Hair Mercury Level is Associated with Anemia and Micronutrient Status in Children Living Near Artisanal and Small-Scale Gold Mining in the Peruvian Amazon

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Abstract. Anemia has been widely studied in global health contexts because of severe nutritional deficiency, and more recently, inflammatory status, but chemical exposures are rarely considered. Until recently, “anemia” was used synonymously with “iron deficiency anemia (IDA)” in global health settings. However, only 50% of anemia cases worldwide are IDA. Environmental toxicology studies of anemia risk have generally focused on populations in developed countries, albeit with high exposure to environmental toxicants, such as lead or cadmium. In the developing world, toxicant exposures commonly coexist with other risk factors for anemia. In particular, artisanal and small-scale gold mining (ASGM) communities are at risk for dietary methylmercury exposure through contaminated fish consumption, and for anemia due to food insecurity and infectious and chronic diseases. Here, we report analysis of total hair mercury content, hemoglobin, and serum micronutrient levels in children < 12 years of age ($N = 83$) near ASGM in the Peruvian Amazon. Forty-nine percent ($N = 29/59$) of those aged < 5 years were anemic (< 11 g/dL) and 52% ($N = 12/23$) of those aged 5–11 years (< 11.5 g/dL). Few children were stunted, wasted, or micronutrient deficient. Median total hair mercury was 1.18 $\mu\text{g/g}$ (range: 0.06–9.70 $\mu\text{g/g}$). We found an inverse association between total mercury and hemoglobin ($\beta = -0.12$ g/dL, $P = 0.06$) that persisted ($\beta = -0.14$ g/dL, $P = 0.04$) after adjusting for age, sex, anthropometrics, and vitamin B₁₂ in multivariate regression. This study provides preliminary evidence that methylmercury exposure is associated with anemia, which is especially relevant to children living near ASGM.

INTRODUCTION

Many low- and middle-income countries (LMICs) are in the midst of an “epidemiological transition,” the classic shift from primarily infectious to chronic disease burden with increasing economic development.^{1,2} This shift in disease burden is often attributed to the “nutrition transition,” a shift toward diets characterized by high-saturated fat, sugar, and refined foods and low in fiber and decreased physical activity.^{1,2} However, the epidemiological transition in disease burden may not be due entirely to nutritional changes. Concurrent, and possibly interacting, with the nutritional transition is a shift in environmental chemical exposure patterns from predominantly traditional hazards, such as vectorborne pathogens and contaminated drinking water that lead to infectious diseases, to new chronic disease risk factors, including urban air pollution and toxic chemicals, such as heavy metals and pesticides.³ Collectively, environmental hazards account for more than 24% of childhood deaths globally.³ More than 300 million children below 20 years of age in Latin America and the Caribbean are exposed to both traditional and emerging contaminants,³ resulting in almost 100,000 annual deaths of children below 5 years of age in the Americas.³ Population health outcomes with both traditional and emerging risk factors deserve special attention in populations that present with combinatorial risk profiles. Specifically, anemia has been widely studied in global health contexts because of severe nutritional deficiency,⁴ and more recently, inflammatory status,^{5,6} but chemical exposures are rarely considered.

Anemia is a significant global health problem that accounts for ~8% of the global burden of disease.⁷ Anemia is a common

but a serious condition in which the oxygen-carrying capacity of circulating red blood cells is insufficient to meet physiologic oxygen needs.⁷ Until recently, “anemia” was used synonymously with “iron deficiency anemia (IDA)” in global health settings. However, the World Health Organization (WHO) estimates that only 50% of anemia cases worldwide are due to iron deficiency.⁷ Although iron deficiency is thought to be the most common nutritional cause of anemia globally, other nutritional deficiencies, including folate and vitamin B₁₂, can cause anemia.^{8,9} More recently, the global health community has recognized the need to discriminate between IDA and inflammatory anemia caused by infectious, enteric, or chronic disease.^{10–12} Low dietary iron in children can impede growth, cognitive development, and immune system functioning.^{13,14} In addition, iron status in the “first 1,000 days” of life is an important predictor of chronic disease risk, cognitive capacity, and economic productivity in adulthood.¹⁴ Iron supplementation in cases of true IDA can prevent slowed growth trajectories and cognitive delays common in iron deficiency.¹⁴ However, provision of iron supplements to anemic children in malaria endemic regions can increase morbidity and mortality due to malaria infection and should be avoided in non-IDA anemia.^{6,12,15,16} Therefore, accurate identification of anemia etiology is essential for safe and effective intervention.

Several large-scale collaborative projects involving the WHO, CDC, NIH, Gates Foundation, and the Global Alliance are addressing the need to distinguish IDA from inflammatory anemia, including the Iron and Malaria Technical Working Group, the Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia Project, and the INSPIRE (Inflammation and Nutrition Science for Programs/Policies and Interpretation of Research Evidence) Project.¹⁰ These projects recognize that identification of the individual causes of anemia is essential. However, none of the Sustainable Development Goals (SDGs) consider or mention any chemical exposures for

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any SDG Target, or in any of the health outcomes to be monitored.^{17,18}

In contrast, environmental toxicology studies of anemia risk have generally focused on populations with disease and nutritional profiles common to developed countries, albeit with high exposure to environmental toxicants, such as lead or cadmium. Only a small number of controlled, laboratory studies and ecotoxicology assessments in animals report evidence of hemotoxicity, including decreased hemoglobin, hematocrit, and red blood cell counts, after exposure to inorganic Hg,^{19,20} elemental mercury,²¹ and methylmercury.²² Prior human epidemiological data suggest the existence of a relationship between environmental inorganic mercury exposure and anemia risk,^{20–24} one of which reported reduced synthesis of heme, an essential component of hemoglobin, by inhibiting the enzyme δ -aminolevulinic acid dehydratase (ALAD), which is a classic mechanism for lead-induced anemia.²⁰ The 1999 Agency for Toxic Substances and Disease Registry report on methylmercury noted that no studies on blood parameters after oral exposure to organic mercury in humans were located in the literature.²⁰ However, based on known toxicokinetics and toxicodynamics of methylmercury, and on known mechanisms for lead- and cadmium-induced anemia, the hypothesis that methylmercury may induce anemia is biologically feasible. Evidence in the literature suggests that methylmercury can induce anemia by triggering red blood cell death secondary to irreparable oxidative damage,^{25,26} mimicking or exacerbating vitamin B₁₂ or folate deficiency,^{8,27} or directly dysregulating iron homeostasis (Figure 1, Discussion).²⁸

