Plasma Choline, Growth, and Development among Malawian Children Age 6-15 Months Enrolled in an Egg Intervention Trial

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Plasma Choline, Growth, and Development among Malawian Children Age 6-15 Months Enrolled in an Egg Intervention Trial

Abstract

Choline is an essential micronutrient that has been positively associated with child growth and development in human and animal studies. Suboptimal choline intake is thought to be common in low and middle income countries (LMICs) because of the relative expense of animal source foods, the major sources of choline. Few studies have examined the role of choline for growth and development in LMICs (reviewed in Chapter 1). This dissertation aimed to help fill this gap using data from the Mazira Project randomized controlled trial, in which 660 children age 6-9 months from rural Malawi were randomized to receive either 1 egg per day or a nonintervention control for 6 months. Despite large improvements in growth from a similar egg intervention trial in Ecuador, which were mediated by improvements in plasma choline, there were few improvements to growth or development with the provision of 1 egg per day in the Mazira Project. This dissertation includes three secondary analyses that explore the role of plasma choline in the Mazira Project.

In the first analysis (Chapter 2), we examined the effect of the egg intervention on plasma choline and several of its metabolites. Plasma choline, betaine, dimethylglycine (DMG), trimethylamine N-oxide (TMAO), and docosahexaenoic acid (DHA) were measured from blood samples collected at enrollment and 6 month follow up. Semi-quantitative lab analyses were conducted in 200 children per group using UPLC-MS/MS, and quantitative lab analyses were measured in a subsample of 60 children using LC-MS/MS. Mean plasma choline values using the quantitative data (n=60) were ~17.0 µmol at enrollment and decreased to ~14.0 µmol at 6 month follow up. Plasma choline, betaine, DMG and DHA were not significantly different by

group at 6 month follow up in minimally or fully adjusted linear regression models using the semi-quantitative data (n=200 per group). However, plasma TMAO was significantly higher (26% [SD 7%, 48%]) in the intervention group at 6 month follow up.

In the second analysis (Chapter 3), we investigated the association between plasma choline and child development. Plasma choline, betaine, DMG, and TMAO were measured as described in Chapter 2. Additionally, measures of child development were collected at enrollment and 6 month follow up. Fine motor, gross motor, language, and personal social normed z-scores were calculated using the Malawi Developmental Assessment Tool (MDAT). Visual attention was measured by response times in an Infant Orienting with Attention (IOWA) task and peak look lengths in a visual paired comparison (VPC) task. Memory was assessed by novelty preference in a VPC task and the number of actions recalled during an elicited imitation task (collected at 6 month follow up only). Plasma choline was not associated with most measures of development in fully adjusted generalized linear models, except a negative association with MDAT fine motor normed z-score (-0.13 SD [95% CI -0.22, -0.04]) and a positive association with IOWA response time (8.84 ms [1.66, 16.03]), both suggesting poorer development with higher plasma choline concentration. Associations with the related metabolites (betaine, DMG, and TMAO) were null, except positive associations of plasma TMAO with peak look length and total actions recalled score.

In the third analysis (Chapter 4), we examined the association between plasma choline and child growth in the Mazira Project. Child length, weight, and head circumference were measured by trained anthropometrists at enrollment, 3 month follow up, and 6 month follow up, then converted to z-scores (length-for-age (LAZ), weight-for-age (WAZ), weight-for-length (WLZ), and head circumference-for-age (HCAZ)) using WHO Growth Standards. Dichotomous

outcomes were calculated using cutoffs of -2 SD. Conditional outcomes were calculated by regressing data from the 6 month follow up on data from the previous time points. The association of plasma choline with continuous and dichotomous growth outcomes was assessed using generalized linear models. Conditional growth outcomes were included in linear regression models, with baseline plasma choline as a predictor. Plasma choline was positively associated with WLZ in a minimally adjusted model, but this was not significant after further adjustment for covariates. Additionally, plasma choline was weakly and negatively associated with LAZ in a fully adjusted model (-0.09 [-0.17, -0.01]). There were no significant associations of betaine, DMG, or TMAO with child growth indicators.

Together, the analyses presented in this dissertation provide a partial explanation for the null results in the Mazira Project. The egg intervention was hypothesized to work in part by improving plasma choline status, which would then lead to improved growth and development. However, the intervention did not significantly improve plasma choline, and plasma choline was not associated with most measures of growth or development. These analyses also suggest that the relationship between choline, development and growth may be context specific, perhaps depending on children's background diet as well as socioeconomic and health factors. Finally, this dissertation highlights the need for sensitive and specific biomarkers of choline status, and their application in the study of young children in LMICs. At this time, choline intake at the Adequate Intake level (AI for 7-12 months: 150 mg/d; 1-3 years: 200 mg/d) should be recommended for young children.

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# Chapter 1: Choline and DHA during the first 1000 days and children's health and development in low- and middle-income countries[1]

[1] This paper has been previously published (Bragg, M. G., Prado, E. L., & Stewart, C. P. (2021). Choline and DHA during the first 1000 days and children's health and development in low- and middle-income countries. Nutrition Reviews. https://doi.org/10.1093/nutrit/nuab050). Reprinted with permission. This version may contain small differences, as it does not include changes made during copyediting.

## 1.1 Abstract

Choline and DHA are nutrients which, when provided during the first 1,000 days from conception to age 2 years, may have beneficial effects on child neurodevelopment, as well as related health factors including birth outcomes, and child growth, morbidity, and inflammation. Because these nutrients are found mainly in animal source foods, they may be lacking in the diets of pregnant and lactating women and young children in low- and middle-income countries, potentially putting children at risk for suboptimal development and health. Prior reviews of these nutrients have mainly focused on studies from high income countries. We present a narrative review of studies describing the pre- and postnatal roles of choline, DHA, and a combination of the two nutrients on child neurodevelopment, birth outcomes, growth, morbidity and inflammation in low- and middle-income countries. Further studies are needed to understand the specific, long-term effects of perinatal choline and DHA intake across various contexts.

## 1.2 Introduction

The period from conception through the first two years after birth (termed the 'first 1000 days') is a time of rapid neurodevelopment when developmental trajectories are malleable to contextual exposures, with outcomes negatively affected by risk factors and positively affected by interventions.[1] Nearly 250 million children younger than five years in low and middle income countries (LMICs) are at risk for not reaching their developmental potential, with adverse effects on future schooling, productivity, and health.[2] Although many environmental conditions influence neurodevelopment, nutrition is a major component.[3] Nutrition also affects factors such as preterm birth, childhood stunting, morbidity and inflammation, which are common in LMICs and linked to impaired neurodevelopment.[4] Choline and docosahexaenoic acid (DHA) are two

nutrients which may influence child neurodevelopment, as well as birth outcomes, growth, morbidity and inflammation.

Both choline and DHA can be endogenously produced from precursors; however, it is unlikely that endogenous production is sufficient to meet needs, so recommended intake levels have been established for pregnant and lactating women and young children (**Table 1**).[5,6] Except DHA requirements for women, these recommendations are based on Adequate Intake (AI) levels and may over or underestimate needs. Choline recommendations, in particular, are based on few studies and do not consider neurodevelopmental outcomes. Whereas the World Health Organization developed the DHA guidelines with the FAO of the United Nations, there are no global guidelines for choline intake.

The main food sources of choline and DHA are of animal origin, such as eggs and fish. Because animal source foods may be expensive,[7] intake of choline and DHA may be limited in many LMICs. Processed foods may also provide choline as lecithin; as processed food consumption increases in LMICs, this may become a significant source. Breastmilk is a rich source of choline and DHA for young children, although concentrations of both nutrients vary based on maternal intake.[8,9] Foods contain different forms of choline (free choline, phosphocholine, glycerophospocholine, phosphatidylcholine and sphingomyelin); each form should be included in estimates of total choline intake.[10]

A lack of representative food composition estimates in national food databases limits assessment of dietary intake; however, it seems intake often falls below recommended levels in LMICs. For example, in a review that reported choline intake in 15 countries, intake among women was lowest in Mexico (263 mg/d) and highest in Sweden (374 mg/d).[10] Another study in The Gambia reported even lower intakes of choline (155.2 mg/d) among 62 non-pregnant

women of reproductive age.[11] Intake of DHA below recommendations is also common in LMICs. Using food balance sheets from 175 countries, per capita availability of DHA among low-income countries was 96 mg/d compared to 184-473 mg/d across high income countries.[12] Using similar data plus breastfeeding rates, the median DHA intake among children age 6-36 months in LMICs was estimated to be 48.7 mg/d, well below recommendations.[13] These nutritional inadequacies may put children at risk for suboptimal development, and may be exacerbated by other common health factors in LMICS. These include inadequate intake of other nutrients, such as iron, zinc, and vitamin B12, required for endogenous production of DHA and choline,[6,14] as well as conditions like gestational diabetes mellitus and an altered composition of the gut microbiota, which may affect DHA and choline availability, respectively.[15,16]

The relationship between poor intake and outcomes is clouded by limitations in assessing choline and DHA status. Plasma choline is poorly correlated with intake across a range of intake levels,[11,17] and may be affected by plasma volume expansion in pregnancy. Lipid soluble forms of choline (such as phosphatidylcholine) are influenced by fat metabolism and transport in lipoproteins. For DHA, red blood cell (RBC) concentration is a better marker of habitual exposure than plasma concentration, although sample collection and storage is more difficult.[18]

Few reviews have examined choline and DHA together. Choline and DHA are present in many of the same food sources and are hypothesized to have similar effects on neurodevelopment, especially memory and learning. Their metabolism is also linked – phosphatidylcholine molecules can incorporate DHA, as described further below – and there is evidence that these nutrients work synergistically to promote neurodevelopment.[19] Several reviews have focused on either choline or DHA, presenting evidence from predominantly high income countries.[20–23] This review presents the evidence relating choline, DHA, and a

combination of the two nutrients during the first 1000 days to children's neurodevelopment, birth outcomes, growth, morbidity and inflammation in the context of LMICs.

## 1.3 Methods

Literature searches were performed in PubMed using the terms: choline, DHA, docosahexaenoic acid, fish, egg; pregnancy, lactation, complementary feeding, formula, infant; child development, neurodevelopment, cognition, memory, vision, visual; child growth, height, weight, head circumference; preterm, birth; morbidity; diarrhea; enteropathy; inflammation; as well as a list of LMICs based on World Bank income categories in 2019-2020. Abstracts and titles were screened for inclusion. Reference lists were scanned for eligible studies.

Selected papers included observational studies or randomized controlled trials (RCTs) in LMICs. Relevant animal studies were included in discussions of mechanisms; however, only human trials were included in discussions of the evidence in LMICs. All types of interventions were eligible, including supplements, foods, or dietary advice to consume foods rich in choline and/or DHA, and studies could include co-interventions, such as arachidonic acid (ARA) or eicosapentaenoic acid (EPA). Studies published in English by August 11, 2020 were eligible.

The outcomes assessed were neurodevelopment (behavioral and physiological measures), physical growth (height, weight, head circumference, measures of adiposity), birth outcomes (preterm birth/gestational age, birth length, birth weight), morbidity (illnesses such as diarrhea, environmental enteropathy), and biochemical markers of inflammation. Visual development was included with neurodevelopmental outcomes, when available. Morbidity and inflammation were presented together due to the limited number of studies identified. Outcomes could be measured at any age; however, the initial exposure assessment must have been within pregnancy or the first

2 postnatal years. Papers were organized by the nutrient of interest (choline, DHA, or both) and the timing of exposure (prenatal, postnatal, or across both time periods).

## 1.4 Choline

### *Proposed Mechanisms*

Choline is an essential micronutrient that is important for early neurodevelopment. Rodent studies show clear improvements in lifelong memory when choline is supplemented during specific pre- and postnatal periods,[24–26] in part by altering rates of mitosis and apoptosis of neural progenitor cells in the hippocampus and the cerebral cortex.[27,28] These effects are epigenetically mediated through conversion of choline to the methyl donor betaine.[29] Betaine donates a methyl group to homocysteine to form methionine and eventually S-adenosyl methionine. These epigenetic changes may affect neurodevelopment in indirect ways, as well. For example, high maternal intake of choline decreases placental expression of cortisol stimulating genes, with potential effects on learning and memory.[30] Choline may also affect development as a precursor for phosphatidylcholine and acetylcholine. Phosphatidylcholine is a major component of cell membranes and a precursor of sphingomyelin, required for myelination of neurons, and the cell signaling molecule diacylglycerol. Acetylcholine is a neurotransmitter involved in the encoding of new memories in the hippocampus; it is also a neuromodulator that influences neurogenesis and synapse formation.[31]

Compared to neurodevelopment, there is less mechanistic evidence for choline's role in birth outcomes, child growth, morbidity and inflammation. In rodent models, prenatal choline supplementation modulates nutrient transport across the placenta, increasing choline availability and altering glucose and amino acid metabolism.[32] As a methyl donor, choline may reduce

homocysteine levels, which are associated with adverse pregnancy outcomes,[33] and increase vitamin B12 availability in pregnant women.[34] Perinatal choline may also influence bone growth and body size. Rodent knockout models without the choline kinase enzyme (which converts choline to phosphocholine) have altered bone formation,[35,36] and phosphatidylcholine is required for the production of new cell membranes. Related to morbidity and inflammation, choline supplementation in rodents reduced markers of inflammation following lipopolysaccharide administration during pregnancy.[37] Different forms of prenatal choline (for example, free choline or phosphatidylcholine) also may affect development of the offspring immune system.[38] Choline's roles outside of neurodevelopment are active areas of research.

*Choline during pregnancy*

Designs of reviewed studies

Two RCTs in LMICs have studied prenatal choline supplementation in human populations. In South Africa, heavy alcohol consumers were randomized to 2g choline per day or placebo from mid-pregnancy until delivery (n=69).[39] Although baseline plasma choline concentration was not reported, mean choline intake at baseline was below guidelines for pregnant women (~370 mg/d in both groups). A trial in Ukraine also examined the effect of choline among women who consumed alcohol during pregnancy (n= 163); however, this trial enrolled abstaining pregnant women, as well (n =204).[40,41] Women were randomized to a daily multiple micronutrient supplement (MMS) with 750 mg of choline, MMS alone, or standard of care (no provision of supplements) from the first prenatal visit till delivery. The subgroup who received choline was small (n= 19 and n=18 among alcohol consumers and abstainers, respectively). Baseline plasma choline was similar across groups (~15 µmol/L). The primary

outcome of both trials was neurodevelopment during the first year of life. One observational study in China reported associations of maternal plasma choline with birth outcomes.[42] No studies in LMICs reported on prenatal choline and infant morbidity or inflammation.

Neurodevelopment

In the South African trial, infants in the choline group had improved eye blink conditioning, an early marker of learning and memory, compared to controls at 6.5 months; however, this was only significant after removing four infants in the choline group whose mothers were considered to have poor adherence. The choline group also had significantly higher mean novelty preference scores on the Fagan Test of Infant Intelligence compared to controls at 12 months (64.5% vs 59.1%, $p<0.05$), demonstrating improved visual recognition memory. There were no effects on information processing speed at 6.5 or 12 months.[39] In the Ukrainian trial, addition of choline to MMS did not significantly affect Bayley Scales of Infant Development (BSID) II Psychomotor Development Index or Mental Development Index scores at 4-11 months.[41] However, infants in the choline group demonstrated improved encoding and memory of visual stimuli, as measured by larger and faster changes in heart rate during habituation and dishabituation tasks at 4-11 months.[40]

Together, these two studies suggest neurodevelopmental benefits in the first year from prenatal supplementation of choline doses from 750mg-2g/d, although this may be primarily generalizable to women who consume alcohol during pregnancy. Further studies are required in abstaining women in LMICs and with prolonged follow up to assess the long term effects of prenatal choline supplementation. Detecting effects of choline may depend on the neurodevelopmental assessment methods used. Assessments of attention and memory based on

eye-blink, eye movements, and heart rate may be more sensitive than assessments based on acquisition of developmental milestones, such as the BSID.

Pregnancy outcomes

In the South African trial, there was no difference between groups in mean gestational age (choline 38.8 weeks vs control 38.9 weeks) or incidence of low birth weight (LBW) (25.0% vs 32.3%), although mean birth length was non-significantly lower in the choline group (47.2 cm [SD: 3.3] vs 48.9 cm [3.7], $p < 0.1$).[39] In Ukraine, birth outcomes were compared by maternal supplementation (MMS vs standard of care; MMS with choline vs MMS alone) and alcohol consumption. Children whose mothers received the MMS with or without choline had significantly higher birth weight compared to the standard of care control group, a pattern that was evident among both those born to women who consumed alcohol during pregnancy and those who abstained. However, when contrasting the group who received MMS + choline to MMS alone, birth weight was significantly lower (-126g among drinking mothers, and -171g among abstaining mothers, $p = 0.048$).[40] In an observational study of 115 pregnant women in China, maternal plasma choline was not associated with birth outcomes, although the choline metabolite betaine was inversely associated with birth weight.[42]

The scant information available suggests additional prenatal choline may be related to smaller birth size; however, this reflects only two small trials enrolling women who consumed alcohol during pregnancy, neither of which were designed to investigate birth outcomes. Future studies should explore the link between prenatal choline supplementation and birth size in LMICs.

Child growth

In the South African trial, the control group decreased in weight, length, and head circumference z-scores over the first year; in contrast, the choline group experienced catch up growth in weight percentile and head circumference percentiles from birth to 12 months.[39] No studies reported on growth after prenatal choline supplementation among abstaining women.

*Choline from birth to 2y*

Designs of reviewed studies

No trials of early postnatal (0-2y) choline supplementation in LMICs were identified. Three observational studies reported on the association of choline and growth within this life stage in Malawi,[43] Brazil,[44] and Bangladesh.[45] No studies reported on early postnatal choline supplementation and child neurodevelopment, morbidity, or inflammation in LMICs.

Child growth

A cross-sectional study of 325 Malawian children aged 12-59 months observed a difference of 0.41 cm in height per 1 SD difference in serum choline (P<0.0001), with a larger magnitude in boys (0.60 cm) than in girls (0.19 cm).[43] Ratios of betaine:choline and TMAO:choline, representing choline conversion to metabolites, were also investigated; both ratios were negatively associated with children's height-for-age z-scores. In Brazil, urinary metabolites were measured among 326 children age 6-24 months with weight-for-age z-score (WAZ) <-2 or >-1. Children in the WAZ <-2 group had lower concentrations of urinary choline metabolites, signifying changes in choline metabolism among underweight children.[44] In a metabolomics study of 130 Bangladeshi children, sphingomyelins and phosphatidylcholine

species were positively associated with change in height-for-age z-score (HAZ) from nine months to four years.[45] Overall, observational trials in LMICs provide evidence that serum or urinary markers of choline concentration are positively associated with child growth, although stronger study designs must test this connection.

*Choline across the first 1000 days*

No trials or observational studies in LMICs have reported on the association between choline intake or plasma choline concentration spanning pre- and postnatal periods and child neurodevelopment, growth, morbidity or inflammation.

*Limitations and Future Directions*

More information is needed regarding the role of perinatal choline in LMICs on all child health outcomes. Both of the reviewed RCTs enrolled alcohol consumers, and neither assessed dose response relationships or stratified by baseline choline intake, limiting the ability to refine choline intake recommendations. Given that choline's influence on neurodevelopment is hypothesized to extend from pregnancy through complementary feeding, potentially up to year four,[25] studies of choline supplementation across this time period are needed. Future studies should also examine the effects of prenatal choline on birth size, as some studies suggest smaller length or weight after prenatal supplementation.

1.5 DHA

*Proposed Mechanisms*

DHA is a long chain polyunsaturated fatty acid (LC-PUFA) highly concentrated in brain and retinal tissues, where it influences neural and visual development. In animal models, perinatal supplementation with DHA improves performance on cognitive tests,[23] and prenatal deficiency is associated with poorer cognitive performance.[46] Comprehensive reviews of DHA's mechanisms may be found elsewhere.[22] Briefly, increased DHA promotes neural development, including formation of hippocampal synapses.[47] Phospholipid-bound DHA in retinal membranes influences visual signaling pathways by interacting with rhodopsin.[48] DHA is also a ligand for cell surface receptors such as GPR120, influencing anti-inflammatory cell signaling pathways,[49] and transcription factors, influencing gene expression in the brain.[50,51] DHA is a precursor for a myriad of anti-inflammatory metabolites, including resolvins and neuroprotectins;[52] in producing these metabolites, DHA blocks metabolism of ARA to pro-inflammatory eicosanoids, including prostaglandins and leukotrienes. Because these metabolites have important physiological functions, balance of DHA/ARA during early life seems necessary for optimal development.[53]

DHA is well known for its anti-inflammatory actions, including creation of anti-inflammatory eicosanoids, decreased production of inflammatory cytokines, and altered cell signaling.[54] These changes affect development of immune function in infants, as well.[55] Prenatal DHA supplementation is associated with a more mature infant immune system (characterized by improved oral tolerance and a more balanced T helper cell 1 (Th1)/Th2 response) in humans.[56]

LC-PUFAs including DHA are associated with longer gestation and larger birth weight,[57] perhaps due to altered production of eicosanoids involved in parturition.[58] DHA may also promote prenatal growth via changes to gene expression. Changes in methylation of genes (*IGF2/H129*) related to fetal growth and development were reported after prenatal DHA

supplementation, only among preterm infants or overweight mothers.[59] It is unclear if these changes in methylation could affect postnatal growth.

*DHA during pregnancy*

Designs of reviewed studies

Seven trials examined the effects of prenatal DHA supplementation in LMICs. Neurodevelopment and pregnancy outcomes were the primary focus of the trials identified. In Mexico, the Prenatal DHA (Omega-3 fatty acid) Supplements on Infant Growth and Development (POSGRAD) trial randomized 1,094 women to 400 mg/d algal DHA vs placebo during the second half of pregnancy.[60] This study reported a range of outcomes and was unique in supplementing DHA alone, without other LC-PUFAs. In Bangladesh, 400 women were randomized to daily fish oil (containing 1.2g DHA, the largest dose among reviewed trials) vs soy oil capsules throughout the third trimester, and neurodevelopmental, growth, and birth outcomes were reported.[61] Five RCTs reported only pregnancy outcomes, including a trial in China,[62] three trials in Iran,[63–65] and one in Egypt.[66] Of note, the trial by Ostadrahimi et al[63] is included in this discussion of pregnancy outcomes because it provided prenatal supplementation; however, supplementation continued after birth, and further discussion of this trial is included in the pre- and postnatal DHA section. This was also the only trial to report DHA status at baseline.

Seven observational studies were identified, including two in Mexico (one reporting only neurodevelopmental outcomes[67] and one reporting pregnancy outcomes, child growth, and inflammation[68]) and five in India which only reported birth outcomes.[69–73]

Neurodevelopment and visual development

In Bangladesh, there was no improvement in infant BSID-II scores at ten months after maternal supplementation with a large dose of fish oil.[61] Similarly, the POSGRAD study in Mexico reported no group differences in brainstem auditory-evoked potentials at 1-3 months, visual-evoked potentials at 3-6 months,[74] BSID-II scores at 18 months,[75] or McCarthy Scales of Children's Abilities scores at five years.[76] However, compared to control, prenatal DHA supplementation improved sustained attention at five years as measured by the percent of children scoring <40 on the omissions subtest of the Conners' Kiddie Continuous Performance Test (14.4% vs 25.7%, $p<0.0001$).[76] No association was found between DHA intake during the third trimester and brainstem auditory-evoked potentials at 1-3 months in an observational study of 76 Mexican women.[67] The scant evidence suggests little effect of prenatal DHA on neurodevelopment or visual processing in LMICs. Studies with prolonged follow up are needed to determine if the delayed benefit to attention reported in the POSGRAD trial is consistent across other studies.

Pregnancy outcomes

Of the seven RCTs which examined the effects of prenatal DHA supplementation on birth outcomes, three reported significant effects (**Table 2**[60,61,71–73,62–66,68–70]). In Iran, healthy pregnant women receiving fish oil had fewer LBW infants compared to control (0% vs 6.7%, $p=0.02$);[63] however, similar studies among Iranian women with gestational diabetes reported null effects.[64,65] In the Mexican POSGRAD study, there were no differences in birth outcomes between groups except after stratification by gravidity. Among primigravid mothers, prenatal DHA supplementation was associated with heavier babies with larger head circumference and lower risk of LBW and intrauterine growth restriction (IUGR).[60] In Egypt, women with

asymmetrical IUGR pregnancy, as measured via ultrasound, were given aspirin with or without omega-3 fatty acids for six weeks during the third trimester. The omega-3 group had greater estimated fetal weight gain during the intervention and larger birth weight at delivery compared to those who received aspirin alone.[66]

Of the six observational reports, five were from prospective studies in India which followed women through pregnancy and delivery. Two reports noted positive associations between maternal plasma or RBC DHA levels and birth size.[69,70] Two others reported lower placental DHA in preterm and LBW babies compared to term and normal weight babies,[70,72] although one found higher cord plasma DHA among LBW newborns.[73] Interestingly, an observational study in Mexico found negative associations between second trimester dietary intake of DHA, EPA and ARA, and birth weight and length.[68] The authors suggest this may be due to concomitant intake of toxins like mercury or substitution of fish in place of other animal source foods, rather than a negative effect of DHA itself.[68]

Overall, evidence suggests a positive effect of prenatal DHA on birth outcomes, especially birth weight, in LMICs. Several trials had relatively small sample sizes (4 studies, $n \leq 150$), perhaps limiting the ability to detect differences in preterm birth or gestational age, although the two largest trials ($n > 1000$)[60,62] also reported null effects on these outcomes. More research is needed to understand the context in which DHA, with or without other nutrients in fish oil, may affect birth outcomes. Effects may vary based on maternal characteristics such as gravidity and pregnancy risk; these characteristics should be recorded in future studies.

Child growth

Three studies have reported on growth outcomes in LMICs. In Bangladesh, mean weight-for-height z-scores (WHZ), WAZ, and HAZ at ten months were moderately low (-0.6 to -1.3) and not different between intervention and control groups.[61] Among primigravid mothers in the POSGRAD study, children in the DHA group were 0.7 cm longer compared to controls at 18 months (95% CI [0.1, 1.3], p=0.02).[77] This effect was lost over time, with no differences in child growth between DHA and control groups at 60 months.[78] In an observational study in Mexico, maternal intake of DHA, EPA, and ARA during the second trimester was negatively associated with child height and BMI z-score at 8-14 years.[68] In each of these studies, the relationship of DHA with postnatal growth closely mirrors the relationship found with birth size; possibly, these results simply reflect altered prenatal growth. Further studies in LMICs may help uncover relationships between DHA and postnatal growth. Considering the opposing effects on linear growth between the two Mexican studies, more information is needed on DHA's effects specific to height.

Morbidity and inflammation

In the POSGRAD trial, Mexican infants whose mothers were supplemented with DHA had fewer cold symptoms at 1 and 3 months compared to controls (37.6% vs 44.6%, $p < 0.05$ and 37.8% vs 44.1%, $p<0.05$).[79] At three months, the DHA group spent 14% less time sick than the control group.[79] Children in the DHA group also had fewer respiratory symptoms through 18 months of age, only among children whose mothers were atopic.[80] In the observational study in Mexico, there was no association between maternal DHA intake and children's C-reactive protein or other markers of metabolic risk at 8-14y.[68] Further studies in varied contexts are needed to better understand this relationship.

*DHA from birth to 2y*

Designs of reviewed studies

Eight trials provided DHA during the early postnatal period (0-2y) in LMICs. Generally, trials provided DHA either directly to breastfeeding infants or via inclusion in infant formula or total parenteral nutrition (TPN). Currently, DHA is recommended for inclusion in infant formulas;[81] however, inclusion is not required and may not occur in some LMICs.[82] Many trials focused on neurodevelopment or visual development (**Table 3**[83,84,93–96,85–92]), including three from Turkey,[84,87,88] and one each from Taiwan,[86] The Gambia,[89] Ethiopia,[83] and Egypt.[85] Of these trials, two provided fish oil directly,[83,89] two provided fish oil via TPN,[84,87] and three supplemented infant formula with DHA, alone[88] or with ARA.[85,86] Additionally, a trial in Malawi reported on gut permeability and growth after supplementation with a micronutrient powder and fish oil.[97] Half of the trials[83,85,89,97] reported DHA status at baseline.

Ten observational studies described DHA in plasma, RBCs, lipid emulsion or breastmilk and neurodevelopment or growth outcomes across a range of LMICs.

