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The Association between Serum/RBC Folate Levels, Serum Vitamin B12 Concentrations, and Malaria in non-Pregnant Women of Reproductive Age in Malawi

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Abstract

The Association between Serum/RBC Folate Levels, Serum Vitamin B12 Concentrations, and Malaria in non-Pregnant Women of Reproductive Age in Malawi

By Ben Redpath

Background

Folate deficiency is a known cause of anemia and severe birth defects of the brain and spine known as neural tube defects. In the setting of vitamin B12 deficiency, there is concern that serum folate measurements may be measured as artificially high. Malaria infection can also raise serum folate measurements. We sought to investigate the association between serum/RBC folate and serum vitamin B12 in women of reproductive age (WRA) in Malawi. We further sought to report the association between malaria and serum/RBC folate measurements and if there is interaction between malaria and vitamin B12 in this association.

Methods

The data analyzed are from the 2015-2016 Malawi Micronutrient Survey, a cross-sectional survey administered by the government of Malawi with support from several other institutions including the Biomarkers Reflecting Inflammation and Nutritional Determinates of Anemia (BRINDA) work group at the Centers for Disease Control and Prevention. Possible serum folate deficiency was defined as serum folate <14.0nmol/L and vitamin B12 deficiency was defined as serum vitamin B12 <150pmol/L. We analyzed crude and adjusted associations between folate and vitamin B12, and folate and malaria using logistic regression. We also tested for interaction between vitamin B12 levels and malaria status.

Results

Of the 742 women studied, prevalence of possible serum folate deficiency, serum folate deficiency, and RBC folate insufficiency were 34.0%, 7.0%, and 81.2%, respectively. Prevalence of serum vitamin B12 insufficiency or deficiency was 18.7% and serum vitamin B12 deficiency was 2.7%. There was no significant association between possible folate deficiency and vitamin B12 deficiency prevalence odds ratio = 0.55 (95%CI = 0.16, 1.92), and there was a near significant association between malaria and possible serum folate deficiency prevalence odds ratios = 0.64 (95%CI = 0.39, 1.04), controlling for wealth.

Conclusions

Our analysis did not show a significant association between vitamin B12 deficiency and serum/RBC folate levels. For both vitamin B12 deficiency and malaria, one must first treat the known underlying condition before an accurate assessment of folate levels can be made. It may be beneficial to recommend supplementing all individuals with low vitamin B12 or malaria infection with 400µg of folic acid daily.

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CHAPTER 1

BACKGROUND AND PUBLIC HEALTH SIGNIFICANCE

Folate deficiency is a public health concern. Many people throughout the world, in both industrialized and developing countries have deficient levels of folate in their blood and tissues. Folate has many important functions in cells including DNA synthesis; folate deficiency is a known cause of anemia, severe birth defects and cardiovascular disease. Conditions arising secondary to low folate levels lead to unnecessary healthcare costs and contribute significantly to childhood mortality (Kancherla, Redpath, & Oakley, 2018). The most important reason for high prevalence of folate deficiency throughout the world is that folate content in most foods is quite low, making it difficult to consume the required amount through diet alone. Additionally, in many resource limited areas, access to folate rich foods such as leafy greens, fruits, vegetables and animal meat is limited. Additionally, natural folates have a bioavailability of only about one-half, whereas folic acid, the synthetic form of folate, used in supplements and for food fortification is 85% bioavailable and less susceptible to breakdown by nitrates and exposure to light (Antony, 2018).

Vitamin B12 is found in primarily animal products including meat, fish, eggs, and milk (Gille & Schmid, 2015). Stores of vitamin B12 in the liver can last 3-4 years, making dietary causes of B12 deficiency rare in industrialized nations. People who follow a strict vegan diet (i.e. do not consume animal products) and those in resource limited areas who consume few animal products are at risk for vitamin B12 deficiency (Shipton & Thachil, 2015).

Vitamin B12 deficiency, like folate deficiency, causes anemia due to perturbation of DNA synthesis, complicating the process of determining causes of anemia in both individuals and populations (Antony, 2018). Vitamin B12 deficiency also causes peripheral neuropathy and is associated with dementia (Moore et al., 2012). Our primary association of interest was between blood folate measurements (both serum and RBC measures) and serum vitamin B12 measurements. We sought to determine if low vitamin B12 levels (either insufficiency or deficiency) are associated with reduced prevalence odds of folate deficiency. Our secondary association of interest is between serum/RBC folate levels and malaria infection. Lastly, we sought to determine whether there is any interaction between vitamin B12 and malaria on the measurement of serum and RBC folate levels. Since both low vitamin B12 and malaria are known to falsely elevate serum folate levels, RBC folate levels, or both, there is concern that national nutrition surveys, such as the 2015-2016 Malawi Micronutrient Survey (MNS) may significantly underestimate the prevalence of folate deficiency in areas with high prevalence of vitamin B12 deficiency or malaria.

CHAPTER II

LITERATURE REVIEW

Folate (also called Vitamin B9) is a general term for a group of more than 100 compounds essential for a variety of intracellular functions including DNA synthesis and one-carbon metabolism in the cytosol (Bailey et al., 2015). Folate deficiency causes widespread impairment of DNA synthesis which manifests clinically as a megaloblastic anemia (Crider, Yang, Berry, & Bailey, 2012) (Ebara, 2017) (Antony, 2018). Megaloblasts are red blood cells (RBCs) that are larger than normal and have certain characteristics which can be seen under microscope. Any process which perturbs DNA synthesis can result in megaloblastic anemia, which can make identifying its cause difficult (Antony, 2017). In addition to anemia, folate deficiency is significantly associated with major birth defects such as severe defects of the brain and spine known as neural tube defects (NTDs) ("Prevention of neural tube defects," 1991). A randomized controlled trial reported in 1991 that folate deficiency is an unequivocal cause of the NTDs spina bifida and anencephaly ("Prevention of neural tube defects," 1991). The current health guidelines recommend that all women of reproductive age consume 400µg of folic acid daily to prevent birth defects (CDC, 2018). The neural tube forms at week 4 of pregnancy(Bailey et al., 2015), when many women are still unaware of their pregnancies. Hence, consuming at least 400µg of folic acid daily *prior* to pregnancy is crucial to prevent folic acid-preventable spina bifida anencephaly.

Folate deficiency is also associated with dementia, rate of first stroke in those with hypertension, and other cardiovascular diseases (CVD) (Huo et al., 2015). A randomized controlled trial with more than 10,000 people with high blood pressure in

each arm showed a dramatic reduction in rates of first ischemic stroke in the group receiving 800µg of folic acid per day and blood pressure medication as opposed to the group who received only blood pressure medication (Huo et al., 2015). This relationship may be in part mechanistically explained by folate's relationship with homocysteine. Folate drives down levels of homocysteine which, has been shown to be a risk factor for cardiovascular disease at high levels (Spence, 2016).

Folate Biomarkers

The most commonly used biomarkers for assessing folate status are serum folate and red blood cell folate (RBC folate; erythrocyte folate) (Bailey et al., 2015). Serum folate is thought to be an indicator of recent folate consumption, whereas RBC folate is an indicator of folate status over the course of the life an RBC, about 120 days (Antony, 2017). However, there are a variety of factors including vitamin B12 deficiency, malaria infection, and any other condition that causes hemolysis of the RBCs that can artificially raise serum folate, and to a lesser extent may affect RBC folate measurements (Antony, 2017) (Antony, 2018). An artificially high laboratory measurement of folate in spite of true *tissue* folate deficiency leaves the potential for underreporting of folate deficiency in population-based studies (Antony, 2017).