In the developing world, toxicant exposures are intensifying rapidly and commonly co-exist with other risk factors for anemia. In particular, individuals used in artisanal and small-scale gold mining (ASGM) are at risk for high occupational inorganic Hg exposure through inhalation of gaseous mercury that is then oxidized internally, and rural communities living near ASGM are at risk for dietary methylmercury exposure through contaminated fish consumption, although mixed exposures are possible for both groups.²⁹ These rural communities are also at high risk for anemia due to food insecurity and infectious and chronic diseases.⁷ Global mercury emissions to the atmosphere from ASGM are estimated to now exceed emissions from large-scale industrial sources such as coal combustion.^{23,30} As ASGM activity intensifies worldwide in response to rising gold prices, the scope of populations with similar anemia risk profiles is likely to expand.

Madre de Dios (MDD), Peru, is a hotspot for ASGM, which can result in release of mercury to local waterways, conversion of inorganic mercury to methylmercury by anaerobic microbes,³¹ subsequent biomagnification of methylmercury in the aquatic food web,¹⁸ and high methylmercury exposures to populations reliant on fish for sustenance. MDD is located in Peru's tropical Amazon, one of the world's most biodiverse ecosystems. ASGM is a major source of income in MDD and mercury use for gold production is widespread. In addition to likely occupational exposure to mercury vapor in gold miners, environmental exposure to methylmercury through fish consumption in MDD communities is also common. Our prior data in 231 individuals in 12 MDD communities along the Rio Madre de Dios indicate that 86% of participants had hair total mercury (total mercury) levels that exceeded the National Research Council/United States Environmental Protection Agency threshold of 1.2 $\mu\text{g/g}$ and hair total mercury was positively associated

with high fish consumption, especially of high trophic level fish (L. Wyatt et al., in review) (Figure 2). Unlike the comprehensive methylmercury exposure assessments in the seafood-consuming Faroe Islands³² and Seychelles Islands,³³ which were performed in populations with reliable health care access and relative economic prosperity, MDD residents have sporadic access to variably equipped health posts or clinics, average household income in MDD is ~\$290/month, or \$3.25 per person per day (L. Wyatt et al., unpublished data), and anemia prevalence is high, particularly in children below 12 years of age (Figure 3, Supplemental Figure 1).

Here, we evaluate the relationship between total mercury levels and anemia status in children below 12 years of age ($N = 83$) living in communities near ASGM in MDD, Peru. Prior studies support hair total mercury as a reliable biomarker of environmental methylmercury exposure in fish-eating populations,²⁰ supporting our use of hair total mercury as a proxy for methylmercury exposure. We also assessed potential roles for select micronutrients in modifying this relationship. Instead of inferring nutritional status from food frequency questionnaires or other nutritional survey instruments, we directly measure serum levels of several micronutrients previously implicated in anemia risk, including vitamin D, vitamin A, vitamin B₁₂, zinc and folate. To our knowledge, the potential relationship between environmental methylmercury exposure and anemia risk has not been assessed previously in a population with high nutritional and inflammatory risk factors.

This study represents a novel assessment of the hypothesis that methylmercury can induce anemia, which is both biologically feasible and especially relevant to children in rural areas of LMICs.

MATERIALS AND METHODS

Study design and participant selection. The parent study, the Amarakaeri Reserve Cohort Study (AmCS), is a baseline survey of 1,221 households across 23 communities ($N = 4,083$ total study participants) surrounding the Amarakaeri Communal Reserve in MDD, a protected area in the southern Peruvian Amazon (Figure 2). Survey administration and sample collection were conducted from March to May 2015. The primary objective of this study is to assess baseline health in communities near resource extractive activities, including ASGM that is prevalent in the eastern border of the reserve (Figure 2), and natural gas extraction planned in the reserve. Twenty-three communities were selected a priori around the reserve. Households with at least one woman of childbearing age (WCBA, aged 15–49) were enrolled. All household members ($N = 4,308$) were offered testing for hemoglobin and total hair mercury. Because of random nonparticipation (mostly because of individuals not at home when field workers visited), $N = 3,128$ study participants received hemoglobin testing and $N = 2,308$ study participants received hair total mercury testing; $N = 2,237$ received both tests. In this study, we focused on children < 12. Data from children < 12 ($N = 1,387$) in the full baseline study participant set comprises the “AmCS Community Children” dataset. A subset of participants were classified as belonging to “sentinel groups,” defined as a WCBA, her spouse, and her child (complete sentinel group), or a WCBA with either spouse or child or neither one (incomplete sentinel group.) Children < 12 ($N = 83$) in sentinel groups were additionally tested for serum micronutrients.

FIGURE 1. Environmental determinants of anemia. Nutritional, inflammatory, and toxicant-induced pathways to anemia. Established mechanisms are shown with solid lines; hypothesized mechanisms or mechanisms with preliminary supporting evidence are shown with dotted lines. Combinatorial, or additive, effects are possible in the context of multiple exposures. Interactive, or multiplicative, effects are possible as well; for example, iron deficiency increases susceptibility to toxicant-induced eryptosis. See Discussion for full description of mechanisms. Excludes genetic hemoglobin disorders, such as sickle cell disease and β -thalassemia, bone marrow disorders leading to aplastic anemia, and hemolytic anemias. This figure appears in color at www.ajtmh.org.

