Evaluation of the Potential for Eggs, Fish, and Meat to Improve Vitamin A, Iron, and Anemia in Young Malawian Children

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ELIZABETH ROCHELLE WERNER
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Approved:

Christine P. Stewart, Chair

Reina Engle-Stone

Marjorie J. Haskell

Committee in Charge

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Evaluation of the Potential for Eggs, Fish, and Meat to Improve Vitamin A, Iron, and Anemia in Young Malawian Children

Abstract

Micronutrient deficiencies are common in young children due to the combination of low dietary diversity and high nutrient needs to support rapid growth and development. Provision of infrequently consumed, nutrient-dense animal-source foods may improve diet quality and fill the nutrient gap between children's nutrient needs and dietary intake. However, few studies have examined the relationships between consumption of these foods on vitamin A and iron status. The studies described in this dissertation stem from the Mazira Project, a randomized controlled trial providing 1 egg/d to young Malawian children. Our objectives were threefold: 1) to evaluate the effect of the intervention providing 1 egg/d on vitamin A deficiency (VAD); 2) to evaluate the effect of the intervention on iron deficiency (ID) and anemia; and 3) to examine the relationship between usual intake of fish and meat on ID and anemia.

The first study examines the impact of supplementing diets of 6-9mo Malawian infants with 1 egg/d for 6mo on the concentration of plasma retinol and retinol binding protein (RBP) as well as the prevalence of VAD (retinol <0.7µmol/L). Venous blood samples were collected at enrollment and 6mo follow-up. Retinol was assessed by HPLC, and RBP, c-reactive protein (CRP), and α-1-acid glycoprotein (AGP) were assessed by ELISA techniques. Prevalence of inflammation (CRP>5mg/L or AGP>1g/L: 62%) and inflammation-adjusted VAD (7%) at enrollment did not differ between groups. At follow-up, the egg intervention group did not differ from the control in inflammation-adjusted retinol [(geometric mean (95%CI); egg: 1.10 µmol/L (1.07, 1.13); control: 1.08 (1.05, 1.12)], RBP [(egg: 0.99µmol/L (0.96, 1.02); control: 0.97 (0.94, 1.00)], or prevalence of VAD [egg: 6%; control: 3%; Prevalence Ratio (PR): 1.87 (0.83, 4.24)].

The second study examines the impact of supplementing diets of 6-9mo Malawian infants with 1 egg/d on 1) plasma ferritin, soluble transferrin receptor (sTfR), body iron index (BII), and hemoglobin (Hb)

concentrations and 2) the prevalence of ID (ferritin <12 µg/L, sTfR >8.3 mg/L, or BII <0 mg/kg), anemia (Hb <11 g/dL), and iron deficiency anemia (IDA). Hemoglobin was assessed from venous whole blood at enrollment and 6mo follow-up. Plasma ferritin, sTfR, CRP, and AGP were measured by ELISA techniques at enrollment and 6mo follow-up. At enrollment, the total prevalence of anemia was 61% and did not differ between groups. At 6mo follow-up, groups did not differ in geometric mean concentration of hemoglobin [mean (95% CI); egg: 10.9 (10.7, 11.1) g/dL; control: 11.1 (10.9, 11.2) g/dL] and inflammation-adjusted ferritin [egg: 6.52 (5.98, 7.10) µg/L; control: 6.82 (6.27, 7.42) µg/L], sTfR [egg: 11.34 (10.92, 11.78) mg/L; control: 11.46 (11.04, 11.89) mg/L] or BII [egg: 0.07 (0.06, 0.09) mg/kg; control: 0.07 (0.05, 0.08) mg/kg]. There were also no group differences in anemia [egg: 46%; control 40%; PR: 1.15 (95% CI: 0.96, 1.38)], ID [PR: 0.99 (0.94, 1.05)], or IDA [PR: 1.12 (0.92, 1.36)].

The third study assesses whether usual intake of small fish, large fish, and meat were associated with plasma ferritin, sTfR, Hb, anemia, ID, and IDA in a population of young Malawian children with a high (>50%) prevalence of IDA. Food frequency questionnaires screening for animal source food intake were conducted weekly; 24-hr dietary recalls and venous blood samples were collected at enrollment and 6mo follow-up. Each food category was consumed by <5% of children at enrollment. By the 6mo follow-up, the prevalence of consumption reported in 24-hr dietary recalls increased to 40% for small fish, 12% for large fish, and 9% for meat. Usual portion sizes were small, ranging from 1.2g/d of meat and large fish to 4.9g/d of small fish. Over the 6mo study, children consumed small fish, large fish, and meat on an average of 25%, 8%, and 6% of days, respectively. Frequency of small fish intake was associated with lower sTfR [geometric mean ratio (95%CI): 0.98 mg/L (0.96, 1.00) per 10 percentage point difference] but was not associated with ferritin [1.03 µg/L (0.98, 1.07)] or Hb [1.01 g/dL (1.00, 1.01)]. Frequency of large fish consumption was associated with a higher prevalence of anemia [PR (95%CI): 1.09 (1.00, 1.19)] and lower prevalence of ID [0.96 (0.93, 1.00)]. Neither frequency of meat consumption over the 6mo

study nor usual gram weight intake of fish or meat at the 6mo follow-up were associated with any iron or anemia indices.

In summary, providing 1 egg/d for 6mo did not impact VAD, ID, anemia, or plasma indices of retinol, RBP, ferritin, sTfR, and hemoglobin among young children in rural Malawi. In this context, high rates of breastfeeding, vitamin A supplementation programs for children, and mandatory fortification of maize flour, wheat flour, sugar, and oil may have contributed to adequate liver stores of vitamin A. Thus, there may have been limited potential for children to benefit from the added vitamin A provided via the eggs. Further, the egg intervention neither alleviated nor exacerbated the high burden of ID in this population. Other more bioavailable and iron-rich foods, like small fish, have greater potential to improve iron stores. In this population, small fish consumption was only weakly associated with iron status, likely due to the small portion sizes consumed. Consuming a variety of animal-source foods including eggs, fish, and meat in sufficient quantities may improve micronutrient status and complement other interventions to reduce the risk of micronutrient deficiencies in young children.

Table of Contents

Acknowledgements ii

Abstract v

Chapter 1. Literature Review 1

1.1 Introduction 2

1.2 Vitamin A background 4

1.3 Iron background 9

1.4 Assessment of usual intake in young children 17

1.5 References 21

Chapter 2. The Effects of One Egg per Day on Vitamin A Status among Young Malawian Children: A Secondary Analysis of a Randomized Controlled Trial 31

2.1 Abstract 32

2.2 Introduction 33

2.3 Methods 34

2.4 Results 40

2.5 Discussion 41

2.6 References 47

Chapter 3. The Effects of One Egg per Day on Iron and Anemia Status among Young Malawian Children: A Secondary Analysis of a Randomized Controlled Trial 57

3.1 Abstract 58

3.2 Introduction 59

3.3 Methods 60

3.4 Results 64

3.5 Discussion 66

3.6 References 71

Chapter 4. Associations of Usual Intake of Fish and Meat with Iron and Anemia in Young Malawian Children: an Observational Cohort Analysis 80

4.1 Abstract 81

4.2 Introduction 82

4.3 Methods 83

4.4 Results 88

4.5 Discussion 91

4.6 References 99

Chapter 1. Literature Review

1.1 Introduction

Micronutrient deficiencies are common in young children due to the combination of low dietary diversity and high nutrient needs to support rapid growth and development. Global estimates indicate that approximately one-quarter of children under five years of age are iron deficient and 29% are vitamin A deficient (1,2). Children under two years of age are at higher risk than older preschool age children because they are frequently fed cereal-based diets that meet energy requirements but have inadequate micronutrient density. Adequate intake of micronutrients for children under two years old can be attained through consuming a minimally diverse diet—one that includes foods from at least five of the following eight food groups: breastmilk; grains, roots, tubers, and plantains; pulses, nuts, and seeds; dairy products; flesh foods; eggs; vitamin A-rich fruits and vegetables; and other fruits and vegetables (3). However, the global estimate of children under two years old who meet the minimum dietary diversity score is 29%, and this rate is even lower in Eastern and Southern Africa (4).

Food-based interventions offer the potential to improve diet quality and fill the nutrient gap between children's nutrient needs and dietary intake. Globally, nutrients of greatest priority and nutritional concern include iron, vitamin A, zinc, calcium, folate, and vitamin B_{12} (5,6). Within most low-and-middle-income countries, these nutrients can be supplied through provision of the following nutrient-dense food sources: organs, small fish, dark green leafy vegetables, goat meat, eggs, and milk (6). While these animal-source foods may be available in low- and middle-income countries, these foods are often infrequently consumed by children and may not be affordable to households with low socioeconomic standing. Nevertheless, provision of these nutrient-dense foods to households with young children may simultaneously address issues of hunger and food insecurity and improve the efficiency of nutrient uptake by delivering the nutrients in a highly bioavailable form within the food matrix (7).

Eggs are a nutrient-dense, animal-source food with high bioavailability of important macro- and micronutrients that have potential to improve diet quality of young children in low- and middle-income

countries (8). Eggs provide a complete amino acid profile and essential fatty acids like omega-6 and omega-3. They are a good source (>10% AI or RDA for 7-12-month-old children) of several micronutrients including choline, vitamin A, folate, vitamin B_{12}, vitamin E, selenium, and zinc, and they also provide some vitamin D_3, calcium, and iron. One 53g commercial, Malawian egg provides 16% [79µg retinol activity equivalent (RAE)] of the AI of vitamin A for a 7-12-month-old child. This vitamin A content is similar to the amount in one 50g USDA egg (75µg RAE or 15% AI), higher than the amount in one 52g Ecuadorian egg (55µg RAE; 11% AI), and more nutrient-dense than the smaller egg (36g) from free-range chickens in Malawian villages (150µg RAE per 100g vs. 102µg RAE per 100g). The iron content in eggs is overall low and comparable between eggs: 0.9mg iron (8% of the AI for a 7-12-month-old child) in one commercial, Malawian egg; 0.6mg iron (5% AI) in one USDA egg; 1.0mg iron (9% AI) in one Ecuadorian egg; and 0.8mg iron (7% AI) in one Malawian village egg (**Table 1.1**).

Though eggs are a good source of vitamin A and provide some non-heme iron, other animal-source foods and leafy green vegetables are also good dietary sources of vitamin A and iron. Organ meats like beef or chicken liver have high bioavailability of both vitamin A and iron. Fish, milk, and dairy products are rich in vitamin A, but milk and dairy products are a poor dietary source of iron unless they are fortified. Conversely, flesh foods like goat meat and chicken contain highly bioavailable heme iron but are low in vitamin A. Dark leafy green vegetables contain pro-vitamin A carotenoids as well as some non-heme iron; however, the bioavailability of vitamin A is limited by the efficiency in forming retinol from carotenoids and the bioavailability of iron is limited by the presence of oxalates, which impede iron absorption. A summary of the vitamin A and iron content in foods available in Malawi is provided in **Table 1.2**.

The purpose of this chapter is to 1) introduce the background on vitamin A, iron, and methods of dietary assessment and 2) highlight gaps in literature that justify a need for conducting the research in the following chapters of this dissertation. The overarching purpose for conducting this research is to

understand the potential for eggs, fish, and meat to improve the micronutrient status of vitamin A and iron in young, Malawian children. All research presented in this dissertation stems from a randomized controlled trial conducted between February 2018 and January 2019 in the Mangochi District of Malawi. In this trial, children 6-9-months-of-age were randomized to receive one egg per day or continue their usual diet for six months. Chapters 2 and 3 examine the effect of the egg intervention on vitamin A and iron, respectively. Chapter 4 examines the relationship between usual intake of fish and meat reported through food frequency questionnaires and 24-hr dietary recalls on iron and anemia status at the six-month follow-up visit.

1.2 Vitamin A background

Primary functions of vitamin A, consequences of VAD, & reason for public health significance

Vitamin A is an essential nutrient that aids in vision, growth, immunity, and gene expression (9,10). Deficiency in vitamin A may result in severe conditions such as xerophthalmia and childhood blindness as well as put young children at higher risk for diarrheal diseases, measles, and all-cause mortality (11,12). Accordingly, the Institute of Medicine established the recommended dietary allowance (RDA) for 1-3 year old children (300µg RAE/d) by extrapolating the requirement for adults on the basis of metabolic weight(10). For 7-12-month-old children, the adequate intake (AI) was set to 500µg RAE/d based on the average intake and vitamin A concentration of breast milk (0.6L x 485µg RAE/L) plus the quantity consumed from foods reported in NHANES III (244µg RAE/d) (10). Infants and young children are at high risk of vitamin A deficiency (VAD) when they do not consume breast milk, have lactating mothers with low vitamin A status, have low intake of vitamin A-rich foods during the complementary feeding period, or live in regions with high burdens of infectious diseases (13).

Dietary intake of vitamin A in breast milk and complementary foods

Infants are born with little reserves of vitamin A and thus rely on maternal breast milk and complementary foods to meet their nutrient needs. The concentration of vitamin A in breast milk

reflects a combination of maternal dietary intake and liver stores (14). Dietary vitamin A is delivered to the mammary tissues through chylomicrons. Higher dietary intake of vitamin A will increase the concentration in breast milk, whereas the amount of vitamin A delivered to the mammary tissues from the liver is constant due to homeostatic regulation of vitamin A in the plasma (15,16). However, mothers with depleted liver stores of vitamin A have lower concentrations of vitamin A in both the plasma and breast milk, and their dietary intake of vitamin A may be preferentially allocated to production of breast milk (17,18). Breast milk is a primary dietary source of vitamin A for young children, and so children who do not consume breast milk or who consume breast milk with low vitamin A concentration are at higher risk of VAD.

Dietary sources of vitamin A in low- and middle-income countries are primarily from pro-vitamin A carotenoids in some staple grains, fruits, and vegetables but may also include some preformed retinol from animal-source foods. Pro-vitamin A carotenoids like β-carotene, α-carotene, and β-cryptoxanthin have a low absorption efficiency of 9-22% (10). The absorption efficiency can be enhanced by the consumption of dietary fats and food processing like cooking, which disrupts the food matrix. After absorption, pro-vitamin A carotenoids are cleaved and converted into retinol, such that 1µg RAE is obtained from dietary consumption of 12µg β-carotene, 24µg α-carotene, and 24µg of β-cryptoxanthin (19). Animal-source foods contain preformed vitamin A as retinol or retinyl esters. Preformed retinol has a high absorption efficiency of 70-90% (20), which can contribute substantially to the body's total vitamin A stores in the liver.

Plasma retinol and RBP as indicators of VAD

The liver stores of vitamin A are critical for maintaining homeostasis of plasma retinol (21). When the liver has adequate stores of vitamin A in the stellate cells, the enzyme lecithin retinol acyltransferase (LRAT) will convert stored retinyl esters into retinol. Retinol will form a complex with two carrier proteins, retinol binding protein (RBP) and transthyretin (prealbumin), prior to release in the

bloodstream and delivery to the tissues. When tissues have sufficient vitamin A, retinol circulates back to the liver to join a pool of vitamin A that is ready for recirculation. Because retinol cycles through the plasma within an average of 2 hours, the liver can rapidly respond to changes in the tissue's utilization of retinol and keep concentrations of plasma retinol relatively constant (22). However, when the liver has inadequate stores of vitamin A, plasma retinol will fall below this homeostatically regulated range. Thus, plasma retinol concentrations below a cutoff of 0.7µmol/L are indicative of VAD (23,24).

Population-level assessment of VAD can be conducted by measuring plasma retinol or RBP (25). While plasma retinol is a direct measure of vitamin A and has an established cutoff for VAD, RBP is an emerging biomarker for the assessment of VAD. Measuring RBP instead of retinol is advantageous because RBP is more resistant to high temperatures and light exposure as well as quicker and less expensive to analyze by using ELISA techniques instead of HPLC. A common assumption for analysis of VAD by RBP is that RBP is present in the plasma samples in a 1:1 molar ratio with retinol (25,26); however, studies that have examined and compared RBP and retinol in the same set of samples have reported the RBP-equivalent of 0.7µmol/L retinol ranging from 0.46-0.83µmol/L (25,27–30).

One factor that may skew the assessment of VAD by RBP or retinol is inflammation. RBP is an acute phase protein that will decrease when inflammation is present (31). Thus, low RBP due to inflammation may also lower plasma retinol concentration and confound the assessment of VAD (32). The assessment of VAD can be improved by adjusting for two markers of inflammation, c-reactive protein (CRP) and α-1-acid-glycoprotein (AGP) using linear regression models. This approach has been recommended by the Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia (BRINDA) working group for the assessment of RBP and retinol in preschool-aged children (33,34). Adjusting for inflammation improves specificity for VAD but also reduces the sensitivity in identifying cases of VAD, thereby reducing the estimate of the burden of VAD (35).

Programmatic interventions addressing VAD in Malawi

Malawi is a country in Sub-Saharan Africa with a historically high prevalence of VAD. Among a national survey of children under 5 years old, the prevalence of VAD (unadjusted RBP<0.7µmol/L) was 59% in 2001, 22% in 2009, and 24% in 2015-2016 (36–38). In the 2015-2016 survey, the inflammation-adjusted prevalence of VAD (RBP<0.7µmol/L) was 10% (38). The government of Malawi and partners have implemented several national programs and policies to reduce the burden of VAD in Malawi: the national food supply is fortified with vitamin A in maize flour, wheat flour, sugar, vegetable oil, and skimmed milk powder; biofortified sweet potatoes and cassava are promoted; and high-dose vitamin A capsules are provided to children under 5 years through biannual child health campaigns (39–41). Monitoring the implementation and coverage of these programs and evaluating the prevalence of VAD over time are essential to understanding the effectiveness of these approaches in Malawi.

Which national or subnational programs a government chooses to employ may be determined, in part, by the goal for programmatic success, targeted population, and cost-effectiveness of interventions. For example, providing high-dose vitamin A capsules to children 6-59 months of age is a lifesaving, morbidity-reducing public health intervention that effectively prevents VAD when capsules are consumed biannually (42,43). However, if the long-term vision is to improve nutriture of children and women of reproductive age sustainably, then other approaches like dietary diversification, biofortification, fortification of the national food supply, and frequent low-dose dietary supplementation should be considered (13,44). If repeated monitoring data suggest that vitamin A intake among preschool children is adequate and the prevalence of VAD is low, biannual high dose vitamin A supplementation programs may be scaled back (45).

Fortification and supplementation are cost-effective methods of increasing micronutrient intake. While national fortification of staple foods has potential to effectively cover adults, such as pregnant or lactating women, the quantity of vitamin A fortified staple foods consumed by infants and young

children may not be sufficient to meet the estimated average requirement for their age group. Biofortification presents with the same concern regarding the vitamin A dosage children receive; additionally, limited bioconversion of carotenoids to retinol may lower the efficacy and utility of biofortification interventions. Home fortification with multiple micronutrient powders did not show mean increase in plasma retinol among 12-18mo old children in South Africa or 14-26mo old children Ghana when provided with 375µg RAE/day or 600µg RAE/day, respectively (46,47). However, an individual participant meta-analysis of small quantity lipid-based nutrient supplement interventions provided to 6-24mo old children in low- and middle-income countries for at least 3mo found a 7% increase in plasma RBP, a 56% decrease VAD (RBP <0.7µmol/L), and no effect on plasma retinol compared to the non-intervention controls (48).

While fortification and dietary supplementation may not directly improve mean plasma retinol concentrations for children under 24 months of age, the overall prevalence of VAD among young Malawian children is low (10%) and these programs are contributing to adequate intakes of vitamin A in children. When VAD is low (≤10%) and most children are regularly consuming small quantities of vitamin A, the Global Alliance for Vitamin A (GAVA) recommends scaling back universal vitamin A supplementation to children 6-59 months of age while conducting careful monitoring of population status (13). In such contexts, countries may elect to rebalance their government assisted nutrition programs by selecting the combination of interventions that are most efficacious and cost-effective in achieving the desired health and nutrition-related outcomes for each population group For example, vitamin A supplementation programs may be targeted towards young children living in regions with a high burden of VAD, thereby preserving resources for implementation of other vitamin A or nutrition programs in regions with a lower burden of VAD. Investing in food-based interventions may be advantageous for targeting adequate intake of multiple nutrients and addressing several Sustainable Development Goals (49). However, before policy makers can make informed decisions among a suite of

options, the evidence base needs to be built from research evaluating an array of dietary interventions on numerous potential health and nutrition-related outcomes.

The evaluation of an egg per day intervention on the vitamin A status of young Malawian children further extends the knowledge of health and nutrition-related outcomes from egg interventions. Other research stemming from the Mazira Project has evaluated the efficacy of an egg intervention on child growth (50), development (51), nutrient adequacy (52), dietary diversity (53), and plasma choline (54). The egg intervention has potential to increase plasma retinol concentrations of young children through daily provision of 79µg RAE (16% of the AI for 7-12-month-old children) delivered in a highly bioavailable form of retinol within the food matrix. The efficacy of this intervention was examined in a rural population of young Malawian children with high potential to benefit: the children in this study have low vitamin A intake (52), low coverage from vitamin A supplement programs (preliminary data), and infrequent consumption of eggs (53) at enrollment. In this context, the evaluation of the effect of the egg intervention on plasma retinol, RBP, and VAD builds a more comprehensive understanding of potential health benefits from providing eggs to young children and explores the potential for eggs to be included in a suite of options addressing VAD in young Malawian children through food-based interventions.

1.3 Iron background

Biological and public health significance of iron

Iron is an important oxygen-binding component of hemoglobin in red blood cells, which transport oxygen to the tissues for use in cellular oxidative respiration pathways like the electron transport chain that generate adenine triphosphate (ATP) for energy. Prolonged iron deficiency may decrease production of hemoglobin, a condition known as anemia that is characterized by microcytic, hypochromic red blood cells, pale pallor, and lethargy. Several studies have suggested that iron deficiency among preschool age children may impair motor and cognitive development (55,56),

however antenatal and early childhood iron intervention studies have not shown consistent effects on children's cognitive development (57). Though iron is a primary contributor to nutritional anemia, other conditions like inflammation, parasitic infections like malaria or hookworm, and hemoglobinopathies may also lead to anemia.

Treating and preventing anemia among young children is a component of the United Nation's Sustainable Development Goals to reduce all forms of malnutrition and ensure healthy lives for all by 2030 (49). In 2019, the global burden of anemia among children 6-59 months of age was 40%, with a higher prevalence in East African countries (53%) (1). Among Malawian children 6-59 months of age, 30% of children 6-59 months of age in Malawi had anemia and 22% had iron deficiency after correcting for inflammation (39). The prevalence of anemia (45%) and inflammation-corrected iron deficiency (43%) is even higher for Malawian children 6-23 months of age (39).

Young children are at high risk of iron deficiency and anemia

Young children are particularly vulnerable to iron deficiency and anemia because of their high physiological requirements, low dietary intake of iron-rich foods, and susceptibility to infections and microbial contaminants. The recommended dietary allowance for infants 7-12 months old is 11mg/d and for children 1-3 years old is 7mg/d, based on the physiological need to support rapid growth, replete basal losses, and replete iron stores (58). Children born at a normal birth weight from mothers with good prenatal iron status have sufficient iron reserves for the first 4-6 months of life. After 4-6 months, breast milk alone is insufficient to meet infants' iron requirements due to a low iron concentration (14), and so iron-rich soft, semi-solid foods need to be introduced to support infants' growth and development (59). Nutrient-dense foods need to be provided to infants because the portion sizes they consume are small. An average desirable iron density of foods is 4mg/100kcals for infants 6-8 months and 2.4mg/100kcals for infants 9-11 months (60).

Diets that are low in iron, lack animal-source foods, and contain high levels of inhibitors of iron absorption put infants and young children at higher risk of iron deficiency (61). Iron-rich foods with high bioavailability include organ meats (e.g., liver) and flesh foods (e.g., beef, fish, poultry, etc.). These foods not only contain highly bioavailable heme iron but also enhance the absorption of non-heme iron from other foods in the meal, like nuts, beans, legumes, cereals, and leafy greens (9). Other enhancers of non-heme iron include nutrients like citric acid and cysteine-peptides or food-processing methods like milling, germination, and fermentation (9). Infants are often fed cereal-based diets with infrequent, small portions of meat, mimicking vegetarian diets with low bioavailability of iron (58). These diets often contain nutritional inhibitors of absorption, such as phytates, polyphenols, oxalates, and tannins (9).

