Effects of Toxic Metal Exposures and Their Mixtures on Adverse Health Outcomes in the Peruvian Amazon

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
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ABSTRACT

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Abstract

Non-essential trace metals (lead, mercury, arsenic, and cadmium) are ubiquitous in our environment and have overlapping routes of exposure, yet mixed trace metal exposures are rarely considered in epidemiological studies. Instead, research often follows a single research question that focuses on a single trace metal of concern and does not incorporate potential co-exposures. The published literature of artisanal small-scale mining in the Amazon is a prime example as it has predominantly focused on mercury exposure, due to its use in the mining process. Once exposures of concern are identified, further studies evaluate health outcomes; however, the health effects cannot be accurately determined without accounting for co-exposures. This verification is becoming more important as there is a growing recognition that mixed trace metal exposures are more common than previously believed.

To address the prevalence of mixed trace metal exposures and their health effects in the Peruvian Amazon region of Madre de Dios, I use epidemiological data from the COhorte de NAcimiento de MAdre de Dios (CONAMAD) birth cohort study (2018-Present), and two cross-sectional epidemiological studies (Amarakaeri Communal Reserve study (ACR, 2015), and Etiology and Toxic Metals study (EATM, 2018)). CONAMAD collected survey data along with maternal and cord blood samples at birth, which were processed for minerals and trace metals. The cross-sectional studies collected venous blood for trace metal analysis and hair samples for total hair mercury. Blood samples from the ACR were also processed for amino acids. In-depth demographic and health survey data were collected in all three studies. Structural equation models and random mixed effect models were used to evaluate research questions.

The cross-sectional studies demonstrate a high correlation of lead and mercury exposure in communities that rely on wild fish and wild game as protein sources, which is prevalent throughout the Amazon. Consuming a meal of wild game resulted in an estimated lead dose of 500 µg, with those who eat wild game (Yes/No) associated with 1.41 µg/dL (95% CI: 1.20 – 1.70) higher blood lead levels compared to those who do not. This furthers the notion that mixed exposures are likely more common than previously believed. Mixed exposures target the same toxicological pathway, which may lead to synergistic or antagonistic effects. My research found that lead disrupts the arginine pathway and is associated with increased blood pressure. Mercury exposure was a modifier of the arginine pathway, with high blood mercury levels changing the effect of global arginine bioavailability from 17.16 (95% CI: 9.09 – 25.84) to -14.17 (95% CI: -31.88 - -0.33) on systolic blood pressure. Interestingly, mercury was not directly associated with the arginine pathway. Results from the birth cohort demonstrate the importance of nutrition and prenatal care for fetal development, which had a large positive effect on birthweight and gestational age. However, even low maternal lead exposure had detrimental effects on fetal health. A 1% increase in maternal blood lead was associated with a shorter gestational age of 0.05 days (β: -0.75, 95% CI: -1.51 - -0.13), even with the CONAMAD birth cohort having lower blood lead levels than other birth cohorts. There is a need for an integrated approach of nutritional and exposure assessments to better understand neonatal health outcomes.

Dedication

I dedicate this work to my parents, Maria André and Bob Berky, for their endless love and gifting me with their creativity and curiosity. I also dedicate this work to the indigenous communities in the region, who have worked with us in studying mercury in the environment and its impact on human health. My experiences working with and in these communities have taught me much about life and the ingenuity and wisdom held in indigenous communities.

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I want to highlight the work and effort contributed by Dr. Ernesto J. Ortiz and Dr. Nelson Rivera to these studies. Ernesto, since my first trip to Madre de Dios, Peru, you have taught me a lot about how to implement scientific studies and how to collaborate with local communities and health officials to improve public health. Nelson, thank you for your patience and willingness to teach me how to prepare blood samples for trace metal analysis. Your expertise and dedication to high quality laboratory data ensures our statistical analyses are well founded.

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Introduction

Non-essential trace metals (lead, mercury, arsenic, and cadmium) comprise four of the top ten chemicals of public concern as designated by the World Health Organization (WHO). There is also a growing awareness that people are exposed to trace metal mixtures, rather than individual trace metals, complicating our current exposure paradigm of a single exposure associated with a single health outcome. This work evaluates mixed trace metal exposures in the Southern Peruvian Amazon region of Madre de Dios, and their effects on public health. Previous research in the Amazon and in communities that practice artisanal small-scale gold mining (ASGM) have focused on mercury exposure and its health effects due to the use of mercury in the mining process. Yet, we are just beginning to understand the extent of mixed trace metal exposures in the Amazon and in ASGM communities, which may share sources of trace metal exposures that are prevalent worldwide.

1.1 Non-essential Trace Metals: Prevalence of Exposures

Exposure to trace metals is pervasive as they are ubiquitous in the environment due to being naturally present in the Earth's crust and commonly used in industry. Geogenic sources have caused arsenic, cadmium, and lead water contamination in India [1-3], Bangladesh [4, 5], the United States (i.e., in North Carolina [6, 7]), Ethiopia [8], Peru [9-11] and Argentina [12]. Although trace metal exposure can occur naturally, elevated human exposures are caused primarily by current and historical industrial pollution [13-16]. Lead used as a gasoline and paint additive is probably the best-known historical metals exposure, resulting in elevated exposures in persons of all ages globally [17, 18].

In the US, trace metals are common contaminants in over 1,322 Superfund and historical mining sites, leading to acid mine drainage and release of metals into waterways [19-22]. Mining is particularly problematic as operations lead to direct and indirect contamination of the environment [23-25] and result in trace metals exposure to human populations [26-29]. Regardless of source, once in the environment, trace metals are generally exposed to people at chronic, low levels via the diet; predominantly in contaminated water [30], rice [31-33], leafy greens [34], yuca [35], potatoes [30, 32, 36-40], hunted wild game [41, 42], fish [43-47] and cigarettes [48, 49]. The ubiquitous nature of trace metals in our environment increases the likelihood of mixed exposures, which may be more prevalent than commonly believed [50]. Nonetheless, few epidemiological studies assess the effects of joint trace metal exposures [51].

These quotidian exposures have tangible health outcomes. In the United States, the attributable fraction of all-cause mortality from lead exposure was estimated to be 18% or an estimated 412,000 deaths annually from 1994 to 2011 [52]. While the WHO recognizes that there is no safe level for lead exposure, regulatory agencies such as the United States Center for Disease Control (CDC) established a threshold of blood lead levels (BLL) at 5 μg/dL, which is associated with adverse health effects in renal, neurological and cardiovascular outcomes in children [53]. In 2021, the CDC updated the BLL threshold to 3.5 μg/dL. Environmental cadmium exposure in the United States is associated with impaired fasting glucose and a higher odds ratio of diabetes [54]. Environmental mercury exposure has been linked to cognitive deficits in children [55], lower neonatal head circumference [56, 57] and increased cardiovascular risk [58]. The pervasiveness and ability of trace metals to cause adverse health outcomes at

exposure levels commonly present in the environment makes them an important public health risk worldwide.

In the Peruvian Amazon, studies have focused on mercury exposure due to its use in artisanal small-scale gold mining (ASGM) and the dependence of fish as a dietary staple for riverine communities [59-62]. Studies in Peru and throughout the Amazon, have shown fish consumption associated with increased mercury exposure [45, 60, 62-66]. In addition to mercury, studies in Northern Peruvian Amazon have also evaluated lead exposure, with potential risk factors being fishing, hunting, ceramics, and oil extraction [67, 68]. Although elevated blood lead levels (BLL) have been found in the Northern Peruvian Amazon, the sources of exposure in the Amazon are still unconfirmed. The potential for dual exposures to mercury and lead complicates the traditional exposure paradigm and requires an approach that can effectively assess their joint as well as individual health effects.

1.2 Non-essential Trace Metals: Shared Toxicological Pathways

Traditional toxicological methods evaluate a single exposure and its effect on a single health outcome. While such an approach may be appropriate in a controlled, laboratory setting, its simplicity fails to incorporate multiple levels of complexity that occur in real world settings where individuals are exposed to mixtures of chemical toxicants and nutrients. Both are essential to evaluating health outcomes. The simplification of toxicological risk assessment to a single exposure of interest assumes either that all other exposures are identical across study participants, or they exhibit no relevant effects such as additive, interactive, or modification on health outcomes. The evaluation of a single health outcome also ignores the complex interdependent relationship between organ systems and the ability of contaminants to target

multiple organ systems. The result being an underestimate of the total effect of exposure on overall health and the inability to identify indirect effects, via alternate organ systems, on the health outcome of interest. Due to overlapping toxicological pathways, shared mechanisms of toxicity, interaction with nutrients, pervasiveness in the environment and high toxicity, trace metals are a valuable class of contaminants to develop and evaluate alternative statistical approaches.

Non-essential trace metals target overlapping organ systems and share toxicological pathways. Mercury, lead, and cadmium target the kidneys and induce toxicity in-part by oxidative stress, but also have unique toxicological pathways that may allow for non-additive toxicity. At the cellular level, lead interferes with calcium, while mercury and cadmium primarily bind to thiol groups [69]. At the organ level, cadmium, mercury, and lead are nephrotoxins that disrupt reabsorption of nutrients in the nephron [34, 70-72]. However, each metal targets different sections of the kidneys' proximal tubule. Mercury targets the proximal straight tubule and the medulla, while cadmium targets the cortex, the medulla, and the proximal convoluted tubule [73, 74]. Lead mainly accumulates in the kidney cortex [75]. These different toxicological endpoints within the kidney, and at the cellular level may promote distinct physiological disturbances that promote non-additive toxicity.

Lead and cadmium are also associated with cardiovascular risk, while the association of mercury exposure and cardiovascular health is still being evaluated. The multiple toxicological pathways of trace metals allow them to potentially cause both direct and indirect effects on health outcomes. For example, the ability of trace metals to damage the kidneys may be a secondary means through which they effect blood pressure and cardiovascular health.

Trace metals also induce toxicity via similar mechanisms such as molecular mimicry and the generation of reactive oxygen species (ROS). In animal models, ROS from both lead [76, 77] and methylmercury [78] have been shown to reduce nitric oxide, an important vasoregulator that allows for muscle relaxation, resulting in cardiovascular risk. Molecular mimicry allows trace metals to replace nutrients in protein transporters and bypass protective barriers that include the blood brain barrier. The potential for trace metals to block transporters may increase the likelihood of additive or synergistic effects with nutrients. Lead is known to mimic calcium allowing it to be stored in bones and remobilized during pregnancy. Similarly, cadmium mimics zinc, while organic mercury resembles glutathione and is therefore able to pass the blood brain and placental barriers.

Trace metal absorption and distribution is also dependent on essential minerals and nutrients. Lead is known to be preferentially absorbed by individuals with low iron and can lower iron absorption [79]. Once absorbed, the distribution of trace metals within the body is also dependent on nutrient status. Selenium has been shown to modify distribution of mercury and cadmium, as well as the ability of the cells and the body to illicit a protective response to trace metal toxicity [80-82]. The effect of nutrient status on trace metals and their toxicity make it a valuable factor that should be adjusted for in risk and health assessments. Furthermore, the shared toxicological pathways and mechanisms of toxicity make trace metals a valuable chemical class to assess mixed exposures.

1.3 Non-essential Trace Metals: Health Metrics

Adequately quantifying health outcomes from trace metal exposures is difficult due to their ability to target multiple organ systems and shared mechanisms of toxicity. This is further

complicated as there are multiple health metrics to assess organ health. The use of a single health metric may ignore the complex interdependent relationship between organ systems and the ability of contaminants to target multiple organs. In comparison, the joint assessment of multiple health metrics may allow for a better understanding of overall health while accounting for the relationship between health metrics. Cardiovascular, renal, and neonatal health are a few prime examples in which evaluating health metrics jointly may expand our understanding of these health outcomes and trace metal exposures.

The cardiovascular system is physiologically dependent on the heart which pumps nutrients throughout the body. This interdependence links the cardiovascular system with the heart and helps explain why high blood pressure is a risk factor for heart attacks. Common cardiovascular health metrics such as mean arterial blood pressure, pulse pressure or systolic and diastolic blood pressure are evaluated individually; even though they have a linear relationship [83, 84]. Each health metric contributes unique information regarding cardiovascular health [85, 86]. When analyzed individually, statistical models are unable to account for any overlap in explained variance, preventing a holistic assessment of cardiovascular health. The joint analysis of these health metrics can broaden our understanding of how trace metals effect different aspects of the cardiovascular system and its overall impact on cardiovascular health.

The renal system is comprised of an intricate system that filters out waste from the blood to be excreted. Multiple metrics have been used to evaluate renal health, that include creatinine levels in serum and urine, cystatin-C, blood urea nitrogen, B2-microglobulin, and estimated glomerular filtration rate [87]. New biomarkers for chronic kidney disease also have

the benefit of being specific to subregions of the kidney, providing a more detailed assessment of renal health [87]. By integrating these metrics, it may be possible to better assess how exposures to trace metals have multifaceted effects on the kidneys.

Similarly, there are numerous metrics for neonatal health that include gestational age; birthweight; Appearance, Pulse, Grimace, Activity, and Respiration (APGAR) score at 1 minute; APGAR score at 5 minutes; length, and head circumference, with each measurement providing unique information regarding a newborn's health. These metrics are often analyzed individually, even though they are correlated, with gestational age being a principal component of newborn health. Gestational age is an outcome of concern, and an outcome to control for to identify other variables of interest. Due to its strong association with birth outcomes, controlling for gestational age in statistical models may lead to overcontrolling and prevent the identification of other variables of interest. The joint analysis of health metrics allows for the covariance between gestational age and other variables of interests to be accounted for, preventing overcontrolling, and better assess variables of interest.

In each presented case, health metrics of each organ system may be correlated and provide valuable, yet distinct information that is not fully utilized when outcomes are assessed individually. The ability to evaluate these health outcomes at the same time while accounting for their correlation is important to adequately estimate their association with the exposures of interest. In addition to the current biomarkers, the field of metabolomics has greatly increased the potential to identify more sensitive health metrics. Continued research in metabolomics will likely identify metabolites that will allow us to better evaluate specific organ systems and overall health.

1.4 Non-essential Trace Metals: Metabolomics

Metabolomics is one of the four emerging fields in biology that includes genomics, transcriptomics, and proteomics. Genomics is the study of genetics and the variation of genes that may predispose an individual to an adverse health outcome. Transcriptomics focuses on ribonucleic acid (RNA) and the conversion from DNA into proteins. Proteomics studies sets of proteins present in the human body or organ system. Metabolomics is the study of metabolites, molecules found within the human body, to evaluate current health status. While the aforementioned emerging fields of biology focus on upstream components of living organisms, metabolomics studies the most immediate biological markers of an organism. Metabolomics is a relatively new approach to study molecular phenotypes resulting from genetic and environmental factors and has grown in step with analytical capabilities. Even in 2015 metabolomics was considered an emerging tool for precision medicine [88]. These methods can quantify hundreds of metabolites in a single sample [89], allowing for a more detailed understanding of how an individual's pathophysiology may be disrupted by xenobiotics. Metabolomics provides an important avenue to study the mechanistic effects of single and joint trace metal exposures and their effect on human health.

In practice, the use of metabolites has been around since the Middle Ages, as healers made inferences on a person's health by the color, smell, and taste of their urine [90, 91]. Today, metabolites are used as biomarkers of exposure, disease progression and disease risk. Common metabolites such as albumin, creatinine, and cystatin-C are used to infer kidney function with high urinary levels associated with chronic kidney disease risk, while HbA1c is used

to monitor diabetes risk. Our knowledge of metabolites and their sensitivity in detecting the health outcome of interest continues to improve in step with our analytical capabilities.

Metabolomics is a breakthrough in the use of metabolites as it allows for hundreds of metabolites to be measured in a single sample, providing a detailed physiological snapshot of an individual. Improved instrumentation has allowed metabolomics to analyze a much wider range of metabolites that may provide a more detailed understanding of disease progression and etiology. This has recently led to new metabolites being linked to various types of cancers [92-95], cardiovascular disease [96, 97] and other chronic diseases [98, 99]. However, only a limited number of studies have assessed the disruption of metabolic profiles from trace metal exposures and health outcomes. The influence of trace metal exposures on metabolite levels, especially amino acids, is not completely understood.

Amino acids are the building blocks of peptides, proteins and enzymes that make up cells. Many amino acids are synthesized in the body, while some are solely derived from the diet. They are also all part of the citric or urea cycles that are critical for proper cellular function. Impacts on these unique molecules could have important downstream consequences on cellular metabolism or the synthesis of peptides. For example, the amino acids methionine and serine are precursors of cysteine, which along with glutamate and glycine form the building blocks of glutathione. Both glutathione and cysteine are known for their antioxidant capabilities that scavenge harmful reactive oxygen species (ROS) produced from trace metal exposures [100, 101]. At the cellular level, the inability to quench oxidative stress can lead to lipid degradation and cellular damage. This can lead to health effects at the individual level as people deficient in glutathione often have chronic hemolytic anemia [100]. Similarly, studies have found arginine

[102-104], tyrosine, lysine, glutamine, alanine, glycine and valine associated with kidney health [105]. The physiological overlap of amino acids at the cellular and organ level with trace metals may make them valuable biomarkers for early cellular damage and an important link in connecting trace metal exposures to adverse health outcomes.

1.5 Non-essential Trace Metals: Dissertation Objectives

The objectives of this dissertation were to determine whether mixed trace metal exposures are occurring in Madre de Dios, Peru and assess their health effects by utilizing structural equation models to analyze multiple endpoints simultaneously, create pathways to test direct and indirect effects and evaluate metal mixtures.

The chapter objectives of this body of work are as follows:

1) **Evaluate the extent of mixed trace metal exposures in Madre de Dios, Peru and identify risk factors**
    a. Previous studies in the region have focused on mercury exposure due to the prevalence of artisanal small-scale gold mining (ASGM); however, little is known about exposures to other trace metals.
2) **Assess the importance of nutritional and toxicological exposures during pregnancy, while accounting for prenatal care**
    a. Few birth cohorts evaluate the joint effects of nutrients and trace metals on neonatal health, even though both effect birth outcomes. This is one of the first birth cohorts in the Amazon to integrate nutrition and trace metal exposures, while adjusting for prenatal care.

3) **Utilize amino acids to better understand how lead and mercury exposure interact to augment cardiovascular risk**

    a. Lead and mercury generate reactive oxygen species that have been postulated to increase cardiovascular risk, with greater evidence being found for lead. This study statistically evaluates the effect of lead and mercury on the arginine pathway, an important regulator of blood pressure.

2. Risk of Lead Exposure from Wild Game Consumption in Madre de Dios, Peru

Lead exposure is classified among the top ten chemicals of major global health concern due to its widespread presence in the environment and its cumulative and often irreversible health impacts on multiple organ systems. The use of lead in industrial products such as gasoline, paint, and piping have been largely phased out globally [106, 107]; however, sources remain from environmental legacy pollution [108], industrial (e.g., oil extraction[109]) and behavioral (e.g., hunting with lead bullets [110]) factors that contribute to elevated lead exposures. Our study evaluated risk factors for lead exposure in Madre de Dios, Peru where oil exploitation, leaded paint and gasoline are unlikely confounders, which may allow us to better evaluate the relationship between lead exposure from consuming wild game.

In remote regions of the Amazon, there is increasing evidence of lead exposure; however, its principal source is still unknown. The lack of exposure to leaded paint [111] and lead pipes, as well as relatively limited gasoline use, have led epidemiological studies to focus primarily on produced waters from oil extraction and oil spills. However, Anticona et al. studied blood lead levels (BLL) in communities impacted by oil spills in the Peruvian Amazon and found no difference in BLL between impacted and non-impacted communities [67, 109]. Although the oil industry is a source of environmental contamination [112], studies in the Amazon have not identified a significant relationship between oil extraction/spills with BLL [67, 109].

An isotopic analysis of hunted wild game in regions with and without oil extraction demonstrated that 86% and 57% of lead, respectively, could be traced to lead bullets [113]. A bullet, upon impact, fragments into hundreds of pieces with 34% being under 0.01 g and are

often microscopic, making it infeasible to remove all the lead from wild game [114]. Hunting as a source of lead exposure has not been well studied in the Amazon, although lead exposure from hunting has been found in the Northern Hemisphere [41, 42]. Hunting with lead ammunition is prevalent throughout the Amazon, potentially making it a common, yet understudied, source of lead exposure.

Many indigenous and rural communities rely on wild game as a food source. In the 1960s, a study by Pierret et al. of 430 households in the Northern Peruvian Amazon found 83% of them ate wild game at least once a week with an estimated 52 grams of wild game consumed daily/capita [115]. Recent studies have estimated an average of 63 kgs/capita/year of wild game are consumed in rural and indigenous communities across the Amazon, equating to 1.3 million tons consumed annually [116]. In 2012, Anticona et al. found 76.9% of indigenous study participants in Northern Peru ate wild game at least once per week.[68] Although wild game consumption is highest in rural regions [117], consumption in urban communities continues throughout South America [116, 118].

The goal of this study is to evaluate risk factors of lead exposure in the Southern Peruvian Amazon region of Madre de Dios where there is currently no gas/oil exploitation. Previous studies in the region have shown elevated mercury exposure from fish consumption [119, 120] but no studies have focused on lead exposure. Indigenous communities predominantly live along the Madre de Dios River with limited or no road access (Figure 1). While several studies in the Amazon mention the potential for lead exposure from hunting [68, 121], this is one of the few to assesses wild game consumption as a potential dietary risk factor for lead exposure.

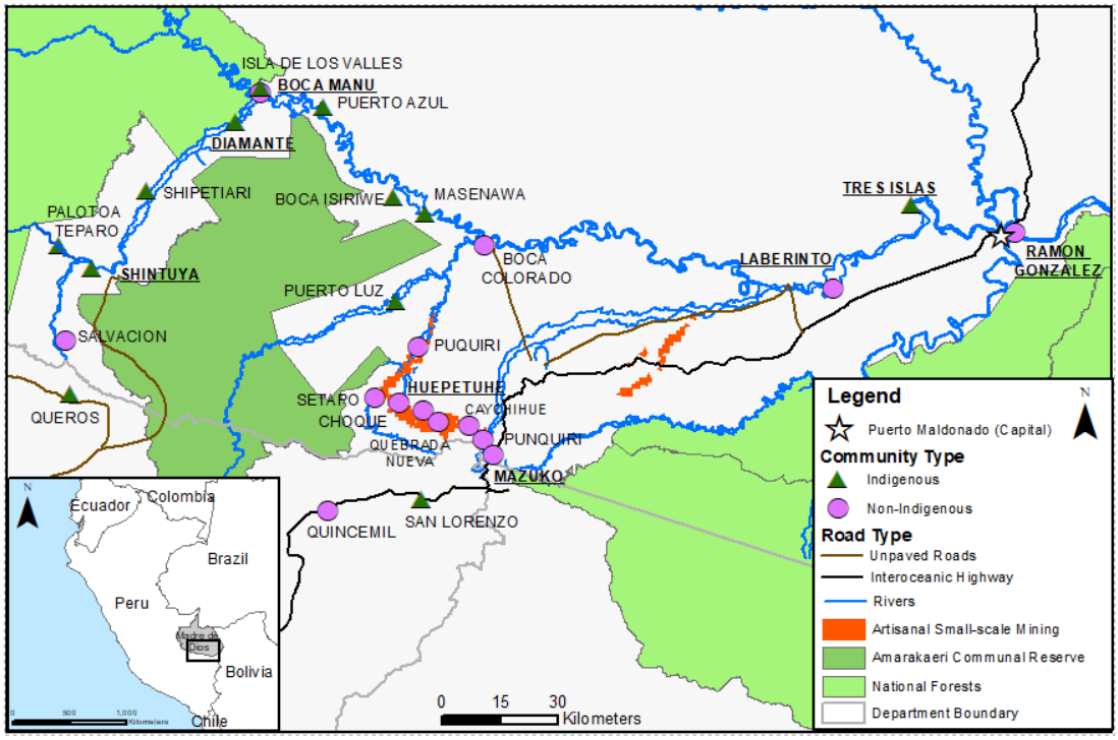


Figure 1: Map of study sites in Madre de Dios, Peru with indigenous and non-indigenous communities portrayed as green triangles and purple circles, respectively. Mining regions are depicted as orange, while national forests, including the Amarakaeri Communal, are green. The interoceanic highway is shown in black, while dirt roads that may become impassible during parts of the year are brown. Bold and underlined community labels are communities visited in 2018 and are part of the data subset.

2.1 Methods

2.1.1 Data Collection

Data were collected in Madre de Dios, Peru in 2016 and in 2018 as part of the Amarakaeri Communal Reserve (ACR) and the Etiology of Anemia and Trace Metals (EATM) studies, respectively (Figure 1). Both studies were approved by the Institutional Review Board of La Universidad Peruana de Cayetano Heredia (IRB: 00001014 and 102134, respectively). The ACR study was designed to evaluate population level health factors such as anemia and trace metal exposures and is described in Weinhouse et al., 2020 [119]. Briefly, indigenous, and non-

indigenous communities surrounding the Amarakaeri Reserve were selected to measure the impact of gas exploration and gold mining. In communities of over 75 households, households with a woman between the ages of 15-49 years old (women of child-bearing age - WCBA) were randomly selected, while in smaller communities, all households with a WCBA were invited to participate. Roughly 10% of the study population were households without a WCBA.

The EATM study communities were selected based on community type (upriver from gold mining, gold mining communities and urban communities), previous participation in Duke University studies, and logistical feasibility. Households were selected based on the anemia status of children under 12 and their mothers, with families invited to enroll if either the child or mother had anemia. Children and mothers were screened for anemia at health posts, schools, and community centers using a HEMOCUE 201+, with written parental consent obtained prior to testing. Enrolled families were administered a similar survey to the ACR study, but with additional information on food portion sizes and food sources. Household drinking water samples were also collected to measure lead concentrations (Appendix A has methods and results from water samples).

Data collection in both studies included anthropometrics (height, weight, body mass index (BMI)), household surveys, hemoglobin measurements taken with a HEMOCUE 201+, and a hair sample for total hair mercury analysis, a biomarker representing methylmercury exposure from fish consumption [122]. The ACR study collected whole blood samples from adults (≥18 years of age), while the EATM study collected whole blood samples from all family members, including children, to be processed for trace metals. Survey administration, sample collection and sample processing were the same across studies.

2.1.2 Laboratory Analysis

2.1.2.1 Blood

Sampling methods for the ACR study are explained in detail in Weinhouse et al., 2020 [119] and were also followed in the EATM study. Briefly, whole blood samples were collected in Trace Metal free EDTA BD Vacutainer tubes and frozen on dry ice in the field to be analyzed for mercury and lead. Blood samples were transported to Duke University on dry ice and stored at -80° C until being processed in Dr Heileen Hsu-Kim's laboratory at Duke University (Durham, North Carolina).

To quantify lead and mercury levels, blood samples were thawed overnight at 4˚C and digested at ambient pressure on a hot block (Environmental Express, Charleston, South Carolina) at 65 °C. The digestions used ultra-trace clean digestion tubes (Environmental Express) and consisted of heating 0.5 ml of blood with 1 ml of 70% HNO_3 (Plasma Pure Plus, SCP Science) and 0.05 ml of 30% HCl (Plasma Pure Plus, SCP Science) for two hours. The samples were cooled, 1 ml of 30% hydrogen peroxide (Plasma Pure Plus, SCP Science) was added to the mixture, and samples were heated again for 1 hour. After cooling, 10 µl of a 4 mg/L gold+2% HCl solution was spiked into the digestate to aid in mercury stability. Each digestion batch consisted of a maximum of 25 blood samples; two samples in each batch were analyzed in triplicate. The relative standard deviation (%RSD) for triplicate digestions (n=33) were 9.2% for Hg (range 0.8-32%) and 10.9% for Pb (range 0.4-36%). (See Appendix A, Supplemental Table A.1). Furthermore, each digestion batch included three blank samples, a NIST whole blood standard reference material (SRM, either 955c level 4 or 955d level 2), an aqueous standard (High Purity

Standards, certified reference material-trace metals in drinking water Mix A (CRM-TMDW-A) + Spex Certiprep Hg), and an IAEA dry blood sample (IAEA-A-13). Mercury recoveries in were 95% for SRM 955c level 4 (n=5), 83% for SRM 955d level 2 n=9, and 99.6% for the aqueous Hg spike (n=16). There are no reported values for mercury with IAEA-A-13. Lead recoveries in were 105% for SRM 955c level 4 (n=5), 101% for SRM 955d level 2 (n=9), 107% for the aqueous Pb spike (n=16), and 80.4% for IAEA-A-13 (n=16, Appendix A, Supplemental Table A.2). The blanks for Hg were an average of 0.06 µg/L and for Pb were 0.1 µg/L.