This subset of children comprises the “AmCS Sentinel Children” dataset.

Biomarker testing. Hair samples were collected by cutting three tufts of hair from the occipital region of the scalp. Hair samples from each participant were stored individually in paper envelopes, held at ambient conditions (e.g., room temperature), and transported to Duke University for total mercury analysis. Total mercury content in the proximal 2-cm segment of hair was determined by direct combustion, gold amalgamation, atomic absorption spectrometry (Milestone DMA-80.) This segment length approximates mercury exposure

over 2 months before sampling.^{20,34} The instrument was calibrated with aqueous Hg^{2+} acidified with 1 M nitric acid, and calibration was verified by the analysis of a hair certified reference material (DB001, European Reference Materials) once per 10 hair samples in the analysis batch. Sample measurements were accepted if the corresponding reference material measurements were within 10% of the certified mean value. The lower limit of quantification was 1 ng total mercury (approximately 0.05 $\mu\text{g/g}$ in hair).

Direct hemoglobin measurements were taken from capillary blood obtained with a finger prick and measured using a

FIGURE 2. The AmCS site. The Amarakaeri Communal Reserve is bordered on the southeast by ASGM (formal mining concessions shown in gold, additional informal mining widespread). This comprehensive baseline health study collected data from $N = 4,083$ individuals from $N = 1,221$ households in $N = 23$ communities (purple dots) around the reserve. This figure appears in color at www.ajtmh.org.

Hemocue Hb201+ point-of-care diagnostic device used by the Peruvian Ministry of Health. Serum was collected in BD Vacutainer serum collection tubes with clot activator (BD Worldwide, cat no. 367382). Serum micronutrients (folate, zinc, vitamin A, vitamin D, and vitamin B₁₂) were measured by Roche Elecsys immunoassays at MedLab Peru, a CLIA-certified clinical laboratory in Lima, Peru.

Statistical analysis. Variables. Biomarker values, including hemoglobin, serum micronutrient levels, total hair mercury, and body mass index (BMI) (calculated from measured height and weight), were treated as continuous variables. Anemia status was determined using WHO age- and sex-specific hemoglobin cutoffs: 11 g/dL for children < 5, and 11.5 g/dL for children aged 5–11 years.³⁵ Demographic variables, including age, sex, and community residence, were self-reported on surveys. Weights and heights were recorded for all participants (recumbent height for children below 24 months of age). Anthropometrics, including BMI, height-for-age z-score (HAZ), weight-for-age z-score (WAZ), and weight-for-height z-score (WHZ), using publicly available SAS code published by the Centers for Disease Control (available from

<https://www.cdc.gov/nccdphp/dnpao/growthcharts/resources/sas.htm>). WHO cutoffs were used to define stunting (HAZ < -2), wasting (WHZ < -2), underweight (WAZ < -2), and overweight (WAZ > 2).³⁶

Linear regression models. *AmCS community children dataset.* We assessed variable distributions using univariate analyses, followed by testing a bivariate association between total hair mercury and hemoglobin with simple linear regression. Our final multivariate linear regression model included hemoglobin as the outcome and total mercury as the primary predictor, adjusted for age, sex, and one of the following four growth metrics: BMI, HAZ, WHZ, or WAZ, for a total of four models. HAZ, WHZ, WAZ, and BMI were not included in a single model because of collinearity; all four metrics were tested to assess robustness of analysis to different growth variables. To account for potential variation by community, we ran generalized linear models (PROC GENMOD with BAYES option in SAS 9.4 with uniform priors for regression coefficients, including community as a class variable). We calculated Bonferroni corrections for multiple comparisons by calculating the number of comparisons for

which we performed inference. For the four models (each containing one of four different growth metrics) using the AmCS Community Children dataset, we counted three comparisons of interest: mercury, age, and sex. The critical value for these models is $(0.05/3 = 0.02)$.

AmCS sentinel children dataset. We assessed variable distributions using univariate analyses, followed by testing a bivariate association between total hair mercury and hemoglobin with simple linear regression. To determine micronutrient variable inclusion in multivariate linear regression models, bivariate associations between predictors vitamins B₁₂, A and D, zinc, and folate and outcome hemoglobin and total mercury were tested. The final multivariate linear regression model (PROC REG in SAS 9.4) included predictor total hair mercury and outcome hemoglobin, adjusted for age, sex, significant micronutrients, and one of the following four growth metrics: BMI, HAZ, WHZ, or WAZ, for a total of four models. HAZ, WHZ, WAZ and BMI were not included in a single model because of collinearity; all four metrics were tested to assess robustness of analysis to different growth variables. Similar to the AmCS Community Children analysis, to account for potential variation by community, we ran four generalized linear models (PROC GEN MOD with BAYES option in SAS 9.4 with uniform priors for regression coefficients, including community as a class variable) with the same outcome and predictors as the multivariate linear model, each model with one of four growth metrics. We calculated Bonferroni corrections for multiple comparisons by calculating the number of comparisons for which we performed inference. For the four models using the AmCS Sentinel Children dataset, we counted eight comparisons of interest: mercury, age, sex, and five micronutrients (vitamins B₁₂, A, and D, folate, and zinc). The critical value for these models is $(0.05/8 = 0.01)$.

