Persky V. Occupational lead exposure and hearing loss. *J Occup Environ Med* 1997;39:658–60.

[63]   Hwang Y-H, Chiang H-Y, Yen-Jean M-C, Wang J-D. The association between low levels of lead in blood and occupational noise-induced hearing loss in steel workers. *Sci Total Environ* 2009;408:43–9.

[64]   Buchanan LH, Counter SA, Ortega F. Environmental lead exposure and otoacoustic emissions in Andean children. *J Toxicol Environ Heal - Part A Curr Issues* 2011;74:1280–93. doi:10.1080/15287394.2011.587106.

[65]   Allen Counter S, Buchanan LH, Ortega F, Laurell G. Normal auditory brainstem and cochlear function in extreme pediatric plumbism. *J Neurol Sci* 1997;152:85–92. doi:10.1016/S0022-510X(97)00149-4.

[66]   Counter SA, Vahter M, Laurell G, Buchanan LH, Ortega F, Skerfving S. High lead exposure and auditory sensory-neural function in Andean children. *Environ Health Perspect* 1997;105:522–6. doi:10.1289/ehp.97105522.

[67]   Counter SA, Buchanan LH, Ortega F, Van Der Velde J, Borg E. Assessment of auditory

brainstem function in lead-exposed children using stapedius muscle reflexes. *J Neurol Sci* 2011;306:29–37. doi:10.1016/j.jns.2011.04.003.

[68] Buchanan LH, Counter SA, Ortega F, Laurell G. Distortion product oto-acoustic emissions in Andean children and adults with chronic lead intoxication. *Acta Otolaryngol* 1999;119:652–8. doi:10.1080/00016489950180586.

[69] Counter SA, Buchanan LH, Ortega F. Neurophysiologic and neurocognitive case profiles of Andean patients with chronic environmental lead poisoning. *J Toxicol Environ Heal - Part A* 2009;72:1150–9. doi:10.1080/15287390903091772.

[70] Steyger PS. Potentiation of Chemical Ototoxicity by Noise. *Semin Hear* 2009;30:38–46. doi:10.1055/s-0028-1111105.

[71] Boettcher FA, Henderson D, Gratton MA, Danielson RW, Byrne CD. Synergistic interactions of noise and other ototraumatic agents. *Ear Hear* 1987;8:192–212.

[72] Li H, Steyger PS. Synergistic ototoxicity due to noise exposure and aminoglycoside antibiotics. *Noise Health* 2011;11:26–32.

[73] Hwang Y-H, Chiang H-Y, Yen-Jean M-C, Wang J-D. The association between low levels of lead in blood and occupational noise-induced hearing loss in steel workers. *Sci Total Environ* 2009;408:43–9. doi:10.1016/j.scitotenv.2009.09.016.

[74] Wu T, Shen C, Lai J, Goo C, Ko K, Chi H-Y, et al. Effects of Lead and Noise Exposures on Hearing Ability. *Arch Environ Heal An Int J* 2000;55:109–14.

[75] Farahat TM, Abdel-Rasoul GM, El-Assy AR, Kandil SH, Kabil MK. Hearing thresholds of workers in a printing facility. *Environ Res* 1997;73:189–92. doi:10.1006/enrs.1997.3700.

[76] Ozcaglar HU, Agirdir B V, Dinc O, Turhan M, Kilinçarslan S, Oner G. Effects of cadmium on the hearing system. *Acta Otolaryngol* 2001;121:393–7. doi:10.1080/000164801300102897.

[77] Agirdir B V, Bilgen I, Dinc O, Ozçağlar HU, Fişenk F, Turhan M, et al. Effect of zinc ion

on cadmium-induced auditory changes. *Biol Trace Elem Res* 2002;88:153–63. doi:10.1385/BTER:88:2:153.

[78] Kim S-J, Jeong H-J, Myung N-Y, Kim M-C, Lee J-H, So H-S, et al. The protective mechanism of antioxidants in cadmium-induced ototoxicity in vitro and in vivo. *Environ Health Perspect* 2008;116:854–62. doi:10.1289/ehp.10467.

[79] Kim S-J, Shin B-G, Choi I-Y, Kim D-H, Kim M-C, Myung N-Y, et al. Hwanggunchungyitang prevents cadmium-induced ototoxicity through suppression of the activation of caspase-9 and extracellular signal-related kinase in auditory HEI-OC1 cells. *Biol Pharm Bull* 2009;32:213–9.

[80] Kim S-J, Myung N-Y, Shin B-G, Lee J-H, So H-S, Park R-K, et al. Protective effect of a Chrysanthemum indicum containing formulation in cadmium-induced ototoxicity. *Am J Chin Med* 2011;39:587–600. doi:10.1142/S0192415X11009056.

[81] Choi YH, Park SK. Environmental exposures to lead, mercury, and cadmium and hearing loss in adults and adolescents: KNHANES 2010–2012. *Environ Health Perspect* 2017;125:67003. doi:10.1289/EHP565.

[82] Hotta S, Itoh T, Matsui T, Yamamura K. An electrophysiological study of the effects of acute methylmercury chloride exposure on the function of the guinea pig cochlea. *Acta Otolaryngol* 1997;117:675–80. doi:10.3109/00016489709113459.

[83] Huang CF, Liu SH, Hsu CJ, Lin-Shiau SY. Neurotoxicological effects of low-dose methylmercury and mercuric chloride in developing offspring mice. *Toxicol Lett* 2011;201:196–204. doi:10.1016/j.toxlet.2010.12.016.

[84] Huang CF, Hsu CJ, Liu SH, Lin-Shiau SY. Ototoxicity induced by cinnabar (a naturally occurring HgS) in mice through oxidative stress and down-regulated Na+/K+-ATPase activities. *Neurotoxicology* 2008;29:386–96. doi:10.1016/j.neuro.2008.01.005.

[85] Schacht J, Hawkins JE. Sketches of otohistory. *Audiol Neurootology* 2006;11:1–6. doi:10.1159/000088850.

[86]	Uchino M, Okajima T, Eto K, Kumamoto T, Mishima I, Ando M. Neurologic features of chronic Minamata disease (organic mercury poisoning) certified at autopsy. *Intern Med* 1995;34:744–7. doi:10.2169/internalmedicine.34.744.

[87]	Counter SA, Buchanan LH, Laurell G, Ortega F. Blood mercury and auditory neuro-sensory responses in children and adults in the Nambija gold mining area of Ecuador. *Neurotoxicology* 1998;19:185–96.

[88]	Saunders JE, Jastrzembski BG, Buckey JC, Enriquez D, Mackenzie TA, Karagas MR. Hearing loss and heavy metal toxicity in a Nicaraguan mining community: Audiological results and case reports. *Audiol Neurotol* 2013;18:101–13. doi:10.1159/000345470.

[89]	Wang L, Chen D, Wang H, Liu Z. Effects of lead and/or cadmium on the expression of metallothionein in the kidney of rats. *Biol Trace Elem Res* 2009;129:190–9. doi:10.1007/s12011-008-8288-3.

[90]	Whittaker MH, Wang G, Chen XQ, Lipsky M, Smith D, Gwiazda R, et al. Exposure to Pb, Cd, and As mixtures potentiates the production of oxidative stress precursors: 30-day, 90-day, and 180-day drinking water studies in rats. *Toxicol Appl Pharmacol* 2011;254:154–66. doi:10.1016/j.taap.2010.10.025.

[91]	Lin X, Gu Y, Zhou Q, Mao G, Zou B, Zhao J. Combined toxicity of heavy metal mixtures in liver cells. *J Appl Toxicol* 2016;36:1163–72. doi:10.1002/jat.3283.

[92]	Stewart WF, Schwartz BS. Effects of lead on the adult brain: A 15-year exploration. *Am J Ind Med* 2007;50:729–39. doi:10.1002/ajim.20434.

[93]	Blanco A, Moyano R, Molina López AM, Blanco C, Flores-Acuña R, García-Flores JR, et al. Preneoplastic and neoplastic changes in the Leydig cells population in mice exposed to low doses of cadmium. *Toxicol Ind Health* 2010;26:451–7. doi:10.1177/0748233710371111.

[94]	Kippler M, Hossain MB, Lindh C, Moore SE, Kabir I, Vahter M, et al. Early life low-level cadmium exposure is positively associated with increased oxidative stress. *Environ Res* 2012;112:164–70. doi:10.1016/j.envres.2011.11.012.

[95]   Gu H, Neal R, Spitz DR, Erc N. Antioxidant effects of N -acetylcysteine and succimer in red blood cells from lead-exposed rats. *Toxicology* 1998;128:181–9.

[96]   Kimitsuki T, Komune N, Noda T, Takaiwa K, Ohashi M, Komune S. Effect of hydrogen peroxide on potassium currents in inner hair cells isolated from guinea pig cochlea. *Neuroreport* 2010;21:1045–9. doi:10.1097/WNR.0b013e32833fcb56.

[97]   Henderson D, Bielefeld EC, Harris KC, Hu BH. The role of oxidative stress in noise-induced hearing loss. *Ear Hear* 2006;27:1–19.

[98]   Gozdzik-Zolnierkiewicz T, Moszyński B. Eight nerve in experimental lead poisoning. *Acta Otolaryngol* 68:85–9.

[99]   Patra RC, Swarup D, Dwivedi SK. Antioxidant effects of alpha tocopherol, ascorbic acid and L-methionine on lead induced oxidative stress to the liver, kidney and brain in rats. *Toxicology* 2001;162:81–8.

[100] Tuncel U, Clerici WJ, Jones RO. Differential ototoxicities induced by lead acetate and tetraethyl lead. *Hear Res* 2002;166:113–23.

[101] Parham K, Sun XM, Kim DO. Distortion product otoacoustic emissions in the CBA/J mouse model of presbycusis. *Hear Res* 1999;134:29–38.

[102] Sha S-H, Kanicki A, Dootz G, Talaska AE, Halsey K, Dolan D, et al. Age-related auditory pathology in the CBA/J mouse. *Hear Res* 2008;243:87–94.

[103] Henry KR. Males lose hearing earlier in mouse models of late-onset age-related hearing loss; females lose hearing earlier in mouse models of early-onset hearing loss. *Hear Res* 2004;190:141–8. doi:10.1016/S0378-5955(03)00401-5.

[104] Sergeyenko Y, Lall K, Liberman MC, Kujawa SG. Age-Related Cochlear Synaptopathy: An Early-Onset Contributor to Auditory Functional Decline. *J Neurosci* 2013;33:13686–94. doi:10.1523/JNEUROSCI.1783-13.2013.

[105] Klimpel KE, Lee MY, King WM, Raphael Y, Schacht J, Neitzel RL. Vestibular dysfunction

in the adult CBA/CaJ mouse after lead and cadmium treatment. *Environ Toxicol* 2017;32:869–76. doi:10.1002/tox.

[106] Abrahams Z, McHiza Z, Steyn NP. Diet and mortality rates in Sub-Saharan Africa: stages in the nutrition transition. *BMC Public Health* 2011;11:801. doi:10.1186/1471-2458-11-801.

[107] Carlson K, Schacht J, Neitzel RL. Assessing ototoxicity due to chronic lead and cadmium intake with and without noise exposure in the mature mouse. *J Toxicol Environ Heal Part A* 2018:1–17. doi:10.1080/15287394.2018.1521320.

[108] World Health Organization (WHO). Prevention of blindness and deafness: Estimates 2018. http://www.who.int/pbd/deafness/estimates/en/ (accessed July 24, 2018).

[109] Rodrigues MA, Amorim M, Silva M V., Neves P, Sousa A, Inácio O. Sound levels and risk perceptions of music students during classes. *J Toxicol Environ Heal Part A* 2015;78:825–39. doi:10.1080/15287394.2015.1051174.