The blood digestates were diluted (10-fold) into an acid matrix (2% (v/v) HNO3 and 0.5% HCl (v/v)) containing 20 µg/L Au. Internal standards (^{193}Ir and ^{209}Bi) were also spiked into the matrix to correct for instrumental drift or matrix effects. The analyses were performed by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS: Agilent 7900) in helium mode to reduce any potential for polyatomic interferences. The ICP-MS was tuned to reduce oxide interferences to less than 2%. The instrument was calibrated with purchased aqueous standards for Hg (Brooks Rand) and Pb (Spex Certiprep mix 2A), and the standard curves were verified with a secondary standard (High Purity Standards (CRM-TMDW-A) + Spex Certiprep (Hg)). Calibration verification checks were performed every 20 samples during a batch run.

2.1.2.2 Hair

Hair samples were attached to self-adhesive notepaper, stored individually in paper envelopes, transported to Duke University, and stored at ambient temperature. The processing of hair samples is explained in detail in Weinhouse et al., 2017 [123]. Briefly, hair segments from the 2 cm closest to the root were analyzed for total Hg content by direct combustion gold

amalgamation atomic absorption spectrometry (Milestone Direct Mercury Analyzer 80), reflecting the last two months of exposure [122].

2.1.2.3 Water

For the 2018 study, a water sample was collected for each enrolled household by filling 125-mL pre-cleaned high density polyethylene bottles from the main faucet or water storage bucket of the household. Additional samples were collected from school tap water and community drinking water sources. Each bottle was labeled, stored in sealable plastic bags, held in cold storage with ice packs in the field, and frozen during international transit to and storage at Duke University. At Duke laboratories, water samples were thawed, acidified to a concentration of 2% v/v concentrated HNO_3, held at 4°C, and analyzed for Pb by ICP-MS.

2.1.1 Statistical Analysis

Data from the ACR (n=245) were pooled with a subset of participants from the EATM study (n=62) to increase the sample size of non-native participants and incorporate a broader age range. Since anemia may increase lead absorption, we used linear regression models to evaluate whether hemoglobin levels were associated with increased BLL (Appendix A, Supplemental Table A.3, n=249). A sub-analysis was also conducted with only EATM data (n=123) to evaluate risk factors that were not included in ACR study, consisting of the risk factor of interest while adjusting for sex. T-tests and Fisher's tests were used to identify potential risk factors of lead exposure and assess for differences between indigenous/non-indigenous participants and between the two studies. Pearson correlations were used to test correlations of mercury concentrations in hair and blood and blood lead levels in the pooled dataset. Linear mixed effect models with community as a random effect were used to evaluate risk factors for

lead exposure. Blood lead levels were analyzed as continuous and as categorical (high/low), with the United States' Centre for Disease Control and Prevention (CDC) threshold of 5 μg/dL being used to differentiate between low vs. high exposures [53]. Lead exposure was also assessed using ACR data to ensure similar results with and without pooling study data (results not shown). Risk factors were first tested individually, adjusting for sex; significant variables were subsequently tested together. Models were tested for heteroskedasticity and multicollinearity. Interactions between final risk factors and study was also evaluated to ensure differences in study design did not have a significant interaction on identified risk factors. Models were compared using the Bayesian Information Criterion (BIC) with the lowest BIC representing the best fit model. We use a classification and regression tree (CART) model, that uses recursive modelling techniques and residual sum of squares in a regression model to identify important variables and cut-off points to determine the ranking of risk factors to identify individuals with higher BLLs. In doing so, we create a classification tree of risk factors (indigenous status, age, sex; and consumption of yuca, fish, and wild game) and BLLs.

Survey data included participant and household information: demographics; smoking status (Yes/No); water source (Treated: municipal water/Untreated: river water, rainwater, well water); cooking fuel (Low: natural gas/High emission: wood); and food frequency consumption of wild game, beef, chicken, and fish (Rarely/Monthly/Weekly). Wild game and fish were also evaluated binomially (Never or Weekly/Monthly and Weekly or non-Weekly), respectively. Age was categorized as adult/child (≥18 years old) and by quartiles for analysis. A variable for study (ACR/EATM) was also evaluated to test whether study design was a significant factor in the analysis. Community variables such as presence of food market (Yes/No), and paved access to

the Interoceanic Highway (Yes/No) were also evaluated. Reference variables for all risk factors was either null, never, or low for ease of model interpretation.

The All-Ages Lead Model (AALM), Version 2.0, created by the United States Environmental Protection Agency (EPA), was used to determine whether periodic consumption of small bullet fragments (<0.01g) in wild game could yield measured blood lead levels [124]. The AALM is a pharmacokinetic model that simulates lifetime lead exposure and predicts lead levels in multiple human tissues based on assumptions of age, sex, and exposure to environmental sources of lead. We apply this model by assuming wild game consumption is the predominate environmental lead source. Blood lead levels of individuals who never ate wild game were used to identify a background lead exposure. An estimated lead dose from wild game was then identified based on mean blood levels and frequency of wild game consumption with dose being held constant. A steady consumption of wild game, as stated in participants' survey response, was assumed, and no other predominant sources of lead exposure were considered. Due to the potential of lead exposure from the use of lead fishing weights and yuca consumption (also known as manioc or cassava), we compared blood lead levels between fishermen and consumers of wild game (monthly or weekly) and evaluated yuca consumption in the EATM dataset (Appendix A, Supplemental Table A.4, n=123). In the same dataset, we evaluated blood lead and the amount of yuca, and wild game consumed. Blood lead, blood mercury and total hair mercury values were log10 transformed to normalize the data. All analyses were done in RStudio Version 1.2.5033.

2.2 Results

The final pooled data set included 307 participants with a mean age of 32 years, 65% female, and 54.6% native (Table 1). Between the two studies, the ACR study had older participants (35.2 years old vs. 22.6 years old, p = <0.001); a larger proportion of men (0.37 vs. 0.23, p = 0.02) and indigenous participants (0.52 vs. 0.19, p = <0.001); and participants with higher BLLs (3.8 µg/dL vs 2.1 µg/dL, p = <0.001, Supplemental Table 5). Participants in the EATM study had significantly lower hemoglobin levels (12.2 vs 13.6, p = <0.001, n = 249) and a higher prevalence of anemia (0.5 vs. 0.0, p = <0.001, n=249, Supplemental Table 3). Hemoglobin was not associated with increased blood lead levels (Appendix A, Supplemental Table A.6).

Table 1: Individual and household risk factors evaluated for lead exposure

	Non-Native (n=168)	Native (n=139)	Overall (n=307)
Age (Years)			
Mean (SD)	32.1 (12.1)	33.4 (12.3)	32.7 (12.2)
Median [Min, Max]	31.5 [2.0, 66.0]	33.0 [3.0, 64.0]	32.0 [2.0, 66.0]
Sex**			
Female	124 (73.8%)	78 (56.1%)	202 (65.8%)
Male	44 (26.2%)	61 (43.9%)	105 (34.2%)
BMI			
Mean (SD)	27.8 (5.56)*	26.7 (4.10)	27.3 (4.98)
Median [Min, Max]	28.4 [13.7, 42.6]	26.6 [16.1, 35.8]	27.3 [13.7, 42.6]
Smoke Status**			
No	148 (88.1%)	103 (74.1%)	251 (81.8%)
Yes	20 (11.9%)	36 (25.9%)	56 (18.2%)
Household Water Source***			
Treated	141 (83.9%)	46 (33.1%)	187 (60.9%)
Untreated	27 (16.1%)	93 (66.9%)	120 (39.1%)

	Non-Native (n=168)	Native (n=139)	Overall (n=307)
Cooking Fuel* **			
High Emissions	24 (14.3%)	74 (53.2%)	98 (31.9%)
Low Emissions	144 (85.7%)	65 (46.8%)	209 (68.1%)
Education Level†			
Elementary	33 (19.6%)	25 (18.0%)	58 (18.9%)
Middle School	31 (18.5%)	41 (29.5%)	72 (23.5%)
High School	78 (46.4%)	61 (43.9%)	139 (45.3%)
Advanced	26 (15.5%)	12 (8.6%)	38 (12.4%)
Highway Access* **			
Highway	75 (44.6%)	0 (0%)	75 (24.4%)
Non-Highway	93 (55.4%)	139 (100%)	232 (75.6%)
Road Access			
Mean (SD)	0.830 (0.172)	0.255 (0.318)***	0.570 (0.379)
Median [Min, Max]	0.750 [0.50, 1.0]	0 [0, 0.75]	0.750 [0, 1.0]
Fish Consumption **			
Rarely	9 (5.4%)	24 (17.3%)	33 (10.7%)
Monthly	86 (51.2%)	58 (41.7%)	144 (46.9%)
Weekly	73 (43.5%)	57 (41.0%)	130 (42.3%)
Beef Consumption* **			
Never	19 (11.3%)	24 (17.3%)	43 (14.0%)
Monthly	64 (38.1%)	86 (61.9%)	150 (48.9%)
Weekly	85 (50.6%)	29 (20.9%)	114 (37.1%)
Daily	0 (0%)	0 (0%)	0 (0%)
Chicken Consumption* **			
Never	0 (0%)	2 (1.4%)	2 (0.7%)
Monthly	9 (5.4%)	35 (25.2%)	44 (14.3%)
Weekly	140 (83.3%)	102 (73.4%)	242 (78.8%)
Daily	19 (11.3%)	0 (0%)	19 (6.2%)
Wild Game Consumption* **			

	Non-Native (n=168)	Native (n=139)	Overall (n=307)
Never	107 (63.7%)	9 (6.5%)	116 (37.8%)
Monthly	58 (34.5%)	93 (66.9%)	151 (49.2%)
Weekly	3 (1.8%)	37 (26.6%)	40 (13.0%)
Blood Pb and Hg Exposure (Low/High)* **			
Low blood Pb and Hg	87 (51.8%)	12 (8.6%)	99 (32.2%)
High blood Hg (>=5.8 µg/L), low Pb	77 (45.8%)	60 (43.2%)	137 (44.6%)
High blood Pb (>=5.0 µg/dL), low Hg	1 (0.6%)	2 (1.4%)	3 (1.0%)
High blood Pb and Hg	3 (1.8%)	65 (46.8%)	68 (22.1%)

Fisher's Exact Test for categorical and T-tests for continuous variables
Significance: <0.001'***'; 0.001 '**'; 0.01 '*'; 0.05 '†'
All large markets are in non-native communities

EATM Data Subset

In the EATM sub-analysis, larger portions of wild game were associated with higher BLLs. Individuals who ate one portion/serving had a 1.32 µg/dL (95% CI: 0.99 – 1.78) increase in BLLs, while those who ate two portions/serving had an increase of 1.66 µg/dL (95% CI: 1.10 – 2.57), compared to those who did not eat wild game (Table 2). No association was found between BLL and yuca consumption. All household water samples were below Environmental Protection Agency's guideline of 15 µg/L (Appendix A, Supplemental Figure A.1) [53].

Table 2: Measured blood lead, blood mercury and total hair mercury by evaluated risk factors and overall.

		Blood Lead Level (µg/dL)		Blood Mercury Level (µg/L)		Total Hair Mercury (µg/g)	
	N	Mean (SD)	Median [Min, Max]	Mean (SD)	Median [Min, Max]	Mean (SD)	Median [Min, Max]
Overall	307	3.47 (3.26)	2.23 [0.25, 17.4]	12.5 (12.3)	9.40 [0.300,	3.34 (3.05)	2.61 [0.0026,

					89.1]		21.4]
Sex							
Female	202	2.85 (2.76)	1.69 [0.25, 15.2]	10.9 (10.0)	8.65 [0.31, 89.1]	3.10 (2.84)	2.55 [0.07, 21.4]
Male	105	4.68*** (3.78)	3.28 [0.52, 17.4]	15.8** (15.2)	11.7 [0.30, 73.7]	3.79. (3.39)	2.91 [0.003, 19.4]
Smoking Status							
No	251	3.08 (2.94)	2.07 [0.25, 15.8]	11.9 (11.9)	8.90 [0.30, 89.1]	3.19 (3.03)	2.50 [0.003, 21.4]
Yes	56	5.22*** (3.99)	4.15 [0.81, 17.4]	15.4. (13.5)	13.1 [0.43, 68.4]	3.98. (3.10)	3.23 [0.09, 13.2]
Indigenous Status							
Non-Native	168	1.60 (1.30)	1.21 [0.25, 8.89]	9.23 (11.9)	5.10 [0.30, 89.1]	2.34 (2.61)	1.43 [0.003, 13.8]
Native	139	5.73*** (3.48)	4.95 [1.02, 17.4]	16.6*** (11.5)	14.1 [1.60, 73.7]	4.54*** (3.11)	3.84 [0.433, 21.4]
Wild Game Consumption[1]							
Never	116	1.50 (1.21)	1.11 [0.25, 7.51]	8.73 (12.5)	4.95 [0.60, 89.1]	2.11 (2.09)	1.33 [0.003, 13.3]
Monthly	151	4.27 (3.50)	3.11 [0.62, 17.4]	14.1 (11.9)	11.4 [0.30, 73.7]	3.62 (2.75)	2.91 [0.09, 13.8]
Weekly	40	6.21*** (3.20)	5.48 [1.59, 14.2]	17.7*** (9.76)	16.9 [2.50, 47.7]	5.84*** (4.44)	4.78 [0.57, 21.4]
Fish Consumption[1]							
Never	33	3.93 (2.57)	3.17 [0.66, 12.1]	12.3 (12.3)	9.60 [0.74, 68.9]	3.15 (2.55)	2.61 [0.35, 12.6]
Monthly	144	3.46 (3.38)	2.15 [0.52, 16.4]	12.8 (12.8)	8.42 [0.31, 73.7]	3.52 (3.12)	2.79 [0.003, 19.4]
Weekly	130	3.37 (3.29)	2.14 [0.25, 17.4]	12.4 (11.8)	10.3 [0.30, 89.1]	3.18 (3.09)	2.32 [0.17, 21.4]

Fisher's Exact Test for categorical and T-tests for continuous variables
Significance: <0.001'***'; 0.001 '**'; 0.01 '*'; 0.05 '†'
[1] ANOVA with consumption frequency of Weekly and Monthly compared to Never

Pooled Data Set

Risk factors were significantly different for individuals living in indigenous and non-indigenous communities. Indigenous communities were more likely to rely on untreated water and high emission fuels, compared to non-indigenous households. Non-indigenous individuals

were predominantly male and younger than indigenous individuals. Overall, 18% of participants smoked, with smoking more prevalent among indigenous (Table 1). Protein sources varied significantly between indigenous and non-indigenous participants. Chicken was consumed most frequently. Beef was more frequently consumed by non-indigenous participants, and wild game by indigenous participants. 63% of non-indigenous participants never ate wild game, whereas 27% of indigenous participants ate it weekly and 66% monthly (Table 1).

Indigenous participants had significantly higher BLLs with an average of 5.7 µg/dL compared to 1.6 µg/dL ($p = <0.0001$, Table 3). Mean blood mercury and total hair mercury were nearly twice as high in indigenous compared to non-indigenous participants ($p = <0.0001$ and <0.0001, respectively). When blood lead and mercury exposure were categorized, 46.8% of indigenous participants had high lead (≥ 5.0 µg/dL) and mercury (≥ 5.8 µg/L) levels. Only 8.6% of indigenous participants had low blood lead and low mercury levels, compared to 51.8% of non-native participants (Table 1). Significant Pearson's correlations of 0.37 ($p < 0.001$) and 0.38 ($p < 0.001$) were found between BLL and mercury levels in blood and hair, respectively.

Table 3: Random mixed effect model results for individuals who eat wild game (weekly or monthly) and log10 blood lead levels (µg/dL) with community as a random effect.

Risk Factor	Estimate	95% Confidence Interval
Sex (Ref: Female)	0.14***	0.08 - 0.20
Native (Ref: Non-indigenous)	0.40***	0.29 - 0.51
Eat Wild Game (Ref: Never)	0.15**	0.08 - 0.23

Significance: <0.001'***'; 0.001 '**'; 0.01 '*'; 0.05 '†'

In random effect models adjusting for sex, higher BLLs were associated with indigenous status, higher frequency of wild game consumption, male sex, blood mercury, smoking and lower beef consumption (Tables 4 and 5). Community as a random effect was significant,

accounting for 12.5% of the variance at the community level. Being indigenous and male was associated with a 2.52 µg/dL (95% CI: 1.95 – 3.24) and 1.38 µg/dL (95% CI: 1.20 – 1.58) increase in BLLs, respectively (Table 4). Eating wild game monthly or weekly were both significantly associated with BLL, with weekly consumption having a larger effect on BLL (monthly: 1.41 µg/dL, 95% CI: 1.19 – 1.65, weekly: 1.70 µg/dL, 95% CI: 1.33 – 2.16). Modifying wild game consumption to weekly/monthly vs. never, improved model fit, resulting in wild game consumption being associated with a 1.42 µg/dL (95% CI: 1.21 – 1.68) increase in BLLs (Table 4). Similar results were found when analyzing only ACR data (results not shown). Smoking was associated with higher BLLs, while beef consumption, access to markets and the highway were associated with lower BLLs but reduced model fit. Age, BMI, water source, cooking fuel, hemoglobin, and study were not associated with lead exposure.

Table 4: Random mixed effect model results for EATM data subset of portions of wild game consumed/meal and log10 blood lead (µg/dL) levels with community as a random effect.

Risk Factor	Estimate	95% Confidence Interval
Sex (Ref: Female)	0.15**	0.06-0.25
Wild Game per Serving (Ref: None)		
Half a Portion	0.12	-0.08-0.33
1 Portion	0.11†	-0.02-0.24
>2 Portions	0.22*	0.05-0.41

Significance: <0.001‘***’; 0.001 ‘**’; 0.01 ‘*’; 0.05 ‘†’

Table 5: Odds ratios and 95% confidence intervals (CI) from random mixed effect logit models for high BLL levels (>5 µg/dL) with community as a random effect.

	Univariate Models		Multivariate Model	
Risk Factor	**Odds Ratio**	**95% CI**	**Odds Ratio**	**95% C.I.**
Sex (Ref: Female)	3.28**	1.51-7.47		
Native (Ref: Non-indigenous)	42.02***	8.95-354.66	24.53***	4.77 – 231.34
Smoke (Ref: No)	4.02**	1.57-11.29	3.16*	1.28 – 8.44
Eat Wild Game (Ref: Never)	6.86**	1.78-33.55	4.10†	1.02 – 20.67
Wild Game Consumption (Ref: Never)				
Monthly	6.51*	1.67-32.08		
Weekly	8.44**	1.83-47.33		
Log10(Blood Hg)	17.14***	4.32-77.21		
Log10(Total Hair Hg)	8.08**	1.93-37.13		

Significance: <0.001'***'; 0.001 '**'; 0.01 '*'; 0.05 '†'

With the CART model, we found indigenous status to be the largest risk factor, followed by wild game consumption, fish consumption and sex (Figure 2). Within indigenous communities, participants with high total hair mercury levels (>2 µg/g) had higher BLL. In non-indigenous communities, participants who ate wild game had higher BLLs, compared to those who did not. Men had higher BLLs than women, regardless of community type. When excluding indigenous status, sex was no longer significant and wild game consumption was the main indicator of BLLs (Figure 3).

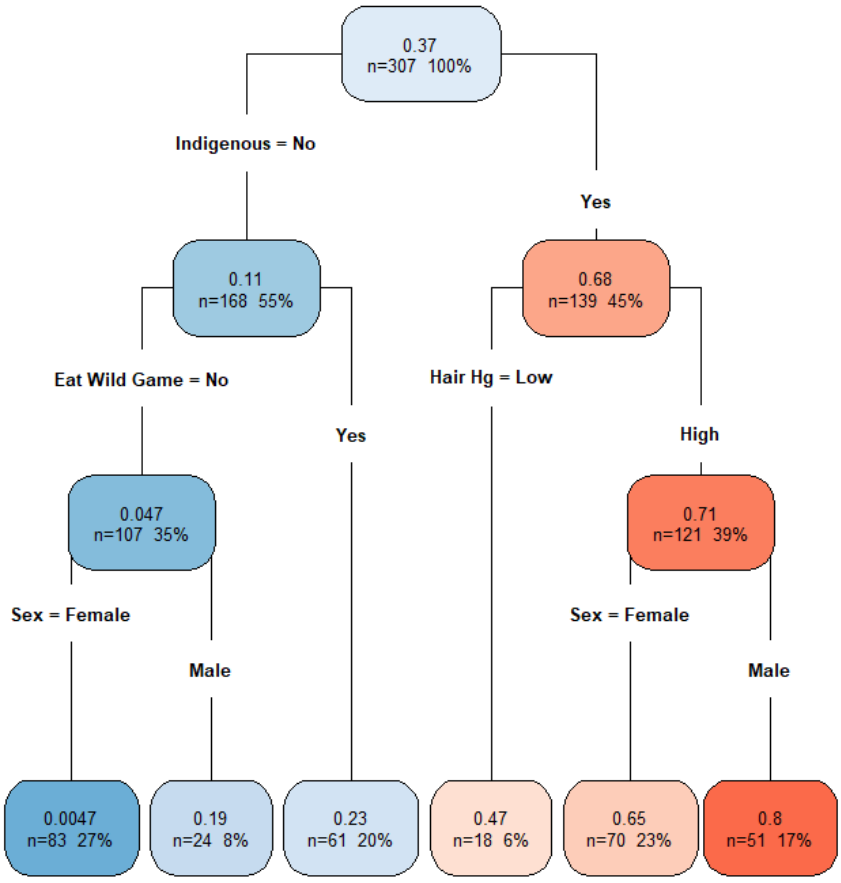


Figure 2: Color coded classification tree of log10(BLL) with higher BLL represented with darker shades of red and lower BLL shown as darker shades of blue. In each box, top number is the predicted log10(BLL) with bottom two numbers representing the number of participants found within the category and its proportion of the total study population, respectively.

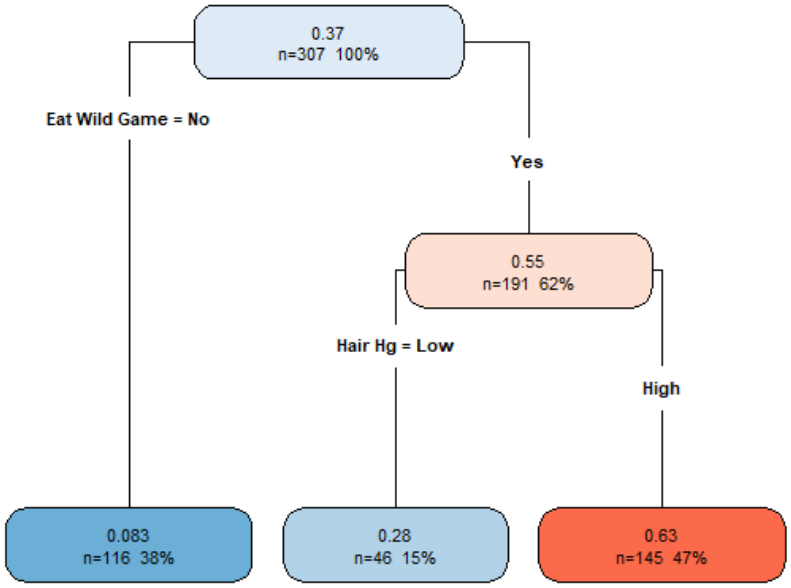


Figure 3: Color coded classification tree of log10(BLL), with indigenous status excluded, with higher BLL represented with darker shades of red and lower BLL shown as darker shades of blue. In each box, top number is the predicted log10(BLL) with bottom two numbers representing the number of participants found within the category and its proportion of the total study population, respectively.

We also evaluated risk factors associated with BLLs above 5 µg/dL. Individually, high BLLs were associated with blood mercury, total hair mercury, smoking, sex, wild game consumption and indigenous status (Table 5). Indigenous participants had 24 times higher odds of having high BLLs than non-native participants, while those who ate wild game had four times higher odds than non-wild game consumers (Table 5, Figure 4). A strong association between BLLs and blood mercury levels was found, with a predicted 50% and 32% of indigenous participants and those who ate wild game (weekly or monthly), respectively, having high BLLs at the 75th percentile of mercury exposure (Figure 5).

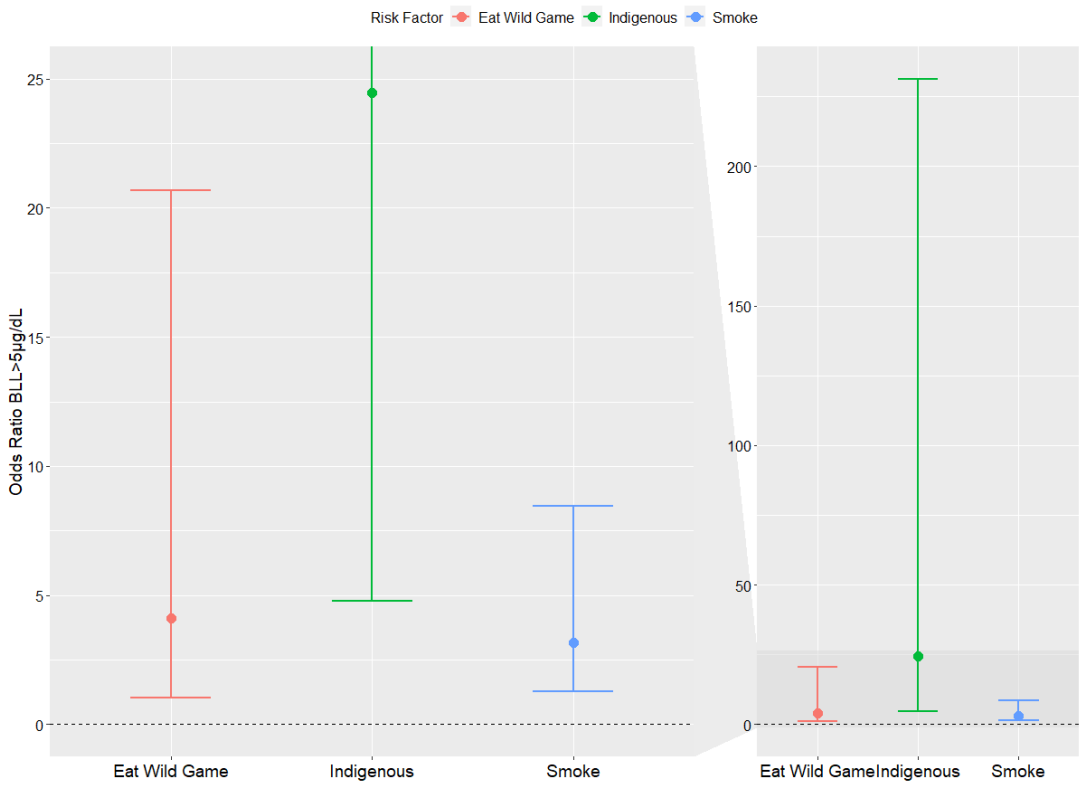


Figure 4: Odds ratio of best fit logit random effects model for high blood lead levels (>5µg/dL) that adjusts for wild game consumption, indigenous status, and smoking with community as a random effect. The split panel on the left is a zoomed in version of the panel on the right.

