Childhood Anemia: Screening Tools, Risk Factors, and Barriers to Treatment in Tumbes, Peru

By

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A DISSERTATION

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CERTIFICATE OF APPROVAL

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ABSTRACT OF THE DISSERTATION

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Anemia impacts nearly a third of the global population, with the highest burden being in developing nations among women and children under the age of 5 years[1]. During early development, anemia can be especially detrimental due to its impact on cognitive and motor development, resulting in lifelong consequences[2]. For this reason, many governments in low and middle income countries (LMICs) have declared anemia to be a national priority[3–5]. Iron deficiency anemia (IDA) is assumed to be the leading cause of anemia and many intervention strategies have been designed under this assumption. However, other contributing causes such as vitamin B deficiency, Pb exposure, blood loss, chronic renal failure, and inflammation exist in the targeted populations and should be considered. More upstream causes, such as access to care should also be explored, especially in places with extensive interventions but minimal changes in childhood anemia prevalence.

Tumbes, Peru, where the estimated prevalence of anemia is 42% in children ages 6-59 months, is a region with little to no change in childhood anemia prevalence despite extensive intervention[6]. Current interventions function on the assumption that iron deficiency anemia and parasitosis are primary causes of childhood anemia in this region, and that screening efforts are successful in

identifying children requiring treatment. However, many communities in Tumbes have documented exposure to heavy metals which could also be contributing to the causes of anemia, and which might not be impacted by current interventions. My dissertation research challenges these assumptions by 1) evaluating the relationship of MeHg, Pb, As and Mn with hemoglobin, 2) exploring factors associated with persistent anemia, and 3) comparing and evaluating the validity of anemia screening tools in two districts of Tumbes, Peru.

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LIST OF ABBREVIATIONS

ALAD	δ-aminolevulnic acid dehydratase
AS	Arsenic
ASGM	Artisanal and Small-scale Gold Mining
ATSDR	Agency for Toxic Substances and Disease Registry
C	Celsius
Cd	Cadmium
CI	Confidence Interval
Coef.	Coefficient
CRP	C-reactive Protein
DMT1	Divalent Metal Transporter
DNA	Deoxyribonucleic Acid
DOHaD	Developmental Origin of Health and Disease
EDTA	Ethylenediamine tetraacetic acid
EPA	Environmental Protection Agency
HB	Hemoglobin
Hg	Mercury
IDA	Iron Deficiency Anemia
INEI	Institución Nacional de Estadistica e Informática
IQR	Interquartile range
LMICs	Low and Middle Income Countries
MCV	Mean corpuscular volume
MeHg	Methylmercury
Mn	Manganese
Pb	Lead
RBC	Red Blood Cell
sABC	Semi-Automated Blood Count
SD	Standard Deviation
THg	Total Mercury
WHO	World Health Organization

CHAPTER 1. INTRODUCTION & RESEARCH AIMS

1.1 Introduction

Globally, nearly half of all preschool aged children are anemic, of which a disproportionate number come from LMIC's[1,2]. While several nations in Latin America, such as Chile and Costa Rica have low prevalence of anemia, much of Latin America still has high levels of anemia, especially in women of childbearing age and children under 6 years of age[3]. In Peru, the average prevalence of anemia in 2020 among children ages six to 35 months was 40.1%, likely resulting in substantial long-term harm[4]. In a report conducted in 2013, the Peruvian government estimated that economic loss due to cognitive and motor deficits, as well as increased attention in health centers from anemia, cost the Peruvian economy approximately 1 billion dollars during that year alone[5]. Because the prevalence of anemia in Peru is high, identifying modifiable risk factors, even with weak associations to anemia, could have notable impacts at the population level.

Anemia during early childhood likely increases risk of impaired neurocognitive development. Although, a majority of existing studies focus on anemia during the perinatal and neonatal periods, epidemiologic studies provide evidence of lower neurocognitive scores later in life for children with anemia at age 12 to 18 months old versus those without.[6,7] Iron deficiency has been proposed as the main mechanism by which neurocognitive impairments among young children with anemia occur. However, mixed results from randomized control studies of iron supplementation warrant the investigation of additional pathways (e.g. infectious, environmental) by which anemia occurs and how it is best treated[8–13]. No matter the pathway, decreased neurocognitive function has life-long implications that diminish quality of life and substantially impact local and regional economies[5]. Because the first three years of life are critical for

neurodevelopment, effective anemia interventions are especially important during early childhood.

Although much of the global burden of anemia is caused by iron deficiency, other common exposures in developing nations likely contribute to this burden and should be considered in prevention and treatment. It is estimated that iron deficiency accounts for approximately 60% of the global anemia burden, and that vitamin B12 and folate deficiency account for another 15%[1,14]. Infectious disease, such as hookworm and malaria are also known to contribute to the global burden of anemia. Twenty-five percent of anemia cases, however, have no specific identified cause. Growing evidence suggests that environmental exposure to heavy metals such as MeHg, Pb, As, and Mn from pollution and mining may also impact hemoglobin levels[15–18]. Because LMIC's bear a greater burden of pollution, malnutrition, and anemia, it is critical to reevaluate the assumption that iron deficiency is the main driver of anemia in these settings. Environmental exposure to heavy metals has the potential to broadly affect populations; therefore, evaluating heavy metal exposure where source activity is known is important.

Artisanal gold mining is a leading source of chronic mercury exposure in many developing nations and has been associated with risk of anemia in small studies[15,19,20]**.** In subsistence agricultural settings like Tumbes, Peru, the presence of mercury in water systems and sediment can lead to high exposure of people to methylmercury through ingestion of fish or crops that have accumulated methylmercury through the process of biomagnification[21]. Recent studies have confirmed that small scale and artisanal gold mining in Portovelo, Ecuador, have resulted in extensive heavy metal contamination of the Tumbes-Puyango River which flows downstream across the border into Tumbes. Because the mining contamination by mercury and other heavy metals such as Pb is chronic and spans decades, the potential for human exposure and adverse

health effects is high [22–24]. The Tumbes-Puyango River serves as the primary source of freshwater and subsistence fish foods for the local population. Studies conducted by the local government and universities have already demonstrated high Pb levels in water and elevated Hg levels in fish in many parts of Tumbes (personal communication Arq. Diana Barreras)[25].

Five years of extensive nutrition and parasite treatment campaigns across Peru since 2009 led to a 10% reduction in anemia prevalence at the national level. However, five regions, including Tumbes, saw little to no change in anemia prevalence[26]. The regional government was tasked with improving anemia intervention strategies, and to effectively move forward to identify gaps in existing interventions and evaluations. The most notable gaps in knowledge include whether heavy metal exposure in communities downstream contributed to anemia prevalence, as standard treatment primarily assumes nutritional deficiencies and not environmental exposure. Another suspected contributing factor was validity of the Hemocue201, the standard screening tool in Peru. Adjusting standard strategies requires a better understanding of both before intervention design.

Anemia is a condition that is highly prevalent worldwide and most prevalent in LMIC's with a greater burden of pollution, malnutrition, and infectious disease. Therefore, evaluating the extent of heterogeneity in anemia etiology across different settings as well as the validity of tools commonly used to screen and monitor anemia are critical for improving anemia control strategies. Understanding these differences are both governmental and community priorities, meaning that there is ample opportunity for community engaged research that holds potential for developing strategies that not only decrease anemia prevalence but also bridge inequities. My dissertation research was developed in collaboration with local leaders, and aims to address the above knowledge gaps to facilitate future intervention.

1.2 Dissertation Overview & Research Aims

My dissertation begins with a review of current literature on anemia including its various etiologies, treatments, risk factors, and screening tools in Chapter 2 (Review of Literature). I begin by providing an overview of the global burden of anemia and its implications. I then explore the complex etiology of anemia and provide background on heavy metal toxicology and its implications on hemoglobin. I end with a summary and discuss the relevance of the research that is described in later chapters.

In chapter 3, I describe the distribution of heavy metals, anemia, and hemoglobin concentration in a population where chronic exposure from ASGM is suspected, adjusting for individual and combined multiple-heavy metals (THg, Pb, As, and Mn) and controlling for biomarkers of micronutrients, inflammation, and infection. I measure individual associations by conducting univariate analysis of hemoglobin concentration with THg, Pb, As, and Mn in hair as well as serum ferritin, serum folate, serum vitamin B12, blood Pb, serum C-reactive protein, presence of giardia in stool, sex, age, breastfeeding status, and birthweight. I then estimate multiple metal adjusted associations with hemoglobin concentration using linear regression to build two models; one of THg, Pb, As, and Mn in hair as continuous log-transformed variables and another as categorical tertiles to explore non-linear associations. I then evaluate effect modification by sex, district, serum ferritin tertiles, folate tertiles, and vitamin B12 tertiles by examining statistically significant interactions with continuous measures and clinically significant differences by stratification of tertiles.

In chapter 4, I evaluate anemia persistence 4 months post-diagnosis and referral among children who screen positive, in order to identify risks for persistent anemia and to evaluate barriers to effective treatment after referral. I achieve this by conducting univariate analysis of factors

described in the previous chapter with persistent anemia and conducting t-test for continuous variables and chi-squared tests for categorical variables for statistical significance. I also explore factors associated with treatment completion based on child demographics and parental responses on a survey about accessing and adhering to treatment.

In chapter 5, I compare the accuracy of the Hemocue 301 with the standard point-of-care Hemocue 201 gravity test in screening for anemia, and explore potential effect modifiers. In the same study population described in previous chapters, I compare Hemocue 301 validity against the conventionally used Hemocue 201 finger prick test. I use the semi-automated blood count hemoglobin results as a gold standard and conduct a non-inferiority test of the Hemocue 301 sensitivity compared to the Hemocue 201. I discuss implications of these results for standard screening methods.

In chapter 6, I summarize findings of each analysis and the overall implications for improving anemia control strategies. I discuss findings in the context of anemia screening, anemia treatment, and anemia monitoring over time. I provide recommendations for the local government where we conducted our studies, and discuss relevance of our findings to other similar settings. Finally, I describe ongoing work and future directions that have been built from the dissertation research described.

CHAPTER 2. REVIEW OF THE LITERATURE

2.1 Burden of Anemia

Over a third of the world has anemia, with the greatest burden being in women of reproductive age and children under the age of five years[1]. Eighty-nine percent of the global burden is in low resource settings, meaning that on a global scale, strategies to decrease anemia prevalence should be practical and cost-effective. The overall prevalence of anemia in South America is 33%, but the burden is not equally distributed across the continent[27].

Peru is a nation with moderately high prevalence of anemia in young children. An estimated 40.1% of children age 6-59 months have anemia in Peru, and approximately 12% have severe to moderate anemia (INEI report)[4]. This high prevalence of anemia has long-term implications for neurocognitive development and future work productivity of the population[28]. A 2013 economic report estimated that combined short and long term costs of anemia in Peru represented 0.62% of the GDP[5].

Anemia has been a national priority since 2009, and the Peruvian government as well as other health organizations have implemented extensive prevention efforts primarily focused on nutritional deficiencies and mass treatment of parasites. Despite large-scale efforts, there have been limited and heterogenous changes in anemia prevalence across Peru. The region of Tumbes, in particular, has had little to no improvement over the past decade [26,29]. A recent report indicated that anemia prevalence in this region increased in recent years to 47% in young children[26,30]. In Tumbes and the rest of Peru, the exploration of multiple strategies to decrease prevalence is warranted. Research to inform new strategies should include unaddressed causes of anemia, limitations to prevention efforts, treatment access, adherence, and effectiveness.

2.2 Implications of Anemia

It is clear that anemia affects a large proportion of the global population; therefore, even a small impact on health at the individual level may have large population implications. The short-term effects of severe anemia in children are most evident, with signs and symptoms including fatigue, pallor, and dyspnea[31]. Children from age 6 to 36 months are at greatest risk for severe anemia, which interrupts participation in learning activities that are especially critical during early development. Existing studies provide evidence of the negative long-term effects on neurocognitive function in children with anemia during their first few years of life[28,32,33]. Although neurocognitive delay or decreased neurocognitive function in relation to anemia has primarily been attributed to iron deficiency, there is evidence that all anemia contributes to decreased neurocognitive function[7,8,12,34]. For example, there are many studies demonstrating an association between anemia from malaria and decreased neurocognitive function later in life[16,35,36]. Lead (Pb) exposure is also a well-established cause of neurocognitive delay, as well as an environmentally induced anemia[37–39]. In addition, recent literature on iron supplementation has shown mixed results on the relationship between iron supplementation and neurocognitive function later in life[10]. Although the causes of anemia can be multifactorial, the long-term implications appear to be universal across etiologies. Therefore, it is critical to conduct research that informs and facilitates anemia intervention, such as descriptive studies of anemia etiology in diverse settings, evaluation of best methods for screening, and exploration of risk factors contributing to anemia persistence.

2.3 Anemia Tests and Screening

Mild to moderate anemia is typically asymptomatic, so most affected children remain undiagnosed[31]. However, due to growing evidence of long-term adverse health and productivity outcomes from anemia, guidelines for screening for anemia have changed over time. A decade

ago, screening was only conducted in children with high risk for anemia, such as premature infants, children breastfeeding past 6 months without iron supplementation, or children drinking cow's milk before age one[31]. Now, the World Health Organization guidelines call for universal screening of all children at one year of age, although the position of the U.S. Preventive Services Task Force is that there is insufficient evidence to evaluate benefit versus risk of this approach[40,41]. It is important to note, that because the positive predictive value for most screening tests is low, all positive screens for anemia should be followed up with diagnostic tests to confirm anemia status and to determine etiology. However, in the United States only 25% receive such tests, and follow-up is even less likely in low and middle-income countries (LMICs)[42]. For this reason, to ensure that screening benefit outweighs risk, it is important to develop tests and organize healthcare systems to minimize barriers to participation and identify groups that will benefit most from treatment.

The gold standard for identifying anemia is an automated blood count (ABC) from venipuncture, which provides the best measure of circulating hemoglobin[43]. In practice, this blood test is typically only conducted for diagnostic purposes when children have symptomatic anemia or have received positive results from a screening. ABC is rarely if ever used for screening purposes due to limited access to labs in many rural settings and invasiveness of the process[44]. The standard screening tool in many countries is the Hemocue 201. This point-of-care test requires a drop of blood and provides results within seconds[45,46]. A recent study in Rwanda provided evidence that validity of the Hemocue 201 was dependent on techniques used to draw blood such as gravity and wicking, and when applied correctly, maximum sensitivity and specificity was approximately 80% compared to the ABC[45]. However, the chemical reactives in the Hemocue 201 can degrade in temperatures greater than 30^{o}C, a common occurrence in many

LMIC settings where screening is most needed, which may lead to inaccurate results[4]. Infrastructure to maintain the required temperature may not be available in these resource limited settings. Alternative tests to the Hemocue 201 continue to be explored for screening purposes, including a updated device (Hemocue 301) which has the benefit of a higher maximum operating temperature of 40^{o}C [48(p301)].

2.4 Anemia: Broadening the Differential

While low hemoglobin concentration is the defining characteristic of anemia, additional tests are needed to define the source of anemia. In general, anemia can result from nutritional deficiencies, inflammation, genetics, chronic blood loss (e.g. gastrointestinal bleeding), and environmental toxicants. Two main classification systems exist for establishing anemia etiology[49]. The traditional Wintrobe classification system uses observation of cell size through mean corpuscular volume (MCV) to identify microcytic or macrocytic anemia[50]. A MCV of <80fL (microcytic anemia) indicates defects in hemoglobin synthesis that is characteristic of iron deficiency anemia, anemia of inflammation, thalassemia or defects in heme synthesis. A MCV >100fL (macrocytic anemia) is evidence of red cell membrane defects and DNA synthesis defects that can indicate liver disease, hypothyroidism, megaloblastic anemia, reticulocytosis, or vitamin B-12 or folate deficiency. Microcytic and normocytic anemia can also be caused by Pb and cadmium (Cd) exposure, and some evidence suggests that methylmercury (MeHg) exposure can cause anemia as well[51]. Problems with the Wintrobe classification system are that early stages of deficiencies may not modify cell size, and multiple etiologies may result in a cell size within the normal range. Another classification system uses reticulocyte count to determine the underlying mechanism. If reticulocyte count is increased, anemia is likely due to increased red blood cell loss. If the reticulocyte count is low, anemia is likely due to decreased red blood cell

production. Both classifications are broad and typically require further analyses for confirmatory diagnosis[49].

Nutritional Deficiency. Nutritional deficiencies are the most common cause of anemia[52]. The three most common micronutrient deficiencies resulting in anemia are iron, vitamin B12, and folate[49]. Serum tests specific for each of these micronutrients are available in standard clinical practice and can indicate the presence of nutritional deficiencies. The interpretation of serum ferritin, a marker of iron deficiency, is more nuanced as iron sequestration occurs with inflammation and this process can affect ferritin levels. Therefore, in the presence of inflammation and infection, ferritin levels may suggest iron deficiency when the true cause of anemia is inflammation from chronic disease or infection. The Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia (BRINDA) project is currently working to establish best practices for anemia evaluation[53].

Inflammatory and Infectious Causes. Identifying specific types of inflammation and infectious disease is difficult; however, there are standard practices that allow the general evaluation of inflammations' contribution to anemia. Two tests for general markers of inflammation are C-reactive protein and α1-acid glycoprotein[53]. Both can be used to identify inflammation. Measures of both can be applied to different strategies such as regression or alternative thresholds that help parse out whether anemia is due in part to iron deficiency or primarily from an inflammatory condition. When inflammation is present, follow-up tests for chronic diseases known to cause anemia, such as obesity, kidney disease, and infectious diseases, are warranted. The presence of parasitic infections that result in blood cell destruction or loss, such as malaria and hookworm, are important. Recent population studies suggest that other parasites may contribute to anemia either through decreased nutrient absorption or inflammation in the GI tract[35,36].

Chronic Blood Loss. Another common cause of anemia, specifically iron deficiency anemia, is chronic blood loss. Menorrhea is a common cause of iron deficiency. In children, potential causes of chronic blood loss include ulcers, intestinal infection with hookworm species, or other conditions such as celiac that lead to gastrointestinal bleeding[54,55]. Although less common than other anemias, failure to identify sources of chronic blood loss will result in ineffective treatment.

Genetic Causes. Anemia may result from genetic disorders that are common in many parts of the developing world[56]. Thalassemia and sickle cell disease are known to be most common in people with Middle Eastern, Asian, and African descent. While there is no solid evidence base establishing a common genetic cause of anemia in Peru, there has been limited research in this population.

Environmental/Toxicological causes of Anemia. Anemia caused by environmental toxics has received less attention in research[15]. Because hemoglobin production is heavily reliant on iron homeostasis, the presence of other heavy metals can interrupt the production of red blood cells. It is well established that high levels of Pb and Cd exposure result in anemia[39,57]. There is also growing evidence of MeHg and arsenic (As) as contributors to anemia[15,18]. However, much of the existing literature is based on studies in occupational settings where adults suffer more acute exposures, whereas there are few studies establishing the relationship of metals and adverse health outcomes from sub-acute and chronic exposures in children[58]. In the next section, I will expand on MeHg as another potential environmental cause of anemia.

2.5 Mercury a Potential Cause of Anemia.

Hg Source: Hg is considered the third most toxic substance on the planet[59]. Although naturally occurring, exposure to environmental mercury has tripled in recent years with the primary cause being artisanal and small-scale gold mining (ASGM). In ASGM, mercury is used to separate ore

from other sediment, and is later burnt off in a process called mercury amalgamation. The extremely volatile form of mercury, elemental mercury, is then released into the environment.

Hg types and exposure: Hg in its various forms has major implications for population health including adverse hematological outcomes[59,60]. Elemental Hg is primarily an occupational hazard where workers or people in close proximity to them inhale elemental mercury released into the atmosphere through activities such as ASGM[61,62]. Once released into the environment, elemental Hg is transformed via oxidation into inorganic Hg which can enter water systems where biomagnification results in the bioaccumulation of MeHg in fish and humans who consume fish[60,63]. It is important to note that exposure to MeHg does not require proximity to mining activities; therefore, exposure can go undetected for long periods resulting in adverse health outcomes as was the case in Minamata[64].