RESULTS

Median total hair mercury in AmCS Sentinel Children, the subset of children < 12 with micronutrient testing, was 1.18 µg/g (range: 0.06–9.70 µg/g), with 58% ($N = 48/83$) above the current EPA threshold of 1.0 µg/g (using a benchmark dose derived from prenatal exposures during a grain poisoning event in Iraq),³⁷ 49% ($N = 41/83$) above the updated threshold of 1.2 µg/g recommended by the National Research Council (using a benchmark dose derived from prenatal exposures due to contaminated seafood consumption in the Faroe Islands),³⁴ and 43% ($N = 36/83$) above the WHO guideline of 2.0 µg/g³⁸ (Figures 4 and 5). Median total hair mercury in AmCS Community Children dataset was 1.03 µg/g (range: < LOD–21.3 µg/g; 25% ($N = 350/1,387$) exceeded 1.0 µg/g, 22% ($N = 307/1,387$) exceeded 1.2 µg/g, and 15% ($N = 204/1,387$) exceeded 2.0 µg/g (Supplemental Figure 2). Total hair mercury was not significantly different between the AmCS Sentinel Children dataset and children < 12 in the larger AmCS Community Children dataset.

In the AmCS Sentinel Children sample, 49% ($N = 29/59$) of children aged < 5 years were anemic (WHO anemia threshold 11 g/dL) as well as 52% ($N = 12/23$) of those aged 5–11 years (WHO anemia threshold 11.5 g/dL) (Figure 3). Only 12% of children were stunted, indicating impaired growth in early childhood (Table 1). Only 2% of children presented with wasting, and only 5% presented as underweight, indicating good nutritional status at the time of sampling (Table 1). An additional 5% of children were overweight (Table 1). Fifteen children were deficient in zinc, seven in vitamin B₁₂, three in vitamin A, and one in folate; three children had two deficiencies and the remaining 22 children had only one deficiency (Table 2). None were deficient in vitamin D (Table 2). In the larger AmCS Community dataset, 45% of children < 5

FIGURE 3. Anemia rates in sentinel group children < 12. Anemia prevalence (red) by age group in a subset of children < 12 ($N = 83$) chosen from sentinel households (defined as comprising a WCBA, a spouse/partner, and at least one child in the household) and selected for micronutrient testing. Anemia thresholds per WHO guidelines (blood hemoglobin < 11 g/dL for children below 5 years and < 11.5 g/dL for children aged 5–11 years). This figure appears in color at www.ajtmh.org.

FIGURE 4. Total hair mercury levels by anemia status in sentinel group children < 12. Violin plots of total hair mercury levels (µg/g) by age group and anemia status in a subset of children < 12 ($N = 83$) chosen from sentinel groups (defined as comprising a WCBA, a spouse/partner, and at least one child in the household) for micronutrient testing. Aqua lines refer to WHO (2 µg/g) and USEPA/NRC (1.2 µg/g) hair reference levels. Anemia thresholds per WHO guidelines (blood hemoglobin < 11 g/dL for children below 5 years and < 11.5 g/dL for children aged 5–11 years). This figure appears in color at www.ajtmh.org.

FIGURE 5. Total hair mercury is inversely associated with hemoglobin in sentinel group children < 12. Unadjusted bivariate association ($\beta = -0.12$, $P = 0.06$) is shown here. The inverse relationship between mercury and hemoglobin remained significant in the final model ($\beta = -0.14$, $P = 0.04$), adjusted for age, sex, HAZ, WHZ, and vitamin B₁₂. Sentinel group children are a subset of all children < 12 in the parent study (AmCS) that were classified as belonging to “sentinel groups,” defined as comprising a WCBA, a spouse/partner, and at least one child in the household. This figure appears in color at www.ajtmh.org.

($N = 199/439$) and 37% of children 5–11 years ($N = 259/709$) were anemic (Supplemental Figure 1). Anemia prevalence was slightly lower in the larger AmCS Community Children dataset; 45% ($N = 199/439$) of children < 5 were anemic and 37% ($N = 259/708$) of children aged 5–11 years were anemic (Supplemental Figure 1). This group difference may be due to a difference in average age between the groups; hemoglobin levels and age were both significantly, if slightly, lower in the AmCS Sentinel Children dataset (average hemoglobin 11.1 g/dL and average age 4 years old) as compared with children < 12 in the AmCS Community Children dataset (average hemoglobin 11.6 g/dL and average age 6 years old).

In the AmCS Community Children dataset, the initial bivariate association between total hair mercury and hemoglobin was not statistically significant (total mercury $\beta = -0.04$,

$P = 0.10$, critical value 0.02), and remained nonsignificant after adjustment for age, sex, and BMI (total mercury $\beta = -0.03$, $P = 0.19$, critical value 0.02) (results similar for all four growth metrics). However, age was a strong predictor of hemoglobin level ($\beta = 0.14$, $P < 0.0001$, critical value 0.02), supporting an age group-specific effect. We observed similar results in generalized linear models (with BMI as growth variable; total mercury $\beta = -0.03$, 95% CI: -0.08 , 0.02), with results again similar for all four growth metrics.

When we tested the same bivariate association between total mercury and hemoglobin in the AmCS Sentinel Children dataset, which contained a higher proportion of anemic children, we observed an inverse relationship between total hair mercury and hemoglobin ($\beta = -0.12$, $P = 0.06$, critical value 0.01) (Figure 5). Of the five serum micronutrients tested, mercury was significantly associated with vitamin B₁₂ ($\beta = 20.2$, $P = 0.04$) and vitamin A ($\beta = 0.01$, $P = 0.06$, critical value 0.01), but not with zinc, folate, or vitamin D. No micronutrients were associated with hemoglobin level. Vitamin B₁₂ has a known biological link to regulation of red blood cell counts.^{7,8} A positive relationship between vitamin A and hemoglobin has been reported previously, but the biological link between the two is unclear.³⁹ The inverse relationship between mercury and hemoglobin remained significant (total mercury $\beta = -0.18$, $P = 0.01$, critical value 0.01) in the model adjusted for age, sex, BMI, and vitamin B₁₂, although vitamin B₁₂ was no longer significant in the model (Figure 5). However, this association disappeared when vitamin A was added to the model (total mercury $\beta = -0.09$, $P = 0.23$, critical value 0.01). We observed a similar association between total mercury and hemoglobin in a generalized linear model (including total mercury, age, sex, BMI, and vitamin B₁₂) (total mercury $\beta = -0.18$, 95% CI: -0.31 , -0.046 ; vitamin B₁₂ $\beta = 0.003$, 95% CI: -0.002 , 0.003) that was no longer evident after addition of vitamin A (total mercury $\beta = -0.09$, 95% CI: -0.22 , 0.044). Results for both sets of models were very similar when any of the alternate three growth metrics (HAZ, WAZ, or WHZ) were included in the model.