Neurodevelopment and visual development

Of 7 studies that measured visual development, only two reported a significant relationship between DHA and visual development (**Table 3**). A trial in Turkey reported that addition of fish oil to TPN emulsions reduced risk for retinopathy of prematurity among very low birth weight (VLBW) preterm infants.[84] However, null results were reported in three similar studies of preterm infants on TPN in Turkey and Iran.[87,91,96] In Argentina, malnourished infants who consumed standard formula had poorer retinal response to light stimuli compared to those

who consumed LC-PUFA supplemented formula or breastmilk;[95] however, the study was small (n = 28), observational, and did not correct for potentially confounding factors, such as socioeconomic status or maternal education. The one study involving healthy, term children found null associations between plasma, RBC, or breastmilk DHA concentrations and visual development in Cuba.[93]

Of 8 studies reporting neurodevelopmental outcomes, three RCTs and two observational studies reported significant results (**Table 3**). Among RCTs, the 3 that supplemented infant formula with DHA, with or without other fatty acids, reported significant improvements in neurodevelopment.[85,86,88] Both RCTs which directly supplemented breastfeeding infants or lactating women reported null results.[83,89]

An observational study in Indonesia found that although genotype of the *FADS* gene cluster, involved in endogenous production of LC-PUFAs, was not related to the BSID-II Mental Development Index at 12-17 months, the plasma DHA:EPA ratio was positively associated with this score.[90] In Tanzania, RBC DHA was positively associated with movement patterns at 10-20 weeks of age.[94] However, there was no association between RBC DHA in infancy and neurodevelopment at 5 years in Nepal.[92]

Overall, there is little evidence that DHA supplementation improves visual or neural development for healthy, breastfeeding children in LMICs. However, benefits to visual development were seen among malnourished or hospitalized infants, and there is supportive evidence for including DHA in infant formula. There may be a relationship between plasma or RBC DHA and neurodevelopment, limited to specific populations or developmental domains.

Child growth

Seven studies in LMICs included measures of child growth. Among trials, changes in body composition and adiposity were commonly noted. In The Gambia, infants who received fish oil had larger mid-upper arm circumference (MUAC)-for-age and triceps skinfold thickness-for-age compared to controls.[89] In Ethiopia, fish oil provision to breastfeeding infants, but not to lactating women, was associated with increased monthly WHZ gains compared to control.[98] In a Malawian trial, children age 12-35 months who received micronutrient powder with fish oil gained more weight over 24 weeks compared to control (1.3 kg vs 1.1 kg, p=0.01).[97] There was no difference in linear growth, and no other anthropometrics were reported. In Taiwan, a trial of preterm infants found no differences in child height, weight, or head circumference with DHA supplemented versus traditional formula; no measures of body composition were reported.[86]

Three observational studies have reported a relationship between DHA in serum or breastmilk and child height and weight; none included other anthropometric indices. In Malawi, serum DHA and ARA concentrations were positively associated with HAZ among 400 Malawian children age 12-59 months.[99] In a small sample in China (n=41), breastmilk DHA was positively related to postnatal length gain at one month (r=0.83) and three months (r=0.76; both $p<0.01$) and weight gain at three months (r=0.46, $p<0.05$).[100] In the Congo and Burkina Faso, children's monthly weight gain from birth to five months was examined in association with breastmilk fatty acid content. Monthly weight gain decreased as the n-6:n-3 ratio increased until a cutoff of 15:1, at which point weight gain remained at a steady low.[101] Although not specific to DHA, it suggests that a substantial intake of omega 3 fatty acids is needed among lactating women with high intake of n-6 rich oils to optimize child weight gain.

Although observational studies in LMICs have noted links between DHA and child length and weight, RCTs more commonly report effects on body composition and adiposity. Few

trials included supplementation of preterm infants or lactating women; future studies should investigate these populations and include a variety of anthropometric measures.

### Morbidity and inflammation

Five studies reported on morbidity or inflammation related to postnatal DHA in LMICs. In Ethiopia, prevalence of inflammation (based on elevated C-reactive protein levels) and morbidity was not different between groups after supplementing lactating women or infants with fish oil versus control; the authors suggest this may be due to the low prevalence of inflammation and morbidity in this study compared to others.[98] In Malawi, all participants had high (>0.1) lactulose:mannitol ratios at baseline, reflecting increased gut permeability, and there was no difference among children who received micronutrient powder with or without fish oil for 24 weeks compared to control.[97] Similarly, in The Gambia, the average lactulose:mannitol ratio was 0.22, and nearly half of children had elevated C-reactive protein levels; children who received fish oil had no differences in lactulose:mannitol ratio, inflammatory markers, or morbidity compared to controls.[89] In Turkey, there were no changes in pro- or anti-inflammatory cytokine levels among preterm children randomized to receive fish oil versus standard lipids in TPN. However, there was a lower prevalence of bronchopulmonary dysplasia in the fish oil group, and total antioxidant capacity was higher after 7 days, but not 14 days, of treatment.[87] In a similar study in Turkey, provision of fish oil did not reduce morbidity or mortality, and total antioxidant capacity was higher in the fish oil group compared to controls, but this difference disappeared after treatment ended.[96] Overall, there is little support for an effect of postnatal DHA on inflammation, gut permeability or morbidity in LMICs.

*DHA across the first 1000 days*

Only one study has described pre- and postnatal DHA provision in a LMIC. In Iran, 150 women were randomized to receive fish oil or liquid paraffin placebo from 20 weeks gestation to 30 day postpartum.[102] Pregnancy outcomes are reported in Table 3. Developmentally, there were no differences across the five domains of the Ages and Stages Questionnaire at four or six months, except higher communication scores in the fish oil group at four months.[102] No differences in infant length, weight, or head circumference were noted between groups from birth to six months.[102] Morbidity and inflammation were not reported.

*Limitations and Future Directions*

The literature in LMICs suggests positive effects of prenatal DHA supplementation on birth weight and morbidity, with a potential delayed benefit to attention at age 5 years. Additionally, studies in LMICs support the addition of DHA to infant formula for improved neurodevelopment. Across life stages, conclusions have been limited by variations in dose, timing, vehicle, context and co-interventions. The effects of DHA may vary with baseline DHA status; however, few trials reported this information. Many trials also lacked endline measures of status, relying on maternal report or pill counts for adherence data. Future trials should explore pre- and postnatal supplementation, including to preterm infants or lactating women.

## 1.6 Choline and DHA

*Proposed Mechanisms*

Beyond the individual effects of choline and DHA, the two may work together to improve neurodevelopment.[19] Among malnourished pigs, addition of dietary DHA, methyl donor

nutrients including choline, or both attenuated losses in fetal brain weight compared to controls.[103] Combined choline and DHA administration decreases brain inflammation[104] and oxidative stress[105] in mouse models. In fact, these nutrients may work synergistically: offspring of dams supplemented with choline and DHA had more hippocampal neurons compared to those given either nutrient alone.[106]

This synergy reflects the interconnected nature of choline and DHA metabolism. Phosphatidylcholine incorporates DHA via the phosphatidylethanolamine N-methyltransferase pathway and is the main carrier of DHA in plasma, including among preterm infants.[107] Lysophosphatidylcholine-DHA is the main form of DHA transported into the brain and eye, via the Mfsd2a transporter.[108] Maintenance of phosphatidylcholine-DHA levels is important for neural progenitor cell proliferation.[109] Additionally, choline and DHA affect each other's transport and metabolism. Prenatal choline supplementation increases placental transcript abundance of DHA transporters in mice.[32] Likewise, DHA increases choline uptake in retinal cells[110] and stimulates production of acetylcholine in cultured cholinergic cells.[111] When choline and DHA are provided together, circulating levels of each nutrient increase more than when provided separately.[112,113] Although this synergy has been linked to improved neurodevelopment, its relationship with birth outcomes, growth, morbidity and inflammation is unclear.

*Choline and DHA during pregnancy*

No studies specific to prenatal choline and DHA were identified in LMICs. Several studies of fish or egg intake were identified and included in this review, as both are sources of choline and DHA. Although typically studied for its omega 3 fatty acids, fish also contains choline. (Fish oil, on the other hand, does not.) Eggs also provide these nutrients, although the

DHA content varies. Both provide other food components as well, including neuroprotective factors like iodine or iron and toxins like mercury; to understand the unique effects of choline and DHA, studies specific to these nutrients are needed.

Several observational studies have reported a link between maternal fish or egg consumption and birth outcomes in LMICs. Prospective cohort studies in Iran,[114,115] Turkey,[116] and India[117] link increased fish intake during pregnancy to decreased odds of low birth weight, although one study in India found the opposite relationship.[118] Risk for preterm birth was also inversely related to fish consumption in Iran[114,115] and Pakistan.[119] Maternal consumption of eggs was positively associated with birth weight in Iran and India.[115,118] No studies reported on neurodevelopment, child growth, morbidity or inflammation.

*Choline and DHA from birth to 2y*

Designs of reviewed studies

Although no studies in LMICs have examined postnatal choline and DHA directly, nine studies investigated foods containing choline and DHA along with other nutrients. Three RCTs compared supplements fortified with choline, DHA, and other nutrients to traditional supplemental foods and non-supplement controls in Guinea-Bissau, South Africa, and Cambodia.[120–122] Two RCTs examined the provision of one egg per day during the early complementary feeding period (6-15 months) versus a nonintervention control in Ecuador ("The Lulun Project") and Malawi ("The Mazira Project").[123,124] One study in China compared the effects of nutrition education including recommendations to provide daily egg yolks as an infant's first food versus a nonintervention control on children's growth.[125] Only one trial

presented baseline measures of choline and DHA status, in a separate article.[126] An observational study in Haiti examined neurodevelopment,[127] and two in India and Zambia studied growth.[128,129]

Neurodevelopment

In Guinea-Bissau, children <4 years had improved working memory and better cerebral blood flow with consumption of a supplement containing DHA, choline, and other nutrients compared to a traditional meal, but there was no difference compared to a common fortified food (Corn Soy Blend++).[120] No other domains of development were measured. In the South African trial, a fortified small-quantity lipid-based nutrient supplement ('SQ-LNS-plus') was associated with improved locomotor development compared to controls as measured by the Kilifi Developmental Inventory at 12 months.[121] The standard SQ-LNS was not different from controls, suggesting the additional nutrients were responsible for these findings. In the Mazira Project in Malawi, daily egg consumption did not affect children's memory, attention, language, or personal social scores, but there were fewer children with delayed fine motor development compared to controls (prevalence ratio [95% CI]: 0.59 [0.38-0.91]).[130] Children's egg intake was also associated with motor but not language development in an observational study of 583 infants in Haiti; other developmental domains were not measured.[127]

Together, the limited evidence from LMICs suggests a benefit to neurodevelopment, especially motor development, from postnatal intake of choline and DHA containing foods. No studies provided these nutrients to lactating women; this may be an area for future research.

Child growth

Eight studies reported on child growth in LMICs (**Table 4**[120–126,128,129,131]**)**. In Guinea-Bissau, the fortified supplement was associated with decreased WAZ, BMI-for-age, fat tissue accretion and increased lean tissue accretion compared to the corn soy blend among children <4y.[120] In Cambodia, consumption of a novel ready-to-use supplemental food with choline, DHA, and other nutrients was associated with increased MUAC compared to nonintervention controls, but there were no differences in HAZ, WAZ, or WLZ.[122]

Several trials noted an effect on linear growth. In South Africa, HAZ was higher in the SQ-LNS-plus group compared to controls at eight and ten months, but not twelve months; the standard SQ-LNS group was not different from controls.[121] Large increases in HAZ (effect size [95% CI]: 0.61 [0.45,0.77]) and WAZ (0.61 [0.37, 0.77]), as well as increases in WHZ and BMI-for-age, were noted after egg provision in Ecuador;[123] however, these effects were absent two years later, suggesting a longer intervention may be needed to sustain benefits.[132] In Malawi, despite a similar study design, no effects on HAZ, WAZ, or WHZ were reported after egg provision, although head circumference-for-age was higher in the intervention group.[124] This difference in response may be due to the high rates of fish consumption in Malawi;[124] perhaps eggs improve growth only in the absence of choline and DHA containing foods in the usual diet. Baseline stunting rates were also lower in the Malawi study (14%) compared to the one in Ecuador (38%).[123,124] In China, 12 month old children in townships where eggs were recommended for child feeding had larger WAZ and HAZ, but not weight-for-height z-score, compared to control townships.[125] However, these townships were not randomly selected and received additional messages about other health practices, such as breastfeeding. In India and Zambia, non-consumption of eggs and fish, respectively, among children age 6-23 months was associated with increased risk of stunting.[128,129] Overall, these studies suggest a beneficial effect

of foods containing choline and DHA on child growth in LMICs, perhaps limited to certain contexts.

Morbidity and inflammation

In South Africa, the SQ-LNS-plus group had decreased longitudinal prevalence of fever, coughing, and wheezing, and increased longitudinal prevalence of diarrhea, vomiting, and rashes/sores compared to control. These effects were not specific to choline and DHA, as the standard SQ-LNS group had similar results.[121] In the Lulun Project in Ecuador, prevalence of diarrhea in the past seven days was higher in the egg group than control; however, the data were from parental report which the authors speculate may have been biased.[123] The Mazira Project has not yet reported child morbidity outcomes. No trial has reported on inflammation.

*Choline and DHA across the first 1000 days*

No trials in LMICs have reported on pre and postnatal relationships between choline, DHA and child neurodevelopment, growth, morbidity or inflammation.

*Limitations and Future Directions*

The literature on perinatal choline and DHA in LMICs is sparse, and no studies assessed the effects of choline and DHA independently of other nutrients. When possible, the specific effects of these nutrients, independent of other dietary factors, should be assessed. Postnatal choline and DHA doses were generally below recommendations; however, improvements to neurodevelopment and growth were evident even at these levels. Given these promising findings, further trials in diverse contexts should be prioritized.

## 1.7 Discussion and key questions

Overall, limited data suggests improvements in child development, birth outcomes, growth, morbidity and inflammation related to perinatal provision of choline, DHA, and a combination of the two nutrients in LMICs. There is evidence to suggest that supplementation with these nutrients may be beneficial for pregnant and lactating women and young children. However, more research is needed to address the following questions.

*What are the specific, long-term effects of choline and DHA during early life in LMICs?*

Additional studies are required to understand the effects of varying doses of choline and/or DHA on child health in LMICs. Future trials should use high quality physiological measures of child development, such as eye movement response time and heart rate, and accurate biomarkers. Measures such as eye tracking have been shown to be feasible in LMICs,[133] but may require more funding and training than assessments based on acquisition of developmental milestones. Accurate biomarkers of intake and status will be required, across all settings, for better measurement of exposure and understanding of biological effects. Controlled feeding trials with varying dosages in multiple arms, although challenging, would provide high quality evidence and are lacking in LMICs. Studies with prolonged follow up are needed to understand the long-term impacts on health and productivity.

*In what settings would choline and DHA supplementation be beneficial?*

Although intake of choline and DHA is thought to be low in many LMICs, this is not the case in all settings. Coastal populations may have substantial intake of fish, regardless of

income.[12] A useful example of this concept is a comparison between the Mazira and the Lulun Projects. Both trials provided eggs to young children in LMICs, but the results on child growth were strikingly different between populations.[123,124] The investigators have suggested several possible reasons for this contrast, including differences in background fish intake (high intake near Lake Malawi; low intake in highland Ecuador).[124] Indeed, in Malawi, breastmilk DHA concentrations among women living near the lake are higher than the global average.[134] Especially in areas with adequate intake of animal source foods, choline and DHA may not be limiting nutrients for children's growth and development. There is a need for more information on population choline and DHA status as well as usual dietary intake. Incorporation of choline and DHA into national nutrition monitoring systems and food composition databases is needed to inform future interventions. Databases should include the five chemical forms of choline, which may have variable effects on children's health,[38] as well as betaine, a separate dietary component that may have a choline-sparing effect and is worthy of further research.

*How might choline and DHA fit into local, sustainable, and affordable diets?*

Considering the perinatal benefits of choline and DHA, efforts to increase maternal and infant intake of these nutrients are needed in LMICs. Breastmilk is a good source of these nutrients, and should be recommended as the only food for infants up to six months; however, the concentrations in breastmilk vary by maternal diet,[8,9] and complementary food sources of these nutrients are needed after six months. The main food sources of these nutrients are often relatively expensive, and there are concerns about sustainability and environmental issues related to their production. Alternative food products, such as biofortified foods, may be needed to meet global maternal and infant needs affordably and sustainably.

Where food sources are unavailable or inappropriate, supplementation is an option. Choline is required and DHA is recommended for inclusion in infant formula,[81] and choline is recommended in prenatal supplements,[135] but products meeting these recommendations may not be available or affordable in LMICs. Choline is supplemented as choline salts, such as choline bitartrate, or phosphatidylcholine. DHA is often supplemented as either fish oil or algal oil. Krill oil contains DHA linked to phospholipids including phosphatidylcholine and has similar bioavailability to fish oil; however, it is expensive and has similar sustainability constraints.[136]

## 1.8 Conclusion

More research is needed on the role of choline and DHA during the first 1,000 days on child outcomes in LMICs. Dose response trials are necessary to refine nutrient intake requirements, and measures of population status should be incorporated into national nutrition programs. This would enable better monitoring of global dietary adequacy as well as improved formulation of fortified or supplementary foods. At this time, adequate intake of foods rich in choline and DHA should be recommended for pregnant and lactating women and their young children, including breastmilk for infants.

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Table 1.1 – Recommended intake levels for choline and docosahexaenoic acid in pregnant or lactating women and young children

| | Choline[a] | DHA[b] |
|---|---|---|
| Pregnant women | 450 mg/d | 200 mg/d |
| Lactating women | 550 mg/d | 200 mg/d |
| Infants 0-6 months | 125 mg/d | 0.10-0.18% of total energy |
| Infants 7-12 months | 150 mg/d | 10-12 mg/kg |
| Children 1-2 years | 200 mg/d | 10-12 mg/kg |

[a] Adequate Intake levels set by the United States Institute of Medicine[5]

[b] Average nutrient requirement levels (for women) and Adequate Intake levels (for infants and children) set by the FAO of the United Nations/World Health Organization [6]

Table 1.2 – Studies describing the role of prenatal docosahexaenoic acid (DHA) on pregnancy outcomes in low- and middle-income countries[a]

| Study | Participants | Type of exposure | Timing of exposure measurement | Pregnancy outcomes | Results |
|---|---|---|---|---|---|
| Randomized controlled trials | | | | | |
| *Ali et al. (2017)*[66] | 80 pregnant women in Egypt with asymmetric IUGR | Omega 3 capsules (1g fish oil) + aspirin (n=40) vs aspirin alone (n=40) | From gestational weeks 28-30, for 6 weeks | Gestational age; birth weight | Increased birth weight in the group receiving omega 3s with aspirin (2022 [SD 25] vs 2324 [SD 19] g, p<0.01). |
| *Jamilian et al. (2016)*[64] | 56 pregnant women in Iran with gestational diabetes | Omega 3 pearls (120mg DHA, 180mg EPA) (n=27) vs placebo (n=27) | From gestational weeks 24-28, for 6 weeks | Gestational age; birth weight, length, head circumference | No significant differences between groups. |
| *Olsen et al. (2019)*[62] | 5118 pregnant women in China | 2g fish oil (n=1706) vs 0.5g fish oil (n=1695) vs placebo (n=1717) | Gestational weeks 16-24 till gestational week 37 | Gestational age; preterm birth, early preterm birth, early term birth | No significant differences between groups. |
| *Ostadrahimi et al. (2017)*[63] | 150 pregnant women in Iran | Fish oil (120mg DHA, 180mg EPA, 400mg ALA)(n=75) vs liquid paraffin placebo (n=75) | Gestational week 20 till delivery | Gestational age; preterm birth; birth weight, length, and head circumference; LBW | Fewer LBW infants in the fish oil group compared to placebo (0% vs 6.7%, p=0.02). |
| *Ramakrishnan et al. (2010)*[60] | 1094 pregnant women in Mexico | 400mg algal DHA capsules (n=547) vs placebo (n=547) | Gestational weeks 18-22 till delivery | Gestational age; preterm birth; birth weight, length, and head circumference; IUGR | Among primigravid mothers, birth weight was 99.4g (95% CI 5.5-193.4g) higher, head circumference was 0.5cm (95% CI 0.1-0.9cm) larger, and risk of LBW and IUGR were lower in the DHA group. |

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| *Razavi et al. (2017)*[65] | 120 pregnant women in Iran with gestational diabetes | Randomized 1:1:1:1 to omega 3 capsules (240mg DHA, 360mg EPA), 50,000 IU vitamin D, both, or control | From gestational weeks 24-28, for 6 weeks | Gestational age; preterm birth; birth weight, length, head and circumference | No significant differences between groups. |
| *Tofail et al. (2006)*[61] | 400 pregnant women in Bangladesh | 4g fish oil (1.2g DHA, 1.8g EPA) (n=200) vs soy oil placebo (n=200) | Gestational week 25 till delivery | Gestational age; preterm birth; birth weight, length, head circumference | No significant differences between groups. |
| Observational studies | | | | | |
| *Al-Hinai et al. (2018)*[68] | 236 pregnant women in Mexico | Intake of fatty acids | Median 13.0 weeks gestation;<br>24.7 weeks gestation;<br>37.0 weeks gestation | Birth weight and length; gestational age | Second trimester DHA intake was negatively associated with birth weight (-0.07kg per SD [95% CI -0.12, -0.02]) and length (-0.34cm [95% CI -0.59, -0.09]). |
| *Dhobale et al. (2011)*[72] | 102 pregnant women in India, categorized into preterm or term | Placental fatty acids | At delivery | Preterm vs term; birth weight, length, head and chest circumference | Placental DHA was lower in the preterm group than in the term group (2.05 [SD 0.97] vs 3.19 [SD 0.94] g/100 g fatty acids, $p<0.01$). |
| *Kilari et al. (2011)*[73] | 235 pregnant women in India, categorized into LBW or NBW | Maternal and umbilical plasma and RBC fatty acids | At delivery | LBW vs NBW | Higher cord plasma DHA in LBW group ($p=0.022$). Among females, lower plasma and RBC DHA in the LBW group ($p=0.031$). |

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| *Meher et al. (2016a)*[70] | 111 pregnant women in India, categorized into LBW or NBW | Maternal and umbilical plasma and RBC fatty acids | 16-20 weeks gestation; 26-30 weeks gestation; At delivery | LBW vs NBW; birth weight, length, head and chest circumference | Positive associations between maternal RBC DHA levels at 16-20 weeks and birth weight (r=0.222, p=0.025), and maternal RBC DHA at delivery and baby head circumference (r=0.241, p=0.027). |
| *Meher et al. (2016b)*[71] | 78 pregnant women in India, categorized into LBW or NBW | Placental fatty acids | At delivery | LBW vs NBW; birth weight, length, head and chest circumference; gestational age | Placental DHA was lower in the LBW group compared to NBW (2.18 [SD 0.56] vs 2.53 [SD 0.78] g/100g fatty acid, p = 0.032). Positive association between placental DHA and birth weight (r=0.325, p=0.011). |
| *Wadhwani et al. (2015)*[69] | 109 pregnant women in India | Maternal and umbilical plasma fatty acids | 16-20 weeks gestation; 26-30 weeks gestation; At delivery | Birth weight, length, head and chest circumference | Positive association between maternal plasma n-3 fatty acids at 16-20 weeks gestation and baby chest circumference (r=0.236, p<0.05). |

[a] ALA – alpha-linolenic acid; IUGR – intrauterine growth restriction; LBW – low birth weight; NBW – normal birth weight

Table 1.3 – Studies describing the role of postnatal DHA on neurodevelopment and visual development in low- and middle-income countries[a]

| Study | Participants | Type of exposure | Timing of exposure | Developmental measure(s) | Timing of measurement | Results |
|---|---|---|---|---|---|---|
| Randomized controlled trials | | | | | | |
| *Argaw et al. (2019)*[83] | 360 mother-child dyads in Ethiopia | Randomized 1:1:1:1 to maternal intervention (fish oil [215mg DHA, 285mg EPA]), infant intervention (169mg DHA, 331mg EPA), both, or control | Starting at 6-12 mos old, for 1 year | Culturally adapted Denver II Developmental Screening Test (Denver II-Jimma);<br>Ages and Stages Questionnaire: Social Emotional domain | 18-24 mos old | No significant difference across groups. |
| *Beken et al. (2014)*[84] | 80 VLBW preterm infants (<32 weeks gestation) in Turkey | SMOFlipid[b] (n=40) vs standard lipid emulsion (n=40) | Birth till weaning from TPN (mean age 14 days) | Retinopathy of prematurity diagnosis;<br>need for laser photocoagulation of the retina | Birth till hospital discharge (mean age 34 days) | Control group had higher odds of retinopathy of prematurity compared to the group receiving SMOFlipid (OR 9.1 [95% CI 1.9-43.8]). No difference between groups in need for laser photocoagulation. |
| *El-khayat et al. (2007)*[85] | 42 term infants in Egypt with WHZ <-2 | Infant formula supplemented with 0.01g/100 mL DHA, 0.02g/100 mL ARA (n= 21) vs standard formula (n=21) | Starting at 6-25 mos old, for 8 weeks | BSID II (MDI and PDI scores) | Baseline (6-25 mos old);<br>Endline (8-27 mos old) | Larger mean change in MDI and PDI scores in the supplementation group vs controls. Positive correlations between plasma DHA and MDI (r=0.52) and PDI (0.50, both p<0.05). |

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| *Fang et al. (2005)*[86] | 27 preterm infants (30-37 weeks gestational age) in Taiwan | Infant formula supplemented with 0.05% DHA, 0.10% ARA (n=16) vs standard formula (n=11) | Birth till 6 mos old | Visual acuity: visual evoked potentials, Lea grating acuity cards, Hiding Heidi low contrast "FACE" cards; BSID (MDI and PDI scores) | 4 mos old;<br>6 mos old;<br>1 year old; | No significant differences in visual acuity measures between groups. There was a significant difference in MDI and PDI scores between groups via repeated measures ANOVA, with higher scores in the supplemented group. |
| *Ozkan et al. (2019)*[87] | 89 preterm infants (<32 weeks gestation) in Turkey | SMOFlipid[b] (n=42) vs standard lipid emulsion (n=47) | Birth till weaning from TPN (mean age 13 days) | Retinopathy of prematurity diagnosis | Birth till hospital discharge (mean age not provided) | No significant difference between groups. |
| *Unay et al. (2004)*[88] | 54 term newborns in Turkey who received formula, + 26 breastfeeding control infants | Infant formula supplemented with 0.5g DHA/100g lipids (n=28) vs standard formula (n=26) vs breastmilk (n=26) | Birth till 16 weeks old | Brainstem auditory evoked potentials (absolute wave and interpeak latencies describe response to auditory stimuli) | 1 week old;<br>16 weeks old | All latencies decreased from birth to 16 weeks; the group receiving standard formula had smaller decreases than the DHA-supplemented or breastfed groups ($p<0.05$ for all). |
| *van der Merwe et al. (2013)*[89] | 183 infants in The Gambia | Fish oil containing 200mg DHA, 300mg EPA (n=92) vs olive oil placebo (n=91) | Starting at 3 mos old, for 6 mos | Willatt's Infant Planning Test; Toddler attention assessment | 1 year old | No significant difference between groups. |
| Observational studies | | | | | | |

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| *Fahmida et al. (2015)*[90] | 240 children of Sasak ethnicity in Indonesia, categorized by genotype | FADS genotype (involved in LC-PUFA production); Child plasma fatty acids | 12-17 mos old | BSID II (MDI score) | 12-17 mos old | Genotype was not significantly associated with MDI score; however, the log DHA:EPA ratio was associated with MDI score (beta 1.75 [95% CI 0.08, 3.41]). |
| *Gharehbaghi et al. (2020)*[91] | 341 preterm infants (<2000g, <34 weeks gestational age) in Iran | SMOFlipid[b] vs standard lipid emulsion | Birth till weaning from TPN (mean age 14 days) | Retinopathy of prematurity diagnosis | Birth till final follow up (age not provided) | No significant difference between groups. |
| *Henjum et al. (2018)*[92] | 320 infants in Nepal | Infant RBC fatty acids | 2-11 mos old | Ages and Stages Questionnaire (ASQ)-3; NEPSY II subtests | 5 years old | No significant association between RBC DHA and neurodevelopmental scores. |
| *Krasevec et al. (2002)*[93] | 56 infants in Cuba | Infant and maternal plasma and RBC fatty acids; breastmilk fatty acids | 2 mos old | Visual acuity measured via Teller acuity cards | 2 mos old | No significant associations between visual acuity scores and fatty acid concentrations. |
| *Luxwolda et al. (2014)*[94] | 97 infants from three tribes in Tanzania + 15 Dutch infant controls | Tribal fish intake level (low, intermediate, high); Infant RBC fatty acids | 10-20 weeks old | General movement quality measured via Assessment of Motor repertoire | 10-20 weeks old | Children in the high fish intake tribe had improved observed movement patterns compared to Dutch controls; no difference between tribes. RBC-DHA was associated with observed |

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| | | | | | | movement patterns score (beta 0.304 [95% CI 0.061, 0.547]). |
| *Marin et al. (2000)*[95] | 28 term moderately underweight (WAZ between -2 and -3) infants in Argentina | LC-PUFA supplemented formula vs standard formula vs breastmilk; Infant RBC fatty acids | 45-90 days old | Full field flash electroretinography (the b wave latency describes retinal response to light stimuli) | 45-90 days old | Standard formula group had longer b wave latencies (mean ± SD: 73.8 ± 7.4 milliseconds) compared to LC-PUFA or breastmilk groups (52.0 ± 5.4 and 51.3 ± 1.0). Correlation between infant RBC DHA and b wave latency ($r^2 = 0.96$, $p<0.0001$). |
| *Unal et al. (2018)*[96] | 227 VLBW preterm infants (25-32 weeks gestational age) in Turkey | SMOFlipid[b] vs standard lipid emulsion | Birth till weaning from TPN (mean age 7 days) | Retinopathy of prematurity diagnosis | Birth till hospital discharge (mean age 45d [fish oil] and 48d [control], p=0.317) | No significant difference between groups. |

[a]ARA – arachidonic acid; BSID – Bayley Scales of Infant Development; LC-PUFA – long chain polyunsaturated fatty acids; MDI – Mental Developmental Index; PDI – Psychomotor Developmental Index; SNP – single nucleotide polymorphism; TPN – total parenteral nutrition; VLBW – very-low-birth-weight; WAZ – weight-for-age z-score; WHZ – weight-for-height z-score

[b] In contrast to standard emulsions, SMOFlipid (Fresenius Kabi) adds fish oil, medium-chain triglycerides and higher levels of α-tocopherol.

