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Late-Pregnancy Salivary Cortisol Concentrations of Ghanaian Women Participating in a Randomized Controlled Trial of Prenatal Lipid-Based Nutrient Supplements[1–3]

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Abstract

Background: High circulating cortisol is associated with miscarriage, preterm birth, and low birth weight. Research in nonpregnant individuals suggests that improved nutrition may lower cortisol concentrations. It is unknown whether nutritional supplementation during pregnancy lowers cortisol.

Objective: Our objective was to determine whether women receiving a lipid-based nutrient supplement (LNS) throughout pregnancy would have lower salivary cortisol at 36 wk gestation compared with women receiving other nutrient supplements.

Methods: We conducted a randomized controlled trial in 1320 pregnant Ghanaian women at ≤20 wk gestation who were assigned to receive daily throughout pregnancy: *1*) 60 mg iron + 400 μg folic acid (IFA), *2*) multiple micronutrients (MMNs), or *3*) 20 g LNS (containing 118 kcal, 22 micronutrients, and protein). Morning salivary cortisol was collected from a subsample at baseline and at 28 and 36 wk gestation.

Results: A total of 758 women had cortisol measurements at 28 or 36 wk gestation. Salivary cortisol at 36 wk gestation did not differ between groups and was (mean ± SE) 7.97 ± 0.199 in the IFA group, 7.84 ± 0.191 in the MMN group, and 7.77 ± 0.199 nmol/L in the LNS group, when adjusted for baseline cortisol, time of waking, and time between waking and saliva collection (P = 0.67). There was an interaction between supplementation group and women's age (continuous variable, P-interaction = 0.03); and when age was dichotomized by the median, significant differences in salivary cortisol concentrations between groups were seen in women ≤26 y of age (IFA = 8.23 ± 0.284 nmol/L, MMN = 8.20 ± 0.274 nmol/L, and LNS = 7.44 ± 0.284 nmol/L; P = 0.03) but not in women >26 y old (IFA = 7.71 ± 0.281 nmol/L, MMN = 7.50 ± 0.274 nmol/L, and LNS = 8.08 ± 0.281 nmol/L; P = 0.13).

Conclusions: We conclude that supplementation with LNSs or MMNs during pregnancy did not affect the cortisol concentration in the study population as a whole, in comparison with IFA, but that LNS consumption among younger women may lead to lower cortisol at 36 wk gestation. This trial was registered at clinicaltrials.gov as NCT00970866. *J Nutr* doi: 10.3945/jn.115.219576.

Keywords: lipid-based nutrient supplements, micronutrient supplements, cortisol, pregnancy, Ghana

Introduction

Fetal growth restriction is estimated to occur in 27% of births in low- and middle-income countries, with 32.4 million infants in 2010 being identified as small for gestational age (SGA)[9] (1). Defined as a birth weight below the 10th percentile of a reference population at the same gestational age, SGA accounts for ~11.8% of all deaths in children under age 5 in low- and middle-income countries (2). SGA also increases the risk of a child being stunted (2) or having developmental deficits in early childhood (3).

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[3] The contents of this manuscript are solely the responsibility of the authors and do not represent the official view of the USDA, which is an equal opportunity provider and employer.
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[9] Abbreviations used: AGP, α1-acid glycoprotein; CRP, C-reactive protein; IFA, iron-folic acid; iLiNS-DYAD, International Lipid-Based Nutrient Supplement Project; LNS, lipid-based nutrient supplement; MMN, multiple micronutrient; SGA, small for gestational age; sTfR, soluble transferrin receptor; ZPP, zinc protoporphyrin; 11-β-HSD-2, 11-β-hydroxysteroid dehydrogenase 2.

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Although there are a number of risk factors for fetal growth restriction, maternal undernutrition, including micronutrient deficiencies, has been associated with an increased risk of SGA (2). The mode in which undernutrition leads to SGA is unclear. Most research has focused on fetal nutrient restriction; however, there may be several pathways involved. One possibility is that undernutrition might act as a physical stress, with the elicited stress response restricting fetal growth. Physical or mental stress stimulates the hypothalamus-pituitary-adrenal axis, which leads to increased circulating cortisol, a glucocorticoid hormone. The majority of studies in animal models have shown that protein or energy restriction during pregnancy results in increased circulating maternal cortisol (4–10). Late pregnancy is a critical time period of sensitivity to cortisol. More than 90% of fetal fat deposition occurs in the last 10 wk of pregnancy (11), and a number of studies found an association between elevated cortisol in late pregnancy and lower birth weight (12, 13). Dexamethasone and betamethasone, 2 potent synthetic corticosteroids, are administered in the third trimester to pregnant women at risk of preterm delivery to accelerate maturation of the fetal lungs, but these drugs are associated with reduced birth weight, regardless of the gestational age at birth (14–16).