[110] Seidman MD, Standring RT. Noise and quality of life. *Int J Environ Res Public Health* 2010;7:3730–8. doi:10.3390/ijerph7103730.

[111] US Environmental Protection Agency (EPA). Information on Levels of Environmental Noise Requisite to Protect Public Health and Welfare with an Adequate Margin of Safety. 1974. doi:550/9-74-004.

[112] Fechter LD. Promotion of noise-induced hearing loss by chemical contaminants. *J Toxicol Environ Heal - Part A* 2004;67:727–40. doi:10.1080/15287390490428206.

[113] Guthrie OW, Wong BA, McInturf SM, Reboulet JE, Ortiz PA, Mattie DR. Inhalation of hydrocarbon jet fuel suppress central auditory nervous system function. *J Toxicol Environ Heal - Part A Curr Issues* 2015;78:1154–69. doi:10.1080/15287394.2015.1070389.

[114] Schacht J, Talaska AE, Rybak LP. Cisplatin and aminoglycoside antibiotics: hearing loss and its prevention. *Anat Rec* 2012;295:1837–50. doi:10.1002/ar.22578.

[115] Schwartz J, Otto D. Blood lead, hearing thresholds, and neurobehavioral development in children and youth. *Arch Environ Health* 1987;42:153–60. doi:10.1080/00039896.1987.9935814.

[116] Vyskocil A, Truchon G, Leroux T, Lemay F, Gendron M, Gagnon F, et al. A weight of evidence approach for the assessment of the ototoxic potential of industrial chemicals. *Toxicol Ind Health* 2012;28:796–819. doi:10.1177/0748233711425067.

[117] Guan H, Piao F-Y, Li X-W, Li Q-J, Xu L, Yokoyama K. Maternal and fetal exposure to four carcinogenic environmental metals. *Biomed Environ Sci* 2010;23:458–65.

[118] Yorita Christensen KL. Metals in blood and urine, and thyroid function among adults in the United States 2007-2008. *Int J Hyg Environ Health* 2012.

[119] Pichery C, Bellanger M, Zmirou-Navier D, Glorennec P, Hartemann P, Grandjean P. Childhood lead exposure in France: benefit estimation and partial cost-benefit analysis of lead hazard control. *Environ Health* 2011;10:44.

[120] Greenway JA, Gerstenberger S. An evaluation of lead contamination in plastic toys collected from day care centers in the Las Vegas Valley, Nevada, USA. *Bull Environ Contam Toxicol* 2010;85:363–6.

[121] Berger Ritchie JA, Gerstenberger SL. An evaluation of lead concentrations in imported hot sauces. *J Environ Sci Heal - Part B* 2013;48:530–8. doi:10.1080/03601234.2013.774226.

[122] Cosselman KE, Navas-Acien A, Kaufman JD. Environmental factors in cardiovascular disease. *Nat Rev Cardiol* 2015;12:627–42. doi:10.1038/nrcardio.2015.152.

[123] Tellez-Plaza M, Navas-Acien A, Menke A, Crainiceanu CM, Pastor-Barriuso R, Guallar E. Cadmium exposure and all-cause and cardiovascular mortality in the U.S. general population. *Environ Health Perspect* 2012;120:1017–22.

[124] Hwang Y-H, Chiang H-Y, Yen-Jean M-C, Wang J-D. The association between low levels of lead in blood and occupational noise-induced hearing loss in steel workers. *Sci Total Environ* 2009;408:43–9.

[125] Counter SA, Buchanan LH. Neuro-ototoxicity in andean adults with chronic lead and noise exposure. *J Occup Environ Med* 2002;44:30–8.

[126] Farahat TM, Abdel-Rasoul GM, El-Assy AR, Kandil SH, Kabil MK. Hearing thresholds of workers in a printing facility. *Environ Res* 1997;73:189–92.

[127] Jones LG, Prins J, Park S, Walton JP, Luebke AE, Lurie DI. Lead exposure during development results in increased neurofilament phosphorylation, neuritic beading, and temporal processing deficits within the murine auditory brainstem. *J Comp Neurol* 2008;506:1003–17.

[128] Fortune T, Lurie DI. Chronic low-level lead exposure affects the monoaminergic system in the mouse superior olivary complex. *J Comp Neurol* 2009;513:542–58. doi:10.1002/cne.21978.

[129] Prins JM, Brooks DM, Thompson CM, Lurie DI. Chronic low-level Pb exposure during development decreases the expression of the voltage-dependent anion channel in auditory neurons of the brainstem. *Neurotoxicology* 2010;31:662–73.

[130] Rice DC. Effects of lifetime lead exposure in monkeys on detection of pure tones. *Fundam Appl Toxicol* 1997;36:112–8.

[131] Whitworth CA, Hudson TE, Rybak LP. The effect of combined administration of cadmium and furosemide on auditory function in the rat. *Hear Res* 1999;129:61–70.

[132] Sharma R, Mogra S. Lead as a developmental toxicant: A review. *Int J Pharm Sci Res* 2014;5:636–42.

[133] Taylor CM, Emond AM, Lingam R, Golding J. Prenatal lead, cadmium and mercury exposure and associations with motor skills at age 7 years in a UK observational birth cohort. *Environ Int* 2018;117:40–7. doi:10.1016/j.envint.2018.04.032.

[134] Reynolds RP, Kinard WL, Degraff JJ, Leverage N, Norton JN. Noise in a laboratory animal facility from the human and mouse perspectives. *J Am Assoc Lab Anim Sci* 2010;49:592–7.

[135] Ohlemiller KK, Rybak Rice ME, Rellinger EA, Ortmann AJ. Divergence of noise vulnerability in cochleae of young CBA/J and CBA/CaJ mice. *Hear Res* 2011;272:13–20. doi:10.1016/j.heares.2010.11.006.

[136] Willott JF, VandenBosche J, Shimizu T, Ding D-L, Salvi R. Effects of exposing C57BL/6J mice to high- and low-frequency augmented acoustic environments: auditory brainstem response thresholds, cytocochleograms, anterior cochlear nucleus morphology and the role of gonadal hormones. *Hear Res* 2008;235:60–71. doi:10.1016/j.heares.2007.10.006.

[137] Vicente-Torres MA, Schacht J. A BAD link to mitochondrial cell death in the cochlea of mice with noise-induced hearing loss. *J Neurosci Res* 2006;83:1564–72.

[138] Sha S-H, Kanicki A, Dootz G, Talaska AE, Halsey K, Dolan D, et al. Age-related auditory pathology in the CBA/J mouse. *Hear Res* 2008;243:87–94. doi:10.1016/j.heares.2008.06.001.

[139] Wu WJ, Sha SH, McLaren JD, Kawamoto K, Raphael Y, Schacht J. Aminoglycoside ototoxicity in adult CBA, C57BL and BALB mice and the Sprague-Dawley rat. *Hear Res* 2001;158:165–78. doi:10.1016/S0378-5955(01)00303-3.

[140] Hurd EA, Adams ME, Layman WS, Swiderski DL, Beyer LA, Halsey KE, et al. Mature middle and inner ears express Chd7 and exhibit distinctive pathologies in a mouse model of CHARGE syndrome. *Hear Res* 2011;282:184–95. doi:10.1016/j.heares.2011.08.005.

[141] Karolyi IJ, Dootz GA, Halsey K, Beyer L, Probst FJ, Johnson KR, et al. Dietary thyroid hormone replacement ameliorates hearing deficits in hypothyroid mice. *Mamm Genome* 2007;18:596–608. doi:10.1007/s00335-007-9038-0.

[142] Matt T, Ng CL, Lang K, Sha S-H, Akbergenov R, Shcherbakov D, et al. Dissociation of antibacterial activity and aminoglycoside ototoxicity in the 4-monosubstituted 2-deoxystreptamine apramycin. *Proc Natl Acad Sci* 2012;109:10984–9. doi:10.1073/pnas.1204073109.

[143] Vos T, Barber RM, Bell B, Bertozzi-Villa A, Biryukov S, Bolliger I, et al. Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic

diseases and injuries in 188 countries, 1990-2013: A systematic analysis for the Global Burden of Disease Study 2013. *Lancet* 2015;386:743–800. doi:10.1016/S0140-6736(15)60692-4.

[144] Charles River Laboratories International. Research Models: C57BL/6 Mice. 2011.

[145] McGinn MD, Bean-Knudsen D, Ermel RW. Incidence of otitis media in CBA/J and CBA/CaJ mice. *Hear Res* 1992;59:1–6. doi:10.1016/0378-5955(92)90094-4.

[146] Mäkitie AA, Pirvola U, Pyykkö I, Sakakibara H, Riihimäki V, Ylikoski J. The ototoxic interaction of styrene and noise. *Hear Res* 2003;179:9–20. doi:10.1016/S0378-5955(03)00066-2.

[147] Mauderly JL. Toxicological approaches to complex mixtures. *Environ Health Perspect* 1993;101:155–65. doi:10.1289/ehp.93101s4155.

[148] Vandamme TF. Use of rodents as models of human diseases. *J Pharm Bioallied Sci* 2014;6:2–9. doi:10.4103/0975-7406.124301.

[149] Kujawa SG. Acceleration of Age-Related Hearing Loss by Early Noise Exposure: Evidence of a Misspent Youth. *J Neurosci* 2006;26:2115–23. doi:10.1523/JNEUROSCI.4985-05.2006.

[150] Laborde A, Tomasina F, Bianchi F, Bruné MN, Buka I, Comba P, et al. Children's health in Latin America: The infuence of environmental exposures. *Environ Health Perspect* 2015;123:201–9. doi:10.1289/ehp.1408292.

[151] Centers for Disease Control and Prevention (CDC). Response to Advisory Committee on Childhood Lead Poisoning Prevention Recommendations in "Low Level Lead Exposure Harms Children: A Renewed Call of Primary Prevention." vol. 61. 2012.

[152] Centers for Disease Control and Prevention (CDC). Adult Blood Lead Epidemiology and Surveillance -- United States, 2005-2007. *MMWR Morb Mortal Wkly Rep* 2009;58:365–9.

[153] Spivey A. The weight of lead. Effects add up in adults. *Environ Health Perspect*

2007;115:A30-6. doi:10.1289/image.ehp.v115.i01.

[154] Mesnage R, Defarge N, Rocque L-M, Spiroux de Vendômois J, Séralini G-E. Laboratory Rodent Diets Contain Toxic Levels of Environmental Contaminants: Implications for Regulatory Tests. *PLoS One* 2015;10:e0128429. doi:10.1371/journal.pone.0128429.

[155] Adamse P, Van der Fels-Klerx HJ (Ine., de Jong J. Cadmium, lead, mercury and arsenic in animal feed and feed materials–trend analysis of monitoring results. *Food Addit Contam - Part A* 2017;34:1298–311. doi:10.1080/19440049.2017.1300686.

[156] Vicente-Torres MA, Schacht J. A BAD link to mitochondrial cell death in the cochlea of mice with noise-induced hearing loss. *J Neurosci Res* 2006;83:1564–72. doi:10.1002/jnr.20832.

[157] Minami SB, Yamashita D, Ogawa K, Schacht J, Miller JM. Creatine and tempol attenuate noise-induced hearing loss. *Brain Res* 2007;1148:83–9. doi:10.1016/j.brainres.2007.02.021.

[158] Kujawa SG, Liberman MC. Adding Insult to Injury : Cochlear Nerve Degeneration after " Temporary " Noise-Induced Hearing Loss 2009;29:14077–85. doi:10.1523/JNEUROSCI.2845-09.2009.