Vitamin B12 and its Impact on Folate Concentrations

Due to interconnected cellular pathways, a deficiency of vitamin B12 can leave folates from the diet unmetabolized and elevate serum folate levels despite tissue folate deficiency. The majority of natural folate is absorbed into the blood and irreversibly

enzymatically converted into the form a 5-methyl-tetrahydrofolate (5-methyl-THF), which is the primary form transported into cells(Antony, 2018). 5-methyl-THF is then converted to THF by the enzyme methionine synthase. THF is the preferred substrate for the enzyme folylpolyglutamate synthase, which adds multiple glutamate moieties to THF (Antony, 2018). This polyglutamated form of THF then goes on to participate in one-carbon metabolism, which is crucial for purine de-novo synthesis and for synthesis of thymidylate(Antony, 2018).

Cobalamin (vitamin B12) is a co-factor necessary for the function of methionine synthase (Antony, 2018). When cobalamin levels in the blood are low, methionine synthase function is reduced, leading to a buildup of 5-methyl-THF in cells. Because 5-methyl-THF is formed in an irreversible reaction and cobalamin levels are too low for the forward reaction to take place, this buildup of 5-methyl-THF is known as "methyl-folate trapping" (Antony, 2017). The excess 5-methyl-THF then leaks out of cells into the bloodstream, elevating levels of folate in the serum. So, even if intracellular levels of THF are deficient, serum folate levels may be normal or high in the setting of concomitant vitamin B12 deficiency. Only when B12 is replaced in the diet or through injection will serum folate drop and reveal the underlying folate deficiency (Antony, 2017). However, RBC folate is largely *unaffected* by vitamin B12 status, so it is a better measure of folate status in this setting (Antony, 2018). In those with B12 deficiency, folic acid supplied through food fortification or vitamin pills, will still replenish THF stores, because dihydrofolate reductase, not methionine synthase, converts folic acid to THF (Antony, 2018). Even in regions with high rates of vitamin B12 deficiency, folic acid consumption through food fortification should adequately raise tissue THF levels.

Malaria and its Impact on Folate Concentrations

Malaria is highly prevalent in many developing countries and is an especially serious health concern in Africa. This parasitic infection of RBCs still kills around 2,000 people per day worldwide, most of whom are children in Africa (White et al., 2014). The malaria parasite directly infects RBCs causing structural damage and lysis of the cells (i.e. hemolytic anemia). Since intracellular folate concentrations are much higher than that of the serum, hemolysis leads to an artificially high serum folate level (Antony, 2017). Thus, there is a potential for the underestimation true folate deficiency prevalence in areas with high malaria prevalence.

While RBC folate may give a more accurate measure of folate status in those with low serum vitamin B12, this may not be the case for those with malaria. The malaria parasite can synthesize folate in RBC cultures in vitro and has been shown to raise RBC folate levels in mice with high parasite burden (Reid & Friedkin, 1973). Also, erythropoiesis is generally upregulated in hemolytic anemia to replace the destroyed RBCs, which also increases the demand for folate and can cause a megaloblastic anemia (Antony, 2018). Hemolysis due to any cause, including malaria, generally induces a compensatory reticulocytosis. Premature RBCs known as reticulocytes leave the bone marrow and enter the bloodstream to compensate for the loss of RBCs. These reticulocytes have a higher concentration of folate than mature RBCs, and thus this reticulocytosis can lead to an increased serum folate (Antony, 2017). Paradoxically, certain severe malaria infections may also *suppress* erythropoiesis, decreasing the production of RBCs and thus measured value of RBC folate(Antony, 2017). In these

cases, RBC folate may appear normal or decreased (Antony, 2017). In those with malaria, both serum and RBC folate measurements may yield misleading results (**Figure 1**). Given the increased folate demands during malaria infection and the geographic overlap between areas with high malaria prevalence and areas with dietary folate deficiency, it is likely safe to assume that a person with malaria is at risk for folate deficiency, regardless of their folate levels measured in serum or RBCs.

Historically there has been concern with giving folate to those with malaria infections. Folate is necessary for the lifecycle of the malaria parasite. In fact, many anti-folate drugs are used to treat malarial infections. Extremely high doses of folic acid (5-10g/day) are generally contraindicated in malaria infection as it may inhibit clearance of malaria with anti-folate drugs(Nzila, Okombo, & Hyde, 2016). This is less clear for dose of folic acid one would consume through food fortification (about 150µg/day) and prenatal vitamins (about 400µg/day) (Nzila et al., 2016) (Kupka, 2015). The benefits of preventing anemia, spina bifida, and anencephaly must be weighed against the theoretical risk of worsening malaria infection, especially in areas with high malaria prevalence.

Micronutrient Malnutrition in Malawi

Malnutrition including micronutrient malnutrition ("hidden hunger") is common in Malawi. Reasons for this include high rates of rural poverty, low dietary diversity, and chronic seasonal food insecurity(Kerr, Chilanga, Nyantakyi-Frimpong, Luginaah, & Lupafya, 2016). The 2015-2016 Malawi Micronutrient Survey (MNS), a national survey administered by the government of Malawi to provide current data on the status of various biomarkers, indicated that 34% of pre-school children (PSC) (ages 6-59 months)

in Malawi were stunted, or too short for their age, 18% were underweight and 12% were overweight (*Malawi Micronutrient Survey 2015-2016*, 2017). Additionally, 30% of PSC were anemic, defined as hemoglobin level below 11.0g/dl, and 21% of non-pregnant women of reproductive age (WRA) were anemic, defined as hemoglobin below 12.0g/dL (*Malawi Micronutrient Survey 2015-2016*, 2017). The prevalence of malaria was 28% in PSC and 16% in non-pregnant WRA (*Malawi Micronutrient Survey 2015-2016*, 2017).

The baseline prevalence of NTDs in the setting of optimal prevention of folic acid-preventable cases of spina bifida and anencephaly (FAP-SBA) is estimated to be estimated to be 5/10,000 births (Cordero, Crider, Rogers, Cannon, & Berry, 2015). The March of Dimes models estimate the NTD prevalence in Malawi to be 15/10,000 live births, which may be an underestimate due to the assumptions in the models, as well as general underreporting of stillbirths and early fetal losses (Christianson et al., 2006). A recent study in Tigray region in northern Ethiopia reported an NTD prevalence of 131/10,000 births (Berihu et al., 2018). We expect that the prevalence in Malawi is greater than 20/10,000 births due to severe malnutrition.

According to the food fortification initiative (www.ffinetwork.org), in Malawi, there is a policy for mandatory fortification of wheat and maize flour with vitamins including folic acid, iron, zinc, B12, niacin, riboflavin, thiamine and vitamin A (Food Fortification Initiative, 2019). However, many people do not eat centrally processed grains and rely on their own food production for consumption, so fortified products may not be part of their usual diet(Kerr et al., 2016). Given the possibility of low dietary intake of folate in poor areas and reason to believe that centrally processed grains may

not be reaching many Malawians, there is concern for a high prevalence of folate deficiency in Malawi, especially in WRA.