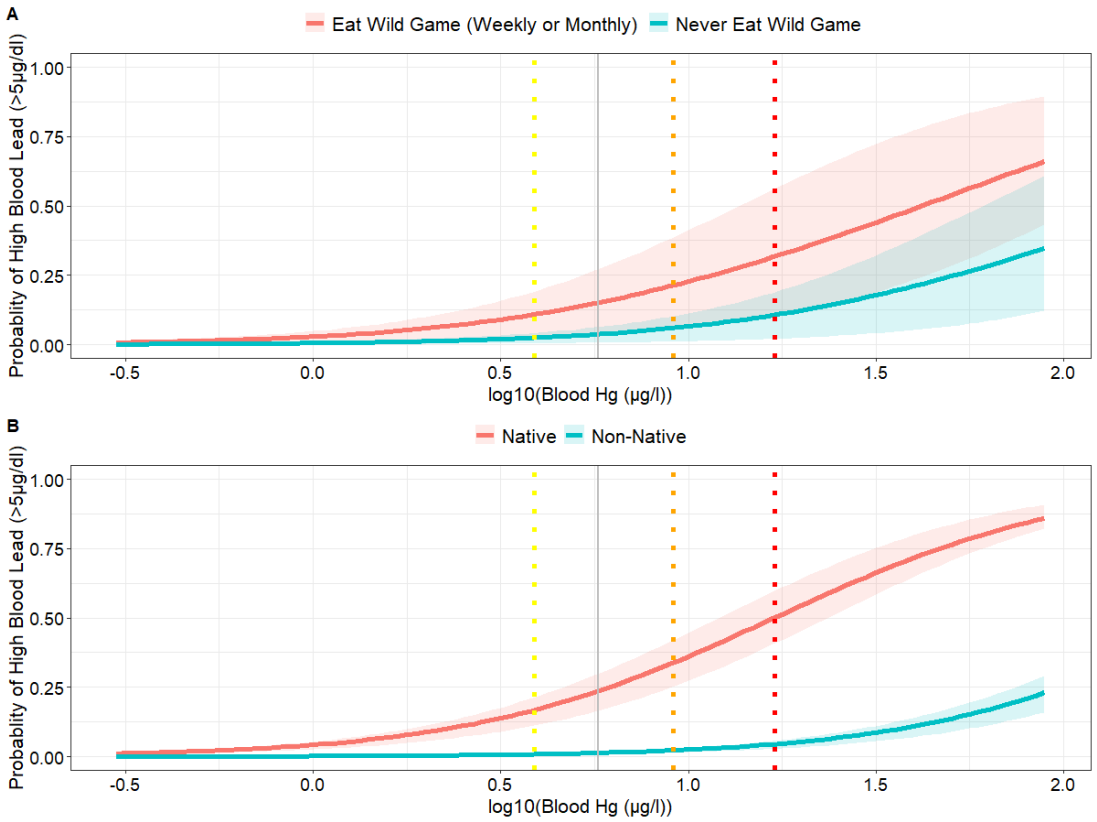


Figure 5: Probability of a blood lead level above the recommended guidelines of 5 µg/dL adjusting for log10 blood mercury and community as a random effect. Panel A also adjusts for wild game consumption, while panel B adjusts for native status. Dotted vertical lines represent the 25th, 50th and 75th percentiles of log10 blood mercury levels, shown as yellow, orange, and red, respectively. The solid gray line is the recommended limit of mercury in blood, 5.8 µg/L.

Blood lead exposure modelling using the AALM demonstrated that periodic lead exposures of 500 µg/meal of wild game and a background lead exposure of 20 µg/day replicated mean BLLs measured in the study (Figure 6). The model assumes an estimated 500 µg of lead are ingested at each meal where wild game is consumed, which correlates with the fragmentation of bullets once they impact wild game.

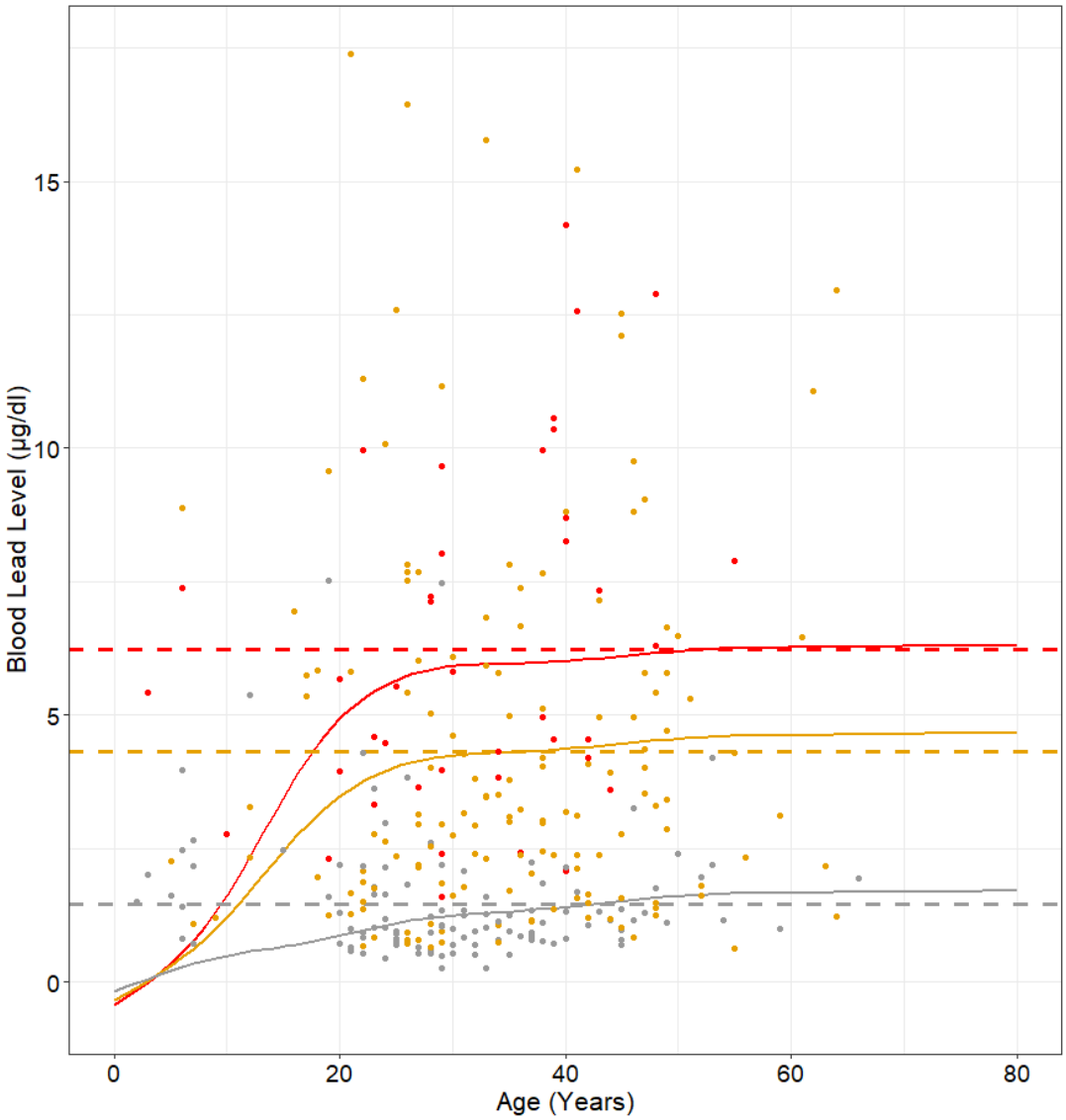


Figure 6: Modeled blood lead levels (solid lines) using All-Age Lead Model for baseline (no wild game consumption), monthly game consumption and weekly game consumption, shown in grey, yellow, and red, respectively. Baseline model lead exposures consist of a 20 µg/day background exposure starting at age three. Lead exposure with consumption of wild game includes the 20 µg/day background and 500 µg/day every 9 days or 14 days for weekly and monthly wild game consumption, respectively. Horizontal dotted lines demonstrate the mean blood lead levels in the study population by wild game consumption (Grey-Never (mean: 1.50 µg/dL, 95% CI: 0.48 – 4.43), Yellow-Monthly (mean: 4.27 µg/dL, 95% CI: 0.74 – 13.53), Red-Weekly (mean: 6.21 µg/dL, 95% CI: 2.06 -12.91)). Points represent study data shown by frequency of wild game consumption.

2.3 Discussion

This study found a strong indication, in a region without confounding lead sources of oil extraction, leaded paint, and leaded gasoline, that hunting is the predominant source of lead exposure, resulting in a 1.41 ug/dL higher BLL compared to individuals who do not consume wild game. It is also the first study in Madre de Dios to associate both increased frequency and larger portion sizes of wild game to higher BLLs. Using CART models, we identify indigenous communities as most at risk with male indigenous having the highest BLL of any subpopulation. Furthermore, the AALM model demonstrates that measured BLLs and frequency of wild game consumption (monthly and weekly) match, with an estimated dose of 500 µg Pb/meal. The estimated 500 µg/meal is reasonable with bullet fragments known to be microscopic and 34% less than 0.01 grams [114]. The evidence found in this study and the literature provide ample support that wild game consumption is an important route of lead exposure in hunting communities.

Results from the CART and logit models demonstrate that mercury and lead exposures are correlated, which may be due the use of lead fishing weights or the dependence of indigenous communities on wild fish and wild game as protein sources. Lead exposure is predominantly via ingestion and inhalation as lead absorption from handling is low. The percent transfer from hand to mouth in adults is ~24% with an estimated average lead exposure of 15.5 µg after handling lead sinkers for 15 seconds [125]. In our study, we find no association with age or age class and BLLs, which would be expected if lead exposure were from handling fishing weights as children have more frequent hand to mouth contact [126].

Indigenous and rural communities that rely on natural resources to meet nutritional needs face a dual exposure burden of lead and mercury [127]. Communities that depend on wild game or wild fish to meet nutritional needs have been found to substitute fish or wild game, depending on which is easier to obtain. Thus, as one resource becomes scarcer, dependence shifts to that which is more prevalent, depending on cultural, and personal preferences [128]. This is especially important as fishing and hunting have traditionally been and continue to be two main sources of protein in the Amazon. It may also help explain the significant correlation between lead and mercury exposure. Future epidemiological studies in the region should consider evaluating both metals due to shared toxicological outcomes.

It is important to note that this study has several limitations. The study is cross-sectional and cannot evaluate how lead exposures vary across time. Although we find a strong correlation between wild game and BLLs, it is possible that other dietary exposures exist. Previous studies in Brazil have identified consumption of farofa, toasted yuca/manioc used as a topping, to be a potential source of lead exposure, finding elevated concentrations of lead in yuca [121, 129]. Yuca is a food staple, eaten boiled or fried, in Madre de Dios and may be a source of lead exposure; however, lead levels in yuca are likely site specific as soils of clay licks have been found to have higher lead levels [113]. Although we find no association between yuca consumption and BLLs in our sub-analysis, our sample size may be too small (n=123). Fermented yuca is used to make Masato, a traditional drink, which was not included in our survey, potentially limiting our ability to adequately determine yuca consumption. The EATM data was not randomly selected; thus, these results may not be reflective of the overall population. The

pooling of data can create biases in results; however, we found similar results when analyzing the datasets separately.

Lead shot has been linked to the lead poisoning of wildlife; however, demand for lead-free ammunition is low even at comparable prices [130]. Worldwide, hunting is the main source of lead in the environment, with an estimated 80% of lead in European soils being attributable to hunting by 2030 [131]. Yet, very few studies on lead exposure have been conducted in the Amazon. Due to the ubiquitous use of lead ammunition, consuming wild game may be an underestimated source of lead exposure worldwide. The United Nations' Convention of Migratory Species, of which Peru is a member, seeks to eliminate lead shot in hunting. Fulfilment of this objective will likely not only improve the health of wildlife, but also of the many indigenous and rural communities that rely on wild game to meet nutritional and cultural needs.

3. *In-Utero* Exposure to Lead and Mercury Differentially Influence Gestational Age and Birthweight in an ASGM Birth Cohort in Madre de Dios, Peru

The *in-utero* and neonatal periods are critical windows of an individual's life, during which exposures to toxic metals such as lead (Pb), mercury (Hg), and cadmium (Cd), or a lack of minerals (calcium, zinc, iron, magnesium) may have lifelong consequences [132-136]. Many studies have evaluated newborn health outcomes from nutritional or toxicological perspectives, but few have evaluated both simultaneously [137], especially in developing countries (Appendix B, Supplemental Table B.1). This is of special importance as nutritional status and toxic exposures are known to interact, causing neonatal health to be dependent on both minerals and toxic metals. Birth cohorts in developed nations benefit from strong healthcare systems and food security, often precluding the ability to evaluate interactions of malnutrition and toxic metal exposure. A lack of minerals is known to adversely affect fetal development: low levels of calcium can lead to poor skeletal formation and arrythmias in newborns [138]; low levels of iron during pregnancy increases risk of preterm birth [139, 140]; and low levels of magnesium are associated with small for gestational age and preterm labor [141]. From a toxicological perspective, maternal exposure to toxic metals during pregnancy is associated with decreased birthweight [142-147], lower ponderal index, shorter gestational age, smaller head circumference, lower APGAR score [148], and a thinner placenta[149]. Maternal lead exposure has also been found to shorten gestational age [150]. Maternal and fetal mercury exposure has been linked to decreased head circumference [56, 57] and cognitive deficits in children [55, 151].

Assessment of exposures to toxic metals and minerals in epidemiological analyses on neonatal health is important as they influence maternal absorption, cellular processes, and nutrient transfer to the fetus [152-156]. For example, lead and cadmium absorption is increased when iron levels are low. Adequate nutrition could mitigate the harmful effects of toxic metals as selenium may sequester harmful free radicals from mercury exposure [157, 158]. Toxic metals also compete with minerals for binding sites through molecular mimicry, disrupting cellular functions [36, 157, 159, 160], and potentially impeding nutrient transfer to the fetus [36, 157]. Cadmium has been shown to impede the transfer of zinc [153, 161] and calcium [161], while increasing levels of maternal selenium has been suggested as a means to lower cadmium levels in cord blood [147]. The potential for toxic metals to interfere with nutrient transport to the fetus may have important biological consequences when minerals and toxic metals are analyzed separately. Not integrating maternal nutrition in statistical analyses may incorrectly infer the association between toxic metal exposures and neonatal health as effect sizes may be greater in malnourished individuals (or non-existent in those well-nourished). This may be of special importance for nutritionally non-replete populations.

Fetal risk of exposure to minerals and toxic metals exists initially with the maternal environment, or what we refer to as the maternal (*in-utero*) exposome. Minerals, metals, and other nutrients are transferred to the fetus in this environment, representing a physiological hierarchy that is commonly not accounted for in statistical analyses. Birth cohort studies predominantly use standard linear regression to evaluate the direct relationship of either maternal or cord blood measurements to neonatal health. The use of maternal exposures as direct effects on neonatal health does not account for any modifications that may occur in the

transfer from mother to child (Appendix B, Supplemental Table B.1). While there is a direct association between cord blood and neonatal health, policy and healthcare recommendations are implemented at the maternal level, requiring an understanding of how the maternal exposome relates to the fetal environment and neonatal health. NIEHS has recently supported development of new statistical methods for analyzing effects of complex mixtures that move beyond traditional regression (PRIME - Powering Research Through Innovative Methods for Mixtures in Epidemiology). Structural equation models (SEMs) and Factor Analysis approaches have been proposed to evaluate multivariate exposures and endpoints [162, 163] as they can account for known physiological processes and allows hypothesized structures to be tested statistically. SEMs also permit dependent variables to be evaluated jointly, which prevents over-fitting. This issue arises in models evaluating correlated (and endogenous) factors associated with birthweight, such as gestational age, whose inclusion in linear models limit the amount of remaining variance available to detect true effects of other variables [164]. SEMs also define unmeasurable (latent) variables that can be constructed and evaluated with newborn health outcomes, such as the maternal exposome (ME) and the fetal environment (FE). Latent variables can provide a better model fit than linear models [165]. In this study, we use SEMs to test a novel conceptual framework for neonatal health that incorporates the transfer of minerals and toxic metals from mother to child while jointly evaluating gestational age and birthweight.

Few birth cohorts have been implemented in low- and middle-income countries (LMIC). Data collection in LMICs is considerably more difficult compared to similar studies in high income countries for a number of reasons, including resource and infrastructure constraints, building community trust, and local logistical issues. In the Amazon, we could not identify any

previous birth cohorts that have jointly evaluated nutrients and toxic metal exposures. This is especially important as results from previous birth cohorts in high income countries may not be translatable to the Amazon due to differences in disease burdens, environmental exposures, and healthcare access [166]. This is the first birth cohort in the Amazon to evaluate how nutritional status and toxic metal exposures may jointly influence neonatal health outcomes, which are particularly relevant to LMICs currently undergoing nutritional transitions to a western diet [167, 168]. Specifically, we measure direct and indirect effects of toxic metals and minerals to determine their effect on birthweight and gestational age, while adjusting for hypertension, maternal age, prenatal care visits, number of previous births and newborn's sex. In doing so, we contribute valuable information in an understudied population on maternal well-being, minerals, and toxic metal exposures. Data are from the COhorte de NAcimiento de MAdre de Dios (CONAMAD) in Madre de Dios, Peru that was collected from 2017-2018 (Figure 7) [169].

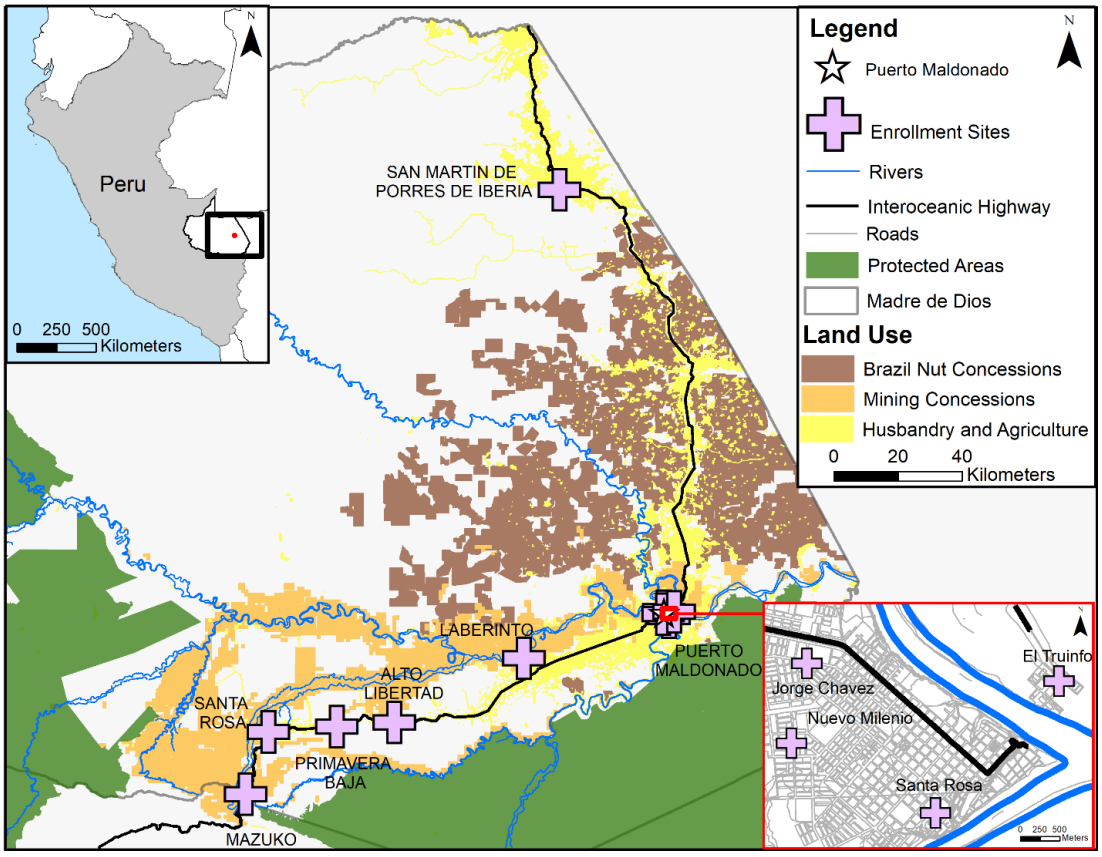


Figure 7: Project map of enrollment centers in Madre de Dios, Peru for the CONAMAD study. The capital of Madre de Dios, Puerto Maldonado, labeled with a star and shown in the red inset map in the lower right.

3.1 Methods

3.1.1 Data Collection

Data were collected in 2017-2018 as part of the CONAMAD birth cohort based in Madre de Dios, Peru and was approved by the IRB of Universidad Peruana Cayetano Heredia, SIDISI 66471 [169]. The cohort is described in detail in Pan et al., 2021 [169]. Madre de Dios is a hotspot of global biodiversity as well as global mercury pollution due to the rapid expansion of artisanal and small-scale gold mining (ASGM) over the past two decades. CONAMAD is one of

the few birth cohorts conducted in Latin America and the Caribbean that collected prenatal exposure data and the only birth cohort of which we are aware that is based in an ASGM region. Briefly, 270 mothers were enrolled during a prenatal visit before their third trimester. Enrollment sites were in 4 zones differentiated by land use and presence of ASGM. Inclusion criteria included multiparous women with at least one other child from the same father and a planned birth in Madre de Dios. Women were excluded if medically diagnosed with type II diabetes prior to pregnancy. Data collected include an enrollment and birth survey, with hemoglobin, anthropometry and hair samples for total mercury taken at both times. The birth survey also included birthweight (kilograms), gestational age (weeks), and APGAR score [170]. A HemoCue® Hb 201+ was used for in-situ hemoglobin measurements. Compete data were available for 200 mother-child dyads consisting of maternal and whole cord blood samples collected in trace element royal blue topped blood collection tubes. Both blood samples were collected to better understand trans-placental transfer of minerals and toxic metals, many of which are bound to red blood cells [144]. The number of prenatal visits was missing for 2 women, resulting in 198 mother-child pairs included in the analysis. Blood samples were initially stored and shipped at -20˚C to Duke University, and subsequently stored at -80˚C.

3.1.2 Laboratory Analysis

Cord and maternal blood samples were analyzed for lead, mercury, cadmium, arsenic, iron, magnesium, zinc, sodium, calcium, and selenium. The lower limits of quantification are reported in Appendix B, Supplemental Table B.2. All analytes were above detection level, except

for cadmium (<0.5 µg/L) and arsenic (<0.9 µg/L). For samples with concentrations below the lower limit of quantification, a value of half of this limit was assigned to that sample.

For blood digestions, samples were thawed over night at 4˚C, and digested on a hot block at 65 °C. The digestions used ultra-trace clean digestion tubes (Environmental Express) and consisted of heating 0.5 ml of blood with 1 ml of 70% HNO3 (Plasma Pure Plus, SCP Science) with 0.05 ml of 30% HCl (Plasma Pure Plus, SCP Science) for two hours. The samples were cooled and 1 ml of 30% hydrogen peroxide (Plasma Pure Plus, SCP Science) was added to the mixture and heated again for 1 hour. After cooling, 10 µl of a 4 mg/L gold+2% HCl solution was spiked into the digestate to aid in mercury stability. Each digestion batch consisted of 25 blood samples including two samples analyzed in triplicates. Furthermore, each batch of 25 blood digestions included three blank samples, a NIST blood SRM (levels 2-4), an aqueous standard (High Purity Standards, CRM-TMDW-A + Spex Certiprep Hg), and an IAEA dry blood sample (IAEA-A-13). Analytes were background corrected by subtracting the average of the three blanks. Recoveries for the SRMs can be found in Appendix B, Supplemental Table B.3.

The blood digestates were diluted (10-fold) into an acid matrix (2% (v/v) HNO3 and 0.5% HCl (v/v)) consisting of 20 µg/L Au. Internal standards (^{45}Sc, ^{89}Y, ^{103}Rh, ^{115}In, ^{159}Tb, ^{193}Ir, and ^{209}Bi) were also spiked into the matrix to correct for instrumental drift or matrix effects. The analyses were performed by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS: Agilent 7900) in helium mode to reduce any potential for polyatomic interferences. The ICP-MS was tuned to reduce oxide interferences to less than 2%. The instrument was calibrated for Hg (Brooks Rand) and Pb (Spex Certiprep mix 2A) with the standard curves verified against second

source standards (High Purity Standards (CRM-TMDW-A) + Spex Certiprep (Hg)). Continuing calibration verification checks were performed every 20 samples during a batch run.

3.1.3 Variables

3.1.3.1 Outcomes

Gestational age (weeks) and birthweight (kg) were the outcomes of concern. Both were normally distributed. Gestational age was determined by the Capurro somatic method [170]. Birthweight was measured at birth by medical staff. Only singleton births were included for analysis.

3.1.3.2 Predictors

Minerals (Zn, Mg, Ca, Se, Fe) and toxic metals (Pb, Hg, As, Cd) measured in maternal and cord blood were the tested predictors of gestational age and birthweight. Analyte measurements were normalized with a log10 transformation. Two latent variables, maternal exposome (ME) and fetal environment (FE), were constructed from measured minerals and toxic metal concentrations in maternal and cord blood, respectively.

3.1.3.3 Confounders

Confounders include survey data consisting of mother's age, nutritional supplements (yes/no), number of previous births, number of prenatal care visits, smoking status (yes/no), and the development of diabetes (yes/no), hypertension (systolic blood pressure >140 mm Hg, yes/no), and pathologies of concern (risk of abortion, previous cesarian delivery, urinary infection, bleeding, and hyperemesis gravidarum) determined by medical staff (yes/no) during pregnancy. The dataset did not include maternal body mass index prior to pregnancy. Age was

evaluated continuously and as a binary variable to compare the top and bottom 50th percentiles. Number of births had a non-normal distribution and was evaluated as a binary variable categorized as multiparous (2-4 births), and grand-multiparous (≥5 births). We also evaluated socioeconomic status, which was determined using estimated household annual income from partner's occupation and classified as below minimum wage (<12,000 Peruvian Nuevo Soles (PNS)) , low (10,200–14,000 PNS, and moderate (>14,000 PNS), with 1 PNS equal to 0.295 USD (November 2016). It is important to note that Peru set the minimum wage in 2016 at 850 PNS/month (or 10,200 PNS annually), indicating that incomes in Madre de Dios are low compared to national standards. Anemia status and hemoglobin measurements at enrollment and at birth were also assessed. Hemoglobin was measured using a HemoCue® Hb 201+ with anemia classification dependent on the estimated gestational week [171]. The potential for outcomes to be modified by newborn sex was also assessed.

3.1.4 Statistical Analysis

3.1.4.1 Measurement Model

To incorporate the transport of minerals and toxic metals from the mother to the fetus, latent variables reflecting the maternal exposome (ME) and the fetal environment (FE) were created from maternal blood at birth and cord blood, respectively. ME and FE were comprised of minerals and toxic metals that loaded onto the latent variable, using a p-value<0.05. Preliminary models demonstrated an association between ME and FE, results not shown.

3.1.4.2 Model Identification

SEM models were used to test the causal structure of the physiological hierarchy with the ME indirectly affecting gestational age and birthweight through the FE (Figure 8). We then

individually evaluated the direct effect of risk factors and toxic metals on gestational age and birthweight. Since birthweight is dependent on gestational age, we evaluated them jointly.

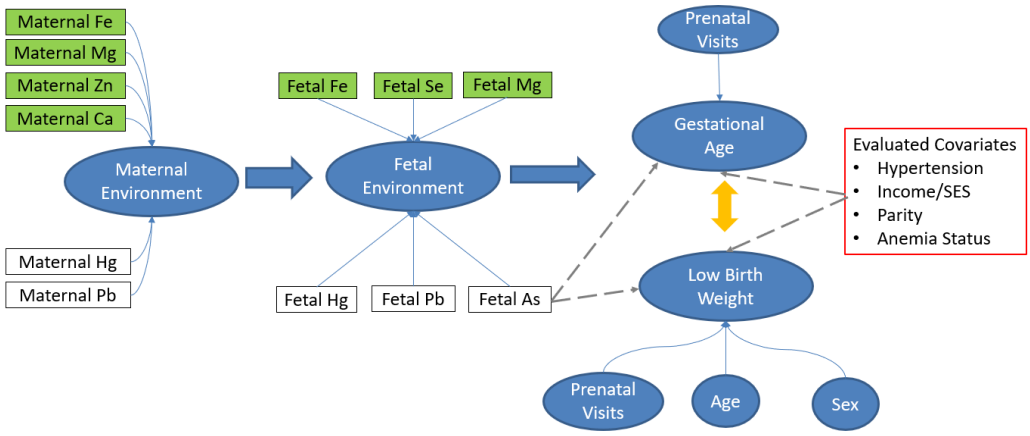


Figure 8: Theoretical latent model with evaluated covariates

While we considered maternal age as a continuous and categorical variable in initial evaluations, we found no nonlinear effects with a continuous maternal age variable and used this for subsequent models. The direct effect of the ME, toxic metals and minerals on gestational age and birthweight was also assessed. We used Pearson correlation statistics to evaluate the correlations of toxic metals and minerals within and between the maternal and cord blood.

