iLiNS-DYAD-G2 Statistical Analysis Plan: Effect of LNS on Child and Maternal Cortisol in Hair and Saliva at Preschool Follow-Up

November 8, 2017

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**Background and Objectives**

Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis, a neuroendocrine system that responds to mental and physical stress by releasing cortisol, can occur during pregnancy and early childhood. Poor maternal nutrition can lead to a greater amount of cortisol crossing the placenta into fetal circulation, potentially contributing to permanent alteration of the fetal HPA axis (1, 2).

In the iLiNS-DYAD trial in Ghana, 1,320 pregnant women $\leq$ 20 wk gestation were enrolled and randomized to three groups: 1) small-quantity lipid-based nutrient supplements (SQ-LNS) for mothers during pregnancy and the first six months postpartum, and for their infants from 6 to 18 months of age; 2) multiple micronutrients (MMN) for mothers during pregnancy and the first six months postpartum; no supplementation for infants; and 3) Iron and folic acid (IFA) for mothers during pregnancy; placebo (200 mg calcium) for mothers for the first six months postpartum; no supplementation for infants. Previously we reported that among younger women, those provided with LNS during pregnancy had lower salivary cortisol (a marker of stress) in late pregnancy compared to younger women in the other two intervention groups (there were no group differences among older women) (3). It is therefore plausible that the offspring in the LNS arm will have lower chronic stress and a better regulated stress response.

Preliminary data analysis of salivary cortisol concentrations from infants in the iLiNS-DYAD trial in Malawi, which has a parallel study design to iLiNS-DYAD-Ghana, shows no difference in basal or stress-response salivary cortisol concentrations among the three trial arms. However, there were significant interactions between maternal cortisol and intervention group. Among mothers with high cortisol at 36 wk gestation, infants in the IFA group had a blunted response to venipuncture at 6 months of age as compared to infants in the LNS or MMN groups, and a higher basal cortisol concentration at 18 months of age.

The primary objectives of this analysis are as follows:

1) Evaluate the effects of maternal and child supplementation with SQ-LNS on child cortisol in hair and saliva
2) Evaluate the effects of maternal and child supplementation with SQ-LNS on maternal cortisol in hair and saliva

**Hypotheses**

1) Children born to women in the SQ-LNS group will have lower hair cortisol concentration than children born to women in either the IFA or MMN groups.

2) Children born to women in the SQ-LNS group will have a salivary cortisol response to a stressor that is consistent with a regulated HPA axis (an increase in cortisol in response to the stressor followed by a decrease in cortisol to pre-stressor concentration), while children born to women in either the IFA or MMN groups will have a blunted salivary cortisol response to a stressor, indicative of a dysregulated HPA axis. Specifically, children from the SQ-LNS group will have a larger area under the curve (AUC) of salivary cortisol concentrations in response to the stressor than children from the IFA or MMN groups.

**Description of Variables**

Outcome Variables

**Hair cortisol (long-term cortisol exposure):** Hair cortisol will be analyzed from hair samples collected from women and children, primarily from the middle of the back of the head. Hair grows in people of African descent at a rate of approximately 0.8 cm per month. We were able to collect hair samples that will give us total hair cortisol concentration over the last 2-4 months, which will serve as an indicator of circulating cortisol concentrations over this time period. Individuals who regularly have high cortisol will be identified with high hair cortisol concentrations. Hair cortisol will be analyzed as a continuous variable as well as with dichotomous variables. Those above the median will be identified as high cortisol. Secondarily, we will explore the possibility of defining high cortisol at the $75^{th}$ or $90^{th}$ percentile.

**Salivary cortisol (cortisol response to a stressor):** Salivary cortisol was collected from women and children at four times:

1) during a home visit (saliva sample #1)
2) upon arrival to the clinic visit (saliva sample #2)
3) 15 minutes after a finger prick (saliva sample #3)
4) 30 minutes after a finger prick (saliva sample #4)

The clinic arrival sample (saliva sample #2) will be the primary indicator of baseline salivary cortisol concentration, with the home visit sample (saliva sample #1) serving as a secondary indicator of baseline salivary cortisol concentration. The saliva samples 15 and 30 minutes post-finger prick will be used to create an area under the curve (AUC) measurement in response to the stressor. Secondarily, change from baseline cortisol at 15 and 30 minutes will be analyzed as continuous and dichotomous variables for each time point. Those above the median change in cortisol will be identified as high cortisol. Secondary definitions of high cortisol will include cortisol at the $75^{th}$ and $90^{th}$ percentiles.

Covariates

All models will include child's age since not all children were measured during the follow-up at the same age. The following variables will be considered as covariates for hair and salivary cortisol analyses of both the mother and the child:

- Child sex
- Maternal age

- Maternal education
- Gestational age at enrollment
- Maternal parity (nulliparous vs. parous)
- Maternal cortisol at enrollment
- Maternal AGP at enrollment
- Maternal CRP at enrollment
- Maternal BMI at enrollment adjusted for gestational age
- Season at enrollment
- Season at time of cortisol measurement

Each variable that shows a statistically significant association with cortisol ($P<0.1$) will be included in the model.

The following variables will be included in all salivary cortisol analyses of both the woman and the child:

- Child's age
- Time between waking and time of saliva collection for saliva sample #2
- Time between last food or drink and time of saliva collection for saliva sample #2
- Mood of child before saliva collection of saliva sample #2 (Categorical variable based on survey question at clinic visit: What was the child's mood like before collecting saliva? 1] Neutral (not upset, not happy), 2] Little upset (unhappy face, hesitant, shy), 3] Medium upset (some crying), 4] Very upset (uncontrollable crying, screaming), 5] Happy (smiling))

Effect Modifiers

The following variables will be considered as significant effect modifiers at $p < 0.10$:

- Child sex
- Maternal cortisol at enrollment
- Maternal age
- Maternal parity (nulliparous vs. parous)
- Season at time of cortisol measurement

**Statistical Analysis Methods**

Data Cleaning

Data collected during the main trial have been cleaned previously. The majority of data cleaning for iLiNS-DYAD-G2 occurred concurrently during data collection, with queries identified using SAS and relayed to the local home visit team manager. Queries were then resolved by seeking clarification from the field worker who completed the form and/or by re-contacting the respondent. Data cleaning will be double checked before beginning analysis by producing stem-and-leaf plots, histograms and/or scatterplots of variables.

Outliers

Outliers will be identified by visually inspecting histograms and/or scatterplots of variables. Outliers that are clearly impossible or implausible values will be corrected if possible and otherwise recoded as missing. Outliers which are plausible or possible will be retained. In cases where extreme outliers are retained, sensitivity analysis will be performed to determine whether the extreme outliers have undue influence on the results.

Data transformation

We will inspect the distribution of outcome variables for normality and transform as necessary. If no suitable transformation is found, normalized ranks will be calculated, or categories will be created.

Model Assumptions

Models will be checked to ensure that the residuals are normally distributed and the heteroscedasticity assumption is met through the residual versus fit plot.

Software

Data will be analyzed using SAS version 9.4.

Analysis of the Effect of the Intervention

The analysis will be by intent-to-treat. That is, by-group analysis will be according to group assignment regardless of any protocol violations. Secondarily, two per protocol analyses will be performed based on self-reported adherence to supplementation during the main trial. The first per protocol analysis will include women with ≥ 80% adherence to supplement during pregnancy. The second per protocol analysis will include women with ≥80% adherence during the entire period of pregnancy up to 6 months postpartum. To address the issue of the supplement switch that occurred during the main trial between IFA and MMN, data will be analyzed both according to: 1) supplement received at enrollment and 2) intended supplement at enrollment.

For continuous outcome variables, the difference between the three groups will be tested with ANCOVA and null-hypothesis of no difference between groups will be rejected if $p < 0.05$. If the null-hypothesis is rejected, post-hoc pairwise comparisons of the three intervention groups will be performed using the Tukey-Kramer test for ANOVA. For all pairwise comparisons with $p < 0.05$, the null-hypothesis of no difference in means between groups will be rejected.

For dichotomous outcome variables, global null hypothesis of no differences between groups will be tested with chi-square test or Fisher's exact test. Pairwise comparisons between groups will be done in the context of log-binomial regression if global null-hypothesis is rejected with $p < 0.05$. Prevalence ratios between intervention groups will be presented.

If there are no differences between the IFA and MMN groups in the above outcomes, a two-group analysis (LNS vs. non-LNS) will also be conducted.

## References


1. Christian P, Nanayakkara-Bind A, Schulze K, Wu L, LeClerq SC, Khatry SK. Antenatal micronutrient supplementation and third trimester cortisol and erythropoietin concentrations. Matern Child Nutr 2016 Jan;12(1):64-73.

2. Lesage J, Blondeau B, Grino M, Breant B, Dupouy JP. Maternal undernutrition during late gestation induces fetal overexposure to glucocorticoids and intrauterine growth retardation, and disturbs the hypothalamopituitary adrenal axis in the newborn rat. Endocrinology 2001;142:1692-702.

3. Oaks BM, Laugero KD, Stewart CP, Adu-Afarwuah S, Lartey A, Ashorn P, Vosti SA, Dewey KG. Late-Pregnancy Salivary Cortisol Concentrations of Ghanaian Women Participating in a Randomized Controlled Trial of Prenatal Lipid-Based Nutrient Supplements. J Nutr 2016;146:343-52.