The current categorization for low folate (Pfeiffer et al., 2016), calibrated for the laboratory method performed by the Center for Disease Control and Prevention (CDC) laboratory in Atlanta, Georgia, United States are: serum folate deficiency based on the basis of hematologic indicator (anemia) is <6.8nmol/L, possible deficiency on the basis of a metabolic indicator (homocysteine) <14.0nmol/L and RBC folate insufficiency on the basis of elevated NTD risk <748nmol/L. The MNS reported the prevalence of serum folate deficiency, possible serum folate deficiency, and RBC folate insufficiency to be 7.6%, 34.5%, and 81.4% respectively. However, these data do not address the possible impact of vitamin B12 deficiency or malaria infection on folate measurements. More accurate reporting has the potential to lead to more targeted interventions increased prevention of NTDs, anemia, and possibly first stroke.

We sought to determine if low vitamin B12 levels (either insufficiency or deficiency) are associated with reduced prevalence odds of folate deficiency. Our secondary association of interest is between serum/RBC folate levels and malaria infection. Lastly, we sought to determine whether there is any interaction between vitamin B12 and malaria on the measurement of serum and RBC folate levels. Since both low vitamin B12 and malaria are known to falsely elevate serum folate levels, RBC folate levels, or both, there is concern that national surveys such as the MNS may be significantly underestimating the prevalence of folate deficiency in areas with high prevalence of vitamin B12 deficiency or malaria.

CHAPTER III

METHODS

We examined data from the 2015-2016 Micronutrient Survey (MNS) in Malawi accessed from the Biomarkers Reflecting Inflammation and Nutritional Determinates of Anemia (BRINDA) project (www.BRINDA-nutrition.org). The MNS survey was conducted jointly with the 2015-2016 Malawi Demographic Health Survey (MDHS) with the goal of providing current data to improve the monitoring and evaluation of nutrition interventions (*Malawi Micronutrient Survey 2015-2016,* 2017). The MNS was used to produce estimates for important nutrition indicators for the country, as well as stratified by region (North, Central, South) and residence (urban, rural). A subsample of 105 clusters (35 in each region) was randomly selected from the total 850 MDHS clusters. Twenty households per urban cluster and 22 households per rural cluster were included in the MNS. The MNS was conducted in 2262 residential households (480 in urban areas and 1782 in rural areas). The sample size for the MNS was calculated based on predicted change in prevalence of vitamin A deficiency in preschool aged children from 22% in 2009 to 16% in 2015-2016 using a confidence level of 95%, power of 80% and design effect of 2.0. Using this calculation, MNS predicted they would enroll 750 women of reproductive age (WRA) (*Malawi Micronutrient Survey 2015-2016,* 2017).

The BRINDA project is a workgroup originally formed in 2012 as a multi-agency and multi-country collaboration with the goal of improving micronutrient assessment and determine causes of anemia (“BRINDA,” 2017). The Malawi government consulted with BRINDA and made certain measurement and methodological adjustments to their

protocols and final reports based on BRINDA's suggestions (*Malawi Micronutrient Survey 2015-2016,* 2017).

Outcome Variables

There were three primary outcomes in our analysis: serum folate deficiency based on the basis of hematologic indicator (anemia) is <6.8nmol/L, possible deficiency on the basis of a metabolic indicator (homocysteine) <14.0nmol/L and RBC folate insufficiency on the basis of elevated neural tube defect risk <748nmol/L (Pfeiffer et al., 2016). Serum and RBC folate cutoffs are based on World Health Organization's (WHO) 2015 guidelines, calibrating the CDC's detection methods for folate (Pfeiffer et al., 2016).

Predictor Variables

We examined serum B12 deficiency as a dichotomous variable, based on risk for megaloblastic anemia (<150 pmol/L vs. ≥150 pmol/L). We also examined serum B12 insufficiency, based on risk for B12 deficiency, as a dichotomous variable (<220 pmol/L vs ≥220 pmol/L). Malaria infection status (positive vs. negative) was determined based on a rapid malaria test kit.

Laboratory Analysis for Nutritional Biomarkers and Anemia

Nutritional biomarker analyses were performed in a Centers for Disease Control and Preventions (CDC) laboratory in Atlanta, Georgia, USA. Serum and RBC folate concentrations were measured using a microbiologic technique with the bacteria *L. rhamnosus* (formerly *L. casei*) with 5-methyl-THF as the calibrator(Pfeiffer et al., 2011).

Previous WHO folate cutoff recommendations were also based on experiments using *L. casei,* but due to calibration differences the cutoff values were adjusted to those used in this study (*Malawi Micronutrient Survey 2015-2016*, 2017). Serum vitamin B12 concentration was measured using an immunoassay method. The inflammatory markers C-reactive protein (CRP) and α-1-acid glycoprotein were measured through enzyme-linked immunosorbent assay (ELISA) at VitMin Labs (Willstaett, Germany). Anemia was established using hemoglobin analysis, measured using a Hemocue 301 hemoglobin field test kit(*Malawi Micronutrient Survey 2015-2016*, 2017).

Co-variables

Age of the participant (in years) was examined (15-19, 20-29, and 30-49). Marital status was classified as either married or unmarried. Body mass index (BMI) (kg/m^2) categories were examined by CDC classification(CDC, 2019): underweight (<18.5); normal weight (18.5 to <25); overweight (25 to <30); obese (≥30). Schistosomiasis infection was based on the presence or absence of hematuria (blood in the urine). Participants were dichotomized into area of residence: urban or rural. Inflammation status was deemed positive if the participant had either an elevated CRP > 5mg/L (acute inflammation) or elevated α-1-acid glycoprotein > 1.0g/L (chronic inflammation), or both. The wealth index of each respondent was determined based on the number and kinds of consumer goods they own. The respondents were divided in to five equal quintiles, each containing 20% of the population. Our analyses use wealth in quintiles as well as grouped into three categories: the lowest two quintiles forming one group, the

third and fourth quintiles forming the second group, and the wealthiest quintile is its own group. Anemia for non-pregnant women of reproductive age was defined as <12.0g/dL.

Statistical Analysis

All statistical analyses were performed in SAS 9.4 (SAS Institute, Cary, NC). In the MNS, there were 838 women of reproductive age (ages 15-49.) We a priori made our analysis group only those who had a known pregnancy status, were not pregnant, and had known values for serum folate, RBC folate, and serum vitamin B12. Of the 838, 39 were removed first due to missing serum folate levels, none were removed for missing vitamin B12 levels, 14 were removed due to missing RBC folate levels, 31 women were then removed due to pregnancy, and lastly 12 were removed due to missing pregnancy status (**Figure 2**).

We a priori identified residence (urban vs. rural), malaria status, wealth index, body mass index (BMI), age, anemia status, schistosomiasis status, marital status, and inflammatory markers as potential confounders based on prior literature. We tested residence, wealth, and malaria status for effect modification with serum vitamin B12 levels and with each other; none were found to be significant effect modifiers using the Breslow-Day test for interaction at $\alpha < 0.05$.

All analyses were conducted accounting for the complex survey sampling design of the MNS. We conducted a descriptive analysis to examine the difference between the main predictor variable and the other selected co-variables by serum and RBC folate categories using Rao-Scott chi-square tests. We estimated crude and adjusted prevalence odds ratios (cPORs and aPORs) and 95% confidence intervals (CI) using logistic

regression analysis. Models 1 and 2 report the association between serum/RBC folate and vitamin B12 concentrations. Model 1 is adjusted for covariables which were identified as significantly associated with the exposure and the outcome and backward logistic regression with a 10% change-in-estimate criterion. Model 2 was determined a priori based on previous literature includes as covariables family wealth, malaria infection status, residence, and BMI. Models 3 and 4 report the associations between serum/RBC folate and malaria status. Models 3 reports the cPOR and model 4 reports aPOR, controlling for wealth.