In addition to low-iron diets, infants may also be at risk of secondary iron deficiency and anemia from infection and inflammation. As infants begin to crawl and explore their environment, they have greater potential for exposure to microbial contaminants (59). These pathogens require iron to survive and replicate. Accordingly, one of the host's defense mechanisms is to sequester iron from the bloodstream (62). However, this defense mechanism also prevents the mobilization of iron stores to the host's tissues that require iron, resulting in a functional iron deficiency. The host's response to infection also decreases dietary iron absorption and promotes retention of iron in the spleen through hepcidin-mediated degradation of ferroportin. Under normal physiological conditions, red blood cells are degraded in controlled apoptosis by macrophages in the spleen, and iron from the red blood cells is recycled to the tissues. However, some parasitic infections like malaria will lyse circulating red blood cells and release iron. Provision of oral iron supplements to treat secondary iron deficiency or anemia can lead to severe adverse events including death because the supplemental iron may support pathogen proliferation (63). Thus, the WHO recommends that provision of iron supplements in malaria-endemic regions is integrated with measures to control malaria (64).

Indicators and methods of assessing iron status

Considering the complex interaction between the host and invading pathogens, a variety of indicators are needed to measure iron status, especially among children living in areas with high burdens of infectious diseases. Plasma ferritin reflects tissue iron stores and is the most common indicator for iron assessment of populations. The WHO recommends monitoring ferritin at a population-level in response to dietary interventions (65). Soluble transferrin receptor (sTfR) is an indicator of cellular or tissue-level need for iron; thus, sTfR increases during iron deficiency. Both ferritin and sTfR may change in response to inflammation and infection, however sTfR may be less impacted by acute inflammation (66). A function of the ratio of sTfR to ferritin known as "Cook's formula" provides a body iron index in which positive values for iron balance reflect net iron storage in the tissue whereas negative values reflect net iron depletion of the tissues (67). Iron deficiency may be defined by ferritin <12µg/L (65), sTfR >8.3mg/L (68), or body iron index <0mg/kg (67).

Because ferritin and sTfR are both proteins impacted by inflammation, the prevalence of iron deficiency is artificially lowered in populations with high burdens of inflammation unless statistical correction factors are applied to adjust for inflammation. Measuring two additional markers of inflammation, CRP and AGP, helps determine the stage of inflammation and the magnitude of the correction factor to apply (69,70). The BRINDA working group recommends applying correction factors to linear regression models to adjust ferritin and sTfR for values exceeding the first decile of CRP and/or AGP as well as for the presence of malarial antigens(66,71), since malaria can induce inflammation and erythropoiesis in response to hemolysis (66,72,73). Adjustments for biomarkers of inflammation are only applied above the first decile to not over-adjust the model, and researchers can select the cutoff for the first decile based on their own datasets or by using predefined percentile cutoffs from the external reference groups for preschool age children and women of reproductive age that were used to develop the BRINDA method.

Iron deficiency and nutritional anemia in Malawi

Iron deficiency and anemia are common among Malawian children and may arise from persistent low intake of iron-rich foods. Of 6-23 month old children sampled in the 2015-2016 Malawian Demographic and Health Survey, the prevalence of iron deficiency anemia was 25%, and iron deficiency was associated with approximately half of the cases of anemia (39). Foods typically provided to rural, young Malawian children are low in iron, predominantly containing small amounts of non-heme iron from plant-based foods like grains, vegetables, and legumes. Animal-source foods were infrequently reported in 24-hr dietary recalls for rural, 6-23 month old children in the 2015-2016 Malawian Demographic and Health Survey: 32% flesh foods (19% from fish), 12% eggs, and 8% dairy (74,75). In 2018, similar patterns of animal-source food consumption were observed among 6-9 month old infants randomized to the control group of an egg intervention trial in rural Malawi: 23% flesh foods, 4% eggs, and 9% dairy (53). The percentage of dietary recalls reporting consumption of iron-rich flesh foods in the control group increased over the study period [23% at enrollment (6-9 months old), 68% at 3-month follow-up (9-12 months old), and 67% at 6-month follow-up (12-15 months old)] (53); however, the total dietary intake of iron for children remained low (1.9mg/d at enrollment [overall]; 2.5mg/d at 3-month follow-up [control]; 2.8mg/d at 6-month follow-up [control]) (52).

Though recent national survey of preschool age children showed 30% anemia and 22% iron deficiency (inflammation-corrected ferritin <12µg/L), Malawi has few ongoing iron interventions in place as protective measures for this age group. The national food supply of wheat and maize flour have mandatory fortification of iron (41). The proposed food fortificant level for sodium iron EDTA in wheat flour is 30mg/kg at the factory level and a minimum of 27mg/kg at the point of sale (76). According to the African Organization for Standardization (ARSO), milled maize meal is fortified with 20mg/kg at the factory level, with a minimum requirement of 10mg/kg (77). However, infants and young children in rural Malawi may not benefit much from fortified maize meal if they consume small portions or

predominantly consume maize meal that was not commercially processed. Home fortification with multiple micronutrient powder or lipid-based nutrient supplements may be viable methods for increasing dietary iron intake of young Malawian children, but coverage of these programs is very low. Iron deficiency and anemia in young children could potentially be prevented through national or community-wide iron supplementation programs or food-based interventions.

Evaluation of egg intervention on iron status

Providing an egg per day to young children is one example of a food-based intervention which may impact the iron status of young children. One egg contains approximately 1mg non-heme iron (52,78), primarily concentrated in the yolk (79,80). One study in Australia provided 4 egg yolks per week to 6-month-old infants for 6 months and found the egg intervention group had higher plasma iron and transferrin saturation percentage points than the non-intervention control group, but the study found no group-wise differences in ferritin, transferrin, or hemoglobin (81). However, the iron content in eggs has limited bioavailability because it is tightly chelated with phosvitin (79). Additionally, phosvitin and proteins like ovotransferrin that are found in the egg whites may bind to iron from other dietary sources consumed in the same meal (82,83). Some single-meal studies in adults have shown that eggs reduce dietary absorption of iron (80,84,85), however the long-term effects of routine consumption of eggs on bioavailability of total dietary iron has not been studied and the net impact of providing whole eggs to young children is unknown. In the context of Malawi, where young children are at high risk for iron deficiency and inflammation, it is important to examine whether an egg intervention may further exacerbate ongoing iron deficiency or provide marginal improvements to iron status.

Potential for flesh foods to improve iron status

Dietary consumption patterns and nutrient content of flesh foods may also influence iron status. Fish, chicken, and red meat are the most widely consumed iron-rich flesh foods in Malawi (74,75). In the 2015-2016 Demographic and Health Survey in Malawi, fish intake was the most prevalent animal source

food and was consumed by 19% of young children (74). The iron content of fish differs by species (86). Though food composition tables have limited data on freshwater fish species, the edible portion of small fish, which are often consumed whole including the organs, is typically more iron-dense than large fish, which are often filleted (87). The iron from fish fillets is 30-40% heme iron, whereas the iron from chicken and red meat is 50-60% heme iron (85). The high bioavailability of nutrients in flesh foods are important qualities that can improve nutriture of malnourished children (88) and help young children meet their high nutrient needs (5).

Patterns of flesh food consumption are determined by geographic and socioeconomic factors that favor fish consumption in Malawi. Fish are widely available in informal markets due to the proximity of many communities to inland lakes, quick proliferation of small fish, preservation of caught fish through smoking or drying, and extensive fish-trading networks that reach rural communities (86). Dried, small fish are the least expensive flesh foods, with stable prices across seasons (89,90). In Malawi, 1kg of fish can be purchased for less than $2 international USD, which is more affordable than red meat (approximately $3 international USD) or poultry (approximately $4 international USD) (86). In regions with developing formal markets, wealthier households may have higher intake of fish and terrestrial animal-source foods due to their higher purchasing power, likelihood of owning livestock, and ability to afford direct consumption of household-produced, animal-source foods (75,91). Less wealthy households that own livestock or fish may elect to sell choice animal-source foods in the market and instead purchase cheaper animal-source foods like small fish, other non-animal source foods, or other essential household goods (91).

Within the household, cultural values, religious or cultural beliefs, and knowledge or preferences of household decision-makers may influence animal-source food consumption of infants and young children. In traditional households, men contribute to food procurement primarily through provision of finances for general household expenses and appropriating money for a food budget, whereas women

purchase, prepare, and serve foods for the household (92). To manage and stretch the household budget, women may purchase cheaper cuts of meat, purchase smaller portions of animal source foods, or buy some more expensive foods on credit (92). At meals, men may be served the first and larger portions of choice meat, young children may be served less desirable portions like legs and offals or different types of animal-source foods, and women may serve themselves last (92). For example, in a fishing community in Bangladesh, children under three years old were more likely to consume eggs, dairy, and organ meat than their mothers; however, mothers were more likely to consume fish than their young children (93). Small fish and meat may be withheld from infants and young children due to parental beliefs about age- and texture-appropriate foods, particularly concerning the bones in small fish and the ability to chew meat without teeth (91,93). While small fish may be more regularly consumed in fishing communities, more expensive animal-source foods like large fish, meat, and poultry may be reserved for celebrations (91). Lastly, some foods like pork or certain species of large fish may be avoided for religious reasons or concerns about contaminants (86,91). While existing literature suggests that small fish in Malawi are available in rural communities, accessible to households, and consumed by infants and young children (53,74,75,86,94), little information is known about whether small fish or other flesh foods are efficacious in improving iron and anemia status of infants and young children in low- and middle-income countries. Much of the existing information is on commercially produced infant porridges fortified with small fish powder (95,96). While data on anemia may be attainable through portable devices that can analyze hemoglobin at the point of care, iron status requires laboratory analyses and maintenance of cold chain systems that may be cost-prohibitive. Thus, there is a dearth of information linking dietary consumption patterns or intake of specific food groups to iron status of young children in low- and middle-income countries.

Examining the association between usual intake of small fish, large fish, and meat with iron and anemia status would begin to fill in some existing gaps in the literature with high quality, quantitative data. The

information from this analysis also creates opportunity to compare the relative effectiveness of various flesh foods to improve iron and anemia status of young children. This data could serve as formative research or provide estimates of effect sizes for future research studies designed to examine efficacy of flesh food interventions. Quantification of the effect size can be translated into estimates of cost-effectiveness, such as the monetary investment required to avert one case of anemia and iron deficiency, which is of interest to those engaged in public policy. Additionally, this information may be of value to nutrition education and behavioral change interventions that are designed to promote the consumption of iron-rich foods.

1.4 Assessment of usual intake in young children

While biomarker data may be used to assess subclinical nutrient deficiencies and biological response to nutrition interventions, dietary data provides an important linkage between nutrition interventions or observational studies and health outcomes, forming the evidence base for national dietary guidelines and nutritional recommendations to promote population health (97). Dietary data collection is non-invasive and versatile, consisting of several types of assessment methods such as 24-hr dietary recalls, food records, food frequency questionnaires, short screeners, and list-based recalls which lend themselves towards applications for a variety of quantitative, semi-quantitative, or qualitative research questions. Collection of dietary data is a component of national surveillance systems designed to examine trends over time for the purpose of understanding and informing federal food and nutrition policy (98). Collection of dietary data can also be used to estimate usual intake of various foods or nutrients in a population and examine their relationship with risk of nutritional deficiencies, diseases, or other health outcomes of interest (99).

Usual intake, defined as long-run average daily consumption, can be assessed using either food frequency questionnaires or repeat 24-hr dietary recalls (100). Food frequency questionnaires may assess usual intake by capturing intake over a longer time frame, such as the past month or year, or by

averaging the responses across multiple questionnaires administered over a shorter time frame, such as the past week. Food frequency questionnaires are prone to systematic errors, including those induced by the cognitive burden placed on respondents to recall foods consumed retrospectively over a long time frame and then average the amount consumed among those days. Conducting 24-hr dietary recalls reduces systematic error but introduces more random error, such as day-to-day variability in intake. However, repeating the 24-hr recalls on a non-consecutive day can help statistically adjust for day-to-day variability of measured intake because, in theory, overestimations and underestimations from individuals' true dietary intake will average over a large number of recalls to produce an unbiased estimate of usual intake (99). Though daily variability of individual intake does not bias the estimate of the mean usual intake in a population from 24-hr recalls, adjusting for day-to-day variability helps refine the distribution of usual intake among a population.

The National Cancer Institute developed a method to statistically model usual intake distributions by correcting for within-person measurement error in short-term diet assessments such as 24-hr dietary recalls or food records (101). The operational definition of usual intake consists of two components: the probability to consume a food, beverage, or nutrient on a given day and the amount that is consumed on a consumption day. Some foods are typically consumed nearly daily by nearly every individual, in which case an amount-only model will suffice. For foods that are episodically consumed, i.e., for which at least 10% of the sampled population did not consume that food on a given day, a two-part model jointly estimates the probability of consumption on a given day and the usual amount consumed on consumption day. These models also allow for the specification of additional covariates. The modeled measurement error for person-specific effects will be correlated if amount and probability of consumption are associated with each other or if they share covariates, such as sex, age, illness, day of week, and market day (101,102). The modeling procedures provided through a publicly-available SAS macro, *MIXTRAN* (103), use repeat recalls to produce parameter estimates for the fixed effects of the

probability and amount models for each individual as well as population-level estimates for the random effects of the probability and amount models, the covariance between the two-part models, the within-person error term from the amount model, and the Box-cox transformation to attain normal distribution of dietary intake.

The *INDIVINT* macro (103) is used to perform regression calibration, which predicts usual intake from the best mean square error estimate of observed 24-hr dietary recalls, conditioned on a mix of fixed and random effects. Subsequently, using the predicted usual intakes in linear regression models reduces the effect of random error on the slope estimate for the relationship between usual intake and health outcomes (102). For proper calibration of regression models that aim to use usual intake as a predictor for health outcomes, it is critical that all covariates intended for inclusion in the health outcome regression model are included as covariates in the probability and amount models in the *MIXTRAN* macro. This is because predicted values of usual intake are conditional on the covariates in the *MIXTRAN* model. The estimates of usual intake from the *INDIVINT* macro are only intended for regression calibration of usual intake for the population; they do not reflect true usual intake for specific individuals (102,104,105). Therefore, only the β-coefficient for usual intake in the health outcome regression models is correct. To obtain a measure of variability for the β-coefficient for usual intake, the entirety of the *MIXTRAN*, *INDIVINT*, and regression models can be bootstrapped to simulate conditions as if all individuals provided multiple, replicate 24-hr dietary recalls from which to sample. The β-coefficient for usual intake and any other desired output are saved for each iteration and may subsequently be used to calculate a confidence interval to draw inference from the mean usual intake.

The assessment of usual dietary intake of young children poses some additional considerations. First, usual intake may include both solid food and breast milk. If daily estimates of human milk intake are not available for each child, a “shrink then add” approach to dietary assessment is recommended (106). In this approach, usual intake of solid foods and breast milk are estimated separately and then added

together to capture total intake. Second, quantitative analysis of breast milk intake involves additional forms of primary data collection such as test-weighing the infant pre- and post-feeding or “dose-to-mother” isotope dilution techniques (107), which may not be available in population surveys. Third, typical dietary patterns and foods offered to infants and young children are interdependent with child age; children recently introduced to solids consume small quantities of a limited set of foods, whereas larger portions, new textures, and a greater variety of foods are offered to older infants and young children (59). In this context, short-term, repeated dietary recalls spaced several days apart are advantageous for assessing unbiased estimates of usual intake (99). Lastly, foods that are infrequently offered to infants like meat, fish, poultry, and eggs (4) require an extensive number of dietary recalls to capture usual intake at a population level. Dietary assessment of long-term exposure may provide a more accurate measure of usual intake of infrequently consumed foods (99). Thus, the combination of short-term, 24-hour dietary recalls and long-term food frequency questionnaires provides comprehensive and robust metrics for assessing usual dietary intake of infants and young children.

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Table 1.1 – Comparison of nutrient composition of whole, hard-boiled eggs

	RDA or AI for 7-12 months[1]	Malawian Village Eggs[2,3]		Mazira Project Eggs[2,4]		Lulun Project Eggs[2.5]		USDA Eggs[6]	
		Per 100g	*Per 36g egg*	*Per 100g*	*Per 53g egg*	*Per 100g*	*Per 52g egg*	*Per 100g*	*Per 50g egg*
Energy, kcal	769-858	162	59	143	76	158	81	155	78
Protein, g	11	12.5	4.5	12.5	6.6	13.1	6.8	12.6	6.3
Fats, g	30*	12.3	4.4	10.3	5.4	11.3	5.9	10.6	5.3
Linoleic acid, g	4.6*	1.85	0.67	1.84	0.98	1.35	0.71	--	--
Linolenic acid, g	0.5*	0.17	0.06	0.20	0.11	0.04	0.02	--	--
DHA, g	--	0.08	0.03	0.08	0.04	0.05	0.03	0.04	0.02
EPA, g	--	0.06	0.02	0.05	0.03	0.05	0.03	0.01	0.00
Calcium, mg	260*	43	15	47	25	58	30	50	25
Iron, mg	11	2.1	0.8	1.7	0.9	2.0	1.0	1.2	0.6
Zinc, mg	3	1.4	0.5	1.1	0.6	1.1	0.6	1.1	0.5
Selenium, µg	20*	24	9	21	11	37	19	31	15
Vitamin A, µg RAE	500*	102	37	150	79	104	55	149	75
Folate, µg DFE	80*	60	22	64	34	29	15	44	22
Vitamin B_{12}, µg	0.5*	1.9	0.7	1.6	0.9	1.8	1.0	1.1	0.6
Vitamin D3, µg	10*	2.9	1.0	0.8	0.4	--	--	2.2	1.1
Vitamin E, mg	5*	4.1	1.5	2.3	1.2	3.6	1.9	1.0	0.5
Choline, mg	150*	314	113	238	126	368	193	294	147

AI, Adequate Intake; DFE, Dietary Folate Equivalent; RAE, Retinol Activity Equivalent; RDA, Recommended Dietary Allowance

[1]RDA or AI values are taken from healthy, breastfed infants from the Institute of Medicine. AIs are marked with an asterisk (*).

[2]Nutrient analysis conducted by Eurofins Scientific Nutrition Analysis Center (Des Moines, IA).

[3]Eggs purchased from two households in Lungwena, Malawi in July 2018, hard-boiled, and shipped on ice for analysis of a single, pooled sample of 17 eggs (108)

[4]Eggs purchased in Mangochi, Malawi in July 2018, hard-boiled, and shipped on ice for analysis of a single, pooled sample of 11 eggs (52)

[5]Eggs purchased in Ecuador in 2016 (109)

[6]FoodData Central record 173424 for Egg, whole, cooked, hard-boiled accessed May 23, 2022 (78)

Table 1.2 – Vitamin A and iron content per 100g edible portion of animal-source foods available in Malawi[1]

	Energy (kcal)	Protein (g)	Fats (g)	Iron (mg)	Vitamin A (µg RAE)
Beef liver, cooked, pan-fried[5]	175	26.5	4.7	6.2	7,730
Chicken liver, all classes, cooked, pan fried[5]	172	25.8	6.4	12.9	4,300
Chicken meat, free range[2]	129	21.1	4.9	1.0	7
Fish, catfish, fresh, fried (Mlamba)[2]	207	18.1	11.8	1.7	38
Fish, lake sardine, whole, dried (Usipa wowuma)[2]	364	67.2	10.1	6.2	66
Fish, whole, par-boiled, sun dried (Usipa ofutsa)[2]	194	27.1	9.5	9.2	--
Fish, whole, sun dried (Utaka wadzuwa)[2]	328	39.8	18.1	20.3	--
Fish, whole, sun dried (Usipa wadzuwa)[2]	354	45.7	19.0	40.6	[0]
Fish, lake sardine stew with groundnut flour (Usipa wotendera)[2]	250	31.2	11.6	5.6	36
Fish, tilapia, fresh fried (Chambo)[2]	225	12.1	15.8	4.4	--
Goat meat, raw[2]	165	17.5	10.6	2.4	0
Milk, powder, full fat, vitamin A, D, and Fe enriched[3]	504	25.7	28.0	6.0	450
Samosa, beef filling, fried[2]	180	15.9	11.2	7.0	--

[1]RAE, Retinol Activity Equivalent. Square brackets [ ] indicate the value is based on an assumption. Double dashes – equate to missing data from either the absence of reliable information or data that did not meet quality criteria for inclusion.

[2]Malawi Food Composition Table (87)

[3]Food Composition Tables for South Africa (110)

[4]Sustaining and improving the contribution small-scale fisheries make to healthy and sustainable food systems in Malawi (86)

[5]USDA FoodData Central Legacy Foods (chicken liver: 174491; beef liver: 168627) (78)

Chapter 2. The Effects of One Egg per Day on Vitamin A Status among Young Malawian Children: A Secondary Analysis of a Randomized Controlled Trial

2.1 Abstract

Objectives: Vitamin A deficiency (VAD) is common in populations with limited dietary diversity and access to vitamin A-rich foods. The objective of this analysis was to determine the impact of supplementing children's diets with 1 egg/day on the concentration of plasma retinol and retinol binding protein (RBP) and prevalence of VAD. **Methods:** Children age 6-9mo living in the Mangochi district of Malawi were individually randomized to receive 1 egg/day for 6mo (n=331) or continue their usual diet (n=329) in the Mazira trial (clinicaltrials.gov; NCT03385252). This secondary analysis measured plasma retinol by HPLC and RBP, c-reactive protein (CRP), and α-1-acid glycoprotein (AGP) by ELISA techniques at enrollment and 6mo follow-up. Retinol and RBP were adjusted for inflammation and mean concentrations were compared between groups using linear regression models. Prevalence ratios (PR) of VAD (retinol<0.7µmol/L) were compared between groups using log binomial or modified Poisson regression models. **Results:** After 6mo of study participation, 489 were assessed for retinol (egg: n=238; control: n=251) and 575 (egg: n=281; control: n=294) were assessed for RBP. Prevalence of inflammation (CRP>5mg/L or AGP>1g/L: 62%) and inflammation-adjusted VAD (7%) at enrollment did not differ between groups. At follow-up, the egg intervention group did not differ from the control in inflammation-adjusted retinol [(geometric mean (95%CI); egg: 1.10 µmol/L (1.07, 1.13); control: 1.08 (1.05, 1.12)], RBP [(egg: 0.99µmol/L (0.96, 1.02); control: 0.97 (0.94, 1.00)], or prevalence of VAD [egg: 6%; control: 3%; PR: 1.87 (0.83, 4.24)]. **Conclusions:** Provision of 1 egg/day did not impact VAD, plasma retinol, or RBP among young children in rural Malawi where the prevalence of VAD was low.

2.2 Introduction

Vitamin A is an essential nutrient supporting growth, vision, and immunity that can be obtained from dietary supplements or vitamin A-rich foods like animal products, dark orange fruits and vegetables, leafy greens, or fortified foods (1). Vitamin A deficiency (VAD) puts young children at higher risk for blindness, measles, and diarrhea (2). Globally, VAD affects 29% of children under 5 years old (3), and in sub-Saharan Africa, nearly half of children under 5 years old are estimated to have VAD (3). The government of Malawi and partners have implemented several ongoing vitamin A interventions including high-dose vitamin A supplementation, micronutrient powders for home fortification, mandatory fortification of maize and wheat flour, sugar, and oil, and the promotion of biofortified sweet potato and cassava (4–6). Among Malawian children under 5 years, national micronutrient surveys have shown VAD declined from 22% in 2009 to 4% in 2015-2016 (6,7). This suggests that the combination of multiple vitamin A programs carries the promise of controlling VAD in Malawi.