DISCUSSION

In this study, we investigated a potential link between hair total mercury exposure and anemia in a highly exposed population ($N = 2,237$) and assessed potential roles for selected micronutrients in a subset of exposed children ($N = 83$). The hair total mercury exposure levels that we observed are

TABLE 1

Baseline characteristics of sentinel group children aged < 12 years ($N = 83$) from the AmCS selected for micronutrient testing

		Proportion	%
Sex	Male	38/82	46%
	Female	44/82	54%
Anemia*	Yes	40/82	49%
	No	42/82	51%
Growth metrics*	Stunted	10/82	12%
	Wasted	2/82	2%
	Underweight	4/82	5%
	Overweight	4/82	5%

* Anemia thresholds and growth metrics as per WHO guidelines. Hemoglobin data on $N = 82/83$ children. Sentinel group children are a subset of all children < 12 in the parent study (AmCS) who were classified as belonging to “sentinel groups,” defined as comprising a WCBA, a spouse/partner, and at least one child in the household.

TABLE 2

Serum micronutrient values in sentinel group children aged < 12 years ($N = 83$) from the AmCS

	<i>n</i>	Mean	Standard Deviation	Minimum	Maximum
Hemoglobin (g/dL)	82	11.1	1.3	6.5	13.5
Zinc (µg/dL)	52	86	32	41.4	191.4
Vitamin A (µg/dL)	67	36	11	18	70
Vitamin D (pg/mL)	78	760	130	470	1,090
Vitamin B ₁₂ (pg/mL)	83	373	200	126	768
Folate (ng/mL)	83	11.6	5.08	4.9	40

WHO guidelines for micronutrient deficiencies: serum folate < 5 ng/mL, serum vitamin B₁₂ < 203 pg/mL, serum zinc < 65 µg/dL, serum vitamin A < 20 µg/dL, and serum vitamin D < 300 pg/mL. Anemia thresholds as per WHO guidelines (blood hemoglobin < 11 g/dL for children below 5 years and < 11.5 g/dL for children aged 5–11 years). Proportions of anemic children in each age group are shown in Figure 2. Sentinel group children are a subset of all children < 12 in the parent study (AmCS) who were classified as belonging to “sentinel groups,” defined as comprising a WCBA, a spouse/partner, and at least one child in the household.

comparable to reported exposures in WCBA (aged 15–49) and children in populations in the Faroe Islands^{32,40} and the Republic of Seychelles^{33,40} with high dietary exposure through seafood consumption. Neurocognitive delays and dysfunctions have been reported in children in both populations,^{32,33} supporting the public health significance of our reported exposures. The anemia rates that we observed are notably higher than those in the 2012 Demographic Health Survey in Peru,⁴¹ which reported 17.7% anemia in WCBA (as compared with rates ranging from 30% to 47% in all individuals aged 15–65 in our data, Supplemental Figure 1) and 28% in children below 6 years of age (as compared with 45% in children below 5 years of age in our data, Supplemental Figure 1). We observed that total hair mercury level was negatively associated with Hb in children. Few children were stunted, wasted, or micronutrient deficient, suggesting anemia was not due to malnutrition. In addition, we noted an inverse relationship between Hb and vitamin B₁₂, as well as a positive association between hemoglobin and vitamin A. Vitamin B₁₂ deficiency is an established risk factor for anemia and may be mimicked or exacerbated by methylmercury exposure^{7,42–44}; our data confirm a previous report of a positive link between hemoglobin and vitamin A, which may play a role in iron absorption or in hepcidin-mediated iron homeostasis.^{39,45} Our observation that the relationship between hair total mercury and hemoglobin shifted with inclusion of one or more micronutrients to the model emphasizes the importance of accounting for micronutrient status in similar studies of anemia. To the best of our knowledge, these results represent the first report of a link between hair total mercury exposure and anemia in a highly exposed human population that has higher rates of infectious and chronic disease and lower socioeconomic status than well described populations with comparable exposures, such as those in the Faroe Islands or Seychelles.^{32,33}

Although we did not observe many micronutrient deficiencies in this study, low folate and vitamin B₁₂ are highly feasible risk factors for anemia in Peruvian populations. Peru currently has a national folic acid fortification program, but Peruvian legislative requirements fall below WHO standards of 2.6 mg/kg flour for prevention of neural tube defects.⁴⁶ Folic acid supplementation impacts risk for both folate and vitamin B₁₂ deficiencies.^{47–49} A recent review of folate and vitamin B₁₂ deficiencies in Latin America and the Caribbean reported folate deficiency rates of 2–54% in children (up to 80% in adults) and vitamin B₁₂ deficiency rates of 5–61% in children (up to 49% in pregnant women).⁸

We included measures of body mass in this analysis as measures of nutritional adequacy, but growth metrics or BMI may function also as confounders because body fat percentage is likely linked to the internal dose of methylmercury. In the past, methylmercury was thought to be lipid soluble and to accumulate in fat depots in both fish and humans, perhaps explaining high methylmercury accumulation in lipid-rich brain tissue and subsequent neurotoxicity.⁵⁰ However, newer evidence suggests that methylmercury is transported in the body primarily in water-soluble complexes with cysteine and is likely restricted to lean body mass,⁵⁰ suggesting that lower lean body mass may be protective against high internal methylmercury doses. Methylmercury–cysteine has a chemical structure very similar to the essential amino acid methionine.⁴⁰ In this form, methylmercury can cross the blood–brain barrier “disguised” as an amino acid using neutral amino acid carrier

transport.⁴⁰ In fact, methylmercury uptake in the brain is inhibited by the presence of other amino acids,⁴⁰ suggesting the potential for increased brain uptake of methylmercury in the context of protein deficiency. Although the relatively low observed rates of stunting and wasting in this subsample suggests our study participants have protein-replete diets, we suggest consideration of protein deficiency as a potential risk factor for methylmercury exposure in food-insecure populations.