The protocol was reviewed by the Emory University Institutional Review Board and was deemed not to be human subjects research.

CHAPTER IV

RESULTS

Our analytic sample included non-pregnant women who had measured values for serum folate, serum vitamin B12, and pregnancy status. We excluded women who were pregnant or had missing data for one or more of the following variables: serum folate, RBC folate, serum vitamin B12, pregnancy status (**Figure 2**). Of the 838 women surveyed by MNS, 742 (90.6%) were in our final analytic sample. The prevalence of possible serum folate deficiency, serum folate deficiency, and RBC folate insufficiency was 34.0%, 7.0%, and 81.2% respectively. Prevalence of serum vitamin B12 insufficiency or deficiency was 18.7% and serum vitamin B12 deficiency was 2.7%.

Descriptive analysis examining the association between vitamin B12 status or other covariables and serum folate status insufficiency (**Table 1A**) or deficiency (**Table 1B**) or RBC folate insufficiency (**Table 1C**). Malaria status (cPOR = 0.52; 95%CI = 0.32-0.86) and lowest wealth status (cPOR = 0.32; 95%CI = 0.15-0.68) were associated with decreased prevalence odds of possible serum folate deficiency while being obese (cPOR= 3.48; 95%CI = 1.19-10.18) and having any inflammation (cPOR = 1.78; 95%CI = 1.04-3.05) were associated with increased prevalence odds of serum folate deficiency. Of the covariables examined, only wealth was associated with both possible serum folate deficiency and vitamin B12 insufficiency at $p < 0.05$ (**Tables 1A and 2**).

Table 2 presents results from unadjusted analysis examining the association between selected covariables and vitamin B12 insufficiency and deficiency. Residence and wealth were significantly associated with vitamin B12 status ($p<0.05$).

Our multivariable analysis showed that there was no significant association between serum/RBC folate status and serum vitamin B12 concentration; however, the effect estimates for serum folate levels were <1.0 indicating an inverse association between serum folate status and vitamin B12 status (**Table 3**). Results were presented based on two regression models. Model 1 adjusted for only wealth category. Model 2 was determined a priori based on published literature and adjusted for wealth, malaria status, residence, and BMI. We found null association between RBC folate status, which is a marker for long-term folate status, and vitamin B12 status with all estimates near unity and wider confidence intervals.

We also performed a secondary analysis of the association between serum/RBC folate and vitamin B12 status stratified by malaria status (positive vs. negative) (**Table 4**). Model 3 shows the cPOR for the association indicating an inverse association between having malaria and serum folate insufficiency or deficiency. However, this association attenuated after adjusting for wealth in model 4 (aPOR = 0.64; 95%CI = 0.39-1.04 & aPOR = 0.35; 95%CI = 0.10-1.20, for possible folate deficiency and frank deficiency respectively). We noted a significant inverse association for RBC folate deficiency and malaria status, controlling for wealth (aPOR=0.48; 95%CI=0.26-0.89).

CHAPTER V

DISCUSSION

Our analyses did not show a significant association between low serum vitamin B12 (insufficiency or deficiency) and low serum folate (possible deficiency or frank deficiency) adjusting for wealth. Our primary association of interest was between possible serum folate deficiency and serum B12 deficiency. It is our assumption that at deficiency levels of vitamin B12, methionine synthase will have and even lower level of activity than at vitamin B12 levels in the insufficiency range. If our assumption is true, we expect that those with vitamin B12 levels below 150 pmol/L to have an even lower odds of being folate deficient than those with blood folates 150-220 pmol/L. In Model 1 we compared possible folate deficiency with vitamin B12 deficiency we observed an aPOR of 0.55 (95% CI=0.17-2.07). An aPOR <1.0 is the expected result if vitamin B12 deficiency is truly masking folate deficiency. Additionally, this trend is seen only for serum folate levels and not with RBC folate levels, supporting that this may be a true association, even though our analysis is not statistically significant.

To our knowledge, this is the first study based on a national micronutrient survey that directly investigates the association between serum folate and vitamin B12. Antony highlighted many older studies that overlooked the possible effect of low vitamin B12 and malaria infection on serum folate measurements, but he did not directly measure these associations (Antony, 2017). A similar cross-sectional national micronutrient survey of WRA was conducted in Belize in 2011. An analysis of this data by Rosenthal et al. showed a significantly higher geometric mean RBC folate in those without vitamin B12 deficiency compared to those with vitamin B12 deficiency (Rosenthal et al., 2016).

Our study did not show this association. The Belize study reported the prevalence of vitamin B12 insufficiency and deficiency as 17.2% and 33.2% respectively, using similar laboratory methods and cutoffs as the MNS survey(Rosenthal et al., 2016). This is a much higher prevalence than reported in Malawi. Furthermore, Rosenthal et al. did not directly comment on the association between serum folate and serum vitamin B12.

A case report in 2015 of a patient presenting with a generalized tonic-clonic seizure thought to be secondary to vitamin B12 deficiency, highlighted the association between serum folate and B12 measurements (Lubana, Alfishawy, Singh, & Atkinson, 2015). This patient had a serum vitamin B12 level of 142pg/ml (105pmol/L) secondary to gastrectomy surgery for cancer and had very high serum folate of >25ng/ml (57nmol/L). While vitamin B12 deficiency is not a common cause of seizure, this patient received injections of vitamin B12 and subsequently his folate level dropped into the normal range and he remained seizure free. The drop in folate level into the normal range immediately following repletion of vitamin B12 argues that vitamin B12 deficiency can increase serum folate measurements in vivo.

The crude association we observed between malaria test positivity and decreased prevalence odds of serum folic acid deficiency is expected. Our models with malaria as the primary predictor of serum/RBC folate show a trend and are close to statistical significance at $p<0.05$. Malaria can cause lysis of the red blood cells, spilling the high folate contents of the cytosol into the bloodstream. We also see that malaria is associated with decreased prevalence odds of RBC folate insufficiency. This relationship is less clear, but it may be due to increased percentage of reticulocytes (precursor RBCs) in the blood as a compensatory response to hemolysis(Antony, 2017). We further analyzed the

relationship between folate and B12, stratified by malaria status, and found no difference in estimated PORs and no interaction between malaria and vitamin B12 with regards to folate status using the Breslow-Day test for interaction. While malaria is significantly associated with a decreased prevalence odds of folate deficiency, it seems that this association is present regardless of vitamin B12 status.

Although not our main association of interest, we observed a statistically significant association between wealth category and possible serum folate, (cPOR = 0.32; 95%CI = 0.15-0.68) indicating that those in the poorest wealth group have the lowest prevalence of folate deficiency. The poorest wealth category also had the highest prevalence of vitamin B12 insufficiency. This is possibly due to dietary reasons: fewer wealthy people tend to eat more folate-rich leafy greens and may not be able to afford vitamin B12 – rich animal proteins. Wealth must be adjusted for as a confounder in the relationship between serum/RBC folate and vitamin B12.

Strengths of this study include that the MNS projected surveyed thousands of homes in areas throughout the country of Malawi, the collected blood samples and tested them for a large variety of biomarkers, and very little data was missing. The survey was designed to cover many regions of Malawi, including urban, rural, and underserved areas, making our findings generalizable to the whole country of Malawi and possibly to similar nearby nations. Participants who were missing data on one variable were more likely to be missing data on many variables and not be included in our analysis. If there is a significant difference in B12 and folate status amongst the people missing data for both, then selection bias would be present. However, we were able to include more than 90% of the total WRA participants in our analysis, making this type of selection bias unlikely.