However, children under 2 years old may be at high risk for VAD due to the high nutrient needs to support rapid growth, low dietary intake of vitamin A-rich foods, or ineffective coverage of existing vitamin A programs. Young children have a small gastric capacity and need to consume nutrient-dense foods to meet their nutrient requirements (8,9). Animal-source foods like milk, small fish, and eggs are highly bioavailable, vitamin A-dense foods but are infrequently consumed by infants (10). For infants consuming predominantly cereal-based diets, breast milk is often the primary source of vitamin A and may be an important contributor to vitamin A adequacy of Malawian children through 23mo of age, the median the duration of breastfeeding in Malawi (10,11). However, the concentration of vitamin A in breast milk depends on the vitamin A intake of the mother (12), and after 6mo of age breast milk is no longer adequate to meet child vitamin A requirements. Among breastfed children in Malawi, 24% of children under 2 years old achieved minimum dietary diversity (MDD)—a proxy for adequate micronutrient density of the diet—and consumption of vitamin A-rich food groups was reported in the

following percentage of 24-hr dietary recalls: 5% dairy; 12% eggs; 30% meat, fish, or poultry; and 74% vitamin A-rich fruits or vegetables (10).

One food-based solution for improving vitamin A intake and decreasing VAD in Malawian children is through the provision of eggs. Eggs contain highly bioavailable, pre-formed retinol in concentrations that vary based on the vitamin A content of the hens' diet (13). One 50g USDA commercial egg contains an estimated 75µg retinol activity equivalent (RAE) (25% RDA for children 1-3yrs old) (14) and one 52g commercial Malawian egg contains 79µg RAE (26% RDA for children 1-3yrs old) (15). Thus, supplementing the diets of young children with 1 egg/day may improve their vitamin A status.

This study is a secondary analysis of a 6mo egg intervention trial conducted in Malawi which evaluates the impact of providing 1 egg/day to young children on vitamin A status. Previously, the population of children in this study location has been shown to have mild to moderate VAD (11). Children enrolled in this trial are at risk of VAD from low intake of vitamin A-rich foods (15), low vitamin A density in breast milk from inadequate maternal dietary intake of vitamin A (11), and low coverage of vitamin A supplementation programs (10). The hypotheses of this analysis were that children receiving the egg intervention would have higher plasma retinol and retinol binding protein (RBP) concentrations and lower prevalence of VAD than children in the control group at 6mo follow-up.

2.3 Methods

Study design, participants, and randomization

The Mazira Project enrolled young children in the Mangochi district of Malawi between February 2018 and July 2018 (clinicaltrials.gov registry NCT03385252). Children were individually randomized to the intervention group that received 1 egg/day or the control group that continued their usual diet for 6mo. Age-eligible children were identified through child listings and recruited from villages near the Lungwena and Malindi health care centers. Children were eligible for study participation if they enrolled between the ages of 6-9.9 months, were of singleton birth, and their family intended to remain in the area for the

duration of the study. Children were excluded from the study based on presence of wasting (mid-upper arm circumference <12.5cm), severe anemia (hemoglobin ≤5g/dL), bipedal edema, egg allergy, recent hospitalization, or other morbidities that may affect growth and development. Children were referred to local health facilities when they screened positive for malaria, wasting, severe anemia, bipedal edema, or other symptoms warranting immediate medical care.

Caregivers of study participants were informed of the study design, research purpose, measures of assessment, rights to withdraw, and incentives to study participation. They were provided with opportunity to ask staff members questions in groups as well as individually in a private environment prior to enrollment. Caregivers provided written, informed consent by signature or thumbprint to confirm their participation in the study and allow collected data and samples to be used for future research. All procedures were reviewed and approved by the Institutional Review Board at the University of California, Davis and the Research Ethics Committee at the University of Malawi College of Medicine.

The study was designed to enroll 662 children based on the ability to detect a 0.25 SD difference between groups in length-for-age z-score with two-sided hypothesis testing, α=0.05, β=0.2, and 20% attrition. After completing initial assessments at the clinic, participants were individually randomized to either the egg intervention or non-intervention group in a 1:1 allocation ratio within blocks of 10. Caregivers randomly selected one opaque, sealed envelope and opened the envelope containing a card with a unique code to reveal their group assignment. Participants' group assignment was masked to staff conducting outcome assessments.

Intervention

A full description of the egg intervention and control group has been published elsewhere (16). Briefly, households in the egg intervention group received 1 egg/day for 6mo for the study child as well as an

additional 7 eggs/week for household sharing. Twice per week, eggs were delivered to households and caregivers were asked to serve the child the egg during the home visits. If the child had already eaten the egg, then caregivers reported the last time the egg was fed to the enrolled child. Children randomized to the control group continued their usual diet for the study duration. They were visited twice per week to maintain the same schedule of staff visits as the egg group. Caregivers in the control group were compensated with non-perishable goods like wash tubs, buckets, and plastic bins during the study and a basket of eggs, other foods, and kitchen goods at the end of the study. After completing the 6mo follow-up visit, all participants received fabric, sugar, and soap tablets.

Data and Sample Collection

Anthropometric measurements, cognitive development, and dietary intake were assessed at enrollment and 6mo follow-up. Recumbent length and weight were measured and converted into z-scores using the WHO growth standards (17). At enrollment, demographic information about the household, parents, and index children was collected, including surveys regarding household assets and food security (18). Vitamin A supplementation was recorded from the child's health passport or by parental report of attending national child health week campaigns during enrollment, 3mo, and 6mo follow-up visits.

Venous whole blood was collected in 5mL lithium heparin tubes at enrollment and 6mo follow-up. At the point of care, hemoglobin was assessed using Hemocue Hb 201 devices (HemoCue Inc., Angelholm, Sweden) and the presence of malarial antigens was determined using a rapid diagnostic test kit (SD Bioline Malaria Ag P.f/Pan, Abbott Diagnostics, Lake Forest, IL). Blood collection tubes were wrapped in foil, placed on ice, and centrifuged at 3000rpm (1040 x *g*) for 15 min at room temperature. Plasma was placed into multiple aliquots as sample volume allowed, filling foil-wrapped cryovials designated for retinol analysis prior to cryovials used for RBP analysis. All aliquots were stored in a -80°C freezer and shipped on dry ice to the laboratories conducting analyses.

Laboratory Analysis

Retinol was measured by HPLC (1260 Infinity II LC, Agilent Technologies, Santa Clara, United States) at the University of California, Davis in pairs of samples collected at enrollment and 6mo follow-up visits for each child (19). An internal standard of retinyl acetate (100ng) in ethanol was added to each analytical sample of 100µL plasma before extraction in hexane. Hexane was evaporated under nitrogen gas, and the residue was dissolved in methanol. Using a mobile phase of 95:5 methanol to water, the sample passed through a 2.7µm reverse phase C18 column (InfinityLab Poroshell 120, Agilent Technologies, Santa Clara, United States) with a 5µm guard column (Zorbax Eclipse Plus-C18, Agilent Technologies, Santa Clara, United States), and absorbance was measured at 325nm. Each tray of study samples was analyzed along with three controls of pooled defibrinated plasma (Utak, Valencia, United States) with a known retinol concentration based on calibration with a certified National Institute of Standards and Technology (NIST) 1950 plasma control. Replicate analysis was conducted when the coefficient of variation (CV) of the controls exceeded 5%. The average intraday CV was 2.0% and the average interday CV was 5.1%.

RBP, c-reactive protein (CRP), and α-1-acid glycoprotein (AGP) were measured at the VitMin laboratory in Germany by combined sandwich techniques with enzyme-linked immunosorbent assay (ELISA) methods (20). Analysis by ELISA was performed on 50-75µL aliquots from all children who provided a minimum plasma volume of 450µL at enrollment or 6mo follow-up. For quality control, the VitMin laboratory selected a subset of 16 samples with RBP ranging from 0.38-1.38 µmol/L for calibration against retinol measured by HPLC, and these retinol measures from study samples had been calibrated against CDC and NIST certified values. Pooled plasma samples were run with each tray with the following coefficient of variation (CV) for each index: RBP (3.6%), CRP (5.8%), and AGP (8.1%).

Statistical Analysis

A statistical analysis plan was prepared prior to analysis and posted online (https://osf.io/vfrg7). All data cleaning and analysis was performed in Stata (version 15; StataCorp LLC) (21). All values for retinol and RBP were within the limit of detection. At endline, 17% of samples had CRP values below the limit of detection. These values were replaced with zeroes for descriptive statistics and converted to 90% of the lower limit of detection for inflammation-adjusted models performed on the log-transformed scale.

Retinol and RBP were adjusted for inflammation on the log-transformed scale using a linear regression approach adapted from Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia (BRINDA) working group (22,23). Retinol and RBP were assessed for bivariate associations ($p<0.1$) with CRP and AGP at enrollment and 6mo follow-up. Only CRP retained significance ($p<0.1$) with retinol and RBP in multivariable regression models and was used for inflammation adjustment. When CRP exceeded 0.1 mg/L, the first decile for preschool age children in the external BRINDA reference group, a linear correction factor specific to the study timepoint was applied to observed retinol and RBP values. Dichotomous variables were created for VAD. Per WHO recommendations, the primary definition for VAD was retinol <0.7µmol/L (24), and secondarily, the prevalence of VAD was assessed using a RBP cutoff of <0.7µmol/L. Retinol and RBP were compared through simple linear regression and by comparing the prevalence estimates of VAD using either biomarker.

Descriptive statistics were calculated for demographic characteristics, vitamin A indices, and inflammation (CRP >5 mg/L or AGP >1 g/L) at enrollment by group assignment. Linear regression models assessed groupwise differences in mean concentration of plasma retinol and RBP. Prevalence ratios were assessed using modified Poisson models in instances when binomial family models with a logarithmic link function failed to converge. For descriptive purposes prevalence differences for VAD were assessed through linear probability models with heteroscedasticity-consistent standard errors.

All models controlled for baseline values of the outcome variables, and the primary inference was drawn from models using inflammation-adjusted values. Fully adjusted models included covariates demonstrating significant bivariate associations with the outcome variable ($p<0.1$) among the following set of *a priori* identified variables: child age, child sex, maternal education, household asset index, number of children under five years in the household, malaria, month of assessment, minutes between blood collection and aliquot storage, and receipt of vitamin A supplementation during the study period. Malaria was included as a potential covariate because malaria may be significantly associated with VAD even after adjusting for inflammation in malaria-endemic areas (25,26). Vitamin A supplementation was proposed as a covariate because recent receipt of high-dose vitamin A supplements may elevate the concentration of plasma retinol. Breastfeeding was not included as a covariate because the percentage of breastfeeding children (>98%) lacked variation and intake of breast milk was not quantified in this study.

Missing values were imputed using linear regression models for 20% of children missing RBP and CRP at enrollment and 13% of children missing CRP at 6mo follow up. Missing RBP at enrollment was imputed using the strongest predictive variables available: first using baseline retinol and date of HPLC analysis (n=25), then baseline hemoglobin (n=31), and lastly ownership of goats (n=60). Missing CRP at enrollment was imputed using the presence of malarial antigens and hemoglobin concentration, and CRP at 6mo follow-up was imputed using the presence of malarial antigens and maternal education. All variables in the imputation models demonstrated a bivariate association with RBP or CRP and retained significance ($p<0.1$) in multivariable models. Demographic characteristics of participants with missing measures were compared to children with complete measures. As a robustness sensitivity analysis, models were rerun with inverse probability of censoring-weighting to match the demographical characteristics of the original sample and compared the results from this model to those from the principal models.

2.4 Results

A total of 660 children were enrolled and randomized to the egg intervention group (n=331) or the control group (n=329). Participant and household characteristics were generally balanced between groups, though the egg group had a higher percentage of children who reported consuming an animal source food (fish, meat, egg, or milk) during the 24-hr recall period prior to enrollment (40%) compared to the control group (31%). Study participants were typically from households with 6 members and experienced moderate to severe food insecurity. Most of the children's mothers had not completed primary education and were unable to read or write. Children were enrolled at an average age of 7mo, all except 1 child were breastfeeding, and 3 children received high-dose vitamin A capsules prior to enrollment (**Table 2.1**). By the 6mo follow-up, 45% of children had received a high-dose vitamin A supplement since enrollment with no differences between the egg and control groups (data not shown). Among children with measured indices at enrollment, 62% had one or more elevated markers of inflammation. Median plasma retinol was 0.89µmol/L, and the prevalence of VAD (retinol <0.7µmol/L) was 21%. After adjusting for inflammation, the geometric mean for plasma retinol was 1.05 µmol/L and the prevalence of VAD decreased to 7% (Table 2.1).

This analysis includes 575 children with RBP at the 6mo follow-up (egg group: n=281; control group: n=294) and 489 children with matching retinol samples from enrollment and 6mo follow-up timepoints (egg group: n=238; control group n=251; **Figure 2.1**). The number of missing values at 6mo follow-up did not differ between the egg and control groups for RBP (15% vs 11%) or retinol (28% vs 24%). Participants with missing retinol or RBP data tended to have enrolled earlier in the study and resided closer to the Lungwena health center (**Supplemental Table 2.1**). Children with missing RBP data also tended to be from households with less maternal education, literacy, and employment in the service industry. These children also lived in households with more food insecurity and in homes constructed with lower quality building materials such as mud, straw thatching, or unburnt bricks (**Supplemental Table 2.2**).

Retinol and RBP were strongly correlated with each other (r=0.9) and provided good internal agreement. RBP values measured by VitMin laboratories were lower than retinol values measured at UC Davis: the RBP equivalent of 0.7 µmol/L retinol was 0.68 µmol/L at enrollment and 0.66 µmol/L at follow-up (**Supplemental Figure 2.1**). The distribution of retinol by treatment group at enrollment and 6mo follow-up is shown in **Supplemental Figure 2.2**.

After 6mo of study participation, 51% of children had at least one elevated marker of inflammation with no differences in group assignment (data not shown). The unadjusted prevalence of VAD was 21%, which decreased to 5% after adjusting for inflammation. The egg and control groups did not differ in the mean concentration of retinol (1.09 µmol/L) or RBP (0.98 µmol/L); nor did the prevalence of VAD and low RBP differ (**Table 2.2**). Inflammation-adjusted estimates of VAD were similar for both indicators: retinol <0.7 µmol/L (5%) and RBP <0.7 µmol/L (8%). Using the cutoff of RBP <0.66 µmol/L (the equivalent of 0.7 µmol/L retinol at 6mo follow-up) did not change the estimate of the prevalence of VAD (data not shown). Results did not differ between the principal models and sensitivity analysis models using inverse probability censoring-weighting (data not shown).

2.5 Discussion

In regions historically impacted by VAD, vitamin A adequacy may be attained for infants and young children through promotion of vitamin A-rich animal-source foods, fruits, and vegetables in dietary guidelines or food-based interventions, such as daily provision of eggs. In this study, mean retinol concentration and prevalence of VAD did not differ between children receiving the 1 egg/day intervention and control groups after 6mo, however the prevalence of VAD was low in this cohort. As previously reported, the egg intervention group had a higher usual dietary intake of vitamin A than the control group; however, both groups had a low proportion of children with inadequate intake of vitamin A (egg: 9%; control: 15%) at the 6mo follow-up (15). Therefore, the lack of intervention effect may be

attributable to the low risk of deficiency since plasma retinol is not sensitive to changes in dietary intake of vitamin A when liver stores are adequate.

Several factors may contribute to a low prevalence of VAD in this study population, including dietary intake of vitamin A from breast milk and coverage of high-dose vitamin A supplements. At enrollment, the unadjusted prevalence of VAD was 21% and decreased to 7% after adjusting for inflammation using the BRINDA regression approach with CRP. Usual intake of vitamin A from the combination of breast milk and solid foods at enrollment was 353µg RAE/d, and the prevalence of nutrient intake inadequacy was 94%, based on 500µg RAE/d for adequate intake for 7-12mo since an EAR has not been established for this age group (15). Breast milk, dark leafy green vegetables, and fish were commonly reported in the 24-hour recalls completed at enrollment (27). After 6mo of study participation, usual intake of vitamin A in the control group (335µg RAE/d) was similar to baseline intake; however, the prevalence of inadequate intake was lowered to 15%, reflecting the lower EAR for 1–3-year-old children of 210µg RAE/d (15). While persistent low intake of vitamin A-rich complementary foods may be suggestive of VAD, the study population may have been protected from VAD through a combination of high breastfeeding rates and provision of high-dose vitamin A supplements to children during the study. Though only 3 children received vitamin A supplementation prior to study enrollment, 45% reported receiving high-dose vitamin A supplementation during the study period from health centers or biannual campaigns occurring in June 2018 in Malindi and December 2018 in Lungwena. This could have contributed to the low prevalence of VAD at the 6mo follow-up visit.

The prevalence of VAD among young children in Malawi has been assessed by multiple studies within the last 10 years, providing consensus of a low prevalence of VAD that poses a mild public health problem (VAD: 2-9%; WHO (24)). The Malawian micronutrient survey was conducted in 2015-2016 and reported VAD of 4% among preschool children using the RBP equivalent (<0.46µmol/L) to a retinol cutoff of <0.7µmol/L, without adjusting for inflammation. Since these retinol and RBP values had a poor

linear relationship, the prevalence of VAD was later reassessed using the cut-off of <0.7µmol/L RBP, yielding a 24% prevalence of VAD (unadjusted) and 10% prevalence after adjusting for inflammation using the BRINDA method (28). In 2011-2012, a small quantity lipid-based nutrient supplement trial (iLiNS-DYAD (29)) that provided 800µg RAE/day to pregnant and postpartum women and 400µg RAE/day to children from 6- to 18-months of age was conducted in the same region of Malawi as the present study. The overall prevalence of VAD (<0.7µmol/L) among children in the iLiNS-DYAD (6mo of age: unadjusted 22%; inflammation-adjusted 10%) in 2011 (11) was similar to VAD among children in the Mazira Project (enrollment at 6-9mo: unadjusted 21%; inflammation-adjusted 7%) in 2018. However, the overall geometric mean for inflammation-adjusted plasma retinol concentrations of children in the Mazira Project [1.05 µmol/L at enrollment (6-9mo of age); 1.09 µmol/L at 6mo follow-up (12-15mo of age)] was higher than children in the iLiNS-DYAD (0.97 µmol/L at 6mo of age; 1.00 µmol/L at 18mo of age).

The results in the present study are comparable to observations in a similar trial providing 1 egg/day to children 6-9 months of age in Ecuador: mean retinol concentrations did not differ between the egg (656.35ng/mL or 2.30µmol/L) and control (643.79ng/mL or 2.25µmol/L) groups after 6mo (30). However, in that trial no children were vitamin A deficient (retinol <0.7µmol/L) and only 3% of children had retinol <1.05µmol/L at baseline. Compared with children in the study in Ecuador, children in the Mazira Project study had greater potential to benefit from the egg intervention based on the historically higher prevalence of VAD and higher retinol concentration of the eggs in this trial (150µg RAE/100g) versus the Ecuadorian trial (107µg RAE/100g) (30,31). Additionally, the present study had greater power to detect an effect due to a larger sample size enrolled (n=660) and analyzed (n=489) for retinol than the Ecuadorian trial (enrolled: n=169; analyzed: n=139). Despite the large sample size and potential for a high response to the intervention among vitamin A deficient children, the Mazira Project in Malawi also

did not observe a difference in mean plasma retinol concentration between the egg intervention and control groups (30,31).

Other dietary intervention trials have similarly observed a lack of biological response in plasma retinol and reduced power to detect an intervention effect from enrolling a study population with a lower prevalence of VAD than expected. In Zambia, 4-8-year-old children were provided with approximately 50% of the RDA of vitamin A (RAE) from maize meal biofortified with 15-20μg β-carotene/g for 6 days/week for 6mo (32). The biofortified maize intervention group had a 0.14μmol/L higher mean β-carotene concentration compared to the white maizemeal control (<2μg β-carotene/g), but mean serum retinol (0.99μmol/L) and VAD (17%; retinol<0.7μmol/L) did not differ between study arms after 6mo. While a lack of bioconversion from β-carotene to retinol may partially explain the lack of effect of the biofortified maize intervention on serum retinol concentrations, bioconversion is not a concern for the egg intervention since the vitamin A found in Mazira Project eggs was predominantly preformed retinol and the concentration of β-carotene was below the limit of detection (31). However, mean baseline retinol concentrations were within normal ranges for both the maize and egg intervention studies, and this may explain the lack of intervention effects on serum or plasma retinol concentrations.

This secondary analysis has several limitations. First, there is missing data on vitamin A for 13% of children, primarily due to blood draw refusals or insufficient sample volume. Though plasma aliquots for assessment of retinol were prepared before aliquots for RBP, fewer children were assessed for retinol due to the exclusion of those who did not provide a blood sample at enrollment (n=91). Children at enrollment had more missing blood draws due to the difficulty in locating the small veins of 6-9-month-old children and higher refusal rate among caregivers (33). This missing data could have contributed to selection bias. To mitigate the impact on this analysis, inverse probability of censoring-weighted analyses was conducted. Missing data could also reduce power to detect differences in groups.

However, this study retained adequate power to detect small differences in vitamin A status because of the large number of enrolled children.

Second, plasma retinol is homeostatically regulated over a broad range of liver reserves and may not be sensitive to the effect of dietary interventions when the prevalence of VAD in the studied population is low. Measuring the change in liver stores through modified relative dose response (MRDR) or isotope dilution techniques would provide a more sensitive assessment of vitamin A status for an intervention providing eggs for daily consumption over 6mo. However, these techniques for vitamin A assessment require administration of a vitamin A tracer prior to conducting the blood draw, which was not included as part of the initial design for primary study objectives. Thus, this secondary analysis was limited to plasma retinol and RBP with the available plasma samples.

Lastly, both the amount and concentration of vitamin A in breast milk, which is influenced by maternal dietary intake, may contribute to liver stores of vitamin A in young, breastfed children. Thus, controlling for vitamin A content from breast milk or maternal diet could refine the estimate of the impact of the egg intervention on measures of children's vitamin A status. However, quantitative data collection on breast milk intake or maternal diet was not included as part of the study design.

The strengths of this study are its design as a randomized controlled trial and strong quality controls. First, the study had a high adherence to the assigned intervention (15,16,27,33–35). Through 24-hr dietary recalls conducted at follow-up visits, consumption of eggs was higher among children in the egg group (3mo: 85%; 6mo: 71%) than the control (3mo: 7%; 6mo: 7%) (16,27). Additionally, analysis of plasma metabolites indicated that biomarkers in the choline pathway were elevated in the egg group compared to the control group, which suggests that egg intake was higher in the egg group than control, although this does not provide a direct measure of the amount of eggs consumed (35). Second, vitamin A was assessed by retinol and RBP. Laboratory analysis of plasma retinol and RBP had good reliability,

with the average intraday CV of 2.0% for retinol and 3.6% for RBP. Lastly, analysis of differences between groups were conducted using three scenarios: controlling for baseline measures only, adding adjustment for inflammation, and adding additional variables for demographics and laboratory analyses. No differences were observed through any of these models.

In conclusion, the provision of 1 egg/day in a population with a low prevalence of VAD did not impact vitamin A status. Results may differ in populations with a higher risk of VAD. The efficacy of using eggs to improve vitamin A status among at-risk populations would benefit from assessment by MRDR or isotope dilution instead of plasma concentrations of retinol and RBP. However, when plasma retinol and RBP concentrations are viewed together with prior studies in this community and national trends in vitamin A status, this study provides further evidence that the vitamin A situation in Malawi has been improving over time. Nevertheless, deficiencies in other micronutrients remain common and there is continued need for interventions focused on improving dietary quality and micronutrient adequacy of young children's diets.