[159] Liu S, Zhang K, Wu S, Ji X, Li N, Liu R, et al. Lead-induced hearing loss in rats and the protective effect of copper. *Biol Trace Elem Res* 2011;144:1112–9. doi:10.1007/s12011-011-9142-6.

[160] Bachmanov AA, Reed DR, Beauchamp GK, Tordoff MG. Food intake, water intake, and drinking spout side preference of 28 mouse strains. *Behav Genet* 2002;32:435–43. doi:10.1023/A:1020884312053.

[161] Kim S-J, Jeong H-J, Myung N-Y, Kim M, Lee J-H, So H, et al. The protective mechanism of antioxidants in cadmium-induced ototoxicity in vitro and in vivo. *Environ Health Perspect* 2008;116:854–62. doi:10.1289/ehp.10467.

[162] Adefegha SA, Omojokun OS, Oboh G. Modulatory effect of protocatechuic acid on

cadmium induced nephrotoxicity and hepatoxicity in rats in vivo. *Springerplus* 2015;4:619. doi:10.1186/s40064-015-1408-6.

[163] Barden EK, Rellinger EA, Ortmann AJ, Ohlemiller KK. Inheritance Patterns of Noise Vulnerability and "Protectability" in (C57BL/6J × CBA/J) F1 Hybrid Mice. *J Am Acad Audiol* 2012;23:332–40. doi:10.3766/jaaa.23.5.4.

[164] Johnson A-C. The ototoxic effect of toluene and the influence of noise, acetyl salicylic acid, or genotype. A study in rats and mice. *Scand Audiol Suppl* 1993;39:1–40.

[165] Venet T, Campo P, Thomas A, Cour C, Rieger B, Cosnier F. The tonotopicity of styrene-induced hearing loss depends on the associated noise spectrum. *Neurotoxicol Teratol* 2015;48:56–63. doi:10.1016/j.ntt.2015.02.003.

[166] Payne BJ, Saunders LZ. Heavy metal nephropathy of rodents. *Vet Pathol Suppl* 1978;15:51–87.

[167] Fels LM, Herbort C, Pergande M, Jung K, Hotter G, Roselló J, et al. Nephron target sites in chronic exposure to lead. *Nephrol Dial Transpl* 1994;9:1740–6.

[168] Karri V, Schuhmacher M, Kumar V. Heavy metals (Pb, Cd, As and MeHg) as risk factors for cognitive dysfunction: A general review of metal mixture mechanism in brain. *Environ Toxicol Pharmacol* 2016;48:203–13. doi:10.1016/j.etap.2016.09.016.

[169] Andrade VM, Aschner M, Marreilha dos Santos AP. Neurotoxicity of metal mixtures. In: Aschner M, Costa LG, editors. Adv. Neurobiol., vol. 18, Cham: Springer International Publishing; 2017, p. 227–65. doi:10.1007/978-3-319-60189-2_12.

[170] Fernandez KA, Jeffers PWC, Lall K, Liberman MC, Kujawa SG. Aging after Noise Exposure: Acceleration of Cochlear Synaptopathy in "Recovered" Ears. *J Neurosci* 2015;35:7509–20. doi:10.1523/JNEUROSCI.5138-14.2015.

[171] Dorsey ER, Brown AW, Cavanagh JB, Nolan CC, Richards HK, Wylie SP. Functional/metabolic modulation of the brain stem lesions caused by 1,3-dinitrobenzene in the rat. *Neurotoxicology* 1992;13:379–88.

[172] Abajobir AA, Abate KH, Abbafati C, Abbas KM, Abd-Allah F, Abdulkader RS, et al. Global, regional, and national disability-adjusted life-years (DALYs) for 333 diseases and injuries and healthy life expectancy (HALE) for 195 countries and territories, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet* 2017;390:1260–344. doi:10.1016/S0140-6736(17)32130-X.

[173] Tharpe AMM, Sladen DPP, Dodd-Murphy J, Boney SJJ. Minimal hearing loss in children: Minimal but not inconsequential. Semin. Hear., vol. 30, 2009, p. 80–93. doi:10.1055/s-0029-1215437.

[174] Boss EF, Niparko JK, Gaskin DJ, Levinson KL. Socioeconomic disparities for hearing-impaired children in the United States. *Laryngoscope* 2011;121:860–6.

[175] Passchier-Vermeer W, Passchier WF. Noise exposure and public health. *Environ Health Perspect* 2000;108:123–31. doi:10.2307/3454637.

[176] Carlson A, Norwitz ER, Stiller RJ. Cytomegalovirus infection in pregnancy: should all women be screened? *Rev Obstet Gynecol* 2010;3:172–9. doi:10.3909/riog0131.

[177] Emery VC, Lazzarotto T. Cytomegalovirus in pregnancy and the neonate. *F1000Research* 2017;6:138. doi:10.12688/f1000research.10276.1.

[178] Anniko M, Sarkady L. Cochlear pathology following exposure to mercury. *Acta Otolaryngol* 1978;85:213–24.

[179] Kurland LT, Faro SN, Siedler H. Minamata disease. The outbreak of a neurologic disorder in Minamata, Japan, and its relationship to the ingestion of seafood contaminated by mercuric compounds. *World Neurol* 1960;1:370–95.

[180] Rothwell JA, Boyd PJ. Amalgam dental fillings and hearing loss. *Int J Audiol* 2008;47:770–6. doi:10.1080/14992020802311224.

[181] Schell LM, Denham M, Stark AD, Ravenscroft J, Parsons P, Schulte E. Relationship between blood lead concentration and dietary intakes of infants from 3 to 12 months of age. *Environ Res* 2004;96:264–73. doi:10.1016/j.envres.2004.02.008.

[182] Berry MJ, Ralston NVC. Mercury Toxicity and the Mitigating Role of Selenium. *Ecohealth* 2008;5:456–9. doi:10.1007/s10393-008-0204-y.

[183] Spankovich C, Le Prell CG. Healthy diets, healthy hearing: National Health and Nutrition Examination Survey, 1999-2002. *Int J Audiol* 2013;52:369–76. doi:10.3109/14992027.2013.780133.

[184] Spankovich C, Hood LJ, Silver HJ, Lambert W, Flood VM, Mitchell P. Associations between Diet and Both High and Low Pure Tone Averages and Transient Evoked Otoacoustic Emissions in an Older Adult Population-Based Study. *J Am Acad Audiol* 2011;22:49–58. doi:10.3766/jaaa.22.1.6.

[185] Kang WS, Lim HW, Suh JK, Chung JW. Effects of a zinc-deficient diet on hearing in CBA mice. *Neuroreport* 2012;23:201–5. doi:10.1097/WNR.0b013e328350235f.

[186] Chung S-D, Chen P-Y, Lin H-C, Hung S-H. Sudden Sensorineural Hearing Loss Associated With Iron-Deficiency Anemia. *JAMA Otolaryngol Neck Surg* 2014;140:417. doi:10.1001/jamaoto.2014.75.

[187] Therrell BL, Hannon WH, Bailey DB, Goldman EB, Monaco J, Norgaard-Pedersen B, et al. Committee report: Considerations and recommendations for national guidance regarding the retention and use of residual dried blood spot specimens after newborn screening. *Genet Med* 2011;13:621–4. doi:10.1097/GIM.0b013e3182147639.

[188] Olshan AF. Meeting report: the use of newborn blood spots in environmental research: opportunities and challenges. *Environ Health Perspect* 2007;115:1767–79. doi:10.1289/ehp.10511.

[189] Michigan Early Hearing Detection and Intervention, Michigan Department of Community Health. Program Report 2011 (2004-2008 Data). 2011.

[190] Michigan Early Hearing Detection and Intervention, Michigan Department of Community Health; Program Report 2013 (2007-2011 Data). 2013.

[191] Michigan Department of Community Health, Childhood Lead Poisoning Prevention

Program. 2010 Data Report on Blood Lead Testing and Elevated Levels. 2010.

[192] Nriagu J, Burt B, Linder A, Ismail A, Sohn W. Lead levels in blood and saliva in a low-income population of Detroit, Michigan. *Int J Hyg Environ Health* 2006;209:109–21. doi:10.1016/j.ijheh.2005.11.005.

[193] Evers DC, Wiener JG, Basu N, Bodaly RA, Morrison HA, Williams KA. Mercury in the Great Lakes region: bioaccumulation, spatiotemporal patterns, ecological risks, and policy. *Ecotoxicology* 2011;20:1487–99. doi:10.1007/s10646-011-0784-0.

[194] US Environmental Protection Agency (EPA). National Listing of Fish Advisories 2011. 2018.

[195] US Census Bureau. Estimated Population of Michigan Regions and Statistical Areas, 2000-2011. 2012.

[196] Brown G. NICU noise and the preterm infant. *Neonatal Netw* 2009;28:165–73.

[197] Basu N, Eng JWL, Perkins M, Santa-Rios A, Martincevic G, Carlson K, et al. Development and application of a novel method to characterize methylmercury exposure in newborns using dried blood spots. *Environ Res* 2017;159:276–82. doi:10.1016/j.envres.2017.08.021.

[198] Nam DH, Basu N. Rapid methods to detect organic mercury and total selenium in biological samples. *Chem Cent J* 2011;5:3. doi:10.1186/1752-153X-5-3.

[199] Grandjean P, Landrigan PJ. Neurobehavioural effects of developmental toxicity. *Lancet Neurol* 2014;13:330–8. doi:10.1016/S1474-4422(13)70278-3.

[200] Hong Y-S, Kim Y-M, Lee K-E. Methylmercury exposure and health effects. *J Prev Med Public Health* 2012;45:353–63. doi:10.3961/jpmph.2012.45.6.353.

[201] Murata K, Weihe P, Budtz-Jørgensen E, Jørgensen PJ, Grandjean P. Delayed brainstem auditory evoked potential latencies in 14-year-old children exposed to methylmercury. *J Pediatr* 2004;144:177–83. doi:10.1016/j.jpeds.2003.10.059.

[202] Rice DC, Gilbert SG. Exposure to methyl mercury from birth to adulthood impairs high-

frequency hearing in monkeys. *Toxicol Appl Pharmacol* 1992;115:6–10.

[203] Choi Y-H, Park SK. Environmental exposures to lead, mercury, and cadmium and hearing loss in adults and adolescents: KNHANES 2010–2012. *Environ Health Perspect* 2017;125:1–8. doi:10.1289/EHP565.

[204] Dutra MDS, Cavadas M, Jesus IM de, Santos EO, Silva EA da, Câmara V de M. Hearing thresholds in children exposed to mercury in the prenatal period. *J Soc Bras Fonoaudiol* 2012;24:322–6. doi:10.1590/S2179-64912012000400006.

[205] Lasky RE, Luck ML, Parikh NA, Laughlin NK. The effects of early lead exposure on the brains of adult rhesus monkeys: A volumetric MRI study. *Toxicol Sci* 2005;85:963–75. doi:10.1093/toxsci/kfi153.

[206] Lilienthal H, Winneke G. Lead effects on the brain stem auditory evoked potential in monkeys during and after the treatment phase. *Neurotoxicol Teratol* 1996;18:17–32.

[207] Lasky RE, Luck ML, Torre P, Laughlin N. The effects of early lead exposure on auditory function in rhesus monkeys. *Neurotoxicol Teratol* 2001;23:639–49. doi:10.1016/S0892-0362(01)00175-1.

[208] Osman K, Pawlas K, Schütz A, Gazdzik M, Sokal JA, Vahter M. Lead exposure and hearing effects in children in Katowice, Poland. *Environ Res* 1999;80:1–8. doi:10.1006/enrs.1998.3886.