Additionally, the wide range of available blood biomarkers allowed us to assess many variables for possible interaction and confounding.

This study has several limitations and areas for further inquiry. Firstly, this study is based on cross-sectional data, so we are only able to look at prevalence and prevalence odds ratios. Although there is a known enzymatic pathway between vitamin B12 deficiency and possible falsely elevated serum folate measurements, we are unable to show causation in this study. Furthermore, given that the prevalence of our primary outcomes of low serum and RBC folate are common, odds ratios may significantly overestimate the true association.

Another limitation is lack of data indicating clinically significant folate and B12 deficiency such as homocysteine and methylmalonic acid (a biomarker in the blood used to differentiate between vitamin B12 deficiency and folate deficiency) levels as well as symptoms of peripheral neuropathy. These variables were not measured by the MNS. Blood levels of folate and vitamin B12 are important, but this additional information is needed to clarify the picture and determine whether a patient has clinically significant folate or vitamin B12 deficiencies. However, even in the setting of no clinically significant signs or symptoms of folate or B12 deficiency, a WRA may still be at increased risk for having a baby with an NTD. Blood folate and vitamin B12 measurements are an important clue but must be interpreted in the context of other indicators. Dietary data could also contribute to the assessment of an individual's risk for folate deficiency, given that laboratory measurements may be misleading. Additionally, while the MNS survey was a wide-reaching and thorough survey, unmeasured confounders such as hemoglobinopathies or other infections may be a source of bias.

The 2015-2016 MNS reported serum folate and erythrocyte folate (RBC folate) levels in only women of reproductive age (WRA) ages 15-49 years. This study was powered to detect a change in vitamin A status in pre-school children. With the relatively low prevalence of frank vitamin B12 deficiency and the sample size of 742 WRA, we were unable to detect a significant association between low B12 and high serum folate if one exists. The prevalence of vitamin B12 deficiency was low (2.7%), especially in those who also had low folate levels. Among participants with serum folate deficiency, there were 0 (0.0%) participants who were vitamin B12 deficient and among those with possible folate deficiency only 5 (1.6%) of participants were vitamin B12 deficient. It is likely that as vitamin B12 levels in an individual become lower and lower, the activity of methionine synthase, which is needed to metabolize food folates, decreases more and more. Thus, people with very low serum vitamin B12 are most likely to have falsely elevated serum folate measurements. This may explain the low sample size in these subgroups. Given the trend we observed in WRA in Malawi, and the known biochemical pathways, a true causal relationship may exist. An analysis of a larger sample, including more participants with both low folate and vitamin B12, children and men, is warranted.

In addition to the current health guidelines recommending at least 400µg of folate daily for WRA, we recommend that women with vitamin B12 insufficiency/deficiency or malaria infection be considered at risk for folate deficiency. In the setting of hemolysis serum folate measurements are unreliable, and in the setting of hemolysis due to malaria infection, both RBC and serum folate measurements may be unreliable. Our analysis did not show a significant association between vitamin B12 deficiency and serum/RBC folate levels, yet to accurately assess a patient or populations folate status, one must first replete

vitamin B12, then assess folate status. For both vitamin B12 deficiency and malaria, one must first treat the known underlying condition before an accurate assessment of folate levels can be made. It may be beneficial to recommend supplementing all individuals with low vitamin B12 or malaria infection with 400µg of folic acid daily.

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Figure 1. Measurements of Serum/RBC folate in the Setting of Vitamin B12 Deficiency and/or Malaria infection.[a]

Type of Deficiency	Serum Folates	RBC Folates	Serum Vitamin B12
Folate Deficiency	Low	Low	Normal/Low
Vitamin B12 Deficiency[b]	Normal/High[b]	Low[b]	Low
Folate Deficiency plus Vitamin B12 Deficiency	Normal	Low	Low
Malaria Infection[c]	Normal/High[c]	High[d]	Normal
Malaria Infection plus Folate Deficiency	Normal[c]	Normal/High[d]/Low[e]	Normal
Malaria Infection plus Vitamin B12 Deficiency	Normal/High[c]	Normal/High[d]	Low
Malaria Infection plus Folate Deficiency plus Vitamin B12 deficiency	Normal/High[c]	Low/Normal[d,e]	Low

a. Adapted from (Antony, 2017)

b. Vitamin B12 is a necessary co-factor for the enzyme methionine synthase which is needed to metabolize food folates. In low vitamin B12 states, the folate pathway backs up, and folate leaks out of RBCs, causing elevated serum folate and decreased RBC folate measurements.

c. Malaria causes hemolysis (destruction of RBCs) releasing the 30-fold times higher folate contents of the RBC cytosol into the serum. The subsequent compensatory reticulocytosis also contributes to elevated folate measurements as reticulocytes have high folate concentrations than mature RBCs. Lastly, malaria may directly damage hepatocytes, the primary storage site of folate in the body, releasing folates into the blood stream.

d. Certain malaria species can synthesize folates in RBC cultures in vitro, raising RBC folate measurements in animal models with high parasite burden.

e. Some malaria infections will paradoxically suppress hematopoiesis directly via cytokine release or indirectly secondary due combined folate and vitamin B12 deficiency, negating an expected rise in RBC folate levels.

Figure 2. Flow Chart of Inclusion and Exclusion Criteria - Malawi Micronutrient Survey, 2015-2016