Table 1.4 – Studies describing the role of postnatal foods containing choline and DHA on child growth in low- and middle-income countries[a]

| Study | Participants | Type of exposure | Timing of exposure | Growth measure(s) | Timing of measurement | Results |
|---|---|---|---|---|---|---|
| Randomized controlled trials | | | | | | |
| *Borg et al. (2020)*[122] | 485 children from 28 clusters in Cambodia | Fish-based RUSF (n=128) vs CSB++ (n=123) vs micronutrient powder (n=107) vs control (n=127) | Starting at 6-11 mos old, for 6 mos | HAZ, WAZ, WHZ; MUAC | Baseline (6-11 mos of age); Endline (12-17 mos of age) | The fish-based RUSF group had higher MUAC (0.04cm, 95%CI 0.01 - 0.06) compared to control, but was not different from the CSB++ or micronutrient powder groups. |
| *Guldan et al. (2000)*[125] | 495 children from four townships[b] | Nutrition education including recommendation of egg yolks for infants (n=250) vs control (n=245) | 1 yr intervention aimed at pregnant women and infants <1y | HAZ, WAZ | Endline (measured infants age 4-12 months only) | HAZ (-1.32 vs -1.96, p=0.022) and WAZ (-1.17 vs -1.93, p=0.004) were higher in the nutrition education townships compared to controls, only among 12 month old children. |
| *Iannotti et al. (2017)*[123] | 163 infants in Ecuador | One egg per day (143.6mg choline, 30mg DHA;[c] n=83) vs control (n=80) | Starting at 6-9 mos old, for 6 mos | HAZ, WAZ, WHZ, BMIz; stunting and underweight | Baseline (6-9 mos old); Endline (12-15 mos old) | The egg group had increased HAZ, WAZ, WHZ, and BMIz compared to controls. Lower prevalence of stunting and underweight in the egg group compared to controls. |
| *Roberts et al. (2020)*[120] | 1059 children in Guinea-Bissau | 'NEWSUP'[d] (22.1mg choline, 534mg n-3 fatty acids; n= 368) vs CSB++ (n= 350) vs traditional rice meal (n=341) | Starting at 15 mos to 7 years old, for 23 weeks | HAZ, WAZ, BMIz; MUAC; lean tissue area; fat tissue area | Baseline (15 mos – 7y old); Endline (~20 mos-7.5y old) | Among children <4y, the group receiving NEWSUP had decreased WAZ, BMI-for-age, fat tissue area, and increased lean tissue area compared to the corn soy blend. Compared to control, WAZ and MUAC were decreased. |

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| *Smuts et al. (2019)*[121] | 750 infants in South Africa | 'SQ-LNS-plus' (7.8mg choline, 75mg DHA; n=250) vs SQ-LNS (n=250 ) vs control (n=250) | Starting at 6 mos old, for 6 mos | HAZ, WAZ, WHZ; MUAC; head circumference | 8 mos old; 10 mos old; 12 mos old; | Compared to control, the SQ-LNS-plus group had higher HAZ at 8 mos (effect size [95% CI]): 0.11 [0.01, 0.22]) and 10 mos (0.16 [0.04, 0.27]), but not 12 mos (0.09 [-0.02, 0.21]). |
| *Stewart et al. (2019)*[124] | 660 infants in Malawi | One egg per day (126mg choline, 40mg DHA,[c] n=331) vs control (n=329) | Starting at 6-9 mos old, for 6 mos | HAZ, WAZ, WHZ, HCAZ; stunting, underweight, wasted, small head size | Baseline (6-9 mos old); Endline (12-15 mos old) | No difference in growth between groups except improved HCAZ (adjusted mean difference [95% CI]: 0.12 (0.49,1.42) and lower prevalence of small head size in the egg group compared to control. |
| Observational studies | | | | | | |
| *Aguayo et al. (2016)*[129] | 2561 children in India | Feeding practices, including consumption of eggs | 0-23 mos old | HAZ; stunting status | 0-23 mos old | Children age 6-23 months who did not consume eggs had increased odds of stunting after adjustment (OR [95%CI]: 2.073 [1.191-3.606]). |
| *Marinda et al. (2018)*[128] | 714 children in Zambia | Feeding practices, including consumption of fish | 6-59 mos old | HAZ, WAZ, WHZ | 6-59 mos old | Among children age 6-23 months, there was a positive correlation between fish consumption and HAZ (r=0.139, p=0.008). |

[a] CSB++ – corn soy blend++; HAZ – height-for-age z-score; HCAZ – head circumference-for-age z-score; MUAC – mid-upper arm circumference; RUSF – ready-to-use supplementary food; SQ-LNS – small-quantity lipid-based nutrient supplements; WAZ – weight-for-age z-score; WHZ – weight-for-height z-score

[b] Townships were not randomly selected.

[c] Nutrient values were presented in separate manuscripts for the Lulun[126] and Mazira[131] Projects

[d] 'NEWSUP' was a novel food supplement fortified with choline, DHA, and other nutrients including polyphenols, chromium, and molybdenum

# Chapter 2: Plasma choline concentration after a six month egg intervention among 6-9 month old Malawian children: results from a randomized controlled trial

## 2.1 Abstract

Background: Eggs are a rich source of choline, an essential nutrient important for child growth and development. In a randomized trial of one egg/day among young children in Ecuador, the egg intervention led to significant improvements in growth, which were partially mediated by increased plasma choline concentration. A similar trial in Malawi (clinicaltrials.gov: NCT03385252) found little improvement in child growth or development.

Objective: We aimed to evaluate the effect of one egg/day for 6 months on plasma choline concentrations among Malawian children enrolled in a randomized trial.

Methods: Children age 6-9 months in rural Malawi were randomized to receive one egg/day (n=331) or nonintervention control (n=329) for 6 months. Anthropometric, developmental, and dietary data were collected at baseline and 6 month follow up, along with a blood draw. Plasma choline, betaine, dimethylglycine, trimethylamine N-oxide (TMAO), and docosahexaenoic acid were measured at both time points using UPLC-MS/MS (n=200 per group). Linear regression analysis was used to determine the difference in plasma choline and related metabolites between groups after 6 months of intervention.

Results: Plasma choline, betaine, dimethylglycine, and docosahexaenoic acid concentrations did not differ between groups at 6 month follow up. Plasma TMAO was significantly (26% [95% CI: 7%, 48%]) higher in the egg intervention group in a fully adjusted model.

Conclusions: Provision of one egg/day for 6 months did not result in increases in plasma choline or related metabolites, except TMAO. This may partially explain the lack of effect on

growth and development. Additional interventions are needed to improve choline status, growth, and development in this population.

## 2.2 Introduction

Choline is an essential nutrient needed for optimal child growth and development, especially memory development.[1] Animal foods are the main sources of choline, but are relatively expensive.[2] Therefore, choline intake is likely low in many low and middle income countries (LMICs).[3] Suboptimal intake of choline in the pre- and postnatal periods may put children at risk for poor growth and development.

Eggs are a rich source of choline, along with other nutrients important in early life.[4] In Ecuadorian children age 6-9 months, significant improvements in growth were measured after provision of one egg per day for six months compared to control.[5] Plasma concentrations of choline, betaine, trimethylamine N-oxide (TMAO), and docosahexaenoic acid (DHA), were also increased in the egg intervention group, and the improvement in length-for-age z-score (LAZ) was partially mediated by increased plasma choline concentration.[6] However, a replication study performed in Malawi found no significant effects on growth, except larger head circumference-for-age (HCAZ) among children in the egg intervention group.[7] Measures of child development were included in the Malawian study, with mostly null results, except for a smaller percentage of children with fine motor delays in the egg intervention group.[8]

It is unclear why the egg intervention had so little effect on growth in Malawi compared to Ecuador. Reported adherence was high in the Malawian trial. Eggs were reported to be consumed by 71% of children in the egg intervention group on a 24-hour dietary recall

performed at the 6 month follow up.[7] However, dietary recall data revealed inadequate intakes of several nutrients, including choline, even with frequent egg consumption.[9] It is unclear whether the increase in choline intake in the Malawian trial was sufficient to improve plasma choline concentration. Considering the mediating role of plasma choline in improvement of growth in Ecuador, the effect of the egg intervention on plasma choline in the Malawian study may help explain the drastic difference in results.

The primary objective for this analysis was to evaluate the effect of provision of one egg per day for six months on plasma choline concentration among Malawian children age 6-9 months at baseline. We hypothesized that children in the egg intervention group would have higher plasma choline compared to children in the control group. We also tested for group differences in several related metabolites: betaine (a methyl donor and the oxidized product of choline), dimethylglycine (DMG) (a metabolite of betaine), TMAO (produced from choline by gut microbiota), and DHA (another nutrient important for child growth and development and linked to choline metabolism[10]). In secondary exploratory analyses, we tested several sociodemographic factors as moderators in the relationship between intervention group and plasma choline or its metabolites, as well as the role of plasma choline or its metabolites as mediators for the two primary outcomes that were significantly different by intervention group (HCAZ and fine motor delay). Finally, in order to provide evidence of adherence to the egg intervention, preliminary metabolomics analyses of the intervention effect are presented.

## 2.3 Methods

### *Study Design*

The Mazira Project (clinicaltrials.gov: NCT03385252) was a randomized trial of 660 Malawian children that took place from February 2018 to January 2019. The primary growth outcomes of the trial have been published previously.[7] Children age 6-9 months were individually randomized to intervention or control for six months. Randomization occurred using a 1:1 allocation ratio in blocks of 10, based on a random sequence generated by a researcher independent from the field team. Study staff invited caregivers to choose one sealed envelope, which contained the allocation code, under monitoring by an independent community member. Families in the intervention group received weekly egg deliveries, with instructions to feed one egg per day to the enrolled child. Eggs were procured from a local distributor. On average, eggs were 53 grams and provided 126 milligrams of choline per egg.[9] Families in the control group were asked to feed the child his or her typical diet. Both groups received instruction on food hygiene and handwashing during home visits.

*Participants*

Singleton infants age 6-9.9 months residing in the Lungwena Health Center and St. Martins Rural Hospital in Malindi catchment areas in Mangochi District were eligible to participate. Study staff identified age-eligible children using listings generated by community health workers, and recruited these children during household visits. Infants were excluded for egg allergy, history of serious allergic reactions requiring emergency care, congenital defects or conditions which affect growth and development, severe anemia (hemoglobin <5 g/dL), low mid-upper arm circumference (<12.5cm), presence of bipedal edema, acute illness or injury warranting hospital referral, or if the family planned to leave the area in the next 6 months.

*Data collection*

Study staff, who were blinded to group assignment, collected an array of dietary, anthropometric, and sociodemographic data during study visits at enrollment and at 6 month follow up. Anthropometric data were converted to z-scores using WHO Growth Standards,[11] with the cutoff of z-score ≤ -2 used to define stunting (length-for-age), underweight (weight-for-age), wasting (weight-for-length), and low head circumference (head circumference-for-age). The Malawi Developmental Assessment Tool (MDAT) was administered at enrollment and 6 month follow up. This tool measures fine motor, gross motor, personal social, and language development using a series of pass/fail tasks. Developmental delay in each domain was defined as failing ≥ 2 tasks which 90% of children at the same age would pass, using a Malawian reference population. The MDAT has been validated for use in Malawi and has high sensitivity (97%) and specificity (82%) to detect neurodevelopmental impairment in this context.[12]

Soon after enrollment, staff visited the home to collect information about housing materials and animal ownership for the creation of a housing and asset index. During the same home visit, staff administered the Household Food Insecurity Access Scale (HFIAS).[13] Additionally, caregivers reported morbidity symptoms at weekly home visits. Detailed information about data collection procedures is available in prior publications.[7,8]

*Plasma Analysis*

Study nurses collected venous blood into lithium heparin tubes at enrollment and 6 month follow up. Venous whole blood samples were used to identify anemia (Hemocue 201, HemoCue Inc., Angelholm, Sweden; anemia defined as hemoglobin < 11 g/dL) and malaria antigens (DF Bioline Malaria Ag P.f/Pan, Abbott Diagnostics, Lake Forest, IL). Afterwards, samples were centrifuged within a mean 28 (SD 42) minutes of collection and aliquoted and stored in a local -

20℃ freezer within a mean 37 (SD 14) minutes of centrifugation. Each afternoon, aliquots were transported for storage in a laboratory facility at -80℃.

Plasma choline was quantitated using two independent assays. First, it was included in a semi-quantitative metabolomics analysis by Metabolon Inc (Morrisville, NC). For this analysis, 200 children per group (n=400) with adequate blood samples at enrollment and follow up were randomly selected for inclusion. More than 800 other metabolites were measured, including plasma betaine, DMG, TMAO, and DHA. After precipitation of proteins with methanol, addition of recovery standards, and centrifugation, sample extracts were inserted onto a C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 µm) flushed with water, methanol, 0.05% perfluoropentanoic acid and 0.1% formic acid. Samples were dried and then reconstituted in solvent containing standards at fixed concentrations. Ultrahigh performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was performed using a Waters ACQUITY ULPC with Thermo Scientific Q-Exactive high resolution mass spectrometer, paired with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer. Injection order of the samples was randomized on the instrument and quality of the run was monitored using evenly spaced control samples and internal and recovery standards. Metabolites were identified by Metabolon, Inc. using authenticated standards. For data measured over several days, the median was set to 1.00 for each run-day block, and data points were normalized proportionally to adjust for day-to-day instrument variation. Missing values were imputed with the minimum. These data are semi-quantitative and reported in 'relative intensity' units, which provide information about the distribution of plasma choline as well as within-study comparisons. However, because these data are not reported in absolute concentrations, they cannot be compared to other studies.

In order to determine the absolute concentrations, a second, targeted quantitative assay of plasma choline, betaine and TMAO was performed in a subsample (n=60) of participants randomly chosen from the metabolomics sample. These analyses were performed at the USDA Western Human Nutrition Research Center using liquid chromatography tandem mass spectrometry (LC/MS/MS) as described by Wang et al[14] with modifications. DMG was not included in these analyses. Briefly, 20 µl plasma samples were aliquoted to a 2 ml Eppendorf tube and mixed with 80 µl of 10 µM surrogate standard (deuterated analytes in methanol) then vortexed for 30 seconds and centrifuged at 18,000 g at 10°C for 10 min. Supernatant was transported to glass inserts in HPLC vials. Similarly, standards were produced from 0 µM to 100 µM of non-deuterated analytes in methanol. Then, 20 µl of each standard and 80 µl of 10 µM surrogate standard were transferred to 150 µl glass inserts in HPLC vials. All standards were purchased from Sigma-Aldrich (St. Louis, MO). All reagent solvents were purchased from Fisher Scientific (Waltham, MA) and were mass spectrometry grade. Samples (5 µl) were injected onto a silica column (2.0 by 150 mm, 5 um Luna silica; Cat. No. 00F-4164-B0, Phenomenex, Torrance, CA) at a flow rate of 0.25 ml/min using a Waters Acquity UPLC (Waters, Milford, MA) outfitted with an API 4000 Q-TRAP mass spectrometer (AB SCIEX, Framingham, MA). Then, a discontinuous gradient of 0.1% acetic acid in water with 0.1% acetic acid in methanol at varying ratios was introduced. Electrospray ionization in positive-ion mode with multiple reaction monitoring (MRM) was used to measure analytes. Integration and quantification of values was completed using Analyst 1.6.2 software. Linear regression models were used to calculate standard linearity. These data were used to estimate the mean concentration of plasma choline, betaine, and TMAO in our study. However, due to the small sample size, they were not used for intervention group comparisons.

Additionally, plasma C-reactive protein (CRP) and alpha-1 acid glycoprotein (AGP) were measured using enzyme-linked immunoassay by the VitMin lab in Germany.[15]

*Statistical Analysis*

A detailed statistical analysis plan was developed prior to analysis and posted publicly (https://osf.io/azf7q/). The main outcome was the difference in mean plasma choline between groups after 6 months of intervention, as calculated in linear regression models using intention-to-treat analysis. Minimally adjusted analyses included baseline plasma choline values as a covariate. Fully adjusted analyses adjusted for sociodemographic factors, including: child age, sex, and birth order; maternal age, height, education, occupation, literacy, marital status, tribe, and religion; housing and asset index; animal ownership (chickens, cows, and goats); food insecurity score; distance to water source; number of children under 5y in the household; and village location (Lungwena vs Malindi health center catchment areas). Additionally, month of blood sample collection and time since last intake of foods other than breastmilk, water, or tea were considered. Covariates were retained in the model if they were associated with plasma choline with $p < 0.1$. Each model was assessed for normality and homoscedasticity of residuals using the Shapiro Wilks and Breush-Pagan tests, respectively. Non-normal variables were log transformed. Outliers were assessed using boxplots, histograms, scatterplots and Cooks' D; outliers which were considered impossible were corrected or removed before analysis. Similar analyses were performed to test for group differences in plasma betaine, DMG, TMAO, and DHA.

A sample size of 200 per group is sufficient to detect an intervention effect on plasma choline and its metabolites as small as 0.28 standard deviations with 80% power and an alpha of

0.05.This effect size is similar to the effect on plasma choline demonstrated in the egg intervention trial in Ecuador (0.35 [95% CI: 0.12, 0.57]).[6]

In exploratory analyses, the following characteristics were assessed as moderators in the relationship between group assignment and plasma metabolites: child sex, birth order, baseline maternal age and education, baseline household food insecurity, baseline housing and asset index, and baseline LAZ. Each moderator was tested as a dichotomous variable by including an interaction term in the minimally adjusted models. Additionally, for the two main effects that were significant in primary analyses (HCAZ and fine motor delay), structural equation modeling was used to assess the mediating role of plasma choline and related metabolites.

To investigate whether there was metabolomic evidence of adherence to the egg intervention, the metabolites from the semi-quantitative UPLC-MS/MS metabolomics analysis were analyzed using linear regression models to estimate mean difference between groups, adjusting for the baseline metabolite concentration and child age and sex, using intention-to-treat analysis. Metabolites with >20% of samples missing were excluded from the analysis; in general, these were metabolites of medications or other chemicals that were expected to be missing in this infant population. Normality was checked using a Shapiro-Wilk test statistic cut-off of 0.95. Non-normal metabolites were log transformed, and ranked data were used if normality was still not achieved. Outcomes were summarized with geometric means and confidence intervals regardless of transformation used for analysis. Fold changes were calculated as the ratio of geometric means. Volcano plots and p-value histograms were used to assess the overall intervention effect, and heatmaps were used to explore patterns of significance among metabolic pathways. Although there are a large number of outcomes, only the uncorrected p-values were reported since these analyses were exploratory in nature. The statistical analysis plan for the

semi-quantitative metabolomics analysis was developed a-priori and is published at https://osf.io/r6amq/.

All analyses were two-sided tests with an alpha of 0.05. Given the number of exploratory tests, significant p-values should be interpreted with caution.

## 2.4 Results

### *Participant characteristics*

This analysis included 200 children per group (**Figure 1**). At baseline, children in both groups were similar in terms of age, consumption of animal source foods, and measures of growth and development (**Table 1**). There were higher levels of maternal education in the egg intervention group, with more mothers having completed primary school and able to read compared to control. In addition, households in the egg intervention group were more likely to own chickens. Of note, chicken ownership was not significantly associated with plasma choline values.

Children included in this analysis were similar to those in the main trial who were excluded from analysis (n = 260) (**Supplemental Table 1**), with some exceptions. Among children who were excluded, there was a lower prevalence of malaria (8.6 vs 14.5%, p = 0.042) and small head size (HCAZ ≤ -2; 15.1 vs 23.5%, p = 0.008), and a higher prevalence of fish consumption at baseline (32.7 vs 23.6%, p = 0.010).

The semi-quantitative (n=400) and quantitative (n=60) choline measures were well correlated (r = 0.92, **Supplemental Figure 1**), as were the betaine (r=0.98) and TMAO (r=0.98) measurements. Plasma choline and betaine concentrations were similar between groups at

baseline (n=60, **Table 2**), whereas plasma TMAO was higher in the control group at baseline. Plasma choline decreased across the study period, whereas plasma betaine and TMAO levels increased.

*Intervention effect on plasma choline and related metabolites*

At 6 month follow up, plasma choline and betaine were higher and plasma DHA was lower in the egg intervention group compared to control; however, the differences were not statistically significant (n=400, **Table 3**). Plasma TMAO was higher in the egg intervention group, an effect which remained significant after adjustment. Because plasma TMAO was log transformed, the fully adjusted beta value of 0.23 (95% CI: 0.07, 0.39) represents a 26% (7%, 48%) increase in TMAO in the egg intervention group compared to control. Adjustment for covariates did not substantively change the results. Plasma TMAO was not significantly associated with markers of inflammation (CRP: r= 0.42, p = 0.41; AGP: r = 0.17, p = 0.17).

*Effect modification and mediation exploratory analyses*

Seven variables were tested as effect modifiers in the relationship between intervention group and plasma metabolites. Out of 35 tests for effect modification (**Supplemental Table 2**), none were significant at the 0.05 level.

Plasma metabolites were also tested as mediators in the relationship between intervention group and the two outcomes that were significant in primary intervention effect analyses (fine motor delay and HCAZ). Tests for mediation were similarly null, with no significant mediation by plasma choline or related metabolites (**Supplemental Table 3**).

*Semi-quantitative metabolomics analysis*

A total of 689 metabolites were included in the analysis after excluding those metabolites for which >20% of samples were missing, and 87 metabolites had a statistically significant difference between the egg and control groups (n=400, **Supplemental Figure 2**). Visualizing the data using a volcano plot (**Supplemental Figure 3**), 22 metabolites were identified as both statistically significant ($p<0.05$) and having a large magnitude fold change (|log2 fold change| >0.25). Eleven of the 22 metabolites, including TMAO, were part of the lipid metabolic pathway, and 4 were part of the amino acid pathway (**Table 4**).

## 2.5 Discussion

In this sample of Malawian children, plasma choline was not significantly increased after a six month intervention of one egg per day compared to control. Mean plasma choline concentrations were ~17 µmol/L at 6-9 months of age, and ~14 µmol/L at 12-15 months of age, and did not differ by group. Similarly, neither plasma betaine, DMG, nor DHA were increased with the intervention, although plasma TMAO was significantly higher in the egg intervention group. In exploratory analyses, none of the plasma metabolites significantly mediated the primary intervention effects on growth or development.

Our findings contrast with the previous egg intervention trial in Ecuador ("The Lulun Project"), in which plasma choline, betaine, TMAO, and DHA were significantly higher in the group receiving eggs, with effect sizes ranging from 0.29 SD (betaine) to 0.43 SD (DHA). The concentrations of plasma choline, betaine, and TMAO were similar (within ~15% of each other) across studies, after pooling Lulun Project values across groups and converting to µmol/L.[6] The eggs provided in both studies were rich sources of choline.[6,9]

However, there were differences between the trials that may explain these findings. First, background choline status may have differed between study sites. In Ecuador, the prevalence of egg consumption was higher at baseline (~40%,[5] compared to ~4% in Malawi[7]). Although choline intake was not reported in the Lulun Project, the mean choline intake among children in the Mazira Project was only 102 mg/day at baseline.[9] At the 6 month follow-up, 97% of children in the Mazira Project intervention group and 100% of children in the control group had choline intakes below the Adequate Intake (AI) level for their age (AI for 7-12 months: 150 mg/d; 1-3 years: 200 mg/d).[9] It is possible that the background choline status was higher in Ecuador, where children (and perhaps mothers) routinely consumed eggs, even if this was not reflected by higher plasma choline values. Plasma choline concentration may not reflect small to moderate changes in intake.[16] Plasma choline is homeostatically regulated, with increased recycling through phosphatidylcholine and decreased oxidation to betaine during deficiency.[17] Perhaps in the context of very low dietary choline intake in Malawi, the addition of one egg per day was below the threshold needed to significantly change plasma choline levels.

Background consumption of nutrients related to choline metabolism may also have differed between studies. Fish consumption was more prevalent in the Mazira Project, with ~25% of children consuming fish at baseline and ~65% at 6 month follow up (compared to ~17% and ~20% in the Lulun Project).[5,7] Fish are a rich source of DHA, a nutrient that is thought to act synergistically with choline to improve neural development.[10] Breast milk of women near Lake Malawi is also rich in DHA due to maternal fish consumption,[18] and nearly every child in this analysis consumed breast milk at baseline. Given their synergy, high DHA seems unlikely to have prevented increases in plasma choline; however, the high background intake may explain why plasma DHA was not significantly increased by the egg intervention in Malawi. In contrast,

fish are also a direct source of TMAO, and concentrations of this nutrient did significantly increase with the egg intervention in Malawi. This may be related to a third factor which likely differed between study sites: the gut microbiome. Although not measured in either study, it seems feasible that differences in diet, environmental pathogens, and access to sanitation between study sites would be associated with differences in the composition of the microbiome. Since this composition influences the conversion of choline to TMAO,[19,20] differences in the microbiome might help explain the contrasting findings in the Mazira Project compared to the Lulun Project. Although TMAO is linked to atherosclerosis and inflammation in adults,[21] its role in the health of young children is unknown. In our study sample, there was no link between plasma TMAO and markers of inflammation, and plasma TMAO concentrations were lower than those linked to poor health outcomes in adults.[22]

To our knowledge, no other studies have evaluated change in plasma choline after an egg intervention in young children; however, several studies of egg consumption have been conducted in adults. The majority of these studies reported increases in plasma choline without changes in plasma TMAO with daily consumption of eggs.[23–26] However, one small study noted a significant increase in plasma TMAO within hours of ingesting eggs, with large inter-individual differences in change in TMAO.[27] There is a need for more studies of the effects of eggs on concentrations of plasma choline and related metabolites, especially among young children in diverse settings. Our study may only be generalizable to young children in LMICs with a similar background diet (low animal source foods, except relatively high fish consumption).

Given the lack of effects on growth, development, and plasma choline concentration in the Mazira Project, the question of adherence becomes important. Egg intake was reported by

caregivers, rather than directly observed during the study. While exploratory, the 22 metabolites identified in the semi-quantitative metabolomics analysis as being impacted by group assignment provide evidence for adherence to the egg intervention. These metabolites also provide insight into potential markers of egg consumption and metabolic pathways that may be sensitive to changes in dietary egg intake in children. There are currently no metabolites that have been identified as biomarkers of egg intake,[28] although this may change as larger metabolomics analyses become more common. One recent study in adult men using nontargeted metabolic profiling found several metabolites associated with egg intake.[29] Although there were no shared metabolites identified between our study and that one, the major metabolic pathways that were impacted in our study (amino acid, peptide, and lipid) are not unexpected given the nutrient content of eggs. Our findings could be used in future egg intervention studies to identify metabolites to target for analyses.

This analysis has several strengths, including a randomized design with low losses to follow up, as well as measurement of several metabolites related to choline in a relatively large sample of 400 children. Also, analyses were pre-specified in a statistical analysis plan. This analysis also has several weaknesses. First, growth and development, not plasma choline concentration, were the primary outcomes for this trial. The trial was not specifically designed to answer this question, although plasma choline was specified as an *a priori* secondary outcome.[30] Also, plasma choline may not be a reliable biomarker of choline status, as it is insensitive to small to moderate changes in intake,[16] and represents only a small fraction of choline found in the body.[17] Many studies, including this analysis, include measurements of choline metabolites, such as betaine and DMG, as a way to clarify choline status. Identification of sensitive biomarkers of choline status is an active area of research.[31] Choline may also be converted to

acetylcholine, phosphatidylcholine, and sphingomyelin; however, these metabolites were not included in this study. Future studies with a broader suite of biomarkers may help reveal the relationship between egg intake and choline status.

## 2.6 Conclusion

Unlike in a similar trial in Ecuador, our trial of one egg per day did not result in increases in plasma choline or its metabolites, except plasma TMAO, in young Malawian children. These contrasting findings may help explain differences in the primary growth outcomes, since choline was responsive to intake and mediated effects on growth in Ecuador. Additional interventions are needed to improve choline status and growth in this population. More research is needed to understand the role of eggs on choline status among young children in diverse contexts, including identification of sensitive biomarkers of choline status.