During pregnancy, circulating cortisol begins to increase around the 11th week of gestation, and by the third trimester total circulating cortisol is ~3 times that of nonpregnant circulating cortisol (17). Both the mother and the fetus appear to have defenses against this period of hypercortisolism. Pregnant women have a dampened cortisol response to stressors, compared with nonpregnant women, presumably as a mechanism to cope with the high concentrations of circulating cortisol (18). For the fetus, protection from the maternal circulating cortisol is provided by the placenta via production of the enzyme 11-β-hydroxysteroid dehydrogenase 2 (11-β-HSD-2), which converts cortisol into the inactive metabolite, cortisone (19). However, several factors have been shown to decrease the production or activity of 11-β-HSD-2, including maternal anxiety (20, 21), infection (22), inflammation (23), licorice consumption (24), and poor nutritional status (25, 26), leading to an imperfect barrier to high maternal cortisol. A study in London in 51 pregnant women showed that whereas fetal cortisol concentrations were only 7–9% of maternal cortisol concentrations, maternal cortisol was positively correlated with fetal cortisol and may account for up to 40% of its variance (27, 28).

Reducing nutritional deficiencies is a possible way to reduce maternal cortisol concentration. A recent study in pregnant Nepalese women showed that multiple micronutrient (MMN) supplementation decreased third-trimester serum cortisol concentration (29). Previous research has shown that nonpregnant individuals with an energy-restricted diet have increased circulating cortisol, which decreases when nutrition is improved (30–32). Omega-3 FA supplementation was found to decrease cortisol concentrations in randomized controlled trials in healthy men and abstinent alcoholics (33, 34). Lipid-based nutrient supplements (LNSs) are new forms of supplements being studied as an alternative to MMN supplements for pregnant women (35–37). LNSs deliver micronutrients in a food base typically consisting of vegetable fat, peanut paste, milk powder, and sugar. Therefore, in addition to providing micronutrients, LNSs also provide energy, protein, and essential FAs (38).

In the International Lipid-Based Nutrient Supplement Project (iLiNS-DYAD) in Ghana, conducted between 2009 and 2014, we tested the efficacy of a small quantity of an LNS for improving maternal and infant nutrition and reported previously that, compared with iron and folic acid (IFA) or an MMN capsule, the LNS promoted fetal growth in vulnerable women such as primiparas (39).

For the analyses reported herein, our objective was to determine whether morning salivary cortisol measured at 28 or 36 wk gestation would differ among women receiving LNSs, MMNs, or IFA. We hypothesized that women receiving small-quantity LNSs throughout pregnancy would have lower cortisol at 36 wk gestation compared with pregnant women receiving IFA or MMNs.

Methods

Study design. We conducted this study as an add-on to the iLiNS-DYAD trial in Ghana, a randomized intervention trial assessing the effects of LNSs on maternal and child outcomes, with the primary outcome being child length at 18 mo of age (clinicaltrials.gov; ID: NCT00970866). Detailed methods have been published elsewhere (39). Briefly, we recruited pregnant women attending a prenatal examination at 1 of 4 health facilities in the semiurban districts of Manya Krobo and Yilo Krobo, ~70 km north of Accra, Ghana. Women were eligible if they were at ≤20 wk gestation, ≥18 y of age, had an antenatal health card complete with history and prenatal examination, and signed or thumb-printed informed consent. We excluded women if they were HIV positive; had asthma, epilepsy, tuberculosis, or a chronic disease that required medical attention; did not reside in the defined catchment area; had a milk or peanut allergy; or were already participating in another clinical trial.

TABLE 1 Nutrient composition of supplements used in the iLiNS-DYAD trial: IFA capsules, MMN capsules, and LNS[1]