2.6 References

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Table 2.1 – Enrollment characteristics of children in the Mazira Project, Malawi, 2018-2019, by intervention group[1]

	Egg		Control	
Characteristic	*n*	value	*n*	value
Maternal				
Maternal age, y	329	25.9 ± 6.7	325	26.1 ± 6.8
Maternal primary education[2], %	331	24	329	16
Maternal literacy, %	322	50	321	42
Household				
Number of children under 5 y	319	1.7 ± 0.8	319	1.7 ± 0.8
Number of household members	321	5.8 ± 2.6	320	6.0 ± 2.7
Moderate or severe food insecurity[3], %	331	75	329	81
Child				
Child age, mo	331	7.4 ± 1.2	329	7.3 ± 1.2
Female, %	331	48	329	48
Breastfeeding, %	330	100	329	100
Consumed animal source food in past 24hrs, %	330	40	329	31
Received high-dose vitamin A capsule, %	331	0	329	1
Prevalence of stunting (LAZ <-2), %	331	13	329	14
Prevalence of underweight (WAZ <-2), %	331	7	329	9
Prevalence of wasting (WLZ <-2), %	331	1	329	1
Inflammation				
CRP >5 mg/L[4], %	265	34	260	36
AGP >1 g/L[4], %	265	60	260	61
Hemoglobin, g/dL	292	10.5 (9.5, 11.5)	290	10.6 (9.3, 11.5)
Anemia (hemoglobin <11 g/dL), %	292	60	290	61
Plasma retinol[5], µmol/L	236	0.88 (0.73, 1.10)	251	0.89 (0.73, 1.08)
Plasma RBP[4], µmol/L	265	0.84 (0.70, 1.01)	260	0.80 (0.68, 1.01)
Vitamin A deficiency (retinol[5] <0.7µmol/L), %	236	20	251	22
Inflammation-adjusted				
Plasma retinol[5], µmol/L	236	1.06 (0.89, 1.26)	251	1.03 (0.86, 1.25)
Plasma RBP[4], µmol/L	265	0.96 (0.83, 1.13)	260	0.94 (0.81, 1.13)
Vitamin A deficiency (retinol[5] <0.7µmol/L), %	236	7	251	8

[1]Values are %, mean ± SD, or median (P25, P75). AGP = α-1-acid glycoprotein; CRP = c-reactive protein; LAZ = length-for-age z-score; RBP = retinol binding protein; WAZ = weight-for-age z-score; WLZ = weight-for-length z-score.

[2]Percent completed primary or greater

[3]Food insecurity assessed using Household Food Insecurity Access Scale (18)

[4]Reasons for missing AGP, CRP, and RBP measures: 4% refused consent, 8% incomplete blood draws, 9% insufficient plasma volume

[5]Reasons for missing retinol measures: 4% refused consent, 8% incomplete blood draws, 5% insufficient plasma volume, 9% excluded from analysis because a matching sample at 6mo follow-up was not obtained

Table 2.2 – Differences in means, prevalence ratios, and prevalence difference of vitamin A indices between treatment groups after 6mo of participation in the Mazira Project, Malawi, 2018-2019

	n		Geometric mean (95% CI)		Geometric mean ratio (95% CI)
Retinol and RBP	Egg	Control	Egg	Control	
Plasma retinol, µmol/L					
Minimally adjusted[1]	238	251	0.90 (0.87, 0.94)	0.89 (0.86, 0.92)	1.01 (0.96, 1.06)
Inflammation-adjusted[1,2]	238	251	1.10 (1.07, 1.13)	1.08 (1.05, 1.12)	1.02 (0.97, 1.06)
Fully adjusted[1,2,3]	238	251	1.10 (1.07, 1.14)	1.08 (1.05, 1.11)	1.02 (0.98, 1.06)
Plasma RBP, µmol/L					
Minimally adjusted[1]	281	294	0.81 (0.79, 0.84)	0.80 (0.77, 0.82)	1.02 (0.98, 1.07)
Inflammation-adjusted[1,2]	281	294	0.99 (0.96, 1.02)	0.97 (0.94, 1.00)	1.02 (0.97, 1.06)
Fully adjusted[1,2,3]	281	294	0.99 (0.96, 1.02)	0.97 (0.94, 1.00)	1.02 (0.98, 1.06)

	n		Prevalence		Prevalence ratio (95% CI)	Prevalence difference (95% CI)
Vitamin A deficiency	Egg	Control	Egg	Control		
Retinol < 0.7µmol/L, %						
Minimally adjusted[1]	238	251	21.2	20.1	1.06 (0.76, 1.49)	1.34 (-5.64, 8.33)
Inflammation-adjusted[1,2]	238	251	6.2	3.3	1.87 (0.83, 4.24)	3.36 (-0.37, 7.09)
Fully adjusted[1,2,3]	238	251	6.1	3.4	1.80 (0.79, 4.08)	3.50 (-0.23, 7.23)
RBP < 0.7µmol/L, %						
Minimally adjusted[1]	281	294	26.7	33.2	0.80 (0.63, 1.03)	-6.31 (-13.52, 0.90)
Inflammation-adjusted[1,2]	281	294	8.2	8.3	0.99 (0.56, 1.75)	0.11 (-4.71, 4.94)
Fully adjusted[1,2,3]	281	294	8.5	8.1	1.05 (0.59, 1.85)	-0.02 (-4.74, 4.70)

RBP = retinol binding protein

[1]adjusted for continuous baseline measures

[2]inflammation-adjusted using methods adapted from the BRINDA (Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia) approach (23)

[3]adjusted for covariates selected based on a bivariate association (p>0.1) with the outcome among the following list: child sex, maternal education, number of children under 5yrs in the household, malaria, month of assessment, and minutes between blood collection and completion of aliquoting

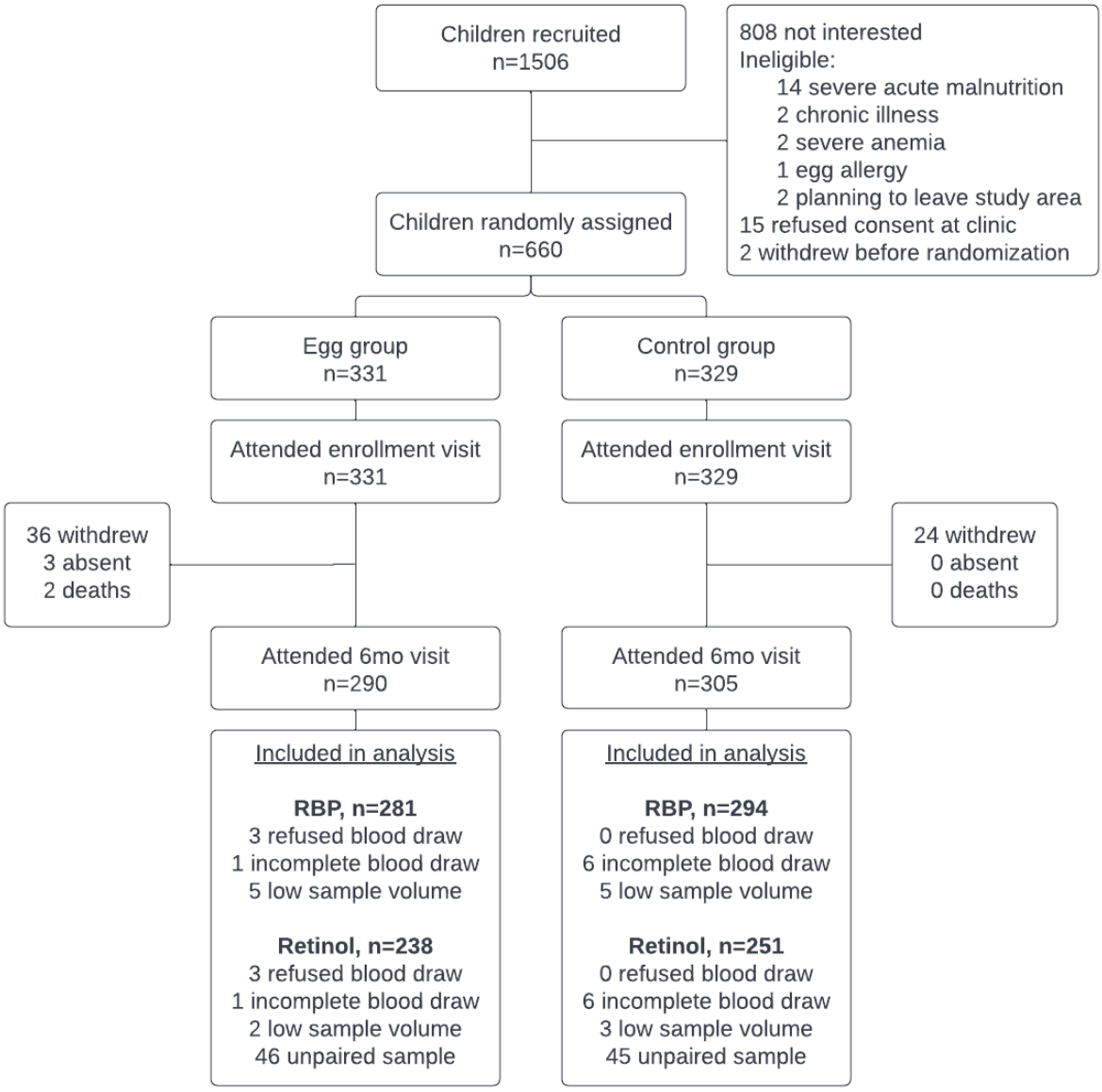


Figure 2.1 – Participant flow diagram for the vitamin A analyses of the Mazira Project, Malawi, 2018-2019

RBP = Retinol Binding Protein. Retinol samples at 6mo follow-up were analyzed if the child also provided a matching sample from the enrollment visit.

Supplementary Table 2.1 – Characteristics of participants missing vs without missing retinol data at 6mo follow-up of the Mazira Project, Malawi, 2018-2019

	Missing (n=171)		Complete (n=489)		
characteristic	*n*[1]	% or mean ± SD	*n*[1]	% or mean ± SD	p-value
Maternal age, y	165	25.4 ± 6.6	489	26.2 ± 6.8	0.228
Maternal primary education[2]	171	18	489	21	0.352
Maternal literacy	157	42	486	47	0.267
Maternal tribe					
Chewa or other	157	13	486	15	0.656
Yao		87		85	
Maternal occupation					
Farming	156	50	486	41	0.1348
Service		22		24	
Housewife		28		35	
Paternal occupation					
Farming or fishing	129	50	387	48	0.760
Service		50		52	
Muslim religion	157	87	486	88	0.735
Enrollment date, days into study period	171	71.9 ± 51.5	489	90.6 ± 54.5	**<0.001**
Health center					
Lungwena	171	61	489	51	**0.020**
Malindi		39		49	
Poor floor quality[3]	157	78	486	76	0.535
Poor roof quality[3]	157	62	486	61	0.809
Poor wall quality[3]	157	44	486	44	0.979
Household assets					
own latrine	157	97	486	96	0.761
own cows	157	4	486	3	0.463
own goats	169	17	489	20	0.351
own chickens	170	31	489	33	0.711
Number of children under 5 y	156	1.7 ± 0.7	482	1.7 ± 0.8	0.631
Number of household members	155	5.6 ± 2.7	486	6 ± 2.7	0.182
Moderate or severe food insecurity[4]	171	78	489	78	0.971
Child					
child age, mo	171	7.4 ± 1.2	489	7.4 ± 1.2	0.364
female, %	171	49	489	48	0.810
prevalence of stunting (LAZ <-2)	171	13.5	489	14	0.934
prevalence of underweight (WAZ <-2)	171	5	489	9	0.145
prevalence of wasting (WLZ <-2)	171	1	489	1	0.490
ever received vitamin A supplement	111	41	489	45	0.482

LAZ = length-for-age z-score; WAZ = weight-for-age z-score; WLZ = weight-for-length z-score

[1]Number of children with available demographical characteristics or assessment at enrollment

[2]Percent completed primary or greater

[3]Poor quality defined as straw, grass, mud, or unburnt brick

[4]Food insecurity assessed using Household Food Insecurity Access Scale (18)

Supplementary Table 2.2 – Characteristics of participants missing vs without missing RBP data at 6mo follow-up of the Mazira Project, Malawi, 2018-2019

	Missing (n=85)		Complete (n=575)		
Characteristic	*n*[1]	% or mean ± SD	*n*[1]	% or mean ± SD	p-value
Maternal age, y	79	25.2 ± 7.4	575	26.1 ± 6.6	0.276
Maternal primary education[2]	85	12	575	21	**0.046**
Maternal literacy	71	37	572	47	**0.099**
Maternal tribe					
Chewa or other	71	10	572	15	0.246
Yao		90		85	
Maternal occupation					
Farming	71	59	571	41	**0.013**
Service		13		25	
Housewife		28		34	
Paternal occupation					
Farming or Fishing	58	41	458	49	0.254
Service		59		51	
Muslim religion	71	92	572	88	0.335
Enrollment date, days into study period	85	60.0 ± 46.8	575	89.6 ± 54.3	**<0.001**
Health center					
Lungwena	85	71	575	51	**0.001**
Malindi		29		49	
Poor floor quality[3]	71	83	572	76	0.168
Poor roof quality[3]	71	72	572	60	**0.049**
Poor wall quality[3]	71	54	572	43	**0.084**
Household assets					
own latrine	71	96	572	97	0.755
own cows	71	4	572	3	0.506
own goats	83	13	575	20	0.157
own chickens	84	35	575	32	0.644
Number of children under 5 y	70	1.7 ± 0.8	568	1.7 ± 0.8	0.845
Number of household members	70	5.8 ± 3.1	571	5.9 ± 2.6	0.718
Moderate or severe food insecurity[4]	85	86	575	77	**0.060**
Child					
child age, mo	85	7.5 ± 1.2	575	7.4 ± 1.2	0.199
female, %	85	47	575	49	0.801
prevalence of stunting (LAZ<-2)	85	13	575	14	0.841
prevalence of underweight (WAZ<-2)	85	6	575	8	0.466
prevalence of wasting (WLZ<-2)	85	1	575	1	0.911
ever received vitamin A supplement	25	36	575	45	0.416

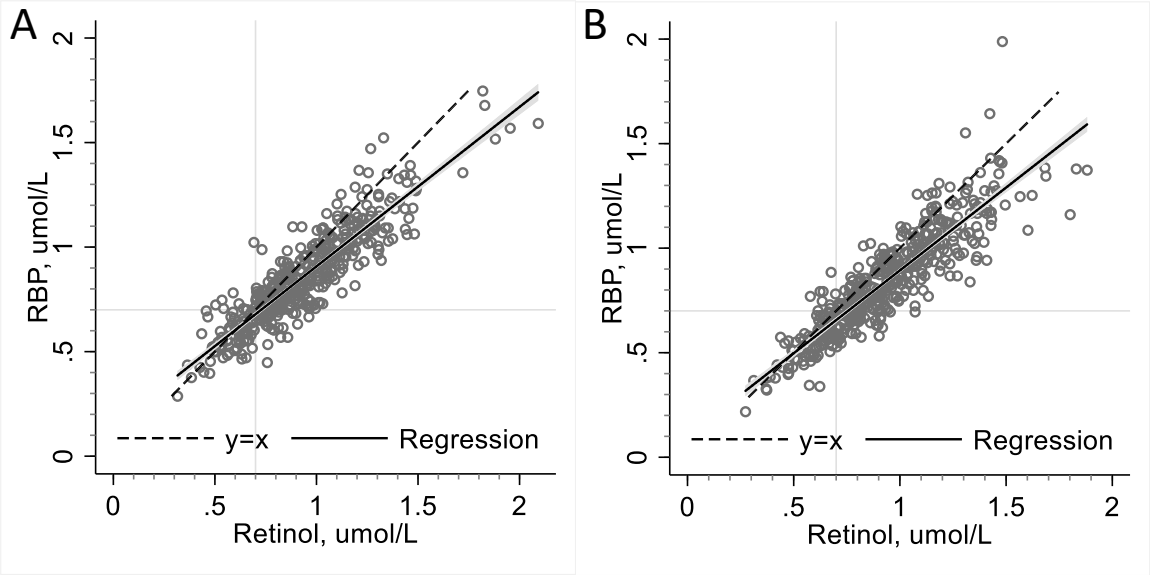
LAZ = length-for-age z-score; WAZ = weight-for-age z-score; WLZ = weight-for-length z-score

[1]Number of children with available demographical characteristics or assessment at enrollment

[2]Percent completed primary or greater

[3]Poor quality defined as straw, grass, mud, or unburnt brick

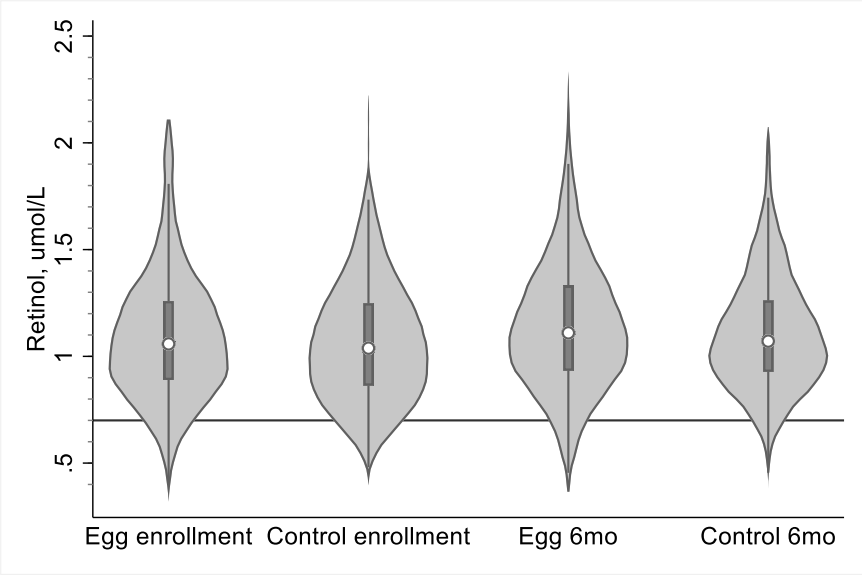
[4]Food insecurity assessed using Household Food Insecurity Access Scale (18)



Supplementary Figure 2.1 – Retinol and RBP equivalency at enrollment and 6mo follow-up in the Mazira Project, Malawi, 2018-2019

Supplementary Figure 1A: Enrollment; regression line (RBP = 0.7628*retinol + 0.1449); RBP equivalent of 0.7µmol/L = 0.6789.

Supplementary Figure 1B: 6mo follow-up; regression line (RBP = 0.7925*retinol + 0.1011); RBP equivalent of 0.7µmol/L = 0.6559



Supplementary Figure 2.2 – Inflammation-adjusted retinol by treatment group and timepoint of the Mazira Project, Malawi, 2018-2019

Chapter 3. The Effects of One Egg per Day on Iron and Anemia Status among Young Malawian Children: A Secondary Analysis of a Randomized Controlled Trial[1]

[1] This paper has been previously published [Werner ER, Arnold CD, Caswell BL, Iannotti LL, Lutter CK, Maleta KM, Stewart CP. The Effects of 1 Egg per Day on Iron and Anemia Status among Young Malawian Children: A Secondary Analysis of a Randomized Controlled Trial. Curr Dev Nutr. 2022 Jun 21;6(6).]. Reprinted with permission. This version may contain small differences, as it does not include changes made during copyediting.

3.1 Abstract

Objectives: Young children with diets lacking diversity with low consumption of animal source foods are at risk of iron deficiency anemia (IDA). Our objectives were to determine the impact of supplementing diets with 1 egg/day on: (1) plasma ferritin, soluble transferrin receptor (sTfR), body iron index (BII), and hemoglobin concentrations; and (2) the prevalence of iron deficiency (ID), anemia, and IDA. **Methods:** Malawian 6-9mo old infants in the Mazira trial (clinicaltrials.gov; NCT03385252) were individually randomized to receive 1 egg/day for 6mo (n=331) or continue their usual diet (n=329). In this secondary analysis, hemoglobin, plasma ferritin, sTfR, c-reactive protein (CRP), and α-1-acid glycoprotein (AGP) were measured at enrollment and 6mo follow-up. Iron biomarkers were corrected for inflammation. Ferritin, sTfR, BII, and hemoglobin were compared between groups using linear regression. Prevalence ratios (PR) for anemia (hemoglobin<11g/dL) and ID (ferritin<12µg/L, sTfR>8.3mg/L, or BII<0mg/kg) between groups were compared using log binomial or modified Poisson regression. **Results:** A total of 585 children were included in this analysis (egg: n=286; control: n=299). At enrollment, the total prevalence of anemia was 61% and did not differ between groups. At 6mo follow-up, groups did not differ in geometric mean concentration of hemoglobin [mean (95%CI); egg: 10.9g/dL (10.7, 11.1); control: 11.1 (10.9, 11.2)] and inflammation-adjusted ferritin [egg: 6.52µg/L (5.98, 7.10); control: 6.82 (6.27, 7.42)], sTfR [egg: 11.34mg/L (10.92, 11.78); control: 11.46 (11.04, 11.89)] or BII [egg: 0.07mg/kg (0.06, 0.09); control: 0.07 (0.05, 0.08)]. There were also no group differences in anemia [egg: 46%; control 40%; PR: 1.15 (95% CI: 0.96, 1.38)], ID [PR: 0.99 (0.94, 1.05)], or IDA [PR: 1.12 (0.92, 1.36)]. **Conclusions:** Providing eggs daily for 6mo did not affect iron status or anemia prevalence in this context. Other interventions are needed to address the high prevalence of ID and anemia among young, Malawian children.

3.2 Introduction

Iron deficiency is a major underlying cause for anemia, which can lead to impaired motor and cognitive development in children (1–3). Globally, half of children under 5 years of age have anemia, and one-quarter of the world's children are estimated to have iron deficiency anemia (4). In a 2015-2016 survey of Malawian children under 2 years of age, 45% had anemia and 25% had iron deficiency anemia (5). Young children are at high risk for iron deficiency and anemia because they have high nutrient needs to support rapid growth (6) and the foods prepared for young children often lack adequate nutrient density. Diversifying diets of young children by including more animal-source foods can help children reach their nutrient requirements.

Eggs are a nutrient-dense food with potential to improve the dietary adequacy of many nutrients for young children (7,8). However, the potential impact of eggs on iron status is unclear. One chicken egg contains 0.9mg of non-heme iron (9), equivalent to 8% of the RDA for infants 6-12 months old (11mg/d) or 13% of the RDA for children 1-3 years of age (7mg/d) (10). In eggs, iron is primarily concentrated in the yolk (11) with traces found in ovotransferrin in the egg whites (12). The iron content in eggs has limited bioavailability (13) because it is tightly bound to phosvitin (11), which is not readily degraded by proteolytic enzyme digestion (14). Moreover, whole eggs and egg whites inhibit iron bioavailability (15–17), reducing dietary absorption in adults by up to 27% (18). Nevertheless, one study from Australia has shown some potential for eggs to increase iron status among infants. After providing 4 egg yolks per week to 6-month-old infants for 6 months, plasma iron and transferrin saturation was higher in the egg yolk group compared to the non-intervention control but concentrations of ferritin and hemoglobin were similar between groups (19). Thus, the net effect of providing a whole egg to young children is unknown, and particularly to children living in areas with a high burden of iron deficiency and inflammation.

We recently conducted a study, entitled the Mazira Project, evaluating the impact of one egg per day on early child growth and development in Malawi (20). In this secondary analysis, we aimed to evaluate the impact of providing one egg per day to young children on indicators of iron status and anemia. We hypothesized that the egg intervention group would have higher mean concentrations of hemoglobin and ferritin, lower mean soluble transferrin receptor, and lower prevalence of iron deficiency, anemia, and iron deficiency anemia as compared to the control group after the intervention period.

3.3 Methods

Study design, participants, and sample size

The Mazira Project was conducted between February 2018-January 2019 in the Mangochi District of Malawi (clinicaltrials.gov registry NCT03385252). Children were randomly assigned to an intervention group, receiving one egg per day for 6 months, or a control group that did not receive additional eggs. Details of the study design have been reported previously (20,21). The study was promoted through community outreach events and study participants were recruited by home visits from household listings. Children were eligible if they were between the ages of 6.0 and 9.9 months, were of singleton birth, and planned to reside in the catchment areas of the Lungwena or Malindi health centers for the study duration. Children were excluded based on wasting (mid-upper arm circumference ≤12.5 cm), severe anemia (hemoglobin ≤5 g/dL), bipedal edema, acute illness warranting hospital referral, history of egg allergy, congenital defects, or other morbidities which may impede growth or development. Children were referred a health center if they presented with signs of severe dehydration or screened positive for wasting, bipedal oedema, malaria, or severe anemia during any study visits.

Caregivers were oriented to the clinic facilities, activities, purpose, and procedures of the research study and had opportunities to ask questions and discuss concerns in a group setting and privately with staff members. They provided written informed consent at enrollment by signature or thumbprint to confirm their study participation, consent to future use of collected blood samples, and right to withdraw at any

time. This study followed principles of ethical conduct approved by the Institutional Review Board at the University of California, Davis and the Research Ethics Committee at the University of Malawi College of Medicine.

Randomization and masking

The target sample size for the main trial was 662 children, based on the desire to detect a 0.25 SD difference between groups in the primary outcome measure of length-for-age z-score with α=0.05 and 80% power. Children were block-randomized in groups of 10 and allocated to the egg intervention or non-intervention group in a 1:1 ratio after enrolling and completing baseline assessments. From the current block, caregivers randomly selected one opaque, unmarked envelope containing a card with a unique randomization code to reveal their group assignment. Study staff conducting assessments were masked to group assignments.

Intervention

A full description of the intervention groups has been published elsewhere (20). Briefly, each week caregivers in the egg intervention group received 7 eggs to feed the enrolled child 1 egg per day, plus 7 additional eggs to share with other household members. Study staff delivered eggs to intervention households twice per week and conducted recalls on the most recent egg feeding. The control group continued their usual diet, and their households were visited twice per week to report on the child's most recent meal. They received wash tubs, buckets, and plastic bins as participation incentives during the study and a mixed basket of foods, including eggs, at the completion of the study. This package of goods was selected to be of equal value to that of the eggs provided to the intervention group. All study participants received fabric cloth, sugar, and soap tablets after completing each visit.

Data collection

During the initial clinic visit and 6-month follow-up, children were assessed for growth, development, and dietary intake. Child recumbent length and weight were converted to z-scores using the sex and

age-specific WHO Growth Standards (22). Enrollment surveys and initial household visits assessed demographic characteristics of the study child and household members, including household assets and food insecurity using the Household Food Insecurity Access Scale (HFIAS) (23) and home environment using the Home Observation for Measurement of the Environment (HOME) (24) indicator. At clinic visits, trained nurses collected venous blood samples to measure hemoglobin concentration using a portable spectrophotometer (Hemocue Hb 201, HemoCue Inc., Angelholm, Sweden) and presence of malaria antigens using a rapid diagnostic test kit (SD Bioline Malaria Ag P.f/Pan, Abbott Diagnostics, Lake Forest, IL) with >85% sensitivity and ≥90% specificity for *P. falciparum (25,26)*. Blood samples were collected in lithium heparin tubes, immediately placed in a cooler with ice, centrifuged for 15 minutes at 1040 x *g*, and aliquoted on site. Plasma samples were temporarily stored at -20°C and transported in coolers at the end of each day to a storage freezer maintained at -80°C. Aliquots were shipped on dry ice to laboratories completing plasma analyses.

Plasma ferritin, soluble transferrin receptor (sTfR), c-reactive protein (CRP), α-1-acid glycoprotein (AGP), and retinol binding protein (RBP) were measured by combined sandwich techniques with enzyme-linked immunosorbent assay (ELISA) methods by the VitMin Lab (27). All analytes were measured from a single well containing 50-75µL plasma for all children who provided a minimum of 450µL plasma sample from blood draws. A 10% subset of samples was reanalyzed for quality assurance. Replicates of pooled plasma samples were run with each tray, and the coefficient of variation (CV) for each indicator was calculated as the following: ferritin (2.3%), sTfR (3.6%), CRP (5.8%), and AGP (8.1%).

Statistical analysis

A detailed statistical analysis plan was developed and posted (https://osf.io/vfrg7) prior to analysis and analyst unblinding. All data cleaning, management, and analyses were performed using de-identified data in Stata (version 15; StataCorp LLC) (28). Indices above the upper limit of detection were replaced by the maximum observed values, and indices below the lower limit of detection were replaced by

zeroes and converted to half the limit of detection as needed for analytical models performed on the log scale.

Ferritin and sTfR were corrected for sub-clinical inflammation on the log-transformed scale using the Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia (BRINDA) regression approach. This method adjusts for elevated CRP and AGP above the lowest decile set by an external reference group of preschool-aged children (29,30). Body iron index was calculated according to Cook's formula (31) by applying constants to the ratio of sTfR:ferritin using inflammation-adjusted values, such that the quantitative estimates of iron stores are indicated by positive values and the magnitude of iron deficit is depicted with negative values (32). Dichotomous variables were created for anemia (hemoglobin<11g/dL), iron deficiency (ferritin<12µg/L, sTfR>8.3mg/L, or body iron index<0mg/kg), and iron-deficiency anemia (both anemia and iron deficiency) (27,33–35).

Descriptive statistics were calculated for demographic characteristics, iron indices, and inflammation (CRP>5mg/L or AGP>1g/L) (36) at enrollment by group assignment. Linear regression models assessed group-wise differences in mean concentrations of hemoglobin and inflammation-corrected ferritin, sTfR, and body iron index. The prevalence of anemia, iron deficiency, and iron deficiency anemia were compared by group assignment using prevalence ratios estimated using logistic regression with a logarithmic link function and prevalence differences estimated using linear probability models with heteroscedasticity-consistent standard errors (37). Modified Poisson models were used when log binomial models failed to converge (38). Our primary inferences were drawn from minimally adjusted models that controlled for baseline values of the outcome variable. For fully adjusted models, covariates were selected based on a bivariate association with the outcome variable ($p<0.1$) from the following set of *a priori* identified variables: child age, child sex, maternal education, household asset index, number of children under five in the household, month of assessment, blood processing time, and inflammation-adjusted retinol binding protein. Malaria was examined for inclusion as a covariate based on a bivariate

association ($p<0.1$) with hemoglobin and anemia but not for ferritin, sTfR, body iron index, iron deficiency, or iron deficiency anemia, since these indicators included malaria in the correction for inflammation.

Linear regression models were used to impute missing baseline values, which affected 11% of hemoglobin and 20% of ferritin, sTfR, CRP, and AGP covariates included in analytical models. Demographic variables were evaluated for bivariate associations with each biomarker and were used for imputation of missing baseline measures when they retained significance ($p<0.1$) in multivariable linear regression models. Additional sensitivity analyses were conducted excluding children missing baseline data and imputing with the mean value. Participant characteristics of children lost to follow-up or missing outcome measures were compared to children with complete measures. We used an inverse probability of censoring-weighted approach to reweight the analytic sample to match the enrolled sample and then compared these results to those from the principal models.

3.4 Results

Children were randomized to either the egg intervention group (n=331) or the control group (n=329). At enrollment, sociodemographic characteristics were balanced by treatment group (**Table 3.1**). On average, children were 7.4 months old and lived with 5 other household members. Twenty percent of mothers completed primary education, and 46% of mothers were literate. Most households (78%) reported moderate or severe food insecurity. Almost all children were breastfeeding, and 29% reported consuming a flesh food during the 24-hours preceding enrollment.

After 6 months of study participation, data were available on hemoglobin concentration from 585 children and iron status from 575 children (**Figure 3.1**). Overall, 13% of children had missing data due to study withdrawal, blood draw refusal, or insufficient sample volume, with similar rates of missing values in the egg intervention group (15%) and control group (11%). Compared to baseline characteristics of participants with complete data, the participants excluded from analysis due to missing hemoglobin or

iron biomarkers had mothers with lower levels of education and literacy, and they lived in households with poorer quality housing, greater food insecurity, and rural residency (farming occupation and primary healthcare center). They were also enrolled earlier in the study (**Supplementary Table 3.1**).

At enrollment, 582 (88%) children completed a blood draw and were assessed for hemoglobin and 525 (80%) children provided sufficient sample volume for assessment of inflammation and iron indices. Reasons for missing data did not differ by group: 4% refused consent, 8% incomplete blood draws, and 9% insufficient sample volume for analyses. Among participants assessed at enrollment, 61% were anemic, 35% had elevated CRP (>5mg/L), 60% had elevated AGP (>1mg/L), and 13% tested positive for malaria antigens. Iron deficiency, as defined by one or more of the inflammation-adjusted iron biomarkers, affected 77% of children, and the overall prevalence of iron deficiency anemia was 52%. Iron indices and prevalence of iron deficiency at enrollment without inflammation-correction are listed in **Supplementary Table 3.2**.

After 6 months of study participation, the prevalence of anemia declined to 43%, while the prevalence of iron deficiency increased to 89% (**Table 3.2**). Neither anemia nor iron deficiency differed by intervention group [anemia PR (95%CI): 1.15 (0.96, 1.38); iron deficiency: 0.99 (0.94, 1.05)], and 93% of children with anemia were also iron deficient. The overall prevalence of positive tests for malaria antigens (6%), elevated CRP (28%), and elevated AGP (46%) did not differ between groups. There were also no group-wise differences in mean hemoglobin [geometric mean ratio (GMR) (95%CI): 0.99 (0.97, 1.01)], inflammation-adjusted ferritin [GMR (95%CI): 0.96 (0.85, 1.08)], sTfR [GMR (95%CI): 0.99 (0.94, 1.04)], and body iron index [GMR (95%CI): 1.06 (0.79, 1.42)]. Adjusting for additional covariates did not impact the group-wise comparisons. Findings did not differ in sensitivity analyses using inverse probability weighted analysis or excluding children missing enrollment measures of hemoglobin or iron indices (data not shown).

3.5 Discussion

In this study population of young children with a high prevalence of iron deficiency and anemia at enrollment that exceeded the WHO threshold (≥40%) for a problem of severe public health significance (33,34), it is important to understand how a dietary intervention such as eggs may influence iron status. Eggs may inhibit bioavailability of iron in the diet, thus exacerbating the problem, or the provision of a small amount of iron through eggs could improve iron status. We found that providing eggs for daily consumption for 6 months did not affect hemoglobin, ferritin, sTfR, or body iron index; nor was there an effect on the prevalence of iron deficiency or anemia.

Our estimates of the prevalence of anemia and iron deficiency in the Mangochi District are similar to those reported in a national sample of young children included in the Malawi Micronutrient Survey in 2015-2016 (5). The prevalence of anemia among 6–23-month-old children (45%) in the survey was similar to the prevalence of anemia among Mazira Project participants at study completion (43%). However, the Mazira Project participants were notably more iron deficient (76%) than the national average of 6–23-month-old children (43%) following the same BRINDA linear regression approach and cutoff of inflammation-adjusted ferritin at <12µg/L.

Iron requirements among infants and young children are high to meet the demand for rapid growth. Had this study found more rapid growth in the intervention group, there could have been even greater demand for iron than among control children. Nevertheless, the high burden of iron deficiency in both groups is likely due to multiple compounding factors, including low dietary intake of iron-rich foods, high intake of foods containing phytates that could inhibit iron absorption, and prevalence of inflammation and malaria. The usual mean intake of iron was 1.9mg at enrollment, and after 6 months of study participation, the usual mean intake of iron did not differ between the egg intervention group (3.0mg) and control group (2.8mg) (39). Inadequate iron intake was nearly ubiquitous among children at both time points. Maize is the staple food and predominant dietary source of phytates among Malawian

infants. Legumes, leafy green vegetables, and tea were also commonly consumed (40); and the phytates, oxalates, and tannins in these foods may also impede iron absorption (32). Absorption of dietary iron is also reduced in response to sustained inflammatory response, which was highly prevalent among Mazira Project participants. At enrollment, most children had at least one elevated marker of inflammation, and 13% tested positive for malarial antigens.

The lack of effect of the egg intervention on iron indices among Malawian children somewhat contrasts with the results from an egg yolk intervention trial among Australian infants (19). That study provided 4 egg yolks per week to 6-month-old infants for 6 months and found that the intervention significantly increased plasma iron (egg: 10.5μmol/L; control: 8.3 μmol/L; $p<0.05$) and transferrin saturation percentage points (egg: 14.3%; control 10.8%; $p<0.05$) but had no impact on ferritin, transferrin, or hemoglobin. In comparison to Australian infants, Mazira Project participants assigned to the non-intervention control group had lower hemoglobin (11.1 vs 12.0g/dL), lower ferritin (6.8 vs 20.6μg/L), and greater prevalence of iron deficiency [77% (inflammation-adjusted ferritin <12μg/L) vs 17% (ferritin <10μg/L)] after 6 months of study participation. The differences in burden of iron deficiency and anemia may be explained by differential intake of dietary iron and prevalence of inflammation. At study completion, the breastfed Australian children in the control group consumed 6.9oz of flesh foods per week (or 28g/d), whereas Malawian children in the control group consumed fewer flesh foods (estimated usual intake of 24kcals/d, or 6g/d assuming 4kcal/g) and likely had lower total iron intake as well (39,40). Inflammation was highly prevalent among Malawian study participants and was not reported in the Australian study. Despite differences in study context, neither study detected significant groupwise differences in hemoglobin or ferritin concentration. The Malawian study had a larger enrollment and was powered to detect smaller differences in mean hemoglobin and ferritin between groups than the Australian study. The Australian trial reported significant differences in plasma iron and transferrin saturation; however, the Mazira Project did not measure transferrin saturation or find

groupwise differences in plasma iron (Lora Iannotti, Washington University in St. Louis, personal communication, 2021).

Prior short-term, single-meal studies in adults have shown potential for eggs to inhibit absorption of non-heme iron from other foods in a meal (13,15), which is hypothesized to be through the binding action of phosvitin and ovotransferrin (8,14). However, the long-term effects of habitual consumption of eggs on iron bioavailability from the total diet have not been examined. Some studies of high-phytate diets on iron bioavailability have found that single-meal studies overestimate the inhibitory effect of phytates on iron absorption compared with longer-term, whole-diet assessments (41,42). While our study was not designed to directly assess iron bioavailability, it is important to note the egg intervention did not negatively impact infants' iron status or exacerbate the ongoing problem of iron deficiency in this study sample.

This study had some limitations inherent to the selected measures included in analysis. Adherence would have been best evaluated through daily feeding observations; however, caregivers frequently fed eggs to their children in the early morning and it was only feasible to observe a portion of these feedings each week. Nevertheless, adherence was measured by caregiver report during the twice-weekly home visits as well as through 24-hour dietary recalls at 3- and 6-month follow-up. The reported consumption of eggs during 24-hr recall interviews was higher in the egg group (3-months: 85%; 6-months: 71%) than the control (3-months: 7%; 6-months: 7%) (20,40). The usual energy intake from eggs at 3-month and 6-month follow-up was approximately 30 kcals/day in the egg group and 1 kcal/day in the control group (40). We also compared plasma metabolites between groups and noted several markers related to egg consumption differed between the egg intervention and control group (43). These biomarkers are less susceptible to reporting bias and suggest that the eggs were indeed consumed by the index child, but they cannot provide information on dose or frequency of egg consumption.

Eggs were fed to children according to the caregivers' preference, reflecting real-world usual feeding practices instead of standardized cooking methods. This introduced greater variability in interactions between eggs and other nutrients or bioactive components of the food matrix, which could potentially affect iron absorption. A comprehensive suite of iron biomarkers would be needed to better understand iron absorption and metabolism in this study population with a high prevalence of dietary iron inadequacy, inflammation, and malaria. Data on enteric diseases, hemoglobinopathies, and other genetic conditions were not collected to determine multifaceted underlying etiologies of anemia. Nevertheless, since 93% of study participants with anemia had concurrent iron deficiency, it is apparent that iron deficiency is an important contributor to anemia in this context.

The high level of missing biomarker data from children at enrollment (12% hemoglobin; 20% iron indices) and 6-month follow-up (11% hemoglobin; 13% iron indices) also presents a limitation and could have introduced selection bias. Conducting blood draws on young children presents with several challenges, including difficulty in locating small superficial veins as well as caregivers' consent to complete the blood draw despite the child's discomfort. A greater number of caregiver refusals were reported at enrollment than at 6-month follow-up. Because we included baseline values as a covariate in our models, we imputed missing baseline values using multivariate linear regression. Results did not differ when compared to multiple sensitivity analyses.

The strengths of our study include its design as a randomized controlled trial, execution with high follow-up rates, and ability to detect small effect sizes with its large sample size. The Mazira Project had high adherence (>70% reported consuming eggs on the previous day) at the 3- and 6-month follow-up (20). Analysis of dietary recalls revealed a high prevalence of inadequate iron intake and provides general agreement with the high prevalence of iron deficiency reported in this paper. The analysis of ferritin and sTfR met high standards for quality control, and the data analysis was conducted according to a prespecified statistical analysis plan. Therefore, it is likely that the lack of difference in measured

iron and anemia status between the egg intervention and control groups accurately depicts the true effect.

We conclude that providing eggs did not affect iron deficiency or anemia prevalence among young children in a population with a high burden of these conditions. While eggs are rich in other nutrients, including choline, vitamin A, and essential amino acids, promotion of eggs will not address the problem of iron deficiency among young children. One egg per day does not provide enough iron to meet the requirements in this population. Low iron stores from birth and iron-deficient diets put young children at increased risk for iron deficiency and anemia. The high burden of iron deficiency and anemia among Malawian infants and young children is concerning, and other interventions such as multiple micronutrient powders (MNP) (44), lipid-based nutrient supplements (LNS) (45), or promotion of other iron-rich foods such as small fish or chicken liver (46–48) are needed to address these issues. Additionally, any future nutrition-related interventions designed to address iron deficiency and anemia in this population are recommended to be implemented in conjunction with measures to control malaria and reduce inflammation.

3.6 References

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Table 3.1 – Enrollment characteristics of children in the Mazira Project, Malawi, 2018-2019, by intervention group

	Egg		Control	
Characteristic	n	value[1]	n	value[1]
Maternal				
Maternal age, y	329	25.9 ± 6.7	325	26.1 ± 6.8
Maternal education[2], %	331	78 (24)	329	54 (16)
Maternal literacy, %	322	161 (50)	321	134 (42)
Household				
Number of children under 5 y	319	1.7 ± 0.8	319	1.7 ± 0.8
Number of household members	321	5.8 ± 2.6	320	6.0 ± 2.7
Moderate or severe food insecurity[3], %	331	247 (75)	329	267 (81)
Child				
Child age, mo	331	7.4 ± 1.2	329	7.3 ± 1.2
Female, %	331	160 (48)	329	159 (48)
Breastfeeding, %	330	329 (100)	329	329 (100)
Meat consumption reported in 24-hr recall, %	330	111 (34)	329	77 (23)
Prevalence of stunting (LAZ<-2), %	331	44 (13)	329	46 (14)
Prevalence of underweight (WAZ<-2), %	331	24 (7)	329	28 (9)
Prevalence of wasting (WLZ<-2), %	331	3 (1)	329	4 (1)
Inflammation				
CRP >5 mg/L, %	265	91 (34)	260	93 (36)
AGP >1 g/L, %	265	158 (60)	260	159 (61)
Positive malaria test (RDT), %	299	38 (13)	296	37 (13)
Hemoglobin, g/dL	292	10.5 (9.5, 11.5)	290	10.6 (9.3, 11.5)
Plasma ferritin[4], µg/L	265	13.1 (7.5, 23.8)	260	15.1 (8.7, 26.7)
Plasma sTfR[4], mg/L	265	10.2 (8.0, 13.6)	260	9.5 (7.7, 12.3)
Body iron index[4], mg/kg	265	-0.6 (-3.0, 1.9)	260	0.2 (-2.3, 2.4)
Anemia (hemoglobin < 11g/dL), %	292	175 (60)	290	178 (61)
Iron deficiency[4] (ferritin < 12µg/L), %	265	121 (46)	260	102 (39)
Iron deficiency[4] (sTfR > 8.3mg/L), %	265	186 (70)	260	176 (68)
Iron deficiency[4] (body iron index < 0mg/kg), %	265	151 (57)	260	120 (46)
Any iron deficiency[4], %	265	209 (79)	260	196 (75)
Iron deficiency anemia[4], %	265	138 (52)	259	132 (51)

AGP = α-1-acid glycoprotein; CRP = c-reactive protein; LAZ = length-for-age z-score; RDT = rapid diagnostic test; sTfR = soluble transferrin receptor; WAZ = weight-for-age z-score; WLZ = weight-for-length z-score

[1]Values are n (%), mean ± SD, or median (P25, P75)

[2]Percent completed primary or greater

[3]Food insecurity assessed using Household Food Insecurity Access Scale (23)

[4]Inflammation corrected using the BRINDA (Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia) approach (29,30)

Table 3.2 – Difference in means and prevalence ratios of iron and anemia indices between treatment groups after 6 months of participation in the Mazira Project, Malawi, 2018-2019

	Egg		Control		Minimally adjusted models[1]	Fully adjusted models[2]
Variable	n	geometric mean (95%CI)	n	geometric mean (95%CI)	GMR (95%CI)	GMR (95%CI)
Hemoglobin, g/dL	286	10.90 (10.74, 11.05)	299	11.06 (10.90, 11.21)	0.99 (0.97, 1.01)	0.98 (0.97, 1.00)
Plasma ferritin[3], µg/L	281	6.52 (5.98, 7.10)	294	6.82 (6.27, 7.42)	0.96 (0.85, 1.08)	0.96 (0.85, 1.07)
Plasma sTfR[3], mg/L	281	11.34 (10.92, 11.78)	294	11.46 (11.04, 11.89)	0.99 (0.94, 1.04)	0.99 (0.94, 1.05)
Body iron index[3], mg/kg	281	0.07 (0.06, 0.09)	294	0.07 (0.05, 0.08)	1.06 (0.79, 1.42)	1.05 (0.79, 1.40)
Variable	n	%	n	%	PR (95%CI)	PR (95%CI)
Anemia (hemoglobin < 11 g/dL), %	286	46	299	40	1.15 (0.96, 1.38)	1.18 (0.99, 1.42)
Any iron deficiency[3], %	281	90	294	89	0.99 (0.94, 1.05)	1.00 (0.94, 1.05)
Ferritin[3] < 12µg/L, %	281	79	294	77	1.02 (0.93, 1.11)	1.02 (0.94, 1.11)
sTfR[3] > 8.3mg/L, %	281	79	294	79	1.00 (0.92, 1.08)	1.00 (0.93, 1.08)
Body iron index[3] < 0 mg/kg, %	281	84	294	81	1.04 (0.96, 1.11)	1.04 (0.97, 1.12)
Iron deficiency anemia[3], %	281	42	294	37	1.12 (0.92, 1.36)	1.16 (0.95, 1.41)

GMR = geometric mean ratio; MD = mean difference; PR = prevalence ratio; sTfR = soluble transferrin receptor

[1]adjusted for continuous baseline measures

[2]adjusted for continuous baseline measures, and covariates selected based on a bivariate association (p<0.1) with the outcome among the following list: child sex, maternal education, number of children under 5yrs in the household, month of assessment, minutes between blood collection and completion of aliquoting, and malaria (for hemoglobin and anemia only)

[3]inflammation corrected using the BRINDA (Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia) approach (29,30)