Measures of Hg Exposure: Multiple studies have established that fish can have high levels of MeHg and have treated fish consumption as a marker for MeHg exposure[65,66]. Although fish consumption has been validated as a marker of methylmercury exposure, it is important to note that MeHg exposure does not come solely from fish and can be found in high concentrations in rice[67–69]. Biomarkers are a more valid measure of MeHg exposure. Common biological measures of Hg exposure in epidemiologic studies are Hg content in blood, urine, hair, toenails, and cord blood[70,71]. Hg in blood and urine samples can have a half-life as short as a few days, so these substrates are typically better markers of recent toxic elemental mercury exposure. Cord blood samples can serve as markers for up to 2 months of previous in-utero MeHg exposure. Hair and toenails are the best markers for chronic MeHg exposure and can reflect up to 5 months of exposure prior to sample collection. It is worth noting that approximately a centimeter of hair from the scalp reflects a month of exposure[72]. Although THg tests of hair are limited because

they measure both inorganic and organic Hg, a study demonstrated that exogenous Hg can be removed by washing hair with 0.1% beta-mercaptoethanol and rinsing three times with Milli-Q water, thereby providing an improved measure of estimated MeHg expxosure[67].

Biologic Feasibility of Methylmercury and Anemia: There are multiple plausible mechanisms through which Hg exposure may cause anemia[60,73,74]. Mechanisms include initiating red blood cell death from irreparable oxidative damage, mimicking or exacerbating vitamin B12 and folate deficiency, and direct dysregulation of iron homeostasis[15]. In addition, toxicological studies using bioassays such as tilapia, provide strong evidence of a relationship in hematologic outcomes including anemia with sub-chronic exposure[75]. The wealth of evidence for biological feasibility of an association between chronic methylmercury exposure and hemoglobin necessitates the expansion of studies at the population level.

Population-based Evidence of Methylmercury and Anemia: Hg is a well-established neurotoxic. Growing population-based evidence also supports MeHg as a cause of anemia[76]. An ecological study of THg and environmental mercury in a Filipino population near an abandoned gold mine afflicted with a syndrome including anemia provided initial impetus to explore the association of mercury and anemia[63]. Weinhouse and colleagues further explored the association through a cross-sectional study of hemoglobin and THg among 95 children under the age of 12 years in Madre de Dios, Peru, a region with extensive ASGM activity[15]. The study provided an estimate of the association of hemoglobin and total hair mercury, and evaluated biomarkers of iron, vitamin B12, folate, vitamin A, and vitamin D as potential confounders. In the final model, adjusting for age, sex, anthropometrics and vitamin B12 in multivariate regression, they estimated that for every one μg/g THg increase, there was a 0.14 g/dL decrease in hemoglobin. While this study significantly advanced literature, it had certain limitations. First, no measures of

infectious or inflammatory markers were evaluated in the regression as is suggested by the Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia (BRINDA) project [53]. Second, co-exposure to heavy metals known to cause anemia (e.g. Pb) is common in ASGM settings and these were not evaluated. Third, the validity of hemoglobin measures was uncertain as capillary blood was processed in a Hemocue 201 rather than the gold standard of venous blood and an ABC. Further studies that address these limitations are warranted.

2.6 Toxicological Pathways between Pb, Cd, MeHg, and Anemia:

Pb: The Agency for Toxic Substances and Disease Registry (ATSDR) concludes that Pb is a cause of anemia, and the Environmental Protection Agency (EPA) has set the benchmark dose for blood Pb that may cause anemia to be 20-40 μg/dL[77]. While two main mechanisms are described for how Pb causes anemia, most data informing this guideline is based on research in occupational settings where exposure is typically a higher dose in adults. The first mechanism is that Pb interferes in absorption of iron[78]. In the small intestines, proteins that typically absorb iron also absorb Pb, resulting in lead at high levels secondarily blocking iron through competitive inhibition[78]. The second is disruption of chemical processes during heme synthesis through the inhibition of the enzyme δ-aminolaevulinic acid dehydratase (ALAD)[36]. Although the benchmark dose is 20 μg/dL, two large studies provide evidence of a significant association of anemia and blood Pb as low as 10 μg/dL[79,80]. It is therefore plausible that other factors, such as chronic exposure to Pb at lower levels in more vulnerable populations may impact the level at which anemia may occur for Pb exposure. High levels of Pb exist in the water systems surrounding our study site; therefore, it will be important to consider Pb in evaluating toxic exposures in relation to anemia.

Cadmium: Although there is an established relationship between Cd and anemia, there is no established benchmark dose for anemia[81]. Few epidemiologic studies exist for the association of

Cd and anemia, but rat and mouse models demonstrate mechanisms by which cadmium likely results in anemia[57,82]. The first mechanism, observed at lower exposure levels to Cd, is hemolysis through the elevated presence of reactive oxygen species[82]. At even higher levels of exposure to Cd, evidence of decreased red blood cell production is also observed and is likely to occur due to secondary competitive inhibition, as is the case for Pb. More population level research of the relationship between Cd and anemia is warranted and may perhaps provide data necessary for exploring minimal thresholds. High levels of Cd in the main water source for our study site warrants the evaluation of how Cd might affect anemia prevalence, especially in our evaluation of the direct effects of Hg.

Methylmercury: Although there is some evidence to suggest that MeHg exposure may cause anemia, this association has not been definitively established. It is hypothesized that similar to Pb, Hg interrupts heme synthesis through inhibition of ALAD which leads to anemia[15,78]. Hg may also exacerbate or imitate vitamin B12 or folate deficiency because the structure of both allow for rapid exchange of methyl groups that affects the absorption of both[15]. Furthermore, there is evidence in the literature of MeHg triggering anemia through early red blood cell death and dysregulation of iron homeostasis[83,84]. There is also some evidence of mercury inducing inflammation, which could lead to anemia. However, in models exploring metal-induced inflammation in relation to anemia, no association has been observed[82,85]. Therefore, the most plausible mechanisms by which MeHg could cause anemia are interruption of heme synthesis, exacerbation or mimicking of vitamin B12 or folate, and signaling of early cell death through increased reactive oxygen species. ATSDR has yet to recognize mercury as a potential contributor to anemia; however, there is growing evidence of such an association. Our study will further contribute to this growing body of literature, not only examining the association of Hg

with anemia, but also controlling for other known causes through the measurement of biomarkers[60].

Probable Collinearity of Mercury with other Heavy Metals: Chronic MeHg exposure from upstream gold mining is frequently discussed. However, it is important to consider that other heavy metals resulting from ore processing are likely released into the environment with Hg[15]. Pb, Cd, manganese (Mn), copper (Cu), zinc (Zn), and arsenic (metalloid, As), have been measured at high levels downstream from small-scale gold mining centers in multiple locations including Zaruma, Ecuador and Tumbes, Peru[22]. Pb and Cd exposure are well-established causes of anemia at high concentrations; therefore, in evaluating anemia in relation to Hg, it is critical to evaluate the presence of these toxics[15,86–88]. Because these metals are from a point source in Tumbes, Peru, and resulting metal exposures are likely collinear in nature, evaluating the direct effects of Hg, Pb, and Cd will likely require special techniques such as weighted quantile sum regression to address collinearity[89]. In addition to direct effects, because there are probable shared mechanisms of Pb, Cd, and Hg in the relationship to anemia, it is worth considering if co-exposure to these metals, even at all levels, may result in an additive effect. Co-occurring metals may also result in multiplicative interactions, for which there is still limited data with sufficient power to evaluate them[90]. Any work conducted to evaluate an association between Hg and anemia should measure Pb and Cd, assess metal collinearity, and evaluate potential additive effects as well as interactions.

2.7 Anemia Treatment

Historically, the term anemia has been used interchangeably with iron deficiency anemia. Therefore, it is not surprising that presumptive iron supplementation for anemia has been a common focus of prevention[31,91,92]. With increasing awareness of iron sequestration and the potential adverse outcomes of iron supplementation in malaria endemic settings, tests for

inflammatory status of anemia are beginning to be more commonly applied and new standards of treatment are being developed[53]. In cases where there is no evidence of inflammation and iron levels are low, oral iron supplementation is the standard treatment for moderate to severe anemia. However, in cases where there is inflammation, further tests should be done to determine the source of inflammation, as this may modify the approach to clinical management[53]. For anemia in which poisoning is present, recommendations for behaviors that will help stop the exposure to Pb can also be applied. In sum, when the etiology of anemia is well characterized, physicians are able to provide more targeted and effective treatment.

Barriers of adherence to iron supplementation. The majority of research regarding anemia treatment is focused on iron deficiency anemia, because it is the most common cause. Although iron supplementation has been shown to be effective in optimal conditions, problems may arise in others setting due to barriers to access and adherence. Studies in both India and Canada have shown that children with food insecurity or low socioeconomic status had the greatest levels of anemia persistence[93,94]. Economic aspects may limit the ability of families to obtain iron supplementation at all, or to provide it for a sufficient amount of time for the treatment to be effective. In many randomized controlled trials, this barrier is addressed by providing the supplement to participants. In this context, the acceptability of treatment, especially in small children who are often a difficult audience to persuade, is key. One study evaluated various methods of iron supplementation dosing and applications, and although supplements proved to be the most effective when taken separate from meals, children were most likely to accept those that were added to food[95]. Because iron supplementation requires prolonged application, social and socioeconomic factors contributing to adherence are especially important to identify and address in settings where anemia burden is high.

Micronutrient Interactions. Treating anemia due to nutritional deficiencies is also nuanced because of various chemical balances and interactions that exist. For example, absorption of iron requires the intake of sufficient vitamin C; without sufficient vitamin C in the diet, oral iron supplementation may not be effective. An additional nuance is that milk or any food with high levels of calcium consumed with iron supplements or iron rich foods will inhibit absorption[94]. Because dietary practices vary across cultures, it is critical to evaluate how these might impact micronutrient levels and adjust interventions appropriately.

2.8 Summary and Specific Aims

The above review establishes that anemia impacts billions of lives and that children in resource-poor settings bear a large proportion of this burden in addition to being some of the most vulnerable to its impact. Children in areas where there is extensive ASGM are at increased risk because environmental contaminants within these settings often include a mix of metals known and suspected to impact hemoglobin levels through both heme synthesis pathways and inflammatory processes. Because anemia is often asymptomatic, this condition can go unnoticed leading to long-term adverse outcomes. Therefore, exploring tools that may facilitate the identification of anemia in high risk and low resource settings is critical.

Anemia is a highly prevalent condition because of the multifactorial pathways by which it can occur. Beyond screening for anemia in communities exposed to multiple risk factors, establishing and describing the distributions of probable etiologies is important for both adequate treatment and improved prevention at the population level. Furthermore, due to elevated risk of exposure to Hg, Pb, and Cd in regions downstream from ASGM, evaluating their potential role in anemia is important for development of policies and regulations within regions such as Tumbes, Peru. Finally, anemia is a condition that can persist if children do not receive the right treatment. For this reason, it is important to explore the various factors that may influence

anemia persistence after referral to treatment such as treatment access, adherence, and effectiveness.

Tumbes, Peru is an ideal setting to investigate the problems described above. Nearly a third of children in the region are estimated to have anemia and many of the rural settings have limited access to health resources. Upstream contamination from approximately 20+ years of nearly 200 small scale gold mining organizations in Zaruma, Ecuador have also been confirmed through isotopic mercury analysis, warranting exploration of toxic effects on the population[22,23]. Finally, although there have been extensive public health efforts to decrease childhood anemia in the region, the prevalence has remained the same. The objective of the dissertation research proposed in the following pages is to provide information that facilitates efforts to decrease anemia prevalence in low resource settings, such as those in Tumbes, Peru through the evaluation of heavy metals as a risk factor, exploration of barriers to anemia treatment, and evaluation of alternative screening tools.

CHAPTER 3: RESEARCH PAPER #1

Exploration Of Heavy Metals in Relation to Anemia In Tumbes, Peru

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Running head: Anemia and Heavy Metals in Peru

Key words: Anemia, Downstream Mining, Heavy Metals, Peru

3.1 Abstract

Background and Aim: Anemia occurs when there is insufficient hemoglobin to transport oxygen in the body. It can result in both short and long-term neurocognitive deficiencies. Mass iron supplementation is a standard intervention in many low to middle-income countries (LMIC's). However, iron deficiency is only one possible cause. Other causes of anemia that prevalent in LMIC's include vitamin deficiencies, chronic inflammation, and exposure to Pb and Cd. In this study we describe the distribution of potential causes of anemia across two districts with and without exposure to mining runoff and evaluate the association of known risk factors of anemia, as well as Hg, As, and Mn.

Methods: We conducted a cross-sectional study among children 6 to 59 months old in Tumbes, Peru in February 2021. The study site covered two districts, one with documented heavy metal exposure and one without. We visited homes and recruited children to participate in venous blood, stool, and hair collection. Participants' guardians completed a survey on clinical history, diet, and household characteristics. We measured hemoglobin concentration and anemia status using semi-automated blood count, micronutrient levels and C – reactive protein in blood. We tested stool for parasites and measured THg, Pb, As, and Mn in hair. We compared the distribution of measures across district and anemia status by running chi-squared and t-tests. We estimated univariable associations of hemoglobin concentration with potential causes of anemia and built a multivariable linear regression model to estimate direct effects of Pb, Mn, As, and MeHg.

Results: We recruited 264 children. Seventeen percent had anemia (44/264) and average hemoglobin concentration was 11.8 (Range: 9.3-14.5). Hemoglobin concentration and anemia prevalence did not differ significantly across districts. Serum ferritin was significantly lower, and

folate was significantly higher in the community with documented metal exposure. Birth weight and age were significantly associated with hemoglobin. THg, Pb, and Mn were not significantly associated with hemoglobin in our final model, however As was highly correlated with folate and had a small and significant positive association with hemoglobin.

Conclusions: More studies in communities downstream from mining should be conducted to determine if average population iron stores may be impacted at the community level. While we did not observe direct inverse associations of hemoglobin concentration with THg, Pb, and Mn larger studies are required to capture smaller associations and permit the use of more advanced statistical techniques to capture effects of multi-metal exposure. Finally, the correlation of folate with arsenic, and arsenic's positive association with hemoglobin concentration warrants the investigation of sources of exposure and whether co-exposure to folate through certain foods such as fish and vegetables may mitigate negative outcomes.

Keywords: Anemia, Hemoglobin, Heavy Metals, Risk profiles

3.2 Introduction

Anemia is a condition that results from insufficient hemoglobin that is necessary to transport oxygen. A third of children across the globe are estimated to have anemia, which left untreated childhood anemia affects both short and long-term neurodevelopment and immunologic function[1,32,96]. Iron supplementation remains one of the most common prevention strategies in many regions and works under the assumption that a majority of anemia cases are caused by insufficient dietary iron[97]. Although it is estimated that 60% of anemia is iron deficiency anemia, there are multiple causes of anemia including infection, chronic inflammation from conditions such as kidney deficiencies or obesity, and prenatal and postnatal environmental

factors such as heavy metal exposure[1,15]. It is also worth noting that beyond dietary deficiency, iron deficiency can be induced by many of the aforementioned factors. Multiple factors can simultaneously contribute to anemia among children; therefore, characterizing causes of anemia in populations and identifying risk factors for the types of anemia in communities is critical for designing and improving anemia control strategies.

Historically, dietary deficiencies have been a primary focus of anemia control strategies, but the high global prevalence of community heavy metal exposure warrants further consideration of their role in anemia burden. A recent report by UNICEF and Pure Earth estimates that globally one in three children have actionable blood lead (Pb) levels (5 µg/dL)[98]. Another article estimates that more than a million illnesses worldwide are due to dietary ingestion of Pb, arsenic (As), cadmium (Cd), and methylmercury (MeHg)[99]. LMIC's are most at risk for both metal exposure as well as high prevalence of anemia as compared to other countries [100].

Pb and Cd are known to cause anemia through competitive iron uptake by DMT-1, inhibition of heme production via ALAD inhibition, and oxidative death[57,77,81]. As is also a less established yet biologically plausible cause of anemia through displacement of zinc in a zinc finger transcription factor that is involved in erythropoiesis[101]. In addition, there are a handful of epidemiologic studies in which exposure biomarkers of Hg and Mn are inversely associated with hemoglobin. A further nuance is that iron deficiency exacerbates Pb, Cd, and Mn uptake by DMT-1 upregulation; therefore, it is likely that children with dietary iron deficiency in areas with chronic exposure to heavy metals may be more likely to suffer toxic effects. There may also be opportunities for intervention based on these complex interactions, for example, population studies suggests that increasing folate could decrease Pb uptake, therefore considering

supplementation beyond iron supplementation could be a feasible intervention in certain settings [102]. **Figure 3.1** summarizes these biologic pathways.

Tumbes, Peru is approximately 100 kilometers downstream on the Puyango-Tumbes River from Portovelo, Ecuador, where there are 87 small-scale gold mining sites and documented artisanal gold mining[103–105]. This river serves as a main water source for communities in Tumbes. Multiple studies have demonstrated levels of Pb, Cd, As, and Mn in water and sediment of the Puyango-Tumbes river that exceed EPA recommended levels for water systems[22,24]. Studies that are more recent have traced Hg isotopes back to the Portovelo region[24,106,106]. Fish from the river, crops irrigated by the river, and seafood from coastal waters near the river mouth are commonly consumed in the region and have had notably high metal levels in previous studies[25,107]. Knowledge of heavy metal contamination in local rivers exist within communities in the region and are considered both a topic of concern for the community and for the local government. In this same region, childhood and maternal anemia are a health priority because of high prevalence and minimal changes in prevalence observed over the past decade despite expansive dietary education, dietary supplementation, and parasite treatment interventions to prevent and treat anemia[108]. Since 2015, anemia prevalence in the Tumbes region has ranged from 42-48% among children 6-35 months[4].

Because industrial pollution is prevalent across the globe, specifically evaluating the association of biomarkers for metals with childhood anemia could have wide-reaching implications for anemia control strategies. Unlike Pb and Cd, which are well-established causes of anemia, there are fewer epidemiologic studies with biomarkers measuring the association of MeHg, As, and Mn with hemoglobin. Quantifying these associations based on biomarkers will help evaluate the potential role of MeHg, As, and Mn on childhood anemia prevalence, and

whether incorporating new strategies to minimize or mitigate these exposures should be implemented. In this study, we evaluated the association of heavy metals with hemoglobin concentration and anemia in the region of Tumbes, Peru, including a district with established chronic exposure to heavy metals.

3.3 Methods

Study Design and Population: We conducted a cross-sectional study among 264 children between 6 to 59 months of age from 28 villages in two rural districts (San Jacinto and Casitas) of Tumbes in February 2021 to evaluate the association of micronutrients, parasitosis, heavy metals with hemoglobin concentration and anemia. We selected San Jacinto and Casitas districts based on similar socioeconomic and rural status and differing exposure to mining runoff. San Jacinto (exposed) is near the Puyango-Tumbes River, whereas Casitas (control) is over 50km away from the river with an alternative water source **(Figure 3.2)**. The Regional Office of Housing and Development has previously measured higher levels of Pb and lower levels of As in water from San Jacinto than Casitas and in a pilot we conducted in August 2019 among a convenience sample of adults and children (n=41) in San Jacinto and Casitas, we measured a broad range of Pb (0.15-11.21µg/g), As (0-1.17µg/g**)**, Hg (0.29-4.2µg/g**)**, and Mn (0.42-14.47µg/g**)** levels in hair establishing feasibility to evaluate toxicity of metals in a larger study. In this study we describe differences in heavy metal concentration and micronutrients across districts and evaluate metals and other known risk factors association with anemia and hemoglobin concentration.

Sample Size: We designed our study to have 80% power to detect a linear relationship of 0.07 or greater between Pb, THg, As, or Cd with hemoglobin (α=0.05, maximum of 15 covariates). The logic behind powering to detect this effect size was based on association of total hair mercury and hemoglobin observed in a study in the Peruvian Amazon[15]. A minimum of 200 participants

were necessary to achieve this power. Therefore, we selected 18 villages from both districts in which data from local health centers suggested an approximate population of 300 children between 6-59 months.

3.3.1 Data Collection

Ethics Statement: The study protocol was reviewed and approved by the Institutional Review Boards of Oregon Health & Sciences University and the Universidad Peruana Cayetano Heredia. During door-to-door recruitment, all guardians of participants provided written informed consent prior to children participating in any study activities.

Survey: We interviewed guardians about the child's clinical history as well as household characteristics. Questions included birth weight, current weight, breastfeeding status, previous anemia testing, anemia diagnosis and anemia treatment in the past year. The dietary frequency survey included questions about weekly consumption of meat, fish, vegetables, and citric fruit that are commonly grown in the region and in existing literature are associated with anemia and metal exposure. Additional information about parent occupation, maternal anemia during pregnancy, and crops grown for personal consumption were also ascertained in the survey.