2.7 References


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**Figure 2.1** – Participant flow diagram for this secondary analysis of the Mazira Project

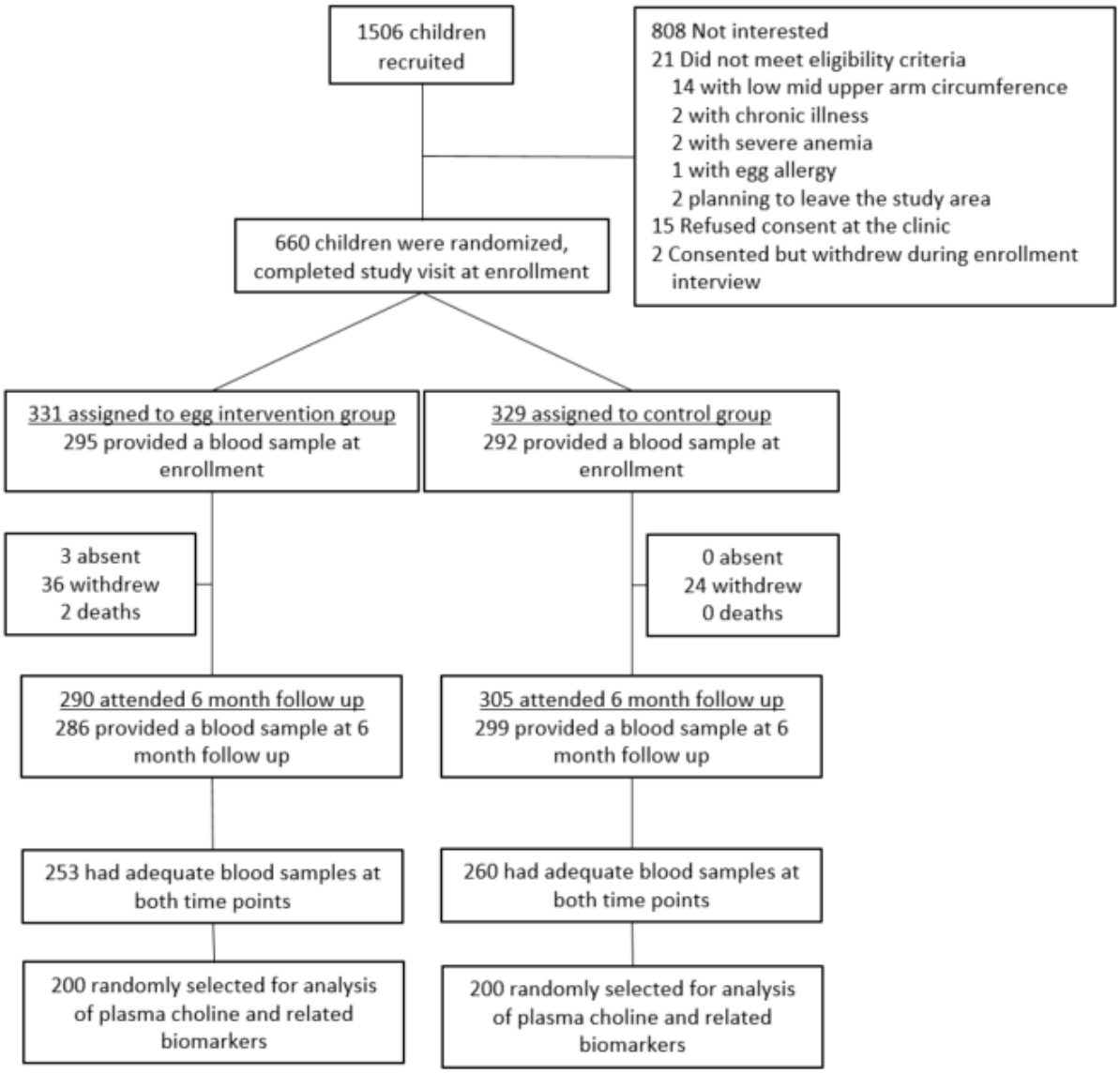


**Table 2.1** – Baseline characteristics of Mazira Project participants included in this analysis (n= 400)

| | **Intervention (n = 200 )** | **Control (n = 200 )** |
|---|---|---|
| | **Mean (SD) or %** | **Mean (SD) or %** |
| *Maternal and child characteristics* | | |
| Child age (mo) | 7.5 (1.2) | 7.3 (1.2) |
| Male (%) | 53.5 | 53.0 |
| First born (%) | 31.2 | 24.5 |
| Animal source food consumption (%)[a] | | |
| Consumed dairy | 10.6 | 9.0 |
| Consumed meat | 3.5 | 1.5 |
| Consumed fish | 27.1 | 20.0 |
| Consumed eggs | 3.5 | 4.0 |
| Any breast milk (%) | 99.5 | 100.0 |
| Prevalence of anemia (Hgb < 11 g/dL) (%) | 62.3 | 62.0 |
| Prevalence of malaria (%) | 14.0 | 15.0 |
| Prevalence of stunting (LAZ ≤ -2) (%) | 13.5 | 14.0 |
| Prevalence of wasting (WLZ ≤ -2) (%) | 1.5 | 1.5 |
| Prevalence of underweight (WAZ ≤ -2) (%) | 6.5 | 10.0 |
| Prevalence of small head size (HCAZ ≤ -2) (%) | 21.5 | 25.5 |
| Prevalence of developmental delay (%)[b] | | |
| Gross motor | 0.5 | 0.0 |
| Fine motor | 2.5 | 2.6 |
| Personal social | 1.5 | 1.5 |
| Language | 0.5 | 0.5 |
| Maternal age (y) | 26.0 (6.6) | 26.0 (6.9) |
| Maternal BMI ($kg/m^2$) | 21.7 (3.3) | 21.8 (2.7) |
| Mother completed primary school (%) | 28.5 | 22.5 |
| Mother can read (%) | 52.8 | 42.7 |
| | | |
| Household characteristics | | |
| Number of household members | 5.8 (2.5) | 6.2 (2.8) |
| Moderate to severe food insecurity (%)[c] | 77.5 | 78.5 |
| Owns latrine (%) | 95.5 | 97.5 |
| Owns cow(s) (%) | 2.5 | 2.5 |
| Owns goat(s) (%) | 17.0 | 23.0 |
| Owns chicken(s) (%) | 28.0 | 38.5 |
| Less than 10 minutes to water source (%) | 55.3 | 51.8 |

HCZ – head circumference-for-age z-score; Hgb – hemoglobin; LAZ – length-for-age z-score; WAZ – weight-for-age z-score; WLZ – weight-for-length z-score

[a] As reported by the caregiver on a 24-hour dietary recall

[b] As defined by the Malawi Developmental Assessment Tool (MDAT)[12]

[c] As defined by the Household Food Insecurity Access Scale[13]

**Table 2.2** – Mean plasma concentrations of selected nutrients at enrollment and 6 month follow up among participants in the Mazira Project egg intervention trial

| | Baseline | | | | 6 month follow up | | | |
|---|---|---|---|---|---|---|---|---|
| | Intervention | | Control | | Intervention | | Control | |
| | n | Mean (SD) | n | Mean (SD) | n | Mean (SD) | n | Mean (SD) |
| Plasma choline (µmol/L) | 30 | 17.0 (3.3) | 30 | 17.2 (3.8) | 30 | 15.5 (4.3) | 30 | 13.7 (2.6) |
| Plasma betaine (µmol/L) | 30 | 86.3 (34.8) | 30 | 83.8 (28.4) | 30 | 101.6 (34.3) | 30 | 91.7 (35.1) |
| Plasma TMAO (µmol/L)[a] | 30 | 1.0 (0.6, 2.9) | 30 | 2.3 (1.3, 4.7) | 30 | 3.8 (2.5, 6.7) | 30 | 3.3 (1.8, 4.5) |

*TMAO – trimethylamine N-oxide*

[a] Variable was skewed; data presented are median (IQR).

**Table 2.3** – Minimally and fully adjusted group differences in plasma choline and selected nutrients at 6 month follow up among young Malawian children enrolled in the Mazira Project egg intervention trial (n=400)

| | Minimally adjusted[a] | | Fully adjusted[b] | |
|---|---|---|---|---|
| | Estimate (95% CI)[c] | p value | Estimate (95% CI)[c] | p value |
| Plasma choline | 0.15 (-0.03, 0.34) | 0.11 | 0.17 (-0.02, 0.35) | 0.08 |
| Plasma betaine | 0.07 (-0.09, 0.24) | 0.38 | 0.09 (-0.07, 0.26) | 0.28 |
| Plasma DMG[d] | -0.01 (-0.07, 0.05) | 0.75 | -0.01 (-0.07, 0.05) | 0.66 |
| Plasma TMAO[d] | **0.26 (0.10, 0.43)** | **0.002** | **0.23 (0.07, 0.39)** | **0.01** |
| Plasma DHA | -0.07 (-0.27, 0.12) | 0.45 | -0.05 (-0.24, 0.15) | 0.62 |

*DHA – docosahexaenoic acid; DMG – dimethylglycine; TMAO – trimethylamine N-oxide*

[a] Adjusted for baseline plasma values.

[b] Additionally adjusted for child age, sex, and birth order; maternal age, height, education, occupation, literacy, marital status, tribe, and religion; housing and asset index; animal ownership; food insecurity score; distance to water source; number of children under 5y in the household; village location (Lungwena vs Malindi health center catchment areas); month of data collection; time since last intake. Covariates were included in the model if they were associated with plasma variable with $p < 0.1$.

[c] Estimates are beta coefficients representing the effect of egg intervention group assignment, in SD units. These estimates use relative intensity data, and cannot be directly translated to effect per absolute concentration (ie, µmol/L).

[d] Outcome variable was log transformed. Estimates represent the difference in the means of the log outcome; after exponentiation, this value can be interpreted as the ratio of the geometric means.

**Table 2.4** – Metabolites for which there was a statistically significant intervention effect with large magnitude fold change after Malawian children consumed one egg/day for 6-months (n=400)[a]

| Code | Biochemical[b] | Super Pathway | Sub Pathway | *p value* | Fold change[c] |
|---|---|---|---|---|---|
| x40473_6 | hydantoin-5-propionate | Amino Acid | Histidine Metabolism | 0.016 | 0.821 |
| x57687_6 | N,N,N-trimethyl-5-aminovalerate | Amino Acid | Lysine Metabolism | 0.003 | 1.21 |
| x541_6 | 4-hydroxyphenylacetate | Amino Acid | Phenylalanine Metabolism | 0.028 | 1.23 |
| x27672_6 | 3-indoxyl sulfate | Amino Acid | Tryptophan Metabolism | 0.015 | 1.30 |
| x37092_6 | gamma-glutamyl-2-aminobutyrate | Peptide | Gamma-glutamyl Amino Acid | 0.001 | 1.27 |
| x48425_6 | phenylacetylcarnitine | Peptide | Acetylated Peptides | 0.006 | 1.35 |
| x35126_6 | phenylacetylglutamine | Peptide | Acetylated Peptides | 0.003 | 1.30 |
| x396_6 | glutarate (C5-DC) | Lipid | Fatty Acid, Dicarboxylate | 0.046 | 1.21 |
| x54907_6 | hexanoylglutamine | Lipid | Fatty Acid Metabolism (Acyl Glutamine) | 0.003 | 0.834 |
| x40406_6 | trimethylamine N-oxide | Lipid | Phospholipid Metabolism | 0.004 | 1.25 |
| x35625_6 | 1-myristoylglycerol (14:0) | Lipid | Monoacylglycerol | 0.002 | 0.787 |
| x32506_6 | 2-linoleoylglycerol (18:2) | Lipid | Monoacylglycerol | 0.009 | 0.827 |
| x54966_6 | diacylglycerol (16:1/18:2 [2], 16:0/18:3 [1])* | Lipid | Diacylglycerol | 0.014 | 0.816 |
| x57373_6 | palmitoyl-docosahexaenoyl-glycerol (16:0/22:6) [1]* | Lipid | Diacylglycerol | 0.010 | 0.838 |
| x37198_6 | 5alpha-pregnan-3beta,20alpha-diol disulfate | Lipid | Progestin Steroids | 0.000 | 1.33 |
| x32425_6 | dehydroepiandrosterone sulfate (DHEA-S) | Lipid | Androgenic Steroids | 0.008 | 1.27 |
| x38168_6 | 16a-hydroxy DHEA 3-sulfate | Lipid | Androgenic Steroids | 0.019 | 1.19 |
| x63731_6 | glycoursodeoxycholic acid sulfate (1) | Lipid | Secondary Bile Acid Metabolism | 0.009 | 1.38 |
| x601_6 | dihydroorotate | Nucleotide | Pyrimidine Metabolism, Orotate containing | 0.006 | 0.818 |
| x15753_6 | hippurate | Xenobiotics | Benzoate Metabolism | 0.014 | 1.37 |
| x62533_6 | (2,4 or 2,5)-dimethylphenol sulfate | Xenobiotics | Food Component/Plant | 0.017 | 0.793 |
| x48698_6 | 6-hydroxyindole sulfate | Xenobiotics | Chemical | 0.025 | 1.32 |

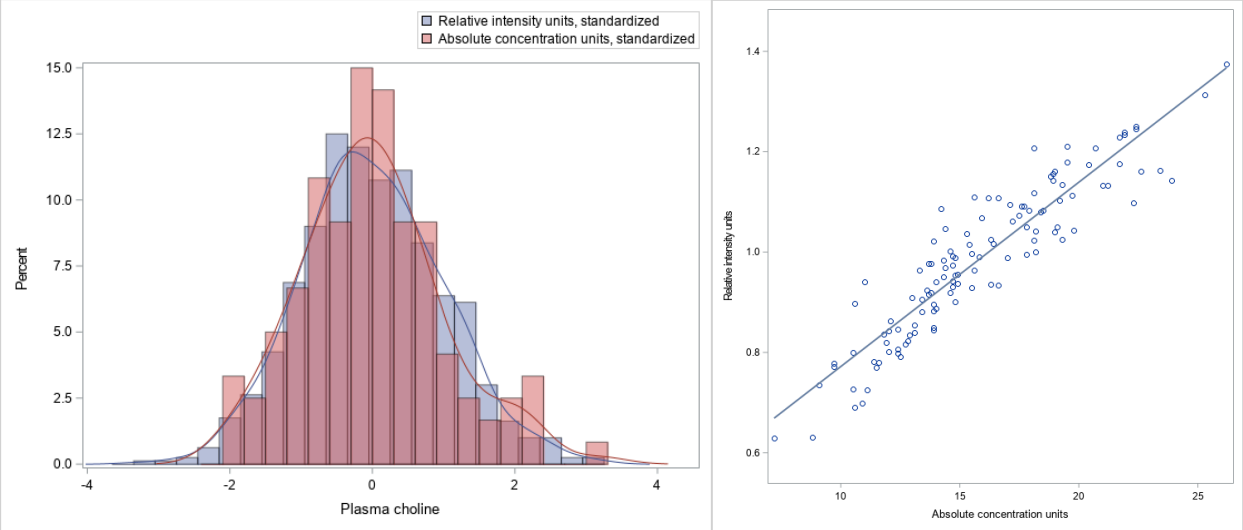
[a] Metabolites with statistically significant *p*-values ($p < 0.05$) from linear regression models adjusted for baseline measures of the outcome variables and child age and sex, and large magnitude fold changes ($|\log2 \text{ fold change}| > 0.25$) which were calculated as the ratio of geometric means. Biochemical compound codes, names, super pathways, and sub pathways provided by Metabolon Inc.

[b] An asterisk (*) indicates compounds that have not been officially confirmed based on a standard, but for which Metabolon Inc is confident in the identity.

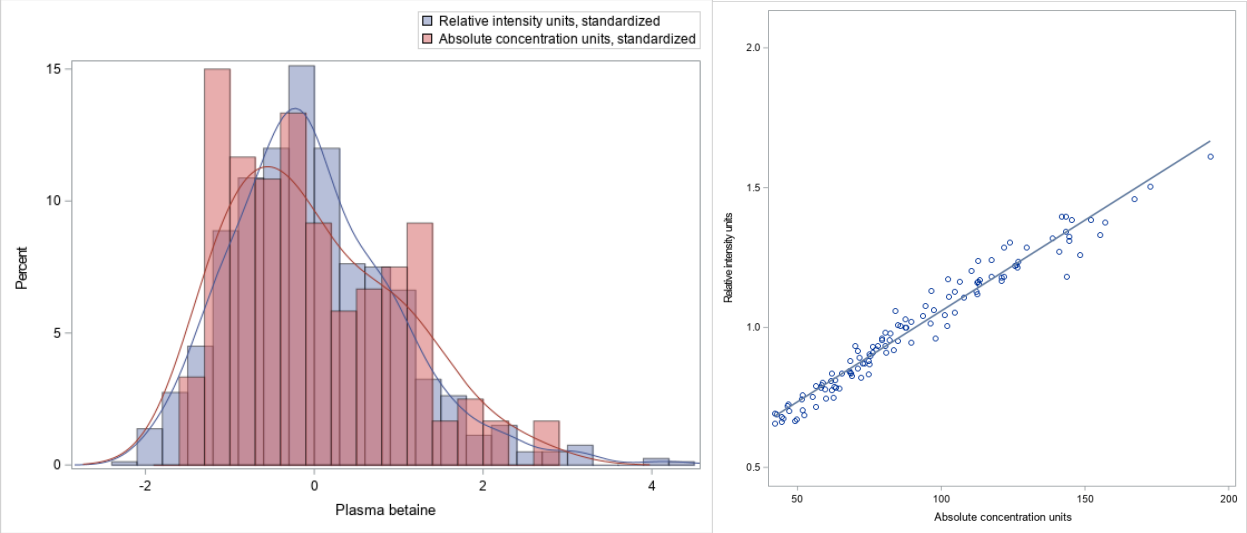
[c] Fold changes for which the intervention egg group had a higher mean are >1 and fold changes for which the control group had a higher mean are<1.

**Supplemental Figure 2.1 –** Correlation between two measures of plasma choline, betaine, and trimethylamine N-oxide among Mazira Project participants[a]

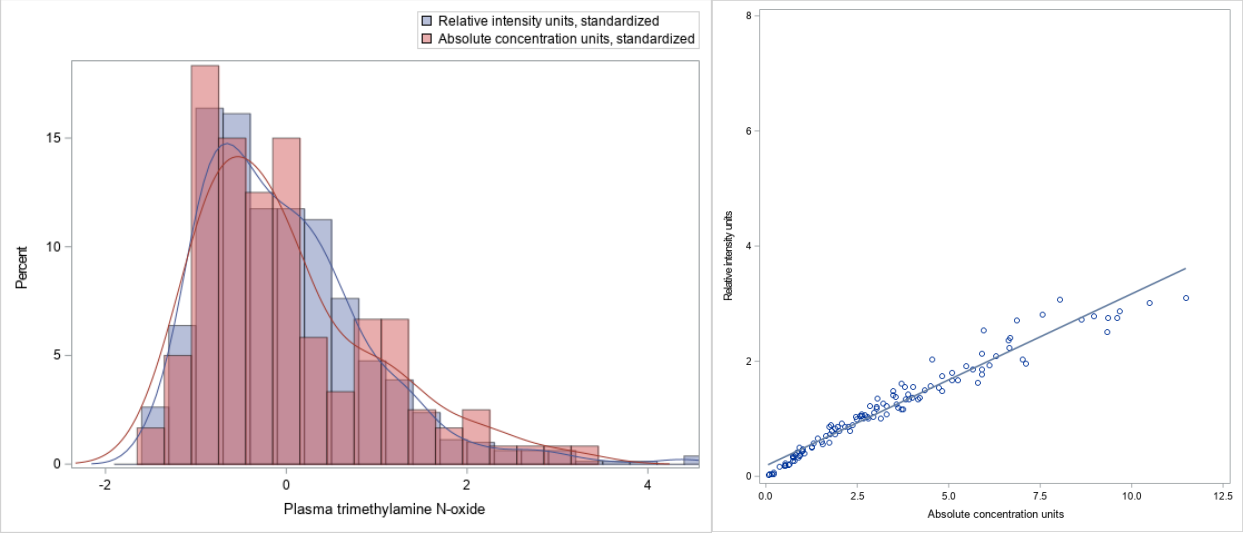
a) Plasma choline (r = 0.92)




b) Plasma betaine (r = 0.98)

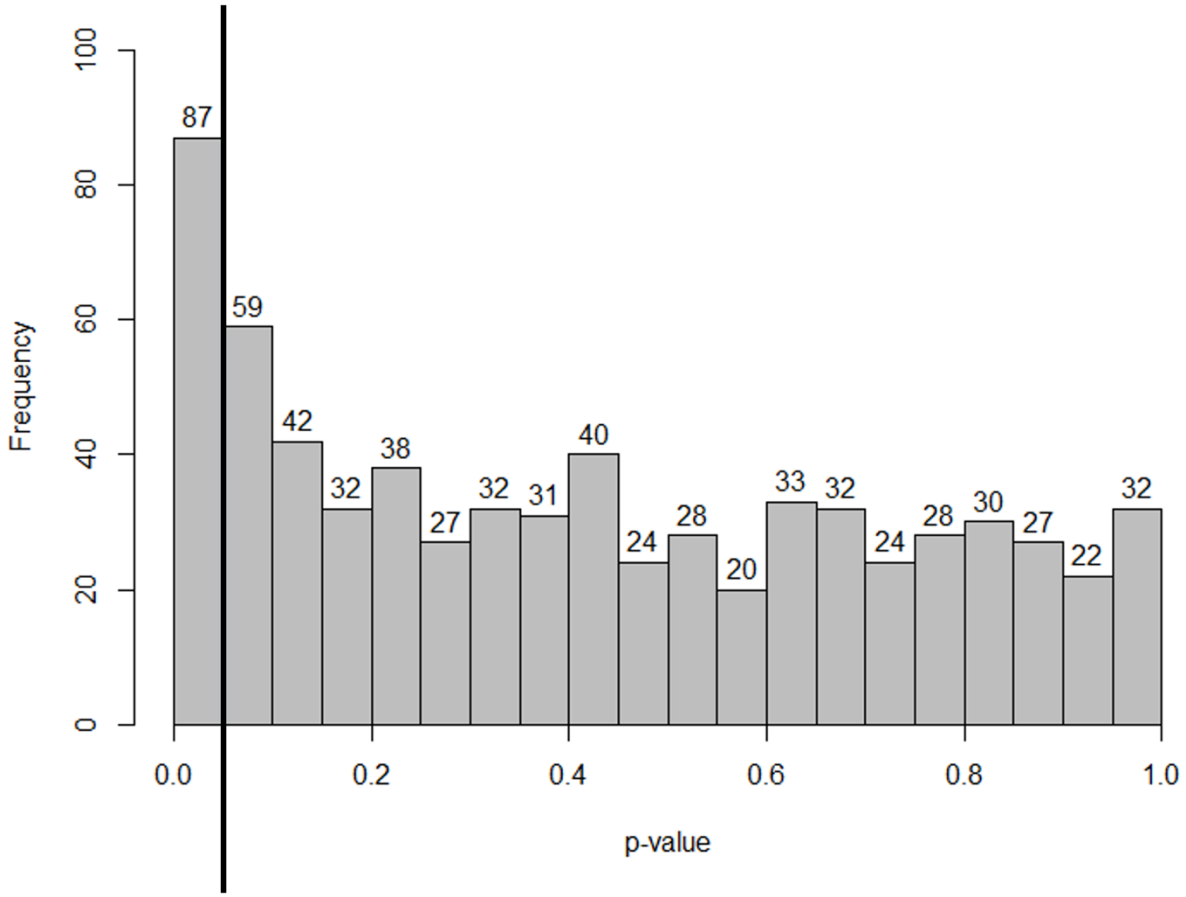


c) Plasma trimethylamine N-oxide (r = 0.98)



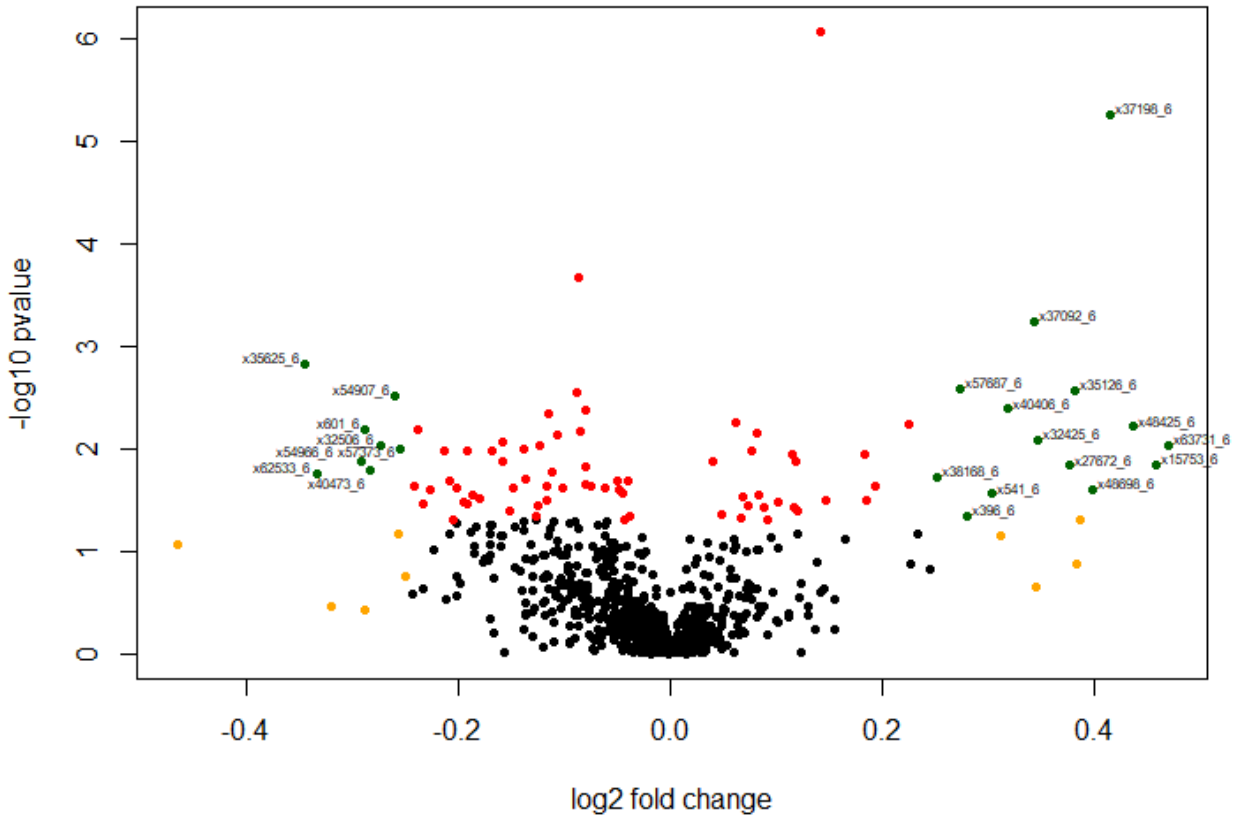

[a] Relative intensity units were measured using UPLC-MS/MS during metabolomic analysis among 400 participants (200 per group). Absolute concentration units were measured quantitatively using LC-MS/MS among 60 participants (30 per group). Data are standardized for comparison.

**Supplemental Figure 2.2 –** Histogram of p-values from a semi-quantitative metabolomic linear regression analysis of 689 metabolites by intervention group among Mazira Project participants (n=400)[a]




[a] The black vertical line indicates a p-value of 0.05.

**Supplemental Figure 2.3** – Volcano plot from a semi-quantitative metabolomic linear regression analysis of 689 metabolites by intervention group among Mazira Project participants (n=400)[a]




[a] Each dot represents a single metabolite. Red dots indicate metabolites for which p<0.05, yellow dots indicate metabolites for which log2 fold change >0.25, and green dots indicate metabolites for which p<0.05 and log2 fold change >0.25. Green metabolites are labeled with their assigned codes. Individual metabolite names are provided in Table 4.