Although we observed relationships between only two of five tested serum micronutrients (vitamin B₁₂ and vitamin A) and either hemoglobin levels or total hair mercury in this study, the micronutrients tested should be considered as covariates in future assessments of environmental determinants of anemia risk. Some micronutrients have clear, unidirectional relationships with anemia status, albeit undetected in this analysis, including vitamin D and zinc. Prior epidemiologic studies have shown that vitamin D levels were inversely associated with anemia risk,^{27,51,52} perhaps by inhibiting inflammatory cytokine production in the bone marrow and subsequent iron sequestration in body tissues stimulated by the peptide hormone hepcidin.²⁷ In the context of deficiency, vitamin D supplementation may protect against anemia by decreasing circulating hepcidin.⁴ However, excess vitamin D may not be beneficial. A high vitamin D diet has been linked to low immature red blood cell counts and plasma levels of erythropoietin, which stimulates red blood cell production.⁵² Zinc is directly involved in heme synthesis via activation of ALAD, an essential enzyme in the heme biosynthetic pathway,⁵³ and zinc deficiency is an established risk factor for anemia.⁵³ Dietary zinc supplementation may be an effective intervention in regions with high enteric disease; zinc supplements increased hemoglobin concentrations in rats infected with anemia-inducing intestinal worms.⁵³ Micronutrients that are coregulated with iron or those impact growth and neurocognitive outcomes independently of iron or methylmercury likely have more complex roles in anemia risk. Iron,⁵⁴ vitamin B₁₂,⁹ and methylmercury^{34,37} can all impact neurocognitive status in children independently. In addition, iron aids absorption of provitamin A carotenoids from foods, suggesting that low iron may inhibit absorption of vitamin A, another micronutrient essential for normal neurocognitive development.³⁹ In this study, few children were deficient in either folate or vitamin B₁₂, but these two micronutrients are tightly coregulated with iron status, and a deficiency in one of these three can mask deficiencies in the others. Alternatively, iron, folate, and vitamin B₁₂ deficiencies often occur in the same individuals,^{4,7,9,48,49,55,56} suggesting that micronutrient-replete participants in this study are less likely to be iron deficient. Because we did not directly measure biomarkers of iron status in this study, we cannot rule out potential roles for any of these three micronutrients in the observed anemia cases. Based on this prior evidence, we suggest that future studies incorporate measures of these and other biologically relevant micronutrients in assessment of hemoglobin status and anemia risk.

Some discussion of the use of total hair mercury as a biomarker is warranted, particularly in a study of ASGM communities where multiple routes of mercury exposure are possible. The current scientific consensus is that the majority (80–90%) of total mercury in hair is monomethylmercury in communities exposed primarily to methylmercury (e.g., via fish consumption)^{20,34,40,57} and that hair total mercury is an

excellent biomarker for chronic environmental methylmercury exposure. Hair total mercury is a much poorer biomarker for occupational or environmental inorganic mercury exposure^{20,34,40,57} due to inconsistent or much lower incorporation rates of inorganic mercury relative to methylmercury in hair follicles.⁵⁷ Nevertheless, total mercury in hair has the potential to reflect both methylmercury exposure (via ingestion) and inorganic mercury exposure (via inhalation, ingestion) as well as ex situ routes of incorporation (e.g., adsorption of gaseous mercury onto hair). Because participants in this study may potentially have mixed exposures, and because fish consumption for each participant is not known, it is possible that hair total mercury is reflecting both inorganic mercury and methylmercury exposure. However, because of the age range of the study cohort, we believe it unlikely that they were participating in the amalgamation process. Regardless, because inorganic mercury and methylmercury are equally biologically feasible risk factors for anemia (see next section), we are confident in using hair total mercury as a biomarker in this study.

Methylmercury-induced anemia is biologically feasible.

Importantly for interpretation of results and consideration of future steps, the observed association between methylmercury exposure and anemia status is biologically feasible. Based on known chemical properties and prior evidence in the literature, we have formed four main hypotheses for a mechanistic link between methylmercury exposure and anemia risk (Figure 1).

Methylmercury can induce eryptosis due to excessive oxidative damage. Erythrocytes, or red blood cells, can undergo senescence and clearance with age.²⁶ They can also be cleared by suicidal red blood cell death, or eryptosis, if they are defective, overly abundant, or potentially harmful, such as malaria-infected cells.²⁶ Eryptosis involves engulfment and degradation of damaged cells, allowing elimination without release of intracellular proteins, which would trigger inflammation.²⁶ Methylmercury accumulates in red blood cells via high-affinity binding to hemoglobin. Methylmercury also binds and inactivates thiol group-containing antioxidant defense enzymes that scavenge ROS, leading to passive increases in intracellular reactive oxygen species (ROS) and oxidative damage to DNA, membrane lipids, and intracellular proteins.^{19,20,58} Mature erythrocytes lack nuclei and most organelles and do not produce proteins; therefore, irreparably damaged cellular proteins cannot be replaced and result in loss of cell function.⁴⁰ Because hemoglobin constitutes more than 90% of the cell's total protein, it is a critical target of oxidative damage.^{40,59} Severe damage leads to eryptosis; sufficient levels of eryptosis lead to anemia. Eryptosis secondary to oxidative damage is a documented effect of exposure to cadmium,^{26,60} lead,^{25,26} aluminum,²⁶ copper,²⁶ nickel,²⁶ and inorganic mercury.²⁶ Therefore, the typical anemia seen after lead intoxication is at least partly due to enhanced eryptosis.²⁵