Model estimates were quantified using maximum likelihood, and evaluated using Chi squared (>0.05), Tucker-Lewis Index (TLI >0.95), root mean square error of approximation (RSMEA <0.07) and standardized root mean residual (SRMR <0.08). Models that met these requirements were further evaluated and compared using the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC), with the lowest values demonstrating the best fit model (Table 6). Analysis was done in RStudio, version 1.4.1717. Model fitting involved the selection of a base model that adjusted for common risk factors (hypertension, sex, age, and

number of prenatal visits), and then the addition of lead and mercury to the base model. Base model results with lead or mercury are identical to the base model, thus, only the final model results are shown here (base model can be found in Appendix B, Supplemental Table B.4 and Supplemental Figure B.1).

Table 6: Global fit measures of models

Model	DF	X^2 (p)	CFI/TLI	RMSEA (C.I.)	SRMR	AIC	BIC
Base Model	116	0.08	0.985/ 0.981	0.031 (0.000 - 0.049)	0.074	-3314.6	-3166.6
Maternal Lead	116	0.06	0.984/ 0.980	0.032 (0.000 - 0.050)	0.073	-3311.6	-3163.5

3.2 Results

The study analyzed 198 mother-child pairs of singleton births. Most women presented no pathologies of concern (82%) and had full term pregnancies (96%). No women self-identified as smokers. Only 6% of women became hypertensive (systolic blood pressure >140 mmHg or diastolic >90 mm Hg) and 0.5% were diagnosed with diabetes during pregnancy. Women had an average of 7.3 prenatal visits, 80% took some type of nutritional supplement and most were pregnant with their second child (Table 7). Most women had moderate and higher household incomes (43% and 32%, respectively). 28.8% of mothers had blood mercury levels that surpassed the United States' Center of Disease Control and Prevention (CDC) guidelines of 5.8 µg/L [70]. Two mothers surpassed CDC's 5 µg/dL limit for total blood lead (Table 8) [53].

Table 7: Characteristics of maternal and newborn health.

	Overall (n=198)
Mother's Age (Years)	
Mean (SD)	27.5 (4.16)
Median [Min, Max]	27.0 [18.0, 35.0]
Hypertension	
No	186 (93.9%)
Missing	12 (6.1%)
Diabetes Status	
No	196 (99.0%)
Yes	1 (0.5%)
Missing	1 (0.5%)
Pathologies of Concern	
No	163 (82.3%)
Yes	17 (8.6%)
Missing	18 (9.1%)
Socioeconomic Status	
High	63 (31.8%)
Low	31 (15.7%)
Moderate	86 (43.4%)
Missing	18 (9.1%)
Number of Previous Births	
Mean (SD)	1.95 (1.09)
Median [Min, Max]	2.00 [1.00, 7.00]
Missing	16 (8.1%)
Prenatal Care Visits	
Mean (SD)	7.25 (2.12)
Median [Min, Max]	7.00 [2.00, 12.0]
Took Supplements	
No	40 (20.2%)
Yes	158 (79.8%)
Maternal Hemoglobin (g/dL)	
Mean (SD)	11.7 (1.25)
Median [Min, Max]	11.6 [7.00, 15.7]

	Overall (n=198)
Missing	8 (4.0%)
Presented Anemia During Pregnancy	
No	131 (66.2%)
Yes	48 (24.2%)
Missing	19 (9.6%)
Newborn's Sex	
Female	87 (43.9%)
Male	111 (56.1%)
Birthweight	
Mean (SD)	3.54 (0.454)
Median [Min, Max]	3.56 [1.65, 5.05]
Gestational Age (Weeks)	
Mean (SD)	39.2 (1.22)
Median [Min, Max]	39.0 [33.0, 42.0]
Preterm Pregnancy	
Yes	7 (3.5%)

Table 8. Minerals and trace metals measured in maternal and cord blood at birth.

	Cord Blood (n=198)	Venous Blood (n=198)	p-value
Mercury (µg/L)			
Mean (SD)	8.98 (10.2)	5.85 (8.63)	0.001
Median [Min, Max]	5.96 [0.399, 79.7]	3.47 [0.159, 93.9]	
Lead (µg/dL)			
Mean (SD)	1.28 (1.27)	1.65 (1.36)	0.006
Median [Min, Max]	1.00 [0.237, 14.7]	1.33 [0.433, 16.8]	
Cadmium (µg/L)			
Mean (SD)	0.282 (0.272)	0.392 (0.512)	0.008
Median [Min, Max]	0.250 [0.008, 3.19]	0.250 [0.14, 6.41]	
Arsenic (mg/L)			
Mean (SD)	0.736 (0.575)	0.782 (0.604)	0.442
Median [Min, Max]	0.450 [0.45, 3.29]	0.450 [0.450, 3.44]	
Iron (mg/L)			

Mean (SD)	533 (92.6)	389 (92.0)	<0.001
Median [Min, Max]	530 [276, 835]	385 [153, 737]	
Calcium (mg/L)			
Mean (SD)	61.6 (13.2)	66.6 (9.39)	<0.001
Median [Min, Max]	60.8 [30.4, 122]	65.7 [43.0, 92.6]	
Magnesium (mg/L)			
Mean (SD)	35.2 (5.11)	34.1 (4.42)	0.03
Median [Min, Max]	34.2 [26.0, 55.9]	33.4 [22.2, 45.9]	
Zinc (mg/L)			
Mean (SD)	2.01 (0.971)	5.04 (1.22)	<0.001
Median [Min, Max]	1.80 [1.15, 8.22]	5.01 [1.79, 9.81]	
Selenium (µg/L)			
Mean (SD)	165 (33.4)	160 (33.4)	0.15
Median [Min, Max]	165 [91.5, 289]	158 [89.9, 294]	

We found correlations ($p<0.05$) among toxic metals and minerals within maternal blood and cord blood as well as between maternal blood and cord blood (Figures 9 and 10). Overall, metals grouped by sample type (maternal or fetal), with the notable exceptions of mercury, lead, cadmium, and magnesium which were not part of a larger correlated group (Figure 10). Mercury and lead were not correlated with other metals.

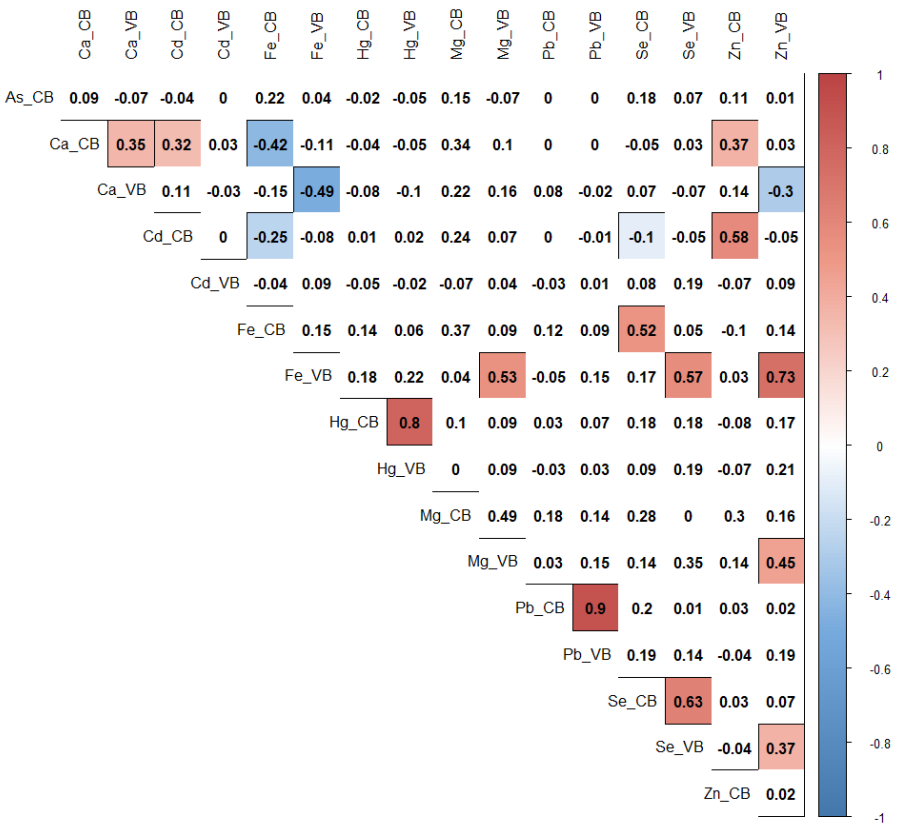


Figure 9: Heat map of minerals and trace metal correlations in maternal (VB) and cord (CB) blood. The Pearson correlation value is shown for maternal and fetal minerals/trace metals with significant positive and negative correlations represented by red and blue shading, respectively. Non-significant correlations are white.

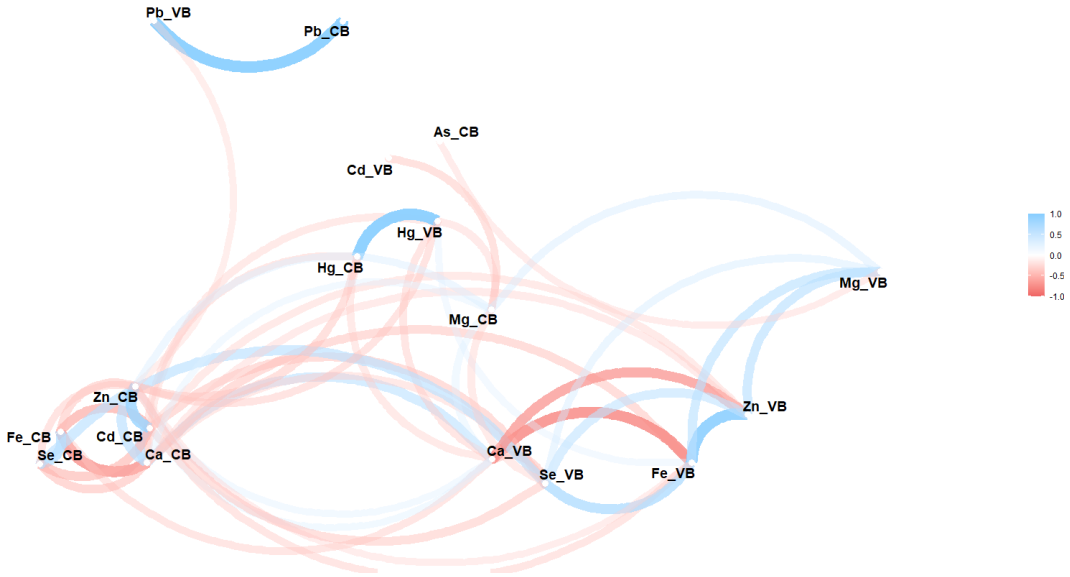


Figure 10: Correlations of metals greater than |0.3| with the location of each mineral/trace metal dependent on a multidimensional scaling of absolute correlation values. Wider and less transparent lines represent stronger correlations, while the colors blue (positive) and red (negative) represent the direction of the correlation.

3.2.1 Relationship of Variables with the Maternal Exposome

In maternal blood, iron was positively correlated with selenium, zinc, and magnesium. Calcium was inversely correlated with zinc and iron (Figure 9). Levels of calcium, selenium, mercury, and lead were positively correlated between maternal and fetal blood, with lead and mercury having the strongest correlations (0.90 and 0.80, respectively, Figure 9).

In establishing ME, mineral concentrations had the greatest influence on the ME (largest loading factors), with toxic metals having a secondary contribution. Iron, zinc, lead, mercury, magnesium, and calcium contributed to the latent variable ME, with loading values of 1.25, 1.24, 1.22, 1, 0.46 and -0.30, respectively. ME values ranged from -.21 – 0.20. All toxic metals and minerals were positively associated with ME, except for calcium. Cadmium did not load onto ME.

3.2.2 Relationship of Variables with the Fetal Environment

Within cord blood, cadmium was correlated with zinc and calcium, and inversely correlated with iron and selenium. Iron was correlated with selenium and inversely associated with calcium.

A different set of toxic metals and minerals was found to load onto FE, which was comprised of mercury, iron, arsenic, selenium, lead, and magnesium with loading values of 1.0, 0.96, 0.93, 0.71, 0.53, 0.36. FE values ranged from -0.22 – 0.19. Cadmium and calcium did not load onto FE.

3.2.3 Relationship between Maternal and Fetal Environments with Neonatal Outcomes

3.2.3.1 Gestational Age

The latent variable ME was associated with FE in both the base and final SEMs (Appendix B, Supplemental Table B.4 and Supplemental Figure B.1). After adjusting for hypertension, age, sex, prenatal visits, and maternal blood lead, the FE had a positive effect on birth outcomes as it was associated with gestational age (β: 2.31, 95% CI: -0.30-4.51), with an additional unit increase in the FE lengthening gestational age by two weeks. Hypertension was associated with a shortened gestational age of four days (β: -0.60, 95% CI: -1.25 - 0.003, Table 9, Figure 11). Number of prenatal care visits was associated with longer gestation (β: 0.13, 95% CI: 0.05 - 0.23), with each prenatal care visit adding almost an extra day of gestation (Appendix B, Supplemental Figures B.2 and B.3).

Table 9: Final model beta estimates and covariance structure with 95th confidence interval (C.I.) that adjusts for hypertension, age, sex, and maternal blood lead with latent variables for the maternal environment (ME) and the fetal environment (FE) (n=198).

Base Model with Maternal Lead	Beta[a] (C.I.)	Std. lv[b]	Std.all[c]
Fetal Environment (FE)			
ME	0.24 (0.05 – 0.45)*	0.22	0.22
Gestational Age (Weeks)			
FE	2.31 (-0.30 – 4.51) †	0.18	0.15
Prenatal Visits	0.13 (0.05 – 0.23)**	0.13	0.23
Hypertension (Ref: No)	-0.60 (-1.25 – 0.003) †	-0.60	-0.12
Log10 Maternal Lead	-0.75 (-1.51 - -0.13)*	-0.75	-0.14
Weight (kg)			
Prenatal Visits	0.05 (0.02 – 0.09)**	0.05	0.24
Mother's Age (years)	0.02 (0.07 – 0.30)**	0.02	0.17
Sex (Ref: Male)	-0.13 (-0.25 - -0.02)*	-0.13	-0.14
Raw Covariance Structure			
Gestational Age~~ Birth weight	0.146 (0.026-0.266)*	0.146	0.296

Significance: <0.001'***'; 0.001 '**'; 0.01 '*'; 0.05 '†'

a -Raw coefficients

b- Beta coefficient only standardizing the latent variables

c- Completely standardized solution

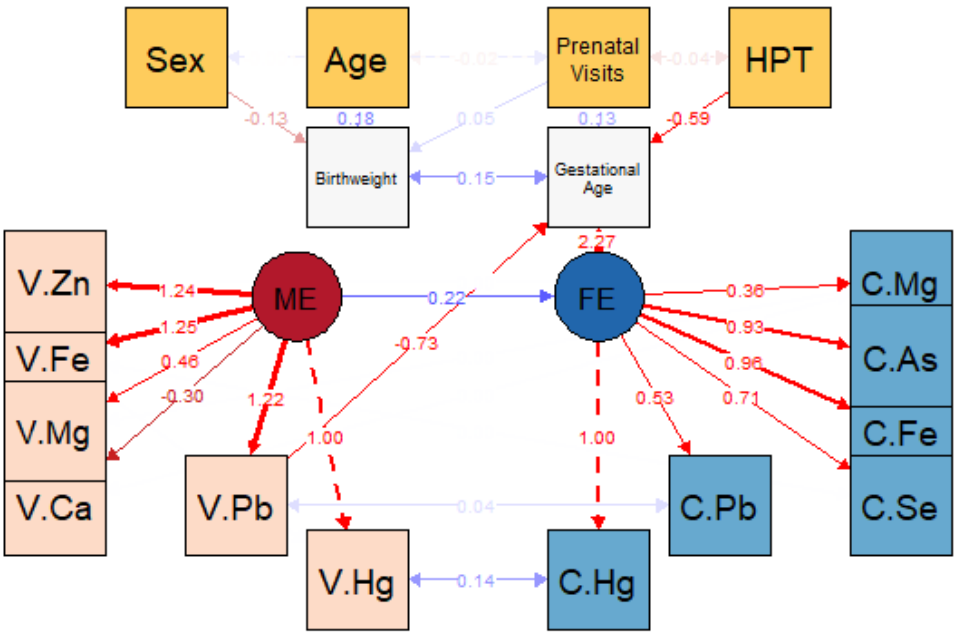


Figure 11: Final model diagram with hypertension (HPT), age, sex, prenatal visits, and maternal blood lead levels (µg/dL) with the maternal and fetal environments as latent variables (ME and FE, respectively). Values shown are unstandardized beta values with

transparency dependent on p-value. Boxes are colored by maternal blood (pink), cord blood (light blue), traditional covariates (orange), ME (red), FE (blue) and neonatal health outcomes (white).

A 1% increase in maternal lead was associated with a shorter gestational age of 0.05 days (β: -0.75, 95% CI: -1.51 - -0.13, Table 9, Figure 11), which at the 5 µg/dl threshold results in a loss of 3.6 gestational days (95% CI: 0.6 - 7.4) and 76.5 grams (95% CI: 13.6 – 139.4) birthweight for newborns. Compared to the base model, the inclusion of maternal blood lead level did not modify the effect of hypertension status (Table 9), potentially indicating independent mechanisms through which lead and hypertension effect gestational age.

Mothers with hypertension and average blood lead levels had a shorter gestational age of 5.3 days (95% CI: 0.22 – 11.05). Similar results were found even when excluding women with outlying lead exposure. According to the standardized SEM solution, a one standard deviation increase in the FE score has an equivalent and opposite effect to a one SD increase in the Log10 maternal lead exposure, indicating that a positive increase in FE may counterbalance the negative effects of maternal lead exposure on neonatal birth outcomes. For example, mothers who had a FE score in the 75th percentile could have 34% higher blood lead levels before a negative loss in gestational days compared to mothers with a FE at the 25th percentile (Figure 12).

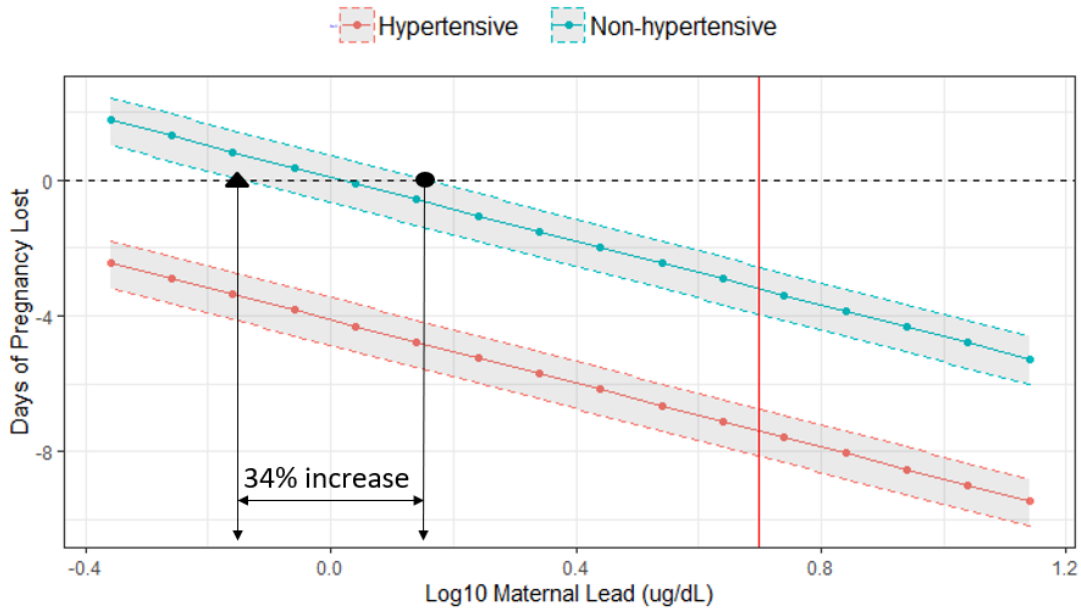


Figure 12: Days of pregnancy lost from log10 lead maternal blood concentration (µg/dL) for mothers with an average fetal environment (50th percentile, center, dotted lines) with the 25th and 75th quartiles for fetal environment (shaded region and dashed lines). Horizontal, black dashed line represents no days of pregnancy lost. Hypertensive and non-hypertensive mothers are shown in red and blue, respectively. The triangle and oval demonstrate where days of pregnancy lost become negative for the 25th and 75th percentiles of the fetal environment, respectively, demonstrating a 34% increase in lead exposure before the 75th percentile suffers days of lost pregnancy. Vertical red line represents the 5 µg/dL reference level set by the United States Center of Disease Control and Prevention.

3.2.3.2 Birthweight

Birthweight was associated with mother's age (β: 0.02, 95% CI: 0.07 – 0.30), number of prenatal visits (β: 0.05, 95% CI: 0.02 – 0.09), and newborn's sex, with females weighing 0.13 kg less than males (β: -0.13, 95% CI: 0.02-0.25). For each additional year of age, a mother's child weighed an additional 0.02 kg. Each prenatal visit was associated with an increase of 0.05 kg (95% CI: 0.02-0.09), with a doubling of visits from 6 to 12 associated with 5.5 more gestational days (95% CI: 1.6 – 9.4). and 300g of birthweight (95% CI: 111.8 – 488.2). The updated World

Health Organization's guidelines of increasing prenatal health visits from four to eight was associated with a 0.2 kg (95% CI: 0.08-0.32) increase in birthweight.

Although mercury loaded on the latent variables ME and FE, mercury in maternal or cord blood was not directly associated with gestational age or birthweight. Nor did we find a direct relationship between ME and gestational age or birthweight. Hemoglobin and anemia status were also not associated with birth outcomes. Lastly, we did find an important covariance between birthweight and gestational age (β: 0.15, 95% CI: 0.03 – 0.27, p = 0.02), with birthweight increasing 0.15 kg for each additional gestational week, providing further support to analyze these outcomes jointly.

3.3 Discussion

In this study, we used structural equation models to develop a novel approach that integrates the physiological hierarchy present during gestation and evaluates the joint effect of toxic metals and minerals on neonatal health, while adjusting for prenatal care and other confounding factors. We find that maternal lead exposure below the current threshold of 5 µg/dL is associated with shorter gestational age and lower birthweight. This finding was robust to measures of the maternal exposome, fetal environment and other known determinants of neonatal health outcomes that were evaluated within a realistic physiological hierarchy. This finding supports the idea that even low levels of lead exposure can have important consequences for fetal health. Unexpectedly, maternal mercury levels were not directly associated with either birth outcome, although mercury levels did load into the maternal exposome and fetal environment. The specification of the latent variables maternal exposome and fetal environment is unique in toxicological and epidemiological approaches and offers an

important tool to evaluating joint effects of nutritional and toxic metal exposure levels. Our data suggest that minerals are the main determinants of the maternal exposome and the fetal environment, with toxic metals having a secondary role. Using this physiological-based approach, we find the maternal exposome is associated with the fetal environment, which, in turn, may mediate the effects of stressors such as lead and/or maternal hypertension.

An important finding from this study is the beneficial effect of prenatal care on neonatal health. We find that increasing prenatal care visits from 6 to 12 would increase gestation by 5.5 days and increase birthweight by 0.3 kgs. Although Peru recommends 6 – 8 prenatal care visits, the number of visits in the CONAMAD cohort ranged from 2 to 12. Guidelines for prenatal care vary across countries. The United States recommends 12 prenatal care visits [172], while Japan and the World Health Organization recommend 14 and 8 prenatal care visits, respectively, for low-risk pregnancies. Although age, sex and hypertension are known covariates for neonatal health, the number of prenatal care visits is often underappreciated in the toxicological studies (Appendix B, Supplemental Table B.1), regardless of the well-found benefits of prenatal care, which include prescription of nutritional supplements, maternal and fetal assessment, and increased knowledge on strategies to alleviate physiological symptoms [173]. In Peru, prenatal care visits are free, covered by national healthcare, reducing the possibility that the number of prenatal care visits is a proxy for wealth. However, the CONAMAD study demonstrates that there remains considerable variability in access and utilization of prenatal care, which is a potential area of focus as Madre de Dios and Peru overall seek to improve neonatal health.

This study also reveals numerous metal and mineral correlations within and across maternal and cord blood, demonstrating their interconnected nature. In birth cohort studies,

this complexity poses a methodological limitation when toxic metals or minerals are evaluated individually as it omits important changes occurring in other analytes that may be associated with health outcomes. A prime example of this interaction is the role of iron levels on lead exposure, as individuals with low iron are known to absorb greater amounts of lead than iron replete individuals. Minerals are also known to interact with each other. Previous epidemiological studies found negative correlations between serum ferratin and calcium [174] and calcium supplementation was found to decrease heme and nonheme iron absorption in humans [175] and rodents [176]. While calcium is expected to not limit iron absorption in populations that consume a Western diet, less is known for those who eat a non-Western diet and for pregnant women [177]. Maternal calcium levels in the CONAMAD cohort were lower than high income countries where 86.4 - 92 mg/L calcium were measured at birth, compared to 66.6 mg/L [178-180]. We found maternal calcium loaded negatively onto ME and may reflect the relationship between iron and calcium. The negative effect of calcium on ME, may provide evidence that calcium is limiting iron absorption and that calcium supplementation should not be taken with main meals to limit any suppression of iron absorption [181]. Given this complexity, the single toxic metal-single health outcome paradigm may not be appropriate. To better understand maternal and neonatal health outcomes, the effects of minerals and toxic metals and their potential interactions need to be further evaluated.

This is one of the first birth cohorts in Peru and the Amazon to focus on minerals, toxic metals, prenatal care, and neonatal health. It is also unique as it was conducted in a region where artisanal gold mining is prevalent, and women live in relatively rural, remote areas. Compared to birth cohorts in high income countries, CONAMAD had lower levels of zinc [182-

184] but were similar levels to other middle income countries [185] and not associated with adverse health outcomes [186]. We also found lower levels of selenium, cadmium, and lead, but much higher levels of mercury in our population compared to the Boston Cohort [187]. Cadmium levels in CONAMAD are lower than other studies that found adverse neonatal health effects [188-190]. Although we did not find any correlations between maternal cadmium and maternal zinc or selenium less than $p<0.05$, previous studies in high-income countries found an inverse correlation between selenium and cadmium [191]. Interestingly, selenium and cadmium were positively correlated in birth cohorts in low income communities in the United States [192], in smoking populations in Eastern Europe [193], and in healthy Japanese women [190], exemplifying the importance of studying populations with different diets and confounding factors.

This is also one of the first birth cohorts studying in-utero metal concentrations using SEMs [164, 194], to model their transfer from mother to child and jointly determine their effects on gestational age and birthweight, while accounting for antenatal care and other confounding factors. By creating latent variables for ME and FE, we demonstrate the feasibility and utility of SEMs to evaluate metal mixtures and their effect on neonatal health. Compared to linear regression, SEMs allow for a more complete understanding of the structural associations present in maternal-fetal health by assessing direct and indirect effects, limiting overcontrolling, and allow outcomes to be modeled jointly to evaluate the entire physiological system. These characteristics make SEMs a useful analytical tool in evaluating the causal links that determine health outcomes. In contrast to dose-response models, where a single toxin or exposure is evaluated against a single health outcome, the SEM allows us to integrate multiple toxins,

minerals, or other nutrients within a hierarchical physiological framework. Some studies have discussed the tradeoffs between SEMs and traditional dose-response models in toxicological risk assessment [162, 195, 196].

Although mercury loaded onto ME and FE, we did not find a direct association between maternal or cord blood mercury levels with evaluated birth outcomes. We also evaluated maternal total hair mercury at birth, reflecting the last two months of exposure, which was also not associated with health outcomes (results not shown). The lack of a direct mercury exposure effect may be due to the exposure assessment (cord and maternal blood) being related to exposure time periods around birth rather than during conception or other earlier periods of fetal growth. It is possible that a segmented analysis of maternal hair, which approximates methylmercury exposure during different periods of the pregnancy, would have been a more appropriate biomarker of exposure. Other possible reasons for lack of effect are the beneficial effects of polyunsaturated fatty acids from fish consumption [197], high prevalence of fruits with elevated levels of antioxidants in the diet [198, 199] and mercury exposures are predominantly methylmercury, targeting the central nervous system, that are not captured by gestational age or birthweight.

