Brietta Oaks - Statistical Analysis Plan, October 15, 2013

Comparison of the main effect of treatment group on change in maternal salivary cortisol during pregnancy (iLiNS-DYAD-Ghana)

Outline

1) **Study Objective.**

The primary aim is to determine the effect of LNS during pregnancy, compared with two other nutritional supplements (iron + folic acid and a multiple micronutrient powder), on maternal cortisol levels, a stress biomarker. This analysis will measure the change in salivary cortisol from baseline (before 20 wk gestation) through 36 wk gestation.

If a relationship is found between treatment group and change in maternal cortisol, a secondary objective will be to determine if the change in cortisol is mediated by inflammation.

Study Description. Pregnant women were randomly assigned to receive one of three nutritional supplements throughout pregnancy:

1. Daily tablet of 60 mg iron & 400 µg folic acid
2. Daily multiple micronutrient tablet (20 mg iron)
3. Daily 20 g of lipid-based nutrient supplement for pregnant & lactating women (LNS-P&L, 20 mg iron)

Baseline blood, urine and saliva samples were collected at the time of enrollment (<20 wk gestation) and final time point samples were collected at 36 wk gestation. In addition, a saliva sample was collected at 28 wk gestation. Gestational age was determined by ultrasound at time

of enrollment. Cortisol levels were determined by enzyme-linked immunosorbent (ELISA) assay, and inflammation biomarkers (CRP and AGP) were measured from maternal blood samples.

2) **Hypotheses to be tested**
   a) Hypothesis A: The positive change in cortisol will be reduced in pregnant women receiving LNS as compared to the groups receiving either Fe+FA or MMN.
   b) Hypothesis B: The positive change in cortisol will be reduced in the group of pregnant women receiving MMN as compared to the group receiving Fe+FA.

3) **Definition of the substudy outcomes**

Outcomes
   a. Change in salivary cortisol (μg/dL), from baseline (< 20 wk gestation) to 36 wk gestation.
   b. Change in salivary cortisol (μg/dL), from baseline (< 20 wk gestation) to 28 wk gestation.
   c. Change in inflammation status, as defined by a combination of CRP (mg/L) and AGP (g/L), from baseline to 36 wk

4) **Basis for the analysis: Intention to treat and per protocol**

The basis for the analysis will be the same as that for the primary outcomes. Saliva samples will be collected at baseline, 28 wk gestation and 36 wk gestation. Subjects missing both the 28 wk gestation and 36 wk gestation will be considered lost to follow-up and will not contribute data to the final time point. However, subjects that have the 28 wk gestation sample and/or the 36 wk gestation sample will be included in the analysis. Subjects that complete the study will be included in the analysis regardless of adherence to the study protocol.
In addition to the intention to treat analysis, a per protocol analysis will be performed including subjects meeting minimum criteria for 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 $\geq$ 70% of supplement days and minimum adherence will be defined as consumption on > 50% of supplement days.

5) **Time points for the analyses**

Salivary samples will be collected at baseline (<20 wk gestation), at 28 wk gestation, and at 36 wk gestation.

6) **Presentation of the study findings and hypothesis testing**

6.1. Comparison of the change in salivary cortisol during pregnancy between intervention groups

Hypotheses A and B will be addressed as follows: The group means and standard deviations for salivary cortisol at each of the three time points will be presented as indicated in Table 1. An overall ANCOVA p-value will be provided and pairwise differences will be denoted by superscript. In addition, group means and standard deviations for the change in cortisol (μg/dL)

throughout pregnancy will be presented as indicated in Table 2. Similarly, an overall ANCOVA p-value will be provided and pairwise differences will be denoted by superscript.

Outcome variables will be assessed for conformance to the normal distribution and transformed if needed. Mean change in biomarkers in each of the intervention groups will be compared using ANCOVA (SAS GLM procedure). Post-hoc comparisons will be analyzed by Tukey's HSD test. Correlation analysis will be performed to determine if potential covariates are linearly related to the outcome variable at a 10% level of significance. All variables which are related to the outcome variable will be included as covariates.

Potential Effect Modifiers

The following variables will be considered as potential effect modifiers

a. Baseline cortisol
b. Baseline CRP
c. Baseline AGP
d. Proxy variable for household food insecurity
e. Proxy variable for household socio-economic status or wealth
f. Primiparity (i.e whether woman had her first childbirth)
g. Maternal height
h. Maternal BMI at enrolment (adjusted for gestational age)
i. Either Hb concentration at baseline, or maternal anemia at baseline
j. Gestational age at enrolment
k. Season at enrolment being dry season (Nov-Apr)
l. Maternal age
m. Maternal education
n. Sex of child (in analyses involving children)
o. One or several proxy indicators for diet quality

6.2. Assessing whether inflammation acts as a mediator between treatment group and maternal cortisol

A series of ANCOVA models will be run to construct a path analysis to determine if inflammation mediates the relationship between treatment group and change in cortisol (Figure 1). The first ANCOVA model will evaluate the relationship between main effect of treatment group and change in inflammation status, including potential covariates. The residual from the first model will be calculated for inclusion in the second model, an ANCOVA evaluating the relationship between main effect of treatment group and change in salivary cortisol. A third ANCOVA model will be generated to include the change in inflammation status as an independent variable instead of the residual from the first model. This will provide an estimate of the main effect of treatment group on salivary cortisol if change in inflammation status is part of the causal pathway.

It is possible that change in cortisol might increase inflammation by increasing morbidity. It is understood that the ability to make statements of causation for this part of the path analysis will not be possible, but morbidity data may be examined to explore that potential relationship (see Figure 1).

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7) General notes on statistical methods

Software. Data will be analyzed using SAS 9.3.