Nutrient	IFA	MMN	LNS (20 g)
Iron (ferrous sulfate), mg	60	20	20
Folic acid (pteroyl monoglutamic acid), μg	400	400	400
Vitamin A (retinyl acetate), μg REs	0	800	800
Vitamin B-12 (cyanocobalamin 0.1%), μg	0	5.2	5.2
Vitamin B-6 (pyridoxine hydrochloride), mg	0	3.8	3.8
Vitamin C (L-ascorbic acid), mg	0	100	100
Vitamin D-3 (cholecalciferol), IU	0	400	400
Zinc (zinc sulfate), mg	0	30	30
Thiamin (thiamin hydrochloride), mg	0	2.8	2.8
Riboflavin, mg	0	2.8	2.8
Niacin (niacinamide), mg	0	36	36
Vitamin E (dl-α-tocopherol acetate), mg	0	20	20
Vitamin K (phylloquinone 5%), μg	0	45	45
Pantothenic acid (calcium pantothenate), mg	0	7	7
Copper (encapsulated copper sulfate), mg	0	4	4
Iodine (potassium iodate), μg	0	250	250
Manganese (manganese sulfate), mg	0	2.6	2.6
Selenium (sodium selenite 1.5%), μg	0	130	130
Calcium (tricalcium phosphate), mg	0	0	280
Phosphorus (tricalcium phosphate), mg	0	0	190
Potassium (potassium chloride), mg	0	0	200
Magnesium (magnesium citrate), mg	0	0	65
Energy, kcal	0	0	118
Protein, g	0	0	2.6
Fat, g	0	0	10
Linoleic acid, g	0	0	4.59
α-Linolenic acid, g	0	0	0.59
Phytate, mg	0	0	24.7

[1] IFA, iron-folic acid; iLiNS-DYAD, International Lipid-Based Nutrient Supplement Project; LNS, lipid-based nutrient supplement; MMN, multiple micronutrient; RE, retinol equivalent.

For the current study, we added the collection of saliva samples for cortisol to the study protocol after enrollment had already commenced. Of the 1320 women enrolled in iLiNS-DYAD, we enrolled 436 before saliva collection began, and we removed 74 from this substudy because they were enrolled during a period when there was an error in the labeling of the IFA and MMN supplements, resulting in mixed exposure (39). In total, 799 women provided a baseline saliva sample; we limited analysis for the current study to the 758 women with a baseline cortisol measurement and a 28- or a 36-wk cortisol measurement. We enrolled women for the add-on study between October 2010 and January 2012.

We randomly assigned women to receive daily throughout pregnancy *1*) an IFA capsule (IFA group), *2*) an MMN capsule (MMN group), or *3*) a sachet of 20 g LNS (LNS group; **Table 1**). The details of the 3 supplements (39) and the rationale for LNS formulation and design have been reported previously (38). Group allocations were determined by a statistician who used a computer-generated randomization scheme in blocks of 9 (3 codes for each of the 3 interventions) and were placed in sealed opaque envelopes. A woman chose an envelope from a stack of 9 envelopes to determine her group allocation.

Procedures. Cortisol is highly variable and associated with both physical and psychosocial stressors, such as malaria (40) and socioeconomic status (41). Therefore, data for a number of variables were included in this add-on study to reduce the within-group variance and to increase the precision of the estimate of the treatment effect in data analysis (42). At enrollment, study nurses determined gestational age by ultrasound (Aloka SSD 500) and anthropometrists measured weight and height (SECA 874 flat scale and SECA 217 stadiometer; Seca GmbH). Trained fieldworkers ascertained sociodemographic information. At both enrollment and at 36 wk gestation, study nurses collected blood samples by venipuncture. Laboratory technicians assayed hemoglobin (HemoCue AG) and malaria parasitemia (Clearview Malarial Combo; Vision Biotech) using venous blood and zinc protoporphyrin (ZPP; hematofluorometer; Aviv Biomedical) in washed RBCs (39). They also measured soluble transferrin receptor (sTfR), C-reactive protein (CRP), and α1-acid glycoprotein (AGP) in plasma by immunoturbidimetry on the Cobas Integra 400 Plus Automatic Analyzer (Roche Diagnostics).

Study nurses collected saliva samples at the clinic visits at enrollment (mean gestational age: ~16 wk) and at 36 wk gestation before any other measurements or sample collection, and fieldworkers collected a saliva