This study has several important limitations. First, as noted above, the blood metal concentrations only reflect those at time of birth, and we do not account for maternal or in-utero exposures to minerals or toxic metals during the first and second trimester. Although we did obtain data on nutritional supplements and hemoglobin levels at two time periods during pregnancy, both were unrelated to birth outcomes. This is important as the critical window of exposure may occur early in pregnancy, depending on the mineral/metal of concern. Maternal

blood lead levels follow a U-shaped curve as they decline early in pregnancy from plasma volume expansion and increase until delivery due to increased absorption and mobilization of lead stored in bone, coinciding with increased calcium demand for the fetus in the third trimester [150, 200, 201]. In agreement with our findings, Rabito et al. found third trimester blood lead levels associated with gestational age and not weight. However, they did find second and third trimester blood lead levels to be associated with preterm birth [150]. There is also evidence that the first trimester maternal lead levels are more strongly associated with adverse cognitive outcomes in children than the third trimester; however, both were associated with child cognition [202]. Lastly, due to limitations in the field, we rely on the Capurro somatic method to determine gestational age, which has been shown to overestimate gestational age for newborns less than 39 weeks in Brazil; however, it can have high specificity (96%) [203-205].

The complexity of mineral and toxic metal transfer to the fetus during pregnancy makes evaluating both the maternal and fetal environment difficult, especially due to the inability to monitor nutrient status of the fetus in-utero. The placenta is a vital organ that regulates nutrient and oxygen transfer to the fetus. Its impairment by toxic metals is yet another route through which toxic metals induce harmful outcomes [206-208] . Future research that incorporates the placenta and its ability to regulate minerals and toxic metal transfer may provide valuable insight on neonatal health. Additionally, data that incorporates dietary, supplemental, and maternal toxic metal exposures throughout pregnancy as well as ultrasound measurements and placenta characteristics may provide important insight into the dynamic changes that occur during pregnancy.

SEMs also provide the framework to evaluate different groups to determine if they fit the same data structure. Although this study does not have a sufficient sample size to conduct such analyses, evaluating how anemia status (yes/no) at term or toxic metal exposure status (high/low) may provide valuable insight on how perturbations of a single metal effect the rest of the covariance structure.

4. Connecting the Dots: Linking Sources of Lead Exposure to Metabolite Disruption and Cardiovascular Risk in Madre de Dios, Peru

Cardiovascular disease is the leading cause of death globally with multiple behavioral and environmental factors determining disease progression. Environmental exposures to lead and mercury are often overlooked as important risk factors [209, 210]. Metabolites, such as amino acids, provide a novel approach to evaluating toxicological pathways and mechanisms through which trace metal exposures increase the risk of cardiovascular disease. Amino acids are the building blocks of proteins and many neurotransmitters, cell signaling molecules that regulate multiple physiological functions, allowing their disruption to have numerous adverse health effects. Arginine, a component of the arginine pathway, is an important regulator of the cardiovascular system [211, 212]; however, there is limited epidemiological research on how trace metals disrupt the arginine pathway to impede cardiovascular health.

Blood pressure and amino acids are dependent on multiple organ systems. In healthy individuals, the kidneys help regulate blood pressure and store certain amino acids, such as citrulline. Citrulline participates in several major pathways of human nitrogen metabolism, including its use to synthesize arginine in the kidney cortex as part of the intestinal-renal axis [213-215]. Citrulline is the rate limiting metabolite in the de novo synthesis of arginine, and it is tightly coupled with arginine in the biosynthesis of nitric oxide (NO) in endothelial cells, where it has a major role in regulating blood pressure [216, 217]. Proper functioning of the kidneys and intestines is vital for amino acid cycling as renal failure limits de novo arginine synthesis [218, 219] and synthesis of arginine from citrulline [211, 220, 221]. Hypertension is associated with increased arginine levels, potentially due to limited arginine transport [222].

Once arginine is synthesized from citrulline, it has multiple pathways in the human body. Arginine is an important upstream component of the major human metabolic pathways of creatine, proline, glutamine, ornithine, urea, and nitric oxide. Derangements in the metabolism of arginine can have profound effects on cardiovascular health, immunology, and the nervous system [217, 223, 224] An estimated 20-30% of arginine is used for creatine synthesis, while 1.5% is used to form NO isoforms used as neurotransmitters (NOS1), inflammatory response mechanisms (NOS2) and vasodilators (NOS3) [217, 225]. Arginine is the rate limiting factor de novo NO synthesis [216], with higher concentrations of plasma arginine resulting in increased NO, regardless of cellular arginine concentrations (known as the "arginine paradox") [226]. A stable isotopic tracer study in healthy men found that plasma arginine accounted for 54% of NO synthesis in the human body [225]. Nitric oxide synthesized by NOS3 in the endothelium is an important regulator of vascular homeostasis, the sodium/water balance in blood, and blood pressure [224, 227-229]. Thus, plasma arginine, via its conversion into NO, is likely an important and perhaps predictive biomarker of cardiovascular health.

Lead (Pb) and mercury (Hg) share toxicological pathways, and both exert toxicity by generating reactive oxygen species (ROS) that may cause co-exposures to have non-additive effects on the arginine pathway. Pb targets the hepatic, renal, neurological, and cardiovascular systems. Its adverse effect on the cardiovascular system includes nephron damage, disruption of the renin-angiotensin pathway and the suppression of NO. In comparison, mercury damages renal and neurological systems, with conflicting evidence of its effect on cardiovascular health. While Pb may directly and indirectly, via renal toxicity, effect blood pressure, Hg's direct and indirect effects are less understood. Assessments of joint exposures to Pb and Hg that evaluate

their direct and indirect effects on cardiovascular health are often not evaluated, even though they share toxicological endpoints and mechanisms of toxicity that may result in non-additive effects.

Both Pb and Hg generate ROS that can disrupt amino acid profiles throughout the body [230, 231]. ROS reacts with NO to form peroxynitrite, a potent vasoconstrictor associated with increased blood pressure in mice [232] and multiple adverse health outcomes [233]. In animal models, ROS from methylmercury [78] and lead [76, 77] exposure reduced levels of NO that were associated with increased blood pressure and hypertension [58, 78, 234-237]. However, there is evidence that Pb and Hg impact the arginine pathway and nitric oxide synthesis differently. Pb exposure upregulated nitric oxide synthase [77], while methylmercury exposure reduced NO synthesis [238-240], potentially by limiting the conversion of arginine to citrulline [241-243]. The joint effect of Pb and Hg on the arginine pathways in humans is not well understood, yet the conflicting effects of Pb and Hg on NO synthesis demonstrates the potential for a non-additive response from dual exposures.

The theoretical pathways through which joint metals exposures influence cardiovascular and other health outcomes via the arginine pathway is shown in Figure 13.

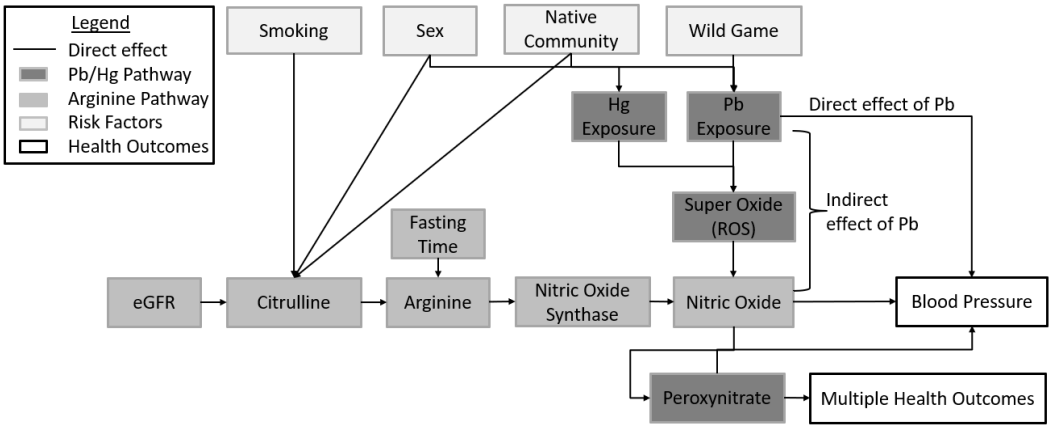


Figure 13: Theoretical framework of lead (Pb) and mercury's (Hg) indirect effect on blood pressure via the arginine pathway. Risk factors for lead exposure are light gray, while components of the arginine pathway are gray.

The objectives of this study are to estimate the indirect effects of joint Pb and Hg exposure on blood pressure via the arginine pathway to elucidate toxicological mechanisms through which Pb and Hg promote cardiovascular disease and compare them to unspecified mechanisms estimated by direct effects. We test these relationships using structural equation models (SEMs), a novel application to test toxicological pathways in epidemiological studies. Specifically, we test the toxicological pathway presented in Figure 13 to estimate the associations between citrulline, arginine and blood pressure, while adjusting for kidney function, lead exposure, mercury exposure, body mass index, triglycerides, and other confounding variables. Data are from Madre de Dios, Peru, an artisanal and small-scale gold mining region where we have previously shown lead and mercury exposures due to wild game and fish consumption, respectively, which are more prevalent in native communities [61, 65, 119, 120, 123]. This is one of the few epidemiological studies that utilizes SEMs to statistically evaluate an

entire toxicological pathway from risk factors of exposure to measured blood levels of toxic metals and metabolites to their effect on cardiovascular health.

4.1 Methods

4.1.1 Data

Data and samples used in this study are a subset from the Amarakaeri Communal Reserve (ACR) study conducted in 2015 in 23 communities in Madre de Dios, Peru [119]. The study was approved by the Institutional Review Board of La Universidad Peruana de Cayetano Heredia (IRB: 00001014). Briefly, households with women of childbearing age (WCBA, 15-49 years) were eligible for enrollment: in large communities (>75 households) WCBA were randomly selected and in smaller communities, all households with WCBA were invited to participate. Every 10^{th} household enrolled did not have the WCBA requirement, thus ~10% of the sample did not have WCBA. Enrolled households were administered a survey that included individual health information and household diet with frequency of consumption of various food items.

Blood samples for trace metals were collected in dark-blue-top EDTA Vacutainer tubes from adult participants (≥18 years) and measured for Hg (BHg), Pb (BPb), and amino acids (arginine, ornithine, and citrulline) at Duke University. Of the 476 blood samples collected in the ACR study, 253 were selected based on mercury and lead exposures to be processed for amino acids to evaluate differences between high (n=175) and low (n=78) exposures. Hair samples were also collected by cutting three hair tufts with stainless steel scissors from the occipital region of the head and were analyzed for total hair mercury (THHg). Due to missing data, a final sample size of 208 was used (n=142 high-, n=66 low- mercury exposure).

4.1.2 Laboratory Methods

4.1.2.1 Sample Processing: Trace Metals

Blood samples were collected in Trace Metal free EDTA BD Vacutainer tubes and frozen on dry ice in the field. Blood samples were transported to Duke University on dry ice and stored at -80° C until processed.

To quantify lead and mercury levels, blood samples were thawed overnight at 4˚C and digested on a hot block at 65 °C. The digestions used ultra-trace clean digestion tubes (Environmental Express) and consisted of heating 0.5 ml of blood with 1 ml of 70% HNO_3 (Plasma Pure Plus, SCP Science) and 0.05 ml of 30% HCl (Plasma Pure Plus, SCP Science) for two hours. The samples were cooled, 1 ml of 30% hydrogen peroxide (Plasma Pure Plus, SCP Science) was added to the mixture, and samples were heated again for 1 hour. After cooling, 10 µl of a 4 mg/L gold+2% HCl solution was spiked into the digestate to aid in mercury stability. Each digestion batch consisted of 25 blood samples including two samples analyzed in triplicates. The relative standard deviation (%RSD) for triplicate digestions (n=33) were 9.2% for Hg (0.8-32%) and 10.9% for Pb (0.4-36%). Furthermore, each batch of 25 blood digestions included three blank samples, a NIST blood SRM (either 955c level 4 or 955d level 2), an aqueous standard (High Purity Standards, CRM-TMDW-A + Spex Certiprep Hg), and an IAEA dry blood sample (IAEA-A-13). Mercury recoveries were 95% for SRM 955c level 4 (n=5), 83% for SRM 955d level 2 n=9, and 99.6% for the aqueous Hg spike (n = 16). There are no reported values for mercury with IAEA-A-13. Lead recoveries were 105% for SRM 955c level 4 (n=5), 101% for SRM 955d level 2 (n=9), 107% for the aqueous Pb spike (n=16), and 80.4% for IAEA-A-13 (n=16).

The blood digestates were diluted (10-fold) into an acid matrix (2% (v/v) HNO3 and 0.5% HCl (v/v)) containing 20 µg/L Au. Internal standards (^{193}Ir and ^{209}Bi) were also spiked into the matrix to correct for instrumental drift or matrix effects. The analyses were performed by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS: Agilent 7900) in helium mode to reduce any potential for polyatomic interferences. The ICP-MS was tuned to reduce oxide interferences to less than 2%. The instrument was calibrated with purchased aqueous standards for Hg (Brooks Rand) and Pb (Spex Certiprep mix 2A), and the standard curves were verified with a secondary standard (High Purity Standards (CRM-TMDW-A) + Spex Certiprep (Hg)). Continuing calibration verification checks were performed every 20 samples during a batch run.

4.1.2.2 Sample Processing: Hair

Three tufts of hair, the width of a pencil, were cut with stainless steel scissors at the hair root from the occipital lobe of study participants. Hair samples were attached to self-adhesive notepaper, stored in paper envelopes, transported to Duke University, and stored at ambient temperature until being analyzed for total Hg content by atomic absorption spectrometry using a Milestone Direct Mercury Analyzer 80. The proximal 2 cm closest to the hair root was analyzed for total hair Hg, reflecting organic mercury exposure, predominantly from fish consumption, for the last two months [244].

4.1.2.3 Sample Processing: Amino Acids

Blood samples were thawed in an ice bath, aliquoted into 1.7 mL tubes and spun for 10 minutes at 15000 relative g force. A 100 µL aliquot of the supernatant was then transferred into 1.7 mL tubes to be processed. Amino acids were analyzed using flow injection electrospray ionization tandem mass spectrometry and measured with isotope dilution techniques described

previously [245]. Briefly, 100 µl of serum were spiked with a mixture of internal standards (Cambridge Isotope Laboratories, MA, USA; CDN Isotopes, Canada). Methanol was then used to deproteinate the solution. Methanol supernatants were then dried and esterified using acidified butanol. Mass spectra for amino acid esters were obtained using a neutral loss scanning method (NTL 102 amu for acidic and neutral amino acids; NTL 119 amu for basic amino acids; and NTL 161 amu for arginine). Data were obtained using a Waters Triple Quadrupole detector with an Acquity™ UPLC system, and a MassLynx 4.1 data operating system (Waters, Milford, MA). Ratios of ion analytes to their respective internal standard, determined from centroid spectra, were transformed into concentrations using calibrations determined from amino acid standards (Sigma, MO, USA) and dialyzed fetal bovine serum (Sigma, MO, USA).

4.1.2.4 Sample Processing: Medlab

Glucose levels in serum were measured using the glucose hexokinase assay, following Roche protocol GLUH2-0120767131322c501V4.0. Glycated hemoglobin was analyzed using capillary electrophoresis using protocol established by Sebia (CAPILLARYS HbA1c, Ref. 2015) and can be found on their website: www.sebia.com/en-us/tests/. Triglycerides, LDL, HDL, Serum creatinine, and cholesterol were measured using colorimetric assays as described in Cobas® protocols TRIGL-0020767107322c501V12.0, LDLC3-0107005717190c501V4.0, HDLC4-0107528566190c501V3.0, and CREJ2-004810716190c501v20.0.

4.1.3 Statistical Analysis

4.1.3.1 Outcomes

Systolic and diastolic blood pressure was measured for adults using an automated Omron BP785 10 series sphygmomanometer. Blood pressure measurements were taken twice

for each study participant and then averaged. Individuals were classified as having elevated blood pressure if their systolic blood pressure (SBP) was greater than or equal to 120 mm Hg or their diastolic blood pressure (DBP) was greater than or equal to 80 mm Hg [246].

4.1.3.2 Metals

Blood Pb (BPb) and blood Hg (BHg) were measured as μg/dL and μg/L, respectively. Hair samples were measured as μg/g. All biomarkers for trace metals were analyzed in Dr. Heileen Hsu-Kim's lab at Duke University. A log10 transformation was used to normalize BPb ($_L$BP), BHg ($_L$BHg) and THHg ($_L$THHg). Previous studies have linked lead exposure to wild game consumption [247, 248]. Risk factors for lead exposure were obtained from household surveys that included consumption frequencies of wild game (never/weekly/monthly). Wild game consumption was categorized as yes/no.

4.1.3.3 Metabolites

Measured levels of arginine (μM), ornithine (μM), and citrulline (μM) were used to construct the arginine pathway. In addition to arginine, the Global Arginine Bioavailability Ratio (GABR) was calculated as the concentration of arginine divided by the sum of citrulline and ornithine as an assessment of arginine and its catabolic products [211]. A log10 transformation was used to normalize arginine and GABR ($_L$GABR). The time of last meal and time of blood collection was recorded. The variable fasting time was determined by calculating the number of hours between the last meal and blood collection.

4.1.3.4 Confounding variables

Information on participants' age (years), sex, weight (kgs), height (m), medication use (yes/no), smoker status (yes/no), hypertension status (yes/no), and diabetes status (yes/no)

were collected in administered surveys. Body mass index was calculated using survey weights and heights.

Purple- and red- topped Vacutainer tubes were used to collect 3 mL of blood that was processed for biomarkers of cardiovascular risk. Biomarkers include serum creatinine (S_{cr}, µmol/L), glucose (mg/dL), triglycerides (mg/dL), cholesterol (mg/dL), glycated hemoglobin (HbA1C), low (LDL) and high-density (HDL) lipoproteins. Samples were processed by Medlab in Lima, Peru (SGS certified, ISO 9001:2015). Serum creatinine levels along with survey information were used to calculate estimated glomerular filtration rate (eGFR) using the CKD-EPI equation ($eGFR = 141 \times \min(S_{cr}/k,1)^{\alpha} \times \max(S_{cr}/k,1)^{-1.209} \times 0.993^{age}$ x (1.018 if female), where values for k (0.7-female, 0.9-male) and α (-0.329-female, -0.411-male), are sex dependent) [249]. A log10 transformation was used to normalize triglycerides, glucose, serum creatinine.

4.1.3.5 Statistical Methods

Prior to using structural equation models, we used spline models to evaluate the relationship between trace metals and variables of interest to determine if any non-linear relationships were present. We found a non-linear relationship between BHg and systolic blood pressure with a knot located at 15.8 µg/L (See Appendix B, Supplemental Figures B.1 and B.2). Thus, we present the effects of trace metals on the arginine pathway and blood pressure on the two data subsets (above or equal to (n=60) and below (n=148) the 15.8 µg/L BHg threshold). Previous studies in high fish consuming populations have found reduced heart rate variability and increased resting heart rate and blood pressure in populations with mean blood mercury levels ranging from 8.12 µg/L to 29.5 µg/L [250-252], while a higher prevalence of hypertension

was associated with low selenium and high mercury classified at both 7.8 μg/L and 20 μg/L [253].

We implemented structural equation models (SEMs) to evaluate the hypothesized toxicological pathways and their effect on systolic (SBP) and diastolic blood pressure (DBP) (Figure 16). SEMs are a system of equations that allow for the joint modeling of SBP and DBP, and the estimation of direct and indirect effects to compare the toxicological pathways through which Pb influences blood pressure. To account for the non-linear effect of BHg on SBP, we used the group function within SEM to simultaneously test the same model pathways for each group. We also used Piecewise SEMs to identify which variables changed significantly ($p<0.05$) between the BHg groups. SEMs were conducted using the lavaan package with bootstrapping, and Piecewise SEMs were tested using the piecewise package in RStudio Version 1.4.1717.

ANOVA models and T.tests were used to identify significant differences between indigenous status (indigenous/non-indigenous and hypertension (hypertensive/non-hypertensive), with $p<0.05$ being considered significant.

SEMs were evaluated by Chi squared (>0.05), Tucker-Lewis Index (TLI >0.95), root mean square error of approximation (RSMEA <0.07) and standardized root mean residual (SRMR <0.08) to assess model fit. Models that met these requirements were compared using Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC), with the lowest values demonstrating better model fit.

We compare three models 1) $_{L}$BPb-> log10(arginine), 2) $_{L}$BPb-> $_{L}$GABR, and 3) $_{L}$BPb + $_{L}$BHg-> $_{L}$GABR to determine if GABR provides a better model fit and how Hg effects the arginine pathway (Table 10).

Table 10: Model fit characteristics of SEM models split by blood mercury levels.

Model #	BHg	n	DF	X^2	CFI/ TLI	RMSEA (90% C.I.)	SRMR	AIC	BIC
1. Pb->Log10(Agr)	Low	148	48	0.26	0.99/ 0.98	0.034 (0.00-0.075)	0.049	4369	4542
Pb->Log10(Agr)	High	60							
2. Pb->GABR	Low	148	48	0.27	0.99/ 0.98	0.034 (0.00-0.008)	0.051	4348	4522
Pb->GABR	High	60							
3. Pb + Hg ->GABR	Low	148	56	0.20	0.98/ 0.97	0.038 (0.00-0.075)	0.051	4351	4531
Pb + Hg ->GABR	High	60							

Acronyms: Pb: Blood lead; Hg: Blood Hg; Agr: Arginine; GABR: Global Arginine Bioavailability Ratio; BHg: Blood Mercury Group; DF: Degrees of Freedom; X^2: Chi Squared; CFI: Comparative Fit Index; TLI: Tucker-Lewis Index; RMSEA: Root Mean Square Error Approximation; SRMR: Standardized Root Mean Square Residual; AIC: Akaike Information Criterion; BIC: Bayesian Information Criterion

4.2 Results

A total of 208 individuals participated in the study, with an average age of 35 years (range 18-66 years). 48% of participants lived in indigenous communities and nearly two-thirds were women (64%). Participants with higher blood pressure tended to be older (38 vs 34 years old, p = 0.03), male (52% vs. 32%, p = 0.02), and have higher levels of BHg (17.1 vs 11.7 µg/L, p = 0.01) , BPb (4.34 vs. 3.16 µg/dL, p = 0.03), arginine (16.7 vs 13.7 µM, p = 0.06), citrulline (28.3 vs. 25.4 µM, p = 0.02) and triglycerides (183 vs 145 mg/dL, p = 0.01), with significantly lower eGFR (99.0 vs. 106.0, p = 0.009) (Table 11, Appendix C, Supplemental Table C.1). Body mass index was similar between participants with elevated and recommended SBP.

Table 11: Population characteristics of those exposed to higher (BHg ≥ 15.8 µg/L) and lower (BHg < 15.8 µg/L) mercury and those with elevated (SBP ≥ 120 mm Hg or DBP ≥ 80 mm Hg) and recommended blood pressure.

	Elevated Blood Pressure (EBP)		Recommended Blood Pressure		EBP vs. Rec.	Overall		
	Higher BHg (N=18)	Lower BHg (N=26)	Higher BHg (N=42)	Lower BHg (N=122)	P	Higher BHg (N=60)	Lower BHg (N=148)	P
Age (Years)					0.03			
Mean	38.2	37.7	35.0	33.9		36.0	34.5	
(SD)	(10.1)	(9.38)	(9.97)	(10.3)		(10.0)	(10.2)	
95% CI	33.5, 44.8	32.5, 45.3	28.3, 41.0	26.0, 39.8		28.8, 42.3	26.8, 40.3	
Sex					0.02			.04
Male	12 (66.7%)	11 (42.3%)	17 (40.5%)	36 (29.5%)		29 (48.3%)	47 (31.8%)	
Body Mass Index (BMI)								
Mean	28.8	28.7	27.3	28.5		27.7	28.5	
(SD)	(3.89)	(4.05)	(3.04)	(4.79)		(3.37)	(4.65)	
95% CI	25.77, 30.68	25.94, 30.89	25.18, 29.21	25.57, 31.23		25.19, 29.94	25.61, 31.17	
Estimated Glomerular Filtration Rate (eGFR)					0.01			
Mean	96.7	101	108	106		104	105	
(SD)	(16.9)	(18.5)	(15.0)	(14.9)		(16.2)	(15.6)	
95% CI	87.96, 110.01	90.47, 114.91	99.31, 119.01	93.79, 115.02		93.16, 117.34	93.38, 115.11	
Triglycerides (mg/dL)					0.01			
Mean	184	182	142	146		154	153	
(SD)	(123)	(88.7)	(60.4)	(88.7)		(85.4)	(89.5)	
Median	162	189	139	130		145	134	
95% CI	106.5, 197.5	107.25, 224	99.5, 165	92, 174.25		98.75, 177.75	93.0, 190.5	
Smoker Status								
Yes	2 (11.1%)	6 (23.1%)	12 (28.6%)	23 (18.9%)		14 (23.3%)	29 (19.6%)	
Blood Lead Level (µg/dL)					0.03			<0.001
Mean	4.97	3.90	5.08	2.49		5.05	2.74	
(SD)	(2.98)	(3.62)	(3.91)	(2.67)		(3.63)	(2.89)	
Median	4.55	2.68	4.28	1.49		4.45	1.64	
95% CI	2.63, 5.68	1.67, 4.29	2.05, 7.32	0.92, 2.92		2.16, 7.15	1.01, 3.14	
Blood Mercury (µg/L)					0.01			<0.001
Mean	32.2	6.67	25.7	6.88		27.6	6.85	
(SD)	(17.5)	(4.44)	(12.3)	(4.49)		(14.2)	(4.46)	
Median	24.0	6.30	21.7	6.34		23.3	6.34	
95% CI	19.3, 38.6	2.5, 10.1	17.7, 28.2	2.8, 10.8		17.9, 29.5	2.7, 10.7	

Total Hair Mercury (µg/g)							<0.001
Mean	6.68	2.22	6.64	2.17		6.65	2.18	
(SD)	(4.29)	(1.52)	(4.06)	(1.64)		(4.10)	(1.62)	
Median	5.33	2.46	5.00	1.99		5.07	2.04	
95% CI	3.9, 8.2	0.8, 3.2	3.9, 8.1	0.8 ,3.0		3.8, 8.3	0.7, 3.1	
Wild Game Consumption (Weekly or Monthly)					0.004			<0.001
Yes	14 (77.8%)	10 (38.5%)	19 (45.2%)	30 (24.6%)		33 (55.0%)	40 (27.0%)	
Fasting time (Hours)								
Mean	9.65	7.69	10.3	9.48		10.1	9.16	
(SD)	(5.03)	(5.34)	(5.22)	(5.75)		(5.13)	(5.70)	
Median	10.9	10.4	11.0	11.0		11.0	10.9	
95% CI	9.78, 12.38	1.55, 11.25	9.44, 13.00	1.81, 13.00		9.48, 12.58	1.70, 15.50	
Arginine (µM)					0.06			
Mean	14.9	17.9	14.5	13.4		14.6	14.2	
(SD)	(9.61)	(8.52)	(7.56)	(10.2)		(8.15)	(10.0)	
Median	11.5	16.7 [	12.4	11.3		12.0	12.5	
95% CI	8.82, 15.97	10.77, 21.87	10.46, 18.58	8.31, 15.84		9.71, 18.38	8.59, 17.18	
Global Arginine Bioavailability Ratio (GABR)								
Mean	0.048	0.060	0.055	0.048		0.053	0.05	
(SD)	(0.02)	(0.03)	(0.04)	(0.03)		(0.03)	(0.03)	
Median	0.045	0.051	0.045	0.041		0.045	0.043	
95% CI	0.03, 0.06	0.04, 0.07	0.03, 0.06	0.03, 0.06		0.03, 0.06	0.03, 0.06	
Citrulline (µM)					0.02			
Mean	26.0	29.9	24.2	25.8		24.8	26.5	
(SD)	(8.43)	(8.75)	(5.99)	(7.30)		(6.79)	(7.71)	
95% CI	19.16, 32.73	25.06, 32.58	20.01, 28.42	20.71, 30.52		19.5, 30.57	21.18, 31.21	
Systolic Blood Pressure (SBP mmHg)					<0.001			0.06
Mean	125	125	103	102		110	106	
(SD)	(12.2)	(13.6)	(9.4)	(9.6)		(14.5)	(13.6)	
95% CI	116.5, 129.6	116.6, 130.9	95.3, 11.1	94.0, 109.3		99.4, 116.8	95.5, 112.6	
Diastolic Blood Pressure (DBP mmHg)					<0.001			0.004
Mean	84.2	82.1	68.4	65.9		73.1	68.7	
(SD)	(9.9)	(7.0)	(7.0)	(7.2)		(10.7)	(9.5)	
95% CI	80.5, 86.9	77.0, 84.9	65.8, 73.6	62.0, 71.5		66.5, 79.1	63.0, 74.5	
Native Community					0.07			0.02
Native	14 (77.8%)	13 (50.0%)	23 (54.8%)	50 (41.0%)		37 (61.7%)	63 (42.6%)	

Participants with BHg ≥ 15.8 µg/L had higher blood Pb levels (2.71 µg/dL vs. 5.38 µg/dL, p = <0.001), SBP (110 vs. 106 mmHg, p = 0.06), DBP (73.1 vs. 68.7 mmHg, p = 0.004) and were

predominantly male (48.3% vs 31.8%, p = 0.04), indigenous (61.7% vs. 42.6%, p = 0.02), and ate wild game (55.0% vs 27.0%, p = <0.001). Metabolite levels were not significantly different by BHg exposure (Table 11).