**Supplemental Table 2.1** – Baseline characteristics of children enrolled in the Mazira Project and included vs excluded from the current secondary analysis

| | Included (n= 400) | Excluded (n= 260)[a] | |
|---|---|---|---|
| | Mean (SD) or (%) | Mean (SD) or (%) | p value |
| *Child and maternal characteristics* | | | |
| Child age (mo) | 7.4 (1.2) | 7.4 (1.2) | 0.849 |
| Male | 53.3 | 49.2 | 0.313 |
| First born | 27.8 | 27.3 | 0.886 |
| Animal source food consumption[b] | | | |
| Consumed dairy | 9.8 | 6.5 | 0.145 |
| Consumed meat | 2.5 | 0.8 | 0.103 |
| Consumed egg | 3.8 | 4.6 | 0.588 |
| Consumed fish | 23.6 | 32.7 | **0.010** |
| Any breastmilk | 99.7 | 100.0 | 0.419 |
| Anemia prevalence (Hgb < 11 g/dL) | 62.2 | 57.8 | 0.320 |
| Malaria prevalence | 14.5 | 8.6 | **0.042** |
| Prevalence of stunting (LAZ ≤ -2) (%) | 13.8 | 13.5 | 0.916 |
| Prevalence of wasting (WLZ ≤ -2) (%) | 1.5 | 0.4 | 0.172 |
| Prevalence of underweight (WAZ ≤ -2) (%) | 8.3 | 7.3 | 0.661 |
| Prevalence of small head size (HCAZ ≤ -2) (%) | 23.5 | 15.1 | **0.008** |
| Prevalence of developmental delay (%)[c] | | | |
| Gross motor | 0.3 | 0.0 | 0.416 |
| Fine motor | 2.5 | 2.7 | 0.914 |
| Personal social | 1.5 | 2.3 | 0.471 |
| Language | 0.5 | 0.8 | 0.679 |
| Maternal age (y) | 26.0 (6.8) | 25.9 (6.7) | 0.857 |
| Maternal BMI (kg/m2) | 21.8 (3.0) | 21.9 (3.0) | 0.445 |
| Mother completed primary school (%) | 20.5 | 19.1 | 0.656 |
| Mother can read (%) | 47.7 | 42.9 | 0.228 |
| | | | |
| *Household characteristics* | | | |
| Number of household members | 6.0 (2.6) | 5.7 (2.7) | 0.242 |
| Moderate to severe food insecurity[d] | 78.0 | 77.7 | 0.926 |
| Owns latrine | 96.5 | 96.3 | 0.918 |
| Owns cows | 2.5 | 3.7 | 0.399 |
| Owns goats | 20.0 | 17.3 | 0.388 |
| Owns chickens | 33.3 | 31.0 | 0.552 |
| Less than 10 minutes to water source (%) | 53.5 | 59.2 | 0.160 |

HCZ – head circumference-for-age z-score; Hgb – hemoglobin; LAZ – length-for-age z-score; WAZ – weight-for-age z-score; WLZ – weight-for-length z-score

[a] Children enrolled in the main trial were excluded from this analysis if they were missing one or both blood samples (n=147) or were not randomly selected for biochemical analysis (n=113)

[b] As reported by the caregiver on a 24-hour dietary recall
[c] As defined by the Malawi Developmental Assessment Tool (MDAT)[12]
[d] As defined by the Household Food Insecurity Access Scale[13]

**Supplemental Table 2.2 –** Results from exploratory effect modification analyses[a] of the effect of group assignment on choline biomarkers among Mazira Project participants (n=400)

| | p for interaction |
|---|---|
| *Plasma choline* | |
| Child sex (male vs female) | 0.410 |
| First born (first vs not first) | 0.163 |
| Household food insecurity (mild/none vs moderate/severe) | 0.619 |
| Housing and asset score (quintile 1 vs quintiles 2-5) | 0.703 |
| Baseline length-for-age z-score (below vs above <-1) | 0.576 |
| Maternal age (below vs above 20y) | 0.180 |
| Maternal education (incomplete primary vs primary or greater) | 0.870 |
| *Plasma betaine* | |
| Child sex | 0.908 |
| First born | 0.582 |
| Household food insecurity | 0.215 |
| Housing and asset score | 0.821 |
| Baseline length-for-age z-score | 0.773 |
| Maternal age | 0.404 |
| Maternal education | 0.607 |
| *Log plasma dimethylglycine* | |
| Child sex | 0.381 |
| First born | 0.759 |
| Household food insecurity | 0.835 |
| Housing and asset score | 0.180 |
| Baseline length-for-age z-score | 0.815 |
| Maternal age | 0.784 |
| Maternal education | 0.637 |
| *Log plasma trimethylamine N-oxide* | |
| Child sex | 0.100 |
| First born | 0.981 |
| Household food insecurity | 0.514 |
| Housing and asset score | 0.887 |
| Baseline length-for-age z-score | 0.251 |
| Maternal age | 0.858 |
| Maternal education | 0.687 |
| *Plasma docosahexaenoic acid* | |
| Child sex | 0.500 |
| First born | 0.438 |
| Household food insecurity | 0.531 |
| Housing and asset score | 0.747 |
| Baseline length-for-age z-score | 0.209 |
| Maternal age | 0.550 |
| Maternal education | 0.864 |

[a] Each moderator was made dichotomous and tested in regression models adjusted for baseline biomarker value.

**Supplemental Table 2.3** – Exploratory analyses[a] investigating plasma choline and related biomarkers as mediators of the Mazira Project primary results (n=400)

| | Total Effect[b] | Direct Effect | Indirect Effect |
|---|---|---|---|
| Fine motor delay | | | |
| Choline | -4.6 (-11.4, 2.2) | -4.6 (-11.2, 2.3) | 0.03 (-0.3, 0.3) |
| Betaine | | -4.6 (-11.4, 2.2) | 0.05 (-0.6, 0.7) |
| Log DMG | | -4.6 (-11.4, 2.3) | -0.01 (-0.1, 0.1) |
| Log TMAO | | -5.1 (-11.9, 1.7) | 0.5 (-0.4, 1.5) |
| DHA | | -4.5 (-11.3, 2.3) | -0.09 (-0.6, 0.4) |
| | | | |
| Head circumference-for-age z-score | | | |
| Choline | 24.6 (2.1, 47.2) | 27.4 (5.1, 49.6) | -2.7 (-7.0, 1.6) |
| Betaine | | 24.5 (2.0, 4.7) | 0.1 (-0.6, 0.9) |
| Log DMG | | 24.9 (2.5, 47.2) | -0.2 (-3.0, 2.6) |
| Log TMAO | | 24.6 (1.9, 47.4) | 0.04 (-2.9, 2.9) |
| DHA | | 24.7 (2.1, 47.3) | -0.04 (-0.8, 0.7) |

DHA – docosahexaenoic acid; DMG – dimethylglycine; TMAO – trimethylamine N-oxide

[a] Mediation analyses were performed using structural equation modeling to test for mediation of the two primary findings of the Mazira Project (fine motor delay, head circumference-for-age z-score) by plasma choline and related biomarkers.

[b] Total effects are presented as the difference in means (95% CI) between intervention and control in outcome variables. Direct effects are the effect of intervention group after controlling for the mediator. Indirect effects are the effect of intervention group that is acting through choline/mediator (in SD units for choline, betaine, DHA; in log units for DMG and TMAO).

# Chapter 3: Plasma choline and neurodevelopment among Malawian children age 6-9 and 12-15 months

## 3.1 Abstract

Inadequate availability of the essential nutrient choline during the perinatal period has been associated with deficits in neurodevelopment, especially memory and attention, in animal and human studies. Few studies have examined postnatal choline status and neurodevelopment in the context of low and middle income countries, where choline intake is likely low and suboptimal development is common. The aim of this observational study was to examine the cross-sectional and predictive associations between plasma choline concentration and neurodevelopment among young Malawian children enrolled in an egg intervention trial. Plasma choline, betaine, dimethylglycine, and trimethylamine N-oxide (TMAO) were measured at enrollment (when children were age 6-9 months) and 6 month study follow up. Developmental assessments were measured at the same time points, and included the Malawi Developmental Assessment Tool (MDAT), the Infant Orienting with Attention task (IOWA), a visual paired comparison task, and an elicited imitation task. The association of plasma choline (in SD units) and its metabolites with developmental measures was examined using cross-sectional and predictive generalized linear models with adjustment for covariates. Plasma choline was not significantly associated with most measures of development, except a negative predictive association with MDAT fine motor normed z-score (-0.13 SD [95% CI: -0.22, -0.04]) and a positive cross-sectional association with IOWA response time (8.84 ms [1.66, 16.03]), both suggesting poorer development with higher plasma choline concentration. Additionally, plasma TMAO was positively associated with peak look length and elicited imitation total actions score in predictive models. More research, with improved biomarkers of choline status, is needed to understand the role of postnatal choline in neurodevelopment in diverse settings.

## 3.2 Introduction

Nearly 250 million children younger than five years in low and middle income countries (LMICs) are at risk for not reaching their developmental potential.[1] Nutrition is one factor which influences neurodevelopment, with specific nutrients required in adequate doses during critical and sensitive periods for optimal brain maturation.[2] Choline is an essential micronutrient that is needed for brain development and lifelong brain function,[3,4] although the dose required for optimal development is unclear, as current recommendations are based on Adequate Intake levels which did not consider neurodevelopmental outcomes.[5] Suboptimal perinatal choline intake alters neural progenitor cell mitosis and apoptosis,[6,7] acetylcholine availability,[8] and synaptic plasticity in the hippocampus.[9] Choline is particularly important for development of the dentate gyrus region of the hippocampus,[6] which is needed for declarative memory.[10] Rodent studies show consistent improvements in memory with perinatal supplementation of choline above standard feed levels,[11–13] including in models of developmental risk such as prenatal alcohol exposure[14] and iron deficiency.[15–17]

Human studies of perinatal choline for neurodevelopment have mostly focused on prenatal exposure, and most have taken place in high-income settings. Few studies have reported on the relationship between early postnatal (<2y) choline concentration and child neurodevelopment in LMICs.[18] The specific period for choline's effect on neurodevelopment in humans may extend from the prenatal period through early postnatal life, potentially up to four years of age,[11,19] with development of the dentate gyrus peaking in the first two years.[20] Studies in early childhood are needed to test this hypothesis. Studies in LMICs may be particularly relevant due to the high prevalence of suboptimal development and of micronutrient deficiencies. The main sources of choline are animal source foods, such as eggs. Because these foods may be

relatively expensive,[21] intake of choline is likely to be suboptimal in many LMICs.[22] Breastmilk is a rich source of choline for young children, although concentration varies with maternal diet,[23] and in food insecure settings, maternal dietary intake may be low. Children in LMICs may be especially at risk for low choline intake during the complementary feeding period, as they transition from breastmilk to a diet that may lack animal source foods.

The objective of this analysis was to examine the association between plasma choline concentration and neurodevelopment across the early complementary feeding period (6-9 and 12-15 months of age) among Malawian children enrolled in an egg intervention trial. The results of the trial growth and developmental outcomes have been published.[24,25] Overall, there were minimal effects on development, except a lower prevalence of fine motor delays, and a pattern of positive effects among less vulnerable children (i.e. length-for-age z-score [LAZ]>-1 at baseline). In this secondary analysis, we tested the hypothesis that plasma choline concentration is positively associated with neurodevelopment in this sample of young Malawian children. Choline metabolites, namely betaine, dimethylglycine (DMG), and trimethylamine-N-oxide (TMAO), were also tested for their association with development. Finally, exploratory analyses examined potential effect modifiers in the relationship between choline and neurodevelopment.

## 3.3 Methods

This is a secondary analysis using data from a randomized controlled trial (the Mazira Project, clinicaltrials.gov: NCT03385252), which took place in rural Malawi from February 2018 to January 2019. This trial investigated the effect of providing one egg per day versus a nonintervention control among 660 Malawian children. Children age 6-9 months were individually randomized to intervention or control for six months. The intervention group

received weekly batches of eggs, and caregivers were asked to feed the child one egg per day. The control group received no eggs, and caregivers were asked to feed the child as they normally would. Both groups received weekly home visits, which included information about food hygiene and handwashing.

*Participants*

Children residing in the Lungwena Health Center and St. Martins Rural Hospital in Malindi catchment areas of Malawi were eligible to enroll. These areas are rural, with most families engaged in fishing and agricultural labor. Staff recruited children and caregivers at household visits, community meetings, and local football tournaments. Exclusion criteria were: egg allergy, history of serious allergic reactions requiring emergency care, congenital defects or conditions which may affect growth and development, severe anemia (hemoglobin <5 g/dL), low mid-upper arm circumference (<12.5cm), presence of bipedal edema, or acute illness or injury warranting hospital referral. Children of families who planned to leave the study area within the next 6 months were also excluded.

*Data collection*

Anthropometric, dietary, and developmental data, as well as a blood sample, were collected at the study center at enrollment (baseline) and at study end six months later. At the study midpoint, only anthropometric and dietary data were collected. Anthropometric measures were converted to z-scores using WHO Growth standards,[26] and a cutoff of -2 was used to define stunting (length-for-age ≤-2) and wasting (weight-for-length ≤-2). Blood samples were used to measure hemoglobin (Hemocue 201, HemoCue Inc., Angelholm, Sweden); anemia was defined as hemoglobin < 11 g/dL. Caregivers provided information on maternal and household

characteristics, and a housing and asset index was calculated from baseline variables including animal ownership. One week after enrollment, staff administered the Household Food Insecurity Access Scale[27] and the Home Observation Measurement of the Environment (HOME) Inventory[28] during a home visit. At the 6 month follow up, the Family Care Indicators (FCI)[29] interview was administered. Both the HOME Inventory and FCI score assess children's opportunities for stimulation. Detailed descriptions of data collection measures have been previously published.[24,25]

*Developmental Assessments*

Developmental assessment included two behavioral and two eye-tracking based measures, performed by trained assessors at the study site. All were performed at baseline and 6 month follow up except the elicited imitation task, which was administered at follow-up only.

Behavioral measures

The Malawi Developmental Assessment Tool (MDAT) includes 136 items across four domains (fine motor, gross motor, personal social, and language development) which are scored as pass/fail based on the child's performance (or, for the personal social domain, parental report). For each domain, z-scores were calculated based on published Malawian norms.[30] The MDAT has been validated for use in Malawi and has high sensitivity (97%) and specificity (82%) to detect neurodevelopmental impairment in this context.[30]

In the elicited imitation task, children demonstrate recall memory by imitating sequences of actions demonstrated by outcome assessors.[31] In our adaptation of the task, children were asked to imitate eight two-action sequences (16 target actions, 8 ordered sequences) performed using sets of toys. For each set, assessors first recorded which actions the child performed prior

to the demonstration ('spontaneous actions') during a 30 second free play. Then, the assessor performed the sequence twice while the child watched. Immediately afterwards ('immediate imitation') or after a ten minute delay ('deferred imitation'), the assessor scored the child's ability to reproduce the target actions (actions recalled score: 0-16) and sequences (sequences recalled score: 0-8) during two 30 second imitation sessions. Information on the adaptation and piloting of this task has been published.[24] In a few cases, children were unable to complete items due to fussiness, sleepiness, or missing or damaged toys; to correct for this, scores were calculated by multiplying the percent of correct actions or sequences available to the child by the maximum possible score. Children who were offered fewer than 8 actions or 4 sequences were excluded. The elicited imitation task was measured at 6 month follow up only.

Eye tracking measures

Children were seated on their caregiver's lap facing a monitor with an eye tracking system (Tobii Pro X2-60) attached. The eye tracker recorded the x and y coordinates of the child's gaze on the screen 60 times per second using corneal refraction of infrared light. Two eye tracking stations were used at the study site, each including a laptop, monitor, webcam and eye tracker surrounded by 4 black curtains. At baseline, children who enrolled before April 4, 2018 (39%) completed a pilot version of the eye tracking tasks, which was later revised; these children were not included in analysis of baseline eye tracking data.

The visual paired comparison (VPC) task measures children's recognition memory based on the concept of novelty preference, or children's preference to look at unfamiliar items. In this version of the test based on a previous study,[32] children were presented with two identical stimuli (an African face) on the left and right sides of the screen for 20 seconds ('the familiarization period'). Then, the familiar face was shown on one side of the screen paired with a novel face on

the other for 20 seconds, with the position of the faces reversed at 10 seconds ('the recognition memory period'). This was repeated with different faces four times. Two outcome measures were calculated from this data: a novelty preference score and the peak look length during familiarization.

To create novelty preference scores, the number and length of fixations to each side of the screen was calculated using the Tobii I-VT fixation filter. A fixation is a period of time when the gaze is directed to a specific focal point without shifting gaze to a different focal point. For each trial, the novelty preference score is the percent of time spent fixating on the side of the screen containing the novel stimulus compared to total time looking at the screen. Trials with <1 second of looking time during the familiarization or recognition memory periods were excluded (11% of trials), since the child may not have been on-task. The total time spent fixating on the screen during the familiarization period was used as a covariate in models examining novelty preference as an outcome.

Peak look length was calculated as the duration of the longest look during the familiarization phase of each trial. In previous studies, shorter looks were associated with improved attention and faster information processing;[33] however, these studies used human scorers rather than eye tracking devices. To mimic the ability of human scorers to identify eye movements, fixations identified by the Tobii filter were recoded into 'looks,' which were defined as periods of visual attention towards one side of the screen that lasted $\geq$1 second and were not interrupted for longer than 1 second.[34]

In the Infant Orienting with Attention (IOWA) task, children demonstrate their attentional processes by shifting their gaze towards targets appearing on the screen.[35] In this task, children were shown a central image (a smiley face), then a 100ms visual cue (a small black

circle), followed by a 1000ms target (a picture of a colorful everyday object) on one side of the screen. Children's gaze was tracked as it shifted from the central image to the target, and the response time was defined as the time from the appearance of the target to the first fixation on that side of the screen. Trials with <200ms of looking time at the central image were excluded (10% of trials), as the child was not properly fixated on the center of the screen. Trials with response times <100ms or >1000ms were also excluded (1% of trials), as they may reflect eye movements that started prior to the appearance of the target or off-track behavior, respectively. The IOWA task included 96 trials across four conditions which varied by the location of the visual cue (same side as target, opposite side, both sides, or not present). These conditions can be used to examine aspects of children's attentional processes. We did not examine specific conditions, but included the condition as a covariate in statistical models of overall mean response time.

*Plasma Concentrations*

Venous blood was collected into lithium heparin tubes at baseline and 6 month follow up. Samples were centrifuged within a mean of 28 (SD 42) minutes of collection. Plasma and cell samples were separated into aliquots, which were stored in the local freezer at -20℃ within a mean 37 (SD 14) minutes of centrifugation. Each afternoon, the aliquots were transported to the main laboratory for storage at -80℃.

Details of plasma choline measurement for this study have been described (Chapter 2). Briefly, plasma choline was measured at baseline and 6 month follow up using two analysis methods. The relative intensity of plasma choline was measured in a subsample of 400 children using ultra high performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) by Metabolon Inc. These semi quantitative data describe the distribution of plasma choline

concentration and may be used for regression analysis; however, they are in relative intensity units. Betaine, DMG, TMAO, and more than 800 other metabolites were also measured in this way. Additionally, plasma choline was measured quantitatively in a subsample of 60 children using liquid chromatography tandem mass spectrometry (LC-MS/MS) at the USDA Western Human Nutrition Research Center. These data provide the absolute concentration and can be used to compare to other studies; however, due to the small sample size, they were not used in regression analyses. Betaine and TMAO, but not DMG, were measured using similar validated and standardized protocols. Plasma concentrations using the two different methods were well correlated (choline: r=0.92, betaine: r=0.98, TMAO: r=0.98) (Chapter 2).

Other metabolites measured include plasma docosahexaenoic acid (DHA) and ferritin. Plasma DHA was measured in relative intensity units using UPLC-MS/MS by Metabolon Inc. Plasma ferritin was measured using enzyme-linked immunoassay by the VitMin lab in Germany.[36] Ferritin was adjusted for inflammation using C-reactive protein (CRP) and alpha-1 acid glycoprotein (AGP), also measured by the VitMin lab, using the Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia (BRINDA) approach.[37]

*Sample Size*

The sample size for the randomized trial was 660 children, based on the hypothesized difference in LAZ between groups at the end of the intervention, which was the pre-specified primary outcome of the trial. This analysis included a subset of 400 children (200 per group) who provided adequate blood samples at baseline and 6 month follow up and were randomly selected for semi quantitative (UPLC-MS/MS) lab analysis. This sample size is sufficient to detect correlations between plasma metabolites and developmental outcomes as small as 0.14 with 80% power and a 2 sided alpha of 0.05.

*Statistical Analysis*

A statistical analysis plan was developed and posted publicly prior to analysis (https://osf.io/5wcn6/). We pooled data from both time points to examine the cross-sectional association between plasma choline and each developmental outcome using generalized linear models. To account for repeated measures within participant over time, and multiple trials per participant for certain outcomes (VPC novelty preference and peak look length, IOWA response time), participant was included as the independent unit and robust standard errors were used. The time point of data collection (baseline vs 6 month follow up) was included in models that contained both time points. Additionally, the predictive association of baseline plasma choline with developmental outcomes at 6 month follow up was examined in simple linear regression models. Each model was assessed for linearity, outliers, and normality and homoscedasticity of residuals. Children with missing plasma choline values or developmental data were not included. All analyses are reported as the difference in developmental outcome per 1 SD difference in plasma choline.

Minimally adjusted models included variables related to data collection, specifically: time of last meal before blood draw, calendar month of blood draw, developmental outcome assessor, and the child's mood, activity level and interaction with the assessor during tasks. Because eggs are rich in choline, group assignment (intervention vs control) was included. For the elicited imitation actions recalled score, the 'spontaneous actions' score was included to account for actions performed independent of memory. For eye tracking tasks, the eye tracking station was included. For the novelty preference score, the familiarization time in seconds was included. For IOWA response time, condition was included.

Covariates for fully adjusted models were pre-specified based on a theoretical causal model framework, with the following variables assessed for inclusion: child age, sex, and birth order, baseline plasma adjusted ferritin, baseline maternal age and education category, child stimulation (as measured by HOME at baseline and/or FCI at 6 month follow up), baseline household asset index, and baseline food insecurity category. Plasma DHA was not included as a covariate, as a portion of choline's effect on neurodevelopment may be via synergy with DHA.[38] Breastfeeding may affect both plasma choline levels and neurodevelopment; however, it was not included due to nearly universal prevalence of breastfeeding in the study sample. Variables associated with the developmental outcome with $p<0.1$ were included in the final multivariable models.

Additional exploratory analyses tested the cross-sectional and predictive associations of betaine, DMG, and TMAO (in SD units) with neurodevelopment in minimally adjusted models, using the covariates described previously.

Because the primary developmental analysis suggested an effect of the egg intervention on the most advantaged groups,[24] ten characteristics were assessed as potential effect modifiers, with each expressed as a dichotomous variable and examined with an interaction term of the potential effect modifier with plasma choline. These characteristics were: group assignment, child sex, birth order, baseline maternal age and education, baseline household food insecurity, baseline housing and asset index, baseline LAZ, baseline developmental score, HOME score below median, FCI score below median. For the elicited imitation task, total normed MDAT z-score was used as the baseline developmental score. Time point of data collection was also examined as a potential effect modifier (in models with both time points), to see if the relationship between choline and development differed by age range. Additionally, plasma

ferritin and DHA were examined as potential effect modifiers due to their connection to choline metabolism and brain development.[17,38] To limit the number of tests, these effect modifiers were only tested in minimally adjusted models of choline and development.

All analyses used two-sided tests with an alpha of 0.05. Effect modification analyses were considered exploratory, given the large number of statistical tests.

## 3.4 Results

### *Participant characteristics*

A total of 400 children were included in this analysis (**Figure 1**), equally distributed between males and females (**Table 1**). Children in this sample were nearly universally breastfed but few consumed animal source foods, except fish, as reported by caregivers on a 24 hour dietary recall at baseline. Wasting was uncommon (1.5%), and anemia prevalence was high (62.2%). The majority of households reported moderate/severe food insecurity.

Children included in this secondary analysis were compared to children in the main trial who were excluded from this analysis (N=262) (**Supplemental Table 1**). A smaller percentage of children included in this analysis consumed fish at baseline compared to excluded children (23.6 vs 32.7%, p =0.01). Otherwise, there were no significant differences.

Plasma choline concentration decreased over the study period (n=60, **Table 2**), whereas plasma betaine and TMAO increased. Inflammation adjusted ferritin levels decreased from baseline to 6 month follow up.

MDAT norm scores reflected advanced development in this sample compared to the Malawian norm at both time points, as evidenced by mean scores greater than 0, with the exception of the language domain (**Table 2**). On average, children demonstrated recognition memory in the VPC test at baseline and 6 month follow up, as demonstrated by mean novelty preference scores greater than 50%. Both peak look length during the VPC familiarization phase and response time during the IOWA task decreased (became shorter/faster) over the study period. On average, children successfully recalled nearly half of the actions (6.8 of 16) and less than 2 of 8 of the sequences in the elicited imitation task. Due to the uniform low number of sequences performed, this score was not used as a primary outcome.

*Association between plasma choline, its metabolites, and development*

Plasma choline was not significantly associated with most measures of development (**Table 3**). In cross-sectional models, there was a positive association between plasma choline and IOWA response time, suggesting a slower response with higher plasma choline, which remained significant after adjustment. In predictive models, baseline choline was negatively associated with fine motor normed z-scores at 6 month follow up, suggesting higher plasma choline predicted poorer fine motor development.

In models of betaine, DMG, and TMAO with child development, few significant associations were found (**Table 4**). Baseline TMAO was positively associated with peak look length and elicited imitation total actions score at 6 month follow up.

*Effect modification analyses*

Fourteen potential effect modifiers were examined for their role in the relationship between plasma choline and the 8 developmental outcomes. Out of 215 tests, 19 were significant at the 0.05 level (8.8%), which is slightly above that expected by chance (5%) (**Table 5**). Among

children with a Family Care Indicators score above the median, baseline choline was negatively associated with gross motor and language norm z-scores and elicited imitations actions recalled score at the 6 month follow up. Among children with a baseline DHA level above the median, plasma choline was negatively associated with VPC novelty preference and peak look length (cross-sectional) and gross motor norm z-score (predictive). Among children in the control group, plasma choline was negatively associated with language norm z-scores and IOWA response time. Overall, no clear pattern was evident across effect modifiers or developmental outcomes.

## 3.5 Discussion

In this sample of young Malawian children, plasma choline and its related metabolites were not associated with most measures of child development. The two significant associations denoted poorer fine motor skills and slower orienting of attention with higher plasma choline, which was contrary to our hypothesis.

Previous studies on postnatal choline and development have generally yielded positive or null findings. Although human studies on this topic in LMICs are scarce,[18] provision of supplementary foods containing choline, along with other nutrients, to young children led to improvement in working memory in Guinea-Bissau[39] and locomotor skills in South Africa.[40] In a high income country (the US), a trial among young children with fetal alcohol spectrum disorder found improvement in deferred imitation scores among children age 2.5 to <4y (but not children 4-5y) after 9 months of choline supplementation, with larger effect sizes at a 4 year follow up.[41,42] In an observational study in the US, breastmilk choline concentration was positively associated with recognition memory at 6 months.[43] In addition to these positive findings, there

have been other studies with null results. Two trials in neonates at risk for neurological impairment found no improvement in Bayley Scales for Infant Development (BSID) III scores after 2 years of receiving a supplement containing choline and other nutrients.[44,45] Two observational studies found no association between plasma choline and development among young children in the Seychelles and Canada;[46,47] however, both studies found positive associations between plasma betaine and domains of development. Very few studies have reported a negative association between postnatal choline and development. In a case-control study in the US, young children with autism spectrum disorder had higher plasma choline and betaine concentrations, and those with Down syndrome had higher plasma choline and DMG concentrations, compared to typically developing children.[48]

It is possible that the current findings differ from previous literature because of the setting; to our knowledge, this is the first observational study of early postnatal choline and neurodevelopment in an LMIC. Perhaps choline status was poorer in this low income setting than in previous reports. Because plasma choline concentration decreases over the first years of life,[49] it is difficult to compare plasma choline status across studies with various age ranges. However, choline intake was very low in this study population, even with the egg intervention.[50] Much of choline's effect on neurodevelopment is thought to be through conversion to betaine, a methyl donor that influences epigenetics. However, when body stores are low, choline oxidation to betaine is limited.[51] Perhaps there is a threshold effect, such that at very low intakes, choline is not converted to betaine at a rate that affects epigenetic control of neurodevelopment. This might explain why other observational studies found a link between betaine and development, but this analysis did not. This population also had a relatively high prevalence of fish intake, and a previous report noted high DHA concentrations among mothers and infants living near Lake

Malawi related to this fish consumption.[52] Perhaps in the context of high DHA, the role of choline on neurodevelopment is less pronounced.

In LMICs, there may also be a greater number of health and environmental factors that constrain neurodevelopment. In addition to nutrition, child development is affected by a range of factors including maternal health, infectious disease, and environmental toxins.[53] This study population had high rates of anemia and inflammation, with low maternal education and socioeconomic status. Because of its unique setting, this study is mainly generalizable to children in LMICs with low intake of choline and increased risk of suboptimal development.

Of note, plasma TMAO was significantly associated with two measures of development, although the direction of the associations varied. Higher plasma TMAO was linked with improved recall memory (higher elicited imitation actions recalled score) but slower information processing (longer peak look length). These findings may not be directly related to choline metabolism, given the high prevalence of fish consumption in this study population. Fish are a direct source of TMAO, and fish intake is associated with improved cognitive development in other populations.[54] TMAO is linked to inflammation in adults;[55] however, the role of TMAO in young children is unclear, and TMAO was not correlated with markers of inflammation in this study sample (Chapter 2). Few other studies have examined associations between plasma TMAO and neurodevelopment; perhaps further exploration is warranted.

