Comparison of the main effect of treatment group on change in biomarkers of lipid peroxidation during pregnancy (iLiNS-DYAD-Ghana)

Contents

1) **Study Objectives.**

This analysis falls under Objective #4 of the iLiNS-DYAD trials, the primary aim of which is to evaluate the efficacy of lipid-based nutrient supplements (LNS) for pregnant women. A secondary aim is to study the impact of LNS as compared to other iron-containing supplements during pregnancy on lipid peroxidation, a subclinical indicator of oxidative stress. This substudy analysis will measure the change in urinary biomarkers of lipid peroxidation from before 20 wk gestation through 36 wk gestation 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 400 μg folic acid (Fe/FA)
b. 20 mg iron and multiple micronutrients tablet (MMN) or

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

In addition, exploratory objectives of the substudy are to evaluate iron status in early pregnancy and the change in iron status throughout pregnancy as potential effect modifiers of the main effect of treatment group on urinary biomarkers of lipid peroxidation.

Study Description. Pregnant women were randomly assigned to receive one of three daily iron-containing supplements throughout pregnancy. Baseline blood and urine samples were collected at the time of enrollment (<20 wk gestation) and final time point samples were collected at 36 wk gestation as determined by ultrasonography. Levels of the main outcome, urinary 8-isoprostane-PGF_{2a} were quantified by ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) and iron status and inflammation biomarkers were measured in blood. The change in concentration of 8-isoprostane-PGF_{2a} (8-$iPGF_{2a}$) from baseline to 36 wk will be compared between groups using an ANCOVA statistical model.

2) **Hypotheses to be tested**
   a) Primary hypothesis A: The positive change in 8-$iPGF_{2a}$ and its primary metabolite, 2, 3-dinor-8- isoprostane-$F_{2\alpha}$ (2,3-dinor-8-$iPGF_{2a}$) will be reduced in pregnant women in Ghana receiving LNS as compared to similar groups receiving either Fe/FA or MMN.
   b) Primary hypothesis B: The positive change in 8-$iPGF_{2a}$ and 2, 3-dinor-8-$iPGF_{2a}$ will be reduced in the group of pregnant women in Ghana receiving 20 mg iron in MMN as compared to a similar group receiving 60 mg iron in Fe/FA.
   c) Secondary/exploratory hypothesis C: The effect of treatment group on urinary biomarkers of lipid peroxidation will be modified by change in iron status throughout pregnancy.
   d) Secondary/exploratory hypothesis D: The effect of treatment group on urinary biomarkers of lipid peroxidation will be modified by iron status in early pregnancy.

3) **Definition of the substudy outcomes**

Outcomes
   a. Change in urinary 8-isoprostane-F2α\creatinine (μg/g) from baseline (< 20 wk gestation) to third trimester of pregnancy (36 wk gestation),
   b. Change in urinary 2, 3-dinor-8- isoprostane-$F_{2\alpha}$\creatinine (μg/g), the major metabolite of 8-isoprostane-$F_{2\alpha}$ from baseline (< 20 wk gestation) to third trimester of pregnancy (36 wk gestation),
   c. Change in iron status from baseline (< 20 wk gestation) to third trimester of pregnancy (36 wk gestation) defined as hemoglobin, zinc protoporphyrin, soluble transferrin receptor or some combination of these.

Quantification of urinary biomarkers of lipid peroxidation will be analyzed using liquid-liquid extraction and UPLC-MS/MS. Urinary creatinine values will be measured using an automated Jaffe2 colorimetric method. Urinary concentrations of 8-isoprostane-$F_{2\alpha}$ and 2, 3-dinor-8-isoprostane-$F_{2\alpha}$ (μg) will be standardized per gram urinary creatinine to account for individual subject variation in diuresis.

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. A biological sample will be collected at 36 wk gestation and 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.
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**

Biological samples will be collected at baseline (<20 wk gestation) and at term, before delivery (36 wk gestation). The change in urinary biomarkers will reflect the intervention period from baseline to term.

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

6.1. Comparison of the change in urinary biomarkers of lipid peroxidation during pregnancy between intervention groups

Hypotheses A and B will be addressed as follows: The group means and standard deviations for urinary 8-isoprostane-$F_{2\alpha}$ (µg/g creatinine) and 2, 3-dinor-8- isoprostane-$F_{2\alpha}$ (µg/ g creatinine) at each of the two 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 8-isoprostane-$F_{2\alpha}$ (µg/g creatinine) and change in 2, 3-dinor-8- isoprostane-$F_{2\alpha}$ (µg/ g creatinine) 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.

