Efficacy of lipid-based nutrient supplements (LNS) for pregnant and lactating women and their infants (iLiNS-DYAD-G)

Statistical Analysis Plan: The impact of LNS on markers of child immune function.

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I. Study objectives

The primary objective for the main trial is to determine whether a lipid-based nutrient supplement (LNS) consumed by women during pregnancy and the first 6 mo of lactation, and by the child from 6-18 mo, improves fetal and child growth, micronutrient status and neuro-behavioral development to a greater extent than consumption of iron and folic acid during pregnancy only, or a multiple micronutrient (MMN) tablet during pregnancy and the first six months of lactation.

The objectives for the present pilot analyses are to determine the effect of LNS on markers of child immune function, specifically:

1) To determine if maternal plus child LNS supplementation resulted in altered child HDL lipidomic composition at 18 months of age, relative to maternal (only) Iron Folic Acid (IFA) supplementation.
2) To determine if maternal plus child LNS supplementation resulted in altered child HDL cholesterol efflux capacity at 18 months of age, compared to maternal IFA supplementation.

II. Hypotheses

1) Primary: Children who received LNS and were born to women who received LNS will have altered mean surface to core lipids ratio and altered levels of fatty acid unsaturation and chain length in their HDL particles, compared to the IFA group.
2) Primary: The HDL of children who received LNS and were born to women who received LNS will have an altered capacity to efflux cholesterol from macrophage cells compared to the IFA group.

III. Outcome variables

The primary outcome variables are as follows:

1. Surface to core lipids ratio

HDL are isolated and analyzed for lipidomic composition by untargeted LC-MS analysis. HDL lipid species are calibrated from raw MS intensity using the equation below:

$$\mathrm{Conc}_{i,j} = \frac{\mathrm{Int}_{i,j}}{\mathrm{Int\_IS}_{k,j}} \times \mathrm{Conc\_IS}_{k,j}$$

$\mathrm{Conc}_{i,j}$ is the concentration for lipid feature i in sample j. $\mathrm{Int}_{i,j}$ is the MS intensity for lipid feature i in sample j. $\mathrm{Int\_IS}_{k,j}$ is the MS intensity for the internal standard k that belongs to the same lipid class as lipid feature i. $\mathrm{Conc\_IS}_{k,j}$ is the concentration of the internal standard k in sample j. The final unit is ug/ml normalized to the molecular weight of each species to calculate a molar concentration. Total amounts of phosphatidylcholine (PC), sphingomyelin (SM), cholesterol ester (CE), free cholesterol (FC), ceramides, diacylglycerol (DG), lyso-PC (LPC), phosphatidylethanolamine (PE), triacylglycerol (TG), are calculated by adding all corresponding lipid species of a particular lipid class together. The surface to core lipids ratio is then calculated as (PC+LPC+PE+FC+SM+Cer+DG)/(TG+CE).

2. Fatty acid unsaturation

For chain saturation we will calculate the Equivalent Of Double bond per 18 carbons (EOD_{18}) as below:

$$EOD_{18} = \frac{\sum conc_{i,j} \times ndb_{i,j}}{\sum conc_{i,j} \times nc_{i,j}} \times 18$$

The $\mathrm{conc}_{i,j}$ is the abundance of the lipid species. $\mathrm{ndb}_{i,j}$ is the number of double bonds of this lipid species, while $\mathrm{nc}_{i,j}$ is the number of carbons.

3. **Fatty acid chain length**

Average Chain Length (ACL) will be calculated as below:

$$ACL = \frac{\sum conc_{i,j} \times nc_{i,j}}{\sum con_{i,j} \times nfa_{i,j}}$$

$\mathrm{nfa}_{i,j}$ represents the number of fatty acids for the lipid species (for example, a PC or a DG will be 2, and a TG will be 3).

4. **HDL cholesterol efflux capacity**

The cholesterol efflux capacity is in the unit of percentage efflux of fluorescently labeled cholesterol.