Strengths of this analysis include a pre-specified statistical analysis plan, a relatively large sample size with several markers of choline metabolism, and two time points of data collection which allowed for a prospective design. Also, the study included a variety of validated developmental measures across domains. Because plasma choline was on the hypothesized causal pathway of the main trial, several developmental assessments were chosen specifically to

match domains that choline is likely to influence (attention and memory). The study also included eye tracking measures which may be more sensitive to small changes in development than measures that assess acquisition of developmental milestones. However, the validity of these eye tracking measures was established in high income settings, and there is evidence suggesting that infants in this LMIC context use different strategies to direct their visual attention compared to infants in high income countries.[34]

The study also has several weaknesses. Because of its observational design, there is risk for bias and confounding; however, this risk was minimized with the use of blinded outcome assessors, objective plasma measures, a prospective design with high rates of study follow up, and statistical adjustment for a range of covariates. Due to the multitude of statistical tests, some findings may be due to chance, although it is suggestive that both significant findings are in the direction of poorer development with higher plasma choline. Finally, plasma choline is not a sensitive biomarker across small to moderate changes in intake[56] and may not have reflected true differences in choline status. Although several choline metabolites were included as a means to better understand choline status, others such as phosphatidylcholine or sphingomyelin were not. Additionally, plasma concentrations of these nutrients may not reflect concentrations in the brain, which may be more closely related to neurodevelopment. Sensitive and specific measures of choline status, including in the brain, are needed for future studies.

## 3.6 Conclusion

Plasma choline was not associated with most measures of development in this sample of Malawian children. These findings highlight the need for more studies of postnatal choline and development in diverse settings, with improved biomarkers of choline status. Adequate intake of

choline should still be recommended for young children, as it is an essential nutrient with biological roles beyond neurodevelopment.

## 3.7 References


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Figure 3.1 – Flow diagram for this secondary analysis of plasma choline and child development among participants of the Mazira Project

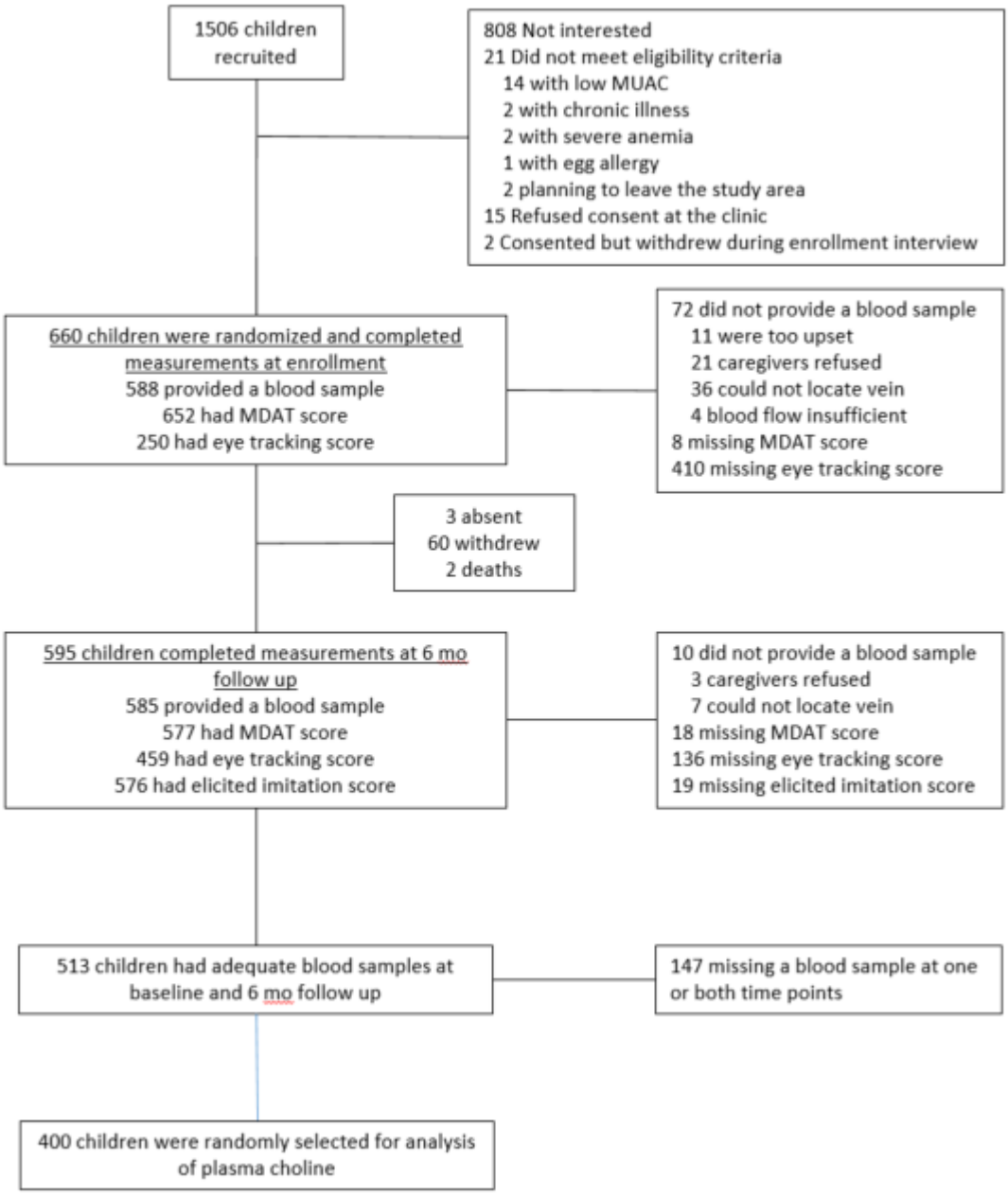

Table 3.1 – Baseline characteristics of children enrolled in the Mazira Project and included in the current secondary analysis

| | N | Mean (SD) or (%) |
|---|---|---|
| *Child and maternal characteristics* | | |
| Child age (m) | 400 | 7.4 (1.2) |
| Male (%) | 400 | 53.3 |
| First born (%) | 399 | 27.8 |
| Animal source food consumption[a] | | |
| Consumed dairy (%) | 399 | 9.8 |
| Consumed meat (%) | 399 | 2.5 |
| Consumed egg (%) | 399 | 3.8 |
| Consumed fish (%) | 399 | 23.6 |
| Any breast milk consumption (%) | 399 | 99.7 |
| Anemia prevalence (Hgb < 11 g/dL) (%) | 399 | 62.2 |
| Stunting prevalence (LAZ ≤ -2) (%) | 400 | 13.8 |
| Wasting prevalence (WLZ ≤ -2) (%) | 400 | 1.5 |
| Maternal age (y) | 400 | 26.0 (6.8) |
| Maternal BMI (kg/m2) | 400 | 21.8 (3.0) |
| Mother completed primary school (%) | 400 | 20.5 |
| | | |
| *Household characteristics* | | |
| Number of household members | 398 | 6.0 (2.6) |
| Mild to no food insecurity (%)[b] | 400 | 22.0 |
| Owns latrine (%) | 398 | 96.5 |
| Owns cows (%) | 398 | 2.5 |
| Owns goats (%) | 400 | 20.0 |
| Owns chickens (%) | 400 | 33.3 |
| HOME Inventory score | 398 | 24.0 (3.5) |
| Family Care Indicators score[c] | 400 | 7.3 (3.6) |

HOME: Home Observation Measurement of the Environment; LAZ: length-for-age z-score; WLZ: weight-for-length z-score

[a]As reported by caregiver on a 24-hour dietary recall
[b]As defined by the Household Food Insecurity Access scale[27]
[c]Measured at 6 month follow up only

Table 3.2 – Plasma concentrations and developmental scores for Mazira Project participants included in this secondary analysis of choline and development

| | Baseline | | 6 month follow up | |
|---|---|---|---|---|
| | N | Mean (SD) | N | Mean (SD) |
| Choline (µmol/L) | 60 | 17.1 (3.5) | 60 | 14.6 (3.6) |
| Betaine (µmol/L) | 60 | 85.1 (31.5) | 60 | 96.7 (34.8) |
| TMAO (µmol/L)[a] | 60 | 1.8 (0.8, 3.8) | 60 | 3.7 (2.2, 5.4) |
| Ferritin (ug/L)[b] | 364 | 19.3 (18.2) | 393 | 10.1 (11.9) |
| | | | | |
| MDAT fine motor norm z-score | 393 | 1.0 (1.3) | 386 | 0.5 (1.1) |
| MDAT gross motor norm z-score | 393 | 0.7 (0.8) | 386 | 0.7 (1.2) |
| MDAT language norm z-score | 393 | -0.002 (0.9) | 387 | 0.2 (0.8) |
| MDAT personal-social norm z-score | 393 | 1.4 (0.9) | 387 | 1.0 (1.1) |
| VPC novelty preference score (%) | 162 (508 trials) | 57 (17) | 303 (950 trials) | 60 (17) |
| VPC peak look length (s) | 164 (529 trials) | 3.9 (2.0) | 302 (931 trials) | 3.7 (2.5) |
| IOWA response time (ms) | 159 (3782 trials) | 442.5 (188.1) | 285 (7560 trials) | 403.7 (178.2) |
| Elicited Imitation actions recalled | - | - | 387 | 6.8 (3.5) |

IOWA – Infant Orienting with Attention task; MDAT – Malawi Developmental Assessment Tool; VPC – visual paired comparison task; TMAO – trimethylamine N-oxide

[a]Variable was skewed; median (IQR) is presented.

[b]Corrected for inflammation according to the BRINDA approach[37]

Table 3.3 - Minimally and fully adjusted, cross-sectional and predictive models of plasma choline concentration (in SD units) in association with measures of development among participants of the Mazira Project (n = 400)

| | Minimally Adjusted[a] | | Fully Adjusted[a] | |
|---|---|---|---|---|
| Outcome | Estimate[b] | 95% CI | Estimate[b] | 95% CI |
| MDAT fine motor norm z-score | | | | |
| *Cross-sectional model, both time points*[c] | 0.01 | -0.06, 0.08 | 0.01 | -0.05, 0.08 |
| *Predictive model*[c] | **-0.11** | **-0.21, -0.02** | **-0.13** | **-0.22, -0.04** |
| MDAT gross motor norm z-score | | | | |
| *Cross-sectional model, both time points* | -0.05 | -0.12, 0.03 | -0.05 | -0.12, 0.03 |
| *Predictive model* | -0.02 | -0.14, 0.10 | -0.01 | -0.13, 0.11 |
| MDAT personal social norm z-score | | | | |
| *Cross-sectional model, both time points* | -0.01 | -0.08, 0.05 | -0.01 | -0.07, 0.06 |
| *Predictive model* | -0.04 | -0.14, 0.05 | -0.04 | -0.14, 0.05 |
| MDAT language norm z-score | | | | |
| *Cross-sectional model, both time points* | -0.02 | -0.08, 0.04 | -0.01 | -0.07, 0.04 |
| *Predictive model* | -0.07 | -0.14, 0.01 | -0.08 | -0.16, 0.001 |
| VPC novelty preference score (%) | | | | |
| *Cross-sectional model, both time points* | 0.5 | -0.5, 1.4 | 0.5 | -0.4, 1.4 |
| *Predictive model* | 0.8 | -0.3, 1.8 | 0.6 | -0.6, 1.7 |
| VPC peak look length (s) | | | | |
| *Cross-sectional model, both time points* | -0.11 | -0.25, 0.03 | -0.10 | -0.24, 0.05 |
| *Predictive model* | 0.08 | -0.11, 0.26 | 0.10 | -0.08, 0.27 |
| IOWA response time (ms) | | | | |
| *Cross-sectional model, both time points* | **8.33** | **0.84, 15.82** | **8.84** | **1.66, 16.03** |
| *Predictive model* | 0.66 | -8.76, 10.08 | 0.46 | -8.90, 9.83 |
| Elicited Imitation actions recalled | | | | |
| *Cross-sectional model, 6 month follow up only* | 0.001 | -0.28, 0.28 | 0.06 | -0.22, 0.35 |
| *Predictive model* | -0.21 | -0.48, 0.06 | -0.18 | -0.45, 0.09 |

IOWA – Infant Orienting with Attention task; MDAT – Malawi Developmental Assessment Tool; VPC – visual paired comparison task

[a]Minimally adjusted models are adjusted for: outcome assessor; child's mood, activity level, and interaction with assessor; minutes since last meal or snack before blood draw, month of blood draw. For eye tracking variables, models also adjusted for eye tracking unit. For novelty preference, models also adjusted for familiarization fixation time. For response time, models also adjusted for

condition. For elicited imitation actions recalled, models also adjusted for spontaneous actions produced. Fully adjusted models are additionally adjusted for: child age, sex, birth order and inflammation-adjusted plasma ferritin; maternal age, BMI, and education; Family Care Indicators score, HOME Inventory, housing and asset index score, food security score.

[b]Estimates are the mean difference in the developmental outcome variable per 1 standard deviation difference in plasma choline.

[c]Cross-sectional models are generalized linear models including baseline and 6 month follow up data with child as repeated subject and robust standard errors (except elicited imitation, which was only measured at 6 month follow up; simple linear regression was used). Predictive models are simple linear regression models with baseline choline as predictor and 6 month follow up development as outcome.

Table 3.4 – Minimally adjusted cross-sectional and predictive linear models of plasma betaine, DMG, and TMAO concentrations (in SD units) as predictors of development among participants of the Mazira Project (n = 400)

| | Plasma betaine[a] | | Plasma DMG[a] | | Plasma TMAO[a] | |
|---|---|---|---|---|---|---|
| Outcome | Estimate[b] | 95% CI | Estimate[b] | 95% CI | Estimate[b] | 95% CI |
| MDAT fine motor norm z-score | | | | | | |
| *Cross-sectional model, both time points*[c] | -0.04 | -0.11, 0.03 | 0.13 | -0.02, 0.28 | 0.002 | -0.06, 0.07 |
| *Predictive model*[c] | -0.09 | -0.19, 0.002 | 0.02 | -0.18, 0.23 | -0.06 | -0.13, 0.01 |
| MDAT gross motor norm z-score | | | | | | |
| *Cross-sectional model, both time points* | -0.05 | -0.12, 0.02 | -0.04 | -0.24, 0.16 | -0.01 | -0.08, 0.06 |
| *Predictive model* | -0.03 | -0.15, 0.10 | 0.17 | -0.09, 0.42 | 0.05 | -0.04, 0.14 |
| MDAT personal social norm z-score | | | | | | |
| *Cross-sectional model, both time points* | 0.02 | -0.05, 0.08 | -0.07 | -0.22, 0.08 | 0.003 | -0.06, 0.06 |
| *Predictive model* | -0.02 | -0.11, 0.07 | -0.12 | -0.32, 0.08 | 0.04 | -0.03, 0.11 |
| MDAT language norm z-score | | | | | | |
| *Cross-sectional model, both time points* | -0.004 | -0.06, 0.05 | 0.02 | -0.10, 0.15 | -0.002 | -0.05, 0.05 |
| *Predictive model* | -0.0002 | -0.08, 0.08 | 0.08 | -0.08, 0.25 | -0.02 | -0.08, 0.03 |
| VPC novelty preference score (%) | | | | | | |
| *Cross-sectional model, both time points* | 0.6 | -0.5, 1.6 | -0.4 | -2.2, 1.3 | 0.5 | -0.5, 1.5 |
| *Predictive model* | 0.6 | -0.8, 2.0 | -1.7 | -3.8, 0.5 | -0.5 | -1.4, 0.4 |
| VPC peak look length (s) | | | | | | |
| *Cross-sectional model, both time points* | 0.09 | -0.06, 0.25 | 0.04 | -0.23, 0.30 | 0.12 | -0.01, 0.24 |
| *Predictive model* | 0.07 | -0.13, 0.27 | -0.04 | -0.38, 0.30 | **0.23** | **0.10, 0.37** |
| IOWA response time (ms) | | | | | | |
| *Cross-sectional model, both time points* | 4.30 | -4.21, 12.80 | 2.36 | -15.70, 20.43 | -4.14 | -12.41, 4.13 |
| *Predictive model* | -2.62 | -12.03, 6.79 | -6.05 | -27.20, 15.09 | -4.66 | -11.56, 2.23 |
| Elicited Imitation actions recalled | | | | | | |
| *Cross-sectional model, 6 month follow up* | -0.03 | -0.30, 0.24 | -0.47 | -1.03, 0.08 | 0.11 | -0.20, 0.41 |
| *Predictive model* | -0.16 | -0.43, 0.11 | -0.17 | -0.74, 0.41 | **0.23** | **0.03, 0.43** |

DMG – dimethylglycine; TMAO – trimethylamine N-oxide; VPC – visual paired comparison; IOWA – Infant Orienting with Attention; MDAT – Malawi Developmental Assessment Tool

[a]Minimally adjusted models are adjusted for: outcome assessor; child's mood, activity level, and interaction with assessor; minutes since last meal or snack before blood draw, month of blood draw. For eye tracking variables, models also adjusted for eye tracking

unit. For novelty preference, models also adjusted for familiarization fixation time. For response time, models also adjusted for condition. For elicited imitation actions recalled, models also adjusted for spontaneous actions produced.
[b]Estimates are the mean difference in the developmental outcome variable per 1 standard deviation difference in plasma betaine, DMG, or TMAO.
[c]Cross-sectional models are generalized linear models including baseline and 6 month follow up data with child as repeated subject and robust standard errors (except elicited imitation, which was only measured at 6 month follow up; simple linear regression was used). Predictive models are simple linear regression models with baseline metabolites as predictor and 6 month follow up development as outcome.

Table 3.5 – Stratified results from statistically significant effect modification analyses[a] of the relationship between plasma choline and development among participants of the Mazira Project

| | Minimally Adjusted | | |
|---|---|---|---|
| | Estimate[b] | 95% CI | p for interaction |
| MDAT fine motor norm z-score | | | |
| *Cross sectional model, both time points*[c] | | | |
| Housing and asset index (quintiles 2-5) | 0.05 | -0.03, 0.13 | **0.014** |
| Housing and asset index (quintile 1) | -0.15 | -0.31, 0.02 | |
| | | | |
| MDAT gross motor norm z-score | | | |
| *Predictive model* | | | |
| FCI score (above median) | **-0.21** | **-0.40, -0.02** | **0.015** |
| FCI score (below median) | 0.13 | -0.03, 0.29 | |
| Baseline gross motor z-score (above median) | 0.17 | -0.01, 0.35 | **0.029** |
| Baseline gross motor z-score (below median) | **-0.18** | **-0.35, -0.01** | |
| | | | |
| MDAT personal social norm z-score | | | |
| *Cross sectional model, both time points* | | | |
| Housing and asset index (quintiles 2-5) | 0.03 | -0.04, 0.10 | **0.011** |
| Housing and asset index (quintile 1) | **-0.16** | **-0.31, -0.02** | |
| Visit code (baseline) | 0.06 | -0.04, 0.15 | **0.014** |
| Visit code (6 month follow up) | -0.09 | -0.19, 0.01 | |
| *Predictive model* | | | |
| Baseline DHA level (above median) | **-0.15** | **-0.29, -0.01** | **0.038** |
| Baseline DHA level (below median) | 0.04 | -0.08, 0.17 | |
| | | | |
| MDAT language norm z-score | | | |
| *Cross sectional model, both time points* | | | |
| Child sex (male) | -0.08 | -0.17, 0.01 | **0.045** |
| Child sex (female) | 0.04 | -0.04, 0.12 | |
| Group assignment (Egg intervention) | 0.04 | -0.04, 0.12 | **0.017** |
| Group assignment (Control) | **-0.10** | **-0.19, -0.01** | |
| Food insecurity score (mild to none) | **-0.12** | **-0.25, -0.004** | **0.019** |
| Food insecurity score (moderate to severe) | 0.004 | -0.06, 0.07 | |
| *Predictive model* | | | |
| FCI score (above median) | **-0.16** | **-0.29, -0.04** | **0.041** |
| FCI score (below median) | 0.01 | -0.10, 0.11 | |
| | | | |
| VPC novelty preference (%) | | | |
| *Cross-sectional model, both time points* | | | |
| Baseline DHA level (above median) | **-2.7** | **-5.5, -0.01** | **0.019** |
| Baseline DHA level (below median) | **3.9** | **1.0, 6.7** | |
| | | | |

| | | | |
|---|---|---|---|
| VPC peak look length (ms) | | | |
| *Cross-sectional model, both time points* | | | |
| Baseline DHA level (above median) | **-0.23** | **-0.42, -0.04** | **0.049** |
| Baseline DHA level (below median) | 0.001 | -0.19, 0.19 | |
| | | | |
| IOWA response time (ms) | | | |
| *Cross-sectional model, both time points* | | | |
| Birth order (not firstborn) | 3.73 | -5.70, 13.17 | **0.038** |
| Birth order (firstborn) | **18.23** | **6.77, 29.69** | |
| Maternal age (over 20y) | 2.63 | -6.55, 11.81 | **0.005** |
| Maternal age (20y or younger) | **16.50** | **6.38, 26.62** | |
| Baseline IOWA response time (above median) | 2.54 | -6.77, 11.86 | **0.049** |
| Baseline IOWA response time (below median) | -3.79 | -9.73, 2.15 | |
| *Predictive model* | | | |
| Group assignment (Egg intervention) | 10.04 | -1.60, 21.67 | **0.014** |
| Group assignment (Control) | **-13.31** | **-26.09, -0.54** | |
| | | | |
| Elicited Imitation actions recalled | | | |
| *Cross-sectional model, 6 month follow up only* | | | |
| Baseline MDAT score (above median) | -0.22 | -0.64, 0.20 | **0.038** |
| Baseline MDAT score (below median) | 0.24 | -0.17, 0.65 | |
| *Predictive model* | | | |
| FCI score (above median) | **-0.55** | **-0.93, -0.16** | **0.032** |
| FCI score (below median) | 0.05 | -0.35, 0.44 | |
| Housing and asset index (quintiles 2-5) | -0.10 | -0.40, 0.20 | **0.031** |
| Housing and asset index (quintile 1) | -0.75 | -1.60, 0.10 | |

[a]Potential effect modifiers included: time point (baseline vs 6 month follow up); group assignment (intervention vs control group); child sex, birth order; child's plasma DHA and adjusted ferritin at baseline; child's LAZ and developmental score at baseline; maternal age and education; child's stimulation opportunities at home (as measured by HOME and FCI scores); household food insecurity and housing and asset index. Each modifier was made dichotomous. Stratified results are only shown above when the p-for-interaction was <0.05. All other tests were not found to be significant.

[b]Estimates are the mean difference in the developmental outcome variable per 1 standard deviation difference in plasma choline.

[c]Cross-sectional models are generalized linear models including baseline and 6 month follow up data with child as repeated subject and robust standard errors (except elicited imitation, which was only measured at 6 month follow up; simple linear regression was used). Predictive models are simple linear regression models with baseline choline as predictor and 6 month follow up development as outcome.

Supplementary Table 3.1 – Baseline characteristics of children enrolled in the Mazira Project and included vs excluded from the current secondary analysis

| | Included (n = 400) | Excluded[a] (n = 260) | |
|---|---|---|---|
| | Mean (SD) or (%) | Mean (SD) or (%) | p value |
| *Child and maternal characteristics* | | | |
| Child age (m) | 7.4 (1.2) | 7.4 (1.2) | 0.849 |
| Male (%) | 53.3 | 49.2 | 0.313 |
| First born (%) | 27.8 | 27.3 | 0.886 |
| Animal source food consumption[b] | | | |
| Consumed dairy (%) | 9.8 | 6.5 | 0.145 |
| Consumed meat (%) | 2.5 | 0.8 | 0.103 |
| Consumed egg (%) | 3.8 | 4.6 | 0.588 |
| Consumed fish (%) | 23.6 | 32.7 | **0.010** |
| Any breastmilk consumption (%) | 99.7 | 100.0 | 0.419 |
| Anemia prevalence (Hgb < 11 g/dL) (%) | 62.2 | 57.8 | 0.320 |
| Stunting prevalence (LAZ ≤ -2) (%) | 13.8 | 13.5 | 0.916 |
| Wasting prevalence (WLZ ≤ -2) (%) | 1.5 | 0.4 | 0.172 |
| Maternal age (y) | 26.0 (6.8) | 25.9 (6.7) | 0.857 |
| Maternal BMI (kg/m2) | 21.8 (3.0) | 21.9 (3.0) | 0.445 |
| Mother completed primary school (%) | 20.5 | 19.1 | 0.656 |
| | | | |
| *Household characteristics* | | | |
| Number of household members | 6.0 (2.6) | 5.7 (2.7) | 0.242 |
| Mild to no food insecurity (%)[c] | 22.0 | 22.3 | 0.926 |
| Owns latrine (%) | 96.5 | 96.3 | 0.918 |
| Owns cows (%) | 2.5 | 3.7 | 0.399 |
| Owns goats (%) | 20.0 | 17.3 | 0.388 |
| Owns chickens (%) | 33.3 | 31.0 | 0.552 |
| HOME Inventory score | 24.0 (3.5) | 24.4 (3.5) | 0.207 |
| Family Care Indicators score[d] | 7.3 (3.6) | 7.3 (3.6) | 0.860 |

HOME: Home Observation Measurement of the Environment

[a]Children enrolled in the trial were excluded from this secondary analysis if they were missing one or both blood samples (n=147) or were not randomly selected for metabolomics analysis (n=113)

[b]As reported by caregiver on a 24-hour dietary recall

[c]As defined by the Household Food Insecurity Access scale[27]