Description of covariates and potential modifying effects

a) initial 8-isoprostane-$F_{2\alpha}$ (µg/g creatinine) or 2, 3-dinor-8- isoprostane-$F_{2\alpha}$ (µg/ g creatinine)
b) initial iron status (ZPP, sTfR, Hb or some combination of those)
c) initial c-reactive protein (CRP)
d) initial alpha-1-glycoprotein (AGP)
e) initial body mass index (BMI)
f) Parity
g) Age

h) Timing of last use of supplement

6.2. Assessing the potential interaction between the main effect of treatment and change in iron status on change in urinary biomarkers of lipid peroxidation

A series of ANCOVA models will be run to construct a Path analysis which will address Hypothesis C. The first ANCOVA model will evaluate the relationship between main effect of treatment group and change in iron status, including the potential covariates identified above. 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 urinary isoprostanes. Again, all potential covariates identified above will be considered. A third ANCOVA model will be generated to include the change in iron 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 urinary isoprostanes if change in iron status is indeed on the causal pathway.

A fourth ANCOVA model will address Hypothesis D, evaluation of the relationship between main effect of treatment group and urinary isoprostanes with inclusion of initial iron status as an effect modifier.

7) **General notes on statistical methods**

7.1 Software

SAS 9.3 software

7.2 Preparing data for analyses

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 urinary biomarkers of lipid peroxidation at baseline (<20 wk gestation) and 36 wk gestation between intervention groups, unadjusted analyses

	LNS	MMN	Fe/FA	Overall	Comparison of	Comparison of	Comparison of

iLiNS-DYAD-Ghana: Sub-study Statistical Analysis Plan-Maternal urinary isoprostanes

				ANCOVA	FE/FA and MMN		Fe/FA and LNS		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 8-iPGF$_{2\alpha}$ (µg/g creatinine)	(mean ± SD)	(mean ± SD)	(mean ± SD)	p-value						
Baseline 2, 3-dinor-8- iPGF$_{2\alpha}$ (µg/g creatinine)	(mean ± SD)	(mean ± SD)	(mean ± SD)	p-value						
36 wk 8-iPGF$_{2\alpha}$ (µg/g creatinine)	(mean ± SD)	(mean ± SD)	(mean ± SD)	p-value						
36 wk 2, 3-dinor-8-iPGF$_{2\alpha}$ (µg/g creatinine)	(mean ± SD)	(mean ± SD)	(mean ± SD)	p-value						

Table 2. Comparison of change in urinary biomarkers of lipid peroxidation 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 8-iPGF$_{2\alpha}$ (µg/g creatinine)	(mean ± SD)	(mean ± SD)	(mean ± SD)	p-value						
Change in 2, 3-dinor-8-iPGF$_{2\alpha}$ (µg/g creatinine)	(mean ± SD)	(mean ± SD)	(mean ± SD)	p-value						
Change in 8-iPGF$_{2\alpha}$ (µg/g creatinine)*	(mean ± SD)	(mean ± SD)	(mean ± SD)	p-value						
Change in 2, 3-dinor-8-iPGF$_{2\alpha}$ (µg/g creatinine)**	(mean ± SD)	(mean ± SD)	(mean ± SD)	p-value						

* Adjusted for covariates x, y and z.

**Adjusted for covariates a, b and c.

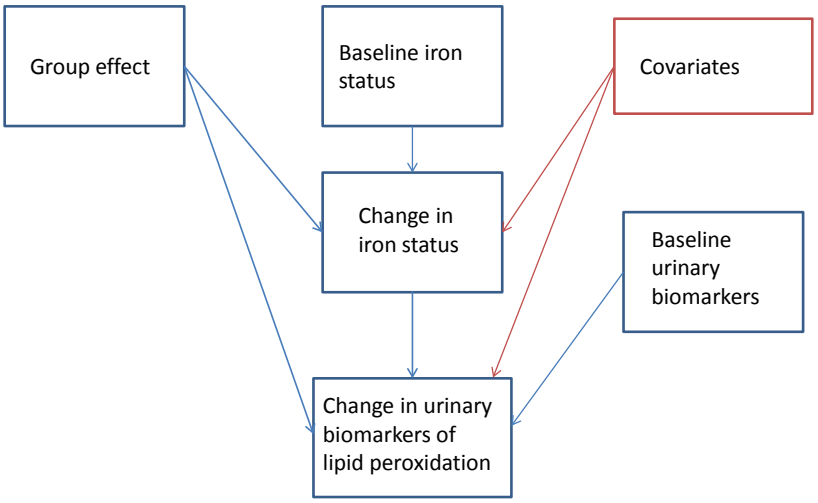

Figure 1. Hypothesized relationships between study variables for multiple regression path analysis

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 iron status as a covariate and Model 2- without change in iron status as covariate.

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