[209] Meites S. Normal total plasma calcium in the newborn. *CRC Crit Rev Clin Lab Sci* 1975;6:1–18.

[210] David L, Anast CS. Calcium metabolism in newborn infants. The interrelationship of parathyroid function and calcium, magnesium, and phosphorus metabolism in normal, "sick," and hypocalcemic newborns. *J Clin Invest* 1974;54:287–96. doi:10.1172/JCI107764.

[211] Dayaratne MWN, Vlajkovic SM, Lipski J, Thorne PR. Kölliker's organ and the development of spontaneous activity in the auditory system: Implications for hearing

dysfunction. *Biomed Res Int* 2014;2014:367939. doi:10.1155/2014/367939.

[212] Gröschel M, Hubert N, Müller S, Ernst A, Basta D. Age-dependent changes of calcium related activity in the central auditory pathway. *Exp Gerontol* 2014;58:235–43. doi:10.1016/j.exger.2014.08.014.

[213] Ohtani I, Schuknecht HF. Temporal bone pathology in digeorge's syndrome. *Ann Otol Rhinol Laryngol* 1984;93:220–4. doi:10.1177/000348948409300306.

[214] Schuffenhauer S, Lichtner P, Peykar-Derakhshandeh P, Murken J, Haas OA, Back E, et al. Deletion mapping on chromosome 10p and definition of a critical region for the second DiGeorge syndrome locus (DGS2). *Eur J Hum Genet* 1998;6:213–25. doi:10.1038/sj.ejhg.5200183.

[215] Ikeda K, Kobayashi T, Kusakari J, Takasaka T, Yumita S, Furukawa Y. Sensorineural hearing loss associated with hypoparathyroidism. *Laryngoscope* 1987;97:1075–9.

[216] Wagner CL, Hollis BW. Beyond PTH : assessing vitamin D status during early pregnancy 2011:285–6. doi:10.1002/jbmr.463/pdf.9.

[217] van der Meer IM, Karamali NS, Boeke AJP, Lips P, Middelkoop BJ, Verhoeven I, et al. High prevalence of vitamin D deficiency in pregnant non-Western women in The Hague, Netherlands1,2. *Am J Clin Nutr* 2006;84:350–3. doi:10.1093/ajcn/84.1.350.

[218] Eyles D, Burne T, McGrath J. Vitamin D in fetal brain development. *Semin Cell Dev Biol* 2011;22:629–36. doi:10.1016/j.semcdb.2011.05.004.

[219] Graven SN, Browne J V. Auditory Development in the Fetus and Infant. *Newborn Infant Nurs Rev* 2008;8:187–93. doi:10.1053/j.nainr.2008.10.010.

[220] Hart PH, Lucas RM, Walsh JP, Zosky GR, Whitehouse AJO, Zhu K, et al. Vitamin D in Fetal Development: Findings From a Birth Cohort Study. *Pediatrics* 2014;135:1–9. doi:10.1542/peds.2014-1860.

[221] Liu XL, Lu YS, Gao JY, Marshall C, Xiao M, Miao DS, et al. Calcium sensing receptor

absence delays postnatal brain development via direct and indirect mechanisms. *Mol Neurobiol* 2013;48:590–600. doi:10.1007/s12035-013-8448-0.

[222] Fleet JC, Gliniak C, Zhang Z, Xue Y, Smith KB, McCreedy R, et al. Serum metabolite profiles and target tissue gene expression define the effect of cholecalciferol intake on calcium metabolism in rats and mice. *J Nutr* 2008;138:1114–20. doi:10.1038/jid.2014.371.

[223] Brookes GB. Vitamin D deficiency--a new cause of cochlear deafness. *J Laryngol Otol* 1983;97:405–20.

[224] Akeson PK, Lind T, Hernell O, Silfverdal SA, Ohlund I. Serum Vitamin D depends less on latitude than on skin color and dietary intake during early winter in northern Europe. *J Pediatr Gastroenterol Nutr* 2016;62:643–9. doi:10.1097/MPG.0000000000001028.

[225] Lee JH, Gadi R, Spertus JA, Tang F, O'Keefe JH. Prevalence of vitamin D deficiency in patients with acute myocardial infarction. *Am J Cardiol* 2011;107:1636–8. doi:10.1016/j.amjcard.2011.01.048.

[226] Krall EA, Dawson-Hughes B. Smoking increases bone loss and decreases intestinal calcium absorption. *J Bone Miner Res* 1999;14:215–20. doi:10.1359/jbmr.1999.14.2.215.

[227] Brot C, Rye Jørgensen N, Helmer Sørensen O, Jorgensen NR, Sorensen OH, Jùrgensen NR, et al. The influence of smoking on vitamin D status and calcium metabolism 1999;53:920–6. doi:10.1038/sj.ejcn.1600870.

[228] Hollis BW, Wagner CL. Vitamin D deficiency during pregnancy: an ongoing epidemic. *Am J Clin Nutr* 2006;84:273. doi:84/2/273 [pii].

[229] US National Institute on Deafness and Other Communication Disorders (NIDCD). NIDCD Statistical Report: Prevalence of Hearing Loss in U.S. Children, 2005 2010. https://www.nidcd.nih.gov/health/statistics/nidcd-statistical-report-prevalence-hearing-loss-us-children-2005 (accessed April 29, 2017).

[230] Eyles DW, Morley R, Anderson C, Ko P, Burne T, Permezel M, et al. The utility of neonatal dried blood spots for the assessment of neonatal vitamin D status. *Paediatr Perinat*

Epidemiol 2010;24:303–8. doi:10.1111/j.1365-3016.2010.01105.x.

[231] Kvaskoff D, Heath AK, Simila HA, Ko P, English DR, Eyles DW. Minimizing matrix effects for the accurate quantification of 25-hydroxyvitamin D metabolites in dried blood spots by LC-MS/MS. *Clin Chem* 2016;62:639–46. doi:10.1373/clinchem.2015.251538.

[232] Shah I, James R, Barker J, Petroczi A, Naughton DP. Misleading measures in Vitamin D analysis: a novel LC-MS/MS assay to account for epimers and isobars. *Nutr J* 2011;10:46. doi:10.1186/1475-2891-10-46.

[233] Sun A-H, Xiao S-Z, Li B-S, Li Z-J, Wang T-Y, Zhang Y-S. Iron deficiency and hearing loss. *Orl* 1987;49:118–22.

[234] Schieffer KM, Chuang CH, Connor J, Pawelczyk JA, Sekhar DL. Association of iron deficiency anemia with hearing loss in US adults. *JAMA Otolaryngol - Head Neck Surg* 2017;143:350–4. doi:10.1001/jamaoto.2016.3631.

[235] Ciorba A, Bovo R, Castiglione A, Pirodda A, Martini A. Sudden bilateral sensorineural hearing loss as an unusual consequence of accidental ingestion of potassium hydroxide. *Med Princ Pract* 2010;19:406–8. doi:10.1159/000316382.

[236] Kim J, Xia A, Grillet N, Applegate BE, Oghalai JS. Osmotic stabilization prevents cochlear synaptopathy after blast trauma. *Proc Natl Acad Sci U S A* 2018;115:E4853–60. doi:10.1073/pnas.1720121115.

[237] Daly K, Farrington E. Hypokalemia and Hyperkalemia in Infants and Children: Pathophysiology and Treatment. *J Pediatr Heal Care* 2013;27:486–96. doi:10.1016/j.pedhc.2013.08.003.

[238] Bhanugopan MS, Fulkerson WJ, Fraser DR, Hyde M, McNeill DM. Carryover effects of potassium supplementation on calcium homeostasis in dairy cows at parturition. *J Dairy Sci* 2010;93:2119–29. doi:10.3168/jds.2009-2703.

[239] Lynch SR. The effect of calcium on iron absorption. *Nutr Res Rev* 2000;13:141. doi:10.1079/095442200108729043.

[240] Spankovich C, Le Prell CG. Associations between dietary quality, noise, and hearing: Data from the National Health and Nutrition Examination Survey, 1999–2002. *Int J Audiol* 2014;53:796–809. doi:10.3109/14992027.2014.921340.

[241] Skalny A V., Berezkina ES, Kiyaeva E V., Alidzhanova IE, Grabeklis AR, Tinkov AA. The effect of alcohol consumption on maternal and cord blood electrolyte and trace element levels. *Acta Sci Pol Technol Aliment* 2016;15:439–45. doi:10.17306/J.AFS.2016.4.42.

[242] Simões H de O, Zanchetta S, Furtado EF. What we know of the central auditory disorders in children exposed to alcohol during pregnancy? Systematic review. *CoDAS* 2016;28:640–5. doi:10.1590/2317-1782/20162015253.

[243] Weijl NI, Elsendoorn TJ, Lentjes EGWM, Hopman GD, Wipkink-Bakker A, Zwinderman AH, et al. Supplementation with antioxidant micronutrients and chemotherapy-induced toxicity in cancer patients treated with cisplatin-based chemotherapy: A randomised, double-blind, placebo-controlled study. *Eur J Cancer* 2004;40:1713–23. doi:10.1016/j.ejca.2004.02.029.

[244] Hoeve LJ, Wensink J, Mertens zur Borg IR. Hearing loss related to zinc deficiency in rats. *Eur Arch Otorhinolaryngol* 1990;247:267–70.

[245] Chmielnicka J, Zareba G, Witasik M, Brzeźnicka E. Zinc-selenium interaction in the rat. *Biol Trace Elem Res* 1988;15:267–76.

[246] Lee MS, Li W. Dried blood spots : applications and techniques. Hoboken, New Jersey: John Wiley & Sons; 2014.

[247] Timmerman P, White S, Globig S, Lüdtke S, Brunet L, Smeraglia J. EBF recommendation on the validation of bioanalytical methods for dried blood spots. *Bioanalysis* 2011;3:1567–75. doi:10.4155/bio.11.132.

[248] Counter SA, Buchanan LH, Ortega F. Blood lead levels in Andean infants and young children in Ecuador: An international comparison. *J Toxicol Environ Heal - Part A Curr Issues* 2015;78:778–87. doi:10.1080/15287394.2015.1031050.

[249] Pirkle JL, Gunter EW, Paschal DC, Matte TD, Kramer RA, Flegal KM, et al. The decline in blood lead levels in the United States: The National Health and Nutrition Examination Surveys (NHANES). *J Am Med Assoc* 1994;272:284–91. doi:10.1001/jama.1994.03520040046039.

[250] Brown MJ, Shenassa E, Matté TD, Catlin SN. Children in Illinois with elevated blood lead levels, 1993-1998, and lead-related pediatric hospital admissions in Illinois, 1993-1997. *Public Health Rep* 2000;115:532–6. doi:10.1093/phr/115.6.532.

[251] Cestari T, Buster K. Photoprotection in specific populations: Children and people of color. *J Am Acad Dermatol* 2017;76:S110–21. doi:10.1016/j.jaad.2016.09.039.

[252] Ariganjoye R. Pediatric Hypovitaminosis D. *Glob Pediatr Heal* 2017;4:2333794X1668550. doi:10.1177/2333794X16685504.

[253] Kim JH, Rodríguez-Vázquez JF, Verdugo-López S, Cho KH, Murakami G, Cho BH. Early Fetal Development of the Human Cochlea. *Anat Rec Adv Integr Anat Evol Biol* 2011;294:996–1002. doi:10.1002/ar.21387.

[254] Lebel C, Beaulieu C. Longitudinal Development of Human Brain Wiring Continues from Childhood into Adulthood. *J Neurosci* 2011;31:10937–47. doi:10.1523/jneurosci.5302-10.2011.