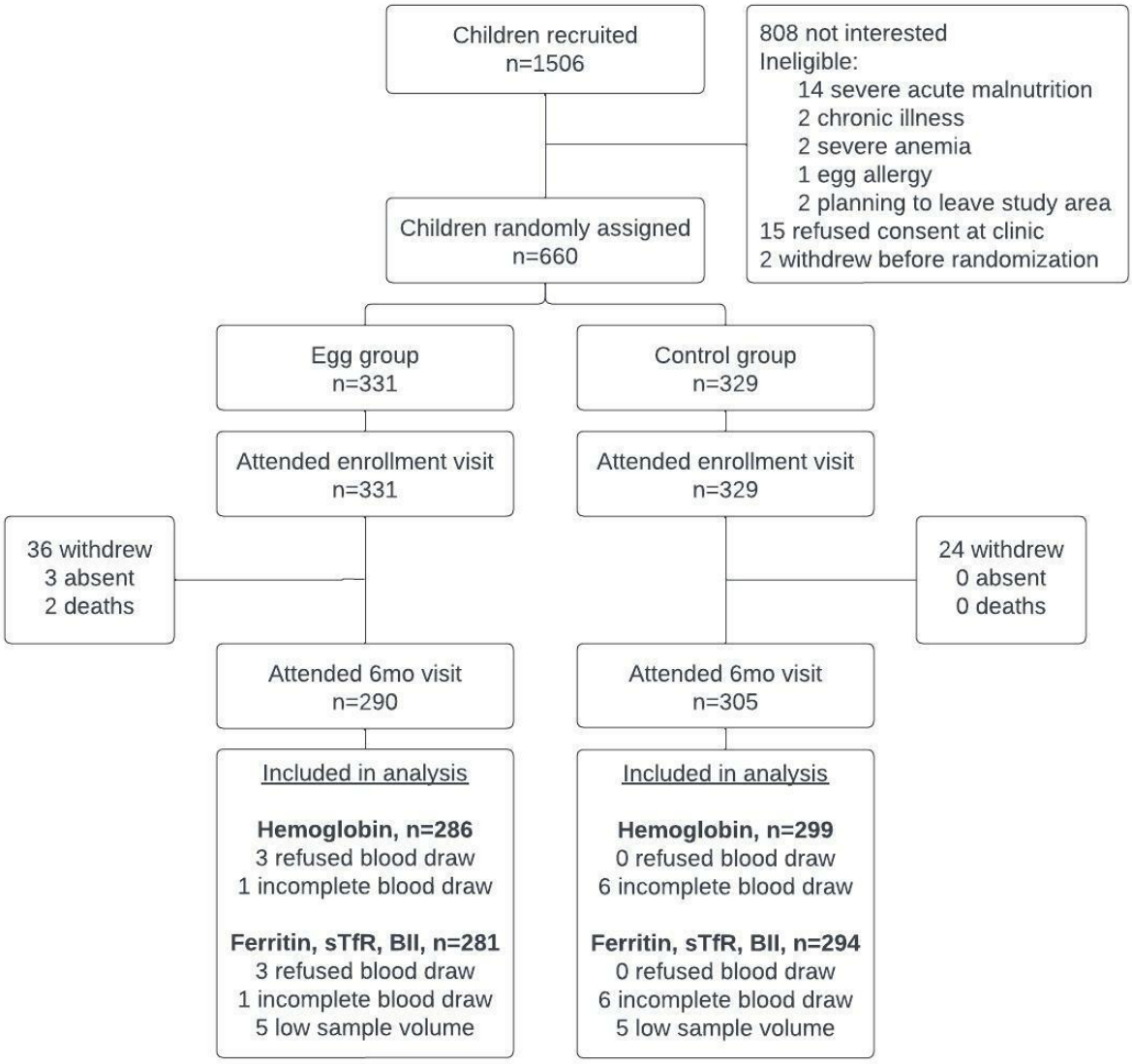

Figure 3.1 – Participant flow diagram for the iron and anemia analyses of the Mazira Project, Malawi, 2018-2019

BII = body iron index; sTfR = soluble transferrin receptor

Supplementary Table 3.1 – Characteristics of participants missing iron indices at 6mo follow-up of the Mazira Project, Malawi, 2018-2019

	Missing (n=85)		Complete (n=575)		
Characteristic	n[1]	% or mean ± SD	n[1]	% or mean ± SD	p-value
Maternal age, y	79	25.2 ± 7.4	575	26.1 ± 6.6	0.276
Maternal education (% completed primary or greater)	85	12	575	21	**0.046**
Maternal literacy (% can read)	71	37	572	47	**0.099**
Maternal tribe					
Chewa or other	71	10	572	15	0.246
Yao		90		85	
Maternal occupation					
Farming	71	59	571	41	**0.0128**
Service		13		25	
Housewife		28		34	
Paternal occupation					
Farming or Fishing	58	41	458	49	0.254
Service		59		51	
Muslim religion	71	92	572	88	0.335
Health center					
Lungwena	85	71	575	51	**0.001**
Malindi		29		49	
Poor floor quality[2]	71	83	572	76	0.168
Poor roof quality[2]	71	72	572	60	**0.049**
Poor wall quality[2]	71	54	572	43	**0.084**
Household assets					
HOME inventory score[3]	71	24 ± 3.1	572	24.2 ± 3.6	0.669
own latrine	71	96	572	97	0.755
own cows	71	4	572	3	0.506
own goats	83	13	575	20	0.157
own chickens	84	35	575	32	0.644
Number of children under 5 y	70	1.7 ± 0.8	568	1.7 ± 0.8	0.845
Number of household members	70	5.8 ± 3.1	571	5.9 ± 2.6	0.718
Moderate or severe food insecurity[4]	85	86	575	77	**0.060**
Child					
child age, mo	85	7.5 ± 1.2	575	7.4 ± 1.2	0.199
female, %	85	47	575	49	0.801
prevalence of stunting (LAZ<-2)	85	13	575	14	0.841
prevalence of underweight (WAZ<-2)	85	6	575	8	0.466
prevalence of wasting (WLZ<-2)	85	1	575	1	0.911
prevalence of malaria	71	11	524	13	0.718

LAZ = length-for-age z-score; WAZ = weight-for-age z-score; WLZ = weight-for-length z-score

[1]Number of children with data at enrollment or first household visit

[2]Poor quality defined as straw, grass, mud, or unburnt brick

[3]HOME, Home Observation for Measurement of the Environment (24)

[4]Food insecurity assessed using Household Food Insecurity Access Scale (23)

Supplementary Table 3.2 – Iron indices for children in the Mazira Project, Malawi, 2018-2019, by intervention group at enrollment without correction for inflammation

Characteristic	Egg (n=265) n (%) or median (P25, P75)	Control (n=260) n (%) or median (P25, P75)
Plasma ferritin, µg/L	23.8 (12.4, 49.86)	28.0 (15.5, 53.09)
Plasma sTfR, mg/L	11.6 (9.1, 16.2)	11.0 (8.8, 14.6)
Body iron index, mg/kg	1.1 (-1.6, 3.6)	1.9 (-0.7, 4.3)
Iron deficiency (ferritin < 12µg/L), %	64 (24)	44 (17)
Iron deficiency (sTfR > 8.3mg/L), %	227 (86)	210 (81)
Iron deficiency (body iron index < 0mg/kg), %	107 (40)	74 (28)
Any iron deficiency, %	234 (88)	212 (82)
Iron deficiency anemia, %	148 (56)	139 (54)

sTfR = soluble transferrin receptor

Chapter 4. Associations of Usual Intake of Fish and Meat with Iron and Anemia in Young Malawian Children: an Observational Cohort Analysis

4.1 Abstract

Objectives: Our objective was to assess whether usual intake of fish and meat was associated with plasma ferritin, soluble transferrin receptor (sTfR), hemoglobin (Hb), anemia, iron deficiency (ID), and iron deficiency anemia (IDA) in a population of young Malawian children with a high (>50%) prevalence of IDA. **Methods:** This secondary data analysis included 585 6-9mo old Malawian infants enrolled in a 6mo egg feeding trial. Small fish, large fish, and meat intake were reported in 7-day food frequency questionnaires administered weekly and 24-hr recalls at 6-9mo, 9-12mo, and 12-15mo of age. Plasma ferritin, sTfR, and Hb at 6mo follow-up were assessed for associations with the percent of consumption days over 6mo and grams of small fish, large fish, or meat intake for each 24-hr recall using linear regression. Prevalence ratios (PR) of anemia (Hb<11g/dL), ID (ferritin<12µg/L or sTfR>8.3mg/L), and IDA (ID and anemia) at 12-15mo were compared for each flesh food category using modified Poisson regression. **Results:** Each food category was consumed by <5% of children at enrollment. Children consumed small fish, large fish, and meat on 25%, 8%, and 6% of days, respectively, over the next 6mo with mean usual intakes of <5g/d. Frequency of small fish intake was associated with lower sTfR [geometric mean ratio (95%CI): 0.98 mg/L (0.96, 1.00) per 10 percentage point difference] but was not associated with ferritin [1.03 µg/L (0.98, 1.07)] or Hb [1.01 g/dL (1.00, 1.01)]. Frequency of large fish consumption was associated with a higher prevalence of anemia [PR (95%CI): 1.09 (1.00, 1.19)] and lower prevalence of ID [0.96 (0.93, 1.00)]. Frequency of meat consumption and usual grams of intake of any of the food group categories were not associated with iron or anemia indices. **Conclusions:** Small fish were a primary contributor to total flesh food intake in this cohort of young Malawian children, but usual portion sizes consumed were small. Fish was associated with modest improvements to iron status, but meat intake was likely too infrequent to be associated with anemia and ID.

4.2 Introduction

Infants and young children are at high-risk for iron deficiency due to low iron stores from birth (1), infrequent intake of iron-rich foods (2), and low bioavailability of iron from plant-based diets (3). In Malawi, over 40% of children 6-23 months of age have iron deficiency (4). Diets typically consist of a maize-based porridge along with some legumes and dark leafy greens, which contain compounds like phytates, polyphenols, oxalates, and tannins that impede iron absorption (5–7). Flesh foods, including meat, organ meat, or fish, are a dietary source of highly bioavailable heme iron that can also improve total iron absorption from foods consumed within the same meal (3,6,7). However, iron-rich flesh foods are infrequently consumed and were reported in only 32% of 24-hr dietary recalls for 6-23mo old children in the 2015-2016 Malawi Demographic and Health Survey (8).

We recently conducted a randomized controlled trial providing 1 egg/d to 6–9-month-old infants at enrollment in the Mangochi District of Malawi (9). At the 6mo follow-up visit when children were 12-15mo old, we found that 89% of children had iron deficiency (inflammation-adjusted ferritin <12µg/L or sTfR >8.3mg/L) and ≥98% of children had inadequate intake of dietary iron (10,11). Overall, the mean total dietary intake of iron at enrollment was 1.9mg/d (17% RDA for infants 6-12mo (6)) and remained low at the 3mo (control: 2.5mg/d; egg: 2.6mg/d) and 6mo follow-up (control: 2.8mg/d; egg: 3.0mg/d) (11). However, the percentage of dietary recalls reporting consumption of iron-rich flesh foods increased over the study period [enrollment (6-9mo): 23% control, 34% egg; 3mo follow-up (9-12mo): 68% control, 65% egg; and 6mo follow-up (12-15mo): 67% control, 72% egg] (12). Thus, this cohort consumed flesh foods at a higher frequency than the national average yet still had a very high prevalence of iron deficiency. Therefore, the objectives of this analysis were to quantify dietary iron intake of Malawian children enrolled in the Mazira Project by source and to determine whether their usual intake of small fish, large fish, and meat is associated with higher ferritin, lower soluble transferrin receptor (sTfR), higher hemoglobin, and lower prevalence of anemia, iron deficiency, and iron deficiency anemia.

4.3 Methods

Study design and participants

The Mazira trial (clinical trials registry, NCT03385252) was conducted in Mangochi District of Malawi between February 2018 and January 2019. The study site was located along the southeastern shore of Lake Malawi (**Figure 4.1**), where fishing and farming are the predominant occupations. Children were individually randomized to receive either 1 egg/d or continue their usual diet for 6mo. A total of 660 children 6-9mo old were enrolled, the sample size selected to detect a 0.25 SD difference in length-for-age z-score between groups (9). Local research staff identified age-eligible children in the Lungwena and Malindi catchment areas from household listings and recruited participants through home visits. Research staff also promoted the project in the communities through outreach events, including dramas and sponsorship of soccer tournaments. During an enrollment visit at the clinic, children were screened for the following eligibility criteria: singleton birth, intent to reside in the catchment area for the duration of the study, mid-upper arm circumference ≥12.5cm, hemoglobin >5g/dL, and absence of bipedal edema, egg allergy, recent hospitalization, or other morbidities that may affect growth or development. Children who screened positive for malaria, wasting, severe anemia, bipedal edema, or other symptoms indicative of need for urgent care were referred to local health facilities.

Study staff informed caregivers of study participants of the research purpose, measurements for assessment, protocols for group allocation, incentives to participate, and rights to withdraw at any time. Caregivers had opportunity to ask study staff questions in group settings as well as individually in a private environment. Caregivers provided written, informed consent by signature or thumbprint to enroll their child in the study and allow collected data and samples to be used for future research. All procedures were reviewed and approved by the Institutional Review Board at the University of California, Davis and the Research Ethics Committee at the University of Malawi College of Medicine.

Timing of assessments

At enrollment, interviewers administered surveys to assess demographic characteristics including household assets, food insecurity (13), parental education, parental occupation, and the child's home environment. Trained staff conducted anthropometric and developmental assessments, blood draws, and 24-hr dietary recalls at the clinic during enrollment, when children were 6-9mo old, and 6mo follow-up visits, when children were 12-15mo old. At 3mo follow-up (9-12mo of age), anthropometric assessments and 24-hr dietary recalls were completed through home visits. Additionally, study staff conducted one repeat dietary recall per timepoint through home visits among a subset of 200 participants. All households were visited weekly at home to complete a food frequency questionnaire (FFQ) screening for intake of animal-source foods over the past 7 days.

Blood collection and laboratory analysis

Nurses collected venous blood from children at 6-9mo and 12-15mo of age into 5mL lithium heparin vacutainers. Hemoglobin in whole blood was measured using Hemocue Hb 201 devices (HemoCue Inc., Angelholm, Sweden) and the presence of malarial antigens was measured using a rapid diagnostic test kit (SD Bioline Malaria Ag P.f/Pan, Abbott Diagnostics, Lake Forest, IL). Vacutainers of blood were wrapped in foil, immediately placed on ice, and centrifuged at 3000rpm (1040 x *g*) for 15 minutes at room temperature. Laboratory technicians placed plasma into aliquots and temporarily stored them in a -20C freezer on-site. At the end of each day, samples were transported on ice to a -80 freezer for storage and later shipped on dry ice by international courier to UC Davis' laboratory. For each child who provided a minimum plasma volume of 450µL, an aliquot of 50-75µL plasma was shipped on dry ice to the VitMin Laboratory in Germany for analysis. Ferritin, soluble transferring receptor (sTfR), c-reactive protein (CRP), and α-1-glyoprotein (AGP) were analyzed by combined sandwich ELISA techniques for each sample in a single well (14). For quality assurance, a subset of 10% of samples were rerun, and each tray was run with replicate controls of pooled plasma. The coefficient of variation of the controls for each index is as follows: ferritin (2.3%), sTfR (3.6%), CRP (5.8%), and AGP (8.1%).

Dietary data collection and analysis

Research staff administered weekly FFQ screeners and asked caregivers to recall the number of days children consumed small fish, large fish, meat, eggs, or milk over the past 7 days as well as the typical portion size on consumption days. Data from FFQs were cleaned so that consecutive interviews did not have 7-day recall periods overlapping by more than 1 day. There were 22 instances of duplicate recalls administered on the same day with discordant responses (0.2% of recalls). In such cases, the recall capturing higher intake of flesh foods, or secondarily greater intake of eggs and milk, was selected. For each child, the percent of days small fish, large fish, or meat were consumed was calculated among all recalls available between study weeks 3-25 as well as within the 3mo prior to attending the follow-up visit at 12-15mo of age. The percent of weeks children consumed any type of flesh foods was also calculated among available recalls over the full 6mo period and the most recent 3mo.

Multi-pass 24-hr dietary recalls were adapted for tablet-based administration with food lists specific for Malawi and administered at enrollment (6-9mo of age), 3mo follow-up (9-12mo of age), 6mo follow-up (12-15mo of age), as well as for a subset of 200 participants who provided repeat recalls at each time point (15). The specific methods of dietary assessment from 24-hr recalls for the Mazira Project have been reported previously (11). The reported portion sizes of consumed foods were converted to gram weights using food density data information from locally collected data and the University of Minnesota Nutrition Data System for Research (NDSR) (16). Mixed dishes were disaggregated into ingredients using recipe data collected through focus groups (15). Food composition tables from NDSR, U.S. Department of Agriculture (USDA), and regional food composition tables linked the gram weight portions to nutrient content for each ingredient (16–23).

Flesh foods from the 24-hr dietary recalls were categorized into subgroups of small fish, large fish, and meat to reflect similar categorization of foods from the FFQ screener. For this analysis, small fish versus large fish were differentiated not by length but by likelihood to consume the small fish whole (e.g., *bonya*, *usipa*, and *kambuzi* fishes) or as a fillet from larger fish species. Meat consisted of chicken, duck,

and goat meat. Organ meats were rarely reported and were thus included in the classification for meat for analytical models. For each recall day, the gram weight and iron content of each ingredient was summed by flesh food category. Additionally, we examined iron intake at each time point by each food group category included in the dietary diversity score for 6-24mo old children: grains, roots, and tubers; legumes, nuts, and seeds; dairy products; flesh foods; eggs; vitamin A-rich fruits and vegetables; other fruits and vegetables; and other snack foods (12,24). We applied correction factors listed in **Supplemental Table 4.1** to observed intake of dietary iron from each food group category to adjust for absorption.

Statistical analysis

A statistical analysis plan was developed and posted online (https://osf.io/vfrg7). All data cleaning, variable creation, and analysis for usual intake from FFQs were performed using de-identified data in Stata (version 15; StataCorp LLC) (25). Analysis for usual intake from 24-hr dietary recalls were conducted in SAS (version 9.4; SAS Institute Inc.) (26).

The primary outcomes of this study were mean concentrations of ferritin, sTfR, and hemoglobin, and the secondary outcomes were anemia (hemoglobin <11g/dL), iron deficiency (ferritin <12µg/L or sTfR >8.3mg/L), and iron deficiency anemia at 12-15mo follow-up. Ferritin, sTfR, and iron deficiency were adjusted for inflammation using the linear regression approach recommended by the Biomarkers Reflecting Nutritional Determinants of Anemia (BRINDA) working group (27,28). When measures of inflammation exceeded the first decile of the external BRINDA dataset for preschool-age children, a correction factor specific to the children and timepoint of this analysis was applied to adjust ferritin and sTfR. For inflammation-adjustment and analytical models performed on the log scale, indices below the lower limit of detection were replaced with half of the limit of detection and indices above the upper limit of detection were replaced with the maximum observed values.

Usual intake of flesh foods was examined by calculating the percent of days caregivers reported that their children consumed small fish, large fish, or meat or the percent of weeks children consumed any type of flesh food over the full 6mo study or over the 3mo preceding the endpoint. Using the 24-hr dietary recall data at 9-12mo and 12-15mo of age, we estimated the usual grams of small fish, large fish, and meat consumed using the NCI method to account for within-person variation in reported intake (29). Briefly, we used the SAS macro, *MIXTRAN* (v.2.21), to estimate the population-level parameters for the amount and probability of consumption through two-part correlated models (30). We used the *DISTRIB* (v.2.2) macro, which uses output from *MIXTRAN* models, to estimate the median and percentiles of usual intake for small fish, large fish, and meat for each timepoint (30). To examine the association between dietary intake and the iron and anemia outcome indicators, we used the *INDIVINT* macro (v.2.3), which adjusts measured dietary intake of individuals for day-to-day variation using population-level parameter estimates for use in regression models with dietary predictors and health outcomes (31). Since predicted dietary intake values were analyzed on a log-transformed scale, we replaced values for 0g intake of flesh foods with one-quarter of the minimum observed value for this analysis. Unique parameter estimates were obtained for each predictor-outcome combination with the inclusion of child age, sex, illness, market day, weekend, number of consumption days reported in the closest FFQ, and any covariates selected for analytical models. These unique population-level parameter estimates were applied within the *INDIVINT* macro for each predictor-outcome combination. Subsequently, we examined the relationship between predicted usual intake of flesh foods and indices for iron and anemia through linear regression models, which produced less biased β-coefficients for usual intake. This procedure was repeated for at least 200 bootstrapped iterations of each predictor-outcome combination to obtain standard errors.

We used linear regression models to examine the relationship between log-transformed ferritin, sTfR, and hemoglobin at 12-15mo of age and usual intake of flesh foods from FFQs and 24-hr dietary recalls. We used modified Poisson regression models with a log link to estimate prevalence ratios of anemia,

iron deficiency, and iron deficiency anemia in relation to usual intake of flesh foods. All analyses controlled for baseline measures of the outcome. Fully adjusted models controlled for child sex, child age, malaria, illness, and month of assessment. Additionally, we examined outcomes for bivariate relationships with the following variables: health center, maternal education, household assets, number of children under 5 years, minutes between blood draw and aliquot processing. Variables demonstrating a bivariate relationship with the outcomes (P<0.1) were included as additional covariates in analytical models.

Demographic characteristics of participants excluded from this analysis due to missing ferritin, sTfR, or hemoglobin at 12-15mo of age were compared to the characteristics of the enrolled population (10). For sensitivity analyses, we also examined results by carrying-forward the last observation to impute missing FFQs as well as dropping participants who completed less than 50% of the weekly FFQs. To better understand the relationship of large fish consumption over 6mo with anemia and iron deficiency, we conducted exploratory post-hoc analyses adjusting for differences in demographic characteristics between participants in the upper and lower quartile of large fish consumption over 6mo as well as variables associated with non-nutritional anemia.

4.4 Results

Demographic characteristics of enrolled children

A total of 660 children enrolled in the Mazira Project at an average age of 7mo. At enrollment, all except 1 child completed a 24-hr dietary recall, and all except 1 child reported current breastmilk consumption. On the day preceding the recall, small fish, large fish, meat, milk, organs, and eggs, were each consumed by less than 5% of children. Approximately one-quarter consumed vitamin-A rich fruits and vegetables; one-third consumed legumes, nuts, or seeds; and one-half consumed other fruits and vegetables, such as tomatoes and onions. At enrollment, 4% refused consent for the blood draw, 8% were unable to complete the blood draw, and 9% did not provide sufficient volume for analysis of iron. This sample of

enrolled children had a high burden of inflammation (62% with CRP >5mg/L or AGP >1g/L), malaria (13%), anemia (61%), and inflammation-adjusted iron deficiency (77%) (**Table 4.1**).

At the 6mo follow-up, children with missing iron outcome data had some notable differences compared to children with complete data (**Supplementary Table 4.2**). Children with missing data tended to have lower indicators of socioeconomic status, have enrolled earlier in the study period, and lived near the Lungwena health center. The primary reason for missing outcome data was due to withdrawing from the study (9% children). Among the 595 children who attended the 6mo follow-up, all provided a 24-hr dietary recall, <1% refused consent for the blood draw, 1% were unable to complete the blood draw, and 2% had insufficient plasma volume for iron analyses (**Figure 4.2**). Among children with outcome data, the average number of weekly, 7-day FFQs per child was 21 ± 4 (data not shown).

Estimated usual intake of iron-rich foods

Over the 6mo study period, children averaged consumption of small fish on 25% of days, large fish on 8% of days, and meat on 6% of days (**Figure 4.3**). The proportion of children consuming any fish or meat in the past 7 days increased with child age: 69% at 6-9mo, 78% at 9-12mo, and 84% at 12-15mo (**Figure 4.4**). Similarly, small fish consumption on 1 or more days within the past week increased with child age: 55% at 6-9mo, 64% at 9-12mo, and 70% at 12-15mo (Figure 4.4). Flesh food consumption was infrequently reported in 24-hr dietary recalls at 6-9mo of age but increased by 9-12mo and 12-15mo of age (**Table 4.2**). However, daily mean portion sizes remained small. At 12-15mo, children consumed a mean usual intake of 1.2 g/d of meat, 1.2 g/d of large fish, and 4.9 g/d of small fish (Table 4.2). Accordingly, the observed mean dietary intake of iron from complementary foods was low: 1.7mg at 6-9mo of age, 2.4mg at 9-12mo of age, and 2.9mg at 12-15mo of age (**Figure 4.5**). Grains, roots, and tubers were the primary source of dietary iron from complementary foods. However, at 12-15mo of age, flesh foods provided 43% of estimated absorbable iron, and the average portion of small fish provided similar absorbable iron to the grains, roots, and tubers food group (Figure 4.4).

Associations between usual intake from FFQs and iron indices

Greater frequency of small fish intake was associated with small improvements in iron status (**Table 4.3**). Over 6mo, each increment of 10% of days (analogous to a difference in frequency of 1 additional day per every 10 days) of small fish intake was associated with a relative 2% lower sTfR concentration, such as a 0.2mg/L decrease from the mean sTfR of 11.4mg/L. Similarly, there was a positive association between plasma ferritin and the percent of weeks during which any type of flesh food was consumed. For each 10% percent of weeks increment of any flesh foods (corresponding to 2.3 weeks), the ferritin concentrations were 3% higher. Over the 3mo preceding the 12-15mo follow-up visit, frequency of small fish intake was not associated with any iron indices but the percent of weeks of any flesh food intake was associated with lower sTfR.

Greater frequency of large fish intake over 6mo was associated with higher anemia prevalence and lower iron deficiency prevalence but not mean hemoglobin, ferritin, or sTfR (Table 4.3). Over 6mo, each 10% of days increment in large fish was associated with a relative 9% higher prevalence of anemia and 4% lower prevalence of iron deficiency. Over the past 3mo, frequency of large fish intake was associated with iron deficiency but not anemia. Frequency of meat consumption over 6mo and the past 3mo was not associated with any iron or anemia indices.

Associations between usual intake from 24-hr dietary recalls and iron indices

Though usual intake reported in FFQs over several months showed some associations between flesh foods and iron indices, usual intake of flesh foods reported in 24-hr recalls at 12-15mo of age was not associated with any iron or anemia indices (**Supplementary Table 4.3**). Each model was analyzed using at least 200 bootstrapped iterations. While models for small fish had a high rate of convergence (≥98%), large fish and meat models had moderate convergence (approximately 75%), primarily due to the high number of zero consumption days. At 9-12mo of age, small fish models converged with ≥96% bootstrap iterations and were not associated with iron or anemia indices (Supplementary Table 4.3). However,

models for large fish and meat at 9-12mo were excluded from analysis due to poor convergence (approximately 50-60%) among bootstrap iterations.

Sensitivity and post-hoc analyses

In sensitivity analyses examining the influence of missing FFQs on our models, we imputed or dropped observations and compared them to models of intake over 6mo (**Supplementary Table 4.4**). Imputing observations from missing FFQs did not substantially impact the point estimates (≤1 percentage-point difference) but did reduce the statistical significance of the association between days of small fish intake and sTfR, days of large fish intake and anemia, and weeks of any flesh food intake and ferritin. Dropping observations from 13 children missing more than 50% of FFQs did not impact the point estimates or significance of associations apart from the large fish intake and anemia model.

We further explored the associations of large fish with anemia and iron deficiency in a series of post-hoc analyses. Frequent consumers of large fish tended to be more likely to consume small fish as well. Additionally, they were more likely to live in Lungwena, own chickens, complete more FFQ recalls, and have malaria at enrollment. Large fish consumption was no longer associated with iron deficiency after adjusting for malaria at enrollment, whereas neither the point estimate nor the significance of association for the large fish and anemia model were impacted by addition of demographic variables as covariates. However, the association between large fish and anemia was no longer present after adjusting for CRP at 12-15mo of age.

4.5 Discussion

In this sample of young children from fishing communities along the southeastern shore of Lake Malawi, small fish were the most consumed animal-source food and the primary dietary source of estimated absorbable iron at 12-15mo of age. Greater frequency of small fish consumption was associated with moderately improved iron status. However, large fish did not show the same potential to improve iron status, and meat was not associated with any iron or anemia indices. Though frequency of fish and meat consumption increased with child age, the average intake remained small (1.2g/d meat, 1.2g/d large

fish, and 4.9g/d small fish at 12-15mo). These small portion sizes may explain the weak association between frequency of consumption over 6mo and iron status as well as the lack of association between usual intake from 24-hr dietary recalls and iron status.

Our finding that small fish are associated with moderate improvements to iron status of young children is consistent with the nutrient profile of small, Malawian fish and findings from dietary modeling studies on optimizing iron intake. Small fish are typically consumed whole, providing a good dietary source of bioavailable heme iron from the fish's head, flesh, and organs. For example, fresh sardines (*Engraulicypris sardella* or "*usipa"*) from Lake Malawi provide 4.6mg iron per 100g edible portion (32). Nutrient-dense foods like small fish are especially important for complementary feeding children who have low caloric requirements but high nutrient needs (1,33). Dietary modeling studies, nutrient analysis of fish products (i.e. fish powder and fish-enriched porridges), and dietary diversity interventions have shown potential for small fish to help optimize nutrient intake of young children in low-and-middle-income countries (34–39). Though some studies have indicated that small fish can be part of a nutritious, locally-sourced, and affordable diet for Malawian children (8,12,40–42), market availability and affordability for some households does not guarantee consumption by children (43).

Our study found that small fish consumption of at least 1 day in the past week increased with the age of the child, from 55% at 6-9mo to 64% at 9-12mo to 70% at 12-15mo, suggesting that allocation of small fish among household members may be influenced by cultural patterns or knowledge of infant and young child feeding practices. In some cultures, infants and young children are provided with foods prepared separately from foods consumed by other household members. For example, infants in fish-farming households in rural Bangladesh were more likely to consume eggs, dairy, and organ meats than their mothers, and their mothers were more likely to consume fish than their children (44). Small fish and meat may be withheld from infants and young children due to parental beliefs about age- and texture-appropriate foods, particularly concerning the bones in small fish and the ability to chew animal

tissue without teeth (43,44). In the present study, caregivers completing 24-hr dietary recalls frequently reported that only the sauce or broth from mixed dishes containing fish were provided to children (11). Thus to increase fish intake of infants, whole small fish containing the head, bones, and organs may be ground into a powder and used to supplement complementary foods provided to infants (37,45).

To date, no studies have directly examined the effect of providing dried fish powder or fresh fish on iron status of infants in low- and middle-income countries in comparison to an unenriched or non-intervention control. However, three trials have examined the efficacy of infant porridges commercially fortified with fish powder on iron status as compared to other enriched infant porridges: when the proportion of fish powder included in blends of complementary foods was at least 12% by dry weight, plasma ferritin, sTfR, percent transferrin saturation, and hemoglobin did not differ between children randomized to receive foods fortified with micronutrients and fish powder (46–48). Among school aged children in Ghana, providing fish meat fortified cowpeas (5.4mg iron per 100g portion) and a vitamin C drink through 6mo of school lunches resulted in higher hemoglobin but similar ferritin to children receiving non-fortified cowpeas (4.1mg iron per 100g portion) with the vitamin C drink or a placebo drink (49). Providing an iron-rich mola fish curry dish to 3-7yr old Bangladeshi children with marginal vitamin A status for 6d/wk for 9wks resulted in a 0.73mg/L lower mean sTfR concentration than children in the rui fish control group supplemented with retinyl palmitate. However, groups did not differ in ferritin or hemoglobin concentrations (50). Together, these studies establish some precedent of potential for fish consumption to contribute towards improved iron and anemia status of young children in low-and-middle income countries, as suggested by the findings from this analysis.

In our analysis, large fish and meat were either not associated with iron indices or the associations were attenuated by controlling for additional covariates. The lack of associations with iron could be related to varying iron densities of foods within the same categories and infrequent consumption of large fish and meat over 6mo. Large fish consumption may not contribute to iron status as much as small fish because

the edible portions of large fish, typically consumed as fillets, are less iron-dense than consumption of small fish, typically consumed whole, including the organ meat. Nevertheless, nutrient analyses for freshwater fish species are limited and nutrient composition tables indicate considerable variation between species (32,51). Children who reported meat consumption in this study primarily consumed chicken, which has less heme and lower iron density than red meats like beef and goat meat. Results from a cluster-randomized efficacy trial conducted in multiple low-and-middle income countries indicated that infants with greater intake of red meat (30-45g/d beef) can maintain their iron status through 18mo of age (52), however we are aware of no similar studies of chicken meat. The lower iron content of chicken combined with small portion sizes and infrequent consumption support the lack of association of meat with iron indices in our study population. Since large fish was not associated with ferritin, sTfR, or hemoglobin, the association of large fish with iron deficiency and anemia may be influenced by other explanatory factors not included as covariates. Post-hoc analyses that adjusted for malaria at enrollment attenuated the relationship between large fish consumption and iron deficiency. Similarly, the association between large fish and higher anemia was attenuated after adjusting for CRP at 12-15mo of age.

Though frequency of flesh food consumption reported through FFQs showed some relationships with iron and anemia, usual intake of flesh foods, as reported in grams of consumption in 24-hr dietary recalls at 12-15mo of age, showed no associations with iron or anemia. The differences in findings may result from several factors pertaining to the definitions and methods of quantifying exposure. First, classification of small fish vs large fish consumption on the FFQ and 24-hr dietary recall may have differed. The FFQ asked about fish consumption based on size but not species or the way in which the fish is typically consumed. Thus, the size classification was determined by the respondent. FFQ respondents who work in the fishing industry, as many in this study population do, may define small fish based on length, such as <25cm (41). In the 24-hr dietary recall, respondents were asked to report the name of the fish and how it was consumed. During dietary analysis, we classified small fish as species

that were likely to be consumed whole, presuming these fish to have higher iron content. Additionally, interviewers conducting 24-hr dietary recalls probed for details on how mixed dishes were consumed, specifically differentiating between intake of broth only versus fish flesh when caregivers reported consumption of fish soup, whereas FFQ respondents may have counted the broth of fish soup towards days of fish consumption. Second, the 24-hr dietary recalls and repeat recalls capture recent short-term exposure, which may differ from usual intake defined by long-term exposure. Accordingly, nutritional biomarkers reflecting iron status and body stores may be more sensitive to measures of cumulative rather than short-term dietary intake. Third, applying the NCI method for assessing usual intake of episodically consumed foods presented with several challenges in this population of young children. Since fish and meat were only reported in 9-40% of dietary recalls, the data set contained many values of 0g intake. For analytical models, these values were replaced with an arbitrarily small number set to one-quarter of the minimum quantity reported in the 24-hr recalls before correcting for within-person variation in dietary intake on the log-transformed scale. However, replacing 60-91% of values with any small number can bias the estimate of mean usual intake as well as artificially reduce the variability of measured dietary intakes, which may additionally bias the relationship with nutritional biomarkers towards the null. Performing sensitivity analyses for various methods of imputation would be ideal, however this is not always feasible due to the long total computing time required to run the full set of analytical models.

This analysis presented with several limitations concerning the analysis and generalizability of findings. Since FFQs prompted respondents for the number of consumption days within the past week without specifying which days were consumption days, we could not aggregate the number of days of any fish or meat consumption and instead counted the number of weeks. Totaling any flesh food consumption by day would be advantageous for comparing usual intake of any flesh foods with recommendations for animal-source food intake among infants and young children as well as for harmonizing the units of interpretation across analytical models in our study. Though examining consumption of flesh foods

quantitatively by milligrams of iron may have a more direct relationship with iron indices, gram weights of flesh food intake were used instead because food composition data for large freshwater fish species is limited and in many cases does not contain genus- or species-specific iron content for fish (51). If heme iron-rich red meat or organs were consumed by a greater proportion of the population, it would have been advantageous to analyze these separately from less heme iron-rich foods like chicken. Conducting additional repeat dietary recalls would capture more consumption days of infrequently consumed large fish and meat, which would improve the precision for estimates of usual dietary intake and their relationship with iron and anemia indices. Our study did not measure other sources of anemia such as hemoglobinopathies and parasites, which does not allow us to distinguish between nutritional anemias and those due to other causes; nor does it allow us to adjust for potential confounding due to these other factors.

Additionally, this analysis may present with some selection bias since approximately 13% of children had incomplete iron measurements, most of whom withdrew from the study prior to 3mo follow-up at 9-12mo of age. Reweighting the analytical sample to match the demographical characteristics of the enrolled population would have necessitated reclassification of the continuous FFQ exposures into deciles and deletion of some covariates for models to converge, resulting in models that were no longer comparable to the analytical methods for the principle FFQ models. For 24-hr dietary recall data, methods have not yet been developed for integrating an inverse probability-weighted analysis into the NCI modeling procedures for usual intake. While weighting the results was not possible for this analysis, we have some indication that the proportion of children missing iron indices was not likely to alter the interpretation of results: the analysis of the 1 egg/d intervention on iron and anemia excluded the same children missing iron indices, and conducting an inverse probability-weighted analysis did not change the findings of the egg intervention on iron (10). Therefore, the results of this present analysis may be generalizable to the original study population as well as children in the two fishing communities within the Mangochi District of Malawi where the study was conducted.

Some of the strengths of this analysis include assessing usual intake by different data sources, conducting multiple sensitivity analyses, and examining the potential for locally available foods to alleviate iron deficiency among children in fishing communities. The 24-hr dietary recall was conducted in such a way as to quantify intake of animal-source foods and to separate consumption of ingredients of mixed dishes. This is particularly important for infants and young children who may only be fed some ingredients of a family meal. Usual intake of flesh foods was assessed by two definitions: the percent of consumption days through FFQs conducted weekly over 6mo and grams of intake reported through 24-hr dietary recalls. Using two different sources of dietary data provides a complementary and more comprehensive assessment of usual intake, since FFQs have more systematic error and less random error whereas 24-hr dietary recalls have less systematic error and more random error due to day-to-day variation in intake (53). Applying the NCI method to statistically adjust for within-person variation in daily intake also strengthens the quantitative assessment of usual intake. Numerous sensitivity analyses were conducted on the FFQ analyses, including the assessment of the percent of flesh food consumption days over the past 3mo and dropping or imputing missing observations. The sensitivity analyses provided consensus on the magnitude and significance of associations as compared to principal models. Importantly, this analysis contributed towards filling a literature gap concerning the relationship between iron-specific biomarkers and iron-rich flesh foods in young children. Contextually, this is significant because young Malawian children enrolled in this study have a high burden of iron deficiency and access to fish as an iron-rich food source.

The potential for small fish to improve dietary adequacy of iron in infants and young children is gaining interest (35,36), yet there is a considerable gap in the literature demonstrating a relationship between fish consumption and iron indices or other nutritional biomarkers (45). This study is one of few to examine the relationship of fish and meat with iron indices among children in low-and-middle income countries. Our findings suggest that even a small increase in small fish consumption, such as 2d/mo, could improve iron status of infants and young children; however, these findings may be specific our

study context in which children who lived in Malawian fishing communities ate small portion sizes of flesh foods and had high burdens of iron deficiency and inflammation (10–12). If the average intake of small fish doubled, such that children consumed small fish every other day, we would expect ferritin to increase by 0.5μg/L and sTfR to decrease by 0.5mg/L, but a high proportion would still be iron deficient. Further research is needed to examine the relationship of fish and meat intake with iron indices in contexts where larger portion sizes are consumed as well as in comparison to non-intervention or control groups provided with unenriched foods (46–48,54). Additionally, future research and health messages concerning infant and young child feeding practices should consider barriers to consumption of fish, meat, and other animal-source foods. Strategies to increase number of days or portion sizes of fish consumption among children may include market-based approaches to increase household purchasing power, reduce market price, and develop innovative products, such as nutritional supplements or ready-to-use foods containing fish powder (46–48,54). Alternatively, community health workers could provide instruction to caregivers on ways to prepare fish for infants with appropriate textures for their developmental stage (39) as well as information on the recommended frequency and quantity of animal-source food consumption to meet infants' nutritional needs. Lastly, dietary strategies to reduce the burden of iron deficiency in young children may vary by context and involve a variety of approaches, such as promoting flesh food intake (55), enhancing the bioavailability of plant-based iron sources (56), and provision of iron-containing nutritional supplements to infants and young children (57,58).

4.6 References

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Table 4.1 – Enrollment characteristics of children in the Mazira Project, Malawi, 2018-2019[1]

Characteristic	n	value
Maternal		
Maternal age, y	654	26.0 ± 6.7
Maternal primary education[2], %	660	20
Maternal literacy, %	643	46
Household		
Number of children under 5 y	638	1.7 ± 0.8
Number of household members	641	5.9 ± 2.7
Moderate or severe food insecurity[3], %	660	78
Child		
Child age, mo	660	7.4 ± 1.2
Female, %	660	48
Breastfeeding, %	659	100
Foods consumed in past 24hrs		
Small fish, %	659	4
Large fish, %	659	1
Meat, %	659	2
Organs, %	659	0
Dairy, %	659	7
Eggs, %	659	4
Legumes and beans, %	659	36
Grains and cereals, %	659	99
Vitamin A-rich fruits and vegetables, %	659	25
Other fruits and vegetables, %	659	52
Inflammation (CRP >5mg/L or AGP >1g/L)	525	62
Positive malaria test (RDT), %	595	13
Hemoglobin, g/dL	582	10.4 (9.5, 11.5)
Plasma ferritin, µg/L	525	25.9 (13.2, 50.4)
Plasma sTfR, mg/L	525	11.4 (9.0, 15.4)
Anemia (hemoglobin <11g/dL), %	582	61
Any iron deficiency[4], %	525	85
Iron deficiency anemia, %	524	55
Inflammation-adjusted[5]		
Plasma ferritin, µg/L	525	14.1 (7.9, 25.5)
Plasma sTfR, mg/L	525	9.7 (7.9, 13.1)
Any iron deficiency[4], %	525	77
Iron deficiency anemia, %	524	52

[1]Values are %, mean ± SD, or median (P25, P75). AGP = α-1-acid glycoprotein; CRP = c-reactive protein; RDT = rapid diagnostic test; sTfR = soluble transferrin receptor

[2]Percent completed primary or greater

[3]Food insecurity assessed using Household Food Insecurity Access Scale

[4]Ferritin <12µg/L and/or sTfR >8.3mg/L

[5]Inflammation-adjusted using the BRINDA (Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia) approach (27,28)

Table 4.2 – Usual intake of flesh foods from 24-hr dietary recalls of 6-15mo old children in Mangochi, Malawi, 2018-2019[1]

	Consumers, *n* (%)			Usual intake, g/d		
	6-9mo	9-12mo	12-15mo	6-9mo[2]	9-12mo	12-15mo
Small fish	25 (4)	144 (24)	239 (40)	--	1.8 (0.6, 4.5)	4.9 (2.3, 8.7)
Large fish	4 (1)	38 (6)	74 (12)	--	0.5 (0.2, 1.3)	1.2 (0.5, 2.6)
Meat	10 (2)	39 (7)	54 (9)	--	0.7 (0.3, 1.6)	1.2 (0.6, 2.1)

[1]Usual intake reported as median (p25, p75) using the *DISTRIB* macro from the National Cancer Institute (29).
[2]Missing values (--) due to failed convergence of the two-part, correlated model from few consumers.

Table 4.3 – Association between percent of days meat and fish were consumed and iron and anemia among 12-15mo old children in Mangochi, Malawi, 2018-2019[1]

	Ferritin, µg/L *Geometric mean (p25, p75)*	sTfR, mg/L *Geometric mean (p25, p75)*	Hgb, g/dL *Geometric mean (p25, p75)*	Anemia, %	ID, %	IDA, %
Inflammation-adjusted value at 12-15mo[2]	7.1 (4.1, 11.4)	11.4 (8.6, 14.7)	11.0 (10.3, 12.0)	43	89	40
	GMR (95%CI)	*GMR (95%CI)*	*GMR (95%CI)*	*PR (95%CI)*	*PR (95%CI)*	*PR (95%CI)*
Intake over past 6mo[3,4]						
Small fish, per 10% of days	1.03 (0.98, 1.07)	**0.98 (0.96, 1.00)**	1.01 (1.00, 1.01)	0.95 (0.88, 1.02)	1.00 (0.98, 1.02)	0.95 (0.87, 1.03)
Large fish, per 10% of days	1.05 (0.98, 1.12)	0.99 (0.96, 1.02)	0.99 (0.98, 1.00)	**1.09 (1.01, 1.19)**	**0.96 (0.93, 1.00)**	1.04 (0.95, 1.15)
Meat, per 10% of days	1.04 (0.94, 1.16)	0.98 (0.93, 1.03)	1.01 (0.99, 1.02)	1.07 (0.91, 1.25)	1.00 (0.96, 1.05)	1.10 (0.93, 1.31)
Any flesh food, per 10% of weeks	**1.03 (1.00, 1.06)**	0.99 (0.98, 1.00)	1.00 (1.00, 1.01)	1.00 (0.95, 1.04)	0.99 (0.98, 1.01)	0.99 (0.94, 1.04)
Intake over past 3mo[3,5]						
Small fish, per 10% of days	1.02 (0.99, 1.06)	0.99 (0.97, 1.00)	1.00 (1.00, 1.01)	0.96 (0.90, 1.02)	1.00 (0.98, 1.01)	0.96 (0.90, 1.03)
Large fish, per 10% of days	1.02 (0.97, 1.08)	0.99 (0.97, 1.02)	1.00 (0.99, 1.01)	1.04 (0.96, 1.12)	**0.97 (0.94, 1.00)**	1.02 (0.94, 1.10)
Meat, per 10% of days	1.02 (0.94, 1.12)	0.98 (0.94, 1.02)	1.01 (0.99, 1.02)	1.06 (0.93, 1.21)	1.02 (0.98, 1.06)	1.10 (0.96, 1.26)
Any flesh food, per 10% of weeks	1.02 (1.00, 1.05)	**0.99 (0.97, 1.00)**	1.00 (1.00, 1.01)	0.99 (0.95, 1.04)	1.00 (0.98, 1.01)	0.99 (0.94, 1.03)

GMR = geometric mean ratio; Hgb = hemoglobin; ID = iron deficiency; IDA = iron deficiency anemia; sTfR = soluble transferrin receptor; PR = prevalence ratio

[1]Ferritin (n=575), sTfR (n=575), Hgb (n=585); anemia (n=585); ID (n=575); IDA (n=568). All models adjusted for malaria, month of assessment, child sex, child age, child illness, and baseline measures. Ferritin, sTfR, ID, and IDA models included adjustment for inflammation. Health center, maternal education, number of children in the household under 5 years, and minutes between blood draw and aliquot completion were included when $P<0.1$.

[2]Ferritin, sTfR, ID, and IDA models adjusted for inflammation using the BRINDA (Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia) approach.

[3]Dietary exposures for small fish, large fish, and meat were calculated as percent of days among available FFQs to include participants with variable number of missing FFQs. Any flesh food intake was totaled by week rather than days because the FFQ asked respondents for the number of consumption days within the past week rather than asking respondents to specify which days foods were consumed.

[4]10% of days refers to increments of 16 days and 10% of weeks refers to increments of 2.3 weeks.

[5]10% of days refers to increments of 9 days and 10% of weeks refers to increments of 1.3 weeks.