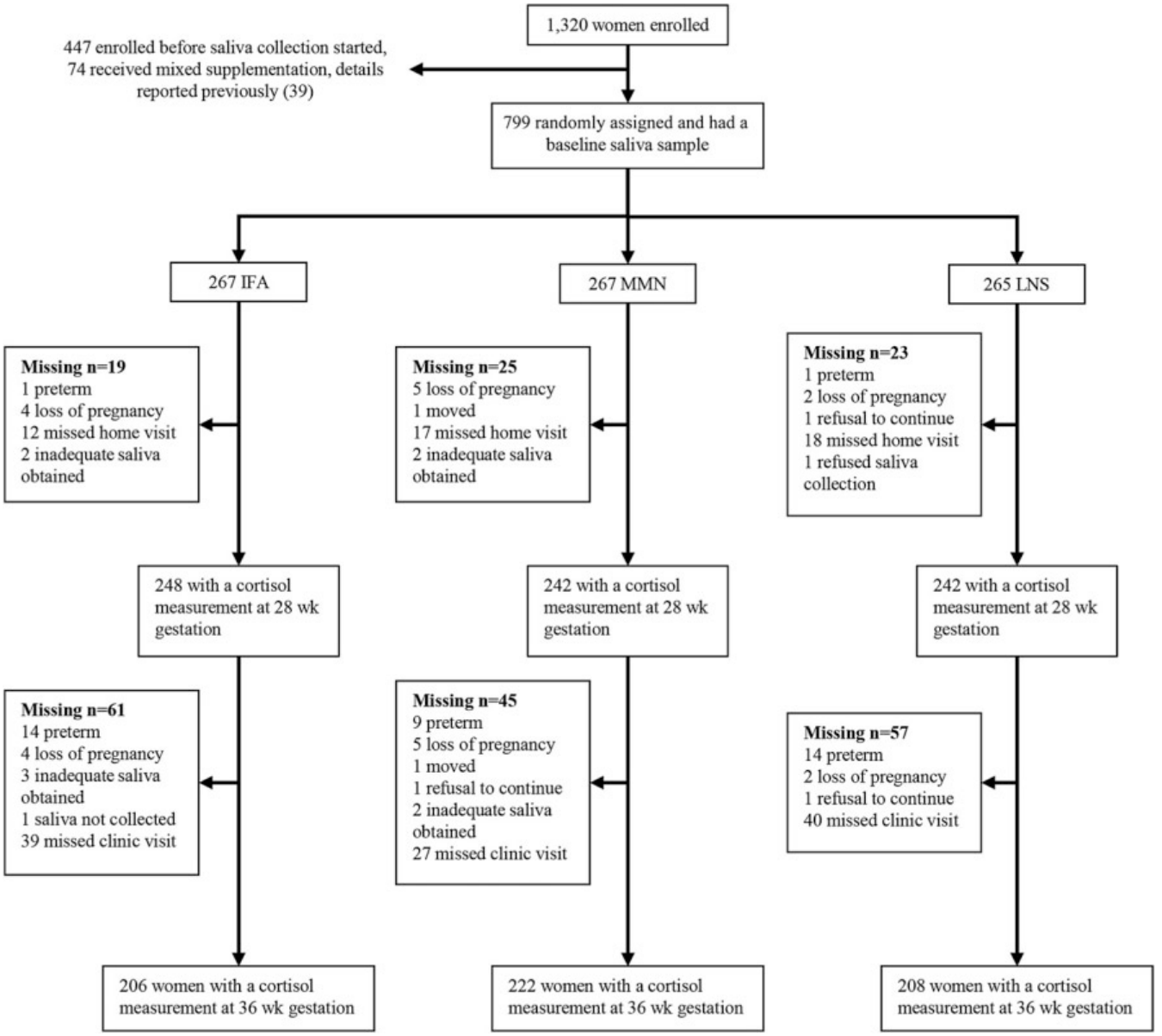

FIGURE 1 Participant flow chart for the iLiNS-DYAD trial in Ghana. IFA, iron-folic acid; iLiNS-DYAD, International Lipid-Based Nutrient Supplement Project; LNS, lipid-based nutrient supplement; MMN, multiple micronutrient.

sample during a home visit at 28 wk gestation. Study nurses and fieldworkers collected saliva samples between the hours of 0800 and 1200, with a mean collection time of ~1030 h. Previous studies have determined that 1 salivary sample collected during the morning is adequate for differentiating women as having high or low cortisol (43). Although many factors can influence cortisol, high morning cortisol has been shown to be a biomarker of physical and mental stress (44, 45), including in pregnant women (46). Workers instructed women not to consume any food or drink with the exception of water for at least 30 min before providing the saliva sample. Study nurses and fieldworkers recorded the time of saliva collection, time of waking, and time of last food or drink.

To collect saliva, each woman placed an inert polymer cylindrical swab (10 × 30 mm; Salimetrics Oral Swab) under her tongue for ~2 min while moving her tongue and jaw as if she were chewing to stimulate saliva. The study nurse or fieldworker then placed the swab in a tube and put it in the refrigerator or on ice packs. Laboratory technicians brought the swabs to room temperature before centrifuging at 1252 × *g* for 15 min. Samples were stored at −20°C within 24 h of collection.

We analyzed the saliva samples in duplicate using Salimetrics Expanded Range High Sensitivity Salivary Cortisol Enzyme Immunoassay Kit (Salimetrics), which can detect cortisol concentrations ranging from 0.193 to 82.8 nmol/L. The intra- and interassay CVs were 4.4% and 7.8%, respectively. The institutional review boards of the University of California, Davis, and the Noguchi Memorial Institute for Medical Research, University of Ghana, approved the study protocol.