[255] Semple BD, Blomgren K, Gimlin K, Ferriero DM, Noble-Haeusslein LJ. Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. *Prog Neurobiol* 2013;106–107:1–16. doi:10.1016/j.pneurobio.2013.04.001.

[256] World Health Organization (WHO). Addressing the rising prevalence of hearing loss. Geneva, Switzerland: World Health Organization; 2018.

[257] Järvelin MR, Mäki-Torkko E, Sorri MJ, Rantakallio PT. Effect of hearing impairment on educational outcomes and employment up to the age of 25 years in northern Finland. *Br J Audiol* 1997;31:165–75.

[258] Punch R. Employment and Adults Who Are Deaf or Hard of Hearing: Current Status and Experiences of Barriers, Accommodations, and Stress in the Workplace. *Am Ann Deaf* 2016;161:384–97. doi:10.1353/aad.2016.0028.

[259] Carroll YI, Eichwald J, Scinicariello F, Hoffman HJ, Deitchman S, Radke MS, et al. Vital Signs: Noise-Induced Hearing Loss Among Adults — United States 2011–2012. *MMWR Morb Mortal Wkly Rep* 2017;66:139–44. doi:10.15585/mmwr.mm6605e3.

[260] Abrams HB, Kihm J. An Introduction to MarkeTrak IX: A New Baseline for the Hearing Aid Market. *Hear Rev* 2015;22.

[261] Forge A, Schacht J. Aminoglycoside antibiotics. *Audiol Neurootology* 2000;5:3–22.

[262] Passchier-Vermeer W, Passchier WF. Noise exposure and public health. *Env Heal Perspect* 2000;108 Suppl:123–31.

[263] Vyskocil A, Truchon G, Leroux T, Lemay F, Gendron M, Gagnon F, et al. A weight of evidence approach for the assessment of the ototoxic potential of industrial chemicals. *Toxicol Ind Health* 2012;28:796–819.

[264] White LD, Cory-Slechta DA, Gilbert ME, Tiffany-Castiglioni E, Zawia NH, Virgolini M, et al. New and evolving concepts in the neurotoxicology of lead. *Toxicol Appl Pharmacol* 2007;225:1–27.

[265] Yan Y, Bian JC, Zhong LX, Zhang Y, Sun Y, Liu ZP. Oxidative stress and apoptotic changes of rat cerebral cortical neurons exposed to cadmium in vitro. *Biomed Environ Sci* 2012;25:172–81. doi:10.3967/0895-3988.2012.02.008.

[266] Robinson BH. E-waste: An assessment of global production and environmental impacts. *Sci Total Environ* 2009;408:183–91. doi:10.1016/j.scitotenv.2009.09.044.

[267] Schmidt CW. Unfair trade: e-waste in Africa. *Environ Heal Perspect* 2006;114:a232-5. doi:10.1289/ehp.114-a232.

[268] United Nation's Environmental Programme. Parties to the Basel Convention 2011.

http://www.basel.int/Countries/StatusofRatifications/PartiesSignatories/tabid/4499/Default.aspx (accessed January 1, 2019).

[269] Basel Convention. Ghana e-waste country assessment: SBC e-waste Africa project. 2011.

[270] Akormedi M, Asampong E, Fobil JN. Working conditions and environmental exposures among electronic waste workers in Ghana. *Int J Occup Environ Health* 2013;19:278–86. doi:10.1179/2049396713Y.0000000034.

[271] Frazzoli C, Orisakwe OE, Dragone R, Mantovani A. Diagnostic health risk assessment of electronic waste on the general population in developing countries' scenarios. *Environ Impact Assess Rev* 2010;30:388–99. doi:10.1016/j.eiar.2009.12.004.

[272] Berglund B, Lindvall T, Schwela DH, World Health Organization: Occupational and Environmental Health Team. Guidelines for community noise. vol. 31. Geneva: World Health Organization; 1999. doi:10.1260/0957456001497535.

[273] Coles RRA, Lutman ME, Buffin JT. Guidelines on the diagnosis of noise-induced hearing loss for medicolegal purposes. *Clin Otolaryngol Allied Sci* 2000;25:264–73. doi:10.1046/j.1365-2273.2000.00368.x.

[274] Centers for Disease Control and Prevention (CDC). Fourth National Report on Human Exposure to Environmental Chemicals. 2009.

[275] American Conference of Governmental Industrial Hygienists (ACGIH). Threshold limit values for chemical substances and physical agents and biological exposure indices. Cincinnati, OH: 2018.

[276] Agency for Toxic Substances and Disease Registry (ATSDR). Toxicological Profile for Mercury. Atlanta, GA, USA: U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention (CDC); 1999.

[277] Taylor V, Goodale B, Raab A, Schwerdtle T, Reimer K, Conklin S, et al. Human exposure to organic arsenic species from seafood. *Sci Total Environ* 2017;580:266–82. doi:10.1016/j.scitotenv.2016.12.113.

[278] International Organization for Standardization (ISO). Acoustics - estimation of noise-induced hearing loss (ISO 1999:2013) 2013.

[279] Ayibani IT. EPA Sensitizes Stakeholders on Noise Pollution n.d. http://www.ghana.gov.gh/index.php/media-center/regional-news/3174-epa-sensitizes-stakeholders-on-noise-pollution (accessed January 2, 2019).

[280] Odoi-Larbi S. EPA sets guidelines for noise-making 2012. https://www.modernghana.com/news/395828/epa-sets-guidelines-for-noise-making.html (accessed January 2, 2019).

[281] Ghana Environmental Assessment Capacity Development Programme. EPA Energy Sector Guidelines Development: Volume 2 Report. 2001. doi:10.1002/gepi.1370050305.

[282] Barceloux DG. Manganese. *Clin Toxicol* 1999;37:293–307.

[283] Myers JE, Thompson ML, Ramushu S, Young T, Jeebhay MF, London L, et al. The nervous system effects of occupational exposure on workers in a South African manganese smelter. *Neurotoxicology* 2003;24:885–94. doi:S0161-813X(03)00081-0 [pii]\r10.1016/S0161-813X(03)00081-0.

[284] US Department of Health and Human Services (DHHS). National Health and Nutrition Examination Survey (NHANES), III 1988-1994, U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention (CDC).; 1997, p. Series 11, No. 1.

[285] Dickson RC, Tomlinson RH. Selenium in blood and human tissues. *Clin Chim Acta* 1967;16:311–21. doi:10.1016/0009-8981(67)90197-0.

[286] Zhu L. Keshan Disease. In: Gawthorne J, McHowell J., White CL, editors. Proc. Fourth Int. Symp. Trace Elem. Metab. Man Anim. (TEMA-4), Canberra: Australian Academy of Sciences; 1981.

[287] Yang G, Wang S, Zhou R, Sun S. Endemic selenium intoxication of humans in China. *Am J Clin Nutr* 1983;37:872–81. doi:10.1016/S0140-6736(83)90575-5.

[288] Bratakos MS, Kanaki HC, Vasiliou-Waite A, Ioannou P V. The nutritional selenium status of healthy Greeks. *Sci Total Environ* 1990;91:161–76.

[289] Minoia C, Sabbioni E, Apostoli P, Pietra R, Pozzoli L, Gallorini M, et al. Trace element reference values in tissues from inhabitants of the European community I. A study of 46 elements in urine, blood and serum of Italian subjects. *Sci Total Environ* 1990;95:89–105. doi:10.1016/0048-9697(90)90055-Y.

[290] Jaakkola K, Tummavuori J, Pirinen A, Kurkela P, Tolonen M, Arstila AU. Selenium levels in whole blood of finnish volunteers before and during organic and inorganic selenium supplementation. *Scand J Clin Lab Invest* 1983;43:473–6. doi:10.1080/00365518309168433.

[291] Kumpusalo E, Karinpaa A, Jauhiainen M, Laitinen M, Lappetelainen R, Maenpaa PH. Multivitamin supplementation of adult omnivores and lactovegetarians: Circulating levels of vitamin a, d and e, lipids, apolipoproteins and selenium. *Int J Vitam Nutr Res* 1990;60:58–66.

[292] Thomson CD, Robinson MF. Selenium in human health and disease with emphasis on those aspects peculiar to New Zealand. *Am J Clin Nutr* 1980;33:303–23.

[293] Rea HM, Thomson CD, Campbell DR, Robinson MF. Relation between erythrocyte selenium concentrations and glutathione peroxidase (EC 1.11.1.9) activities of New Zealand residents and visitors to New Zealand. *Br J Nutr* 1979;42:201. doi:10.1079/BJN19790107.

[294] Oster O, Schmiedel G, Prellwitz W. Correlations of blood selenium with hematological parameters in West German Adults. *Biol Trace Elem Res* 1988;15:47–81. doi:10.1007/BF02990126.

[295] Allaway WH, Kubota J, Losee F, Roth M. Selenium, Molybdenum, and Vanadium in Human Blood. *Arch Environ Health* 1968;16:342–8. doi:10.1080/00039896.1968.10665069.

[296] Yang G-Q, Ge K, Chen J, Chen X. Selenium-related endemic diseases and the daily

selenium requirement of humans. *World Rev Nutr Diet* 1988;55:98–152.

[297] Agency for Toxic Substances and Disease Registry (ATSDR). Toxicological Profile for Selenium. Atlanta, GA, USA: U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention (CDC); 2003.

[298] Heydorn K. Environmental variation of arsenic levels in human blood determines by neutron activation analysis. *Clin Chim Acta* 1970;28:349–57.

[299] Hindmarsh JT, McCurdy RF. Clinical and environmental aspects of arsenic toxicity. *CRC CritRevClinLabSci* 1986;23:315–47. doi:10.3109/10408368609167122.

[300] Valentine JL, Kang HK, Spivey G. Arsenic levels in human blood, urine, and hair in response to exposure via drinking water. *Environ Res* 1979;20:24–32. doi:10.1016/0013-9351(79)90082-3.

[301] Amonoo-Neizer EH, Amekor EM. Determination of total arsenic in environmental samples from Kumasi and Obuasi, Ghana. *Environ Health Perspect* 1993;101:46–9. doi:10.1289/ehp.9310146.

[302] Fosu Gyasi S, Awuah E, Asiedu Lar J, Asumeng Ko G. Arsenic in Water and Soil: A Possible Contributory Factor in Mycobacterium ulcerans Infection in Buruli Ulcer Endemic Areas. *Asian J Biol Sci* 2012;5:66–75. doi:10.3923/ajbs.2012.66.75.

[303] Adokoh CK, Obodai EA, Essumang DK, Serfor-Armah Y, Nyarko BJB, Asabere-Ameyaw A. Statistical evaluation of environmental contamination, distribution and source assessment of heavy metals (aluminum, arsenic, cadmium, and mercury) in some lagoons and an estuary along the coastal belt of Ghana. *Arch Environ Contam Toxicol* 2011;61:389–400. doi:10.1007/s00244-011-9643-5.

[304] Chuttani HK, Gupta PS, Gulati S, Gupta DN. Acute copper sulfate poisoning. *Am J Med* 1965;39:849–54. doi:10.1016/0002-9343(65)90105-1.

[305] Moreno MA, Marin C, Vinagre F, Ostapczuk P. Trace element levels in whole blood samples from residents of the city Badajoz, Spain. *Sci Total Environ* 1999;229:209–15.

doi:10.1016/S0048-9697(99)00074-1.

[306]   Bárány E, Bergdahl IA, Bratteby LE, Lundh T, Samuelson G, Schütz A, et al. Relationships between trace element concentrations in human blood and serum. *Toxicol Lett* 2002;134:177–84. doi:10.1016/S0378-4274(02)00187-X.