Blood Collection: Nursing technicians from our team collected 10mL of peripheral venous blood from the upper extremity of each participating child using a 21-gauge butterfly needle. Approximately 2mL of this sample were extracted directly into BD Vacutainer tubes containing K2 EDTA anti-coagulant (lavender top # 367861) tube and 8mL were extracted into BD Vacutainer serum collection tubes containing clot activators (red top #367815). These were stored on ice in coolers, and then transferred the same day to the main laboratory of the regional government in Tumbes (DIRESA) where samples were refrigerated until processing. During collection, remnant blood drops were tested with point of care hemoglobin meters that are further detailed in *Chapter 5*.

Blood processing: The lavender top vial included whole venous blood that was processed using a semi-automated hematology analyzer (Prokan PE-6100, Guangdon, China) to obtain results for semi-automated blood count including hemoglobin concentration. The red top vial was centrifuged to separate the sera, which was then processed on a biochemical analyzer (iFlash ImmunoAssay Analyzer) to measure ferritin, vitamin B12, folate, and C-reactive protein. Stool samples were sent directly to the Center for Global Health, where 10 grams of stool were placed in 40mL of 5% formal PBS for processing using spontaneous sedimentation and light microscopy[105]. *Giardia Lamblia*, *Entamoeba Coli,* and *Ancilostoma sp.* Were included in analysis for potential factors associated with anemia.

Hair Collection: To measure chronic exposure to Hg, Pb, As, and Mn over the past 2-3 months, members of our research team tied off with string and cut an approximately a 1cm in diameter lock of each child's hair near to the scalp from behind the ear. The 2 cm of hair proximal to the scalp was cut and processed as described below for analysis of Pb, Hg, Mn, and As. Exposure to metals was suspected to be chronic in San Jacinto, therefore hair was selected as the best medium for this analysis. THg in the 2 cm of hair nearest to the scalp is considered a standard by the EPA to estimate previous 2-3 months of MeHg exposure. In populations with likely exposure to metals from a water source and fish, hair has been identified as an acceptable medium for estimating chronic exposure to Pb, As, and Cd [109–111]. Total hair Mn has also been frequently applied in epidemiologic studies[112]. Steel scissors were sanitized between uses and hair samples were placed in a plastic bag with zip closure for transport and storage

Hair Analysis: Hair was sonicated in 1% Triton X-100 solution (Sigma-Aldrich, Inc., St. Louis, MO) and rinsed repeatedly with Milli-Q water (Millipore Corporation, Billerica, MA) to remove external contamination. Samples were split into two equal portions and weighed. One set of hair

samples was air dried for 2-3 days. We measured hair total mercury (THg) concentrations in the air-dried hair with thermal decomposition, amalgamation and atomic absorption spectrophotometry following EPA Method 7473 (USEPA, 2007) using a Lumex(Model RA-915+/PYRO-915+, St. Petersburg, Russia)[67]. For quality control and assurance, we measured standard reference materials [IAEA-086 (hair)], with an average recovery of 99.1% (SD: %, n=28) for IAEA_086 (hair). We measured 9% of hair samples in duplicate with an average relative standard deviation of 8.6% (SD: 7.1%, n=22). All 264 samples exceeded the minimum detection level (0.0095 ppm).

The other set of hair samples was dried at 60°C for 24 hours, digested with nitric acid in a controlled environment, and analyzed by inductively coupled mass spectrophotometry (ICP-MS) for Pb, As, Cd, and Mn[113,114]. Quality assurance included analysis of blanks and certified human hair reference material (CRM Hair; Shanghai Institute of Nuclear Research, China). Average percent recovery was 94% (SD: 4%, n=5) for Pb, 71% (SD:5%, n=5) for As, and 89% (SD: 4%, n=5) for Mn. Levels of metals were all 5 times above calculated limits of detection (Pb: 0.002ppm, As: 0.0037ppm, Mn: 0.0243). Values for calculating recovery rates for CRM standard are under current revision, therefore cadmium concentrations were excluded from data analysis.

Stool Collection: Participants received a 1L plastic lidded container labeled with their name, along with instructions to deposit a whole stool from that day or the following morning into the container. Filled containers were placed inside a plastic bag for collection by the field team the next morning. Samples were transported directly to the Center for Global Health for analysis.

Stool Analysis: Ten grams of stool were placed in 40mL of 5% formal PBS for processing using spontaneous sedimentation and light microscopy to identify *Giardia Lamblia*, *Entamoeba Coli,* and *Ancilostoma sp.*(Hookworm)[115].

3.3.2 Definitions for Analysis

Comparison of Factors across District

District (Primary Exposure): San Jacinto (exposed) and Casitas(control) are both small rural districts. San Jacinto, however, depends on the Puyango-Tumbes River that is known to have mining runoff. Although a smaller pilot study among adults and children demonstrates differences in distribution of metals, I evaluate differences across district in this study to confirm assumptions of differences in metal distribution and similarities of other factors that may contribute to anemia risk.

Heavy Metals (Primary Outcome): I calculated quartiles for the distribution of Hg, Pb, As, and Mn concentration in hair (ug/g) for the entire study population and by district. I ran the Wilcoxon signed-rank test to identify statistically significant (p-value>0.05) differences of hair metal concentration across district and evaluated correlation between metals using the Spearman correlation test to explore if common sources of exposure were likely. I then used a 250:1 conversion factor often applied to convert hair THg to Blood Hg, to convert hair Pb and As to blood Pb and As in order to compare with standard blood reference doses across district.

Contributing causes of Anemia: I evaluated if there were any district differences in the distribution of other factors known to contribute to anemia. For sex, having giardia, and having C-reactive protein, I evaluated difference across districts using a chi-squared test. For normally distributed continuous variables, ferritin, folate and vitamin B12, I ran t-tests.

Evaluation of factors associated with anemia and hemoglobin concentration:

Heavy Metals in Hair (Primary exposure): Hair metals were heavily right skewed and were log transformed to evaluate continuous associations of potential risk factors with anemia and

hemoglobin concentration. I then categorized THg, Pb, Mn, and As into terciles (low, medium, and high) to explore non-linear associations with hemoglobin.

Outcome/ Gold Standard: Hemoglobin (Hb) concentration from semi-automated blood count (continuous, g/dL) was the primary outcome, and anemia status based on hemoglobin concentration (binary, Hb<11g/dL) was also explored as an outcome.

Covariate Measures: I created a directed acyclic graph (**Figure 3.3**) to identify confounders and mediators in the relationship between THg, Pb, Cd, Mn, and As with hemoglobin, and to explore additional risk factors for hemoglobin change in the literature detailed in chapter 2. District and sex, as well as ferritin, folate and vitamin B12 measured in serum were identified as potential effect modifiers in this model. Dietary frequency, previous anemia treatment, and family health history reported by guardians were included as covariates. The presence of giardia measured from stool and C-reactive protein measured in serum as a marker of chronic inflammation, were also included. Iron deficiency was evaluated and defined as a serum ferritin >12µg/L[116,117].

3.3.3 Statistical Analysis

Descriptive: I used Stata SE version 16 for analyses. I calculated anemia prevalence with 95% confidence intervals based on the WHO classification system of mild (10-10.9dg/L Hb), moderate (7-9.9dg/L Hb), and severe(<7dg/L Hb)[118]. I also calculated the medians of hair metals, means of continuous covariates and proportions of categorical covariates across children with and without anemia.

Analysis of Factors Associated with anemia: I conducted chi-squared tests for categorical variables and t-tests for continuous variables to assess associations with anemia. A p-value<0.05 was considered significant.

Analysis of Factors Associated with Hemoglobin Concentration: I conducted univariable linear regression to identify factors significantly associated ($p<0.05$) with hemoglobin concentration. I evaluated models for extreme outliers that were identified for Pb, As, and Mn. I presented results for univariable analysis with and without outliers to determine if there was notable change in measures of association. There was little to no change with the exception of a weakened association of As with hemoglobin so outliers were included in multivariable models.

Analysis of THg, Pb, Mn, and As association with Hemoglobin Concentration: I conducted multivariable linear regression to evaluate individual associations of continuous and categorized (tertiles) THg, Pb, Mn, and As concentrations in hair with hemoglobin concentration while accounting for one another. I based model selection on **Figure 3.3** that incorporates metals into a conceptual model of the metals and hemoglobin association. To create a parsimonious model, I only included covariates, age and birthweight that were significant during univariable analysis. Current breastfeeding status was not included because it was correlated with age. I further evaluated THg, Pb, As, and Mn individually for statistical interaction with sex, district, ferritin, folate, and vitamin B12 to create a third model with interaction terms. The final model included age and birthweight as confounders and an interaction term for THg with district. The crude, minimally adjusted, and final model were created treating metals as continuous and categorical, for a total of six models.

3.4 Results

Comparison of Factors across District

We recruited 265 children between the ages of 6 to 59 months into the study. One participant was excluded due to insufficient blood collected leaving an analytic sample of 264. One-hundred fifty children were from the exposed district bordering the contaminated river and 114 were from

the control district approximately 50 kilometers away (Table 3.1). There were significant differences across the two districts. Giardia was more prevalent in the exposed district (22%) than the control district (5%) and median levels of Pb and Mn were nearly twice as high in the exposed district. Median arsenic was greater in the control district than the exposed (Figure 3.3a). Over 30% of children in exposed district had levels of THg or Pb surpassing levels associated with toxicity in existing literature (Table3.2; Figure 3.4). Pb was moderately correlated with As and Mn. As was moderately correlated to Mn and Folate. Although there were few cases of iron deficiency, the exposed district had significantly lower average serum ferritin and significantly higher average folate than the control (Figure3.6). Both the four participants who had moderate anemia and the four who had iron deficiency anemia, resided in exposed district (Table 3.10).

Evaluation of factors associated with anemia

The overall prevalence of anemia was 16.7 percent (44/264), and mean hemoglobin was 11.8 mcg/dL (Range: 9.3-14.5 mcg/dL). Neither the prevalence of anemia nor the mean hemoglobin concentration was statistically different across districts. Anemia prevalence was 17.3% (26/150) in the exposed district and 15.8% (18/114) in the control, while the mean hemoglobin was 11.8 (SD=0.81) and 11.8 (SD=0.77), respectively.

Children with anemia were significantly ($p<0.05$) more likely to have a higher birthweight, lower vitamin B12, and lower serum iron than those without anemia (Table 3.4). A notably greater proportion of children with anemia were younger, male, positive for CRP, had giardia and lower levels of serum ferritin. Concentrations of Pb, THg, As, and Mn in hair were not significantly different among children with and without anemia. Based on the survey, children with anemia (41/44; 93.2%) were significantly more likely to have been screened for anemia in the past year

than children without (173/220; 78.6%), and four of the cases had previously tested positive for anemia (Table 3.5). Although not statistically significant, children with anemia were more likely to be breastfed; however, this relationship had a significant interaction with age that resulted in less notable associations among younger children. As expected, a lower proportion of children with anemia reported consuming meat other than chicken (p=0.2) or oranges (p=0.07) three or more times per week (Table 3.5). Children with anemia were also more likely to consume vegetables including carrots and beets, and consume oranges at least three times per week (p=0.06).

Evaluation of factors associated with hemoglobin concentration

Based on univariable analyses, vitamin B12 (β (95%CI): 0.001 (0.0001, 0.002), birth weight (β (95%CI): -0.23 (-0.40, -0.07)), breastfeeding (β (95%CI): -0.30 (-0.53, -.062)), and age (β (95%CI): 0.11 (0.04, 0.18)) were all significantly associated with hemoglobin concentration (Table 3.6). Association of log transformed and categorical Hg, Pb, and Mn with hemoglobin were null during univariable analysis, even when excluding outliers. Log transformed As and the highest tertile of As, however had a small but significant positive association with hemoglobin that persisted when excluding outliers (Table 3.7). When evaluating plausible effect modifiers, the only statistically significant interaction term was district and log (Hg) (Table 3.11).

In both the crude model and model 2 of log-transformed THg, Pb, As, and Mn, As was the only metal to have a statistically significant association with hemoglobin concentration (β (95%CI): 0.077 (0.007, 0.15) and 0.11 (0.024, 0.19)). These results were similar when modeling metal concentrations by tertiles with hemoglobin. The only difference was a significant negative association of the second tertile of Hg in the crude model and model 1.

3.5 Discussion

Metals and Anemia in Study

In our population, both within and between communities, we did not observe meaningful associations of Hg, Pb, As and Mn with anemia nor hemoglobin. The lack of association between metals and hemoglobin persisted when adjusting for age and birthweight. Prevalence of anemia across districts was similar and lower than expected, despite significantly higher metal levels in hair of the district downstream from mining. Therefore, we did not see evidence for our main hypothesis that multiple heavy metal exposure would result in lower hemoglobin and greater likelihood of anemia.

It is important to note that lack of association between metals and hemoglobin could be a reflection of limitations in study design and sample size. We selected districts for our study under the assumption that community characteristics were similar, with the exception of greater risk of heavy metal exposure. However, as observed there are notable differences in burden of giardia, folate, and vitamin B12 that influence anemia burden. Unmeasured factors such as micronutrients, including zinc and C-reactive protein as a continuous variable may bias our estimates of association towards the null. In addition, prevalence of anemia was half of what we expected. Therefore, our sample size was also limited for exploring associations of anemia. It is also possible that metal exposure period has not been long enough in young children to affect hemoglobin levels. In addition, with respect to measures of heavy metals, blood is typically the preferred matrix to measure Pb; however, the use of hair in our study allowed us to maximize participation in our study communities and facilitated ease of transport. The choice of hair was also a strength in that measured metals represent approximately 3 months of accumulated previous exposure. Lack of association between Pb and hemoglobin in our models, despite ample epidemiological and toxicological evidence, further supports that our results likely do not represent the true absence of association[119].

There were other factors notably associated with anemia and lower hemoglobin in our study population, including age, extended breastfeeding, higher birthweight, and presence of giardia in stool. Younger children and children breastfed for longer have previously been identified as having greater risk for anemia in other studies[120]. Age and breastfeeding were independently associated with lower hemoglobin during univariable analysis; however, when breastfeeding was treated as an effect modifier, this association notably decreased as children got older. This may be explained by inadequate supplementary feeding. However, we did not ask about frequency or exclusivity of breastfeeding, so were unable to establish this. Although fewer studies have directly linked higher birthweight to lower hemoglobin, our findings are complementary to existing literature that links macrosomia to maternal anemia, which is a risk factor to childhood anemia[121,122]. Therefore, macrosomia or factors leading to it should be considered in future anemia interventions. Giardia is a less established cause of anemia in humans, however, it is a plausible one based on animal studies and because it may lead to decreased absorption of food[123]. It is worth noting that this protozoa is highly prevalent in many LMIC's and not affected by drugs routinely provided for mass treatment of intestinal helminths. Age and birthweight, however were the greatest predictors of hemoglobin in our study therefore, gestational health and a Developmental Origins of Health and Disease (DOHaD) approach in this population may be warranted.

Pathways of Metal-induced Anemia

Although, no direct associations of metals and hemoglobin were observed, we can deduce whether other biological measures are compatible with competitive inhibition of iron uptake, inhibition of heme production or chronic inflammation as pathways for metal-induced anemia.

Competitive inhibition of iron uptake is supported by the significantly lower level of serum ferritin in the community with established exposure to heavy metals than in the community without. Divalent metal transporter 1 is a major iron transporter, however it is also known to take up Pb and Cd, and suspected to take up Mn, As, and Hg[15,119,124]. When there are insufficient iron stores the body upregulates DMT-1[125]. Therefore, metals can inhibit iron uptake and iron deficiency can also trigger increased uptake of metals. Serum ferritin is a marker of iron stores and the significantly lower levels in our metal exposed community as well as significant association of serum ferritin with Pb and As are consistent with this plausible pathway of metal-induced anemia. It is also important to note that iron deficiency alone is associated with neurocognitive development delays. Therefore, if research is applied to set benchmark doses, considering iron deficiency as the main outcome for determining lowest observable adverse effect levels should be considered.

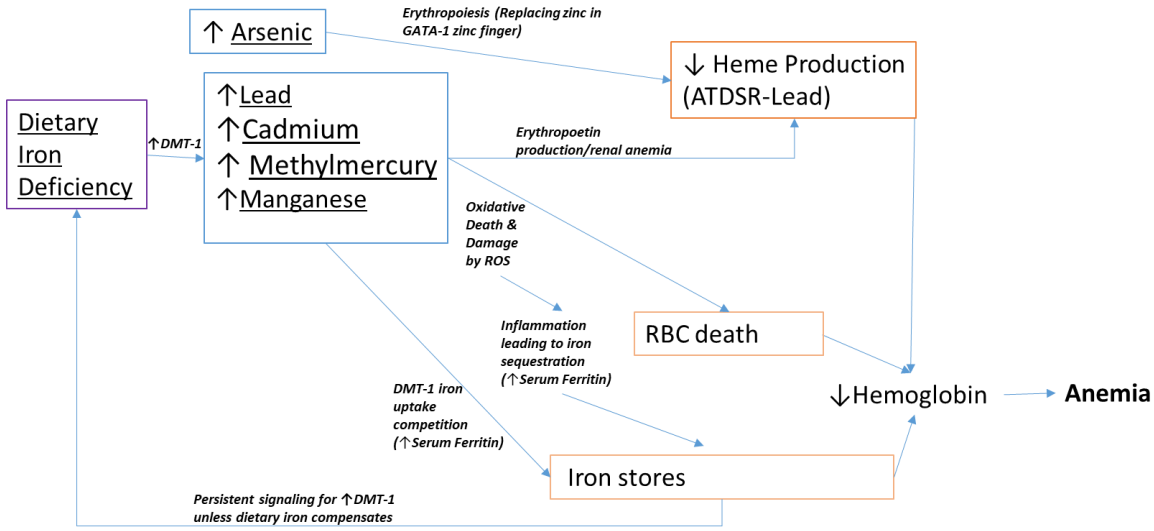
Our results do not support the presence of metal-induced heme inhibition nor inflammatory anemia. Based on similar distributions of hemoglobin across our two study communities, the presence of metals has not led to decreased heme production. If the ALAD enzyme were blocked by metals, we would have anticipated a clear association of metals and hemoglobin. As this is a known pathway for Pb exposure to anemia, it is possible that unmeasured confounders such as zinc may be masking this association. In addition, we were unable to establish the presence of inflammatory anemia due to limitations of data obtained. C-reactive protein serves as a measure of inflammation, however few children were identified as being C-reactive protein positive. Analysis of a continuous variable would have provided further insight. Reticulocyte count would have also allowed us to identify the presence of shorter red blood cell life span from

inflammatory anemia, and inclusion of measures of transferrin in addition to our measures of iron and serum ferritin would have allowed us to identify iron sequestration. Future studies incorporating measures specified may provide more conclusive results about mechanisms of metal-induced anemia in similar communities.

Conclusion: We observed no meaningful association of heavy metals and anemia in this study. However, we observed evidence of decreased iron stores that warrant further exploration on heavy metals role in iron regulation, especially in settings with elevated levels of Pb. Larger studies are warranted to evaluate metal-induced iron deficiency in communities and identify risk factors for iron deficiency progressing to anemia.

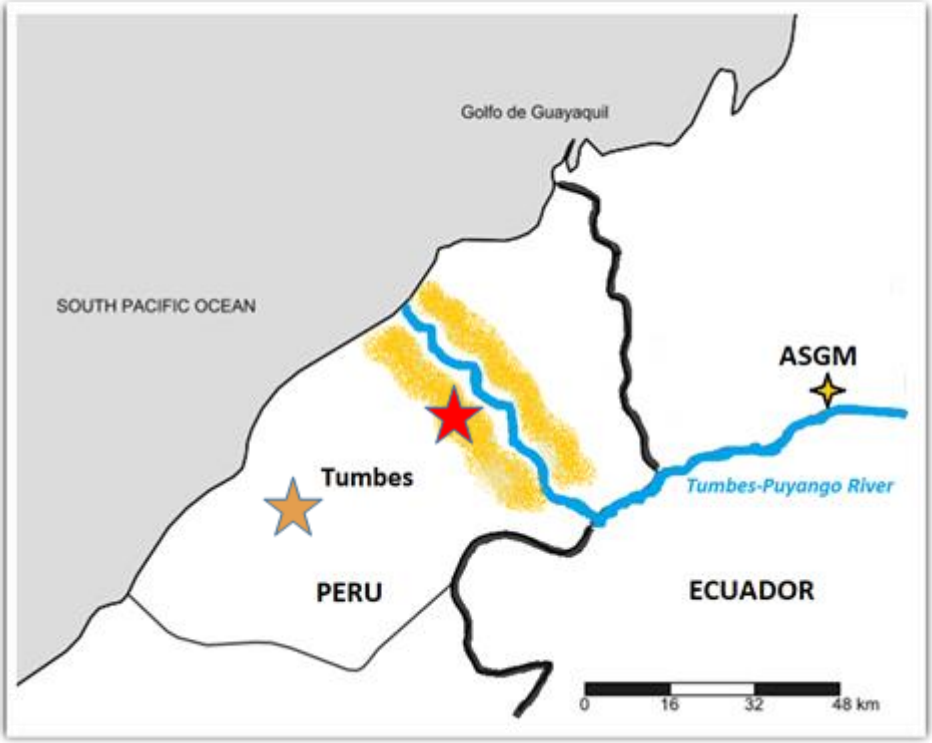
3.7 Tables and Figures

Figure 3. 1 Conceptual Diagram of Metal Association with Anemia (based on literature review described in introduction).