IV. Basis for the analysis: Intention to treat

The analytic subset for this pilot study was selected from the full cohort of children in the DYAD-G study. First, we selected children in the LNS and IFA groups born in period 3 (i.e., to mothers who were not exposed to the supplement mislabeling between IFA and MMN), who had a plasma sample at 18 months, and whose mothers were primiparous and had a BMI at baseline of < 25 kg/m^2. These maternal criteria were imposed for the pilot study to maximize the chances of seeing differences between groups, as children with these characteristics were more likely to respond to the LNS intervention. This list of children was then randomly sorted and we selected the first 40 individuals from the LNS group and the first 40 individuals from the IFA group.

The primary analysis will be by intention-to-treat. That is, results for all children will be analyzed according to the group to which their mothers were assigned regardless of any protocol violations.

Time points
HDL was derived from plasma samples that were collected from children at 18-months of age.

V. Statistics software

Analyses will be performed using SAS version 9.4 and R version 3.5.0.

VI. Outliers

Outliers will be visually inspected by creating box and whisker plots and/or histograms of individual continuous variables, and scatterplots of related variables. Outliers which are clearly impossible or implausible values will be corrected if possible, or recoded to missing if correction is not possible. Outliers which are plausible or possible will be kept. When outliers are not able to be corrected, low outliers will be winsorized to the 2.5th percentile and high outliers will be winsorized to the 97.5th percentile. If winsorization is necessary, a sensitivity analysis will be performed to compare the winsorized results with the non-winsorized results.

VII. Data transformation

Distribution of outcome variables and key baseline variables will be inspected for normality using the Shapiro-Wilks test. A SW statistic larger than 0.95 will be considered normally distributed. Outcome variables will transformed as necessary, with a natural log transformation being tried before others. If no suitable transformation is found, normalized ranks will be calculated, or categories will be created.

VIII. Covariates

The covariates to be included in the ANCOVA 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.

1. Maternal height at baseline
2. Gestational age at enrollment
3. Maternal Inflammatory markers (CRP and AGP) at baseline
4. Malaria at baseline
5. Maternal education
6. Season at enrollment
7. Household food insecurity score at baseline
8. Asset index at baseline
9. Child's sex
10. Maternal Hb at enrollment

IX. Presentation of study findings and FDR correction

Characteristics of the study sample will be shown in Table 1. Group means and standard deviations for HDL cholesterol efflux capacity, as well as HDL surface to core lipids ratio, fatty acid unsaturation and chain length will be tabulated by intervention group and presented in Table 2. The table will also indicate the differences in means and their 95% confidence intervals between the intervention groups.

The difference between the LNS and IFA groups will be tested with ANOVA (model without covariates) and ANCOVA (model with covariates) and the null-hypothesis of no difference between groups will be rejected if $P<0.05$. For all pairwise comparisons with $P<0.05$, the null-hypothesis of no difference in means between groups will be rejected. Correlation analysis will be conducted using Spearman correlations between lipid species and cholesterol efflux capacity.

X. Tables

Table 1. Mean (SD) or % of baseline characteristics

Characteristic	IFA (n=40)	LNS (n=40)
Body mass index (BMI), kg/m2	21.9 (2.1)	21.6 (2.0)
Maternal age, years	XX (X.X)	XX (X.X)
Education, completed years	X (X.X)	X (X.X)
Gestational age at enrollment, weeks	X (X.X)	X (X.X)
Women with anemia (Hb < 100 g/l)	XX%	XX%

Table 2. Mean (SD) outcomes by supplement group

	Result by study group		Unadjusted Models		Adjusted Models	
Outcome	IFA (n=xxx)	LNS (n=xxx)	Difference in means (95% CI)	p-value	Difference in means (95% CI)	p-value
Mean (SD) Cholesterol Efflux Capacity, %	xxx.x (x.x)	xxx.x (x.x)	x.xx (x.xx, x.xx)	0.xxx	x.xx (x.xx, x.xx)	0.xxx
Mean (SD) surface to core lipids ratio	xxx.x (x.x)	xxx.x (x.x)	x.xx (x.xx, x.xx)	0.xxx	x.xx (x.xx, x.xx)	0.xxx
Mean (SD) EOD_{18}	xxx.x (x.x)	xxx.x (x.x)	x.xx (x.xx, x.xx)	0.xxx	x.xx (x.xx, x.xx)	0.xxx
Mean (SD) ACL	xxx.x (x.x)	xxx.x (x.x)	x.xx (x.xx, x.xx)	0.xxx	x.xx (x.xx, x.xx)	0.xxx