Data analysis. We completed our primary data analysis on an intention-to-treat basis. Second, we performed per protocol analysis limited to women who consumed the assigned supplement at least 70% of the days between enrollment and at 36 wk gestation. We tested normality using the Shapiro-Wilk test and log-transformed cortisol, AGP, and CRP and inversely transformed ZPP, sTfR, and BMI to normalize distributions. We used transformed values to obtain *P* values and nontransformed cortisol to obtain means and pairwise differences.

We used the Household Food Insecurity Access Scale to estimate food insecurity (47). We created a housing quality index on the basis of household drinking water supply, sanitation facilities, wall material, flooring material, roofing material, and lighting source using principal components analysis (48). We also created an asset index in a similar manner using household drinking water supply, sanitation facilities, flooring materials, lighting source, and presence of radio, television, refrigerator, cell phone, and stove.

We defined anemia using both the WHO's definition of anemia in pregnant women as hemoglobin <110 g/L (49) and using a cutoff value of hemoglobin <100 g/L on the basis of research suggesting that this cutoff value is more useful and accurate when defining anemia in pregnant black women (50–52). We defined high ZPP as ZPP >60 μmol/mol heme (53) and inflammation as a CRP concentration >5 mg/L or an AGP concentration >1 g/L (54–56). A common cutoff to define high sTfR is 8.5 mg/L, measured by using enzyme-linked immunosorbent assay (57). However, a study determined that the immunoturbidimetric assay estimates sTfR at 30% below the measurement provided by enzyme-linked immunoassay (58). Therefore, we defined high sTfR as >6.0 mg/L, reflecting a 30% reduction in the standard 8.5-mg/L cutoff. We defined iron deficiency as high ZPP or high sTfR. When analyzing differences in maternal characteristics at baseline, we used ANOVA (comparison of means) or Fisher's exact test (comparison of proportions) to obtain *P* values.

Both unadjusted and adjusted analyses were completed, because guidelines for best statistical practices support the use of covariates in analyses of

TABLE 2 Baseline characteristics of pregnant women in the iLiNS-DYAD trial in Ghana with salivary cortisol at 28 or 36 wk gestation, by supplementation group: IFA, MMN, or LNS[1]

Characteristic	IFA (*n* = 252)	MMN (*n* = 256)	LNS (*n* = 250)	*P*[2]
Maternal age, y	27 ± 5.3	27 ± 5.6	27 ± 5.4	0.68
Gestational age, wk	16 ± 3.1	16 ± 3.0	16 ± 3.0	0.74
Primiparous, %	37.3	30.5	34.4	0.26
Total school completed, y	7.6 ± 3.3	7.3 ± 3.4	7.7 ± 3.6	0.29
Married, %	90.9	91.0	91.2	0.99
Household food insecurity index	2.5 ± 4.4	2.2 ± 3.9	1.8 ± 3.5	0.14
BMI,[3] kg/m^2	24 ± 4.2	24 ± 4.7	25 ± 4.4	0.48
≥25 kg/m^2, %	35.1	34.04	39.2	0.45
Salivary cortisol, nmol/L	4.82 ± 2.46	4.72 ± 2.54	4.67 ± 2.47	0.76
Hemoglobin, g/L	112 ± 12.7	111 ± 11.4	112 ± 11.6	0.28
<110 g/L, %	39.3	42.6	38.0	0.55
<100 g/L, %	9.5	15.2	13.6	0.14
ZPP, μmol/mol heme	41.2 ± 27.1	45.2 ± 31.5	43.8 ± 29.1	0.30
>60 μmol/mol heme, %	8.4	14.5	12.0	0.10
sTfR, mg/L	4.0 ± 2.0	4.0 ± 1.9	4.4 ± 4.0	0.24
>6.0 mg/L, %	7.9	9.6	9.9	0.71
Iron deficiency,[4] %	13.2	18.4	16.8	0.26
Malaria-positive (positive antigen or microscopy), %	7.5	8.2	10.0	0.60
Plasma AGP, g/L	0.66 ± 0.23	0.64 ± 0.20	0.64 ± 0.19	0.33
>1 g/L, %	9.1	5.6	3.3	0.03
Plasma CRP, mg/L	8.6 ± 16	6.3 ± 8.6	6.7 ± 9.5	0.08
>5 mg/L, %	41.7	39.0	40.7	0.83
Inflammation,[5] %	42.6	39.8	42.0	0.81