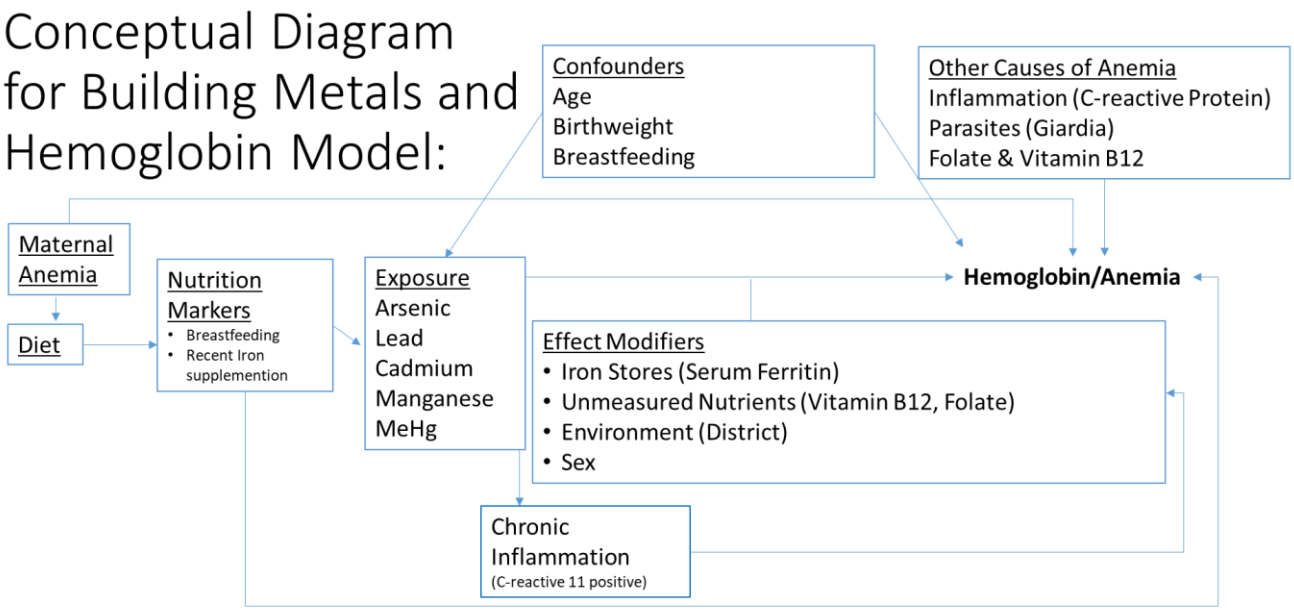
Notes: Lead, cadmium, and arsenic pathways are well established. Methylmercury and manganese pathways supported by some literature.

Figure 3. 2 Map of Study Districts in Tumbes, Peru.



Notes: This map shows the region of Tumbes in South America which borders Ecuador and the South Pacific Ocean. The red star is San Jacinto district, our mining runoff exposed district, that is dependent on the Tumbes-Puyango River and has extensive mining upstream. The yellow star indicates the central point of Casitas, our control district, which is similar to San Jacinto in socioeconomic and rural status.

Figure 3. 3 Directed Acyclic Graph and Models Evaluating Metal and Hemoglobin Association



Evaluation of Linear Association:
Crude Model: Hemoglobin= β0 + βlog(THg) + βlog(Pb) + βlog(As) + βlog(Mn) + €
Model 1: Hemoglobin= β0 + βlog(THg) + βlog(Pb) + βlog(As) + βlog(Mn) + βAge + βBirthweight + €
Model 2: Hemoglobin= β0 + βlog(THg + βlog(Pb) + βlog(As) + βlog(Mn) + βAge + βBirthweight + βDistrict + βDistrict*Hg €

Evaluation of Categorical Association:
Crude Model: Hemoglobin= β0 + βTHg + βPb + βAs + βMn + €
Model 1: Hemoglobin= β0 + βTHG + βPb + βAs + βMn + βAge + βBirthweight + €
Model 1: Hemoglobin= β0 + βTHG + βPb + βAs + βMn + βAge + βBirthweight + βDistrict + βDistrict*Hg €

Figure 3. 4 Total Heavy Metals in Hair by District; Exposed is San Jacinto district downstream from mining and Casitas is the control.

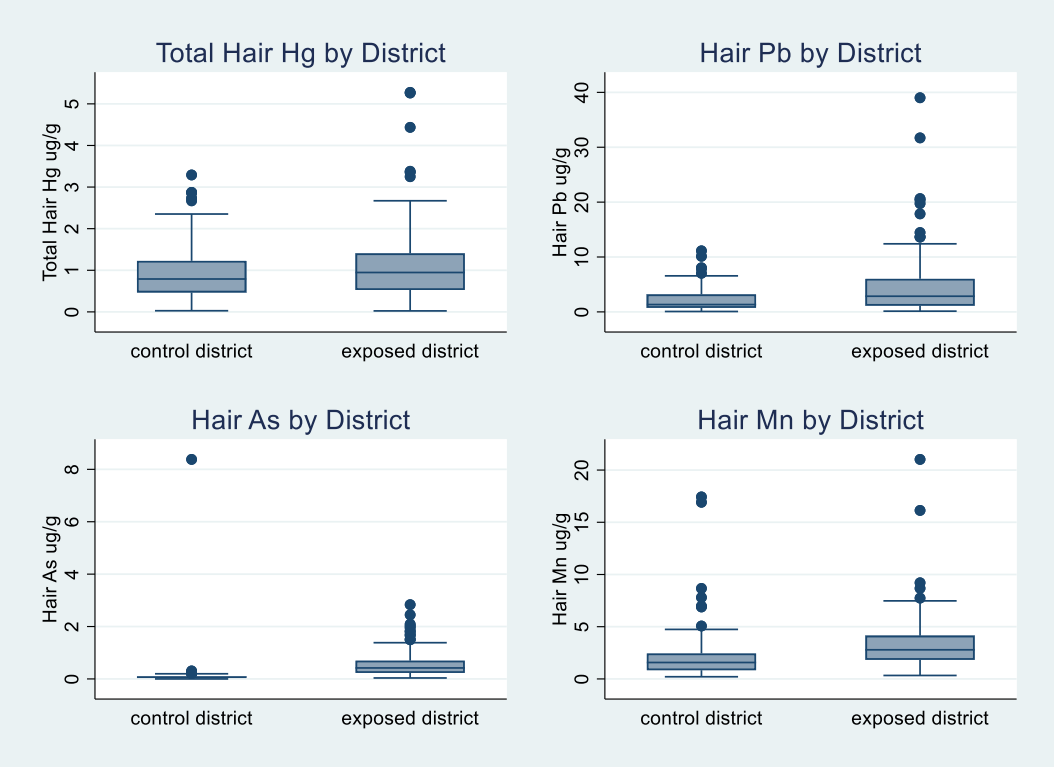
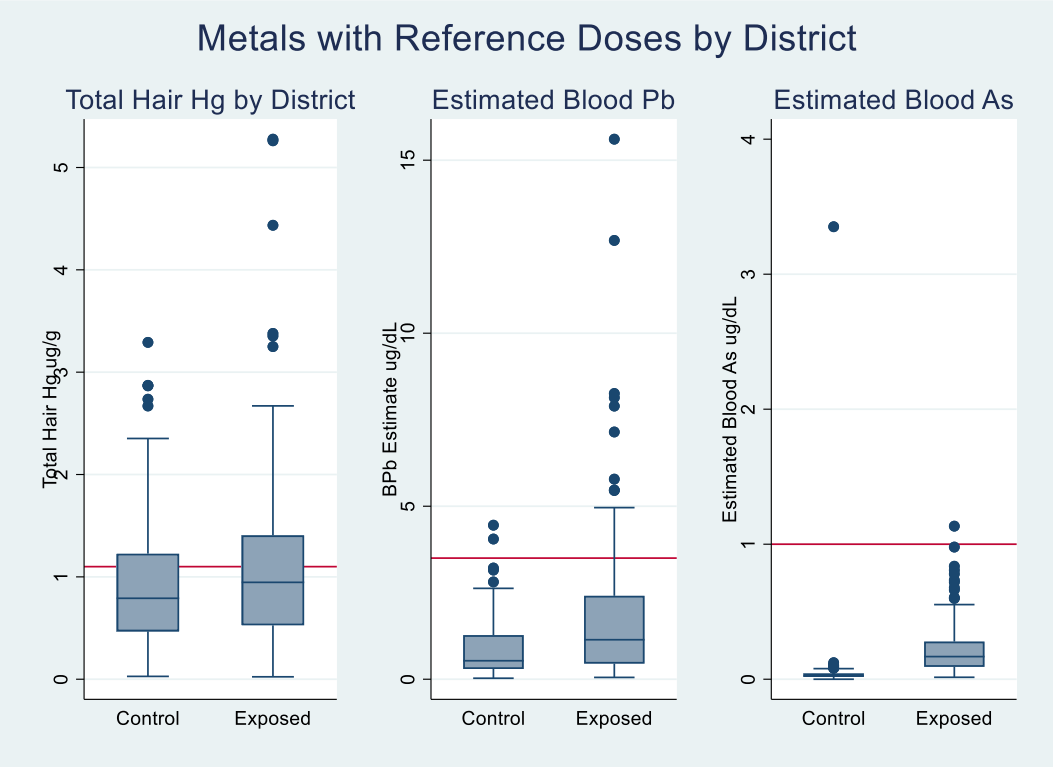
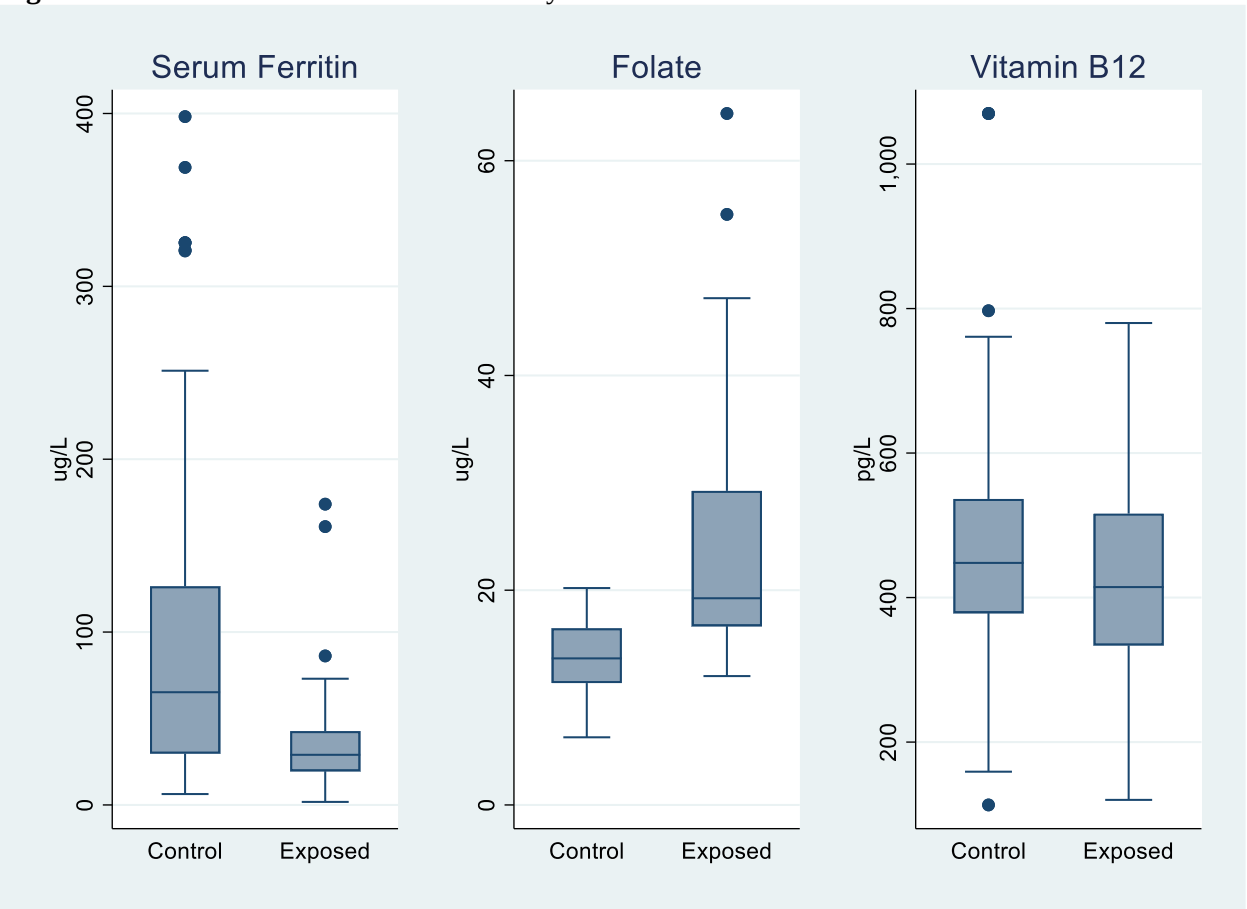


Figure 3.5 THg and estimates of blood Pb and As



Note: hair Pb and As converted using a 250:1 ratio with hair Pb and As by District. District; Exposed is San Jacinto district downstream from mining and Casitas is the control. Red lines indicate the reference values for MeHg (1.1ug/g) and Pb (3.5ug/dL). For Arsenic the reference value of 1 ug/dL for blood has also been applied.

Figure 3. 6 Micronutrient Markers in Serum by District



Note: Casitas is far from the river (control). San Jacinto is downstream from mining (exposed).

Table 3.1
Distribution of Biomarkers by District

	Total N=264	Casitas N=114	San Jacinto N=150	p-value*
	n (%)	n (%)	n (%)	
Male	134 (51%)	57 (50%)	77 (51%)	0.83
CRP Positive	11 (4%)	5 (4%)	6 (4%)	0.13
Giardia lamblia	39 (15%)	6 (5%)	33 (22%)	<0.001
	mean (SD)	mean (SD)	mean (SD)	
Age (years)	2.3 (1.3)	2.2 (1.3)	2.5 (1.2)	0.07
Birth weight (kg)	3.3 (0.6)	3.3 (0.5)	3.3 (0.6)	0.94
Serum iron (mcg/dL)	74 (37)	82 (43)	69 (30)	0.005
Serum ferritin (mcg/L)	58.7 (67.5)	96.7 (90.0)	32.9 (23.3)	<0.001
Folate (mcg/L)	19 (9)	14 (3)	24 (10)	<0.001
Vitamin B12 (pg/mL)	446.9 (137.5)	469.5 (144.7)	429.7 (129.6)	0.03
	median (IQR)	median (IQR)	median (IQR)	
Lead/Pb (µg/g)	1.9 (0.9-4.7)	1.3(0.7-3.2)	2.8 (1.1-6.0)	<0.001
Mercury/THg (µg/g)	0.8 (0.5-1.3)	0.8 (0.5-1.2)	0.9 (0.5-1.4)	0.14
Arsenic/As (µg/g)	0.2 (0.1-0.5)	0.06 (0.04-0.1)	0.4 (0.2-0.7)	<0.001
Manganese/Mn (µg/g)	2.2 (1.3-3.6)	1.6 (0.8-2.4)	2.8 (1.8-4.1)	0.001

Note. SD-Standard Deviation; IQR-Interquartile Range

Table 3.2
Distribution of heavy metals measured in hair among study participants and by community

All Participants										
Metal: ppm	N	Wilcoxn p value	MIN/LOQ*	25th	50th	75th	90th	MAX	Toxic Level	Children N(%)
Hg	264	--	0.02	0.5	0.9	1.3	2	5.3	1.1	91(34)
Pb	263	--	0.07	0.9	1.9	4.7	8	39		
As	263	--	<0.01	0.071	0.18	0.48	0.91	8.4		
Mn	263	--	0.21	1.3	2.2	3.6	5.3	21	NA	NA
Control District										
Hg	114	0.2	0.03	0.5	0.8	1.2	2	3.3	1.1	33(29)
Pb	113	<0.001	0.1	0.7	1.3	3.2	4.8	11.1		
As	113	<0.001	<0.01	0.042	0.066	0.11	0.15	8.4		
Mn	113	<0.001	0.2	0.8	1.6	2.4	4.5	17.4	NA	NA
Exposed District										
Hg	149	0.2	0.02	0.5	0.9	1.4	2.2	5.3	1.1	58 (39)
Pb	151	<0.001	0.1	1.1	2.8	6	10.4	39		
As	151	<0.001	0.036	0.23	0.41	0.7	1.19	2.83		
Mn	151	<0.001	0.3	1.8	2.8	4.1	5.9	21	NA	NA

Note: Exposed is San Jacinto district Downstream from mining and Casitas is the control.

Table 3.3
Correlation Matrix of Metals, Micronutrients, Age, and Birth Weight

	Hb	Hg	Pb	As	Mn	Vitamin B12	Folate	Age	Birth Weight
Hb	1.00								
Hg	0.09	1.00							
Pb	0.05	-0.01	1.00						
As	0.10	0.09	0.63	1.00					
Mn	0.02	-0.06	0.63	0.56	1.00				
Vitamin B12	0.15	0.22	0.01	-0.05	-0.09	1.00			
Folate	-0.11	0.15	0.13	0.51	0.20	-0.04	1.00		
Age	0.14	0.25	-0.27	0.03	-0.13	0.25	0.11	1.00	
Birth Weight	-0.15	0.01	-0.06	-0.11	-0.04	0.02	0.05	0.03	1.00

Table 3.4
Distribution of age, sex and measured biomarkers among children with and without anemia

	Total N=264 n (%)	No anemia N=221 n (%)	Anemia N=44 n (%)	p-value*
Male	134 (51%)	106 (48%)	28 (64%)	0.06
CRP Positive	11 (4%)	7 (3%)	4 (10%)	0.07
Giardia lamblia	39 (15%)	29 (14%)	10 (23%)	0.11
	mean (SD)	mean (SD)	mean (SD)	
Age (years)	2.3 (1.3)	2.4 (1.3)	2.0 (1.3)	0.09
Birth weight (kg)	3.3 (0.6)	3.3(0.6)	3.5 (0.4)	0.02
Serum iron (mcg/dL)	74.3 (36.6)	76.5 (38.4)	63.8 (24.7)	0.04
Serum ferritin (mcg/L)	58.7 (67.5)	61.1 (69.8)	47.9 (55.4)	0.25
Folate (mcg/L)	19.4 (8.9)	19.3 (9.0)	20.0 (8.4)	0.63
Vitamin B12 (pg/mL)	446.9 (137.5)	457.5 (142.9)	398.6 (96.6)	0.011
	median (IQR)	median (IQR)	median (IQR)	
Lead/ Pb (µg/g)	1.9 (0.9-4.7)	2.0 (1.0-4.7)	1.6 (0.8-4.6)	0.56
Mercury/THg (µg/g)	0.9 (0.5-1.3)	0.9 (0.5-1.4)	0.7 (0.5-1.0)	0.11
Arsenic/ As (µg/g)	0.2 (0.07-0.5)	0.19 (0.07-0.5)	0.2 (0.07-0.3)	0.58
Manganese/ Mn (µg/g)	2.2 (1.3-3.6)	2.2 (1.3-3.5)	2.5 (1.1-4.1)	0.54

Note: * from t-tests for continuous variables and chi-squared test for categorical; SD= Standard Deviation; CRP=C-reactive Protein; THg = Total hair mercury; IQR=Interquartile range

Table 3. 5
Distribution of Survey Data across Children with and without Anemia

	Total	No Anemia	Anemia	p-value*
	N=264	N=220	N=44	
Household Member Participates in Mining Activities	21 (8%)	19 (9%)	2 (5%)	0.36
Consume own Crops	192 (73%)	161 (73%)	31 (70%)	0.71
Mother had Anemia during Pregnancy	56 (21%)	45 (20%)	11 (25%)	0.50
Currently Breastfed	55 (20.8%)	42 (19.09%)	13 (29.6%)	0.23
Anemia in the past year	19 (7.2%)	15 (6.8%)	4 (9.1%)	0.60
Tested for Anemia in the past year	214 (81.1%)	173 (78.6%)	41 (93.2%)	0.03
Foods Consumed 3 or more times per week				
Meat other than Chicken	88 (33%)	77 (35%)	11 (25%)	0.20
Fish	212 (80%)	177 (80%)	35 (80%)	0.89
Legumes	207 (78%)	172 (78%)	35 (80%)	0.84
Spinach/Broccoli	116 (44%)	96 (44%)	20 (45%)	0.82
Other Vegetables	260 (98%)	218 (99%)	42 (95%)	0.07
Banana	254 (96%)	211 (96%)	43 (98%)	0.56
Orange	159 (60%)	138 (63%)	21 (48%)	0.06

Note:*from t-tests for continuous variables and chi-squared test for categorical

Table 3. 6
Univariable Analysis of Factors Associated with Hemoglobin Concentration

Potential Risk Factors	N	β (95%CI)	p-value
Giardia	254	0.01 (-0.26, 0.28)	0.96
Vitamin B12	240	0.001 (0.0001, 0.002)	0.031
Folate	240	-0.003 (-0.015, 0.008)	0.55
Birth weight	263	-0.23 (-0.40, -0.07)	0.006
Breastfeeding	264	-0.30 (-0.53, -.062)	0.013
Maternal Anemia	264	-0.090 (0.32,0.14)	0.46
Male	264	-0.12 (-0.31, 0.068)	0.21
Age	264	0.11 (0.04, 0.18)	0.004
District near river	264	-0.41 (-0.24, 0.15)	0.67
Recent iron supplements	68	-0.05 (-0.28, 0.17)	0.65
Serum Ferritin	228	0.001 (-0.002, 0.003)	0.097

Note: CI-Confidence Interval

Table 3. 7
Beta Coefficients from univariable linear regression to explore factors associated with Hemoglobin

	Univariable (N=263)			Univariable Excluding Outliers*		
Heavy Metals	N	β (95%CI)	p-value	N	β (95%CI)	p-value
Log(Hg)	262	0.10 (-0.02, 0.21)	0.092	NA		
Hg T1(<0.597 ug/g)		Ref.				
Hg T2 (0.597-1.12 ug/g)		-0.29 (-0.52, -0.06)	0.013			
Hg T3 (>1.13 ug/g)		0.22 (-0.12, 0.45)	0.063			
Log(Pb)	262	0.04 (-0.05, 0.12)	0.38	260	0.042 (-0.044, 0.13)	0.34
Pb T1 (<1.16 ug/g)		Ref.			Ref.	
Pb T2(1.16-3.6 ug/g)		0.01 (-0.23, 0.25)	0.93		0.011 (-0.23, 0.25)	
Pb T3(>3.6 ug/g)		0.07 (-0.17, 0.30)	0.58		0.072 (-0.17, 0.31)	
Log(As)	262	0.07 (0.010, 0.12)	0.02	261	0.061 (0.003, 0.12)	0.037
As T1 (<0.094 ug/g)		Ref.			Ref.	
As T2 (0.096-0.31 ug/g)		0.032 (-0.20, 0.27)	0.79		0.032 (-0.21, 0.27)	0.79
As T3(>0.92 ug/g)		0.29 (0.052, 0.52)	0.02		0.27 (0.040, 0.51)	0.022
Log(Mn)	262	0.006 (-0.11, 0.12)	0.92	258	0.0012 (-0.12, 0.13)	0.98
Mn T1 (<1.58 ug/g)		Ref.			Ref.	
Mn T2(1.58-3.16 ug/g)		-0.13 (-0.37, 0.10)	0.27		-0.13 (-0.37, 0.10)	0.27
Mn T3(>3.13 ug/g)		0.02 (-0.21, 0.26)	0.85		0.02 (-0.22, 0.26)	0.86

Note: CI: Confidence Interval *Outliers Pb, As, and Mn are Pb>25ug/kg; As>8ug/kg; Mn>15ug/kg

Table 3.8
Estimates of Coefficients for Linear Regression with Continuous Metal Measures

Linear Association	Crude Model		Model 1*		Model 2*	
Beta Estimates	β (95%CI)	p-value	β (95%CI)	p-value	β (95%CI)	p-value
Log(Hg)	0.85 (-0.029, 0.20)	0.14	0.047 (-0.69, 0.16)	0.43	-0.062 (-0.23, 0.10)	0.45
Log(Pb)	0.013 (-0.10, 0.12)	0.82	0.068 (-0.05, 0.19)	0.26	0.036 (-0.083, 0.15)	0.55
Log(As)	0.077 (0.007, 0.15)	0.03	0.047 (-0.023, 0.12)	0.19	0.11 (0.024, 0.19)	0.02
Log(Mn)	-0.081 (-0.24, 0.074)	0.30	-0.081 (-0.23, 0.071)	0.30	-0.061 (-0.21, 0.09)	0.42
Age (years)	********		0.11 (0.026, 0.19)	0.01	0.11 (0.032, 0.20)	0.007
Birth weight (kg)	********		-0.22 (-0.39, -0.056)	0.009	-0.20 (-0.36, -0.032)	0.02
San Jacinto	********		********		-0.24 (-0.49, 0.11)	0.45
Log(Hg)* San Jacinto	********		********		0.22 (-0.0029, 0.44)	0.06

Table 3. 9
Estimates of Coefficients for Linear Regression with Metals as Tertiles

Non-Linear Associations	Crude Model		Model 1*		Model 2*	
Beta Estimates	β (95%CI)	p-value	β (95%CI)	p-value	β (95%CI)	p-value
Hg T1(<0.597 ug/g)	Ref.		Ref.		Ref.	
Hg T2 (0.597-1.12 ug/g)	-0.29 (-0.42, -0.60)	0.014	-0.28 (-0.41,-0.05)	0.019	-0.30 (-0.63, 0.037)	0.081
Hg T3 (>1.13 ug/g)	0.18(-0.57, 0.41)	0.14	0.14 (-0.10, 0.38)	0.26	0.042 (-0.32, 0.40)	0.82
Pb T1 (<1.16 ug/g)	Ref.		Ref.		Ref.	
Pb T2(1.16-3.6 ug/g)	-0.067(-0.31,0.18)	0.48	0.29 (-0.22, 0.28)	0.82	-0.10 (-0.36, 0.15)	0.43
Pb T3(>3.6 ug/g)	-0.13(-0.44, 0.18)	0.58	0.007 (-0.32, 0.32)	0.97	-0.18 (-0.51, 0.16)	0.30
As T1 (<0.094 ug/g)	Ref.		Ref.		Ref.	
As T2 (0.096-0.31 ug/g)	0.06(-0.18, 0.31)	0.61	0.62 (-0.18,0.30)	0.61	0.35 (0.065, 0.64)	0.017
As T3(>0.92 ug/g)	0.32(0.037, 0.61)	0.027	0.20 (-0.095, 0.49)	0.18	0.75(0.32,1.18)	0.001
Mn T1 (<1.58 ug/g)	Ref.		Ref.		Ref.	
Mn T2(1.58-3.16 ug/g)	-0.18(-0.43, 0.07)	0.16	0.20 (-0.45, 0.053)	0.12	-0.16(-0.41, 0.93)	0.216
Mn T3(>3.13 ug/g)	-0.05(-0.34, 0.23)	0.72	-0.021 (-0.31, 0.26)	0.88	-0.007 (-0.29, 0.27)	0.96
Age (years)	********		0.090 (0.0088, 0.17)	0.03	0.9 (0.11, 0.17)	0.03
Birth weight (kg)	********		-0.21 (-0.38, -0.041)	0.15	-0.16 (-0.33, 0.010)	0.064
San Jacinto			********		-0.57 (-0.95, -0.20)	0.003
Hg T2 * San Jacinto			********		0.036 (-0.41, 0.49)	0.16
Hg T3*San Jacinto			********		0.14 (-0.32, 0.60)	0.55

Note: T1-Tertile 1, T2-Tertile 2, T3- Tertile 3; *Coef.=Beta Coefficient; CI=Confidence Interval

Table 3.10
Characteristics of Individuals with Moderate Anemia and Iron Deficiency Anemia

Moderate Anemia	Iron Deficiency Anemia	District	Sex	Hg ppm	Pb ppm	As ppm	Mn ppm	Ferritin (mcg/dL)	Folate (mcg/L)
No	Yes	San Jacinto	F	0.53	1.12	0.20	1.67	5.97	15.40
No	Yes	San Jacinto	F	0.67	12.40	1.70	2.62	10.3	30.9
No	Yes	San Jacinto	M	0.024	2.85	0.27	2.84	6.01	12
No	Yes	San Jacinto	M	0.45	1.00	0.28	1.01	8.48	17.3
Yes	No	San Jacinto	M	0.29	0.21	0.13	2.26	19.4	16
Yes	No	San Jacinto	M	0.94	5.99	0.29	1.66	53.1	20
Yes	No	San Jacinto	M	1.34	0.33	0.14	1.52	37.8	28.3
Yes	No	Casitas	F	0.97	6.13	0.084	1.94	95.3	15.8

Table 3.11
Interaction Term Coefficients with p-values

	Interaction term coefficient and p-value					**Interaction term coefficient and p-value excluding outliers***				
Heavy Metals	β*Male (p-value)	β*San Jacinto (p-value)	β*Ferritin (p-value)	β*Folate (p-value)	β*Vitamin B12 (p-value)	β*Male (p-value)	β*San Jacinto (p-value)	β*Ferritin (p-value)	β*Folate (p-value)	β*Vitamin B12 (p-value)
Log(Hg)	0.13 (0.26)	0.23 (0.043)	-0.0009 (0.24)	-0.004 (0.56)	-0.00005 (0.90)	NA				
Log(Pb)	-0.008 (0.93)	0.06 (0.11)	0.11 (0.096)	0.002 (0.62)	-0.0002 (0.17)	-0.012 (0.90)	0.061 (0.51)	-0.0005 (0.57)	0.003 (0.58)	-0.0002 (0.40)
Log(As)	-0.1 (0.62)	0.07 (0.39)	-0.001 (0.42)	0.0012 (0.82)	-0002 (0.12)	-0.01 (0.09)	0.08 (0.31)	-0.001 (0.42)	-0.24 (0.75)	-0002 (0.17)
Log(Mn)	0.04 (0.74)	0.13 (0.28)	-00048 (0.63)	0.011 (0.22)	-0.0004 (0.94)	0.05 (0.71)	0.08 (0.55)	0.0001 (0.91)	0.009 (0.37)	-0.0003 (0.48)

CHAPTER 4: RESEARCH PAPER #2

A 6 Month Cohort Study Of Anemia Persistence And Potential Risk Factors In Rural Tumbes, Peru

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Running head: A Cohort Study of Anemia Persistence

Key words: Anemia, Persistent Anemia, Iron supplementation, Standard of Care, Peru

4.1 Abstract

Background and Aim: Nearly a third of children across the globe have anemia. A majority are from settings with limited resources. The longer a child has anemia the more likely they are to suffer long-term effects, such as decreased neurocognitive function. For this reason, we explored factors related to anemia persistence and treatment completion 4 months after anemia screening among children 6-59 months in in rural Tumbes, Peru.

Methods: We conducted a small cohort study in two rural districts of Tumbes, Peru. Our cohort consisted of children positive for anemia (Hemoglobin>11g/dL) as part of a larger cross-sectional study and followed-up 4 months afterward. Hemoglobin, serum ferritin, folate, and vitamin B12 were measured at the beginning and end of the study. Pb was measured in hair at baseline and a guardian survey was applied at follow-up to identify barriers to clinical attention, treatment initiation, and treatment adherence.

Results: The cohort comprised 40 children of whom 6 were lost to follow up (n=38). Nine (24%) children had persistent anemia, of whom four reported having completed iron supplementation prescription. Twenty-one received iron supplementation prescription and completed treatment. Among children not receiving clinical attention, multiple guardians cited flooded roads to health posts and lack of personnel in health posts as major issues. Several reported fear of health posts due to the COVID-19 pandemic. Persistent anemia was more common among children from a district (San Jacinto) with mining runoff despite higher rates of treatment completion compared to another rural district (Casitas).

Conclusions: Our study is too small to identify statistically significant risk factors for persistent anemia and treatment. However, results suggest the need to further explore the potential role of Pb in persistent anemia, especially in communities downstream from mining, and to explore barriers to clinical access.

Keywords: Anemia, Hemoglobin, Heavy Metals, Risk profiles

4.2 Introduction:

An estimated 40% of children across the globe have anemia[126]. Although the majority of children with mild anemia have few immediate symptoms, persistent anemia has been linked to long-term neurocognitive deficits[5,29]. For this reason, anemia screening during early childhood is standard in most parts of the world, and in areas with especially high prevalence, mass iron supplementation among this age group is also common. As resources are often limited in regions most affected, identifying children with moderate or severe anemia, or those with greater risk of persistent anemia, is critical so they can be prioritized for follow-up care.

Although childhood anemia is often thought of as a singular disease, it is a condition that can be a result of multiple factors including nutritional deficiencies, chronic disease, environment, and genetics[1]. Based on global estimates, about 60% of anemia cases are due to iron deficiency, and approximately another 25% are estimated to be due to other nutritional deficiencies including vitamin B12 and folate[127]. Exposures to heavy metals, such as Pb, and Cd are also known causes of anemia, although less information exists on their global contribution to childhood anemia prevalence[1,3,128]. It is important to note that causes of anemia are not mutually exclusive, and that many lower-to-middle income countries (LMICs) are more vulnerable to multiple causes of anemia as compared to wealthier nations. Presumptive iron supplementation

alone can be expected, therefore, to inadequately address a large proportion of childhood anemia, even under optimal conditions.

Healthcare access and treatment acceptability can also be a barrier to anemia resolution, even in settings where standard interventions support the early identification of children with anemia and standard treatments address the underlying causes of anemia[129]. LMICs not only have higher prevalence of anemia, malnutrition, and pollution exposure, but healthcare facilities are often understaffed and understocked for medication[9]. This is likely to present challenges for families seeking both initial and follow-up care. Furthermore, clinical management of anemia often requires sustained interventions, such as prescribed supplements or behavioral changes. Barriers such as acceptability of treatment to children or acceptability of cost for the parents can affect efficacy through decreased adherence[5,6]. Identifying and addressing systemic barriers to anemia resolution is critical for public health interventions to be effective and efficient in reducing the burden of childhood anemia.

The Tumbes region in northern Peru has provided iron supplementation to children as a population intervention for over a decade in an effort to address a high burden of childhood anemia[4]. However, despite these programs, the prevalence of childhood anemia has consistently remained above 41%[130]. Because anemia screening and iron supplementation have been made widely available to children in this region, other prominent causes of anemia not addressed by iron supplementation, or unidentified barriers to access and adherence, may have limited the effectiveness of these programs. The objective of the study described here was to evaluate the status of anemia, four months after diagnosis and referral, among a small cohort of children in two rural districts in Tumbes, and to describe factors associated with cases of persistent anemia.

4.3 Methods

Study design: We conducted a small prospective cohort study to evaluate the proportion of persistent anemia, and its associated risk factors, among 44 children aged 6 months to 5 years diagnosed with anemia during a cross-sectional study described in the previous chapter (Chapter 3). We evaluated participating children 4 months after their initial diagnosis and referral to local health center to determine whether their anemia had resolved or persisted. We then used the framework in **Figure 1** to design survey questions about potential barriers to accessing clinical attention, obtaining prescribed supplements, and adhering to treatment regimens, and applied these surveys to the child's parent or guardian.

Study population: The 44 children identified for this study were those who were diagnosed with anemia as part of a cross-sectional study to estimate anemia prevalence in 28 communities across two districts of Tumbes during February 2021. All children 6-59 months residing in the selected communities were invited to participate in the initial screening. Both districts in this study are rural agricultural communities. The San Jacinto (exposed) district is downstream from an active gold mining region and is highly dependent on the Puyango-Tumbes River for household water and crop irrigation. The Casitas (control) district is over 50 km away from the Puyango-Tumbes River and uses alternative water sources. Children recruited for this cohort study were those with a hemoglobin concentration <11g/dL measured by a semi-automated blood count using venous blood as part of the baseline study in February 2021[118].

Procedures: We identified our cohort as all children diagnosed with anemia during the cross-sectional study described in Chapter 3, where procedures to measure semi-automated blood count, serum ferritin, parasitosis, and hair Pb are further detailed. For this cohort study, our team returned one month after anemia screening to the homes of children with hemoglobin

concentrations <11g/dL and referred them to their local health centers for treatment. Standard treatment post screening in the region often includes presumptive iron supplementation. At this time, healthcare facilities also received reports of nutritional deficiencies, parasitosis, and Pb levels >5ppm in hair to consider in the treatment plan as needed. At 4 months after baseline, our team visited participants' homes again and collected a second blood sample. We administered a survey to guardians in which we collected information about barriers to accessing clinical attention (e.g., distance to health post and cost of travel to health post) and recommended supplements (e.g., availability and cost of supplement). We asked about barriers to treatment adherence, issues of treatment acceptability, child's clinical history, and maternal medical history, to identify plausible anemia etiology.

Outcomes: The primary outcomes of interest were the proportion of children with persistent anemia 4 months after initial screening, and mean change in hemoglobin concentration. Hemoglobin concentration of ≤11g/dL was used as the threshold to identify persistent anemia. Baseline hemoglobin concentrations were subtracted from those at 4 months to calculate mean change in hemoglobin. Iron supplementation completion was also treated as a secondary outcome in our study. Treatment type and treatment completion were ascertained by visual examination of supplement bottle and interviews of guardians by our field team. I also explored average change in ferritin 4 months after baseline among children completing supplementation as a secondary outcome.

4.3.1 Statistical analysis:

Descriptive: I characterized the distribution of demographic and clinical characteristics including sex, age, iron deficiency status, moderate anemia, and parasitosis among all study participants

and presented individual characteristics of the 9 children with persistent anemia 4 months after baseline.

Analytic: I conducted Fishers exact tests to explore the association of persistent anemia across district, age category (≤2 years and >2years), sex, clinical history, iron deficiency, moderate anemia, probable Pb exposure, elevated C-reactive protein, giardiasis, and completion of iron supplementation treatment. I also evaluated factors associated with hemoglobin by conducting linear regression to estimate average change in hemoglobin while adjusting for age and sex. Statistical significance was defined as any association with a p-value < 0.05.

I also evaluated treatment regimen completion and associations with district, age category, sex, limited trust in health post, side effects, acceptability of treatment, previous anemia treatment, time to health post, cost of transport to health post, and continuous age using Fishers exact tests for categorical variables and t-tests for continuous variables to identify statistically significant differences between children completing and not completing treatment. I explored open-ended responses about barriers to receiving clinical attention for recurring themes and conducted an additional sub-analysis among children completing treatment to explore supplement efficacy.

4.4 Results

Among the 44 children with anemia in our cohort, we were able to contact 38 at the 4-month follow-up visit. The six whom we could not locate were excluded, leaving an analytic sample of 38 participants (Figure 4.2). The six children lost to follow-up in this study had no nutritional deficiencies or elevated Pb in their hair, and a majority were from the control district (n=4) and male (n=5) but were otherwise similar to those in our analysis. Among participants included in analysis, slight majorities were from the exposed district (n=24), between 6-24 months of age when screened (n=25), or male (n=24) **(Table 4.1)**. Only three children were

positive for C-reactive protein. Giardiasis (n=8, 21%) and high Pb levels in hair (n=11, 29%), were relatively common. No children met clinical criteria for folate or vitamin B12 deficiency anemia. Four participants met clinical criteria for iron deficiency anemia at baseline and another four children had moderate anemia, while the rest had mild anemia (34/38) at baseline.

Twenty-four percent (9/38) children were still anemic 4 months after initial screening. Most (7/9) children with persistent anemia were between 6-24 months at baseline and four of the nine children with persistent anemia completed iron supplementation. There were no statistically significant differences across children with and without persistent anemia. When visually exploring individual data of children with persistent anemia, children in the control district had greater improvement in ferritin after 4 months than children in the exposed district **(Table 4.2)**. Two of the four children with iron deficiency anemia at baseline were girls and both had persistent anemia at follow-up. Although not statistically significant, a greater proportion of children with persistent anemia at follow-up were female, resided in the exposed district, were iron deficient at baseline, and had hair Pb levels >5ppm **(Table 4.3)**. Average change in hemoglobin was also significantly lower among children currently breastfeeding, even when adjusting for sex and age.

Twenty-three of the 38 children reported having received attention at a local clinic (Figure 2). Among those children twenty-two were prescribed iron supplementation and twenty-one completed iron supplementation. Our team was able to visually inspect supplementation containers for all children reported as having completed treatment and copy prescriptions. One child received clinical attention but was prescribed dietary changes instead of supplementation. The child who received a prescription but did not complete treatment discontinued treatment due to side effects. Among the eleven children not receiving clinical attention, four had guardians

who reported specific barriers to receiving care. Open-ended responses to question about additional barriers to receiving treatment included concern about going to the health post due to the COVID-19 pandemic, issues of physical access due to flooding during rainy season, and absence of personnel in the local health post. One parent did not bring their children to the health post because they did not have health insurance.

4.5 Discussion

Due to the small size of our cohort (n=38), our study was likely underpowered to observe any modest risk factors that might otherwise be significantly associated with anemia and hemoglobin level. None of the risk factors explored were statistically significant for persistent anemia and only current breastfeeding was significantly associated with lower change in hemoglobin before adjusting for age and sex. For this reason our discussion primarily focuses on characteristics and distributions of children with persistent anemia and potential barriers to receiving iron supplementation that should be further explored in larger studies.

Our results reflected a hypothesis that there is a natural dip in hemoglobin around the age of one year like that observed in preterm infants [131]. Although not statistically significant, 7 of the 9 children with persistent anemia were one year or less. More notably, the two four-year old's with persistent anemia had hair Pb >5ppm. Universal screening for anemia is recommended between ages 9-12 months by the American Association of Pediatrics and most studies demonstrate that prevalence of anemia is higher during this age. In settings where there is high prevalence of anemia, future studies should evaluate whether hemoglobin thresholds for diagnosing anemia in children age 1 years or less should be the same as in children age 2 years and above. Ensuring that thresholds are clinically significant at this age and intervenable are important for establishing a standard of care. If a natural dip occurs around age 1 year, it may be more

pertinent to promote balanced diet and universal supplementation and have targeted screening around age 2-3 years with more specific tests that facilitate efficient use of resources[13].

Another notable result was differences across districts. Although, a greater proportion of children from the exposed district had persistent anemia, the proportion of children completing iron supplementation was notably higher in the exposed district (77%) than the control (24%). Change in ferritin levels among children receiving iron supplementation were also notably lower among children from the exposed district than the control. Our numbers do not allow us to meaningfully examine effect modification of iron supplementation across districts but do warrant larger studies to better define what factors may modify standard intervention efficacy. Due to the exposed districts greater risk of chronic exposure to mining runoff and differences in crops grown, including cassava, across districts, future studies should include heavy metal exposure as well as more specific dietary surveys. Limited change in ferritin levels among children receiving iron supplementation may indicate competitive uptake of heavy metals or deficiency in other key micronutrients that are essential for iron uptake.

In recent years, the Peruvian government's emphasis on decreasing anemia and malnutrition has included additional programs that include increased maternal and childhood anemia screening, improved childhood nutrition, and mass parasite treatment campaigns[4,130]. Few resources however have been put in place for program evaluation. A pre and post population study of childhood anemia prevalence in Madre de Dios demonstrated that the prevalence of anemia decreased, especially in indigenous communities after the series of governmental interventions. However, the mixture of interventions and lack of follow-up in the same children, limited their ability to determine direct effects of each intervention[130]. Unlike the Madre de Dios study, our study followed the same group of children over time, exclusively applied anemia

screening, and measured anemia using hemoglobin concentrations from blood counts rather than less valid screening tools. This allowed us to explore potential risk profiles as well as characteristics that could contribute to effectiveness of iron supplementation. While study design was very different, we did find parallels with the study in Madre de Dios. Change in anemia prevalence and hemoglobin concentration differed notably by community type. They observed the greatest decrease in anemia prevalence among indigenous communities as compared to others. We observed more persistent anemia from our study in the exposed district where greater heavy metal exposure has been established, despite greater treatment completion and fewer reported barriers to clinical attention than the control district. Both studies present settings where general increased access to clinical attention is associated with childhood anemia in communities suspected to have historically less secure sources of water for consumption and irrigation of crops. Heavy metal exposure or parasitosis such as giardia may contribute to these differences.

While we have discussed treatment efficacy, it is important to acknowledge access to clinical care as a major barrier to treatment in our study. Of 38 children, only 23 children received clinical attention and 21 of those children completed iron supplementation. Therefore, obtaining clinical attention was the greatest barrier to starting treatment in this study. Barriers reported for 12 children obtaining clinical attention included access to the health post due to seasonal flooding, the ongoing COVID-19 pandemic, and lack of personnel at respective health posts. These findings demonstrate that in future research of childhood anemia and many other diseases requiring clinical attention, it is important to consider the role that extreme weather from climate change has on healthcare access, especially in LMIC's. It further demonstrates the need to better understand how pandemic response may have inadvertently affected other chronic conditions by limiting people's access to care, so action can be put into place to remedy this.

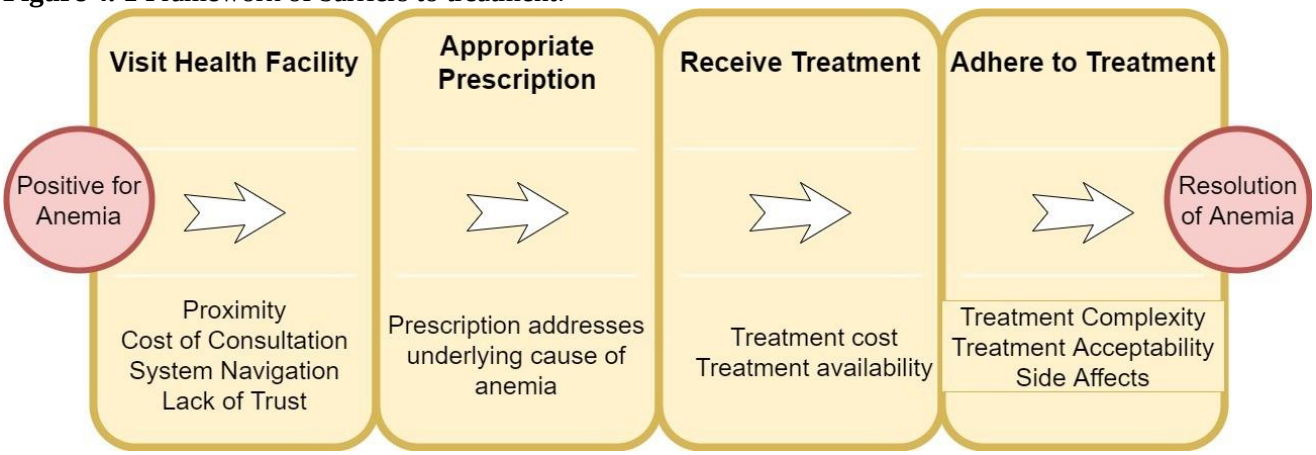
Our study serves as an exploratory precursor to further research. Therefore, several limitations must be noted. Anemia prevalence was lower than anticipated in the region, resulting in our sample size being too small to capture smaller potentially significant associations with explored risk factors. In addition, due to limited resources, Pb was measured in hair rather than the standard matrix of blood for identifying exposure, and C-reactive protein was measured as a binary rather than continuous marker of inflammation. Even so, lead measured in hair, especially in populations with high fish consumption, is used as a marker of exposure across the literature and the binary measure of C-reactive protein provides some preliminary data. In much of the existing literature only micronutrients are measured objectively; therefore, our objective measures of Pb and inflammation are a strength. We also use hemoglobin measures from semi-automated blood counts of venous samples that are a more valid measure of anemia than those taken from capillary blood in standard screening tools such as the Hemocue 201. Furthermore, the longitudinal nature of our data strengthens our ability to identify associated factors as potential risk factors or valuable risk profiles for persistent anemia in larger studies.

4.6 Conclusion:

Our study is too small to draw definitive conclusions about risk factors for persistent anemia and lower hemoglobin levels. It does, however, provide preliminary data demonstrating a need for larger longitudinal studies that further evaluate best methods for frequency and timing of anemia screening, especially in settings where heavy metal exposure is suspected. Furthermore, it provides examples of increasing challenges of climate change and pandemics that are important to consider when evaluating access to care in community settings.

4.7 Tables and Figures

Figure 4. 1 Framework of barriers to treatment.



Notes: The top provides broad categories of where barriers may occur in the continuum of care and bottom are potential barriers that we will be measuring.

Figure 4. 2 Summary of participants in study and their reported clinical management and treatment.

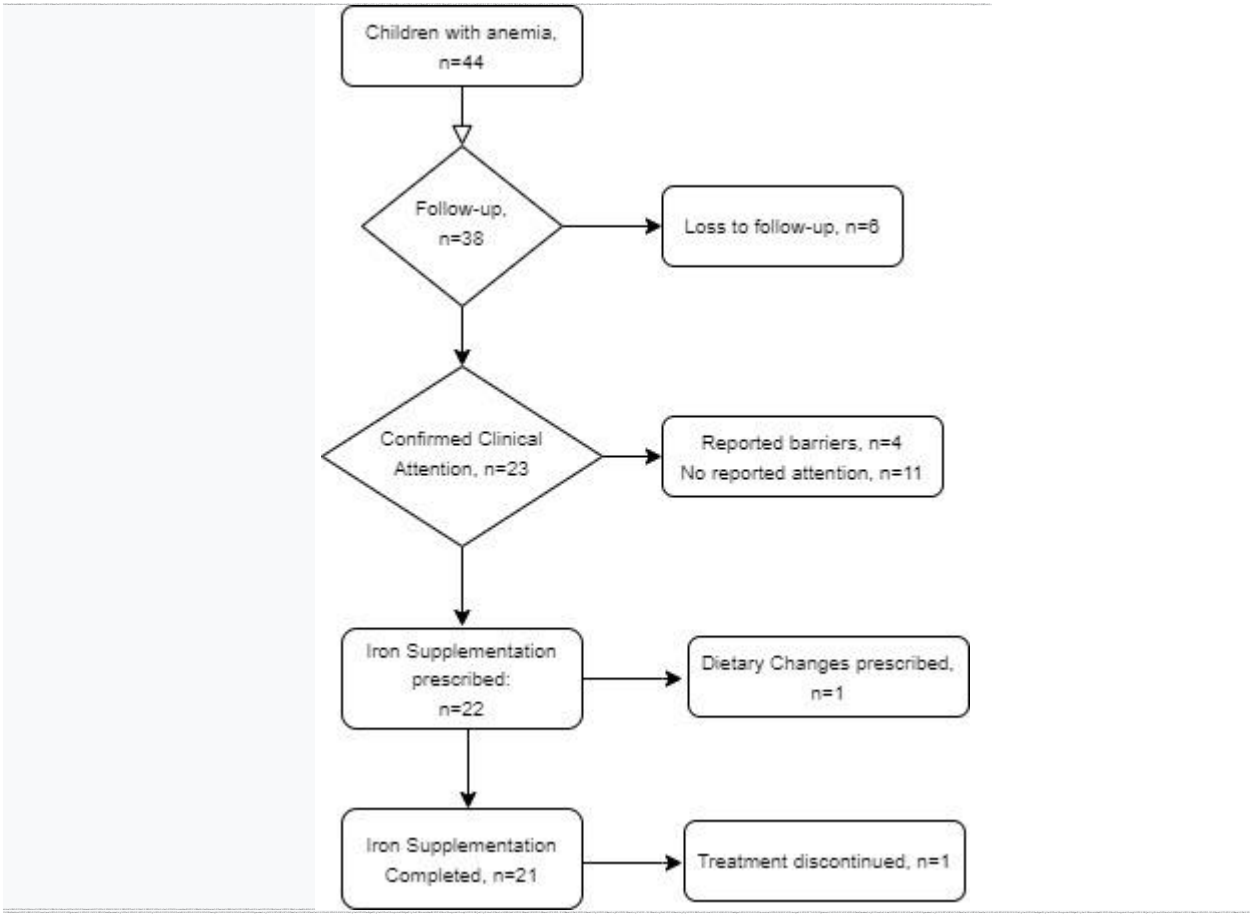


Table 4. 1
Study Population Characteristics by Persistent Anemia Status.

Characteristics	Total (N=38)	Not Anemic at 4 months (N=29)	Persistent Anemia at 4 months (N=9)
	N (%)	N (%)	N (%)
Exposed District	24 (63%)	18 (62%)	6 (67%)
Control District	14 (37%)	11 (38%)	3 (33%)
6-24 months	25 (66%)	18 (62%)	7 (78%)
25-59 months	13 (34%)	11 (38%)	2 (22%)
Male	23 (61%)	19 (66%)	4 (44%)
Female	15 (39%)	10 (34%)	5 (56%)
Prior history of anemia	2 (5%)	1 (3%)	1 (11%)
Prior history of iron supplementation	9 (24%)	7 (24%)	2 (22%)
Moderate Anemia at baseline	4 (11%)	2 (7%)	2 (22%)
Iron deficient at baseline	4 (11%)	2 (7%)	2 (22%)
Probable Lead Exposure (Pb>5)	11 (29%)	7 (24%)	4 (44%)
Elevated C-Reactive Protein	3 (38%)	3 (11%)	0
Giardiasis	8 (21%)	6 (21%)	2 (22%)
Did Not Visit Health Center	15 (39%)	11 (38%)	4 (44%)
Obtained Iron Supplementation	22 (61%)	17 (59%)	5 (55%)
Completed Iron Supplementation	21 (55%)	17 (59%)	4 (44%)

Note: Based on two-sided fisher's exact test there were no statistically significant difference between children with and without persistent anemia.

Table 4.2
Characteristics of Children with Persistent Anemia after 4 months

District	Age (Years)	Sex	Completed Treatment	Treatment *	Fe Deficient at baseline	Fe Deficient at 4 months	Hair Pb >5ppm	Hemoglobin Change g/dL	Ferritin Change mcg/L
Exposed	1	Female	No	FeSO4 20 Drops	Yes	Yes	No	-0.4	-5.47
Exposed	4	Female	Yes	FeSO4 1 Tsp.	Yes	Yes	Yes	-0.1	-7.13
Exposed	4	Male	No	No supplement	No	No	Yes	0	-1.1
Exposed	0	Male	Yes	FeSO4 26 Drops	No	No	Yes	1.2	24.3
Exposed	1	Male	No	FeSO4 1 Tsp.	No	.	No	-0.1	.
Exposed	1	Female	No	No Supplement	No	Yes	No	-0.1	-34.03
Control	0	Male	No	No Supplement	No	Yes	No	0.5	-26.64
Control	0	Female	Yes	FeSO4 13 Drops	No	Yes	Yes	1.2	-92.44
Control	0	Female	No	No Supplement	No	No	No	0.5	-5.42

Note: *Supplement label with daily dose is what was copied from supplement containers in participants homes. "." Data was missing

Table 4.3
Results from bivariate linear regressions to explore associations with average change in hemoglobin

		Average Hemoglobin Change	Adjusted Average Hemoglobin Change
	(n)	β (g/dL)	β (g/dL)
Male	23	Ref.	Ref.
Female	15	-0.4 (-0.8, -0.005)	---
Age 6-24 months	25	Ref.	Ref.
Age 25-59 months	13	0.1 (-0.3, 0.6)	---
Control District	14	Ref.	Ref.
Exposed District	24	0.2 (-0.2, 0.6)	0.1 (-0.3, 0.6)
Not Iron deficient	34	Ref.	Ref.
Iron deficient *	4	-0.4 (-1.0, 0.3))	-0.3 (-1.0, 0.3)
No or Incomplete Supplementation	17	Ref.	Ref.
Complete Supplementation	21	0.1 (-0.3, 0.5)	0.08 (-0.3,0.5)
Pb>5ppm	27	Ref.	Ref.
Pb≤5ppm	11	-0.20 (-0.6, 0.2)	-0.06 (-0.5, 0.4)
No Giardia	29	Ref.	Ref.
Giardia	8	-0.2 (-0.7, 0.3)	-0.2 (-0.7, 0.3)
Not Currently Breastfed	28	Ref.	Ref.
Currently Breastfed	10	-0.5 (-1.0, -0.1)	-0.7 (-1.3, -0.1)

Note: * Serum ferritin <12mcg/L Ref.=Reference; OR=Odds Ratio. Adjusted models include age and sex as covariates.

Table 4.4
Results from bivariate linear regressions to explore associations with average change in hemoglobin

		Completed Supplementation		
	Total (N=38)	No (N=17)	Yes (N=21)	p-value*
	n (%)/ Avg (SD)	n (%)/ Avg (SD)	n (%)/ Avg (SD)	
Control District	14 (37%)	9 (53%)	5 (24%)	0.094
Exposed District	24 (63%)	7 (44%)	17 (77%)	
6-24 months	25 (66%)	12 (71%)	13 (62%)	0.73
25-59 months	13 (34%)	5 (29%)	8 (38%)	
Male	23 (61%)	9 (53%)	14 (67%)	0.51
Female	15 (39%)	8 (47%)	7 (33%)	
Limited trust in Health post	26 (68%)	10 (59%)	16 (76%)	0.73
Side Effect	10 (26%)	0	10 (47%)	1.0
Anemia in Past Year	2 (5%)	2 (12%)	0	0.19
Previous Anemia treatment	8 (21%)	5 (29%)	3 (14%)	0.44
Time to Health Post (minutes)	28 (18)	26(17)	31 (18)	0.32
Cost of Going to Health Post (Soles)	3.8 (0.8)	4.2 (1.3)	3.4 (1.0)	0.64
Age (continuous)	2(1.3)	2 (1.2)	2.1 (1.4)	0.83

Note: * P-values for Fisher's Exact Test

Table 4.5
Sub-analysis of risk factors among children completing treatment

	Total	Not Anemic at 4 months	Persistent Anemia at 4 months	p-value	Average Change in Hemoglobin	Average Change in Serum Ferritin
	N=21	N=16	N=4			
Control District	5 (24%)	4 (25%)	1 (20%)	0.82	0.78 (0.5)	-69 (78)
Exposed District	16 (76%)	12 (75%)	4 (80%)		1.0 (0.7)	-12.4 (27.2)
6-24 months	13 (62%)	9 (56%)	4 (80%)	0.34	0.9 (0.7)	-25.3 (63.9)
25-59 months	8 (38%)	7 (44%)	1 (20%)		1.0 (0.6)	-25.5 (25.2)
Female	7 (33%)	4 (25%)	3 (60%)	0.15	0.8 (0.6)	-37.9 (63.3)
Male	14 (67%)	12 (75%)	2 (40%)		1.1 (0.6)	-14.6 (27.7)
Moderate Anemia at baseline	4 (19%)	2 (13%)	2 (40%)	0.17	1.5 (0.3)	-18.1 (64.6)
Iron deficient	4 (19%)	2 (13%)	2 (40%)	0.17	0.53 (1.0)	-4.1 (3.9)
Probable Lead Exposure (Pb>5)	6 (29%)	3 (19%)	3 (60%)	0.075	0.8 (0.6)	-14.7 (44.6)
C-reactive Protein	1 (5%)	1 (7%)	0 (0%)	0.55	0.9	-58.4
Giardia	5 (24%)	3 (19%)	2 (40%)	0.33	0.6 (0.62)	-15.7 (31.6)

CHAPTER 5: RESEARCH PAPER #3

A Non-Inferiority Study Of Hemocue 301 Versus Hemocue 201 In Screening For Childhood Anemia In Tumbes, Peru

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Running head: Comparison of Hemocue Anemia Screening Tools in Peru

Key words: Anemia, Screening Tools, Validity, Non-Inferiority Test, Peru

5.1 Abstract

Background: The Hemocue 201 hemoglobin meter has been a standard screening tool for anemia used worldwide since 1996. However, multiple studies have demonstrated its limited reliability and validity when compared to automated blood counts (ABC) for identifying childhood anemia. Although its user manual contraindicates application in temperatures over 30 degrees Celsius, this device is often used in warmer settings without temperature control. A newer hemoglobin meter, Hemocue301, can be stored in settings of up to 40 degrees Celsius. The objective of this study was to evaluate the Hemocue301 as an alternative screening tool to the Hemocue201 for childhood anemia.

Methods: We conducted a cross-sectional study of children aged 6 months to 59 months in Tumbes, Peru, during February 2021, and recruited children in a door-to-door fashion. Participants provided a peripheral venous blood sample that was evaluated on site for hemoglobin concentration using the Hemocue201 and Hemocue301. The remaining blood was sent to a nearby laboratory to measure hemoglobin by semi-automated blood count (sABC) as a gold standard. A non-inferiority test of sensitivity was conducted to evaluate the Hemocue301 as an alternative to Hemocue201, with an acceptable margin set at no more than 10 percentage points lower sensitivity.

Results: We recruited 265 children of whom 43 were identified as having anemia using the sABC according to hemoglobin thresholds established by the World Health Organization. The sensitivity, specificity, positive predictive value, and negative predictive value of the Hemocue 201 were 60%, 87%, 47%, and 91%; for the Hemocue 301 they were 23%, 99%, 77%, and 75%. The Hemocue301 sensitivity was 37 percentage points (95% CI: 18, 56) less than Hemocue201 ($p<0.001$) and non-inferiority of the Hemocue301 could not be established.

Correlation with the gold standard for Hemocue201 (r=0.36) and Hemocue301 (r=0.41) were both low.

Conclusions: Hemocue 201 remains a preferred screening tool to Hemocue301 including settings with warmer temperatures. However, given the lower than expected prevalence of childhood anemia in our study populations, additional studies should be conducted in larger populations to confirm our results.

5.2 Introduction

An estimated 293 million children under the age of five years worldwide have anemia[1]. Left untreated, the lack of systemic oxygen delivery resulting from anemia can have long-term effects on child development, especially during early childhood[28]. While the prevalence of childhood anemia in children under the age of 5 is a little under 10% in countries such as the United States and Canada, preschool age children in lower and middle-income countries (LMIC) bear a much larger burden, especially in rural areas [1,132]. Malnutrition is the main cause of childhood anemia in these settings, although infection and other factors also contribute [1].

The Hemocue 201 is a portable screening device that has been used for over 20 years in many countries to identify children with anemia for clinical management, as well as to monitor the population prevalence of anemia [4,133]. Although follow-up testing using an automated blood count (ABC) to quantify venous hemoglobin is recommended in order to rule out false positive screening results, presumptive iron supplementation based on the results from the portable device is common, especially in LMIC's. Although Hemocue201 is considered a valid tool for identifying anemia in the general population, multiple studies have documented sub-optimal validity and reliability of Hemocue201 in young children [134–137]. In one study, the sensitivity of the Hemocue201 for detecting childhood anemia in three populations in the US ranged from

33%-65 %[134]. Differences in hemoglobin meter performance across studies can be attributed in part to operator experience, source of the blood sample (i.e. venous versus capillary), and adherence to storage protocols particularly in settings with extreme heat [138].

The manufacturer's manual indicates that microcuvettes for the Hemocue201 can be stored at room temperature, with a maximum of 30°C [47]. In temperate settings, this makes storage easy and inexpensive. However, many LMIC's have regions with high anemia prevalence where temperatures frequently exceed 30 °C [47]. In these regions, cooling facilities may be required to store Hemocue201 microcuvettes according to manufacturer indications. Temperature-controlled storage space can be expensive, and in these LMIC's settings may not be available or feasible. A newer model (Hemocue 301) is indicated for temperatures of up to 40°C and has been validated in multiple settings [48]. In studies set in warmer locations its hemoglobin results were generally more valid than the traditional Hemocue 201 when compared to measures of hemoglobin from ABC [44,79,135]. In one study simulating high temperature (37°C) and high humid settings, reactives within Hemocue201 microcuvettes degraded within 10 minutes while Hemocue301 reactives were unaffected [139]. This suggests that in high temperature settings, the Hemocue201 microcuvettes should be stored at cool temperatures right up to the point of use, adding both logistical complexity and cost.

We conducted a study to evaluate Hemocue301 as an alternative to the standard Hemocue201 in screening for childhood anemia during the warm and humid summer months in Tumbes, Peru, where childhood anemia is prevalent. We considered that the Hemocue301 would be an acceptable alternative screening tool if its performance was non-inferior to the Hemocue 201, given the potential benefits with respect to temperature stability and necessary storage conditions.

5.3 Methods

Study Design: We conducted a cross-sectional study of anemia among children ages 6-59 months from two rural districts (San Jacinto and Casitas) in Tumbes, Peru, during the month of February 2021. We used a non-inferiority framework to compare the sensitivity of Hemocue301 versus Hemocue201 because the Hemocue301 microcuvettes are more robust to high temperatures, which would reduce cost and logistical complexity for point of care screening [140,141]. We prioritized comparison of sensitivity because micronutrient supplementation is both widely available and safe, so the greatest concern would be missing children with true anemia (i.e. false negative screening results).

Study Setting: Tumbes is the northern most region on the Peruvian coast. Over the past decade the estimated prevalence of childhood anemia has fluctuated between 40 to 55 percent without a clear temporal pattern, despite increased iron supplementation [4,142]. These estimates are based on the Hemocue201, which has been the standard screening tool used in Peru since the 1990's. Tumbes has a tropical climate, with the period from January to April being the hottest time of year during which average temperatures range from 28-32^{o}C. Primary industries in this region are trade, fishing, and agriculture. Tumbes is a coastal region where people consume a substantial amount of fish both from the ocean as well as from freshwater sources. On the inland, rice, cassava, banana, lime, and papaya are among the main crops grown for household use. More details on the study site including risk factors for childhood anemia are found in Chapter 3.

Sample and Data Collection: The sampling frame was the census of children between the ages of 6 to 59 months maintained by the local health posts in the two rural districts included in our study. Recruitment was door to door. After obtaining guardian consent, we applied a survey tool

to collect demographic and clinical information about the participating child. A nursing technician then collected a peripheral venous blood sample from the upper extremity of each participant using a butterfly needle and vacutainer. Immediately upon extraction of the needle, remnant blood droplets from the needle tip were applied to Hemocue 201 and Hemocue 301 microcuvettes, which were then inserted into their respective devices. The order in which blood droplets were applied to the Hemocue 201 and Hemocue 301 was alternated between consecutive participants.

Screening Tools: The Hemocue201 (Hemocue AB, Angelholm, Sweden) [47(p201)] and Hemocue301 (Hemocue AB, Angelholm, Sweden) [48] are portable battery-operated systems. The Hemocue201 device applies azidemethemoglobin determination methods; microcuvettes contain sodium deoxycholate for red blood cell hemolysis, sodium nitrite to convert hemoglobin to methemoglobin and sodium azide to convert methemoglobin to azidemethemoglobin. Hemoglobin is then estimated by reading turbidity compensation at wavelengths of 570nm and 880nm. Storage and use of Hemocue201 microcuvettes are recommended for settings reaching up to 30°C. In warmer settings once packages of Hemocue201 have been opened, they should be used or discarded within a few days[138]. The Hemocue 301 alternatively estimates hemoglobin by absorbance of whole blood at the Hb/HbO2 isosbestic points (506nm and 880nm) for turbidity compensation [44]. Its manual indicates use in settings with temperatures up to 40°C. We stored the devices and cuvettes for both the Hemocue201 and Hemocue301 at ambient temperature, the standard practice for the region.

Diagnostic Gold Standard: Hemoglobin concentration was determined by the regional public health laboratory (DIRESA) using complete blood count results produced by a semi-automated

hematology analyzer (Prokan PE-6100, Guangdon, China), with results reported in g/dL. Results from the semi-automated blood count (sABC) were used to evaluate the Hemocue point of care tools. Venous blood measures by automated hematology analyzers are considered a gold standard for anemia diagnosis [143].

5.3.1 Statistical Analysis

We defined anemia based on the WHO classification system of mild (10-10.9dg/L Hb), moderate (7-9.9dg/L Hb), and severe (<7dg/L Hb), according to hemoglobin concentration measures reported from the sABC[118]. We calculated sensitivity, specificity and negative predictive values of the Hemocue201 and Hemocue301, and used a McNemar test to determine if differences in sensitivity and specificity between the devices were statistically significant [144]. We summarized agreement of anemia diagnosis of portable devices with sABC using kappa coefficients. We then measured concordance, correlation and linear fit of hemoglobin concentration (continuous variable) generated by each portable device with sABC results using Lin's concordance coefficient, Pearson's r, and R-squared values from linear regression.

We conducted a non-inferiority test of sensitivity for Hemocue301 with the Hemocue201. We set an acceptable margin for non-inferiority of 10 percentage points considering that estimates of Hemocue201 sensitivity to childhood anemia range from 75-91%, and expected prevalence for anemia in our study population is high (~40%)[4,137]. We calculated the observed margin of sensitivity by subtracting the sensitivity of the Hemocue301 from that of the Hemocue201 and running a test of equality of proportions in StataSE16 to calculate 95% confidence intervals. We defined non-inferiority of the Hemocue301 to be established if the upper bound of the 95% confidence interval of the margin of difference was less than the acceptable margin of 10

percentage points. We then explored whether applying a correction factor determined by regression and average differences between the Hemocue301 and sABC hemoglobin concentrations might modify our conclusions.

Ethical considerations: This study was approved by the institutional review boards at Universidad Peruana Cayetano Heredia in Lima, Peru and at Oregon Health & Sciences University in Portland, OR.

5.4 Results

A total of 265 children aged 6 months to 59 months from two districts in Tumbes, Peru participated in the study, with 262 providing sufficient blood to acquire results from both screening tools and the sABC. The age distribution was 148 (57%) between 6-24 months and 114 (43%) from 25-29 months. One-hundred twenty-eight (49%) were male. Forty-three (17%) had anemia based on results from the sABC of whom 64% were male, and 64% were 6-24 months of age. A large majority of anemia cases were mild (40/44; 91%), while only 4 had moderate anemia and none had severe anemia according to WHO standards **(Table1)**.

When applying the Hemocue201, 55 (21%) children screened positive for anemia, whereas when applying the Hemocue301 only 13 (5%) screened positive for anemia. Among the 4 moderate cases, Hemocue 201 classified three as having moderate anemia and one as having mild anemia, while Hemocue301 classified one with moderate, two with mild, and one with no anemia. Overall agreement with the sABC for anemia diagnosis was 82% (kappa=0.4) for the Hemocue201 and 86% (kappa=0.3) for the Hemocue301. However, the sensitivity of the

Hemocue 301 was much lower than that of the Hemocue 201 (23% vs 60%; p>0.001) and the specificity was higher (99% vs 87%; p>0.001) (**Table 2**).

The hemoglobin concentration reported by portable devices compared to those reported by the sABC are shown in **Figure 2**. R-squared values were relatively low both for the Hemocue201 (0.36) and Hemocue301 (0.41). The concordance of hemoglobin concentration from the sABC was 0.58 with the Hemocue201 and 0.64 with the Hemocue301. On average the Hemocue201 underestimated hemoglobin by -0.12 g/dL, whereas the Hemocue301 on average overestimated hemoglobin by 0.81g/dL **(Table 2)**. When applying a correction factor of 0.81 g/dL (average difference of hemoglobin concentration between Hemocue301 and the gold standard) subtracted from the hemoglobin concentration reported by Hemocue301, the device had similar sensitivity, specificity, and predictive values to the Hemocue201 (**Table 2**).

Sensitivity of the Hemocue301 was 37 percentage points (95% CI: 18, 56) lower than that of the Hemocue201. Applying the acceptable margin of 10 percentage points to this difference, non-inferiority of the Hemocue301 to the Hemocue201 was not established (Figure 1). Even after applying the correction factor to hemoglobin results, the Hemocue 301 sensitivity, although improved, did not meet our criteria for non-inferiority (percentage point difference: 2% (95% CI: -19, 23).

5.5 Discussion

In this study, the Hemocue301 was not established as a non-inferior tool to the Hemocue201 for screening for children with anemia. The sensitivity of 23% was far lower than that of the Hemocue201, and well outside the acceptable margin of 10 percentage points. These results

occurred even in the hottest months of the year in a tropical region, conditions in which the Hemocue301 should have an advantage. The low sensitivity of the Hemocue301 in our study was the result of a systematic overestimation of the hemoglobin concentration compared to sABC. This finding has been reported in other studies [79,135,136,139]. We found that by applying a correction factor, systematically subtracting 0.81 g/dL from the hemoglobin concentration reported by the Hemocue301, we were able to improve the sensitivity to be comparable with that of the Hemocue201. However, even relying on the adjusted results would not have established non-inferiority. This apparent underestimation of hemoglobin concentration by the Hemocue 301 should be carefully evaluated in larger studies and in different settings.

We also found notable limitations in the performance of the Hemocue201, which on average underestimated hemoglobin by 0.12 g/dL compared with the results of the sABC. Existing literature shows mixed results on the direction and magnitude of average difference in hemoglobin estimated by the Hemocue201 among young children compared against ABC[45,134,136,145,146]. Variability in these differences could be attributed to some studies using capillary rather than venous blood for hemoglobin measurement on portable devices, or to different storage conditions for the microcuvettes. Alternatively, because correlation between Hemcoue201 hemoglobin results and the gold standard is low, this tendency could be a product of chance. The 60% sensitivity of the Hemocue201 in our study is similar to what was observed in a study in Kansas and Mississippi[134]; however, some studies have reported estimated sensitivities of 90% and greater [137,145]. Although the estimated prevalence based on hemoglobin results from Hemocue201 and sABC were similar, the lower than expected correlation presents a

concern about whether children requiring treatment would be correctly identified using the Hemocue201, and what factors contribute to these inconsistencies.

Another unanticipated result of our study was the prevalence of childhood anemia of 17% measured both by the sABC and the Hemocue201, a level less than half of what has been measured for the Tumbes region using Hemocue201 in recent years[4]. Hemoglobin measured from venous blood is typically lower than that of capillary blood[147]; average hemoglobin concentrations among children age 1 to 5 years old measured using venous blood and an ABC were 1-2 g/dL lower than those measured by capillary blood using Hemocue201 and Hemocue301 [145,146]. Therefore, we anticipated measuring a greater prevalence of childhood anemia in our study based on the use of venous blood. Our results may mean that the prevalence in the Tumbes region has recently dropped, although we are not aware of any major changes in anemia prevention programs that would explain this precipitous drop. It is also possible that our small study population does not represent the Tumbes region as a whole. Major differences in the application by end users of screening tools, and the reliability of the Hemocue tools in general, should be further evaluated as plausible reasons for this discrepancy, especially among children.

Our study has several important limitations. It is a relatively small study in two districts of a single region, therefore, results may not be as generalizable as those in larger studies with more diverse populations. The low prevalence of moderate and severe anemia in our study limits our ability to estimate screening tool validity and discuss utility for children with more severe anemia. It should be noted, however, that because moderate to severe cases are more likely to be identified in their clinical phase, our study provides valuable insight into identifying preclinical

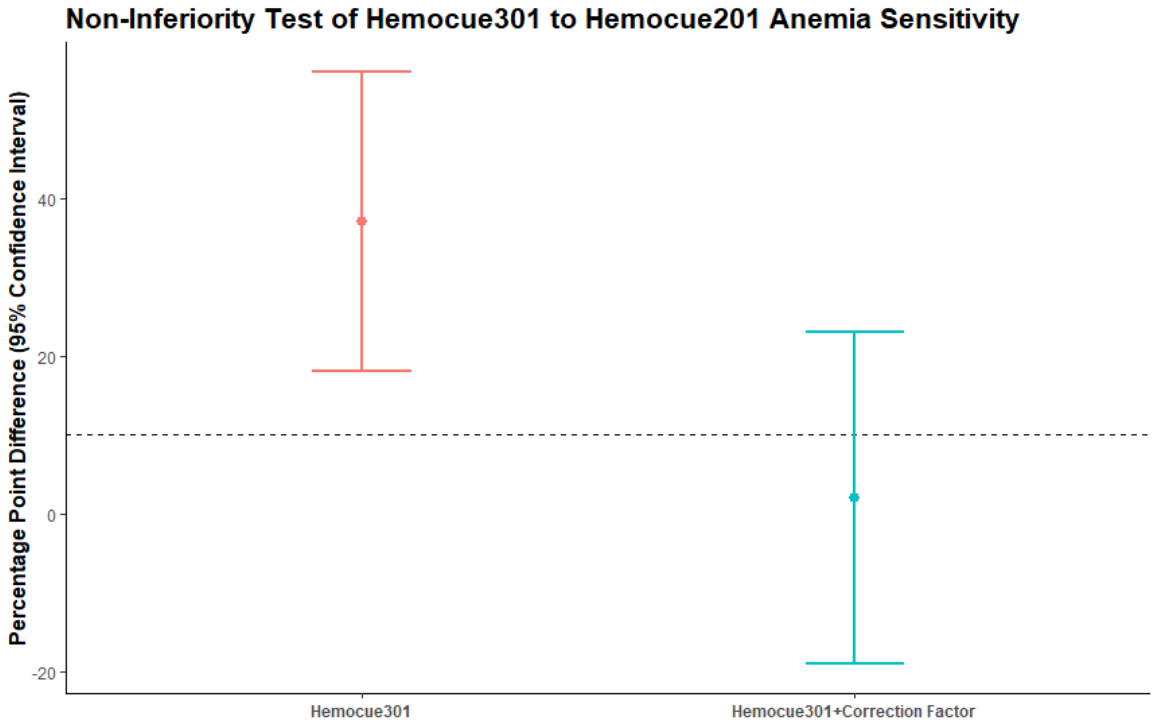
childhood anemia. We also relied on a semi-automated blood count instead of a completely automated blood count, although the difference in reported hemoglobin concentration is likely to be minimal. We also did not record specific temperatures at time of the study so we could not directly evaluate the effect of temperature on results of each tool. Finally, while the Hemocue201 meters had been calibrated before the study start, the Hemocue301 meters were not calibrated according to the user manual indications that calibration is not necessary [48(p301)]. Based on the consistent overestimation of hemoglobin concentration measured by the Hemocue301 in our study, it may be worth reconsidering whether environmental factors or end-user factors might modify the need for calibration. Despite the aforementioned limitations, our study contributes important information regarding the selection and use of hemoglobin screening devices, particularly in warmer climates.

5.6 Conclusion

Based on the results of this study, the Hemocue201 remains a preferred device for anemia screening compared to the Hemocue301, even in warmer settings. The prevalence of childhood anemia measured in our study was less than half than what has been estimated at the regional level of Tumbes in recent years. Larger studies in different settings should be carried out to confirm our findings and to explore alternative screening approaches.

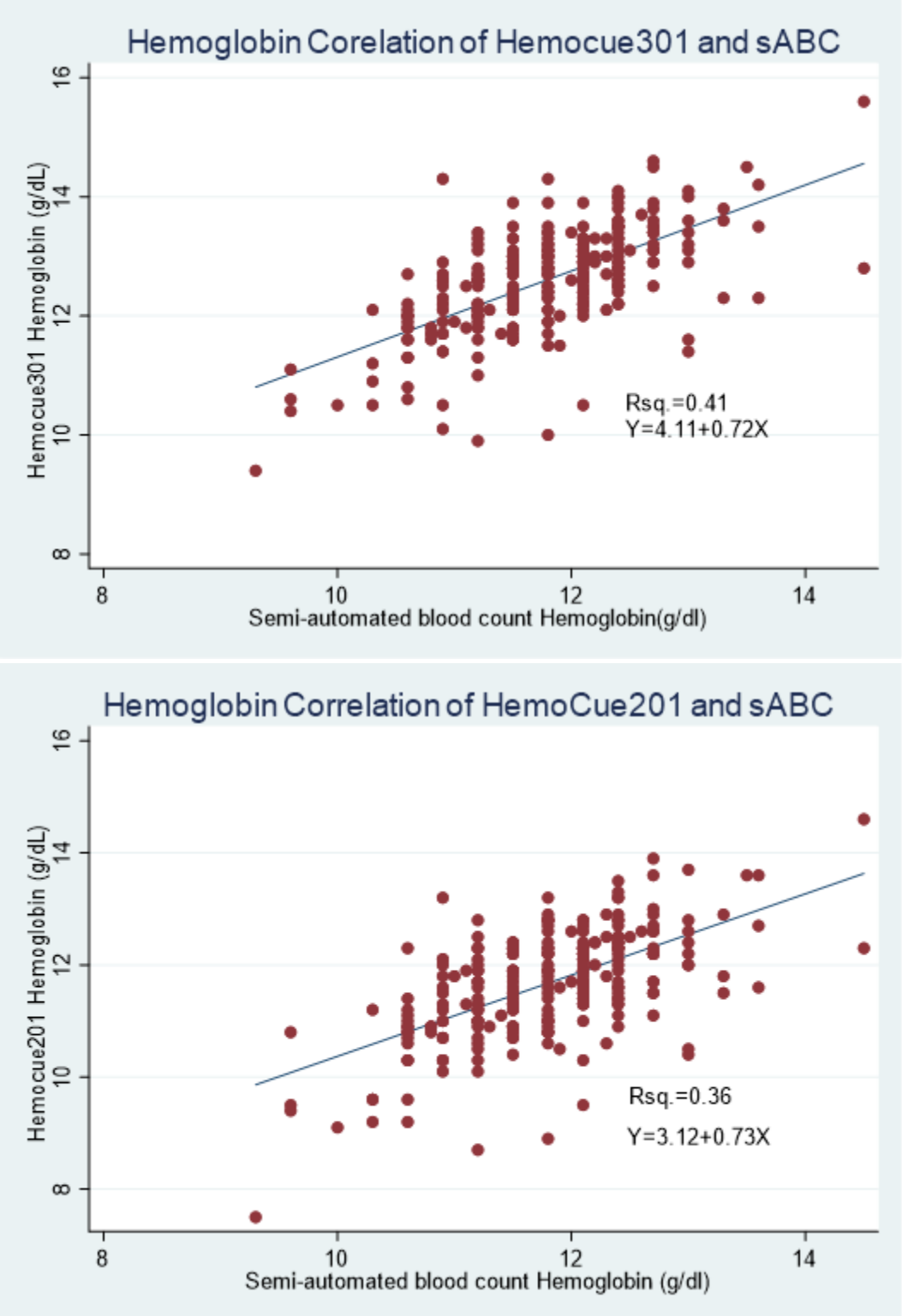
5.7 Tables and Figures

Figure 5. 1 Non-Inferiority Test of Hemocue301 to Hemocue201 Anemia Sensitivity.