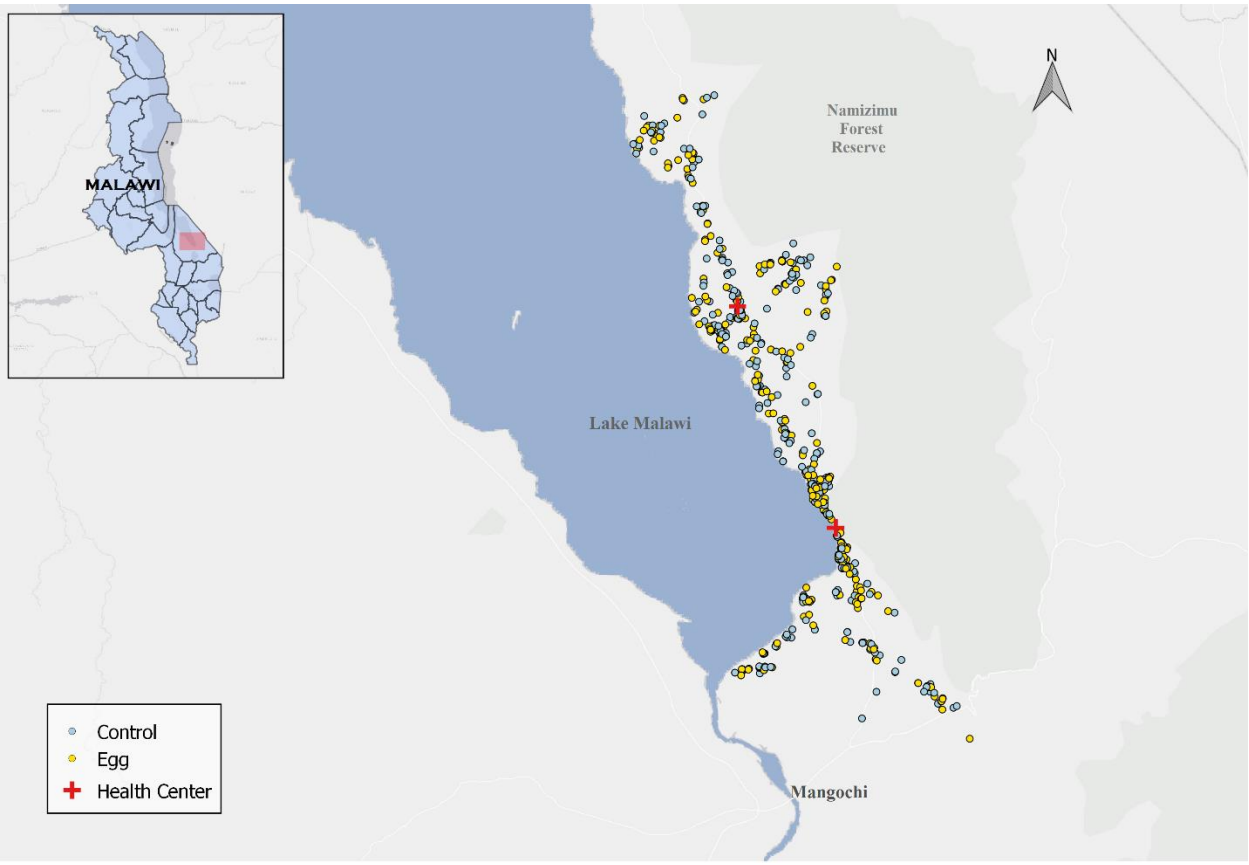

Figure 4.1 – Map of study sites in the Mazira Project, Malawi, 2018-2019

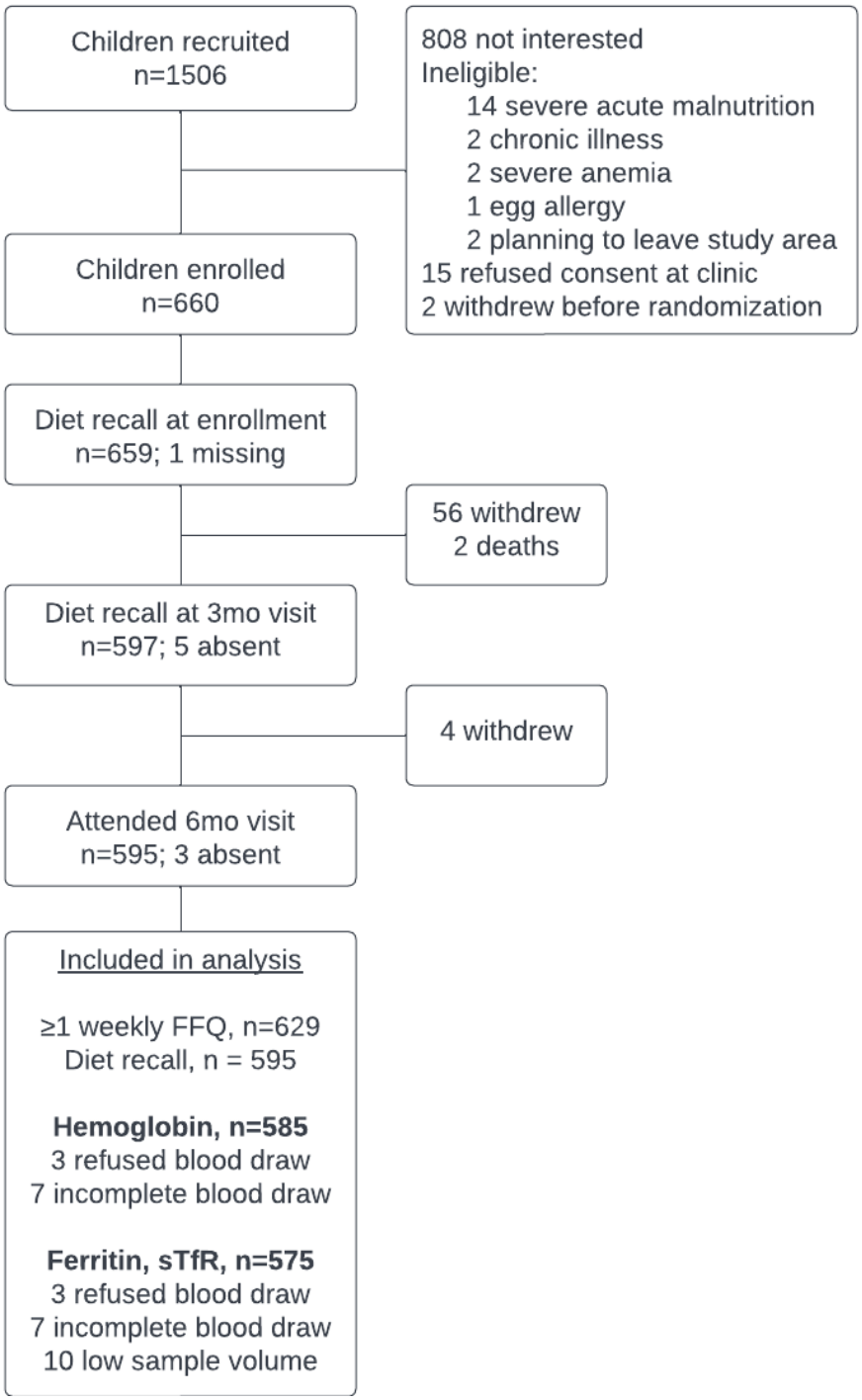

Figure 4.2 – Participant flow diagram for the iron and dietary analyses of the Mazira Project, Malawi, 2018-2019

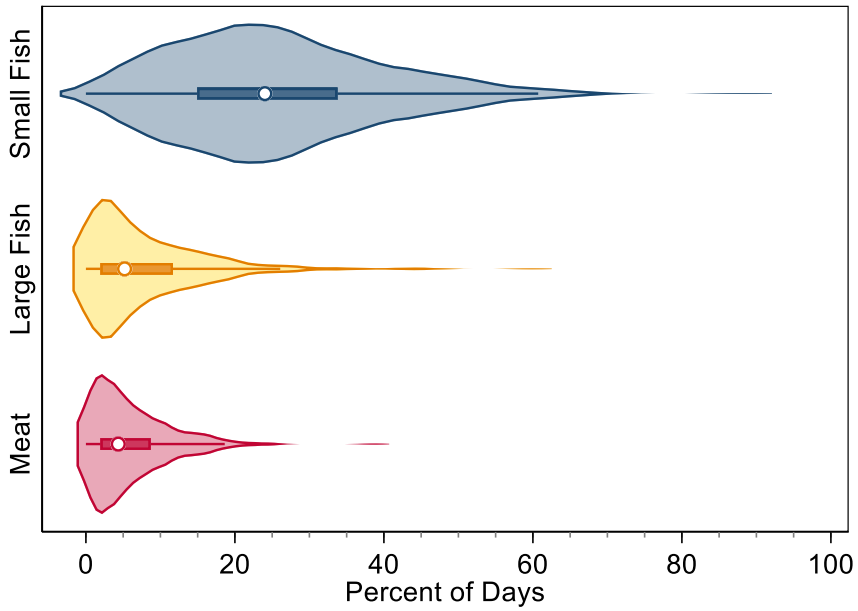

Figure 4.3 – Frequency distribution of the percent of days that 6-15mo old children reported consuming meat and fish in Mangochi, Malawi, 2018-2019

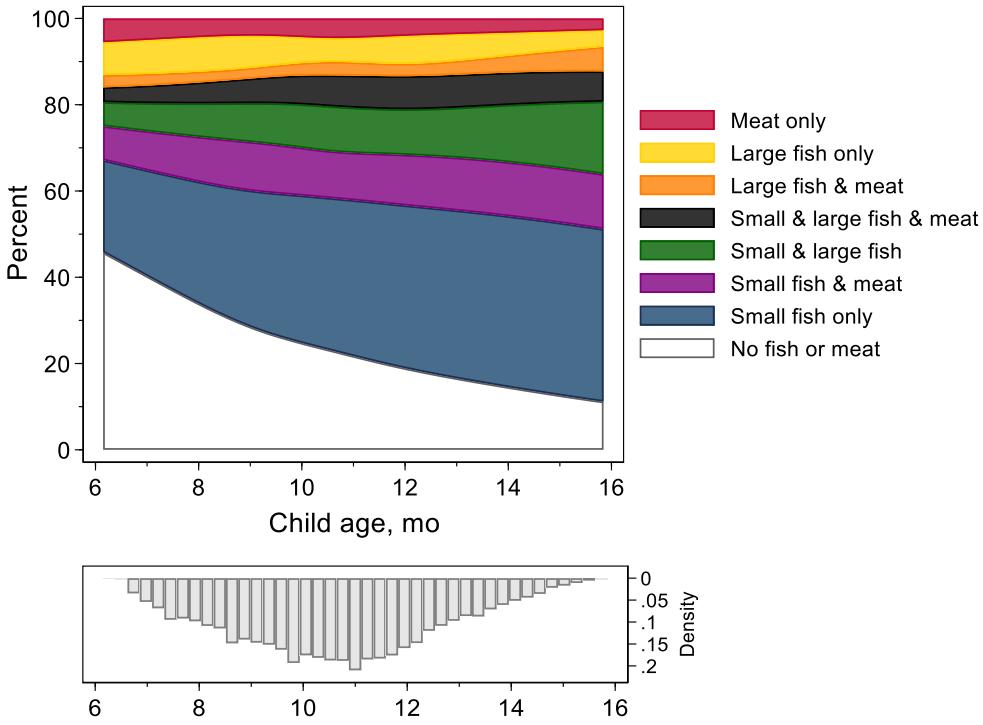

Figure 4.4 – Weekly flesh food consumption among 6-15mo old children in Mangochi, Malawi, 2018-2019

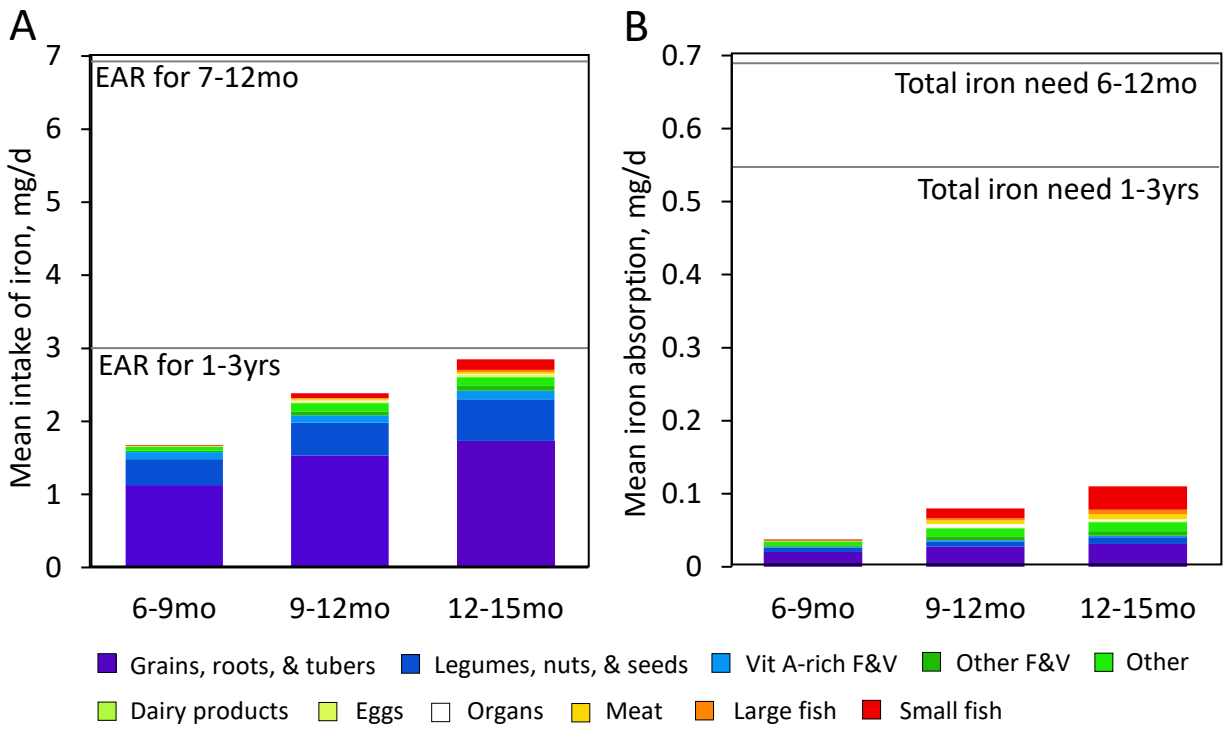

Figure 4.5 – A) observed and B) absorption-adjusted intake of iron by dietary source among 6-15mo old children in Mangochi, Malawi, 2018-2019

Footnote: EAR = Estimated Average Requirement; F&V = Fruits and Vegetables. Children provided 24-hr dietary recalls at ages 6-9mo (n=659), 9-12mo (n=597), and 12-15mo (n=595). Correction factors listed in Supplementary Table 1 were applied to adjust observed dietary iron for absorption. Median total iron need for 6-12mo infants is 0.69mg/d and for 1–3-year-old children is 0.54mg/d (6).

Supplementary Table 4.1 – Correction factors for absorption of dietary iron for children in the Mazira Project, Malawi, 2018-2019

Category	Factor	Reference
Grains	1.8%	1.8% maize (Hurrell, RF et al. *Am J Clin Nutr*. 2003;77:1213-9)
Legumes/nuts	1.6%	1.6% peanuts (Petry et al. *J. Nutr*. 2010;140:1977-1982)
Vit A-rich F&V		
Leafy greens	1.2%	1.2% broccoli w/ 2g phytate (Gillooly, M et al. *British J of Nutr*. 1984;5:37-46)
Dark orange F&V	10%	10% from moderate iron bioavailable infant diet (IOM 2001)
Other F&V	10%	10% from moderate iron bioavailable infant diet (IOM 2001)
Small fish[1]	22%	~30% (up to 40%) heme iron in fish (Cook, JD & Monsen, ER. *Am J Clin Nutr*. 1976;29:859-67)
Large fish[1]	22%	~30% (up to 40%) heme iron in fish (Cook, JD & Monsen, ER. *Am J Clin Nutr*. 1976;29:859-67)
Meat[1]	22%	~30% heme iron in white meat (Hurrell, R. *The mineral fortification of foods*. Leatherhead: Leatherhead International Ltd; 1999. pp. 54–93. [As cited in Lynch, S et al. *J Nutr*. 2018; 148:1001S–1067S])
Organs[1]	30%	~70% heme iron in red meat (Hurrell R. *The mineral fortification of foods*. Leatherhead: Leatherhead International Ltd; 1999. pp. 54–93. [As cited in Lynch, S et al. *J Nutr*. 2018; 148:1001S–1067S])
Milk	10%	10% from moderate iron bioavailable infant diet (IOM 2001)
Eggs	3.7%	3.7% hen eggs (Callender, ST et al. *British J of Haem*. 1970;19:657-65)
Other	10%	10% from moderate iron bioavailable infant diet (IOM 2001)

F&V = fruits and vegetables

[1]Assumes 35% absorption of heme iron (Hurrell, R. *Am J Clin Nutr*. 2010; 91(suppl):1461S–7S.) and 16.8% absorption of non-heme iron from flesh food-rich diets (IOM 2001)

Supplementary Table 4.2 – Characteristics of participants missing iron indices at 12-15mo of age in the Mazira Project, Malawi, 2018-2019

	Missing (n=85)		Complete (n=575)		
Characteristic	n[1]	% or mean ± SD	n[1]	% or mean ± SD	p-value
Maternal education (% completed primary or greater)	85	12	575	21	**0.046**
Maternal literacy (% can read)	71	37	572	47	0.099
Maternal tribe					
Chewa or other	71	10	572	15	0.246
Yao		90		85	
Maternal occupation					
Farming	71	59	571	41	**0.013**
Service		13		25	
Housewife		28		34	
Paternal occupation					
Farming or Fishing	58	41	458	49	0.254
Service		59		51	
Muslim religion	71	92	572	88	0.335
Health center					
Lungwena	85	71	575	51	**0.001**
Malindi		29		49	
Poor floor quality[2]	71	83	572	76	0.168
Poor roof quality[2]	71	72	572	60	**0.049**
Poor wall quality[2]	71	54	572	43	0.084
HOME inventory score[3]	71	24 ± 3.1	572	24.2 ± 3.6	0.669
Number of children under 5 y	70	1.7 ± 0.8	568	1.7 ± 0.8	0.845
Moderate or severe food insecurity[4]	85	86	575	77	0.060
Enrollment date, days into study period	85	60.0 ± 46.8	575	89.6 ± 54.3	**<0.001**
Child					
child age, mo	85	7.5 ± 1.2	575	7.4 ± 1.2	0.199
female, %	85	47	575	49	0.801
prevalence of stunting (LAZ<-2)	85	13	575	14	0.841
prevalence of underweight (WAZ<-2)	85	6	575	8	0.466
prevalence of wasting (WLZ<-2)	85	1	575	1	0.911
prevalence of malaria	71	11	524	13	0.718
total FFQs completed	85	7 ± 9	575	22 ± 2	**<0.001**
consumed small fish, % of days	54	20 ± 15	575	26 ± 14	**0.007**
consumed large fish, % of days	54	9 ± 13	575	8 ± 9	0.547
consumed meat, % of days	54	5 ± 5	575	6 ± 6	0.093
consumed any flesh foods, % of wks	54	67 ± 33	575	76 ± 21	**0.004**

FFQ = food frequency questionnaire; LAZ = length-for-age z-score; WAZ = weight-for-age z-score; WLZ = weight-for-length z-score

[1]Number of children with data at enrollment or first household visit

[2]Poor quality defined as straw, grass, mud, or unburnt brick

[3]HOME, Home Observation for Measurement of the Environment (59)

[4]Food insecurity assessed using Household Food Insecurity Access Scale (13)

Supplementary Table 4.3 – Association between predicted usual intake of flesh foods from 24-hr dietary recalls with iron and anemia at 12-15mo of age in the Mazira Project, Malawi, 2018-2019[1]

	Ferritin, µg/L	sTfR, mg/L	Hgb, g/dL	Anemia, %	ID, %	IDA, %
	GMR (95%CI)	*GMR (95%CI)*	*GMR (95%CI)*	*PR (95%CI)*	*PR (95%CI)*	*PR (95%CI)*
Recall at 12-15mo[2]						
Small fish, per 1g	1.02 (0.97, 1.07)	1.00 (0.98, 1.02)	1.00 (0.99, 1.01)	1.01 (0.95, 1.08)	1.01 (0.99, 1.03)	1.04 (0.97, 1.11)
Large fish, per 1g	1.10 (0.93, 1.30)	0.99 (0.90, 1.10)	0.99 (0.95, 1.02)	1.06 (0.80, 1.40)	0.91 (0.73, 1.14)	1.03 (0.67, 1.57)
Meat, per 1g	1.09 (0.72, 1.66)	0.90 (0.67, 1.21)	1.00 (0.91, 1.10)	0.99 (0.46, 2.14)	1.18 (0.92, 1.51)	1.01 (0.26, 3.95)
Recall at 9-12mo[3]						
Small fish, per 1g	0.99 (0.86, 1.15)	0.96 (0.89, 1.03)	1.01 (0.99, 1.04)	0.9 (0.68, 1.18)	1.02 (0.95, 1.09)	0.95 (0.73, 1.23)

GMR = geometric mean ratio; Hgb = hemoglobin; ID = iron deficiency; IDA = iron deficiency anemia; sTfR = soluble transferrin receptor; PR = prevalence ratio

[1]Ferritin (n=575), sTfR (n=575), Hgb (n=585); anemia (n=585); ID (n=575); IDA (n=568). All results adjusted for malaria, month of assessment, child sex, child age, child illness, and baseline measures. Ferritin, sTfR, ID, and IDA models adjusted for inflammation using the BRINDA (Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia) approach (27,28). Health center, maternal education, number of children in the household under 5 years, and minutes between blood draw and aliquot completion were included when $P<0.1$.

[2]Usual intake of 1g higher than the median corresponds to the following percentiles: 57th for small fish, 70th for large fish, and 76th for meat.

[3]A 1g higher usual intake from the median corresponds to the 62nd percentile for small fish.

Supplementary Table 4.4: Sensitivity analyses for imputed and dropped observations conducted for the association between percent of days of flesh food intake over 6mo and iron and anemia at 12-15mo of age in the Mazira Project, Malawi, 2018-2019[1]

	Ferritin, µg/L GMR (95%CI)	sTfR, mg/L GMR (95%CI)	Hgb, g/dL GMR (95%CI)	Anemia, % PR (95%CI)	ID, % PR (95%CI)	IDA, % PR (95%CI)
Imputed observations[2]						
Small fish, per 10% of days	1.02 (0.98, 1.07)	0.98 (0.96, 1.00)	1.01 (1.00, 1.01)	0.95 (0.89, 1.02)	1.00 (0.98, 1.02)	0.95 (0.88, 1.03)
Large fish, per 10% of days	1.05 (0.98, 1.13)	0.99 (0.96, 1.02)	0.99 (0.98, 1.00)	1.08 (1.00, 1.18)	**0.96 (0.92, 1.00)**	1.03 (0.94, 1.14)
Meat, per 10% of days	1.05 (0.95, 1.16)	0.98 (0.93, 1.02)	1.01 (0.99, 1.02)	1.06 (0.91, 1.24)	1.00 (0.96, 1.05)	1.10 (0.92, 1.30)
Any flesh food, per 10% of weeks	1.02 (0.96, 1.08)	1.00 (0.98, 1.03)	1.00 (0.99, 1.01)	0.99 (0.91, 1.08)	1.01 (0.98, 1.05)	0.93 (0.84, 1.02)
Dropped observations[3]						
Small fish, per 10% of days	1.03 (0.98, 1.07)	**0.98 (0.96, 1.00)**	1.01 (1.00, 1.01)	0.94 (0.88, 1.01)	1.00 (0.98, 1.02)	0.94 (0.86, 1.02)
Large fish, per 10% of days	1.06 (0.99, 1.13)	0.99 (0.96, 1.02)	0.99 (0.98, 1.00)	1.09 (1.00, 1.18)	**0.96 (0.92, 0.99)**	1.04 (0.94, 1.15)
Meat, per 10% of days	1.04 (0.94, 1.16)	0.98 (0.93, 1.03)	1.01 (0.99, 1.02)	1.09 (0.94, 1.27)	1.01 (0.96, 1.06)	1.14 (0.96, 1.34)
Any flesh food, per 10% of weeks	**1.03 (1.00, 1.06)**	0.99 (0.98, 1.00)	1.00 (1.00, 1.01)	0.99 (0.95, 1.04)	0.99 (0.98, 1.01)	0.98 (0.94, 1.03)

GMR = geometric mean ratio; Hgb = hemoglobin; ID = iron deficiency; IDA = iron deficiency anemia; sTfR = soluble transferrin receptor; PR = prevalence ratio

[1]Ferritin (n=575), sTfR (n=575), Hgb (n=585); anemia (n=585); ID (n=575); IDA (minimally-inflammation adjusted: n=575; fully adjusted: n=568). All models adjusted for malaria, month of assessment, child sex, child age, child illness, and baseline measures. Ferritin, sTfR, ID, and IDA models included adjustment for inflammation (27,28). Health center, maternal education, number of children in the household under 5 years, and minutes between blood draw and aliquot completion were included when $P<0.1$.

[2]Last observations carried forward to cover gaps between observed weeks and from the last recall through the end of 6mo study period

[3]Analysis included children who completed 50% or more of 7-day food frequency questionnaires: ferritin (n=568), sTfR (n=568), Hgb (n=578); anemia (n=578); ID (n=568); IDA (n=561)