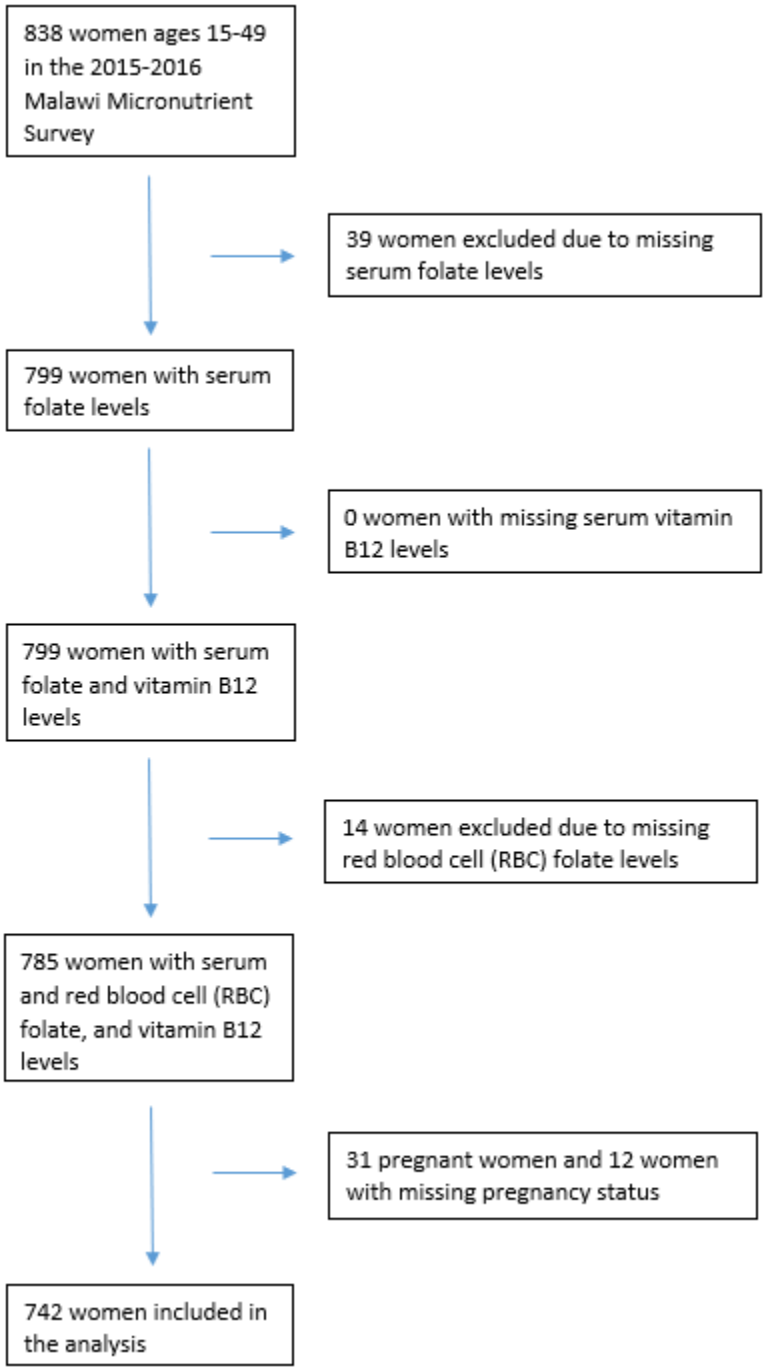

Table 1A. Characteristics of Women of Reproductive Age by Possible Serum Folate Deficiency

	Adequate Serum Folate (≥14.0nmol/L)	Possible Folate Insufficiency (<14.0nmol/L)	Chi Sq*	Crude PORs+ (95% CI)	P Value
	n (%)[1]	**n (%)[1]**			
	460 (66.0%)	282 (34.0%)			
Vitamin B12 (pmol/L)			0.09		
Normal (≥220)	362 (78.6 %)	248 (86.5%)		ref	
Insufficient (150-220)	82 (18.1%)	29 (11.9%)		0.60 (0.32, 1.11)	0.10
Deficient (<150)	16 (3.3%)	5 (1.6%)		0.42 (0.12, 1.44)	0.17
Age (years)					
11-19	101 (21.5%)	58 (18.6%)	0.72	0.89 (0.47, 1.64)	
20-29	162 (38.9%)	106 (38.1%)		ref	
30-49	197 (39.6%)	118 (43.4%)		1.12 (0.72, 1.73)	0.74
Missing	0	0			
Marital Status			0.68		
Married	355 (77.9%)	220 (79.8%)		ref	
Unmarried	105 (22.1%)	62 (20.2%)		0.89 (0.52, 1.54)	0.67
Missing	0	0			
Body Mass Index (kg/m^2)			0.02		
Underweight (<18.5)	35 (7.7%)	27 (11.5%)		1.75 (0.93, 3.29)	0.08
Normal weight (18.5 to <25)	362 (80.6%)	184 (68.9%)		ref	
Overweight 25 to <30	46 (9.2%)	52 (12.2%)		1.56 (0.80, 3.04)	0.19
Obese ≥30	14 (2.5%)	15 (7.4%)		3.48 (1.19, 10.18)	0.02
Missing	3	4			
Malaria Status[2]			0.01		
Negative	374 (80.4%)	243 (88.7%)		ref	
Positive	76 (19.6%)	33 (11.3%)		0.52 (0.32, 0.86)	0.01
Missing	10	6			
Presumed Schistosomiasis[3]			0.53		
Negative	406 (92.3%)	235 (90.3%)		ref	
Positive	27 (7.7%)	26 (9.7%)		1.30 (0.57, 2.96)	0.53
Missing	27	21			
Residence			0.11		
Urban	50 (7.1%)	65 (13.1%)		ref	
Rural	410 (92.9%)	217 (86.9%)		0.50 (0.21, 1.23)	0.13
Missing	0	0			

Wealth Index (3 Level)			0.003		
Lowest	200 (48.8%)	77 (31.1%)		0.32 (0.15, 0.68)	0.004
Middle	181 (41.2%)	129 (49.2%)		0.61 (0.28, 1.32)	0.20
Highest	79 (10.0%)	76 (19.7%)		ref	
Missing	0	0			
Wealth Index (5 Level)			0.005		
Lowest	107 (27.9%)	36 (14.2%)		0.26 (0.12, 0.57)	0.001
Second	93 (20.9%)	41 (16.9%)		0.41 (0.18, 0.91)	0.03
Middle	93 (20.1%)	49 (19.6%)		0.49 (0.21, 1.18)	0.11
Fourth	88 (21.2%)	80 (29.7%)		0.71 (0.30, 1.69)	0.44
Highest	79 (10.0%)	76 (19.7%)		ref	
Missing	0	0			
Any Inflammation[4]			0.03		
No	390 (87.3%)	220 (79.5%)		ref	
Yes	70 (12.7%)	62 (20.5%)		1.78 (1.04, 3.05)	0.04
Missing	0	0			
Anemia[5]			0.08		
No	384 (81.8%)	217 (75.5%)		Ref	
Yes	76 (18.2%)	65 (24.5%)		1.46 (0.94, 2.26)	0.09
Missing	0	0			

*Rao-Scott Chi-Squared Value

+Prevalence Odds Ratio

1. Weighted percentages based on complex survey analysis
2. Based on rapid malaria test kit result
3. Based on presence or absence of hematuria
4. Elevated C-reactive protein > 5mg/L or elevated α-1-acid glycoprotein > 1.0g/L
5. Defined as hemoglobin <12.0 g/dL on Hemocue 301 hemoglobin kit

Table 1B. Characteristics of Women of Reproductive Age by Serum Folate Deficiency

	Adequate Serum Folate (≥6.8nmol/L)	Serum Folate Deficiency (<6.8nmol/L)	Chi Sq*	Crude PORs+ (95% CI)	P Value
	n (%)[1]	n (%)[1]			
	678 (93.0%)	64 (7.0%)			
Vitamin B12 (pmol/L)			N/A		
Normal (>220)	550 (80.5%)	60 (91.7%)		ref	
Insufficient (<220)	107 (16.6%)	4 (8.3%)		0.44 (0.15, 1.32)	0.14
Deficient (<150)	21 (2.1%)	0 (0.0%)		N/A	N/A
Age (years)			0.04		
11-19	152 (21.4%)	7 (8.6%)		0.45 (0.13, 1.55)	0.20
20-29	245 (38.9%)	23 (34.8%)		ref	
30-49	281 (39.7%)	34 (56.6%)		1.59 (0.85, 2.99)	0.15
Missing	0	0			
Marital Status			0.07		
Married	520 (77.7%)	55 (90.1%)		ref	
Unmarried	158 (22.3%)	9 (9.9%)		0.38 (0.13, 1.10)	0.07
Missing	0	0			
Body Mass Index (kg/m^2)			0.47		
Underweight (<18.5)	58 (8.4%)	4 (8.4%)		1.03 (0.29, 3.58)	0.97
Normal weight (18.5 to <25)	506 (77.2%)	40 (70.1%)		ref	
Overweight 25 to <30	83 (9.7%)	15 (16.8%)		1.91 (0.85, 4.31)	0.12
Obese ≥30	25 (4.1%)	4 (4.7%)		1.26 (0.35, 4.46)	0.72
Missing	6	1			
Malaria Status[2]			0.05*		
Negative	560 (82.4%)	57 (93.7%)		ref	
Positive	103 (17.6%)	6 (6.3%)		0.31 (0.09, 1.06)	0.06
Missing	15	1			
Presumed Schistosomiasis[3]			0.12		
Negative	591 (92.1%)	50 (85.5%)		ref	
Positive	43 (7.9%)	10 (14.5%)		1.99 (0.80, 4.94)	0.14
Missing	44	4			
Residence			0.19		
Urban	103 (9.5%)	12 (4.5%)		ref	
Rural	575 (90.5%)	52 (95.5%)		2.20 (0.62, 7.86)	0.22
Missing	0	0			

Wealth Index (3 Level)			0.06		
Lowest	264 (44.2%)	13 (24.7%)		0.49 (0.17, 1.43)	0.19
Middle	276 (42.7%)	34 (60.5%)		1.25 (0.45, 3.51)	0.67
Highest	138 (13.1%)	17 (14.8%)		ref	
Missing	0	0			
Wealth Index (5 Level)			0.09		
Lowest	136 (24.1%)	7 (12.5%)		0.46 (0.14, 1.56)	0.21
Second	128 (20.1%)	6 (12.2%)		0.54 (0.17, 1.75)	0.30
Middle	129 (19.8%)	13 (20.6%)		0.92 (0.31, 2.76)	0.87
Fourth	147 (22.9%)	21 (39.9%)		1.55 (0.50, 4.78)	0.45
Highest	138 (13.1%)	17 (14.8%)		ref	
Missing	0	0			
Any Inflammation[4]			0.68		
No	561 (84.8%)	49 (82.5%)		ref	
Yes	117 (15.2%)	15 (17.5%)		1.18 (0.53, 2.65)	0.68
Missing					
Anemia[5]			0.08		
No	554 (80.4%)	47 (70.2%)		Ref	
Yes	124 (19.6%)	17 (29.8%)		0.58 (0.31, 1.09)	0.09
Missing	0	0			

*Rao-Scott Chi-Squared Value

+Prevalence Odds Ratio

1. Weighted percentages based on complex survey analysis
2. Based on rapid malaria test kit result
3. Based on presence or absence of hematuria
4. Elevated C-reactive protein > 5mg/L or elevated α-1-acid glycoprotein > 1.0g/L
5. Defined as hemoglobin <12.0 g/dL on Hemocue 301 hemoglobin kit

Table 1C. Characteristics of Women of Reproductive Age by RBC Folate Insufficiency (based on elevated NTD risk)

	Adequate RBC Folate, (>748nmol/L)	RBC Folate Insufficiency (<748nmol/L)	Chi Sq*	Crude PORs+ (95% CI)	P Value
	n (%)	n (%)			
	144 (18.8%)	598 (81.2%)			
Vitamin B12 (pmol/L)			0.98		
Normal (>220)	120 (81.8%)	490 (81.2%)		ref	
Insufficient (<220)	20 (15.9%)	91 (16.0%)		1.02 (0.43, 2.44)	0.97
Deficient (<150)	4 (2.4%)	17 (2.8%)		1.19 (0.33, 4.32)	0.80
Age (Years)			0.26		
11-19	34 (26.2%)	125 (19.2%)		0.62 (0.34, 1.14)	0.12
20-29	45 (33.6%)	223 (39.8%)		ref	
30-49	65 (40.2%)	250 (41.1%)		0.86 (0.49, 1.53)	0.61
Missing	0	0			
Marital Status			0.07		
Married	106 (72.7%)	469 (79.9%)		ref	
Unmarried	38 (27.3%)	129 (20.1%)		0.67 (0.43, 1.04)	0.07
Missing	0	0			
Body Mass Index (kg/m^2)			0.66		
Underweight (<18.5)	12 (9.0%)	50 (8.9%)		1.05 (0.54, 2.04)	0.88
Normal weight (18.5 to <25)	115 (80.4%)	431 (75.8%)		ref	
Overweight (25 to <30)	13 (8.0%)	85 (10.7%)		1.42 (0.60, 3.39)	0.42
Obese (≥30)	4 (2.6%)	25 (4.5%)		1.86 (0.48, 7.27)	0.37
Missing	0	7			
Malaria Status			0.004		
Negative	107 (72.4%)	510 (85.7%)		ref	
Positive	34 (27.6%)	75 (14.3%)		0.45 (0.24, 0.81)	0.009
Missing	3	13			
Presumed Schistosomiasis[3]			0.50		
Negative	119 (90.2%)	522 (92.0%)		ref	
Positive	14 (9.8%)	39 (8.0%)		0.80 (0.41, 1.57)	0.51
Missing	11	37			
Residence			<0.0001		
Urban	10 (0.9%)	105 (11.1%)		ref	
Rural	134 (99.1%)	493 (88.9%)		0.07 (0.02, 0.28)	0.0003
Missing	0	0			

Wealth Index (3 Level)			0.22		
Lowest	65 (48.8%)	212 (41.4%)		0.48 (0.20, 1.16)	0.10
Middle	58 (43.0%)	252 (44.2%)		0.58 (0.24, 1.44)	0.24
Highest	21 (8.2%)	134 (14.4%)		ref	
Missing	0	0			
Wealth Index (5 Level)			0.38		
Lowest	28 (23.9%)	115 (23.1%)		0.55 (0.22, 1.41)	0.21
Second	37 (24.9%)	97 (18.3%)		0.42 (0.17, 1.02)	0.05
Middle	30 (21.1%)	112 (19.6%)		0.53 (0.20, 1.43)	0.21
Fourth	24 (21.9%)	140 (24.6%)		0.64 (0.24, 1.70)	0.36
Highest	21 (8.2%)	134 (11.7%)		ref	
Missing	0	0			
Any Inflammation[4]			0.52		
No	117 (86.8%)	493 (84.1%)		ref	
Yes	27 (13.2%)	105 (15.9%)		1.24 (0.63, 2.47)	0.53
Missing	0	0			
Anemia[5]			0.0002		
No	101 (65.6%)	500 (82.9%)		ref	
Yes	43 (34.4%)	98 (17.1%)		0.39 (0.24, 0.64)	0.0003
Missing					

*Rao-Scott Chi-Squared Value

+Prevalence Odds Ratio

1. Weighted percentages based on complex survey analysis
2. Based on rapid malaria test kit result
3. Based on presence or absence of hematuria
4. Elevated C-reactive protein > 5mg/L or elevated α-1-acid glycoprotein > 1.0g/L
5. Defined as hemoglobin <12.0 g/dL on Hemocue 301 hemoglobin kit

Table 2. Characteristics of Women of Reproductive Age by Vitamin B12 Insufficiency and Deficiency in Malawi