Notes: This figure illustrates the difference of sensitivity for the Hemocue201 and Hemocue301 with 95% confidence intervals calculated for difference in proportions. The dashed line indicates the maximum 10% margin of difference we defined to establish non- inferiority. Non-inferiority of the H301 would be established if the upper bound of the 95% CI was less than 10 percentage point margin (shown as dashed line). Sensitivity was calculated using the standard definition of anemia (hemoglobin<11g/dL) for all screening tools. A corrected sensitivity difference was calculated using hemoglobin concentrations from Hemocue301 and adding 0.81g/dL , the average difference between hemoglobin concentrations between Hemocue301 and sABC.

Figure 5. 2 Correlation of Hemocue Hemoglobin Concentrations with sABC Concentrations



Notes: Linear regression of Hemocue301 (top) and Hemocue201 hemoglobin results (bottom) with hemoglobin results from semi-automated blood count (sABC). Blue line indicates the regression line fitted based on association.

Table 5.1
Apparent prevalence of anemia by anemia screening tools

	No Anemia	Any Anemia	Mild Anemia	Moderate Anemia
Semi-Automated Blood Count	84%(219)	17%(43)	15% (39)	2%(4)
Hemocue 201	79%(207)	21%(55)	16% (43)	5%(12)
Hemocue 301	95%(249)	5% (13)	4% (11)	1%(2)
Hemocue 301 corrected	82% (216)	17% (46)	13% (34)	5%(12)

Table 5. 2
Measures of validity for the Hemocue 201, Hemocue301, and Hemocue 301 with a correction factor

	Hemocue 201	Hemocue 301	Hemocue301 – 0.81 g/dL
Sensitivity any anemia	60% (26/43; CI=44,75)	23% (10/43; CI=12, 38)	58% (25/43; CI=42, 73)
Specificity any anemia	87% (190/219; CI=82,91)	99% (216/219; CI=96,99)	90% (198/219; CI=86, 94)
PPV any anemia	47% (26/55; CI=34,61)	77% (10/13; CI=46,95)	55% (25/46; CI=39, 69)
NPV any anemia	91% (190/207; CI=87, 95)	87% (216/249; CI=82,91)	92% (198/216; CI=88, 95)
Sensitivity moderate anemia	100% (4/4)	75% (3/4)	100% (4/4)

Note: Correction Factor= -0.81g/dL (average difference between hemoglobin concentration from Hemocue301 and semi-automated blood count).

CHAPTER 6: SYNTHESIS

6.1 Summary

Anemia, a condition characterized by insufficient hemoglobin for bodily functions, is estimated to affect a third of children across the globe. In the short term, it can lead to symptoms including fatigue, shortness of breath, or dizziness. Long-term, especially in cases of persistent iron deficiency anemia, childhood anemia has been associated with decreased neurocognitive function. Because of anemia's high prevalence among children and potential long-term effects on population health, anemia is a global health priority in many low to middle-income countries (LMIC's), including Peru. Mass iron supplementation for anemia is a common strategy in many countries. However, it has frequently resulted in minor changes in anemia prevalence post intervention and has also increased risk for infectious disease mortality in many settings, making it essential to more closely evaluate the benefits[148]. Potential explanations for minimal change in anemia prevalence include prevalent anemia etiologies beyond nutritional iron deficiency, lack of access to effective treatment, or limited validity of screening tools. My dissertation research investigated reasons for this disparity by evaluating the association of heavy metal exposure with childhood anemia (chapter 3), exploring risk factors for persistent anemia (chapter 4), and by comparing the validity of two anemia screening devices, Hemocue 201 and Hemocue301, for identifying anemia (chapter 5). This research was conducted in the Tumbes Region of Peru, in collaboration with the Centers for Global Health Tumbes, a local academic community research center, as well as with DIRESA, the regional public health authority. The research and collaboration were designed to inform development of prevention strategies for childhood anemia in Tumbes, a top public health priority for the region.

In Chapter 3, I addressed the first aim of characterizing and evaluating the association of heavy metals with anemia and hemoglobin by conducting a cross-sectional study in two districts

of rural Tumbes, Peru. We recruited children 6-59 months and interviewed parents to obtain demographic, household, and clinical history information. We collected participant blood, hair, and stool samples to objectively measure hemoglobin (used to identify anemia), micronutrients, presence of inflammation, exposure to heavy metals, and parasitosis. For analysis, I compared distributions of metals, serum ferritin, vitamin B12, folate, C-reactive protein positivity and giardia across a district with known exposure to mining runoff and a control district. I then presented cross-sectional analysis of characteristics by anemia status and conducted univariable analysis of our measures with hemoglobin. I conducted multiple linear regression analysis with THg, Pb, As, and Mn as independent variables and hemoglobin as the main outcome. One set of models used log transformed metal variables to evaluate linear associations and a second set of model used categorical variables by tertiles for each metal to capture non-linear relationships. I adjusted for age and birthweight and added an interaction term for district and THg for my final model. Interaction for sex, district, serum ferritin, folate, and vitamin B12 with each of the heavy metals was tested but only THg and district were statistically significant.

Among the 264 children we recruited, seventeen percent had anemia (44/264) with an average hemoglobin concentration of 11.8 (Range: 9.3-14.5). Neither anemia prevalence nor hemoglobin concentration was significantly different across district. However, serum ferritin levels were significantly lower in the mining runoff exposed community. Hair Pb, As, Mn, and folate levels were all significantly higher in the mining runoff-exposed district, as compared to Casitas. Age, birthweight, and breastfeeding status were associated with hemoglobin during univariable analyses. In the final model with multiple metals, I observed no significant association of THg, Pb, and Mn with hemoglobin concentration. There was a statistically significant association of As with hemoglobin. However, between the small magnitude of

association and lack of literature to support a positive association of Arsenic with hemoglobin, significance was likely spurious. Alternatively, As exposure may come from a nutrient rich source as it was highly correlated with folate. Overall, I concluded that larger studies were warranted to apply techniques such as structural equation modelling that better account for collinearity of metals and potential multiple metal effects on anemia. I also concluded that there is evidence of competitive inhibition of iron absorption in the mining runoff-exposed community and that identifying potentially intervenable risk factors for iron deficiency and the progression to iron deficiency anemia is a critical next step.

In Chapter 4 we conducted a small cohort study to explore risk factors for persistent anemia and completion of treatment post anemia screening. All children with anemia during our cross-sectional study (Chapter 3) were visited after 4 months and asked to provide another blood sample to measure hemoglobin and micronutrient levels. Guardians were surveyed about their experience accessing clinical attention, obtaining prescribed supplements, and adhering to treatment regimens. I explored suspected risk factors associated with anemia persistence and hemoglobin change by conducting Fishers exact test of potential risk factors across anemia status and linear regression to estimate average hemoglobin change associated with potential risk factors. I applied simple bivariable analysis to evaluate potential factors associated with treatment regimen completion as a secondary outcome using Fisher's exact test for categorical factors and t-test for continuous factors to identify statistically significant differences. I then explored open-ended responses about barriers to receiving clinical attention for recurring themes, and conducted an additional sub-analysis among children completing treatment to explore supplement efficacy.

In this study, I found that children with persistent anemia were more likely to be younger and breastfed. Although not statistically significant, more children with persistent anemia had higher hair Pb levels at baseline. No association was observed between anemia persistence and completion of iron supplementation. When exploring risk factors and barriers for treatment completion, I observed that all but one child, whose parents reported receiving clinical attention, completed prescribed treatment regimens. Therefore, my observations centered on barriers to accessing care. Common themes described by parents were lack of health personnel, limited access to roads during heavy rains, and COVID-19 related restrictions and risk perceptions. Based on this study, it is plausible that minimal changes to childhood anemia prevalence where iron supplementation has increased may be due to multiple anemia etiologies beyond nutritional iron deficiency, and in some settings limited healthcare access to receive appropriate treatment and follow-up. However, as this study was small and exploratory, larger studies investigating alternative or additional anemia treatments as well as barriers to accessing clinical attention are warranted.

In chapter 5, I evaluated the validity of two portable screening devices for identifying anemia and conducted a non-inferiority test of sensitivity to determine if the newer Hemocue301 would be a viable alternative to the Hemocue201, which has been the standard device in practice. The rationale behind this comparison was that the Hemocue 201 user manual indicates that reactives should be stored and tests should be run at a temperature under 30^{o}C, whereas Hemocue301 is indicated for use in settings up to 40^{o}C. In settings such as Tumbes where outdoor temperatures during summer can surpass 30^{o}C, the Hemocue301 might be a better alternative than the Hemocue201 due to the operational complexity and expense required for temperature control for the latter. We conducted rapid tests using both devices at the same time

as initial blood and hair collection described in the earlier chapters. To compare the validity of the tools, I calculated sensitivity, specificity, positive predictive value, and negative predictive value of each apparatus, treating hemoglobin results of <11g/dL from semi-automated blood counts as the gold standard.

I applied a non-inferiority test on sensitivity because standard treatment is affordable and the risk of persistent anemia outweighs the risk of treatment in this region. Based on the results of this test of sensitivity, I determined that Hemocue301 was not a non-inferior tool to Hemocue20. However, correlation of hemoglobin values with automated blood count measures of hemoglobin was low for both Hemocue201 (r=0.36) and Heomcue301 (r=-0.41) devices. I concluded that even in warmer settings the Hemocue201 was preferable to Hemocue301, but that with calibration adjustments the tools might be comparable. Best methods, however, would be to apply targeted semi-automated blood counts to high-risk children, or at a minimum conduct a follow up confirmatory test with an automated blood count.

Collectively, these studies provide a meaningful body of work for informing the development of new strategies to decrease the global burden of childhood anemia in Tumbes by exploring environmental causes (aim1), exploring iron supplementation efficacy and access to care (aim 2), and exploring the validity of tools used to identify anemia (aim 3). Our research provides insight on how we may take a more ecologic approach to decreasing the burden of childhood anemia, especially in settings where populations have been historically underserved, under resourced, and overburdened with industrial pollution. Results from aims 1 and 2 demonstrate that in settings downstream from mining, childhood screening for Pb exposure, especially among children with persistent anemia, is needed. Serum ferritin results further emphasize the need to better understand the impact of iron deficiency and what makes some

children more vulnerable to progressing to iron deficiency anemia. Finally, aim 3 highlights the need for more valid point of care screening tools and at minimum emphasizes the need for increasing access to testing with complete blood count.

6.2 Strengths

This work contains several strengths. The most notable is that we measured multiple biomarkers. This provides us with the unique ability to explore anemia etiology in our study population. The baseline and follow-up measures that include micronutrients such as serum ferritin as well as visual confirmation of treatment access (observation of iron supplement bottles) provide us with objective measures that complement information about treatment on surveys. Open-ended components of the survey also allowed us to inductively explore barriers to care including social and structural barriers to treatment. The overall research addresses a broad range of factors that can contribute to multiple facets of efforts to monitor, screen, and decrease childhood anemia prevalence.

Beyond a multi-faceted data approach, this research was implemented with an equity lens. Our team has worked for many years in the community and as part of an earlier study had asked communities and leaders about their priorities. Persistently high measures of childhood anemia and suspected chronic heavy metal exposure in one community was expressed as a priority in both community focus groups and conversations with local health authorities. For this reason, we believe there were high rates of participation in the community. This approach and the intentional recruitment in a community historically exposed to heavy metals provides us with results that inform how prevention and treatment of anemia in such settings should be approached. This body of research simultaneously informs standards of anemia care in

underserved settings and sets an example of what can be achieved when researchers collaborate with community and governments to meet mutual priorities.

6.3 Limitations

Despite multiple strengths, this body of research has several limitations. Because fewer children than expected had anemia at baseline, results drawn from our analysis about persistent anemia and anemia treatment are limited and would require larger populations to observe statistical significance for smaller associations. Furthermore, while measures of metals in hair are indicative of exposure, the measures are more standardized for certain metals. MeHg is the only metal we measured that has a standard reference dose for hair and this is for fetotoxicity among pregnant women. Reference doses and standard measures of exposure for Pb, Cd, As, and Mn have historically been in matrices such as blood and urine. However, epidemiologic studies do exist for each of these metals that use measures in hair to examine associations, and the added benefit of measuring all metals in hair is that we may infer that markers are representative of the same period of time. It is important to note that this research does not address sources of exposure or causes of malnutrition. It does, however, provide a steppingstone for public health practitioners to broaden the exploration of potential interventions that include both environmental, structural, and behavioral approaches. Finally, our research was conducted during the COVID-19 pandemic; therefore, barriers to health care will have been exacerbated during this event. However, with climate change and globalization, pandemics and other large-scale disruptions of clinical and public health systems are likely to increase; documenting adverse impacts on access during the current event can help governments prepare for future events. Although there are limitations, our research will be useful moving forward.

6.4 Conclusions and public health impacts

Overall Conclusion: Validity of anemia measures likely explain a large part of why many populations similar to ours observe minimal change in childhood anemia prevalence despite major intervention efforts, but population exposure to heavy metals and giardia should also be considered when developing childhood anemia intervention strategies moving forward. It is clear based on the limited validity of the Hemocue201 hemoglobin meter as compared to venous sABC that past estimates have likely been unreliable, therefore, estimates of the impact of increased intervention on childhood anemia, including iron supplementation, are unreliable. Although we observed no significant association of THg, Pb, As, and Mn with hemoglobin, low population level iron stores and significantly higher heavy metals levels in the mining runoff-exposed district, as well as the exclusive presence of all cases of iron deficiency anemia in this district, provide preliminary support for metal induced iron deficiency for which iron supplementation could have some impact. The direct association of Pb and As with serum ferritin at baseline, as well as a general decrease in serum ferritin at 4 months follow-up among all anemia cases, provide further supporting evidence for this mechanism. Another interesting result was the association of anemia with giardia, because most deparasitation campaigns in the region do not provide treatment that is effective against giardia. Four month follow-up among anemia cases showed no association of persistent anemia with giardia; however, we did not directly ask about giardia treatment. Therefore, further evaluation of treating for giardia as an effective intervention against anemia is warranted.

Anemia screening: Based on this body of research, current strategies for anemia screening may need to be reconsidered. Although the main goal of chapter 5 was to evaluate Hemocue 301 as an alternative screening tool to the standard tool in the region (Hemocue201), our results were also valuable for considering how screening might be approached. Both versions

of the Hemocue had low validity; therefore, at minimum for children screening positive for anemia, emphasizing the importance of follow-up testing with automated blood count and re-testing post treatment could provide a more effective approach. This would not only improve specificity and validity of measuring persistent anemia, but it would also provide clinicians with more insight into actual etiology of anemia. An even better scenario would be identifying more valid point-of-care screening tools. Our results from chapter 3 and 4 indicate that children aged 2 years and younger are more likely to have anemia that is persistent than older children. Seven of the children with persistent anemia were age 2 years or less at baseline. Although our sample size was too small to state statistical significance, larger studies should explore if this distribution remains consistent. Standard anemia screening in many places is done for one-year old children but annual screening until age 3 or 4 years should be considered[1]. Therefore, in settings where there is substantial risk of anemia, establishing blood counts as a standard of care during wellness checks from age 1-4 years should also be explored.

Results from our metals and anemias analysis (chapter 3) and from the persistent anemia and treatment completion analysis (chapter 4) indicate that certain children may be more likely to have anemia. Therefore, developing risk profiles for more targeted screening strategies and follow-up should be considered. For example, the notably high Pb levels of an older child with persistent anemia in the district downstream from large scale mining, warrant exploration of Pb screening among anemia cases or at minimum anemia cases that persist. In addition, the heterogeneous distribution of macronutrients and metals in our study warrants further studies that incorporate environmental factors such as chronic heavy metal exposure in affected communities. We did not observe significant differences in anemia prevalence across the two districts. However, in the district with greater heavy metal exposure, folate levels were

significantly higher and reported clinical access was notably greater. Existing literature and our results support that multiple factors contribute to anemia etiology. Therefore, issues of environmental justice including pollution, healthcare access and food security should be considered in building risk profiles for more targeted interventions.

Treatment and Intervention: Based on our results and using standard clinical thresholds for micronutrient deficiencies, iron, vitamin B12 and folate deficiencies were rare in our study population. In most non-malaria endemic settings presumptive iron supplementation is a standard treatment. However, based on the low prevalence of iron deficiency and the lack of association between iron supplementation and anemia resolution, iron supplementation is likely not sufficient for most anemia cases. Beyond the clinical approach, our results warrant exploration of change at the policy level addressing clinical access, and environmental remediation for decreasing anemia prevention.

Implications for anemia surveillance: Childhood anemia is a main priority in the region where we conducted our study due to past measures indicating a high prevalence of anemia that has not changed substantially despite large-scale interventions. Our study, however, resulted in an estimated anemia prevalence that was less than half of what was previously estimated in recent years. This may be due in part to our hemoglobin measures being from semi-automated blood counts of venous blood rather than standard measures of hemoglobin from capillary blood using the hemocue201. While the Hemocue201 continues to be a useful tool for screening for moderate and severe anemia that has the greatest risk of adverse outcomes, it's use for estimating anemia prevalence should be reconsidered due to the limited correlation with the gold standard in this study. Cost would increase and participation might decrease if taking a venous sample for blood count, but estimates would be more accurate and informative for evaluating the impact of

governmental interventions. Taking a smaller but representative sample to evaluate interventions in populations could minimize cost and maximize accuracy. The Hemocue apparatuses are useful for screening purposes but not for monitoring population prevalence.

Recommendations for Tumbes, Peru: Moving forward with the goal to decrease anemia in the region of Tumbes, Peru, I recommend several strategies that the Tumbes government should consider. The first is to explore developing community specific risk indexes based on potential etiologies including exposure to heavy metals and access to care. The second is to explore the use and validity of other point of care screening tools. The third is to have at least annual standardized screening from age 1 to 4 years. I recommend that screening with the Hemocue201 be continued, but with an emphasis on follow-up using an automated blood count for children with anemia. Parents and clinicians should also be educated on the symptoms of moderate to severe anemia and encouraged to request automated blood counts rather than use Hemocue201 to identify anemia. In addition, to accurately monitor and evaluate applied intervention strategies, I recommend a representative sample of children should be invited to receive automated blood counts.

In addition to these recommendations, I urge DIRESA to consider making iron deficiency a priority and supporting efforts to identify and decrease sources of heavy metal exposure. At the population level, the metal-exposed community has been resilient to depleted iron stores and Pb exposure. However, a hallmark of public health and environmental epidemiology is that we protect our most vulnerable. The fact that all cases of moderate anemia and iron deficiency anemia were found in San Jacinto suggests that some children in these community may be more vulnerable to the effects of metals exposure. Beyond being a precursor to iron deficiency anemia, iron deficiency alone has well documented impacts on long-term neurocognition and immunity.

Therefore, addressing this condition and identifying and combatting metal exposures early on that can exacerbate iron deficiency is critical.

6.5 Directions for future research

Our study led to the planning and implementation of a larger regionally representative study of 900 children in Tumbes. We identified similar prevalence measures based on the automated blood count of venous samples lending more evidence to the need for new surveillance strategies with complete blood count of venous blood or new screening tools. Our plan is to examine heterogeneity of markers more closely for plausible anemia etiology and see if risk profiles may be effectively built. By doing this work we hope to provide more tangible environmental interventions for anemia control. Our groups goal with this research is to continue to collaborate with communities involved in this research and apply an environmental justice lens that emphasizes the translation of research to the improvement of population health.

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