[d]Measured at 6 month follow up only

Supplementary Table 3.2 – Interaction tests from effect modification analyses of the relationship between plasma choline and development among participants of the Mazira Project

| | |
|---|---|
| | p for interaction |
| MDAT fine motor norm z-score | |
| *Cross sectional model, both time points*[a] | |
| Visit code (baseline vs 6 mo follow up) | 0.354 |
| Group (intervention vs control) | 0.843 |
| Child sex (male vs female) | 0.290 |
| Birth order (first vs not first) | 0.439 |
| Baseline DHA (below vs above median) | 0.065 |
| Baseline adjusted ferritin (below vs above <12 µg/L) | 0.914 |
| Baseline LAZ (below vs above <-1) | 0.086 |
| Baseline fine motor norm z-score (below vs above median) | 0.985 |
| Maternal age (below vs above 20y) | 0.455 |
| Maternal education (incomplete primary vs primary or greater) | 0.653 |
| FCI score (below vs above median) | 0.751 |
| HOME score (below vs above median) | 0.620 |
| Household food insecurity score (none/mild vs moderate/severe) | 0.804 |
| Household asset index quintile (quintile 1 vs 2-5) | **0.014** |
| *Predictive model*[a] | |
| Group (intervention vs control) | 0.068 |
| Child sex (male vs female) | 0.217 |
| Birth order (first vs not first) | 0.753 |
| Baseline DHA (below vs above median) | 0.477 |
| Baseline adjusted ferritin (below vs above <12 µg/L) | 0.939 |
| Baseline LAZ (below vs above <-1) | 0.901 |
| Baseline fine motor norm z-score (below vs above median) | 0.722 |
| Maternal age (below vs above 20y) | 0.401 |
| Maternal education (incomplete primary vs primary or greater) | 0.654 |
| FCI score (below vs above median) | 0.092 |
| HOME score (below vs above median) | 0.257 |
| Household food insecurity score (none/mild vs moderate/severe) | 0.553 |
| Household asset index quintile (quintile 1 vs 2-5) | 0.466 |
| | |
| MDAT gross motor norm z-score | |
| *Cross sectional model, both time points* | |
| Visit code (baseline vs 6 mo follow up) | 0.898 |
| Group (intervention vs control) | 0.874 |
| Child sex (male vs female) | 0.831 |
| Birth order (first vs not first) | 0.113 |
| Baseline DHA (below vs above median) | 0.244 |
| Baseline adjusted ferritin (below vs above <12 µg/L) | 0.737 |

| | |
|---|---|
| Baseline LAZ (below vs above <-1) | 0.900 |
| Baseline gross motor norm z-score (below vs above median) | 0.758 |
| Maternal age (below vs above 20y) | 0.804 |
| Maternal education (incomplete primary vs primary or greater) | 0.862 |
| FCI score (below vs above median) | 0.337 |
| HOME score (below vs above median) | 0.625 |
| Household food insecurity score (none/mild vs moderate/severe) | 0.760 |
| Household asset index quintile (quintile 1 vs 2-5) | 0.146 |
| *Predictive model* | |
| Group (intervention vs control) | 0.253 |
| Child sex (male vs female) | 0.463 |
| Birth order (first vs not first) | 0.624 |
| Baseline DHA (below vs above median) | 0.715 |
| Baseline adjusted ferritin (below vs above <12 µg/L) | 0.643 |
| Baseline LAZ (below vs above <-1) | 0.502 |
| Baseline gross motor norm z-score (below vs above median) | **0.029** |
| Maternal age (below vs above 20y) | 0.436 |
| Maternal education (incomplete primary vs primary or greater) | 0.664 |
| FCI score (below vs above median) | **0.015** |
| HOME score (below vs above median) | 0.851 |
| Household food insecurity score (none/mild vs moderate/severe) | 0.903 |
| Household asset index quintile (quintile 1 vs 2-5) | 0.156 |
| | |
| MDAT personal social norm z-score | |
| *Cross sectional model, both time points* | |
| Visit code (baseline vs 6 mo follow up) | **0.014** |
| Group (intervention vs control) | 0.616 |
| Child sex (male vs female) | 0.572 |
| Birth order (first vs not first) | 0.258 |
| Baseline DHA (below vs above median) | 0.544 |
| Baseline adjusted ferritin (below vs above <12 µg/L) | 0.796 |
| Baseline LAZ (below vs above <-1) | 0.685 |
| Baseline personal social norm z-score (below vs above median) | 0.481 |
| Maternal age (below vs above 20y) | 0.730 |
| Maternal education (incomplete primary vs primary or greater) | 0.503 |
| FCI score (below vs above median) | 0.598 |
| HOME score (below vs above median) | 0.712 |
| Household food insecurity score (none/mild vs moderate/severe) | 0.425 |
| Household asset index quintile (quintile 1 vs 2-5) | **0.011** |
| *Predictive model* | |
| Group (intervention vs control) | 0.943 |
| Child sex (male vs female) | 0.921 |
| Birth order (first vs not first) | 0.554 |
| Baseline DHA (below vs above median) | **0.038** |
| Baseline adjusted ferritin (below vs above <12 µg/L) | 0.074 |

| | |
|---|---|
| Baseline LAZ (below vs above <-1) | 0.129 |
| Baseline personal social norm z-score (below vs above median) | 0.131 |
| Maternal age (below vs above 20y) | 0.324 |
| Maternal education (incomplete primary vs primary or greater) | 0.363 |
| FCI score (below vs above median) | 0.142 |
| HOME score (below vs above median) | 0.087 |
| Household food insecurity score (none/mild vs moderate/severe) | 0.275 |
| Household asset index quintile (quintile 1 vs 2-5) | 0.298 |
| | |
| MDAT language norm z-score | |
| *Cross sectional model, both time points* | |
| Visit code (baseline vs 6 mo follow up) | 0.848 |
| Group (intervention vs control) | **0.017** |
| Child sex (male vs female) | **0.045** |
| Birth order (first vs not first) | 0.897 |
| Baseline DHA (below vs above median) | 0.606 |
| Baseline adjusted ferritin (below vs above <12 µg/L) | 0.958 |
| Baseline LAZ (below vs above <-1) | 0.373 |
| Baseline language norm z-score (below vs above median) | 0.122 |
| Maternal age (below vs above 20y) | 0.131 |
| Maternal education (incomplete primary vs primary or greater) | 0.488 |
| FCI score (below vs above median) | 0.120 |
| HOME score (below vs above median) | 0.500 |
| Household food insecurity score (none/mild vs moderate/severe) | **0.019** |
| Household asset index quintile (quintile 1 vs 2-5) | 0.786 |
| *Predictive model* | |
| Group (intervention vs control) | 0.918 |
| Child sex (male vs female) | 0.855 |
| Birth order (first vs not first) | 0.963 |
| Baseline DHA (below vs above median) | 0.453 |
| Baseline adjusted ferritin (below vs above <12 µg/L) | 0.075 |
| Baseline LAZ (below vs above <-1) | 0.597 |
| Baseline language norm z-score (below vs above median) | 0.179 |
| Maternal age (below vs above 20y) | 0.199 |
| Maternal education (incomplete primary vs primary or greater) | 0.691 |
| FCI score (below vs above median) | **0.041** |
| HOME score (below vs above median) | 0.548 |
| Household food insecurity score (none/mild vs moderate/severe) | 0.476 |
| Household asset index quintile (quintile 1 vs 2-5) | 0.762 |
| | |
| Novelty preference score | |
| *Cross sectional model, both time points* | |
| Visit code (baseline vs 6 mo follow up) | 0.513 |
| Group (intervention vs control) | 0.351 |
| Child sex (male vs female) | 0.553 |

| | |
|---|---|
| Birth order (first vs not first) | 0.979 |
| Baseline DHA (below vs above median) | **0.019** |
| Baseline adjusted ferritin (below vs above <12 µg/L) | 0.899 |
| Baseline LAZ (below vs above <-1) | 0.352 |
| Baseline novelty preference score (below vs above median) | 0.652 |
| Maternal age (below vs above 20y) | 0.810 |
| Maternal education (incomplete primary vs primary or greater) | 0.362 |
| FCI score (below vs above median) | 0.936 |
| HOME score (below vs above median) | 0.836 |
| Household food insecurity score (none/mild vs moderate/severe) | 0.862 |
| Household asset index quintile (quintile 1 vs 2-5) | 0.690 |
| *Predictive model* | |
| Group (intervention vs control) | 0.732 |
| Child sex (male vs female) | 0.072 |
| Birth order (first vs not first) | 0.793 |
| Baseline DHA (below vs above median) | 0.633 |
| Baseline adjusted ferritin (below vs above <12 µg/L) | 0.366 |
| Baseline LAZ (below vs above <-1) | 0.336 |
| Baseline novelty preference score (below vs above median) | 0.236 |
| Maternal age (below vs above 20y) | 0.401 |
| Maternal education (incomplete primary vs primary or greater) | 0.709 |
| FCI score (below vs above median) | 0.779 |
| HOME score (below vs above median) | 0.648 |
| Household food insecurity score (none/mild vs moderate/severe) | 0.962 |
| Household asset index quintile (quintile 1 vs 2-5) | 0.582 |
| | |
| VPC peak look length | |
| *Cross sectional model, both time points* | |
| Visit code (baseline vs 6 mo follow up) | 0.081 |
| Group (intervention vs control) | 0.302 |
| Child sex (male vs female) | 0.645 |
| Birth order (first vs not first) | 0.078 |
| Baseline DHA (below vs above median) | **0.049** |
| Baseline adjusted ferritin (below vs above <12 µg/L) | 0.637 |
| Baseline LAZ (below vs above <-1) | 0.780 |
| Baseline peak look length (below vs above median) | 0.703 |
| Maternal age (below vs above 20y) | 0.239 |
| Maternal education (incomplete primary vs primary or greater) | 0.833 |
| FCI score (below vs above median) | 0.579 |
| HOME score (below vs above median) | 0.732 |
| Household food insecurity score (none/mild vs moderate/severe) | 0.441 |
| Household asset index quintile (quintile 1 vs 2-5) | 0.359 |
| *Predictive model* | |
| Group (intervention vs control) | 0.757 |
| Child sex (male vs female) | 0.969 |

| | |
|---|---|
| Birth order (first vs not first) | 0.116 |
| Baseline DHA (below vs above median) | 0.843 |
| Baseline adjusted ferritin (below vs above <12 µg/L) | 0.672 |
| Baseline LAZ (below vs above <-1) | 0.241 |
| Baseline peak look length (below vs above median) | 0.801 |
| Maternal age (below vs above 20y) | 0.196 |
| Maternal education (incomplete primary vs primary or greater) | 0.898 |
| FCI score (below vs above median) | 0.118 |
| HOME score (below vs above median) | 0.544 |
| Household food insecurity score (none/mild vs moderate/severe) | 0.197 |
| Household asset index quintile (quintile 1 vs 2-5) | 0.707 |
| | |
| IOWA response time | |
| *Cross sectional model, both time points* | |
| Visit code (baseline vs 6 mo follow up) | 0.475 |
| Group (intervention vs control) | 0.825 |
| Child sex (male vs female) | 0.670 |
| Birth order (first vs not first) | 0.038 |
| Baseline DHA (below vs above median) | 0.675 |
| Baseline adjusted ferritin (below vs above <12 µg/L) | 0.514 |
| Baseline LAZ (below vs above <-1) | 0.084 |
| Baseline response time (below vs above median) | **0.049** |
| Maternal age (below vs above 20y) | **0.005** |
| Maternal education (incomplete primary vs primary or greater) | 0.285 |
| FCI score (below vs above median) | 0.953 |
| HOME score (below vs above median) | 0.394 |
| Household food insecurity score (none/mild vs moderate/severe) | 0.807 |
| Household asset index quintile (quintile 1 vs 2-5) | 0.760 |
| *Predictive model* | |
| Group (intervention vs control) | **0.014** |
| Child sex (male vs female) | 0.264 |
| Birth order (first vs not first) | 0.920 |
| Baseline DHA (below vs above median) | 0.501 |
| Baseline adjusted ferritin (below vs above <12 µg/L) | 0.651 |
| Baseline LAZ (below vs above <-1) | 0.243 |
| Baseline response time (below vs above median) | 0.469 |
| Maternal age (below vs above 20y) | 0.821 |
| Maternal education (incomplete primary vs primary or greater) | 0.893 |
| FCI score (below vs above median) | 0.485 |
| HOME score (below vs above median) | 0.958 |
| Household food insecurity score (none/mild vs moderate/severe) | 0.849 |
| Household asset index quintile (quintile 1 vs 2-5) | 0.714 |
| | |
| Elicited imitations total actions recalled | |
| *Cross sectional model, 6 month follow up only* | |

| | |
|---|---|
| Group (intervention vs control) | 0.701 |
| Child sex (male vs female) | 0.229 |
| Birth order (first vs not first) | 0.813 |
| Baseline DHA (below vs above median) | 0.451 |
| Baseline adjusted ferritin (below vs above <12 µg/L) | 0.058 |
| Baseline LAZ (below vs above <-1) | 0.298 |
| Baseline actions recalled score (below vs above median) | **0.038** |
| Maternal age (below vs above 20y) | 0.511 |
| Maternal education (incomplete primary vs primary or greater) | 0.177 |
| FCI score (below vs above median) | 0.503 |
| HOME score (below vs above median) | 0.400 |
| Household food insecurity score (none/mild vs moderate/severe) | 0.289 |
| Household asset index quintile (quintile 1 vs 2-5) | 0.819 |
| *Predictive model* | |
| Group (intervention vs control) | 0.059 |
| Child sex (male vs female) | 0.193 |
| Birth order (first vs not first) | 0.462 |
| Baseline DHA (below vs above median) | 0.570 |
| Baseline adjusted ferritin (below vs above <12 µg/L) | 0.736 |
| Baseline LAZ (below vs above <-1) | 0.217 |
| Baseline actions recalled score (below vs above median) | 0.984 |
| Maternal age (below vs above 20y) | 0.491 |
| Maternal education (incomplete primary vs primary or greater) | 0.190 |
| FCI score (below vs above median) | **0.032** |
| HOME score (below vs above median) | 0.051 |
| Household food insecurity score (none/mild vs moderate/severe) | 0.734 |
| Household asset index quintile (quintile 1 vs 2-5) | **0.031** |

[a]Cross-sectional models are generalized linear models including baseline and 6 month follow up data with child as repeated subject and robust standard errors (except elicited imitation, which was only measured at 6 month follow up; simple linear regression was used). Predictive models are simple linear regression models with baseline metabolite as predictor and 6 month follow up development as outcome. Each dichotomous effect modifier was tested in cross-sectional and predictive models, except visit code, which was only tested in models which included both visits.

# Chapter 4: Association between plasma choline and growth among Malawian children age 6-15 months enrolled in an egg intervention trial

## 4.1 Abstract

Choline is an essential micronutrient that may influence child growth and affect risk of stunting; however, there are few studies investigating early postnatal choline status and children's growth, especially in low and middle income countries. The aim of this observational analysis was to examine the cross-sectional and predictive associations between plasma choline and measures of growth among young children age 6-15 months in rural Malawi using data from an egg intervention trial. Plasma choline and three of its metabolites (betaine, dimethylglycine, and trimethylamine N-oxide) were measured at baseline and 6 month follow up. Anthropometrics (length, weight, head circumference) were measured at baseline, 3 month follow up, and 6 month follow up. World Health Organization Growth Standards were used to convert values to z-scores. The association of plasma choline (in SD units) and its metabolites with anthropometric measures was tested using cross-sectional generalized linear models with both time points and prospective linear regression models with baseline plasma choline values as the independent variable. Plasma choline was positively associated with weight-for-length z-score in minimally adjusted but not fully adjusted cross-sectional models, and negatively associated with length-for-age z-score (-0.09 SD [95% CI -0.17, -0.01]) in the fully adjusted cross-sectional model. There were no other significant associations between plasma choline or its metabolites and child growth. More research is needed on the role of choline for child growth in varied contexts; future studies will require improved biomarkers and more rigorous study designs.

## 4.2 Introduction

Globally, more than 20% of children under 5y were stunted in 2019, mostly in low and middle income countries (LMICs),[1] putting these children at risk for poor health outcomes and decreased adult productivity.[2] Stunting, or low height-for-age, often develops during the 'first 1000 days' from conception to 2y.[3] Many factors affect risk for stunting, including poor nutrition, chronic illness or infection, and environmental conditions.[4]

Choline is an essential micronutrient that may affect risk of stunting,[5] perhaps through its role as a precursor for important metabolites including phosphatidylcholine, acetylcholine, trimethylamine N-oxide (TMAO), betaine and dimethylglycine (DMG). Because choline is mainly found in animal source foods, which are relatively expensive,[6] intake is likely suboptimal in many LMICs.[7] For young children, breastmilk is a rich source of choline, although concentrations vary with maternal diet.[8] As children in LMICs transition from breastmilk to traditional complementary foods, they may be at risk for low choline intake. This is especially crucial because the complementary feeding period is a period of rapid growth, with stunting often occurring during this time.[9]

There are few studies investigating early postnatal choline status and children's growth, especially in LMICs.[10] However, in a cross-sectional study of Malawian children age 12-59 months, Semba et al found significantly lower serum choline levels among stunted children compared to non-stunted children.[5] Also, there was a significant positive association between serum choline levels and children's height, such that a 1 SD difference in serum choline was associated with a 0.41 cm difference in height.[5] In Brazil, an observational study of children age 6-24 months found a negative association between stunting and urinary levels of betaine and

DMG, suggesting altered choline metabolism among stunted children.[11] Further studies are needed to replicate these findings.

Two studies have examined the effect of eggs, a rich source of choline, on child growth. A randomized trial in Ecuador found that the provision of one egg per day for 6 months to 6-15 month old children significantly reduced the risk of stunting, an effect that was partially mediated by differences in plasma choline concentrations.[12,13] However, a similarly designed trial in Malawi did not observe an effect of eggs on child growth or risk of stunting.[14]

To explore potential reasons for the null effect of the egg intervention in Malawi and to understand the relationship between choline and child growth, we conducted a secondary analysis of data from children in the Malawi trial. The aim of this analysis was to examine the cross-sectional and predictive associations between plasma choline and measures of child growth (height, weight, and head circumference) during the early complementary feeding period (6-15 months) in rural Malawi. We hypothesized that plasma choline would be positively associated with child anthropometric measures across the 6 month study period. In exploratory analyses, we also tested the effect of several potential effect modifiers, as well as the association between choline metabolites (plasma betaine, DMG, and TMAO) and child growth.

## 4.3 Methods

The Mazira Project randomized controlled trial (clinicaltrials.gov: NCT03385252) investigated the effect of providing one egg per day versus a nonintervention control among 660 rural Malawian children. From February 2018 to January 2019, children age 6-9 months were individually randomized to intervention or control for six months. Both groups received weekly

home visits, where study staff provided information about food hygiene and handwashing. In addition, the intervention group received locally produced eggs, and caregivers were asked to feed one per day to the study child. Caregivers in the control group were asked to continue feeding their child as they typically would.

*Participants*

Children and their caregivers residing in the catchment areas of two health centers (Lungwena Health Center and St. Martins Rural Hospital) in Malawi were recruited at household visits, community meetings, and local football tournaments. Children were excluded for: family plans to leave the study area within 6 months; egg allergy or history of serious allergic reactions; congenital conditions which could affect growth and development; severe anemia (hemoglobin <5 g/dL), low mid-upper arm circumference (<12.5cm), or bipedal edema; or acute illness/injury warranting hospital referral.

*Data collection*

Detailed descriptions of data collection for this trial have been previously published.[14] Briefly, children and caregivers came to the study site at enrollment and at the end of the study 6 months later. At both times, staff collected anthropometric, dietary, demographic and developmental data, as well as a blood sample, which was used to measure hemoglobin concentration (Hemocue 201, HemoCue Inc., Angelholm, Sweden) and test for malaria (DF Bioline Malaria Ag P.f/Pan, Abbott Diagnostics, Lake Forest, IL).

Other data were collected during home visits. Soon after enrollment, staff administered the Household Food Insecurity Access Scale[15] questionnaire and collected data on housing materials and animal ownership for incorporation into a housing and asset index. After 3 months

of enrollment, study staff collected anthropometric and dietary data during a home visit. Throughout the study, caregivers reported weekly on child morbidity symptoms, including the number of days with diarrhea. The longitudinal prevalence of diarrhea was calculated as the number of days with reported diarrhea divided by the total number of days of recall.

*Anthropometric Measures*

Trained and standardized pairs of anthropometrists measured children's recumbent length (in cm) using a Holtain length board, weight (in kg) using a Seca 874 digital scale, and head circumference (in cm) using insertion tapes (Health Books International at enrollment and Seca model 212 at 6 month follow up). World Health Organization Growth Standards were used to convert values to z-scores (length-for-age [LAZ], weight-for-age [WAZ], weight-for-length [WLZ], and head circumference-for-age [HCAZ]).[16]

In addition to continuous z-scores, conditional and dichotomous measures were calculated, in line with best practices for linear growth analyses.[17] To calculate conditional variables, anthropometric data collected at 6 month follow up was regressed on data from enrollment and 3 month follow up.[18] The residuals reflect how each child's growth over the 6 month study period differed from what would be expected based on their initial growth status compared to the other study participants. A positive value reflects comparatively faster growth (or slower faltering); a negative value reflects comparatively slower growth (or quicker faltering). Because the insertion tapes were changed during the study, a conditional measure of head circumference was not included. Dichotomous outcomes were defined as being above or below a cutoff: stunted ($LAZ \leq -2$), wasted ($WLZ \leq -2$), underweight ($WAZ \leq -2$), or small head circumference ($HCAZ \leq -2$).

*Plasma Metabolites*

Staff collected a sample of children's venous blood into lithium heparin tubes at enrollment and 6 month follow up. Within a mean 28 (SD 42) minutes of collection, samples were centrifuged; 37 (SD 14) minutes afterward, aliquots were stored in the local freezer at -20°C. Each afternoon, aliquots were transported on ice to the long-term freezers at -80°C.

The measurement of plasma choline in this study has been described elsewhere (Chapter 2). Briefly, plasma choline was measured at baseline and 6 month follow up using two methods. First, ultra high performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) was used to measure the relative intensity of plasma choline, betaine, DMG, TMAO and other metabolites among 400 children. This semi quantitative measurement provided data on the relative distribution of plasma metabolites, and was used for regression analyses; however, the data are in relative intensity units. Second, liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to measure the absolute concentration of plasma choline, betaine, and TMAO among a subsample of 60 children. DMG was not included in the LC-MS/MS analysis. This measurement provided quantitative data, which can be compared to other studies. Plasma concentrations using the two methods were well correlated (choline: r=0.92, betaine: r=0.98, TMAO: r=0.98) (Chapter 2).

Other measured biomarkers include plasma leucine, C-reactive protein (CRP), alpha(1)-acid glycoprotein (AGP), and zinc. Plasma leucine was included in the semi quantitative UPLC-MS/MS analysis and is in relative intensity units. CRP and AGP were measured by the VitMin lab using enzyme-linked immunoassay.[19] Plasma zinc was measured at Washington University in St Louis using inductively coupled plasma mass spectrometry.

*Sample Size*

The original trial included 660 children, based on a sample size calculation for difference in LAZ between groups after 6 months. Of the children who provided adequate blood samples at enrollment and 6 month follow up, 400 (200 per group) were randomly chosen for the semi quantitative UPLC-MS/MS analysis, which allows for detection of correlations between plasma metabolites and growth outcomes as small as 0.14 with 80% power and a 2 sided alpha of 0.05.

*Statistical Analysis*

A statistical analysis plan was developed and shared publicly prior to analysis (https://osf.io/sz7e5/). For each continuous and dichotomous outcome, growth at both time points (enrollment, 6 month follow up) was assessed in a generalized linear model with plasma choline at the corresponding time point as predictor. To account for repeated measures, robust standard errors were used and participant was listed as the independent unit. Each conditional growth outcome was examined in a linear regression model, with baseline plasma choline as a predictor. Each model was assessed for linearity, outliers, and normality and homoscedasticity of residuals. Children with missing anthropometric or plasma choline values were not included.

Minimally adjusted models included covariates related to data collection and study design: time of last meal before blood draw, calendar month of blood draw, anthropometrist, group assignment, and time point of data collection (for models that contained multiple time points).

Fully adjusted models included pre-specified covariates collected at baseline, including: child age, sex, and birth order; plasma leucine and zinc; plasma inflammatory markers (CRP and AGP); longitudinal prevalence of diarrhea; maternal age, height, and education category;

household asset index and food insecurity score. To enter the model, variables must have been associated with the growth outcome with $p<0.1$.

In exploratory analyses, the following characteristics were assessed as potential effect modifiers by inclusion of an interaction term: child sex, baseline stunting status, longitudinal prevalence of diarrhea, baseline maternal age, and baseline housing and asset index. Each potential effect modifier was made dichotomous. Additionally, time point of data collection (for models with multiple time points) and group assignment were assessed as potential effect modifiers, and if significant, stratified results were reported. To limit the number of tests, these effect modifiers were only included in minimally adjusted models of choline and growth.

Additional exploratory analyses tested the association of choline metabolites betaine, DMG, and TMAO with growth in minimally adjusted models, using similar methods as described for plasma choline. All analyses used two-sided tests with an alpha of 0.05. Given the large number of exploratory tests, significant p-values should be interpreted cautiously.

## 4.4 Results

### *Participant characteristics*

A total of 400 children were included in this analysis (**Fig 1**), distributed equally across the sexes (**Table 1**). Rates of breastfeeding were very high, while consumption of animal source foods, except fish, was low. The mean longitudinal prevalence of diarrhea was 0.1 (SD 0.1), meaning on average children were reported to have diarrhea on 10% of recall days. The majority of households reported moderate to severe food insecurity.

Compared to children in the main trial who were excluded from this analysis (n=260), a smaller percentage of children who were included in this analysis consumed any fish at baseline (32.7% vs 23.6, p =0.01) **(Supplemental Table 1**). Otherwise, there were no significant differences between those included and excluded.

Plasma choline decreased with age (n=400, **Fig 2**) and over the study period (n=60, **Table 2**), from a mean (SD) of 17.1 (3.5) µmol/L at baseline to 14.6 (3.6) µmol/L at 6 month follow up. Plasma betaine and TMAO increased with age and across the study period.

The mean LAZ was -0.9 at enrollment and decreased to -1.1 by the 6 month follow up. Stunting was common, ranging from 13.8% at 6-9 months to 28.5% at 9-12 months. The mean WAZ remained relatively constant, while the mean WLZ decreased slightly over the study period. Because of the low prevalence of wasting, this outcome was not included in models of dichotomous outcomes.

*Association between plasma choline, its metabolites, and growth*

Plasma choline was positively associated with WLZ in the minimally adjusted model, but the association was attenuated and no longer statistically significant in the fully adjusted model (**Table 3**). There was a weak but significant negative association between plasma choline and LAZ in the fully adjusted model No other associations between choline and measures of growth were apparent. There were no significant associations of betaine, DMG, or TMAO with growth indicators (**Table 4**).

*Effect modification analyses*

Several variables were tested as potential effect modifiers of the relationship between plasma choline and child growth outcomes. Out of 67 tests (7 effect modifiers with 4 continuous

and 3 dichotomous growth outcomes; 6 effect modifiers with 3 conditional outcomes (**Supplementary Table 2)**), 3 were significant at the 0.05 level (4.5%), which is near that expected by chance (5%) (**Table 5**). Among children in the lowest quintile of the housing and asset index, plasma choline was negatively associated with continuous LAZ; however, there was no association between choline and LAZ among children in other quintiles. Among females, baseline plasma choline was positively associated with conditional LAZ; there was no association between choline and conditional LAZ among males. Finally, among children with a longitudinal prevalence of diarrhea below the median, plasma choline was positively associated with continuous WLZ; however, there was no association between plasma choline and WLZ among children with higher longitudinal prevalence of diarrhea.

## 4.5 Discussion

Contrary to our hypothesis, in this secondary analysis, plasma choline was not associated with measures of child growth, except a small but significant negative association with LAZ. Plasma betaine, DMG, and TMAO were also not associated with child growth.

These null findings are important for understanding the results of the Mazira Project randomized trial, in which there was no impact of an egg intervention on linear growth.[14] In the Mazira Project, the intervention was hypothesized to improve growth in part via improvement in choline status, as plasma choline partially mediated the improvement in growth in a similar egg intervention trial in Ecuador.[13] The lack of effect in Malawi could have been due to a break in one or both of the links in the potential biologic pathway: eggs did not cause a change in plasma choline and/or plasma choline was not associated with growth in this context. Plasma choline was not improved by the egg intervention trial in Malawi (Chapter 2), negating one portion of

the hypothesized pathway to improved growth. In the present analysis, we found no connection between plasma choline and children's growth, negating the other portion of the hypothesized causal pathway.

These findings also suggest that the relationship between plasma choline and growth may vary in different settings and populations, as they are in direct contrast to the study by Semba et al. In the cross sectional study of Malawian children by Semba et al, there was a significant positive association between serum choline and height-for-age z-score, and a negative association between serum choline and stunting.[5] This contrast may be due to differences in the study population. First, although these studies were conducted in the same country, stunting rates varied widely, with more than 60% of children classified as stunted in Semba et al, and <20% in the Mazira Project at baseline. This may reflect the decreasing national stunting rates in Malawi over time,[20] as well as regional differences in stunting rates. With less stunting in the current study, there could be lower potential for choline to beneficially affect growth. Second, the age of the children differed. In Semba et al, children were 12-59 months of age, whereas in the current analysis, children were 6-15 months. It is possible the role of choline in growth varies by age, especially considering the majority of growth faltering occurs before age 2 years.[3] Third, choline concentrations varied by study. In Semba et al, the median serum choline concentration was ~6 µmol/L, whereas in the Mazira Project, plasma choline was ~17 µmol/L at baseline. This may be due to differences in children's age. Plasma choline is high at birth and decreases over the first years of life to adult levels.[21] Without age-matched plasma choline values, it is difficult to compare the choline status of the two populations. We do not have information about choline intake in Semba et al; however, estimated choline intake was very low in the Mazira Project, even with the egg intervention and high prevalence of breastfeeding.[22] Fourth, prevalence of

diarrhea likely differed, as diarrhea was an exclusion criterion in Semba et al, and there was a 10% longitudinal prevalence in the Mazira Project. Chronic diarrhea affects child growth and risk for stunting and wasting,[23] and it is plausible that diarrhea could affect choline absorption or metabolism in the gut via the microbiota. In the current analysis, plasma choline was positively associated with WLZ among children with lower diarrhea prevalence. Finally, the two populations were located in different parts of the country. Regional differences could contribute to differences in sociodemographic factors, such as maternal education or household assets, which could affect children's growth.

Choline is hypothesized to affect growth through several potential mechanisms. First, choline can be used to synthesize phosphatidylcholine, a phospholipid required for the formation of cell membranes, as well as fat absorption and transport. Phosphatidylcholine is also a major component of surfactant in the lung[24] and the mucus layer in the gut.[25] Rodent models provide support for the importance of phosphatidylcholine in growth. Rodents without choline kinase beta, an enzyme on the pathway from choline to phosphatidylcholine, have altered endochondral bone growth with shortened forelimbs.[26,27] Additionally, maternal phosphatidylcholine intake affects offspring immune development in rodents,[28] which may contribute to optimal growth. Choline can also be converted to acetylcholine, oxidized to betaine, or metabolized by gut microbiota to TMAO. There is less evidence for a role of these metabolites in growth, although a few studies are suggestive. In rodent knockout models, a lack of acetylcholine in the perinatal period reduced circulating levels of growth hormone and insulin-like growth factor 1.[29] Betaine is a methyl donor with epigenetic effects; however, these effects may not promote improved growth, as maternal and umbilical betaine levels have been negatively associated with birth

weight.[30,31] Although TMAO is related to atherosclerosis and inflammation in adults,[32] its effects on growth in young children are unclear.

In the current setting, plasma choline was not associated with child growth. This could be a true null association, or may be due to limitations of plasma choline as a marker of choline status. Plasma choline is an imperfect biomarker which may not reflect small to moderate changes in intake.[33] Currently, sensitive and specific markers of choline status are lacking, although this is an active area of research.[34] We included several related metabolites (betaine, DMG, and TMAO) as a means to investigate choline status, but other metabolites, such as phosphatidylcholine, were not included. Although phosphatidylcholine may be important for bone growth, its measurement is affected by fat transport and metabolism. Future studies with improved biomarkers of choline status may help uncover the relationship between choline and growth. If this is a true null association, additional interventions may be needed to improve children's growth in this setting. These interventions may include provision of different nutrients of interest or may focus on lessening other constraints, such as illness and poverty.