[307]   Barnett JB, Dao MC, Hamer DH, Kandel R, Brandeis G, Wu D, et al. Effect of zinc supplementation on serum zinc concentration and T cell proliferation in nursing home elderly: a randomized, double-blind, placebo-controlled trial. *Am J Clin Nutr* 2016;103:942–51. doi:10.3945/ajcn.115.115188.

[308]   The Age-Related Eye Disease Study Research Group. The effect of five-year zinc supplementation on serum zinc, serum cholesterol and hematocrit in persons randomly assigned to treatment group in the age-related eye disease study: AREDS Report No. 7. *J Nutr* 2002;132:697–702. doi:10.1093/jn/132.4.697.

[309]   Jung S, Kim S, Yeo S. Association of Nutritional Factors with Hearing Loss. *Nutrients* 2019;11:307. doi:10.3390/nu11020307.

[310]   Blaksmith Institute, Green Cross. The world's worst 2013: The top ten toxic threats. New York, USA: 2013.

[311]   United Nations Environment Programme. Annual Report 2010: Harmful Substances and Hazardous Waste. 2011.

[312]   Nordic Council of Ministers. Cadmium Review. 2003.

[313]   Hovmand MF, Kemp K, Kystol J, Johnsen I, Riis-Nielsen T, Pacyna JM. Atmospheric heavy metal deposition accumulated in rural forest soils of southern Scandinavia. *Environ Pollut* 2008;155:537–41. doi:10.1016/j.envpol.2008.01.047.

[314]   Oner G, Sentürk UK, Izgüt-Uysal VN. Role of cadmium-induced lipid peroxidation in the kidney response to atrial natriuretic hormone. *Nephron* 1996;72:257–62.

[315]   Sataloff RT, Sataloff J, Virag TM. Occupational Hearing Loss. In: Sataloff RT, Sataloff J,

editors. Hear. Loss. 4th ed., Boca Raton, FL: Taylor and Francis; 2005, p. 403–33.

[316] Phaneuf R, Hétu R. An epidemiological perspective of the causes of hearing loss among industrial workers. *J Otolaryngol* 1990;19:31–40.

[317] Hétu R, Getty L, Waridel S. Attitudes towards co-workers affected by occupational hearing loss. II: Focus groups interviews. *Br J Audiol* 1994;28:313–25.

[318] Emmett SD, Francis HW. The Socioeconomic Impact of Hearing Loss in U.S. Adults. *Otol Neurotol* 2015;36:545–50. doi:10.1097/MAO.0000000000000562.

[319] Bainbridge KE, Ramachandran V. Hearing aid use among older U.S. adults: The national health and nutrition examination survey, 2005-2006 and 2009-2010. *Ear Hear* 2014;35:289–94. doi:10.1097/01.aud.0000441036.40169.29.

[320] Arlinger S. Negative consequences of uncorrected hearing loss--a review. *Int J Audiol* 2003;42 Suppl 2:2S17-20.

[321] Maharani A, Dawes P, Nazroo J, Tampubolon G, Pendleton N, SENSE-Cog WP1 group. Longitudinal Relationship Between Hearing Aid Use and Cognitive Function in Older Americans. *J Am Geriatr Soc* 2018;66:1130–6. doi:10.1111/jgs.15363.

[322] Lataye R, Campo P, Loquet G. Combined effects of noise and styrene exposure on hearing function in the rat. *Hear Res* 2000;139:86–96. doi:10.1016/S0378-5955(99)00174-4.

[323] Jamesdaniel S, Rosati R, Westrick J, Ruden DM. Chronic lead exposure induces cochlear oxidative stress and potentiates noise-induced hearing loss. *Toxicol Lett* 2018;292:175–80. doi:10.1016/j.toxlet.2018.05.004.

[324] Wilpizeski C. Effects of lead on the vestibular system: Preliminary findings. vol. 84. 1974. doi:10.1288/00005537-197405000-00013.

[325] Friedrich LA, Halden NM. Determining Exposure History of Northern Pike and Walleye to Tailings Effluence Using Trace Metal Uptake in Otoliths. *Environ Sci Technol* 2010;44:1551–8. doi:10.1021/es903261q.

[326] Daverat F, Lanceleur L, Pécheyran C, Eon M, Dublon J, Pierre M, et al. Accumulation of Mn, Co, Zn, Rb, Cd, Sn, Ba, Sr, and Pb in the otoliths and tissues of eel (Anguilla anguilla) following long-term exposure in an estuarine environment. *Sci Total Environ* 2012;437:323–30. doi:10.1016/j.scitotenv.2012.06.110.

[327] Conlee JW, Abdul-Baqi KJ, McCandless GA, Creel DJ. Differential susceptibility to noise-induced permanent threshold shift between albino and pigmented guinea pigs. *Hear Res* 1986;23:81–91.

[328] Rivera J, Tessarollo L. Genetic Background and the Dilemma of Translating Mouse Studies to Humans. *Immunity* 2008;28:1–4. doi:10.1016/j.immuni.2007.12.008.

[329] Lanzieri TM, Chung W, Flores M, Blum P, Caviness AC, Bialek SR, et al. Hearing Loss in Children With Asymptomatic Congenital Cytomegalovirus Infection. *Pediatrics* 2017;139:e20162610. doi:10.1542/peds.2016-2610.

[330] Xyrichis A, Wynne J, Mackrill J, Rafferty AM, Carlyle A. Noise pollution in hospitals. *Bmj* 2018;4808:k4808. doi:10.1136/bmj.k4808.

[331] Kitcher ED, Ocansey G, Abaidoo B, Atule A. Occupational hearing loss of market mill workers in the city of Accra, Ghana. *Noise Health* 2014;16:183–8. doi:10.4103/1463-1741.134919.

[332] Basu N, Clarke E, Green A, Calys-Tagoe B, Chan L, Dzodzomenyo M, et al. Integrated assessment of artisanal and small-scale gold mining in Ghana--part 1: human health review. *Int J Environ Res Public Health* 2015;12:5143–76. doi:10.3390/ijerph120505143.

[333] Gyamfi CKR, Amankwaa I, Owusu Sekyere F, Boateng D. Noise Exposure and Hearing Capabilities of Quarry Workers in Ghana: A Cross-Sectional Study. *J Environ Public Health* 2016;2016:7054276. doi:10.1155/2016/7054276.

[334] Srigboh RK, Basu N, Stephens J, Asampong E, Perkins M, Neitzel RL, et al. Multiple elemental exposures amongst workers at the Agbogbloshie electronic waste (e-waste) site in Ghana. *Chemosphere* 2016;164:68–74. doi:10.1016/j.chemosphere.2016.08.089.

[335] Carlson K. Electronic waste worker health: Studying complex problems in the real world. *Proc Eighth Int Conf Inf Commun Technol Dev - ICTD '16* 2016:1–4. doi:10.1145/2909609.2909618.

[336] Lundgren K. The global impact of e-waste: Addressing the challenge. Geneva, Switzerland: 2012.

[337] Rider C V., Boekelheide K, Catlin N, Gordon CJ, Morata T, Selgrade MJK, et al. Cumulative risk: Toxicity and interactions of physical and chemical stressors. *Toxicol Sci* 2014;137:3–11. doi:10.1093/toxsci/kft228.

[338] McGinnis DP, Brownstein JS, Patel CJ. Environment-Wide Association Study of Blood Pressure in the National Health and Nutrition Examination Survey (1999–2012). *Sci Rep* 2016;6:30373. doi:10.1038/srep30373.

[339] Zhuang X, Ni A, Liao L, Guo Y, Dai W, Jiang Y, et al. Environment-wide association study to identify novel factors associated with peripheral arterial disease: Evidence from the National Health and Nutrition Examination Survey (1999–2004). *Atherosclerosis* 2018;269:172–7. doi:10.1016/j.atherosclerosis.2018.01.006.

[340] Liberman MC, Kujawa SG. Cochlear synaptopathy in acquired sensorineural hearing loss: Manifestations and mechanisms. *Hear Res* 2017;349:138–47. doi:10.1016/j.heares.2017.01.003.

[341] Cernichiari E, Brewer R, Myers GJ, Marsh DO, Lapham LW, Cox C, et al. Monitoring methylmercury during pregnancy: Maternal hair predicts fetal brain exposure. *Neurotoxicology* 1995;16:705–10. doi:10.1093/jpids/pix105/4823046.

[342] Keeler GJ, Morishita M, Young L-H. Characterization of complex mixtures in urban atmospheres for inhalation exposure studies. *Exp Toxicol Pathol* 2005;57 Suppl 1:19–29.

[343] Silkworth JB, Mcmartin DN, Rej R, Narang RS, Stein VB, Briggs RG, et al. Subchronic exposure of mice to love canal soil contaminants. *Toxicol Sci* 1984;4:231–9. doi:10.1093/toxsci/4.2part1.231.

[344] Sim M. Transforming Agbogbloshie: From Toxic Dump Into Model Recycling Center - The Pollution BlogThe Pollution Blog. *Pollut Blog* 2015. http://www.pureearth.org/blog/photos-transforming-agbogbloshie-from-toxic-e-waste-dump-into-model-recycling-center/ (accessed December 7, 2018).

[345] Nylund AM. Demolition exercise, forced evictions, fire outbreak and rainfall. *Medium* 2015. https://medium.com/@AfricanSolarLLP/demolition-exercise-forced-evictions-fire-outbreak-and-rainfall-399541864075 (accessed December 7, 2018).

[346] GNA. CHRAJ condemns AMA over demolition | Regional News 2015-06-26. *GhanaWeb* 2015. https://www.ghanaweb.com/GhanaHomePage/regional/CHRAJ-condemns-AMA-over-demolition-364651 (accessed December 7, 2018).

APPENDIX: Survey for Ghanaian e-waste workers

Nutrition, Metals, and Hearing Loss Among E-waste Workers in Accra, Ghana
2014 Survey

Survey no. _____

GPS Waypoint No. _____   Interview Time Start: _ _:_ _ am / pm
LAT: _____
LONG: _____   Interview Time End: _ _:_ _ am / pm

Interviewer Name(s) _____

Interpreter Name(s) _____

Interview Date: Day: _____   Month: _____   Year: _____

Language: ☐₁ Twi   ☐₂ English   ☐₇₇₇ Other: _____

Country of Origin: _____

Part 1: INSTRUCTIONS TO INTERVIEWER

777= Other   999 =Refused to answer
888 = Don't Know   789= Skipped/ Not Available

Part 2: SAMPLES COLLECTED

1=Yes; 2=No; 3=Refused
Krystin=KC, Mozhgon=MR, Rachel=RL, Stephanie=SS, Rick=RN

ID Number	Full Name	Survey	Dosimetry	Hearing Test	Blood	Urine	Heart Rate	Saliva
Compensation (cedis)		2	1	2	2	1	1	1
Compensation given?								

156

Now I will ask you some questions about yourself.