Other risk factors can feasibly interact with methylmercury to increase risk for anemia through an eryptotic mechanism. For example, iron-deficient erythrocytes are more sensitive to eryptosis.²⁵ In addition, iron deficiency leads to a greater relative proportion of newly formed red blood cells, which are particularly vulnerable to cell destruction through a process termed neocytolysis,^{25,26} which enables clearing of excess cells after a rapid increase due to a transient environmental trigger, such as high altitude.²⁶ In addition, age is an important

predictor of eryptosis.⁶¹ Enhanced eryptosis may contribute to anemia in the elderly, due to higher oxidative stress and lower bioavailable glutathione in older persons.⁶¹

Methylmercury can mimic or exacerbate vitamin B₁₂ or folate deficiency. Folate or vitamin B₁₂ deficiencies can lead to anemia by disrupting and reducing DNA synthesis.^{4,7,47,48} Vitamin B₁₂- or folate-deficiency anemia is thought to be less common than IDA,⁷ but this may not be the case in developing countries. Vitamin B₁₂ is a cofactor of methionine synthase, which converts 5-methyl-tetrahydrofolate (5MTHF) to tetrahydrofolate as part of the one-carbon metabolism cycle.^{47,48} Low vitamin B₁₂ can lead to buildup of precursor 5MTHF, or "methyl trapping"; because 5MTHF is often measured as a marker of total body folate, "methyl trapping" can mask underlying folate deficiency.^{48,55} Therefore, although folate levels are normal in this study, that does not rule out possible folate deficiency in study participants. Methylmercury also inhibits methionine synthase,^{42,43} which suggests that methylmercury can induce anemia through a similar mechanism as vitamin B₁₂ deficiency. In addition, transport of 5MTHF into cells requires sulfhydryl-dependent mechanisms.⁴⁴ Because methylmercury exerts much of its toxicity via binding sulfhydryl groups,^{20,34,59} methylmercury exposure may limit 5MTHF transport and its use in intracellular one-carbon metabolism.

Methylmercury can dysregulate iron homeostasis. In addition to the two iron-independent mechanisms described previously, methylmercury feasibly can induce anemia by directly dysregulating iron homeostasis through disrupted hepcidin-ferroportin signaling.⁵⁸ There is no mechanism for iron excretion; therefore, the body regulates iron status by controlling iron uptake into the bloodstream from the gut, and from the bloodstream into body tissues. Ferroportin is a pore that allows intestinal absorption and organ uptake of iron. Hepcidin is a peptide hormone derived from hepatocytes that promotes ferroportin degradation, thereby slowing iron absorption and organ uptake, in response to iron status, hypoxia, anemia, and inflammation.⁵⁸ Therefore, low hepcidin leads to high circulating iron; high hepcidin leads to low circulating iron and limits pathogen access to iron during infections. Low circulating iron can lead to low blood hemoglobin, or apparent anemia, even if total body stores of iron are normal. Chronically high levels of hepcidin and depressed iron absorption in the gut due to chronic low-level inflammation that is a hallmark of chronic disease that can lead to anemia or commonly classified as anemia of chronic disease (ACD).^{6,7,62} Methylmercury exposure leads to passive increases in ROS and resultant inflammation and oxidative damage^{20,26,63}; this low-level inflammatory response to methylmercury may mimic ACD.

Alternatively, methylmercury exposure may directly repress transcription of hepcidin. The hepcidin gene promoter contains an estrogen response element (ERE) that is responsive to endogenous estradiol or endocrine active xenobiotics.^{28,58} Endogenous estradiol can inhibit gene expression by binding estrogen receptor α (ER α), which binds both an ERE in the target gene and a corepressor molecule that represses gene expression.⁶⁴ Estradiol-bound ER inhibits hepcidin expression in vitro^{65,66} and in vivo.^{65,66} Methylmercury competes with estradiol for ER binding in the brain,⁶⁷ suggesting that women, who have higher estradiol levels, would experience some protection from methylmercury toxicity. Polychlorinated biphenyls (PCBs), cadmium, and chlorpyrifos are known to alter expression of hepcidin and ferroportin and disrupt

signaling.⁵⁸ Hepcidin expression is reduced by more than 65% after short-term exposure to PCB-77 in vitro and in vivo, and ferroportin expression is altered after short-term exposure to cadmium and chlorpyrifos.⁵⁸ In particular, PCBs have demonstrated functional interaction with the hepcidin promoter and are sufficient to decrease hepcidin and increase serum iron in vitro and in vivo.^{29,58} Methylmercury may, like PCBs, chlorpyrifos,⁵⁸ and other heavy metals,⁶⁸ bind an ERE in the gene promoter and repress hepcidin. These data suggest the possibility for endocrine active chemicals, including methylmercury, dichloro-diphenyl-trichloroethane, and dioxins, to impact iron homeostasis in two directions. First, endocrine-active chemicals can bind ERs and repress hepcidin, raising serum iron levels, which might stimulate ROS production. Second, these same chemicals can induce oxidative stress through previously described mechanisms, and the resulting inflammation can trigger the activation of hepcidin, lowering serum iron levels. In fact, DDT exposure has been linked to anemia, including reports of aplastic anemia and dose-dependent anemia due to long-term inflammation.⁵⁸

Methylmercury can induce mitochondrial anemia. A fourth possible mechanism that could contribute to anemia risk is development of mitochondrial anemia. Although mature red blood cells lack both mitochondria and nucleus, both are present in immature reticulocytes and have important roles in cell development. Mitochondria are required for iron loading and heme biosynthesis.⁶⁹ When hemoglobin synthesis reaches a threshold, erythrocyte precursors enucleate and exit the bone marrow into the bloodstream, where they undergo final maturation, including clearance of residual mitochondria and transferrin receptors.⁶⁹ Elevated mitochondrial DNA (mtDNA) mutagenesis can impair erythrocyte maturation by delaying mitochondrial clearance, resulting in lipid oxidation and membrane damage and cell removal via eryptosis.⁶⁹ Excessive red blood cell loss through eryptosis can lead to anemia.⁶⁹ Notably, mitochondrial anemia only occurs in animal models that present with mtDNA mutagenesis in hematopoietic stem cells in early life; adult onset mitochondrial diseases do not present with anemia in mice.⁶⁹ These data suggest that developmental, but not adult, exposure to environmentally mediated mtDNA mutagenesis can impact the anemia risk.

In this study, we were unable to assess mitochondrial retention in red blood cells from exposed individuals, or to identify a subset of participants with known exposure limited to fetal or infant development. However, unpublished data from our research group suggest that higher methylmercury exposure is associated with higher levels of mtDNA damage (AJ Berky et al., unpublished data.) If confirmed as a mechanism of methylmercury -induced hemotoxicity, this result suggests administration of dietary or pharmacological antioxidants to methylmercury-exposed individuals during fetal and infant development as a potential mitigating intervention.

In addition to these four potential mechanisms linking organic mercury exposure with anemia, it is worth noting that some inorganic heavy metals can trigger anemia through mechanisms that are relevant to inorganic mercury exposure. These mechanisms may provide small contributions to anemia risk in the context of mixed species mercury exposure, or in the event of low-level methylmercury conversion to inorganic mercury in the body. For example, chronic cadmium exposure can lead to renal anemia by decreasing erythropoietin synthesis; inorganic mercury is known to accumulate in

the kidney and trigger renal anemia at high doses. High oral intake of lead, cadmium,⁶⁰ or cobalt, and possibly arsenic, competes with iron uptake in the gut and leads to reduced iron absorption, potentially leading to IDA.⁵⁸ The reverse is true, as well; low body iron stores can increase intestinal absorption of divalent metals, including lead, cadmium,⁶⁰ and cobalt.⁵⁸

Accurate diagnosis of anemia etiology is essential for safe, effective treatment. Until recently, iron supplementation has been the primary response to suspected IDA recommended by the WHO,¹³ due to the health impacts of low iron, including stunted growth, delayed or impaired neurocognitive function, and impaired immune function.⁴ However, recent observations raise concerns about the safety and efficacy of iron supplementation before definitive diagnosis of IDA, particularly in the context of high infectious disease burden, including enteric disease and malaria.¹³ A large, randomized clinical trial of iron and folic acid supplements in anemic Tanzanian children showed supplemented children, particularly those with no iron deficiency, suffered higher malaria morbidity and mortality.^{13,70} Iron supplementation in infected children likely counteracted the hepcidin-mediated decrease in circulating iron that is a normal physiologic response to infection, to limit iron availability to proliferating pathogens.⁷⁰ These results led to the release of a joint statement by the WHO and the United Nations Children's Fund revising iron and folic acid recommendations in malaria endemic regions and a renewed awareness of the need to distinguish IDA from inflammatory anemia, or ACD.¹³ The Iron and Malaria Project, a collaboration among the Gates Foundation, the National Institute for Child Health and Development, and WHO, has published reports aimed at informing the development of accurate and reliable biomarkers of IDA.¹³ In addition, the Centers for Disease Control and the Global Alliance for Improved Nutrition are using national data from 18 countries to determine the influence of inflammatory status on measures of iron homeostasis, including serum iron and serum ferritin.¹³ The INSPIRE (Inflammation and Nutrition Science for Programs/Policies and Interpretation of Research Evidence) project recommends routine assessment of inflammatory status with C-reactive protein and alpha-1-acid glycoprotein for clinical interpretation of patient and population micronutrient, including iron, status.¹³

Global health studies should include chemical exposure assessment with traditional determinants of anemia. The global health community recognizes the need to distinguish IDA from ACD, and research and policy approaches for addressing this challenge are ongoing. However, none of these large consortia incorporate or consider environmental toxicant exposures as risk factors for anemia, despite documented evidence of heavy metal-induced anemia and rapidly increasing exposures to chemicals that may contribute to anemia, including methylmercury. Based on prior literature and our current data, we urge the global health community to incorporate direct measurements of methylmercury, with validated biomarkers of nutritional status, iron homeostasis, and inflammation, in populations with known or suspected methylmercury exposure, such as ASGM communities, to carefully determine the relative contributions of each risk factor for anemia in these populations. This approach has strong potential for significant impact on integration of chemical exposures in classification and treatment of anemia in global health settings.

This study has several notable strengths, including direct assessment of hair total mercury, which likely represents primarily methylmercury exposure in the context of contaminated fish consumption, and levels of relevant micronutrients in a rural Peruvian population with high anemia risk. Our results provide preliminary evidence that methylmercury exposure increases the likelihood of anemia in high-risk settings. As this study was not designed initially to test for a relationship between methylmercury and anemia, we did not include measurements of iron homeostasis and inflammatory status that would improve our effect estimates. In addition, we did not test for genetic hemoglobin disorders, including thalassemia and sickle cell disease. Although these diseases are thought not to be highly prevalent in this population, data on these disorders in the Amazon are sparse. Future studies that incorporate these measurements will also enable assessment of multiple exposures that impact similar health endpoints. For example, lead exposure is known to induce anemia, stunt growth, and impair neurocognitive function; iron deficiency has similar impacts on the same health endpoints.

This work supports a potential link between methylmercury exposure and anemia and highlights the importance of epidemiology in environmental toxicology studies. In particular, this research highlights the need to expand global toxicology research to include high exposures to chemicals like methylmercury, that preferentially and increasingly impact poor, rural communities in LMIC countries.

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