Procedures for data cleaning.
Two rounds of initial data cleaning are being performed at the Ghana field site. Any gross errors are queried to data collection managers and field team members to correct the error by reference to original questionnaires, data collectors, or study subjects. Additional data cleaning will be performed before statistical analysis by producing stem-and-leaf plots and histograms on individual variables and scatterplots of related variables. Queries will be communicated to the Ghana field site to perform similar clarification steps.

Procedures for breaking code.
Primary and secondary analyses will be performed while the analyst is masked. A preliminary report will be produced using alternatively labeled group names (e.g. A, B, and C).

Procedures for modifying analysis plan.
In the case that new hypotheses arise out of new data that have been collected or added, addendums will be added to the data analysis plan with clear documentation of rationale for the changes made.

8) Tables and Figures

Table 1. Comparison of salivary cortisol at baseline (<20 wk gestation), 28 wk gestation, and 36 wk gestation between intervention groups, unadjusted analyses.

	LNS	MMN	Fe+FA	Overall ANCOVA	Comparison of FE+FA and MMN		Comparison of Fe+FA and LNS		Comparison of LNS and MMN	
	n=	n=	n=	p-value	Difference in means (95% CI)	p-value	Difference in means (95% CI)	p-value	Difference in means (95% CI)	p-value
Baseline cortisol (µg/dL)	(mean ± SD)	(mean ± SD)	(mean ± SD)	p-value						
28 wk cortisol (µg/dL)	(mean ± SD)	(mean ± SD)	(mean ± SD)	p-value						
36 wk Cortisol (µg/dL)	(mean ± SD)	(mean ± SD)	(mean ± SD)	p-value						

Table 2. Comparison of change in salivary cortisol (µg/dL) throughout pregnancy (<20 wk to 36 wk gestation), unadjusted and adjusted analyses

	LNS	MMN	Fe+FA	Overall ANCOVA	Comparison of FE+FA and MMN		Comparison of Fe+FA and LNS		Comparison of LNS and MMN	
	n=	n=	n=	p-value	Difference in means (95% CI)	p-value	Difference in means (95% CI)	p-value	Difference in means (95% CI)	p-value
Change in cortisol (µg/dL), baseline to 28 wk	(mean ± SD)	(mean ± SD)	(mean ± SD)	p-value						
Change in cortisol (µg/dL), baseline to 36 wk	(mean ± SD)	(mean ± SD)	(mean ± SD)	p-value						
Change in cortisol (µg/dL), baseline to 28 wk*	(mean ± SD)	(mean ± SD)	(mean ± SD)	p-value						
Change in cortisol (µg/dL), baseline to 36 wk**	(mean ± SD)	(mean ± SD)	(mean ± SD)	p-value						

* Adjusted for covariates x, y and z.
**Adjusted for covariates a, b and c.

Figure 1. Hypothesized relationships between study variables for multiple regression path analysis.

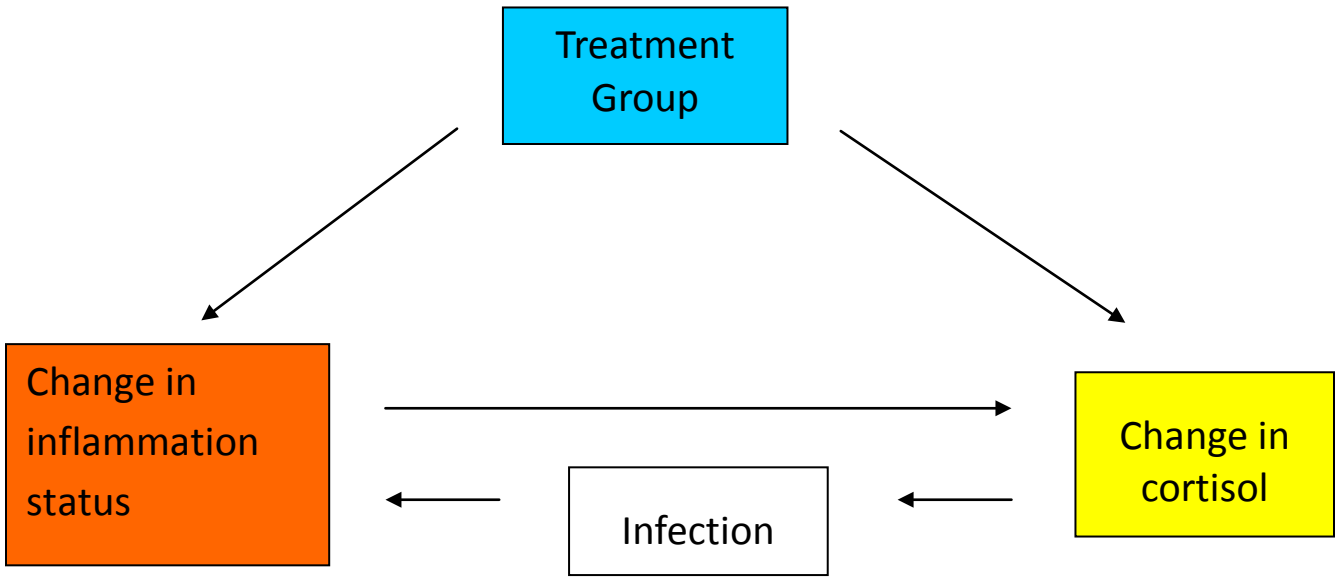

Table 3. ANCOVA results for path analysis comparing least squares means for each group and mean square for the group variable between two models: Model 1- including change in inflammation status as a covariate and Model 2- without change in inflammation status as covariate.

Statistic	Model 1	Model 2
LS Mean LNS		
LS Mean MMN		
LS Mean Fe + FA		
Mean Square Group variable		