Full name of interviewee:
1. Given: _____

2. Surname: _____

3. Other/Christian/nicknames: _____

4. Sex: $\Box_1$ Female $\Box_2$ Male [GO TO 7]

5. Are you pregnant now?
$\Box_{789}$ N/A $\Box_1$ Yes $\Box_2$ No $\Box_{888}$ Don't know

6. Have you breastfed in the last sixth months?
$\Box_{789}$ N/A $\Box_1$ Yes $\Box_2$ No $\Box_{888}$ Don't know

7. What was your age at your last birthday? _____ years (Estimated $\Box_1$)

8. Do you live within 1 km of Agbogbloshie? $\Box_1$ Yes $\Box_2$ No

9. How long have you been living here? _____ years _____months

10.   Just before you lived here, where did you live? _____

11.   Where do you sleep? $\Box_1$ Self Rented Room $\Box_2$ Friend's Rented Room
$\Box_3$ Kiosk/Shop $\Box_4$ Open space $\Box_5$ Other: _____

12.   What is your daily income? $\Box_1$ <GHS10 $\Box_2$ GHS11-20
$\Box_3$ GHS21-30 $\Box_4$ GHS31-40 $\Box_5$ >GHS 40

13.   To which of the following religious groups do you belong?
$\Box_0$ No religion $\Box_1$ Catholic Christian $\Box_2$ Protestant Christian
$\Box_3$ Muslim $\Box_4$ Traditional $\Box_5$ Other _____

14.   What is your marital status?
$\Box_{789}$ N/A $\Box_1$ Single $\Box_2$ Married $\Box_3$ Divorced $\Box_4$ Widowed $\Box_5$ Separated

15.   What is the highest level of school you attended: primary, middle/JSS, secondary/SSS, or higher?
$\Box_{789}$ N/A $\Box_0$ None $\Box_1$ Primary $\Box_2$ Middle/JSS $\Box_3$ Secondary/SSS $\Box_4$ Higher

157

Part 4: SOCIOECONOMIC

Now I will ask you some questions about your life.

16. How often do you walk barefooted?
  $\square_1$ Rarely or never $\square_2$ Occasionally $\square_3$ Always or frequently

17. How often do you sleep under a bed net?
  $\square_1$ Rarely or never $\square_2$ Occasionally $\square_3$ Always or frequently

18. How often do you wash your hands before eating?
  $\square_1$ Rarely or never $\square_2$ Occasionally $\square_3$ Always or frequently

19. How often do you wash your hands after defecation?
  $\square_1$ Rarely or never $\square_2$ Occasionally $\square_3$ Always or frequently

20. How often do you wash your hands with soap?
  $\square_1$ Rarely or never $\square_2$ Occasionally $\square_3$ Always or frequently

Part 5. WORK HISTORY

Now I will ask you some questions about your work history.

21. Are you currently working? $\square_1$ Yes $\square_2$ No [GO TO 25]

22. If yes, what is your current job(s)?_____ _____

23. Of these, which is your primary job? _____

Note to surveyor- please check appropriate job categories below:

$\square_1$ Petty Trading/Hawking	$\square_2$ Electronic Repairer
$\square_3$ Food Vendor	$\square_4$ Artisan (Mason/Carpenter/Electrician/Plumber)
$\square_5$ Scrap Dealer	$\square_6$ Mechanic/Vulcanizer
$\square_7$ Shop owner/ Retailer	$\square_8$ Market Trades
$\square_9$ Shop Attendant	$\square_{10}$ Cart Pusher/Potter
$\square_{11}$ Recycling Activities	$\square_{12}$ Taxi/Minibus/Truck Driver
$\square_{777}$ Other	$\square_{13}$ Factory/industry worker

24. For how long have you worked at your primary job? _____ months _____ years

25. What were you doing most of the past 12 months: working at a job or business, retired, keeping house, going to school, or something else?
  $\square_1$ working at a job or business $\square_2$ looking for work $\square_3$ retired $\square_4$ keeping house
  $\square_5$ going to school or full time studying $\square_6$ other, please specify:
  _____

26. In your lifetime, how many total years have you worked in the following jobs?
$\square_4$ Artisan (Mason/Carpenter/Electrician/Plumber- years: _____
$\square_5$ Scrap Dealer- years: _____
$\square_6$ Mechanic/Vulcanizer- years: _____

158

$\square_{12}$ Taxi/Minibus/Truck Driver - years: _____
$\square_{13}$ Factory/industry worker - years: _____

Part 6. OCCUPATIONAL - E-WASTE TASKS

Now I will ask you about tasks related to electronic waste.

27. **Have you ever been involved in electronic waste activities?** $\square_1$ Yes $\qquad$ $\square_2$ No [SKIP to 38]
Have you ever worked...

28. **Repairing electronic waste?** $\square_1$ Yes $\qquad$ $\square_2$ No [GO TO 29]
How many years have you/did you do this work? _____ years $\qquad$ $\square_{789}$ NA [GO TO
Have you done this work in the last 6 months? $\square_1$ Yes $\qquad$ $\square_2$ No [GO TO **29**]
How long ago did you stop? _____ years $\qquad$ $\square_{789}$ NA

29. **Collecting electronic waste?** $\square_1$ Yes $\qquad$ $\square_2$ No [GO TO 30]
How many years have you/did you do this work? _____ years $\square_{789}$ NA [GO TO
Have you done this work in the last 6 months? $\square_1$ Yes $\qquad$ $\square_2$ No [GO TO **30**]
How long ago did you stop? _____ years $\qquad$ $\square_{789}$ NA

30. **Sorting electronic waste?** $\square_1$ Yes $\square_2$ No [GO TO 31
How many years have you/did you do this work? _____ years $\square_{789}$ NA [GO TO
Have you done this work in the last 6 months? $\square_1$ Yes $\qquad$ $\square_2$ No [GO TO **31**]
How long ago did you stop? _____ years $\qquad$ $\square_{789}$ NA

31. **Removing covering of wires?** $\square_1$ Yes $\qquad$ $\square_2$ No [GO TO 32]
How many years have you/did you do this work? _____ years $\square_{789}$ NA [GO TO
Have you done this work in the last 6 months? $\square_1$ Yes $\qquad$ $\square_2$ No [GO TO **32**]
How long ago did you stop? _____ years $\qquad$ $\square_{789}$ NA

32. **Dismantling electronic equipment?** $\square_1$ Yes $\qquad$ $\square_2$ No [GO TO 33]
How many years have you/did you do this work? _____ years $\square_{789}$ NA [GO TO
Have you done this work in the last 6 months? $\square_1$ Yes $\qquad$ $\square_2$ No [GO TO **3**]
How long ago did you stop? _____ years $\qquad$ $\square_{789}$ NA

33. **Burning activities ?** $\square_1$ Yes $\qquad$ $\square_2$ No [GO TO 34]
How many years have you/did you do this work? _____ years $\square_{789}$ NA [GO TO
Have you done this work in the last 6 months? $\square_1$ Yes $\qquad$ $\square_2$ No [GO TO **34**]
How long ago did you stop? _____ years $\qquad$ $\square_{789}$ NA

34. **Ash/wire collection after burning?** $\square_1$ Yes $\qquad$ $\square_2$ No [GO TO 35]
How many years have you/did you do this work? _____ years $\square_{789}$ NA [GO TO
Have you done this work in the last 6 months? $\square_1$ Yes $\qquad$ $\square_2$ No [GO TO **35**]
How long ago did you stop? _____ years $\qquad$ $\square_{789}$ NA

35. **Lead smelting ?** $\square_1$ Yes $\square_2$ No [GO TO 36]
How many years have you/did you do this work? _____ years $\square_{789}$ NA [GO TO
Have you done this work in the last 6 months? $\square_1$ Yes $\qquad$ $\square_2$ No [GO TO **36**]
How long ago did you stop? _____ years $\qquad$ $\square_{789}$ NA

36. In the past three months, which activity have you primarily conducted (check ONE only)?
☐$_1$ Repairing electronic waste
☐$_2$ Collecting electronic waste
☐$_3$ Sorting electronic waste
☐$_4$ Removing covering of wires
☐$_5$ Dismantling Electronic Equipment
☐$_6$ Burning Activities
☐$_7$ Ash/wire collection after burning
☐$_8$ Lead Smelting
☐$_{777}$ Other, please specify: _____

37. In your lifetime, how many total years have you worked at this or other e-waste sites? _____

Part 7: HEALTH STATUS

Now I will ask you some questions about your health.

38. Would you say your health in general is:
☐$_1$ Excellent   ☐$_2$ Very good ☐$_3$ Good   ☐$_4$ Fair   ☐$_5$ Poor

39. Are you limited in the kind or amount of work you can do because of any impairment or health problem?
☐$_1$ Yes   ☐$_2$ No

If yes, what is that impairment or health problem?
_____
_____
_____

40. How often have you had skin rashes over the past two weeks?
☐$_1$ Rarely or never   ☐$_2$Occasionally   ☐$_3$ Always or frequently

41. How often have you had itching around your anus over the past two weeks?
☐$_1$ Rarely or never   ☐$_2$Occasionally   ☐$_3$ Always or frequently

42. How often have you had a headache over the past two weeks?
☐$_1$ Rarely or never   ☐$_2$Occasionally   ☐$_3$ Always or frequently

43. How often have you had an episode of stomach ache over the past two weeks?
☐$_1$ Rarely or never   ☐$_2$Occasionally   ☐$_3$ Always or frequently

44. How often have you seen blood in your urine over the past two weeks?
☐$_1$ Rarely or never   ☐$_2$Occasionally   ☐$_3$ Always or frequently

45. How often have you seen blood in your stool over the past two weeks?
☐$_1$ Rarely or never   ☐$_2$Occasionally   ☐$_3$ Always or frequently

46. How often have you had a cough over the last two weeks?
☐₁ Rarely or never   ☐₂ Occasionally   ☐₃ Always or frequently

47. How often have you experienced shortness of breath or difficulty in breathing over the last two weeks?
☐₁ Rarely or never   ☐₂ Occasionally   ☐₃ Always or frequently

48. How often have you experienced dizziness over the last two weeks?
☐₁ Rarely or never   ☐₂ Occasionally   ☐₃ Always or frequently

49. How often have you felt your heart beating abnormally over the last two weeks?
☐₁ Rarely or never   ☐₂ Occasionally   ☐₃ Always or frequently

50. How often have you had diarrhea over past two weeks?
☐₁ Rarely or never   ☐₂ Occasionally   ☐₃ Always or frequently

51. How often have in the last two weeks did you have fever?
☐₁ Rarely or never   ☐₂ Occasionally   ☐₃ Always or frequently

52. How often have in the last two weeks did you experience vomiting?
☐₁ Rarely or never   ☐₂ Occasionally   ☐₃ Always or frequently

53. Have you taken any treatment for worms over the last three months?
☐₁ Yes   ☐₂ No   ☐₈₈₈ Don't know

54. Did you experience nausea over the last two weeks?
☐₁ Rarely or never   ☐₂ Occasionally   ☐₃ Always or frequently

55. Have you experienced tremors in the past year? [Specifically, intention tremors of the fingers and hands, making handwriting difficult]
☐₁ Rarely or never   ☐₂ Occasionally   ☐₃ Always or frequently

56. Do you think you've lost weight recently? [Do you think that your clothes are too large for you due to a loss of weight?]
☐₁ Yes   ☐₂ No   ☐₈₈₈ Don't know

57. Did you seek treatment for any of these conditions?
☐₁ Yes   ☐₂ No   ☐₈₈₈ Don't know

58. If yes where did you receive treatment for the most severe condition?
☐₁ Self Medication   ☐₂ Traditional Healer   ☐₃ Drug Store
☐₄ Clinic/Hospital   ☐₇₇₇ Other_____

59. Have you ever been told by a doctor or health professional that you have any of the following medical conditions?

☐₁ High Blood Pressure   ☐₂ Diabetes Melitus
☐₃ Asthma   ☐₄ Sickle cell disease
☐₇₈₉ N/A   ☐₈₈₈ Don't know

60. Are you on any medicine for any of these conditions?
☐₁ Yes   ☐₂ No   ☐₇₈₉ N/A   ☐₈₈₈ Don't know

61. In the past month on the days when you drank, on average, how many drinks did you have?
[A drink= 1 beer, 1 shot of liquor, 1 glass of wine, 2 glasses of palm wine, or 0.5 calabash of pito]
_____ Drinks   ☐₈₈₈ Don't Know   ☐₀ Does not drink

Part 8: TOBACCO USE

Now I will ask you some questions about your smoking history.