4.2.1 Univariate Piecewise SEM

Univariate piecewise SEM demonstrated that estimates of $_{L}$BHg, eGFR, and $_{L}$GABR on blood pressure were significantly different by BHg status (Table 12). $_{L}$BPb was associated with increased SBP and DBP, and was consistent, regardless of BHg group. $_{L}$BHg had a greater effect on DBP for the higher BHg group, compared to the lower BHg group (26.6 vs. 0.05, p = 0.01); however, mercury's effect was not statistically significant after adjusting for additional covariates (Table 12). eGFR had a constant inverse association with SBP, regardless of BHg group. At BHg ≥ 15.8 µg/L, eGFR was more inversely associated with DBP, compared to the lower BHg group (-0.24 vs. -0.02, p = 0.02, Table 13). $_{L}$GABR had a positive associated with SBP and DBP for the lower BHg group but had an inverse association for the higher BHg group (SBP: 18.7 vs. -6.3, p = 0.009; DBP: 14.5 vs. -1.7, p = 0.04; Table 12).

Table 12: Univariate Piecewise SEM for tested variables by blood mercury group (BHg). Beta estimates (β) and standard errors (SE) for variables (rows) associated with outcomes (columns).

	Univariate Piecewise SEM								Statistical Difference by BHg Group (p-value)[2]			
	BHg < 15.8 µg/L				BHg ≥ 15.8 µg/L[1]							
	SBP β (SE)	DBP β (SE)	GABR β (SE)	Cit[3] β (SE)	SBP β (SE)	DBP β (SE)	GABR β (SE)	Cit[3] β (SE)	SBP p	DBP p	GABR p	Cit[3] p
$_L$GABR	18.73 (4.74) ***	14.45 (3.3) ***	-	-	-6.34 (8.74)	-1.74 (6.51)	-	-	0.01 **	0.04 *	-	-
Sex	11.08 (1.86) ***	3.94 (1.41) **	-0.003 (0.03)	5.14 (1.02) ***								
$_L$Tri[4]	12.92 (4.16) **	8.31 (3.00) **	-0.02 (0.07)	1.04 (2.28)								
$_L$BPb	9.68 (2.43) ***	7.52 (1.73) ***	0.20 (0.04) ***	-1.39 (1.34)								
$_L$BHg	0.05 (2.91)	4.24 (1.47) **	0.03 (0.03)	-2.62 (1.11) *	26.59 (10.15) *	4.24 (1.47) **	0.03 (0.03)	-2.62 (1.11) *	0.01 *	0.09 †		
BMI	0.39 (0.22)	0.22 (0.16)	-0.007 (0.003)	-0.07 (0.12)							0.07 †	
eGFR	-0.18 (0.06) **	-0.02 (0.05)	0.0006 (0.001)	-0.15 (0.03) ***	-0.18 (0.06) **	-0.24 (0.08) **	0.0006 (0.001)	-0.15 (0.03) ***		0.02 *		
Cit[3]	0.22 (0.13)	0.04 (0.09)	-0.006 (0.002) **	-								

Fast Time	-0.19 (0.17)	-0.08 (0.13)	-0.007 (0.003) *	-0.03 (0.09)		
Age	0.30 (0.09) **	0.13 (0.07)	-0.001 (0.002)	0.17 (0.05) ***		
Smoke	4.34 (2.38)	1.82 (1.71)	0.05 (0.04)	4.70 (1.24) ***		

Significance: <0.001 ' *** '; 0.001 ' ** '; 0.01 ' * '; 0.05 ' † '
Dash indicates that variables precede the variable of interest in the toxicological pathway
[1] Spaces are blank as the beta estimates are not significantly different than the BHg < 15.8 µg/L group
[2] Statistical comparison is of the standardized beta estimates
[3] Cit: short for citrulline
[4] Tri: short for triglycerides

4.2.2 SEM Model- Lower Hg Exposure Group (< 15.8 µg/L)

Overall, $_{L}$BPb directly and indirectly effected blood pressure with 45.0% and 28.6% of the effect of lead being exerted indirectly on SBP and DBP via the arginine pathway (Table 13). $_{L}$BPb's direct effect increased DBP by 4.97 mmHg (95% CI: 0.89 – 9.01) with no statistically significant effect on SBP. Indirectly, $_{L}$BPb was associated with a 0.20 (CI: 0.10 – 0.29) increase in $_{L}$GABR, which in turn was associated with an increase in SBP and DBP of 17.16 mmHg (95% CI: 9.09 – 25.84) and 10.06 mmHg (95% CI: 3.58 – 16.37), respectively (Table 13, Figures 14A and 15A).

Table 13: SEM model of the effect of Pb on the arginine pathway at lower (< 15.8 µg/L) and higher (≥ 15.8 µg/L) blood Hg (Model #2, Table 10).

	Lower Blood Hg (n= 148)			Higher Blood Hg (n= 60)		
	Beta[a] (S.E.)	Std. lv[b]	Std.all[c]	Beta[a] (S.E.)	Std. lv[b]	Std.all[c]
Log10(Pb µg/dL)						
Eat Wild Game (Ref = No)	0.25 (0.06)***	0.24	0.30	0.16 (0.09) †	0.16	0.24
Native Status (Ref = Non-native)	0.33 (0.05)***	0.33	0.45	0.36 (0.09)***	0.36	0.52
Log10(Citrulline)						
Sex (Ref = Female)	5.95 (1.23)***	5.95	0.36	3.14 (1.76) †	3.14	0.23
eGFR	-0.16 (0.04)***	-0.16	-0.32	-0.07 (0.06)	-0.07	-0.17
Log10(GABR)						
Log10(Pb µg/dL)	0.20 (0.05)***	0.20	0.32	0.31 (0.07)***	0.31	0.48
Fast Time (Hours)	-0.01 (0.003)*	-0.01	-0.18	-0.01 (0.004)*	-0.01	-0.21
Log10(Citrulline)	-0.01 (0.002)**	-0.01	-0.19	-0.004 (0.004)	-0.004	-0.12
Systolic Blood Pressure (SBP)						
Sex (Ref = Female)	10.57 (1.97)***	10.57	0.37	12.36 (3.24)***	12.36	0.43
Log10(GABR)	17.16 (4.00)***	17.16	0.29	-14.17(8.25) †	-14.17	-0.21
Log10(Triglycerides)	16.24 (4.77)**	16.24	0.28	13.36 (9.04)	13.36	0.20
Log10(Pb µg/dL)	3.89 (2.71)	3.89	0.11	11.89 (5.1)*	11.89	0.28
Diastolic Blood Pressure (DBP)						
Sex (Ref = Female)	3.37 (1.34)*	3.37	0.17	3.25 (2.80)	3.25	0.15
Log10(GABR)	10.06 (3.28)**	10.06	0.24	-4.30 (7.69)	-4.3	-0.09
Log10(Triglycerides)	8.31 (3.49)*	8.31	0.21	11.68 (6.50) †	11.68	0.24
Log10(Pb µg/dL)	4.97 (1.92)**	4.97	0.19	5.20 (4.29)	5.20	0.16
Covariance Structure						
SBP~~DBP	75.28 (9.79)***	75.28	0.78	94.27(18.94)***	94.27	0.80
Calculated Indirect Effect of Lead via Arginine on Blood Pressure						
$_{L}$BPb-Arg-SBP	0.09			-0.10		
$_{L}$BPb -Arg-DBP	.08			-0.04		
Calculated Total Effect of Lead on Blood Pressure						
$_{L}$BPb -SBP	0.20			0.18		
$_{L}$BPb -DBP	0.28			0.12		
Calculated Indirect Effect of Citrulline						
Citrulline-> $_{L}$GABR-> SBP	-0.06			0.03		
Citrulline-> $_{L}$GABR-> DBP	-0.05			0.01		

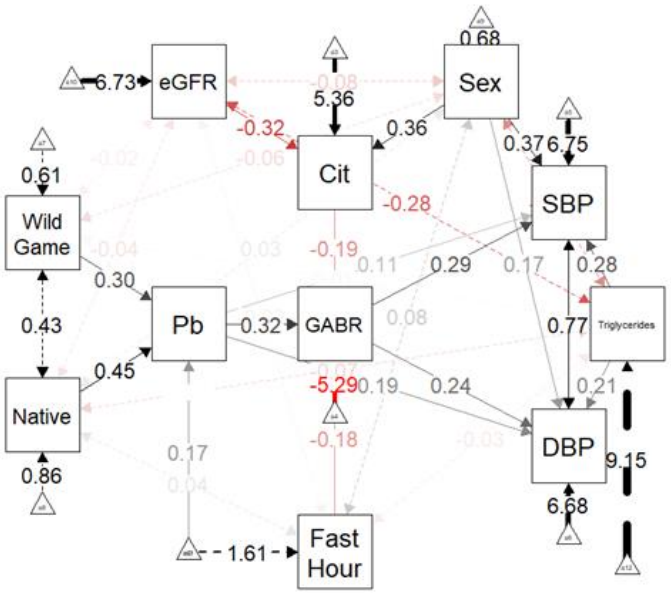
Significance: 0 ' *** '; 0.001 ' ** '; 0.01 ' * '; 0.05 ' † '

[a] Beta estimates that can be compared across studies

[b] Partially standardized beta estimates

[c] Fully standardized beta estimates to compare effect sizes within the model

A) Lower Blood Mercury (< 15.8 µg/l)



B) Higher Blood Mercury ((≥15.8 µg/l)

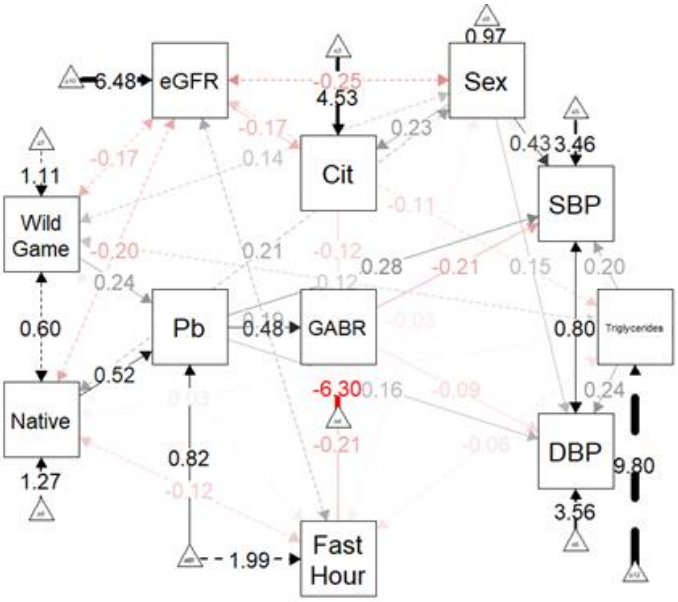


Figure 14: Structural equation model of risk factors for lead exposure and its toxicological pathway through arginine associated with increased systolic blood pressure by blood Hg levels. Fully standardized model coefficients with positive and negative associations shown in black and red, respectively. Statistical p-value is demonstrated by fading of values with lower

p-values having darker lines and values. Labels for variables are as follows: Pb-Log10(Blood Lead), Cit-Citrulline, GABR-log10(Global Arginine Bioavailability Ratio), SBP- Systolic Blood Pressure, DBP-Diastolic Blood Pressure, eGFR-estimated Glomerular Filtration Rate, Fast Hour-Time, in hours, since last meal.

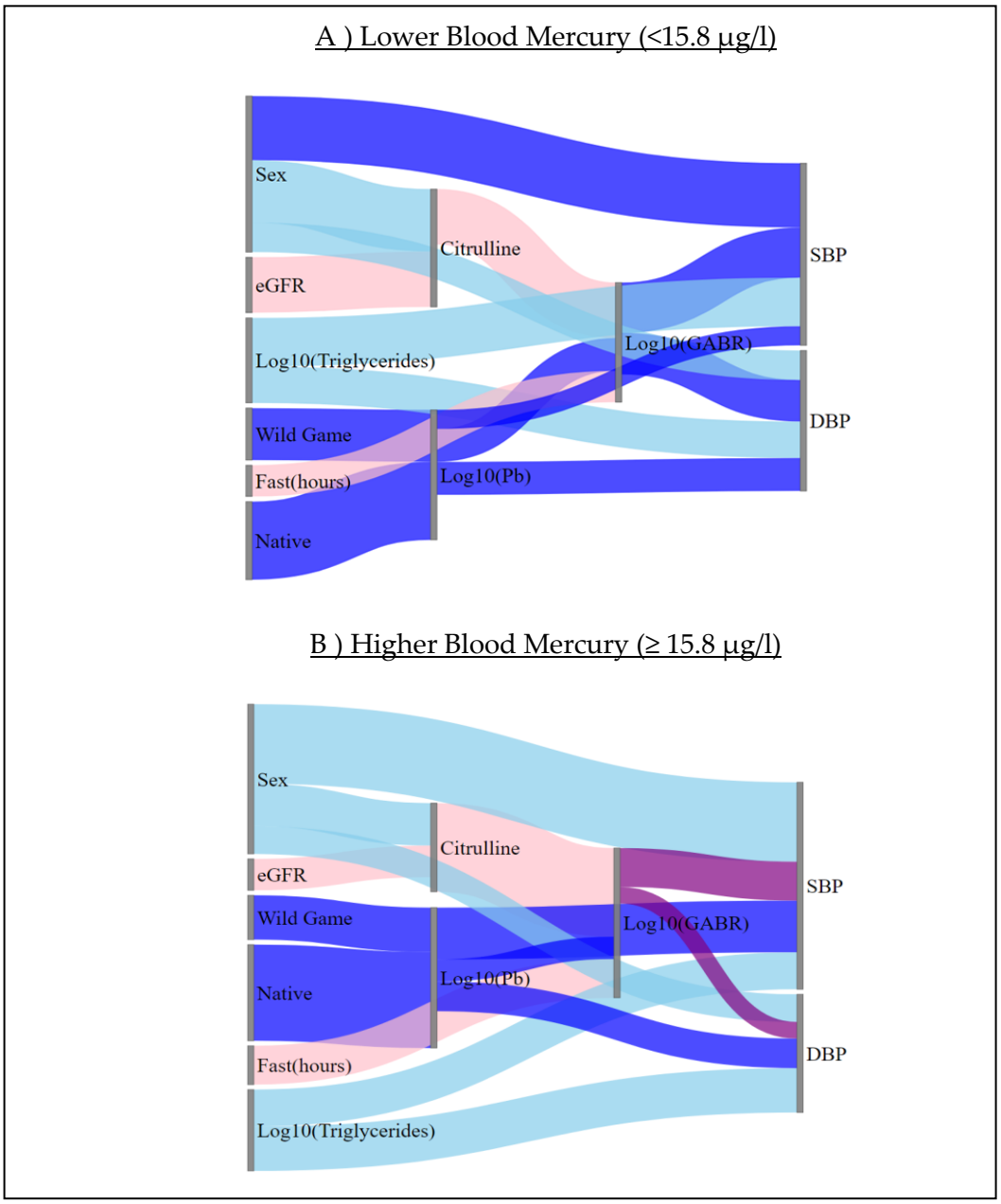


Figure 15: Sankey diagrams linking sources of lead exposure to blood lead levels that directly and indirectly effect blood pressure though the disruption of the arginine pathway by blood mercury group. Line thickness represent the fully standardized beta estimates from Table 14 with dark blue and purple representing positive and negative associations that are part of lead's toxicological pathway in effecting blood pressure. Light blue and pink represent positive and negative associations, respectively of model covariates.

As hypothesized in our theoretical framework (Figure 13), citrulline levels were dependent on eGFR (β: -0.16 μM, CI: -0.23 - -0.07), and were inversely associated with $_{L}$GABR (β: -0.01, CI: -0.01 - -0.002). $_{L}$BPb was associated with wild game consumption and indigenous status, regardless of BHg group (Table 13). Interestingly, while BHg status was found to modify the effect of GABR on blood pressure, neither $_{L}$BHg or $_{L}$THHg were associated with the arginine pathway or directly associated with blood pressure. BMI, smoking status, age and $_{L}$THHg did not improve model fit and were excluded from the final model.

4.2.3 SEM Model- Higher Hg Exposure Group (≥ 15.8 μg/L)

The effect of $_{L}$GABR on blood pressure shifted drastically for individuals with BHg ≥ 15.8 μg/L. The association of $_{L}$GABR on SBP changed from 17.16 mmHg to -14.17 mmHg (95% CI: -31.88 - -0.33) and the association on DBP changed from 10.06 mmHg to -4.30 mmHg (95% CI: -20.0 – 10.49). $_{L}$BPb's effect on $_{L}$GABR (0.31 (95% CI: 0.17 - 0.43)), indirectly lowered blood pressure. Using the fully standardized values, we calculate $_{L}$BPb's indirect effect via $_{L}$GABR on SBP and DBP to be -0.10 and -0.04, respectively, reducing its total adverse effect on blood pressure (Table 13, Figure 14). $_{L}$BPb's direct effect was associated with an increase in SBP of 11.89 mm Hg (95% CI: 1.74 – 22.10), nearly 4-fold greater than those with lower BHg (Table 13). This direct effect resulted in a 6.9 mmHg (95% CI: 1.01 – 12.89) increase for those at the 75th percentile of Pb exposure compared to the 25th percentile (Figure 16). Although the overall effect of $_{L}$BPb was to increase blood pressure, for individuals with BHg ≥ 15.8 μg/L, the arginine pathway reduced the total effect of BPb on SBP and DBP by 35% and 25% (Table 13).

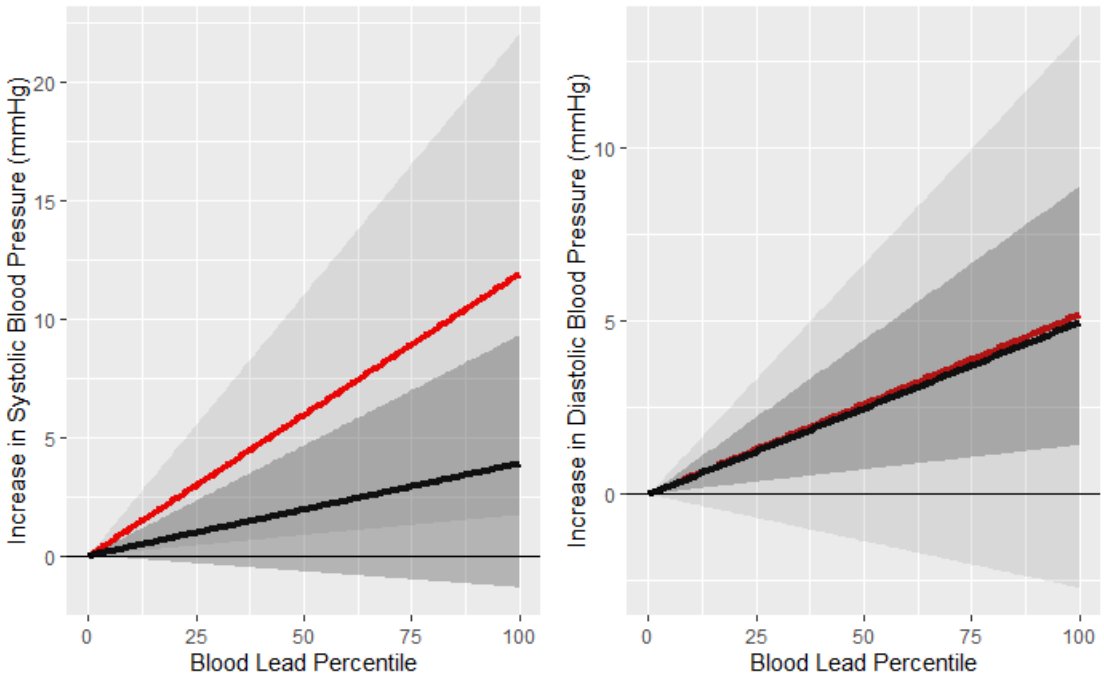


Figure 16: Direct effect of blood lead on systolic and diastolic blood pressure by blood mercury group for blood lead levels measured in the study. Blood mercury ≥ 15.8µg/L is shown in red, while <15.8 µg/L is shown in black. 95% confidence intervals are shown in black. 95% confidence intervals are shown in light and dark gray for blood mercury ≥15.8µg/L and <15.8µg/L, respectively. Blood lead concentrations at the 25th, 50th, 75th and 100th are 1.2, 2.2, 4.4 and 16.4 µg/dL.

Compared to the associations identified in participants with BHg <15.8 µg/L, the hypothesized framework was substantially disrupted in the high BHg group (Table 13, Figures 14B and 15B). Citrulline levels were not affected by eGFR and were no longer associated with $_{L}$GABR. BPb had a greater effect on SBP in individuals with BHg ≥ 15.8 µg/L, even at the same level of Pb exposure. For both Hg exposure groups, we found a significant covariance between SBP and DBP with greater effect for those with higher Hg exposure (Lower: β: 75.51, S.E. 9.30, p = <0.001 vs. Higher: β: 98.17, S.E. 18.88, p = <0.001).

4.3 Discussion

Our SEM models connect and statistically demonstrate that sources of Pb exposure from wild game consumption, due to the incidental consumption of microscopic Pb ammunition, and other exposure sources associated with indigenous status cause perturbations in metabolomic profiles that culminate in higher blood pressure. We also find Hg to have a non-linear effect on SBP, which may be through Hg's ability to modify the effect of the arginine pathway on blood pressure so that at BHg ≥ 15.8 μg/L GABR mitigates the effect of Pb exposure on blood pressure. Our results also show that Pb exerts ~45% and 29% of its toxicity on SBP and DBP, respectively via the arginine pathway when BHg < 15.8 μg/L. At higher BHg levels, Pb stimulates the arginine pathway in a protective response that lowers blood pressure. Not accounting for BPb's indirect effect on GABR would result in underestimating its effect at lower BHg levels and overestimating its effect on SBP at BHg levels ≥ 15.8 μg/L. These results agree with the current literature on the effects of Pb exposure on the arginine pathway [76] and provide further support of the arginine pathway as a toxicological route through which Pb exerts its effect on the cardiovascular system.

We find Hg does not directly affect the arginine pathway, but instead to be an effect modifier, demonstrating the importance of mixed metal exposures. While BPb and BHg exposures are correlated, BPb was found to have a positive linear effect on SBP (Appendix C. Supplemental Figure C.1). At BHg exposures <15.8 μg/L, the SEM model results agree with known literature of the arginine pathway and the effect of Pb exposure. Interestingly, GABR was associated with an increase in blood pressure, which may be due to arginine being used for biological processes other than NO synthesis. At BHg levels ≥ 15.8 μg/L, the established associations are no longer significant, potentially disrupted or not detectable with our sample

size. GABR was associated with decreasing SBP and DBP, as expected with NO formation. This transition of GABR could represent a protective response from Hg exposure, even though Hg was not directly acting on the arginine pathway. It may also reflect the limit of the cardiovascular benefits of fatty acids from fish consumption, the main source of Hg exposure in the region [254, 255]. In rodent studies, unsaturated fatty acids have also been found to lower BPb levels and reduce Pb toxicity [256, 257]. BPb may also promote catabolism, lowering levels of unsaturated fatty acids that are associated with improved cardiovascular health[258]. Yet, there is limited knowledge on how unsaturated fatty acids interact with mercury and lead to effect cardiovascular health, as much of the emphasis has been on neonatal and neurological outcomes [259-261].

This work demonstrates the utility of combining metabolomics and SEM models to test hypothetical pathways and their association with health outcomes. A better understanding of toxicological pathways might also provide valuable insight in evaluating mixtures which may target the same endpoint but via different mechanisms or act on the same pathway. Combining metabolomics and SEMs provides a new method to evaluate how adverse exposures effect toxicological pathways to induce chronic disease. This mechanistic approach could aid in identifying greater than additive effects when cascading effects from xenobiotic exposures overlap and in testing the assumptions commonly made by the dose-addition method to evaluate mixtures; specifically, that no interactions are present, and toxicity is induced by a single pathway. Deconstructing these toxicological pathways is also important for potential therapeutics [262, 263], with the potential of amino-acid panels to guide personalized medicine, such as site-specific environmental control and, where indicated chelation therapy.

These findings also contribute to our understanding of the kidneys and their importance in regulating and storing amino acids such as citrulline. Kidney function, evaluated by eGFR, was significantly associated with citrulline levels, which were in-turn associated with GABR. While the role of the kidneys in amino acid regulation has been demonstrated in patients with kidney disease, this study adds to their importance as regulators of amino acids in the broader population. The associations between amino acids and the kidneys may also prove to be a valuable tool in monitoring kidney function, as current kidney biomarkers often fail to identify kidney damage early enough for preventative measures to be taken.

Lastly, we find Pb exposure from wild game consumption to be associated with increased blood pressure. This is only the second such study, that we are aware of, to find such an association [42]. Although increased blood pressure from Pb exposure is well documented [53], the risk of consuming wild game shot with lead ammunition is often not assessed in lead exposure studies. Our study provides additional evidence to the health risks associated with consuming wild game shot with lead ammunition. Although Peru passed legislation this year banning paint that exceeds 90 ppm Pb (Ley N° 31182), other sources of lead may pose more prominent health risks [106], which include lead ammunition [110, 247], ceramics, fishing weights [68], and food staples such as yuca [129]. Banning lead ammunition may also help populations of the Andean condor [264], an important cultural symbol for many people in the region. Further evaluations of Pb exposure in Peru and across South America would improve the understanding of Pb sources and their prevalence. Meanwhile, legislation to eliminate Pb from ammunition, fishing weights and other products could have tangible improvements on public and environmental health.

It is important to note the limitations of this study. The study is cross-sectional with both metabolite and BPb levels potentially varying in time. We account for metabolite levels and time by adjusting for the number of hours since the last meal; however, this may not adequately account for temporal fluctuations in amino acid profiles. Similarly, BPb levels have a half-life of 1-2 months [53], and we are unable to account for long-term vs. short-term Pb exposure. A longitudinal study accounting for both metabolites and Pb exposure would greatly add to our understanding of amino acid profiles and Pb exposure across time. Due to the high cost of metabolomics, we had to limit the amino acid panel to high and low levels of BPb and BHg exposure which may limit these findings to be representative of the population. It is also important to note that BHg levels in the study population greatly surpassed those in the United States with the 15.8 µg/L threshold representing the 99.9th percentile of BHg exposure in the 2008 National Health and Nutritional Survey. Lastly, serum is traditionally aliquoted shortly after blood collection; however, in this study, serum was obtained at Duke University after whole blood samples had been frozen at -80˚C. While we do not believe this would have modified the amino acid profiles, the deviation from standard protocol used in urban settings is nonetheless a potential limitation in comparing this study to others.

The United States Environmental Protection Agency has emphasized the need for more mixture assessments [265]. A widely used approach is using component-based methods that rely on the ability to perform dose addition of exposures. This approach is used when toxicants exert the same type of toxicity and assumes interactions are not significant at low levels; assumptions that could be made with trace metals. However, the results of this study demonstrate that Hg may modify the effect of Pb on blood pressure. Statistical approaches that

incorporate the inherent physiological biology of amino acids provide a powerful tool to better evaluate the chronic health effects of xenobiotic exposures. This work is one of the few to incorporate SEM models, amino acids, exposure risk factors and trace metals to link exposure risk factors to health outcomes. In doing so, we contribute to our understanding of the effect Pb and Hg have on the arginine pathway and demonstrate the utility of these new methodological approaches to expand our ability to link xenobiotic exposures to chronic disease.

5. Conclusions

The purpose of this dissertation was to evaluate the extent of mixed trace metal exposures and their health effects in Madre de Dios, Peru, a region where previous research has mainly focused on mercury exposures. To do so, I tested blood for trace metals and found high concentrations of lead. I then leveraged my knowledge of the region and survey data to identify risk factors for lead exposure. To assess the health effects of mixed exposures to mercury and lead, I used metabolomics and structural equation models to test toxicological pathways that link lead exposure to increased blood pressure. I also assess how minerals and trace metals are associated with neonatal health that accounts for the transfer of nutrients from mother to child, which is rarely incorporated in birth cohort studies. This work demonstrates that mixed trace metal exposures are more common than previously believed and utilizes structural equation models as an alternative mean to better analyze the effects of mixed trace metal exposures on human health.

Future research should continue to assess the extent of mixed trace metal exposures and their health effects. As shown in chapter 4, mercury modified the effect of lead on blood pressure, demonstrating that interactions may not be only antagonistic or synergistic in

response, but an intermediary response that is still significantly different than a single exposure. In addition to mixtures, there is still a significant gap in our understanding of how a single or mixed trace metal exposure impacts multiple organ systems to harm human health. Organ systems are intricately connected, allowing for an exposure that may target a specific organ to reverberate throughout the body. The ability to incorporate multiple physiological systems and trace metal exposures is the best way to fully quantify the effects of trace metal exposures.

To achieve this level of knowledge, further funding needs to be allocated to studies that assess mixtures. Funding is currently being provided via the National Institute of Environment Health Science's Powering Research Through Innovative Methods for Mixtures in Epidemiology (PRIME) program. In addition to mixtures, a more integrated approach to evaluating health outcomes that accounts for the interdependence of organ systems is also needed but does not have a similar funding program. A better understanding of mixed trace metal exposures, other co-morbidities, and risk factors (blood pressure, body mass index, age, cystatin-C, etc.) and an improved assessment of how these measurements are interrelated to various organ systems could identify organ systems most at risk of failure, potentially allowing for a proactive means to identify future health concerns.

From a public policy lens, identification of mixtures that have a non-additive effect and the exposure levels required for a non-additive effect is a first step in identifying at risk populations. As seen in Chapter 4, the non-linear response mercury had on blood pressure was at 15.8 μg/L, which is the 99.9^{th} percentile of blood mercury levels measured in the 2008 National Health and Nutrition Examination Survey (NHANES). Thus, the levels of mercury present in most US adults would not modify a lead co-exposure found in Chapter 4. Additional

studies comparing non-additive effect thresholds across populations is also important to ensure findings are translatable across populations. Populations should not only be geographical but also demographic since children they have different exposures risks and compared to an adult, the same dose results in a higher blood concentration due to their smaller body size. Once these levels are identified, at risk populations can be identified using population level data such as NHANES in the United States. Unfortunately, similar data sets do not exist in every country and their establishment could be a valuable contribution in monitoring harmful exposures and assessing their health impacts. After being identified, steps to lower exposures of the metal mixtures to within the additive effect range or eliminate the exposure all together can be taken.

Along with new exposure thresholds to mixtures to protect human health, new lower thresholds for entities disposing of hazardous waste should also be considered if the waste contains a hazardous mixture that has non-additive toxicity. This would also apply to Superfund and other contaminated sites, where there is a greater potential for co-exposures. As environmental exposures are the most prevalent, limiting exposures to trace metal mixtures that have a non-additive effect is a secondary means to eliminate co-exposures.

5.1 Mixed metal exposures are more prevalent than commonly believed

Trace metals will continue to be a part of our environment in a myriad of ways. Shifting away from the 1-exposure: 1-outcome paradigm is necessary to account for mixed exposures. Given the ubiquitous nature of trace metals, researchers need to assess mixtures to ensure co-exposures are not occurring. This is especially important as trace metals have overlapping toxicological effects, which may cause incorrect inferences from health assessments if a single trace metal is evaluated in a dual exposure scenario.

In chapter 2, we found important evidence supporting lead exposure through the consumption of wild game. This route of exposure is not often studied yet could be common world-wide, further complicating the 1-exposure, 1 outcome paradigm. In the study region of Madre de Dios, this is a surprising and important finding as nearly all the exposure assessments focus on mercury. Although numerous routes of environmental exposures to trace metals have been identified, there are likely other routes of exposure that need to be better evaluated.

Identifying new sources of exposure may also be limited by research being predominantly conducted by institutions in high income countries and by researchers from wealthier communities. The limited shared experience between researchers and underrepresented communities, domestic and abroad, may prevent research from encompassing the full range of potential exposure sources. Differences in the phasing out or banning of trace metals in various products or limited assessment of trace metals in products can also obfuscate potential exposure sources [266]. The heterogenous nature of trace metals in soil and their bioavailability to plants may also hinder identification of potential food items that can accumulate metals. As a legacy contaminant, trace metals may be from historical uses that are not clearly visible on the modern landscape. A notable example are instances where shooting ranges (for gun owners) were converted to other uses. A playground in Port Richey, Florida was built next to a former shooting range that had elevated levels of lead contamination from spent lead ammunition [267, 268]. Integrating these factors for multiple trace metals is crucial for proper exposure assessment.

New sources of trace metals are also emerging as electronic products are phased out and recycled. The growing demand for rare earth metals that are required by the renewable

energy, electronic and automotive sectors will incentivize more mining that will likely liberate trace metals into the environment. Climate change will also contribute new sources of trace metals. As glaciers and ice caps recede, new source rock will become eroded. The unprecedented forest fires, mudslides and increased erosion on the landscape will also mobilize trace metals previously stored in soils and biomass. Similarly, new development and continued landcover change will liberate trace metals into the environment.

Although trace metals have been banned in many industrial applications, they will continue to be used in certain industrial and non-industrial settings as their banning faces strong opposition. Mercury's use in artisanal small-scale gold mining (ASGM) as a cheap method to extract gold, means its use will likely continue for the foreseeable future. Although there are several alternatives to eliminate or reduce mercury from ASGM, such as the use of retorts, and shaker tables; much work is needed before these methods are commonly used worldwide.

The use of lead shot in the United States will also likely continue due to its politicization in United States' politics. Lead ammunition was banned in the last week of the Obama administration but was rescinded by the Trump administration several weeks later. The National Rifle Association (NRA) has made efforts to demonstrate that lead bullets are not absorbed into the body, even though there is ample evidence of lead poisoning in wildlife that consume the carcasses of hunted animals. The bald eagle being a prime example [269, 270]. While lead ammunition is banned when hunting waterfowl in wetlands, it is commonly used for game hunting, even when non-lead ammunition is available at comparable cost. An important exception is California, which banned lead ammunition in 2013 [271]. The continued use of lead ammunition in the United States may be due to the strong opposition the NRA has adopted to

non-lead ammunition; however, the current Biden administration could act to ban lead ammunition once again [271].

Other countries have begun to phase out lead ammunition with mixed success. The Netherlands passed a lead ammunition ban in 1993, while Norway passed a similar ban in 2005, but overturned it in 2015 [266, 271]. Lead ammunition has become a socio-political issue [271], complicating the role of scientific evidence and raising the political hurdle for bans to be enacted.

5.2 Need to evaluate nutritional factors when assessing trace metals

There is plenty of literature on the interactions between trace metals and minerals. Although nutritionists and toxicologists have unique perspectives, both need to be integrated to better understand health outcomes. As shown in Chapter 3, proper nutrition and prenatal care were the predominant factors in ensuring adequate gestational age and birthweight. Lead exposure, while still adversely associated with birth outcomes, was only a part of a larger exposure profile associated with health outcomes.

The integration of prenatal care, nutrition and toxicological profiles will allow for more thorough assessments of health outcomes and may increase agreement between epidemiological studies, especially when malnutrition or lack of healthcare are pervasive. A more holistic understanding of exposure should be adopted that encompasses nutrients, to which we need to be exposed, and trace metals, that we seek to avoid.

5.3 New statistical methods should be encouraged

Although linear regression is commonly used, new statistical approaches should be adopted to ensure the best statistical method that accounts for the physiological and statistical

complexity is used to evaluate the research question. Statistical methods that allow for health outcomes to be evaluated jointly may allow for a more robust understanding of exposures and health effects. This may especially be useful for research on cardiovascular, renal, and neonatal health outcomes, as they have multiple co-dependent health metrics. For example, systolic and diastolic blood pressure for cardiovascular health (as shown in chapter 4); glomerular filtration rate, creatinine, and cystatin-C for renal health; and as shown in chapter 3, gestational age, and birthweight. These measured outcomes are interdependent and could benefit from being analyzed jointly rather than individually.

Alternative methods to linear regression should also be used when indirect effects may be occurring. The ability to assess direct and indirect effects allows for an improved understanding of toxicological pathways that are needed to better understand how trace metal exposures are associated with adverse health outcomes. As shown in chapters 3 and 4, the use of structural equation models provides a method to evaluate risk factors, exposure biomarkers and health outcomes jointly, allowing the entire hypothesized pathway to be analyzed. The statistical benefits of these models allow for complex research questions to be evaluated that will further our understanding of the health effects from trace metal exposures.

5.4 Metabolomics can help evaluate mixed exposures

Metabolomics are a powerful tool that can provide important insight on how amino acids and other biomarkers are linked to adverse health outcomes. In doing so, they provide a new framework to understand how trace metals and other toxicants disrupt the body's physiology and induce chronic disease. While previous risk assessments linked exposure to trace metals to adverse health outcomes, the exact mechanisms within the body were left

unaccounted. By incorporating metabolomics, we can begin to understand the physiological pathways that trace metals disrupt and in doing so, potentially identify new mechanisms to target for therapeutics. Improving our knowledge of these pathways, may also allow us to better understand how nutrients and minerals may modify trace metal exposures.

While metabolomics has great potential, the cyclical nature of certain metabolites may make it difficult to differentiate between pathways. Currently, metabolite analysis is expensive, and the statistical power needed to evaluate complex mixtures poses an important financial challenge. Once these costs begin to decline, metabolomics will have much to offer to risk assessment and mixed exposure analyses.

5.5 Trace metals can be a steppingstone for complex mixtures

While much of the toxicological paradigm has traditionally focused on 1-exposure, 1-health outcome, there is an increasing focus on understanding complex mixtures that are comprised of tens or hundreds of unique chemicals. This is especially true with inorganic chemicals such as polychlorinated biphenyls (PCBs), per- and polyfluoroalkyl substances (PFAS), flame retardants, chemicals that comprise the indoor environment, and the chemical components of smoke. These chemicals may be molecularly similar but have varying toxicities and concentrations. Approaches to evaluate these mixtures has involved principal component analysis (PCA), sum factor or evaluating each compound individually. However, similar to trace metals, overlapping toxicological pathways are likely present, allowing methods used for mixed trace metal analysis to be applied to complex mixtures. New methodological approaches using mixed trace metals can leverage the comparatively vast body of literature comprising of animal, cellular and epidemiological studies, that does not yet exist for newer chemicals of concern.

Appendix A: Supplemental Materials for Chapter 2

Supplemental Methods

Drinking Water:

For the 2018 study, a water sample was collected for each enrolled household by filling 125-mL pre-cleaned high density polyethylene bottles from the main faucet or water storage bucket of the household. Additional samples were collected from school tap water and community drinking water sources. Each bottle was labelled, stored in sealable plastic bags, held in cold storage with ice packs in the field, and frozen during international transit to and storage at Duke University. At Duke laboratories, water samples were thawed, acidified to a concentration of 2% v/v concentrated HNO_3, held at 4°C, and analyzed for Pb by ICP-MS (Agilent 7900). Analysis of Pb was conducted under a He atmosphere (collision cell) to limit polyatomic interferences. Prior to analysis, standards and study samples were diluted with 18.2 MΩ water to create a uniform 2% HNO3/0.5% HCl (v/v) (Fisher Scientific trace metal grade) matrix. The signals for Pb isotopes ^{206}Pb, ^{207}Pb, and ^{208}Pb were summed to obtain Pb concentrations. ^{209}BI was used as an internal standard to account for variability of analyte signal intensity between samples. Calibration curves were confirmed using a NIST traceable second source standard (CRM-TMDW-A).

Supplemental Tables

Supplemental Table A.1.a Whole blood mercury (Hg) concentrations with relative standard deviation (RSD) for each set of sample triplicates. Batch code is represented by capital letters, followed by sample number.

Blood Digestion Batch	$[Hg]_{rep\ 1}$	$[Hg]_{rep\ 2}$	$[Hg]_{rep\ 3}$	$[Hg]_{average}$	$[Hg]_{SD}$	RSD
	µg/L	µg/L	µg/L	µg/L	µg/L	%
CA 10	47.2	43.4	37.2	42.6	5.0	12
CA 15	15.0	16.8	16.8	16.2	1.0	6.2
AB 10	20.7	20.6	20.4	20.6	0.2	0.8
AB 14	9.8	9.8	9.2	9.6	0.4	4.0
AC 9	18.4	18.4	16.8	17.9	0.9	5.2
AC 17	12.8	12.8	12.5	12.7	0.1	1.2
AD 7	0.3	0.3	0.3	0.3	0.0	4.2
AD 14	0.8	0.9	0.7	0.8	0.1	17
AE 9	1.2	0.7	1.3	1.1	0.3	31
AE 16	1.1	1.1	1.1	1.1	0.0	3.2
AF 8	16.0	17.9	16.6	16.9	1.0	5.8
AF 18	3.3	3.3	5.6	4.1	1.3	32
AG 3	13.2	12.5	13.1	12.9	0.4	2.9
AG 17	18.4	18.7	20.6	19.2	1.2	6.3
AH 5	4.0	3.6	3.3	3.6	0.3	9.5
AH 17	26.1	25.7	25.6	25.8	0.2	0.9
AI 9	17.1	14.3	18.5	16.7	2.1	13
AJ 14	14.7	17.4	15.0	15.7	1.5	9.5
AK 2	69.6	64.8	70.9	68.4	3.2	4.7
AK 13	21.5	19.1	26.2	22.3	3.6	16
AL 3	7.5	6.6	6.5	6.9	0.6	8.3
AL 14	2.0	2.7	2.8	2.5	0.4	17
BA 1	6.9	7.0	7.1	7.0	0.1	1.5
BA 12	13.9	14.8	14.7	14.5	0.5	3
BB 1	5.8	5.9	5.9	5.9	0.1	1.5
BB 12	1.8	1.7	1.8	1.8	0.0	2.5
BC 1	4.6	4.5	4.7	4.6	0.1	2.1
BC 12	2.7	2.9	3.4	3.0	0.4	13.2
BD 1	13.0	15.2	20.2	16.2	3.7	23
BD 12	4.2	4.2	4.3	4.2	0.1	2.2
BF 1	1.7	2.2	1.7	1.9	0.3	15.0
BE 12	6.2	7.1	6.1	6.5	0.5	8.2
Average						8.9
Standard Deviation						8.3
Median						6.0
Minimum						0.8
Maximum						32.3

Supplemental Table A.1.b Whole blood lead (Pb) concentrations with relative standard deviation (RSD) for each set of sample triplicates. Batch code is represented by capital letters, followed by sample number.

Blood Digestion Batch	$[Pb]_{rep\ 1}$	$[Pb]_{rep\ 2}$	$[Pb]_{rep\ 3}$	$[Pb]_{average}$	$[Pb]_{SD}$	RSD
	µg/L	µg/L	µg/L	µg/L	µg/L	%
CA 10	76.1	70.1	62.2	69.5	7.0	10.0
CA 15	44.2	43.5	44.1	43.9	0.3	0.8
AB 10	127.1	125.6	124.5	125.7	1.3	1.0
AB 14	43.8	44.5	40.6	43.0	2.1	4.8
AC 9	81.3	80.0	73.0	78.1	4.5	5.8
AC 17	29.6	29.4	29.6	29.5	0.1	0.4
AD 7	20.5	26.1	24.9	23.8	3.0	12.5
AD 14	17.6	19.9	15.1	17.5	2.4	13.8
AE 9	20.2	10.9	22.7	17.9	6.2	34.8
AE 16	19.1	17.9	18.7	18.6	0.6	3.2
AF 8	46.4	51.1	45.6	47.7	3.0	6.2
AF 18	19.5	20.3	30.5	23.5	6.1	26.2
AG 3	64.4	62.3	66.7	64.5	2.2	3.4
AG 17	55.2	56.5	60.8	57.5	2.9	5.0
AH 5	35.0	30.4	28.0	31.1	3.6	11.4
AH 17	24.2	23.5	24.2	24.0	0.4	1.6
AI 9	95.2	82.3	100.7	92.7	9.4	10.2
AJ 14	14.0	15.9	14.1	14.7	1.0	7.1
AK 2	4.2	2.8	3.3	3.4	0.7	21.1
AK 13	6.6	3.2	4.7	4.8	1.7	35.7
AL 3	5.7	5.2	4.9	5.3	0.4	8.2
AL 14	6.7	8.6	8.4	7.9	1.0	13.0
BA 1	60.1	48.8	53.5	54.1	5.7	10.5
BA 12	72.0	76.8	76.5	75.1	2.7	3.6
BB 1	9.0	9.7	10.0	9.6	0.5	5.4
BB 12	32.0	33.2	33.1	32.8	0.6	2.0
BC 1	57.8	57.2	63.1	59.4	3.3	5.5
BC 12	40.2	46.7	56.4	47.7	8.1	17.1
BD 1	17.5	22.4	34.1	24.7	8.6	34.6
BD 12	14.8	15.2	15.5	15.1	0.3	2.2
BF 1	9.9	15.4	10.4	11.9	3.0	25.3
BE 12	7.8	9.4	7.8	8.3	0.9	11.3
Average						11.1
Standard Deviation						10.3
Median						7.6
Minimum						0.4
Maximum						35.7

Supplemental Table A.2. Standard reference material (SRM) measurements by blood sample batch and National Institute of Standards and Technology (NIST) reference materials.

NIST	955d[a] Level 2		NIST	955c[b] level 2		NIST	955c level 4	
	Hg (µg/L)	Pb (µg/L)		Hg (µg/L)	Pb (µg/L)		Hg (µg/L)	Pb (µg/L)
NIST Certified value	6.83	49.47	NIST Certified value	4.95	139.5	NIST Certified value	33.9	455.3
Batch A_C	6.46	49.85	Batch B_A	4.80	142.4	Batch B_A	32.2	484.3
Batch A_D	5.63	51.50	Batch C_A	4.64	158.2	Batch B_B	34.1	473.6
Batch A_E	5.51	49.46				Batch B_C	30.5	455.1
Batch AF	5.63	49.94				Batch B_D/F	34.0	481.4
Batch AG	5.77	46.40				Batch B_E	29.9	492.8
Batch AH	5.98	53.21						
Batch AIAJ	5.68	51.24						
Batch AK	5.69	46.92						
Batch AL	5.53	50.21						
Average	5.8	49.9	Average	4.7	150.3	Average	32.2	477.4
Standard Deviation	0.3	2.1	Standard Deviation	0.1	11.2	Standard Deviation	1.9	14.3
% RSD	5.1	4.3	% RSD	2.4	7.4	% RSD	6.0	3.0
% Recovery	84.4	100.8	% Recovery	95.4	107.7	% Recovery	94.9	104.9

a-NIST SRM is of whole human blood. b-NIST SRM of whole caprine blood

Supplemental Table A.3. Descriptive statistics of ACR and EATM studies to evaluate blood lead levels and anemia. Fisher's Exact Test for categorical and T-tests for continuous variables were used to evaluate differences between the ACR and EATM study.

	EATM (N=48)	ACR (N=201)	p-value	Overall (N=249)
Age (Years)* **			<0.001	
Mean (SD)	19.6 (13.9)	36.1 (11.1)		32.9 (13.4)
Median [Min, Max]	20.5 [2.00, 49.0]	35.0 [15.0, 66.0]		33.0 [2.00, 66.0]
Sex				
Female	33 (68.8%)	112 (55.7%)		145 (58.2%)
Male	15 (31.3%)	89 (44.3%)		104 (41.8%)
BMI* **			<0.001	
Mean (SD)	23.1 (7.56)	28.1 (4.17)		27.1 (5.36)
Median [Min, Max]	22.1 [13.7, 39.9]	27.9 [17.0, 42.6]		27.3 [13.7, 42.6]
Smoke Status **			0.002	
No	46 (95.8%)	150 (74.6%)		196 (78.7%)
Yes	2 (4.2%)	51 (25.4%)		53 (21.3%)
Household Water Source				
Treated	31 (64.6%)	120 (59.7%)		151 (60.6%)
Untreated	17 (35.4%)	81 (40.3%)		98 (39.4%)
Cooking Fuel				
High Emissions	20 (41.7%)	62 (30.8%)		82 (32.9%)
Low Emissions	28 (58.3%)	139 (69.2%)		167 (67.1%)
Education* **			<0.001	
Elementary	28 (58.3%)	28 (13.9%)		56 (22.5%)
Middle School	4 (8.3%)	46 (22.9%)		50 (20.1%)
High School	14 (29.2%)	103 (51.2%)		117 (47.0%)
Advanced	2 (4.2%)	24 (11.9%)		26 (10.4%)
Highway Access* **			<0.001	
Highway	34 (70.8%)	23 (11.4%)		57 (22.9%)
Non-Highway	14 (29.2%)	178 (88.6%)		192 (77.1%)
Road Access* **			<0.001	
Mean (SD)	0.755 (0.421)	0.507 (0.361)		0.555 (0.385)
Median [Min, Max]	1.00 [0, 1.00]	0.750 [0, 1.00]		0.750 [0, 1.00]
Fish Consumption* **			<0.001	
Rarely	0 (0%)	24 (11.9%)		24 (9.6%)
Monthly	6 (12.5%)	115 (57.2%)		121 (48.6%)
Weekly	42 (87.5%)	62 (30.8%)		104 (41.8%)
Beef Consumption* **			<0.001	
Never	17 (35.4%)	19 (9.5%)		36 (14.5%)
Monthly	6 (12.5%)	117 (58.2%)		123 (49.4%)

Weekly	25 (52.1%)	65 (32.3%)		90 (36.1%)
Chicken Consumption* **			<0.001	
Never	1 (2.1%)	2 (1.0%)		3 (1.2%)
Monthly	0 (0%)	35 (17.4%)		35 (14.1%)
Weekly	31 (64.6%)	164 (81.6%)		195 (78.3%)
Daily	16 (33.3%)	0 (0%)		16 (6.4%)
Wild Game Consumption* **			<0.001	
Never	28 (58.3%)	61 (30.3%)		89 (35.7%)
Monthly	12 (25.0%)	114 (56.7%)		126 (50.6%)
Weekly	8 (16.7%)	26 (12.9%)		34 (13.7%)
Native Status **			0.002	
Non-Native	36 (75.0%)	99 (49.3%)		135 (54.2%)
Native	12 (25.0%)	102 (50.7%)		114 (45.8%)
Haemoglobin Level* **			<0.001	
Mean (SD)	12.2 (1.39)	13.6 (1.17)		13.4 (1.34)
Median [Min, Max]	12.1 [10.5, 15.1]	13.5 [12.0, 17.7]		13.2 [10.5, 17.7]
Anemia Status* **			<0.001	
Did Not Present Anaemia	25 (52.1%)	201 (100%)		226 (90.8%)
Presented Anaemia	23 (47.9%)	0 (0%)		23 (9.2%)
Blood Lead Level (µg/dL) *			0.027	
Mean (SD)	2.72 (2.88)	3.92 (3.49)		3.69 (3.41)
Median [Min, Max]	1.46 [0.250, 12.7]	2.40 [0.520, 17.4]		2.30 [0.250, 17.4]
Blood Mercury Level (µg/L) **			0.001	
Mean (SD)	7.90 (9.09)	14.7 (12.7)		13.4 (12.4)
Median [Min, Max]	4.00 [0.300, 47.0]	12.4 [0.310, 73.7]		11.1 [0.300, 73.7]

Supplemental Table A.4. Descriptive statistics for the Etiology of Anemia and Trace Metals (EATM) study.

	EATM Sub-dataset (N=123)
Age (Years)	
Mean (SD)	19.5 (13.8)
Median [Min, Max]	21.0 [2.00, 69.0]
Sex	
Female	81 (65.9%)
Male	42 (34.1%)
Smoke Status	
No	94 (76.4%)
Yes	3 (2.4%)
Missing	26 (21.1%)
Community Type	
Mining	62 (50.4%)
Native	27 (22.0%)
Urban	34 (27.6%)
Household Water Source	
Treated	86 (69.9%)
Untreated	33 (26.8%)
Missing	4 (3.3%)
Cooking Fuel	
High Emissions	46 (37.4%)
Low Emissions	76 (61.8%)
Missing	1 (0.8%)
Education	
Advanced	9 (7.3%)
Elementary	61 (49.6%)
High school	30 (24.4%)
Middle school	19 (15.4%)
Unknown	4 (3.3%)
Beef Consumption	
Daily	7 (5.7%)
Weekly	54 (43.9%)
Monthly	26 (21.1%)
Never	34 (27.6%)
Missing	2 (1.6%)
Chicken Consumption	
Daily	50 (40.7%)
Weekly	63 (51.2%)

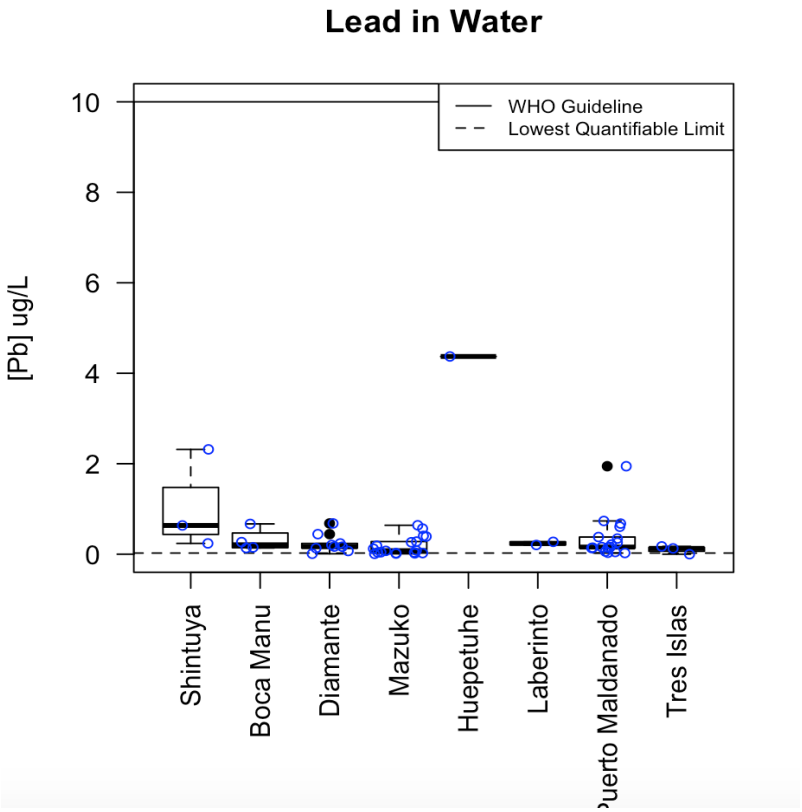
Monthly	8 (6.5%)
Never	2 (1.6%)
Wild Game Consumption	
Weekly	16 (13.0%)
Monthly	40 (32.5%)
Never	67 (54.5%)
Yuca Consumption	
Daily	25 (20.3%)
Weekly	65 (52.8%)
Monthly	21 (17.1%)
Never	12 (9.8%)
Eat Yuca Daily	
No	98 (79.7%)
Yes	25 (20.3%)
Haemoglobin	
Mean (SD)	11.8 (1.25)
Median [Min, Max]	11.3 [9.90, 15.1]
Missing	11 (8.9%)
Blood Lead Level (µg/dL)	
Mean (SD)	2.70 (2.68)
Median [Min, Max]	1.64 [0.250, 14.4]
Blood Mercury Level (µg/L)	
Mean (SD)	7.06 (10.2)
Median [Min, Max]	3.90 [0.300, 89.1]
Total Hair Mercury (µg/g)	
Mean (SD)	1.84 (1.81)
Median [Min, Max]	1.03 [0.128, 7.89]
Missing	22 (17.9%)

Supplemental Table A.5. Descriptive statistics of the Amarakaeri Communal Reserve (ACR) and the Etiology of Anemia and Trace Metals (EATM) studies.

	EATM (N=62)	ACR (N=245)	P-value	Overall (N=307)
Age (Years)				
Mean (SD)	22.6 (12.4)	35.2 (10.8)	<0.001	32.7 (12.2)
Median [Min, Max]	25.0 [2.00, 49.0]	35.0 [15.0, 66.0]		32.0 [2.00, 66.0]
Sex				
Female	48 (77.4%)	154 (62.9%)		202 (65.8%)
Male	14 (22.6%)	91 (37.1%)		105 (34.2%)
Smoke Status			0.001	
No	59 (95.2%)	192 (78.4%)		251 (81.8%)
Yes	3 (4.8%)	53 (21.6%)		56 (18.2%)
Community Type				
Mining	39 (62.9%)	40 (16.3%)		79 (25.7%)
Native	12 (19.4%)	127 (51.8%)		139 (45.3%)
Urban	11 (17.7%)	78 (31.8%)		89 (29.0%)
Household Water Source				
Treated	41 (66.1%)	146 (59.6%)		187 (60.9%)
Untreated	21 (33.9%)	99 (40.4%)		120 (39.1%)
Cooking Fuel			0.08	
High Emissions	23 (37.1%)	75 (30.6%)		98 (31.9%)
Low Emissions	39 (62.9%)	170 (69.4%)		209 (68.1%)
Education level			<0.001	
Advanced	6 (9.7%)	32 (13.1%)		38 (12.4%)
Elementary	24 (38.7%)	34 (13.9%)		58 (18.9%)
High school	21 (33.9%)	118 (48.2%)		139 (45.3%)
Middle school	11 (17.7%)	61 (24.9%)		72 (23.5%)
Beef Consumption			<0.001	
Never	17 (27.4%)	26 (10.6%)		43 (14.0%)
Monthly	11 (17.7%)	139 (56.7%)		150 (48.9%)
Weekly	34 (54.8%)	80 (32.7%)		114 (37.1%)
Chicken Consumption			<0.001	
Never	0 (0%)	2 (0.8%)		2 (0.7%)
Monthly	3 (4.8%)	41 (16.7%)		44 (14.3%)
Weekly	40 (64.5%)	202 (82.4%)		242 (78.8%)
Daily	19 (30.6%)	0 (0%)		19 (6.2%)
Wild Game Consumption			<0.001	
Never	40 (64.5%)	76 (31.0%)		116 (37.8%)
Monthly	16 (25.8%)	135 (55.1%)		151 (49.2%)
Weekly	6 (9.7%)	34 (13.9%)		40 (13.0%)

Blood Lead Level (µg/dL)			
Mean (SD)	2.13 (2.14)	3.81 (3.41)	3.47 (3.26)
Median [Min, Max]	1.22 [0.250, 9.97]	2.45 [0.250, 17.4]	2.23 [0.250, 17.4]
Blood Mercury Level (µg/L)			
Mean (SD)	7.54 (11.8)	13.8 (12.1)	12.5 (12.3)
Median [Min, Max]	4.35 [0.300, 89.1]	11.1 [0.310, 73.7]	9.40 [0.300, 89.1]
Total Hair Mercury (µg/g)			
Mean (SD)	1.92 (1.76)	3.70 (3.20)	3.34 (3.05)
Median [Min, Max]	1.28 [0.167, 6.79]	2.95 [0.00260, 21.4]	2.61 [0.00260, 21.4]

Supplemental Figures



Supplemental Figure A.1. Boxplots of lead levels in household water samples for each study community in the data subset. Blue circles represent lead levels of individual water samples. Solid black dots represent outliers. Indigenous communities are labelled with an asterisk.

Appendix B: Supplemental Materials for Chapter 3

Supplemental Tables

Supplemental Table B.1. Levels of trace metals and Se in maternal blood in µg/L, neonatal health outcomes assessed, statistical analyses conducted, and identified outcomes. Values shown are either geometric mean (GM), mean (M) or median (ME). None of the studies incorporated the number of prenatal care visits.

Location (Value)	Pb	Hg	Cd	Se	Outcome Assessed	Statistical Analysis	Identified Outcomes	Includes Minerals	Ref
Spain (GM)	19.8	3.9	0.53		APGAR, BW, BL	Linear Regression / tobit regression	Cd lowers APAGR score	No	[148]
Saudi Arabia	2.89	3.005	0.986		APGAR, HC, BW, BL, GA, PT, PW	Logistic regression	Hg effects head size. Cd and Pb effects placental thickness	No	[145]
India (Varanasi, M)	11.1	NA	0.095		BW	Logistic linear, stepwise regression	None	No	[272]
China (Multiple, M)	23.1	<LOD	0.9	171	BW	Linear regression	None	Yes (Se)	[273]
USA (Boston, GM)	3.93	2.35	0.86		BW, GA	Linear Regression	Hg reduces birthweight and GA	Yes (Se)	[144]
Japan (Fukuoka,	24.5	7.87	2.81	238	No	Dunn multiple comparison, Spearman rank	Neonatal health outcomes not assessed	Yes (Se)	[274]
Japan (M)	NA	4.21	NA	171.4	HC, BW, GA	Linear and Logistic Regression	Hg slight impact on head circumference	Yes (Se)	[56]
Japan (Multiple, GM)	6.4	3.8	0.71	179	No	Multiple regression	Neonatal health outcomes not assessed	Yes (Se, Mn)	[275]

Sweden (Norrbotten,	14	1.8	0.37		HC, BW, BL	linear spline regression, linear regression, BKMR	Cd and Hg inversely associated with birth weight	No	[276]
Saudi Arabia (Riyadh, M)	28.97	3.005	0.99		No	Linear regression	Did not assess endpoints	No	[277]
Korea	12.7		1.52		MDI, PDI	Multivariable regression	Pb*Cd at early and late pregnancy, Pb effected MDI	No	[278]
Russia (ME)	28.9		0.25		BW, BMIC	Linear regression ANOVA	Pb effected birth weight and child body mass index	No	[279]
Norway (ME))	12.4		0.2		BW, BMIC	Linear regression ANOVA	Pb reduced birth weight and child body mass index	No	[279]
China (Guiyu, GM)	67		1.7		HC, GA, BMI, PI	Logistic regression, BKMR	Pb effects head circumference, Cd lowers infant BMI	Yes (Mn)	[280]
China (Haojiang)	38		1.4		HC, GA, BMI, PI	Logistic regression, BKMR	Cd lowers SGA	Yes (Mn)	[149]
Egypt (M)			0.7		APGAR, GA, HC	Pearson Correlation	As lowers GA. As and Cd lower APGAR score	No	[281]
USA (Boston)	Quintiles				PC	Poisson regression	Cd associated with Preeclampsia	Yes (Se, Mn)	[282]
USA	Hig				BW, GA	Linear and	Pb	No	[283]

(California)	h/ Low					Logistic Regression	associated with gestational age		

Supplemental Table B.2. Limits of quantification for selected elements

Element	Limit of Quantification µg/L
As	0.90
Ca	1000
Cd	0.60
Cr	1.60
Fe	1000
Hg	0.25
Mg	1000
Pb	0.42
Zn	20.0

Supplemental Table B.3. Percent recoveries of elements in various quality control checks. Elements with the denotation of N/A indicates that no SRM value was reported on the certificate of analysis.

	As		Cd		Hg		Pb		
	Reference value[a]	Recovery	Reference value[a]	Recovery	Reference value[a]	Recovery	Reference value[a]	Recovery	Number of Analyses
	µg/L	%	µg/L	%	µg/L	%	µg/L	%	
NIST 955c Level 2	21.9	95	2.14	97	4.95	102	139.5	104	4
NIST 955c Level 3	53.9	99	5.201	101	17.8	99	277.6	105	11
NIST 955c Level 4	77.5	93	9.85	99	33.9	95	455.3	108	4
CRM-TMDW-A+ Hg	55	92	10	97	43.2[b]	98	20	105	17
IAEA-A-13	N/A		N/A		N/A		0.18[c]	82	21

a-values from Certificate of Analysis, b-Hg diluted from a 10 ppm stock from Spex Certiprep, c-µg/g

	Na	Mg	Ca	Fe	Cu	Zn	Se	Rb	V	Cr	Mn	Co	Ni	Analyses
IAEA-A-13 (Reference Values)[a,b]	12600	99	286	2400	4.3	13	0.24	2.3	N/A	N/A	N/A	N/A	N/A	21
IAEA-A-13 (Recovery %)	91	94	94	87	79	74	112	92						
CRM-TMDW-A+Hg (Reference Values)[a,c]	2300	8000	31000	90	20	75	11	N/A	35	20	40	25	60	17
CRM-TMDW-A+Hg (Recovery %)	115	103	107	<LOQ	103	96	83		99	106	120	102	101	

a-values from Certificate of Analysis, b-units are in µg/g, c-units are in µg/L

Supplemental Table B.4. Beta estimates for the base model and covariance structure with 95th confidence interval (C.I.) that adjusts for hypertension, age, and sex with latent variables for the maternal exposome (ME) and the fetal environment (FE).

Base Model (n=198)	Beta[a] (C.I.)	Std. lv[b]	Std.all[c]
Fetal Environment (FE)			
ME	0.24(0.03 - 0.44)*	0.24	0.24
Gestational Age (Weeks)			
FE	2.05 (-0.28 - 4.39).	0.15	0.13
Prenatal Visits	0.13 (0.04 - 0.22)**	0.13	0.23
Hypertension (Ref: No)	-0.61 (-1.23 - 0.02).	-0.61	-0.12
Weight (kg)			
Prenatal Visits	0.05 (0.020 - 0.08)**	0.05	0.24
Mother's Age (years)	0.02 (0.01 - 0.03)**	0.02	0.17
Sex (Ref: Male)	-0.13 (-0.24 - -0.02)*	-0.13	-0.14
Raw Covariance Structure			
Gestational Age~~ Birth weight	0.150 (0.025-0.275)*	0.150	0.300

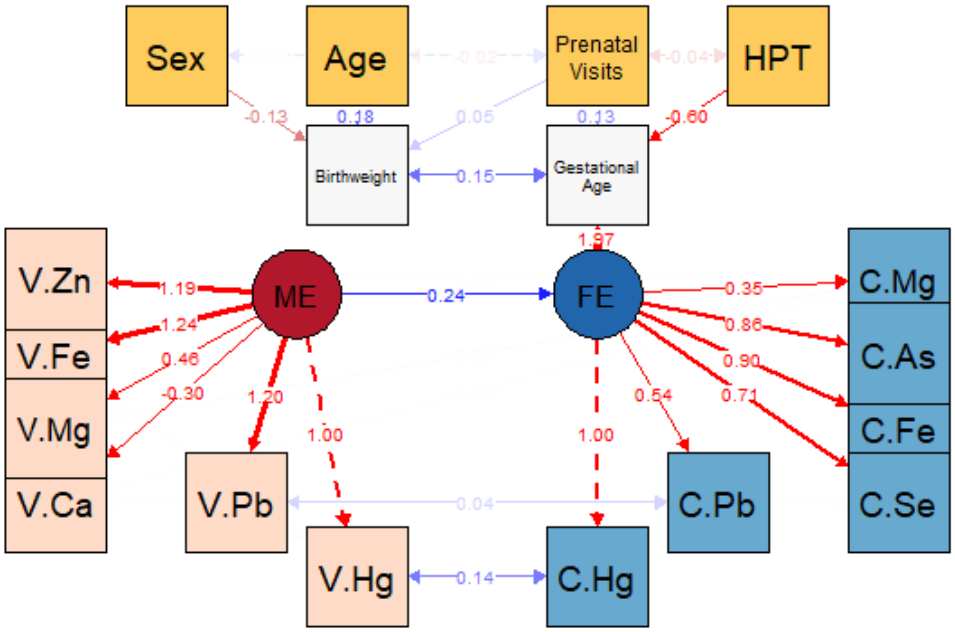
*** p<0.0001; ** p<0.01; * p<0.05; . p<0.1

a- Raw coefficients

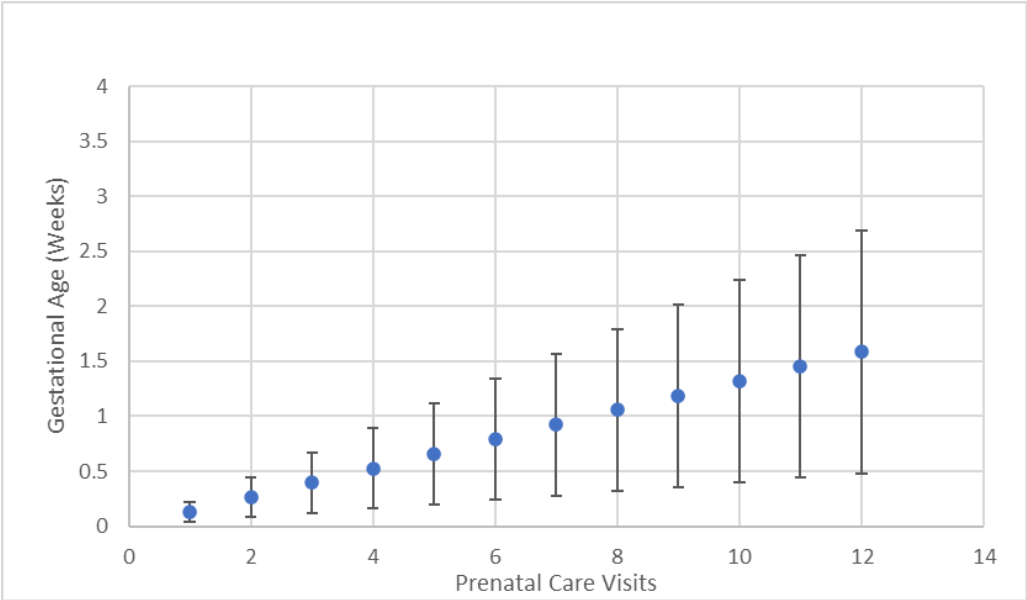
b- Beta coefficient only standardizing the latent variables

c- Completely standardized solution

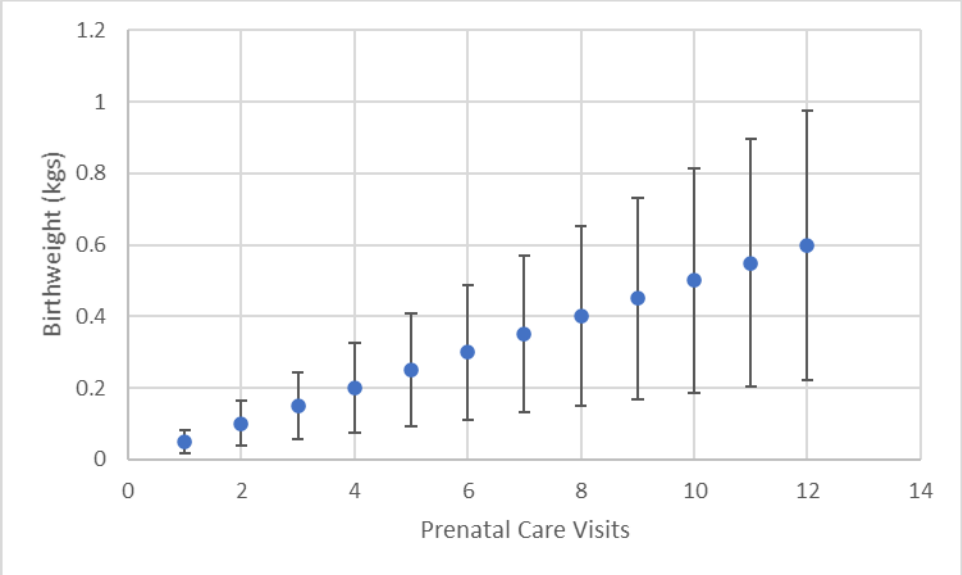
Supplemental Figures



Supplemental Figure B.1. Base model diagram with hypertension (HPT), age, sex, and prenatal visits with the maternal exposome and fetal environment as latent variables (ME and FE, respectively). Values shown are unstandardized beta values with transparency dependent on p-value. Boxes are colored by maternal blood (pink), cord blood (light blue), traditional covariates (orange), ME (red), FE (blue) and neonatal health outcomes (white).



Supplemental Figure B.2. Model estimates of the effect of prenatal care visits on gestational age (weeks) with 95% confidence interval.



Supplemental Figure B.3. Model estimates of the effect of prenatal care visits on birthweight (kgs) with 95% confidence interval.

Appendix C: Supplemental Materials for Chapter 4

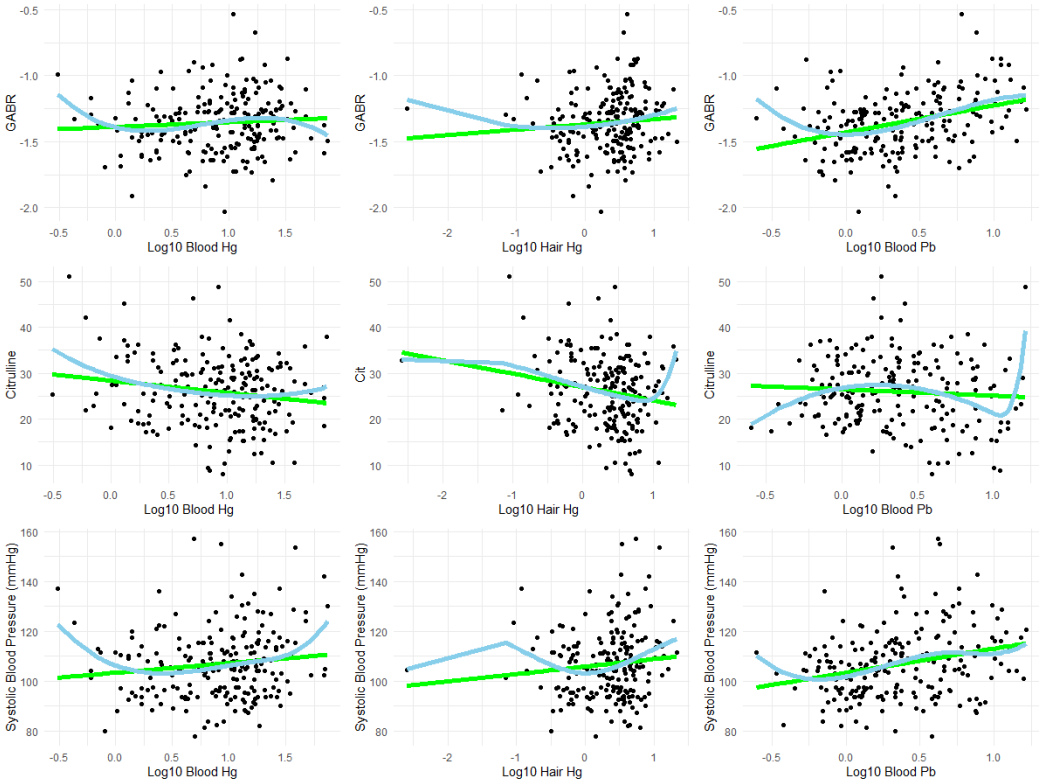
Supplemental Tables

Supplemental Table C.1. Population characteristics by elevated and recommended blood pressure.

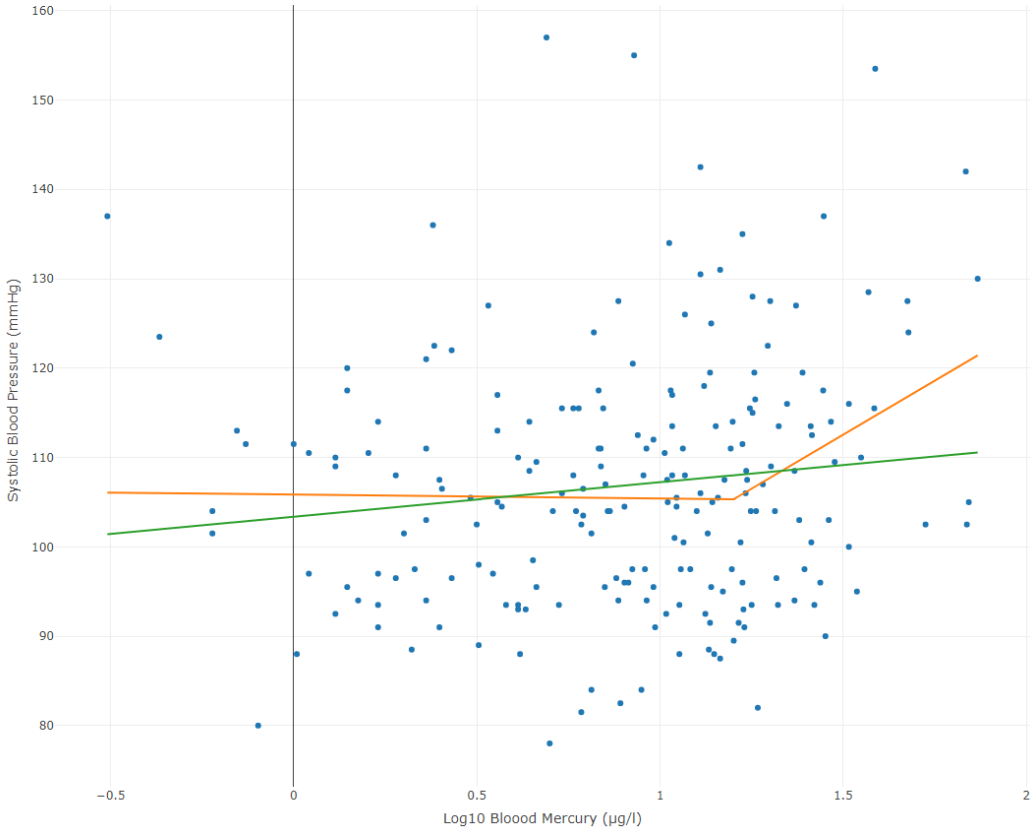
	Elevated Blood Pressure (N=44)	Recommended Blood Pressure (N=164)	Overall (N=208)	P
Age				0.03
Mean (SD)	37.9 (9.57)	34.2 (10.2)	35.0 (10.2)	
Median [Min, Max]	38.5 [19.0, 53.0]	33.0 [18.0, 66.0]	34.0 [18.0, 66.0]	
Sex				0.02
Mean (SD)	0.523 (0.505)	0.323 (0.469)	0.365 (0.483)	
Median [Min, Max]	1.00 [0, 1.00]	0 [0, 1.00]	0 [0, 1.00]	
Body Mass Index (BMI)				0.41
Mean (SD)	28.8 (3.94)	28.2 (4.43)	28.3 (4.33)	
Median [Min, Max]	28.6 [22.2, 37.0]	27.9 [17.0, 42.6]	28.0 [17.0, 42.6]	
Estimated Glomerular Filtration Rate (eGFR)				0.009
Mean (SD)	99.2 (17.8)	106 (14.9)	105 (15.7)	
Median [Min, Max]	98.2 [54.1, 136]	107 [70.8, 145]	105 [54.1, 145]	
Triglycerides (mg/dL)				0.01
Mean (SD)	183 (103)	145 (82.3)	153 (88.1)	
Median [Min, Max]	164 [42.0, 537]	133 [30.0, 694]	137 [30.0, 694]	
Smoker Status				0.8
Mean (SD)	0.182 (0.390)	0.213 (0.411)	0.207 (0.406)	
Median [Min, Max]	0 [0, 1.00]	0 [0, 1.00]	0 [0, 1.00]	
Blood Lead Level (µg/dL)				0.03
Mean (SD)	4.34 (3.38)	3.16 (3.23)	3.41 (3.29)	
Median [Min, Max]	3.38 [0.720, 16.4]	1.81 [0.250, 15.8]	2.15 [0.250, 16.4]	
Blood Mercury (µg/L)				0.01
Mean (SD)	17.1 (17.1)	11.7 (11.0)	12.8 (12.7)	
Median [Min, Max]	12.3 [0.310, 73.7]	9.19 [0.600, 69.7]	9.65 [0.310, 73.7]	
Wild Game Consumption (Weekly or Monthly)				0.004
Mean (SD)	0.545 (0.504)	0.299 (0.459)	0.351 (0.478)	
Median [Min, Max]	1.00 [0, 1.00]	0 [0, 1.00]	0 [0, 1.00]	
Fasting time (Hours)				0.2
Mean (SD)	8.49 (5.25)	9.70 (5.62)	9.45 (5.55)	
Median [Min, Max]	10.7 [1.06, 19.5]	11.0 [1.00, 22.5]	11.0 [1.00, 22.5]	
Arginine (µM)				0.06
Mean (SD)	16.7 (8.99)	13.7 (9.59)	14.3 (9.52)	

Median [Min, Max]	14.6 [5.39, 39.8]	11.8 [3.04, 103]	12.3 [3.04, 103]	
Global Arginine Bioavailability Ratio (GABR)				0.3
Mean (SD)	0.0553 (0.0275)	0.0496 (0.0332)	0.0508 (0.0321)	
Median [Min, Max]	0.0492 [0.0237, 0.135]	0.0417 [0.00936, 0.293]	0.0435 [0.00936, 0.293]	
Citrulline (μM)				0.02
Mean (SD)	28.3 (8.74)	25.4 (7.00)	26.0 (7.48)	
Median [Min, Max]	28.2 [10.6, 51.1]	25.6 [8.06, 46.4]	25.9 [8.06, 51.1]	
Systolic Blood Pressure (SBP mmHg)				<0.001
Mean (SD)	125 (12.9)	102 (9.50)	107 (14.0)	
Median [Min, Max]	125 [104, 157]	103 [78.0, 120]	106 [78.0, 157]	
Diastolic Blood Pressure (DBP mmHg)				<0.001
Mean (SD)	82.9 (8.23)	66.5 (7.22)	70.0 (10.0)	
Median [Min, Max]	82.3 [63.0, 105]	67.0 [47.0, 79.5]	70.0 [47.0, 105]	
Native Community				0.069
Mean (SD)	0.614 (0.493)	0.445 (0.499)	0.481 (0.501)	
Median [Min, Max]	1.00 [0, 1.00]	0 [0, 1.00]	0 [0, 1.00]	

Supplemental Figures



Supplemental Figure C.1. Evaluation of non-linear of effects of trace metals on metabolites and blood pressure.



Supplemental Figure C.2. Evaluation of linear and non-linear fit shown in green and orange, respectively. Non-linear fit with knot at 1.2 has a better model fit compared to the linear model (BIC: 1696 vs 1698).

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Biography

Axel Berky obtained dual bachelor's degrees from the University of Michigan in Political Science and Environmental Science in 2009. He then served as a Peace Corps volunteer in El Salvador, teaching interactive environmental science from 2009-2011. In 2012, he enrolled in the Master of Environmental Management (MEM) program at Duke University in Ecotoxicology and Environmental Health. Upon graduation in 2014, Axel worked as an associate researcher at the Duke Global Health Institute where he led environmental and epidemiological studies in Madre de Dios, Peru. In 2017, he began his doctoral degree with Dr. William Pan.

Axel Berky has led 2 first author publications (Evaluation of Peruvian Government Interventions to Reduce Childhood Anemia, Predictors of mitochondrial DNA copy number and damage in a mercury-exposed rural Peruvian population near artisanal and small-scale gold mining: An exploratory study) and has been a listed coauthor on 14 other peer reviewed articles. He also contributed to the revision of the Agency of Toxic Substances and Disease Registry on mercury in 2021. His fellowships include being a Duke Global Health Scholar (2020-2022) and a Duke Environmental Impacts Fellow Program (2019-2020). In 2019, the Duke Environmental Law and Policy Clinic awarded him the Science Communications Award in recognition of his ability to communicate scientific findings to his peers.