This analysis had several strengths. First, the large sample size allowed for detection of even weak associations. Second, measurement of anthropometry at three time points and plasma choline at two time points allowed for longitudinal analysis. The current analysis builds on prior studies by including predictive, as well as cross-sectional analyses. Third, several measures of anthropometry were included, with pre-specified analysis guided by best practices. This study also had weaknesses. As a secondary analysis, this observational study used data from a trial that was not specifically designed to test the association between plasma choline and growth. As such, it is possible that the results are confounded, although we controlled for an extensive list of potential confounders. These findings are correlations, and statements of causation cannot be

made. Also, given the inconsistencies in findings relating choline and growth even within Malawi, the generalizability of this study is likely limited to LMIC settings with similar anthropometric, dietary, and social characteristics.

## 4.6 Conclusion

In this observational study, plasma choline and its metabolites were not related to most measures of growth among a sample of young Malawian children. More information is needed on the role of choline for child growth in varied contexts; future studies will require improved biomarkers and more rigorous study designs. Adequate intake of choline should still be recommended for young children, as it is an essential nutrient with multiple roles throughout the body.

4.7 References


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Figure 4.1 - Study flow diagram for this secondary analysis of plasma choline and child growth among young Malawian children

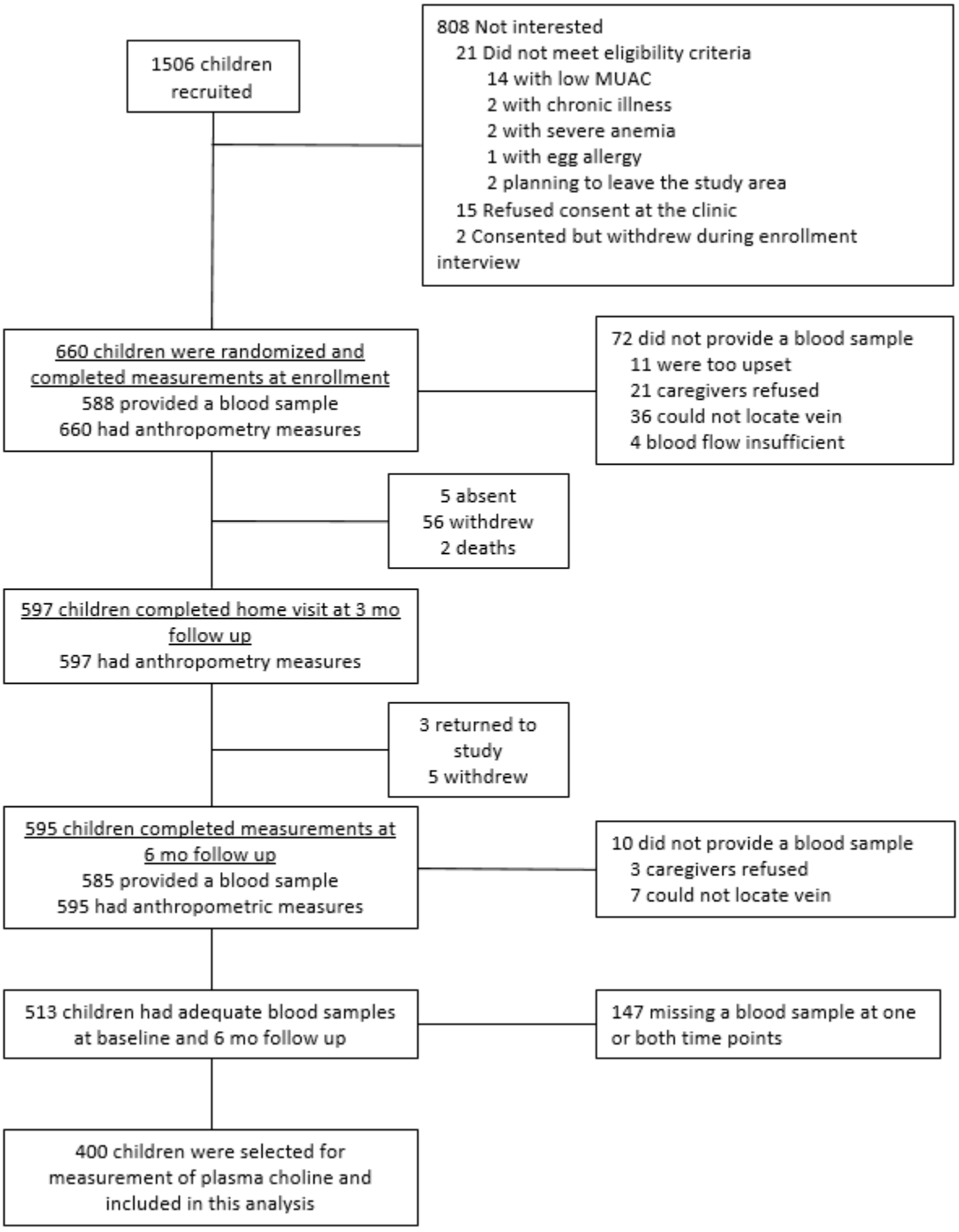
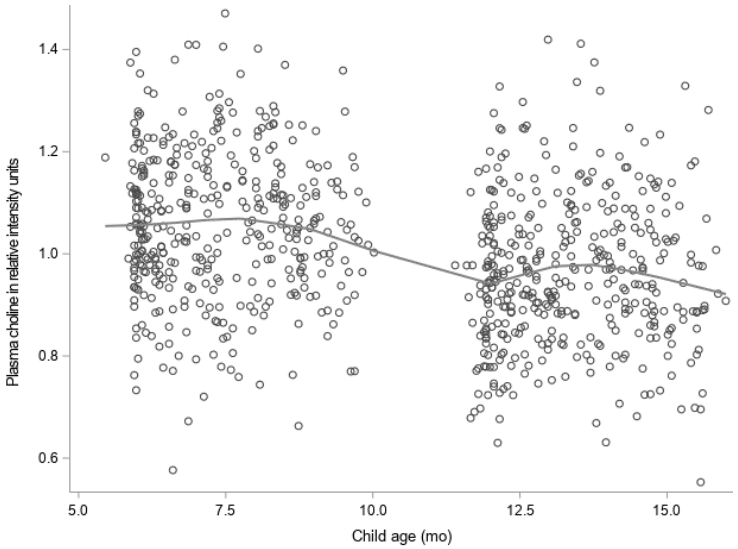
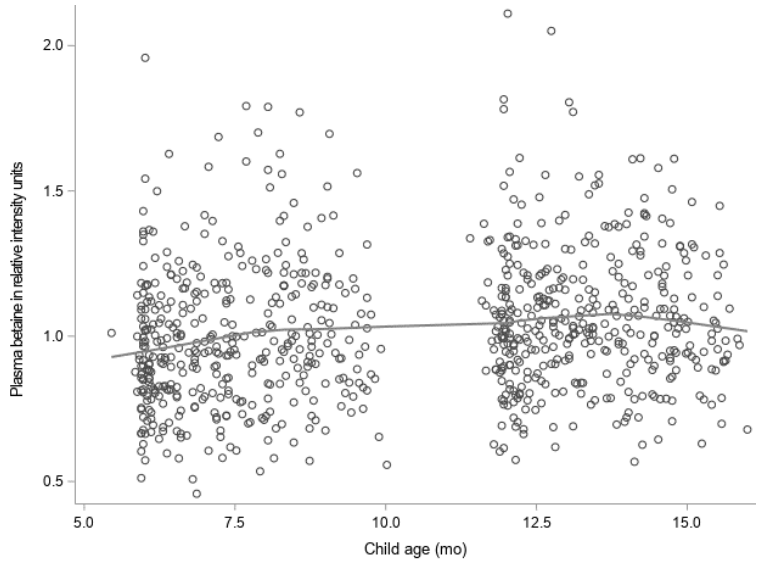


Figure 4.2 - Plasma choline (A), betaine (B), dimethylglycine (C), and trimethylamine N-oxide (D) by child age among children enrolled in the Mazira Project (n=400)[a]

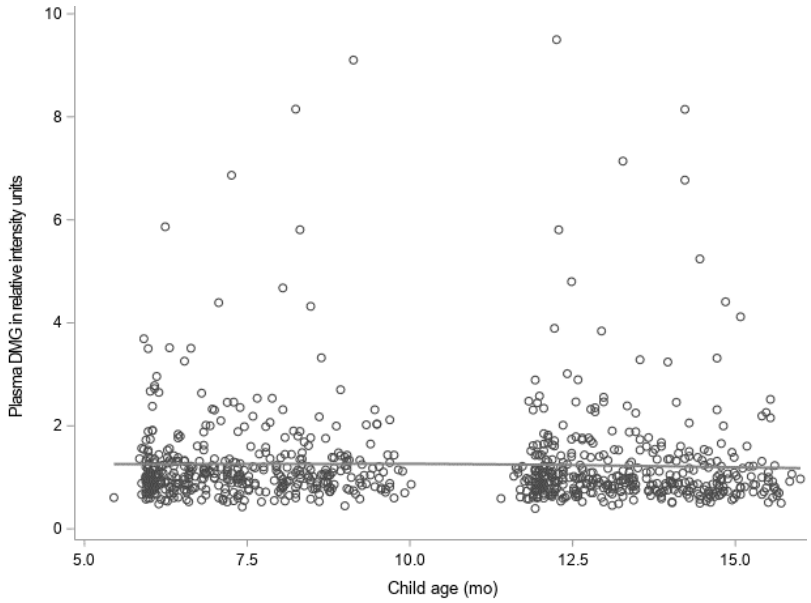
a) Choline



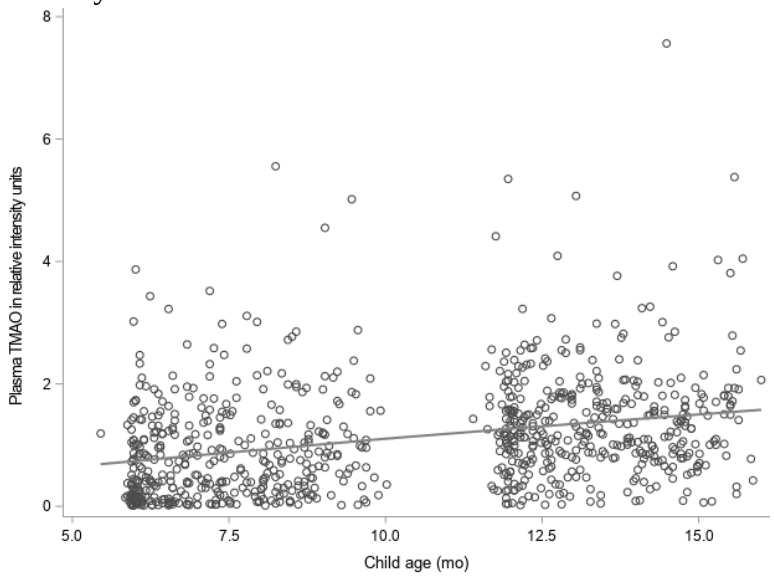

b) Betaine




c) Dimethylglycine




d) Trimethylamine N-oxide




[a] Lines are loess curves.

Table 4.1 – Baseline characteristics of children enrolled in the Mazira Project and included in this secondary analysis of plasma choline and growth (n=400)

| | Mean (SD) or (%) |
|---|---|
| *Child and maternal characteristics* | |
| Child age (mo) | 7.4 (1.2) |
| Male *(%)* | 53.3 |
| First born *(%)* | 27.8 |
| Animal source food consumption *(%)*[a] | |
| Consumed dairy | 9.8 |
| Consumed meat | 2.5 |
| Consumed egg | 3.8 |
| Consumed fish | 23.6 |
| Any breastmilk *(%)* | 99.7 |
| Anemia prevalence (Hgb < 11 g/dL) *(%)* | 62.2 |
| Malaria prevalence *(%)* | 14.5 |
| Longitudinal prevalence of diarrhea (proportion of days) | 0.1 (0.1) |
| Maternal age (y) | 26.0 (6.8) |
| Maternal BMI (kg/m2) | 21.8 (3.0) |
| Maternal height (cm) | 156.9 (5.2) |
| Mother completed primary school *(%)* | 20.5 |
| | |
| *Household characteristics* | |
| Number of household members | 6.0 (2.6) |
| Moderate to severe food insecurity *(%)*[b] | 78.0 |
| Owns latrine *(%)* | 96.5 |
| Owns cows *(%)* | 2.5 |
| Owns goats *(%)* | 20.0 |
| Owns chickens *(%)* | 33.3 |

Hgb: hemoglobin

[a] As reported by caregiver on a 24-hour dietary recall
[b] As defined by the Household Food Insecurity Access scale[15]

Table 4.2 – Plasma concentrations and growth measures for Mazira Project participants included in this secondary analysis of choline and growth

| | Baseline (age 6-9 months) | | 3 month follow up (age 9-12 months) | | 6 month follow up (age 12-15 months) | |
|---|---|---|---|---|---|---|
| | N | Mean (SD) or % | N | Mean (SD) or % | N | Mean (SD) or % |
| Choline (µmol/L) | 60 | 17.1 (3.5) | - | - | 60 | 14.6 (3.6) |
| Betaine (µmol/L) | 60 | 85.1 (31.5) | - | - | 60 | 96.7 (34.8) |
| TMAO (µmol/L)[a] | 60 | 1.8 (0.8, 3.8) | - | - | 60 | 3.7 (2.2, 5.4) |
| Zinc (µg/dL)[a] | 364 | 53.0 (32.2, 82.7) | - | - | 393 | 50.6 (31.1, 95.6) |
| | | | | | | |
| Length (cm) | 400 | 66.8 (2.8) | 399 | 70.2 (3.0) | 400 | 73.7 (2.9) |
| Length-for-age z-score (LAZ) | 400 | -0.9 (1.0) | 399 | -1.4 (1.1) | 400 | -1.1 (1.1) |
| Stunting (LAZ ≤ -2) | 400 | 13.8% | 399 | 28.1% | 400 | 19.5% |
| Weight (kg) | 400 | 7.7 (1.0) | 399 | 8.6 (1.1) | 400 | 9 (1.1) |
| Weight-for-age z-score (WAZ) | 400 | -0.5 (1.1) | 399 | -0.5 (1.1) | 400 | -0.6 (1.1) |
| Underweight (WAZ ≤ -2) | 400 | 8.3% | 399 | 9.8% | 400 | 11.0% |
| Weight-for-length z-score (WLZ) | 400 | 0.1 (1.1) | 399 | 0.3 (1.1) | 400 | -0.2 (1.0) |
| Wasting (WLZ ≤ -2) | 400 | 1.5% | 399 | 1.3% | 400 | 2.3% |
| Head circumference (cm) | 400 | 42.1 (1.6) | 398 | 43.9 (1.5) | 400 | 45.6 (1.6) |
| Head circumference-for-age z-score (HCAZ) | 400 | -1.1 (1.1) | 398 | -0.9 (1.1) | 400 | -0.2 (1.2) |
| Low head circumference (HCAZ ≤ -2) | 400 | 23.5% | 398 | 17.3% | 400 | 7.5% |

TMAO – trimethylamine N-oxide

[a] Variable was skewed. Median (IQR) is presented.

Table 4.3 - Minimally[a] and fully[b] adjusted models of plasma choline concentration (in SD units) as a predictor of growth among participants of the Mazira Project (n=400)

| | Minimally Adjusted[a] | | Fully Adjusted[b] | |
|---|---|---|---|---|
| | Estimate | 95% CI | Estimate | 95% CI |
| Continuous[c] | | | | |
| Length-for-age z-score (LAZ) | -0.08 | -0.17, 0.004 | **-0.09** | **-0.17, -0.01** |
| Weight-for-age z-score (WAZ) | 0.01 | -0.07, 0.10 | 0.01 | -0.07, 0.09 |
| Weight-for-length z-score (WLZ) | **0.08** | **0.0003, 0.17** | 0.07 | -0.03, 0.16 |
| Head circumference-for-age z-score (HCAZ) | -0.02 | -0.11, 0.06 | -0.03 | -0.12, 0.05 |
| | | | | |
| Conditional[d] | | | | |
| Length-for-age z-score (LAZ) | 0.04 | -0.003, 0.08 | 0.03 | -0.02, 0.07 |
| Weight-for-age z-score (WAZ) | 0.01 | -0.03, 0.06 | -0.01 | -0.06, 0.03 |
| Weight-for-length z-score (WLZ) | -0.01 | -0.07, 0.04 | -0.03 | -0.09, 0.02 |
| | | | | |
| Dichotomous[c] | | | | |
| Odds ratio for stunting (LAZ ≤ -2) | 1.09 | 0.90, 1.33 | 1.10 | 0.89, 1.36 |
| Odds ratio for underweight (WAZ ≤ -2) | 1.00 | 0.78, 1.29 | 1.04 | 0.80, 1.36 |
| Odds ratio for low head circumference (HCAZ ≤ -2) | 0.89 | 0.71, 1.11 | 0.90 | 0.71, 1.14 |

[a] Adjusted for: anthropometrist, minutes since last intake before blood draw, month of draw, group assignment, study visit (for models including multiple study visits).

[b] Additionally adjusted for: child age, sex, birth order; child plasma leucine, zinc, C-reactive protein and alpha-acid glycoprotein; malaria, anemia, longitudinal prevalence of diarrhea; maternal age, height, BMI, and education; housing and asset index score, food security score.

[c] Continuous and dichotomous outcomes were assessed in generalized linear models with plasma choline as predictor and robust standard errors to account for repeated measures across study visits.

[d] Conditional measures were calculated by regressing data from the 6 month follow up on data from enrollment and 3 month follow up. The residuals are included in linear regression models, with baseline plasma choline as a predictor.

Table 4.4 – Minimally adjusted[a] regression models of plasma betaine, DMG, and TMAO concentrations (in SD units) as predictors of growth among participants of the Mazira Project (n=400)

| | Betaine | | DMG | | TMAO | |
|---|---|---|---|---|---|---|
| | Estimate | 95% CI | Estimate | 95% CI | Estimate | 95% CI |
| Continuous[c] | | | | | | |
| Length-for-age z-score (LAZ) | -0.02 | -0.11, 0.06 | -0.07 | -0.26, 0.12 | -0.04 | -0.11, 0.04 |
| Weight-for-age z-score (WAZ) | 0.01 | -0.08, 0.09 | -0.15 | -0.35, 0.05 | -0.04 | -0.12, 0.03 |
| Weight-for-length z-score (WLZ) | 0.03 | -0.05, 0.11 | -0.14 | -0.34, 0.05 | -0.03 | -0.11, 0.04 |
| Head circumference-for-age z-score (HCAZ) | 0.02 | -0.07, 0.10 | -0.12 | -0.31, 0.06 | -0.04 | -0.11, 0.04 |
| | | | | | | |
| Conditional[d] | | | | | | |
| Length-for-age z-score (LAZ) | 0.03 | -0.01, 0.07 | 0.07 | -0.02, 0.15 | -0.02 | -0.05, 0.01 |
| Weight-for-age z-score (WAZ) | 0.02 | -0.02, 0.07 | 0.03 | -0.06, 0.13 | -0.02 | -0.05, 0.02 |
| Weight-for-length z-score (WLZ) | 0.02 | -0.04, 0.07 | -0.01 | -0.12, 0.11 | -0.01 | -0.05, 0.03 |
| | | | | | | |
| Dichotomous[c] | | | | | | |
| Odds ratio for stunting (LAZ ≤ -2) | 0.95 | 0.78, 1.16 | 0.86 | 0.57, 1.29 | 0.99 | 0.83, 1.17 |
| Odds ratio for underweight (WAZ ≤ -2) | 0.84 | 0.65, 1.08 | 1.24 | 0.76, 2.02 | 1.23 | 0.96, 1.57 |
| Odds ratio for low head circumference (HCAZ ≤ -2) | 0.94 | 0.76, 1.17 | 1.00 | 0.64, 1.56 | 0.99 | 0.83, 1.18 |

DMG – dimethylglycine; TMAO – trimethylamine N-oxide

[a] Adjusted for: anthropometrist, minutes since last intake before blood draw, month of draw, group assignment, study visit (for models including multiple study visits).

[c] Continuous and dichotomous outcomes were assessed in generalized linear models with plasma choline as predictor and robust standard errors to account for repeated measures across study visits.

[d] Conditional measures were calculated by regressing data from the 6 month follow up on data from enrollment and 3 month follow up. The residuals are included in linear regression models, with baseline plasma betaine, DMG, or TMAO as a predictor.

Table 4.5 – Stratified results from effect modification analyses[a] of the relationship between plasma choline and growth among participants of the Mazira Project

| | Minimally Adjusted | | |
|---|---|---|---|
| | Estimate[b] | 95% CI | p for interaction |
| Length-for-age z-score (LAZ) | | | |
| *Continuous*[c] | | | |
| Housing and asset index (quintiles 2-5) | -0.03 | -0.13, 0.06 | **0.001** |
| Housing and asset index (quintile 1) | **-0.38** | **-0.55, -0.21** | |
| *Conditional* | | | |
| Child sex (male) | -0.01 | -0.07, 0.05 | **0.030** |
| Child sex (female) | **0.08** | **0.02, 0.14** | |
| | | | |
| Weight-for-length z-score (WLZ) | | | |
| *Continuous* | | | |
| Longitudinal prevalence of diarrhea (below median) | **0.17** | **0.06, 0.29** | **0.038** |
| Longitudinal prevalence of diarrhea (above median) | -0.003 | -0.12, 0.11 | |

[a] Potential effect modifiers included: group assignment (intervention vs control group); child sex; longitudinal prevalence of diarrhea; stunting status at baseline; housing and asset index; maternal education. Time point (baseline vs 6 mo follow up) was tested in continuous and dichotomous outcomes only. Each modifier was made dichotomous. Stratified results are only presented for analyses in which the p-for-interaction was statistically significant ($p<0.05$).

[b] Estimates are the mean difference in the growth outcome variable per 1 standard deviation difference in plasma choline.

[c] Continuous outcomes were assessed in generalized linear models with plasma choline as predictor and robust standard errors to account for repeated measures across study visits. Conditional measures were calculated by regressing data from the 6 month follow up on data from enrollment and 3 month follow up. The residuals are included in linear regression models, with baseline plasma choline as a predictor.

Supplementary Table 4.1 – Baseline characteristics of children enrolled in the Mazira Project and included vs excluded from the current secondary analysis

| | Included (n=400) | Excluded (n= 260)[a] | |
|---|---|---|---|
| | Mean (SD) or (%) | Mean (SD) or (%) | p value |
| *Child and maternal characteristics* | | | |
| Child age (mo) | 7.4 (1.2) | 7.4 (1.2) | 0.849 |
| Male | 53.3 | 49.2 | 0.313 |
| First born | 27.8 | 27.3 | 0.886 |
| Animal source food consumption[b] | | | |
| Consumed dairy | 9.8 | 6.5 | 0.145 |
| Consumed meat | 2.5 | 0.8 | 0.103 |
| Consumed egg | 3.8 | 4.6 | 0.588 |
| Consumed fish | 23.6 | 32.7 | **0.010** |
| Any breastmilk | 99.7 | 100.0 | 0.419 |
| Anemia prevalence (Hgb < 11 g/dL) | 62.2 | 57.8 | 0.320 |
| Malaria prevalence | 14.5 | 8.6 | 0.042 |
| Longitudinal prevalence of diarrhea | 0.1 (0.1) | 0.1 (0.1) | 0.345 |
| Maternal age (y) | 26.0 (6.8) | 25.9 (6.7) | 0.857 |
| Maternal BMI (kg/m2) | 21.8 (3.0) | 21.9 (3.0) | 0.445 |
| Maternal height (cm) | 156.9 (5.2) | 156.2 (5.3) | 0.110 |
| Mother completed primary school | 20.5 | 19.1 | 0.656 |
| | | | |
| *Household characteristics* | | | |
| Number of household members | 6.0 (2.6) | 5.7 (2.7) | 0.242 |
| Moderate to severe food insecurity[c] | 78.0 | 77.7 | 0.926 |
| Owns latrine | 96.5 | 96.3 | 0.918 |
| Owns cows | 2.5 | 3.7 | 0.399 |
| Owns goats | 20.0 | 17.3 | 0.388 |
| Owns chickens | 33.3 | 31.0 | 0.552 |

[a] Children enrolled in the trial were excluded from this secondary analysis if they were missing one or both blood samples (n=147) or were not randomly selected for biochemical analysis (n=113)

[b] As reported by caregiver on a 24-hour dietary recall

[c] As defined by the Household Food Insecurity Access scale

Supplementary Table 4.2 – Interaction tests from effect modification analyses[a] of the relationship between plasma choline and growth among participants of the Mazira Project

| | p for interaction |
|---|---|
| Length-for-age z-score (LAZ) | |
| *Continuous*[b] | |
| Child sex | 0.254 |
| Group assignment | 0.478 |
| Housing and asset index | **0.001** |
| Maternal education | 0.704 |
| Longitudinal prevalence of diarrhea | 0.868 |
| Stunting status at baseline | 0.823 |
| Study visit | 0.844 |
| *Conditional*[b] | |
| Child sex | **0.038** |
| Group assignment | 0.716 |
| Housing and asset index | 0.605 |
| Maternal education | 0.707 |
| Longitudinal prevalence of diarrhea | 0.957 |
| Stunting status at baseline | 0.606 |
| *Dichotomous: Stunted (LAZ ≤ -2)*[b] | |
| Child sex | 0.078 |
| Group assignment | 0.143 |
| Housing and asset index | 0.174 |
| Maternal education | 0.381 |
| Longitudinal prevalence of diarrhea | 0.618 |
| Stunting status at baseline | 0.382 |
| Study visit | 0.609 |
| | |
| Weight-for-age z-score (WAZ) | |
| *Continuous* | |
| Child sex | 0.403 |
| Group assignment | 0.450 |
| Housing and asset index | 0.377 |
| Maternal education | 0.268 |
| Longitudinal prevalence of diarrhea | 0.067 |
| Underweight status at baseline | 0.925 |
| Study visit | 0.812 |
| *Conditional* | |
| Child sex | 0.195 |
| Group assignment | 0.684 |
| Housing and asset index | 0.895 |
| Maternal education | 0.896 |
| Longitudinal prevalence of diarrhea | 0.260 |
| Underweight status at baseline | 0.876 |

| | |
|---|---|
| *Dichotomous: Underweight (WAZ ≤ -2)* | |
| Child sex | 0.954 |
| Group assignment | 0.054 |
| Housing and asset index | 0.598 |
| Maternal education | 0.694 |
| Longitudinal prevalence of diarrhea | 0.521 |
| Underweight status at baseline | 0.734 |
| Study visit | 0.448 |
| | |
| Weight-for-length z-score (WLZ) | |
| *Continuous* | |
| Child sex | 0.789 |
| Group assignment | 0.669 |
| Housing and asset index | 0.547 |
| Maternal education | 0.203 |
| Longitudinal prevalence of diarrhea | **0.030** |
| Wasting status at baseline | 0.488 |
| Study visit | 0.918 |
| *Conditional* | |
| Child sex | 0.551 |
| Group assignment | 0.806 |
| Housing and asset index | 0.608 |
| Maternal education | 0.724 |
| Longitudinal prevalence of diarrhea | 0.071 |
| Wasting status at baseline | 0.491 |
| | |
| Head circumference-for-age z-score (HCAZ) | |
| *Continuous* | |
| Child sex | 0.869 |
| Group assignment | 0.907 |
| Housing and asset index | 0.634 |
| Maternal education | 0.635 |
| Longitudinal prevalence of diarrhea | 0.327 |
| Low head circumference status at baseline | 0.189 |
| Study visit | 0.390 |
| *Dichotomous: Low head circumference (HCAZ ≤ -2)* | |
| Child sex | 0.690 |
| Group assignment | 0.600 |
| Housing and asset index | 0.095 |
| Maternal education | 0.079 |
| Longitudinal prevalence of diarrhea | 0.217 |
| Low head circumference status at baseline | 0.427 |
| Study visit | 0.939 |

[a] Each modifier was made dichotomous: child sex (male or female), group assignment (intervention or control), housing and asset index (quintile 1 vs quintiles 2-5), maternal education

(incomplete primary vs primary or greater), longitudinal prevalence of diarrhea (above or below median), stunting/underweight/low head circumference at baseline (z-score ≤ or >-2), and study visit (baseline or 6 month follow up; only for models with multiple time points).

[b] Continuous and dichotomous outcomes were assessed in generalized linear models with plasma choline as predictor and robust standard errors to account for repeated measures across study visits. Conditional measures were calculated by regressing data from the 6 month follow up on data from enrollment and 3 month follow up. The residuals are included in linear regression models, with baseline plasma choline as a predictor.