62. Have you smoked at least 100 cigarettes during your entire life (equivalent to about 5 packs)?
☐₁ Yes   ☐₂ No   ☐₈₈₈ Don't know

63. Do you smoke cigarettes now?
☐₁ Yes   ☐₂ No

64. Are you often around people who smoke?
☐₁ Yes   ☐₂ No

Part 9: NOISE & HEARING

Now I am going to ask you questions about noise and hearing. The term "loud noise" here means loud enough that a person has to raise their voice to talk to someone at arm's length (about three feet away).

65. How often are you exposed to loud noise at work?
☐₀ Never   ☐₁ Almost never   ☐₂ Sometimes
☐₃ Fairly often   ☐₄ Very often   ☐₈₈₈ Don't Know

66. How many years total have you worked in loud noise?

_____ years

67. How much does loud noise bother or annoy you at work?
☐₁ Not at all   ☐₂ A little   ☐₃ A great deal

68. How often are you exposed to loud noise when you are NOT at work?
☐₀ Never   ☐₁ Almost never   ☐₂ Sometimes
☐₃ Fairly often   ☐₄ Very often   ☐₈₈₈ Don't Know

69. How much does loud noise bother or annoy you at night?
☐₁ Not at all   ☐₂ A little   ☐₃ A great deal

162

70. How often does noise around your home affect how well you sleep at night?
$\square_0$ Never $\quad\square_1$ Almost never $\quad\square_2$ Sometimes $\quad\square_3$ Fairly often $\square_4$ Very often

71. Do you have difficulties with your hearing?
$\square_1$ Yes $\quad\quad\quad\square_2$ No SKIP to 75

72. If yes, how long have you had difficulties with your hearing?
$\square_1$ Since birth $\square_2$ Since childhood $\quad\quad\square_3$ Since adolescence $\quad\square_4$ Since adulthood

73. Have you ever been told by a healthcare professional that you have hearing loss or another hearing problem?
$\square_1$ Yes $\quad\quad\quad\square_2$ No

74. How often do you hear ringing or whistling sounds in your ears after spending time in loud noise?
$\square_0$ Never $\quad\square_1$ Almost never $\quad\square_2$ Sometimes $\quad\square_3$ Fairly often $\square_4$ Very often

Part 10: OCCUPATIONAL INJURIES

Now I am going to ask you about injuries at work.

75. How many times have you been injured at work in the past 6 months?

_____times [IF 0, GO TO 86]

76. How many of these injuries occurred while doing electronic waste in the past 6 months?

_____injuries

77. For your worst work injury at work in the past 6 months, what were you doing at the time of injury?
[ ]$_1$ Collecting electronic waste $\quad$ [ ]$_2$ Sorting electronic waste
[ ]$_3$ Removing covering of wires $\quad$ [ ]$_4$ Dismantling Electronic Equipment
[ ]$_5$ Burning Activities $\quad\quad\quad\quad$ [ ]$_6$ Ash/wire collection after burning
[ ]$_7$ Lead Smelting $\quad\quad\quad\quad\quad$ [ ]$_{777}$ Other _____

78. For your worst injury at work in the past 6 months, what type of medical care did you receive?
[ ]$_1$ No medical care $\quad\quad\quad$ [ ]$_2$ Self administered first aid
[ ]$_3$ Treatment by herbalist $\quad\quad$ [ ]$_4$ Treatment in a Chemical shop/Pharmacy
[ ]$_5$ Treatment at hospital/Clinic $\quad$ [ ]$_6$ Other:_____

79. For your worst injury at work in the past 6 months, were you hospitalised? [ ]$_1$ Yes $\quad$ [ ]$_2$No

80. If Yes, for how long were you hospitalized_____

81. How much work did you miss due to your worst injury in the past 6 months?
[ ]$_1$ Did not miss any work and worked regular job
[ ]$_2$ Did not miss any work but could not do regular job

163

[ ]₃ Missed work: _____ days

82. **What was the cause of your worst injury at work in the past 6 months?**
    [ ]₁ Cut from a working tool(screw driver/Knife etc)
    [ ]₂ Cut from a sharp object(other than a working tool)
    [ ]₃ Bruise from a working tool
    [ ]₄ Bruise from a sharp object(other than a working tool)
    [ ]₅ Burns from fire or heat source
    [ ]₆ Burns from a Chemical
    [ ]₇ Crushed by a working tool/Implement
    [ ]₈ Struck/Hit by a working tool/Implement
    [ ]₉ Road traffic Related(Motor Bicycle, Motor Vehicle, Push Cart)
    [ ]₁₀ Choking/Suffocation
    [ ]₁₁ Slip/Trip/Fall
    [ ]₁₂ Physical Assault
    [ ]₁₃ Drowning
    [ ]₁₄Other: _____
    [ ]₈₈₈ Don't Know

83. **What body part or parts were injured? (Check all that apply)**
    [ ]₁ Head   [ ]₂ Eye(s)   [ ]₃ Face   [ ]₄ Mouth/teeth
    [ ]₅ Neck   [ ]₆ Shoulder   [ ]₇ Arm   [ ]₈ Hand
    [ ]₉ Chest   [ ]₁₀ Spine   [ ]₁₁ Waist   [ ]₁₂ Hip
    [ ]₁₃ Thigh   [ ]₁₄ Knee   [ ]₁₅ Lower leg   [ ]₁₆ Ankle
    [ ]₁₇ Foot   [ ]₁₈ Abdomen   [ ]₇₇₇ Other_____

84. **What type of injury did you sustain? (Check all that apply)**
    [ ]₁ Contusions/abrasions   [ ]₂ Burns/scalds   [ ]₃ Concussions
    [ ]₄ Cuts/lacerations   [ ]₅ Punctured wounds   [ ]₆ Amputations
    [ ]₇ Dislocations   [ ]₈ Fractures (simple/compound) [ ]₉ Sprains/strains
    [ ]₁₀ Asphyxiation   [ ]₁₁ Internal bleeding   [ ]₁₂ Electric shock
    [ ]₇₇₇ Other_____

85. **Prior to injury had you received any instructions/training on how to avoid injury while doing your work?**
    [ ]₁ Yes   [ ]₂ No   [ ]₈₈₈ Don't Know

86. **In your job, do you regularly wear safety clothing or equipment like gloves, boots, ear plugs or dust masks?**   ☐₁ Yes **IF YES, SEE BELOW**
    ☐₂ No **IF NO GO TO 87**

Safety glasses, goggles, or other eye protection such as face shields?	☐₁ Yes	☐₂ No
Rubber-soled boots or shoes?	☐₁ Yes	☐₂ No
Gloves?	☐₁ Yes	☐₂ No
Dust mask or respirator?	☐₁ Yes	☐₂ No
Long pants?	☐₁ Yes	☐₂ No
Ear plugs or earmuffs to block sound?	☐₁ Yes	☐₂ No
Hat?	☐₁ Yes	☐₂ No

Is there any other equipment or clothing that you use to protect your health and safety at work?

_____

_____

Part 11: WATER & DIET

Now I will ask you about your water sources and your diet.

87. What is your primary water source for drinking and cooking?
[ ]$_1$ Bottled water   [ ]$_2$ Sachet water   [ ]$_3$ Out of a well
[ ]$_4$ Out of a pipe   [ ]$_{777}$ Other

88. How many meals do you eat in a week? _____ meals

89. In one week, how often do you eat meat? _____ times

Part 12: STRESSORS

Now I will ask you about stress in your life.

90. In the last month, how often have you been upset because of something that happened unexpectedly?
[ ]$_0$ Never   [ ]$_1$ Almost never   [ ]$_2$ Sometimes   [ ]$_3$ Fairly often [ ]$_4$ Very often

91. In the last month, how often have you felt that you were unable to control the important things in your life?
[ ]$_0$ Never   [ ]$_1$ Almost never   [ ]$_2$ Sometimes   [ ]$_3$ Fairly often [ ]$_4$ Very often

92. In the last month, how often have you felt nervous and "stressed"?
[ ]$_0$ Never   [ ]$_1$ Almost never   [ ]$_2$ Sometimes   [ ]$_3$ Fairly often [ ]$_4$ Very often

93. In the last month, how often have you felt confident about your ability to handle your personal problems?
[ ]$_0$ Never   [ ]$_1$ Almost never   [ ]$_2$ Sometimes   [ ]$_3$ Fairly often [ ]$_4$ Very often

94. In the last month, how often have you felt that things were going your way?
[ ]$_0$ Never   [ ]$_1$ Almost never   [ ]$_2$ Sometimes   [ ]$_3$ Fairly often [ ]$_4$ Very often

95. In the last month, how often have you found that you could not cope with all the things that you had to do?
[ ]$_0$ Never   [ ]$_1$ Almost never   [ ]$_2$ Sometimes   [ ]$_3$ Fairly often [ ]$_4$ Very often

96. In the last month, how often have you been able to control irritations in your life?
[ ]$_0$ Never   [ ]$_1$ Almost never   [ ]$_2$ Sometimes   [ ]$_3$ Fairly often [ ]$_4$ Very often

97. In the last month, how often have you felt that you were on top of things?
[ ]$_0$ Never   [ ]$_1$ Almost never   [ ]$_2$ Sometimes   [ ]$_3$ Fairly often [ ]$_4$ Very often

98. In the last month, how often have you been angered because of things that happened that were

outside of your control?
[ ]$_0$ Never   [ ]$_1$ Almost never   [ ]$_2$ Sometimes   [ ]$_3$ Fairly often [ ]$_4$ Very often

99. **In the last month, how often have you felt difficulties were piling up so high that you could not overcome them?**
[ ]$_0$ Never   [ ]$_1$ Almost never   [ ]$_2$ Sometimes   [ ]$_3$ Fairly often [ ]$_4$ Very often

Part 13: RISK FACTORS OF WORK-RELATED STRESS

Job demands and working conditions:
100.   **How often do you feel exhausted after work?**
☐$_1$ Rarely or never   ☐$_2$ Occasionally   ☐$_3$ Always or frequently

101.   **How often are you exposed to unfavourable physical conditions in your work (for example, unfavourable climate, noise, chemicals, sharp or moving objects, slippery surfaces, constant repetitive work, heavy lifting or strenuous work)**
☐$_1$ Rarely or never   ☐$_2$ Occasionally   ☐$_3$ Always or frequently

102.   **How often does someone else decide your work methods, pace, and/or order?**
☐$_1$ Rarely or never   ☐$_2$ Occasionally   ☐$_3$ Always or frequently

103.   **How often do you feel you are not receiving support from your supervisor and/or colleagues?**
☐$_1$ Rarely or never   ☐$_2$ Occasionally   ☐$_3$ Always or frequently

104.   **How often do you experience violence or harassment at work?**
☐$_1$ Rarely or never   ☐$_2$ Occasionally   ☐$_3$ Always or frequently

105.   **How many hours do you work on a typical day? _____**

106.   **How often do you work evenings, nights, and/or weekends?**
☐$_1$ Rarely or never   ☐$_2$ Occasionally   ☐$_3$ Always or frequently

107.   **How often does your work interfere with your family responsibilities or leisure time activities?**
☐$_1$ Rarely or never   ☐$_2$ Occasionally   ☐$_3$ Always or frequently

108.   **How often do you feel your income is not sufficient to support yourself and your family?**
☐$_1$ Rarely or never   ☐$_2$ Occasionally   ☐$_3$ Always or frequently

Part 14. OTHER INFORMATION

Is there anything else you would like to say?

If you answered Yes, please explain: