Supplementing Maternal and Infant Diet with Micronutrient Fortified Lipid-based Nutrient Supplements (LNS) (iLiNS-DYAD-G)

iLiNS-DYAD-G: Statistical Analysis Plan, appendix XX, version 03.0

Statistical Analysis Plan

Appendix 15: Comparison of the main effect of treatment group on change in maternal vitamin B12 and folate status during pregnancy, in maternal and infant B12 and folate status at 6 months postpartum, in infant B12 and folate status at 18 months, and in vitamin B12 in breast milk at 6 months postpartum.

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Table 3. Prevalence of abnormal maternal plasma B12, folate and homocysteine and breast milk vitamin B12 homocysteine at <20 wk gestation, at 36 wk gestation, and at 6 months postpartum.

Table 4. Prevalence of abnormal infant plasma B12 & folate values at 6 and 18 months.

Figure 1: Path analysis of milk B12 at 6 months.

Figure 2: Path analysis of infant B12 at 6 months.

1) **Study Objectives.**

This analysis falls under the iLiNS-DYAD-G trial, the primary aims of which is to evaluate the efficacy of lipid-based nutrient supplements (LNS) for pregnant women and their infants. A secondary aim is to study the impact of LNS on breast milk B12 concentrations at 6 mo postpartum. This sub-study analysis will compare the change in plasma B12, folate and homocysteine from enrolment (before 20 wk gestation) through 36 wk gestation and 6 months postpartum between similar groups of women randomly assigned to receive daily antenatal supplements in one of the following three intervention groups:

a. 60 mg iron and 400g folic acid (IFA)

b. 20 mg iron and multiple micronutrients tablet (MMN) or

c. 20 mg iron and multiple micronutrients in a lipid-based nutrient supplement (LNS)

This sub-study will also compare changes in infant plasma B12 and folate at 6 and 18 months postpartum in the different intervention groups. Infants of mothers who received the LNS intervention were supplemented with LNS between 6 and 18 mo postpartum.

In addition, we will evaluate the strength of the association between maternal status in early pregnancy, at 36 wk gestation, and at 6 mo postpartum and i) B12 breast milk concentrations at 6 mo and ii) infant plasma B12 at 6 mo.

2. Study Description. Pregnant women were randomly assigned to receive one of three daily supplements throughout pregnancy. Blood samples were collected at the time of enrollment (<20 wk gestation) at 36 wk gestation, as determined by ultrasonography, as well at 6 mo postpartum. Breast milk samples were collected at 6 months postpartum. Infant blood samples were collected at 6 and 18 months postpartum. Concentrations of the main outcomes, plasma B12, folate and homocysteine were quantified by chemiluminescence for B12 and folate and high performance liquid chromatography HPLC for homocysteine. Breast milk B12 was assessed by chemiluminescence. The concentrations of B12, folate and homocysteine at each time point will be compared between groups.

3. Hypotheses to be tested

The primary hypotheses A1- D make 1 assumption: that there is not a significant difference between the LNS and MMN groups. If LNS and MMN treatment groups significantly differ, then we will do a 3 group comparison.

. Primary hypothesis A1: The combined MMN and LNS intervention group will have significantly higher maternal plasma B12 concentrations at 36 weeks compared to the IFA group in women in Ghana

. Primary hypothesis A2: The combined MMN and LNS intervention group will have significantly higher maternal plasma B12 concentrations at 6 months postpartum compared to the IFA group in women in Ghana

. Primary hypothesis B1: The combined MMN and LNS intervention group will have significantly lower maternal plasma homocysteine concentrations at 36 weeks gestation compared to the IFA group in women in Ghana

. Primary hypothesis B2: The combined MMN and LNS intervention group will have significantly lower maternal plasma homocysteine concentrations at 6 months postpartum compared to the IFA group in women in Ghana

. Primary hypothesis C1: The combined MMN and LNS intervention group will have significantly higher infant plasma B12 concentrations at 6 months postpartum compared to the IFA group in Ghana

. Primary hypothesis C2: The LNS intervention group will have significantly higher infant plasma B12 concentrations at 18 months postpartum, compared to the IFA and MMN groups in Ghana

. Primary hypothesis D: The combined MMN and LNS intervention group will have significantly higher breast milk B12 concentrations at 6 months postpartum compared to the IFA group in women in Ghana

. Secondary/exploratory hypothesis E: The impact of the treatment on breast milk B12 at 6 months is positively and partially mediated by the change in maternal plasma B12 between baseline and 36 wk gestation and positively and partially mediated by the change in maternal plasma B12 from 36 wk gestation to 6 months postpartum.

. Secondary/exploratory hypothesis F: The impact of the treatment on infant B12 at 6 months is positively and partially mediated by the change in maternal plasma B12 between baseline and 36 wk gestation and positively and partially mediated by breast milk B12 at 6 months postpartum.

Secondary/exploratory hypothesis G: When controlling for <20 wk plasma and 36 wk plasma, B12 in milk at 6 mo is significantly correlated with maternal plasma B12 at 6 mo, and not with maternal plasma at <20 wk or 36 wk gestation.

Secondary/exploratory hypothesis H: When controlling for maternal plasma at <20 wk gestation and at 6 months postpartum, infant plasma B12 at 6 mo is significantly correlated with maternal plasma B12 at 36 wk gestation, and not with maternal plasma at <20 wk or 6 mo postpartum.

4. Definition of the sub study outcomes

Outcomes

a. Concentrations of maternal plasma B12, folate and homocysteine at <20 weeks gestation, 36 wk gestation and 6 months postpartum

b. Concentrations of infant plasma B12 and folate at 6 & 18 months postpartum.

c. Breast milk B12 concentrations at 6 mo postpartum.

d. Percent abnormal values of B12, folate and homocysteine at baseline, 36 wk gestation and 6 months postpartum in mothers.

e. Prevalence of abnormal values of B12 and folate at 6 &18 months postpartum in infants.

Cutoffs used to calculate percent of abnormal values:

	Mothers			Infants	
Biomarker	<20 wk Pregnancy	36 wk Pregnancy	6 mo Postpartum	6 mo	18 mo
Plasma B12	<150 pmol/L	<100 pmol/L	<150 pmol/L	Plasma B12 <150 pmol/L	Plasma B12 <150 pmol/L
Plasma Folate	<10 nmol/L	<10 nmol/L	<10 nmol/L	Plasma folate <10 nmol/L	Plasma folate <10 nmol/L
Plasma Homocysteine Elevated	<10 μmol/L	<10 μmol/L	<10 μmol/L		
Breast milk B12 Deficient			<362 pmol/L		

5. Basis for the analysis: Modified intention to treat and per protocol

The basis for the analysis will be the same as that of the primary outcomes. Subjects lost to follow-up will not contribute data to the final time point. Subjects that complete the study will be included in the analysis regardless of adherence to the study protocol. Subject who received mixed exposure to supplements in Ghana, (participants from the IFA group who received MMN,) will not be included in the analysis. As such, a modified intention to treat method will be used.

In addition to the intention to treat analysis, a per protocol analysis will be performed including subjects meeting criteria for good adherence to study protocol. Adherence is recorded biweekly by interview of study subject and verified by collection and count of remaining intervention supplements. Good adherence will be defined as consumption on > 70% of supplement days. There is no adherence data for infants in the control group at 18 mo. As such, infant adherence at 18 mo will be based upon maternal adherence.

Furthermore, we will test for differences in adherence based upon maternal baseline characteristics.

6. Time points for the analyses

Biological samples will be collected at baseline (<20 wk gestation) at term, before delivery (36 wk gestation), and at 6 months postpartum in mothers and at 6 & 18 months postpartum in infants. Breast milk samples will be collected at 6 mo postpartum.

7.Statistical software

All statistical analyses will be performed using SAS version 9.4 (SAS Institute, Cary, NC, USA).

8. Presentation of the study findings and hypothesis testing

ANOVA(baseline)/ANCOVA (36wk, 6mo) or logistic regression will be conducted to evaluate whether there is a significant difference between the LNS and MMN groups for hypotheses A1-D and for maternal plasma folate. If a significant difference is found between the groups, the groups will be analyzed separately in a 3 group test, rather than a 2 group test.

For Hypotheses A1-D and for maternal plasma folate, group means and standard deviations for each time point will be presented in tables 1 (maternal) & 2 (infant). In addition, the prevalence of abnormal values for mothers at baseline, 36 weeks gestation, and 6 months postpartum and for infants at 6 and 18 mo in

each intervention group will be presented in Tables 3 & 4, respectively. Both risk ratios (RR) and adjusted odds ratios (OR) will be calculated and presented in Tables 3 and 4.

An overall ANOVA(baseline)/ANCOVA (36wk, 6mo) will be conducted to generate the p values for continuous variables, while logistic regression will be used to generate p values for categorical variables. Logistic regression will utilize adjusted OR and differences between groups will be based upon the Wald-chi squared p value. In cases where LNS & MMN groups were not combined, (see note above,) Tukey's test will be conducted to evaluate pairwise differences between the groups for ANOVA/ANCOVA tests.

Hypotheses E and F will then be examined using a path analysis and displayed in Figure 1 and Figure 2, (not shown.) The LNS and MMN groups will be combined for this analysis.

Exploratory hypotheses G and H will be examined using multiple linear regression models. A total of 4 models will be evaluated for each hypothesis: 3 2-factor models and 1 3-factor model. The 2-factor models will include the independent variables i) maternal plasma at <20 wk gestation & 36 wk gestation, ii) maternal plasma at <20 wk gestation & 6 mo postpartum, or iii) maternal plasma at <20 wk gestation & 6 mo postpartum. The 3-factor model will include maternal plasma at <20 wk gestation, 36 wk gestation, and 6 mo postpartum.

Outcome variables will be assessed for conformance to the normal distribution and transformed if needed. If no suitable transformation can be found, non parametric testing will be used.

The covariates to be included in the ANOVA, ANCOVA, and logistic regression models will be derived from the list below. Each variable that shows a statistically significant association with each outcome ($P<0.1$), will be included in the adjusted model. The difference between the three groups will be tested with ANOVA (model without covariates), ANCOVA (model with covariates), or logistic regression (model with covariates) and null-hypothesis of no difference between groups will be rejected if $P<0.05$.

9. Description of covariates

. a) Initial maternal B12, folate and homocysteine (baseline)

. b) Initial maternal C-reactive protein (CRP)

. c) Initial maternal alpha-1-glycoprotein (AGP)

. d) Initial maternal body mass index (BMI)

. e) Maternal malaria at baseline

. f) Maternal HIV status at baseline

. g) Parity

. h) Maternal age

. i) Season of enrollment

. j) Maternal ARV use at baseline

. k) Maternal height

. l) Maternal weight

. m) Maternal education

Table 1. Maternal plasma B12, folate and homocysteine and breast milk vitamin B12.

Variable	Time point	IFA [n]	MMN / LNS [n]	Comparison of IFA and pooled MMN/LNS	
				P-value	Difference in means (95 % CI)
Plasma B12 (pmol/L) (mean (95% CI)) [n]	Baseline	x.xx (x.xx, x.xx) [n]	x.xx (x.xx, x.xx) [n]	x.xx	x.xx (x.xx, x.xx)
	36 weeks gestation	x.xx (x.xx, x.xx) [n]	x.xx (x.xx, x.xx) [n]	x.xxx	x.xx (x.xx, x.xx)
	6 months postpartum	x.xx (x.xx, x.xx) [n]	x.xx (x.xx, x.xx) [n]	x.xxx	x.xx (x.xx, x.xx)
Plasma folate (nmol/L) (mean (95% CI)) [n]	Baseline	x.xx (x.xx, x.xx) [n]	x.xx (x.xx, x.xx) [n]	x.xxx	x.xx (x.xx, x.xx)
	36 weeks gestation	x.xx (x.xx, x.xx) [n]	x.xx (x.xx, x.xx) [n]	x.xxx	x.xx (x.xx, x.xx)
	6 months postpartum	x.xx (x.xx, x.xx) [n]	x.xx (x.xx, x.xx) [n]	x.xxx	x.xx (x.xx, x.xx)
Plasma homocysteine (umol/L) (mean (95% CI)) [n]	Baseline	x.xx (x.xx, x.xx) [n]	x.xx (x.xx, x.xx) [n]	x.xxx	x.xx (x.xx, x.xx)
	36 weeks gestation	x.xx (x.xx, x.xx) [n]	x.xx (x.xx, x.xx) [n]	x.xxx	x.xx (x.xx, x.xx)
	6 months postpartum	x.xx (x.xx, x.xx) [n]	x.xx (x.xx, x.xx) [n]	x.xxx	x.xx (x.xx, x.xx)
Breast milk B12 (pmol/L) (mean (95% CI)) [n]	6 months postpartum	x.xx (x.xx, x.xx) [n]	x.xx (x.xx, x.xx) [n]	x.xxx	x.xx (x.xx, x.xx)

Table 2. Infant plasma B12 & folate

Variable	Time point	IFA [n]	MMN / LNS [n]	Comparison of IFA and pooled MMN/LNS	
				P-value	Difference in means (95 % CI)
Plasma B12 (pmol/L) (mean (95% CI)) [n]	6 months	x.xx (x.xx, x.xx) [n]	x.xx (x.xx, x.xx) [n]	x.xxx	x.xx (x.xx, x.xx)
	18 months	x.xx (x.xx, x.xx) [n]	x.xx (x.xx, x.xx) [n]	x.xxx	x.xx (x.xx, x.xx)
Plasma folate (nmol/L) (mean (95% CI)) [n]	6 months	x.xx (x.xx, x.xx) [n]	x.xx (x.xx, x.xx) [n]	x.xxx	x.xx (x.xx, x.xx)
	18 months	x.xx (x.xx, x.xx) [n]	x.xx (x.xx, x.xx) [n]	x.xxx	x.xx (x.xx, x.xx)
Plasma homocysteine (umol/L) (mean (95% CI)) [n]	6 months	x.xx (x.xx, x.xx) [n]	x.xx (x.xx, x.xx) [n]	x.xxx	x.xx (x.xx, x.xx)
	18 months	x.xx (x.xx, x.xx) [n]	x.xx (x.xx, x.xx) [n]	x.xxx	x.xx (x.xx, x.xx)

Table 3 Proportions of women with abnormal biochemical values

Cutoff	**Time point**	**IFA n (%)**	**MMN / LNS n (%)**	**Comparison of IFA and pooled MMN/LNS**	
				Risk ratio (95 % CI)	**P-value**
Plasma B12 <150 pmol/L	Baseline	x (x.x)	x (x.x)	x.xx (x.xx, x. xx)	x.xxx
Plasma B12 <100 pmol/L	36 wk	x (x.x)	x (x.x)	x.xx (x.xx, x. xx)	x.xxx
Plasma B12 <150 pmol/L	6 months postpartum	x (x.x)	x (x.x)	x.xx (x.xx, x. xx)	x.xxx
Plasma folate <10 nmol/L	Baseline	x (x.x)	x (x.x)	x.xx (x.xx, x. xx)	x.xxx
Plasma folate <10 nmol/L	36 wk	x (x.x)	x (x.x)	x.xx (x.xx, x. xx)	x.xxx
Plasma folate <10 nmol/L	6 months postpartum	x (x.x)	x (x.x)	x.xx (x.xx, x. xx)	x.xxx
Plasma tHcy >10 umol/L	Baseline	x (x.x)	x (x.x)	x.xx (x.xx, x. xx)	x.xxx
Plasma tHcy >10 umol/L	36 wk	x (x.x)	x (x.x)	x.xx (x.xx, x. xx)	x.xxx
Plasma tHcy >10 umol/L	6 months postpartum	x (x.x)	x (x.x)	x.xx (x.xx, x. xx)	x.xxx

breast milk B12 concentrations controlling for baseline B12 status, and assessing the effect of parity and maternal age as covariates. (Table to be completed later).

Table 4 Proportions of infants with abnormal biochemical values

Cutoff	**Time point**	**IFA n (%)**	**MMN / LNS n (%)**	**Comparison of IFA and pooled MMN/LNS**	
				Risk ratio (95 % CI)	**P-value**
Plasma B12 <150 pmol/L	6 months	x (x.x)	x (x.x)	x.xx (x.xx, x. xx)	x.xx
	18 months	x (x.x)	x (x.x)	x.xx (x.xx, x. xx)	x.xxx
Plasma folate <10 nmol/L	6 months	x (x.x)	x (x.x)	x.xx (x.xx, x. xx)	x.xxx
	18 months	x (x.x)	x (x.x)	x.xx (x.xx, x. xx)	x.xxx