	Adequate Serum B12 (≥220nmol/L)	B12 Insufficiency (<220nmol/L)	Chi Sq*	Crude POR+ (95% CI)	P Value	Adequate Serum B12 (≥150nmol/L)	B12 Deficiency (<150nmol/L)	Chi Sq*	Crude POR+ (95% CI)	P Value
	n (%)[1]	n (%)[1]				n (%)[1]	n (%)[1]			
	610 (81.3%)	132 (18.7%)				721 (97.3%)	21 (2.7%)			
Age			0.32					0.82		
14-19	129 (20.8%)	30		1.15 (0.57, 2.31)	0.69	152 (20.3%)	7 (26.4%)		1.28 (0.39, 4.23)	0.68
20-29	230 (32.5%)	38		ref		260 (38.6%)	8 (39.2%)		ref	
30-49	251 (31.9%)	64		1.51 (0.94, 2.73)	0.17	309 (41.1%)	6 (34.4%)		0.82 (0.22, 3.07)	0.77
Missing	0	0								
Marital Status			0.09					0.71		
Married	466 (77.2%)	109 (84.3%)		ref		560	15		ref	
Unmarried	144 (22.8%)	23 (15.7%		0.63 (0.37, 1.08)	0.09	161	6		0.81 (0.26, 2.54)	0.72
Missing	0	0				0	0			
BMI			0.24					0.89		
Underweight <18.5	57 (9.4%)	5 (7.0%)		0.66 (0.22, 1.97)	0.46	61 (8.9%)	1 (10.9%)		1.16 (0.14, 9.93)	0.89
Normal weight 18.5 to <25	436 (74.9%)	110 (84.4%)		ref		529 (76.5%)	17 (80.9%)		ref	
Overweight 25 to <30	86 (11.3%)	12 (5.4%)		0.42 (0.21, 0.87)	0.02	96 (10.4%)	2 (5.0%)		0.46 (0.09, 2.24)	0.33
Obese >=30	24 (4.4%)	5 (3.2%)		0.64 (0.18, 2.33)	0.50	28 (4.2%)	1 (3.2%)		0.72 (0.08, 6.23)	0.76
Missing	7	0				7	0			
Malaria[2]			0.93					0.10		
Negative	508 (83.1%)	109 (83.5%)		ref		602 (83.7%)	15 (67.0%)		ref	
Positive	89 (16.9%)	20 (16.5%)		0.97 (0.46, 2.03)	0.93	103 (16.3%)	6 (33.0%)		2.52 (0.80, 7.94)	0.11
Missing	13	3				16	0			
Presumed Schistosomiasis[3]			0.88					0.67		
Negative	526 (91.5%)	115 (92.0%)		ref		624 (91.6%)	17 (94.0%)		ref	
Positive	41 (8.5%)	12 (8.0%)		0.94 (0.40, 2.20)	0.88	51 (8.4%)	2 (6.0%)		0.69 (0.12, 3.98)	0.68
Missing	43	5				46	2			

Residence			<0.0001					0.003		
Urban	111 (11.1%)	4 (0.6%)		ref		114 (9.4%)	1 (0.8%)		ref	
Rural	499 (88.9%)	128 (99.4%)		19.14 (4.51, 81.29)	0.0001	607 (90.6%)	20 (99.2%)		12.23 (1.33, 112.47)	0.03
Missing	0	0				0	0			
Wealth Index (3 Level)			0.0001					0.26		
Lowest	203 (38.6%)	74 (61.1%)		6.25 (2.22, 17.61)	0.0007	266 (42.3%)	11 (61.6%)		3.35 (0.49, 22.89)	0.21
Middle	261 (46.0%)	49 (35.0%)		3.00 (0.96, 9.34)	0.06	302 (44.3%)	8 (32.6%)		1.69 (0.24, 12.05)	0.60
Highest	146 (15.4%)	9 (3.9%)		ref		153 (13.5%)	2 (5.9%)		ref	
Missing	0	0				0	0			
Wealth Index (5 Level)			0.0006					0.11		
Lowest	100 (19.6%)	43 (39.1%)		7.85 (2.72, 22.66)	0.0002	135 (22.5%)	8 (51.0%)		5.22 (0.70, 3.88)	0.11
Second	103 (18.9%)	31 (22.1%)		4.60 (1.53, 13.78)	0.007	131 (19.8%)	3 (10.6%)		1.23 (0.14, 10.94)	0.85
Middle	117 (20.0%)	25 (19.2%)		3.79 (0.98, 14.69)	0.05	137 (20.0%)	5 (17.6%)		2.03 (0.25, 16.69)	0.51
Fourth	144 (26.0%)	24 (15.7%)		2.39 (0.77, 7.44)	0.13	165 (24.3%)	3 (15.0%)		1.42 (0.16, 12.95)	0.75
Highest	146 (15.4%)	9 (3.9%)		ref		153 (13.5%)	2 (5.9%)		ref	
Missing	0	0				0	0			
Any Inflammation[4]			0.81					0.02		
No	503 (84.5%)	107 (85.5%)		ref		592 (84.3%)	18 (95.8%)		ref	
Yes	107 (15.5%)	25 (14.5%)		0.92 (0.48, 1.78)	0.81	129 (15.7%)	3 (4.2%)		0.24 (0.06, 0.87)	0.03
Missing	0	0				0	0			
Anemia[5]			0.69					0.38		
No	494 (79.3%)	107 (81.1%)		ref		585 (79.4%)	16 (86.6%)		ref	
Yes	116 (20.7%)	25 (18.9%)		0.90 (0.51, 1.56)	0.69	136 (20.6%)	5 (13.4%)		0.60 (0.18, 1.97)	0.39
Missing	0	0				0	0			

*Rao-Scott Chi-Squared Value

+Prevalence Odds Ratio

1. Weighted percentages based on complex survey analysis
2. Based on rapid malaria test kit result
3. Based on presence or absence of hematuria
4. Elevated C-reactive protein > 5mg/L or elevated α-1-acid glycoprotein > 1.0g/L
5. Defined as hemoglobin <12.0 g/dL on Hemocue 301 hemoglobin kit

Table 3: Multivariable analysis examining the association between serum or folate (based on selected categorizations) and serum B12

		Serum Folate <14 nmol/L (aPOR[1]; 95% CI)	Serum Folate < 6.8 nmol/L (aPOR[1]; 95% CI)	RBC Folate <748 nmol/L (aPOR[1]; 95% CI)
	Vitamin B12			
Model 1	Normal (≥220)	Ref	Ref	Ref
	Insufficient or Deficient (<220)	0.71 (0.39, 1.27)	0.43 (0.15, 1.25)+	1.15 (0.56, 2.36)
	Deficient (<150)	0.55 (0.16, 1.92)	N/A*	1.31 (0.33, 5.11)
Model 2	Normal (≥220)	Ref	Ref	Ref
	Insufficient or Deficient (<220)	0.71 (0.40, 1.24)	0.40 (0.14, 1.18)+	1.20 (0.58, 2.50)
	Deficient (<150)	0.59 (0.17, 2.07)	N/A*	1.57 (0.38, 6.49)

[1]Adjusted prevalence odds ratio
Model 1: Adjusted for wealth category (3 levels)
Model 2: Adjusted for urban/rural, wealth category (3 levels), BMI category, and Malaria status
*There are no people in BOTH the lowest folate group and the lowest B12 group
+There are only 4 people in both the Insufficient B12 and <6.8nmol/L serum folate group

Table 4: Multivariable analysis examining the association between serum or folate (based on selected categorizations) and malaria infection status

		Serum Folate <14 nmol/L (POR[1]; 95% CI)	Serum Folate < 6.8 nmol/L (POR[1]; 95% CI)	RBC Folate <748 nmol/L (POR[1]; 95% CI)
	Malaria Test Result[2]			
Model 3	Negative	Ref	Ref	Ref
	Positive	0.53 (0.32, 0.87)	0.31 (0.09, 1.06)	0.45 (0.24, 0.81)
Model 4	Negative	Ref	Ref	Ref
	Positive	0.64 (0.39, 1.04)	0.35 (0.10, 1.20)	0.48 (0.26, 0.89)

[1] Crude prevalence odds ratio in model 1, adjusted prevalence odds ratio in model 2
[2] Based on results from a rapid malaria test kit
Model 3: Unadjusted
Model 4: Adjusted for wealth category (3 levels)