[1] Values are means ± SDs or proportions where unit is %, *n* = 758. Data were missing for AGP, CRP, and sTfR (*n* = 22); ZPP (*n* = 1); BMI (*n* = 19); and household food insecurity index (*n* = 4). AGP, α1-acid glycoprotein; CRP, C-reactive protein; IFA, iron-folic acid; iLiNS-DYAD, International Lipid-Based Nutrient Supplement Project; LNS, lipid-based nutrient supplement; MMN, multiple micronutrient; sTfR, soluble transferrin receptor; ZPP, zinc protoporphyrin.
[2] Obtained by using ANOVA (comparison of means) or Fisher's exact test (comparison of proportions).
[3] BMI at enrollment was adjusted to expected BMI at 10 wk gestation based on regression modeling.
[4] High ZPP or sTfR.
[5] High AGP or CRP.

randomized controlled trials (42). Variables we identified a priori as potential covariates, for both per protocol and intent-to-treat analyses, were baseline cortisol, baseline BMI, parity (primiparous compared with multiparous), household food insecurity, asset index, housing quality index, baseline inflammation (CRP >5 mg/L or AGP >1 g/L), gestational age at enrolment, educational level, maternal age, season (dry or wet), maternal height, sex of infant, baseline hemoglobin, baseline ZPP, and baseline sTfR. All covariates were also examined as effect modifiers in intent-to-treat analyses. We tested variables individually in an ANCOVA model, with baseline cortisol and supplementation group as the other independent variables and 36-wk-gestation cortisol as the dependent variable. We examined each variable as a covariate and as an effect modifier with supplementation group by creating a cross-product term. Variables were included as covariates in the model only if their association with the outcome was statistically significant at $P < 0.10$. All continuous variables were placed in the model in continuous form to determine significance. Effect modifiers were included if the P value for the interaction was <0.10. If a continuous variable was identified as a significant effect modifier, the continuous variable was changed to a categorical variable to examine group differences within subgroups. If there were no biologically based cutoffs available for creating the categorical variable, a continuous variable was dichotomized at the median. We also examined time of saliva collection, time between saliva collection and waking, and time between saliva collection and last food or drink (besides water) and included these if associated with the outcome at $P < 0.10$.

After finding a significant interaction with maternal age (continuous variable, P-interaction = 0.03), we performed ANCOVA with maternal age dichotomized at the median age for the study population (≤26 y, >26 y), because there are no biologically based age cutoffs related to cortisol. We adjusted the analyses for multiple comparisons using the Tukey-Kramer adjustment, and P values <0.05 were considered significant.

Results

Of the 799 women who provided a baseline saliva sample, 732 had a cortisol measurement at 28 wk gestation, 636 women had a cortisol measurement at 36 wk gestation, and 758 had a cortisol measurement at either 28 or 36 wk gestation. Losses in follow-up for both the 28-wk-gestation and 36-wk-gestation time points are described in **Figure 1**. Compared with those without a baseline saliva sample (n = 521), women with a baseline saliva sample (n = 799) were less likely to be iron deficient (16.2% compared with 21.2%, P = 0.02) or have malaria (8.6% compared with 12.5%, P = 0.03) but were generally similar with regard to other baseline characteristics.

Baseline characteristics did not differ significantly between the intervention groups (**Table 2**). The majority of the women in the study population were educated and multiparous. There was a wide range of BMIs represented and a low prevalence of iron deficiency and malaria. The mean ± SD adherence (percentage of days supplement was consumed) from enrollment to 36 wk gestation was higher in the IFA group (76.3% ± 16.9%) than in the MMN (73.8% ± 19.4%) and the LNS (72.5% ± 20.5%) groups (P = 0.047).

Mean cortisol concentrations at 28 and 36 wk gestation by supplementation group are presented in **Table 3**. In unadjusted analyses, there was no significant difference between groups in cortisol measured at either 28 or 36 wk gestation. Adjustment of the models for significant variables related to the diurnal rhythm of cortisol (time of waking, time between waking and saliva collection) or for significant covariates (BMI, maternal education) did not significantly change the results. However, for the outcome of cortisol at 36 wk gestation, there was an interaction between supplementation group and women's age (continuous variable, P-interaction = 0.03); and when age was dichotomized by the median, significant differences in mean cortisol at 36 wk gestation were seen between the MMN and the LNS groups in women ≤26 y (**Figure 2**A) but not in women >26 y (Figure 2B) (**Table 4**); the differences between the LNS and IFA groups and between the MMN and IFA groups were not significant. In per protocol analyses, all of the results

TABLE 3 Salivary cortisol in pregnant women in the iLiNS-DYAD trial in Ghana at 28 and 36 wk gestation by supplementation group: IFA, MMN, or LNS[1]

	Results by study group				Comparison between groups					
					IFA − LNS		MMN − LNS		IFA − MMN	
Outcome	IFA (n = 252)[2]	MMN (n = 256)[3]	LNS (n = 250)[4]	P[5]	Difference in means (95% CI)	P[5]	Difference in means (95% CI)	P[5]	Difference in means (95% CI)	P[5]
Cortisol at 28 wk gestation, nmol/L										
Unadjusted	6.58 ± 3.59	6.37 ± 3.61	6.41 ± 3.16	0.63	0.17 (−0.44, 0.79)	0.89	−0.041 (−0.66, 0.58)	0.87	0.22 (−0.40, 0.83)	0.60
Adjusted model 1[6]	6.34 ± 0.226	6.17 ± 0.226	6.37 ± 0.231	0.68	−0.03 (−0.65, 0.61)	0.68	−0.20 (−0.81, 0.43)	0.98	0.17 (−0.45, 0.78)	0.80
Adjusted model 2[7]	6.31 ± 0.225	6.16 ± 0.225	6.35 ± 0.231	0.80	−0.04 (−0.66, 0.60)	0.80	−0.19 (−0.80, 0.45)	0.98	0.15 (−0.47, 0.76)	0.89
Cortisol at 36 wk gestation, nmol/L										
Unadjusted	8.00 ± 3.12	7.79 ± 3.07	7.77 ± 2.97	0.61	0.23 (−0.36, 0.82)	0.66	0.02 (−0.56, 0.60)	1.00	0.21 (−0.37, 0.79)	0.67
Adjusted model 1[6]	7.97 ± 0.199	7.84 ± 0.191	7.77 ± 0.199	0.67	0.20 (−0.35, 0.76)	0.65	0.064 (−0.46, 0.63)	0.94	0.14 (−0.42, 0.66)	0.83
Adjusted model 2[7]	7.93 ± 0.200	7.86 ± 0.192	7.67 ± 0.200	0.55	0.26 (−0.29, 0.82)	0.53	0.18 (−0.34, 0.75)	0.76	0.070 (−0.48, 0.60)	0.92

[1] Values are means ± SDs for unadjusted analyses and means ± SEs for adjusted analyses; n = 758. IFA, iron-folic acid; iLiNS-DYAD, International Lipid-Based Nutrient Supplement Project; LNS, lipid-based nutrient supplement; MMN, multiple micronutrient.

[2] Missing data for 28 wk cortisol (n = 4), 28 wk time of waking and time between waking and saliva collection (n = 5), 36 wk time of waking (n = 43), 36 wk time between waking and saliva collection (n = 44), 36 wk cortisol (n = 46), and BMI (n = 7).

[3] Missing data for 28 wk cortisol (n = 14), 28 wk time of waking and time between waking and saliva collection (n = 12), 36 wk time of waking and time between waking and saliva collection (n = 32), 36 wk cortisol (n = 34), and BMI (n = 6).

[4] Missing data for 28 wk cortisol (n = 8), 28 wk time of waking and time between waking and saliva collection (n = 10), 36 wk time of waking and time between waking and saliva collection (n = 45), 36 wk cortisol (n = 42), and BMI (n = 5).

[5] P values were obtained from linear regression and adjusted for multiple comparisons by using the Tukey adjustment.

[6] Adjusted for baseline cortisol, time of waking, and time between waking and saliva collection.

[7] Adjusted for baseline cortisol, time of waking, time between waking and saliva collection, BMI, and education.

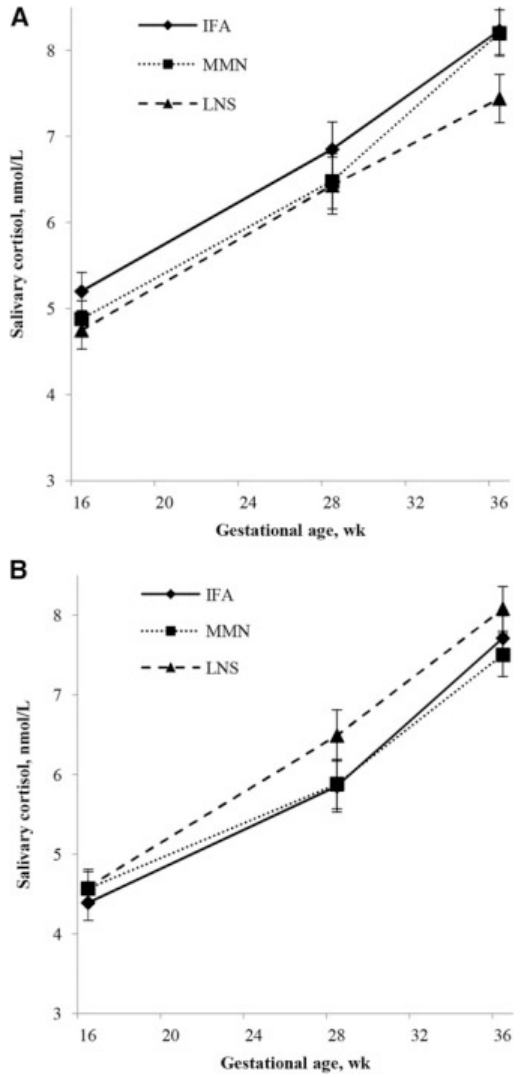

FIGURE 2 Salivary cortisol concentrations in pregnant women in the iLiNS-DYAD trial in Ghana at 28 and 36 wk gestation by supplementation group (IFA, MMN, or LNS) for women ≤26 y (A) and >26 y (B) of age. Data were adjusted for time of waking and time between waking and saliva collection. Cortisol data at 28 and 36 wk gestation also were adjusted for baseline cortisol. Values are means ± SEMs. (A) Baseline: n = 134 (IFA), 124 (MMN), and 128 (LNS); 28 wk gestation: n = 130 (IFA), 115 (MMN), and 123 (LNS); 36 wk gestation: n = 103 (IFA), 111 (MMN), and 104 (LNS). (B) Baseline: n = 118 (IFA), 132 (MMN), and 122 (LNS); 28 wk gestation: n = 118 (IFA), 127 (MMN), and 119 (LNS); 36 wk gestation: n = 103 (IFA), 111 (MMN), and 104 (LNS). Data were stratified by age based on a test for interaction with age, P-interaction = 0.03. IFA, iron-folic acid supplement; iLiNS-DYAD, International Lipid-Based Nutrient Supplement Project; LNS, lipid-based nutrient supplement; MMN, multiple micronutrient supplement.

become nonsignificant, possibly due to the smaller sample size (data not shown).

Younger women differed from older women by having a lower prevalence of overweight and obesity (26.4% compared with 50.0%) and a higher prevalence of anemia (hemoglobin <10 g/dL; 16.9% compared with 8.5%), malaria (12.7% compared with 5.1%), and inflammation (45.7% compared with 32.7%) at baseline. There were no other significant interactions with the potential effect modifiers examined, except for a relation with ZPP at baseline, which was not easily interpretable and became nonsignificant when stratified analyses were completed (data not shown).

Discussion

In this randomized controlled trial in Ghana, salivary cortisol measured in late pregnancy did not differ significantly between intervention groups. However, there was a significant interaction between maternal age and intervention group: when stratified by the median age of the study population, cortisol at 36 wk gestation was significantly lower in younger women (≤26 y) who received the LNS than in younger women who received the MMN capsule. No significant difference in salivary cortisol was found among women >26 y of age. The mean cortisol concentrations in this study population were similar to those found in previous studies in pregnant women (43, 59–62) and followed the expected pattern of increasing in mid- and late pregnancy.

To our knowledge, only 1 other study examined the effects of a nutritional supplement on maternal cortisol in pregnant women (29). Those investigators found somewhat lower serum cortisol in the third trimester among Nepalese women who received MMNs compared with the control group who received vitamin A and women in a third arm who received vitamin A, iron, and folic acid. In that study, the MMNs included both calcium and magnesium, 2 nutrients present in LNSs but absent in the MMNs used in our study. In a trial in Gambia, lactating women who were provided a supplement containing energy, protein, riboflavin, vitamin A, vitamin C, and calcium during lactation had lower maternal cortisol than did controls (63); however, other studies that provided nonpregnant individuals with calcium or milk supplementation found no effect on cortisol (64, 65).

Our results suggest an effect of LNSs during pregnancy among younger women but not older women. In the Nepal study that reported lower serum cortisol in pregnant women who received MMNs, the effect was seen in the study participants as a whole, not just the younger women, although the women were, on average, younger than in our study (mean maternal age: ~23 y) (29). In our study, women aged ≤26 y had a lower prevalence of overweight and obesity and a higher prevalence of anemia, malaria, and inflammation than did women >26 y, which suggests that the younger women had poorer health status and were plausibly in a state that enabled them to be more responsive to the supplement. In bivariate analyses, BMI was the only factor out of this list that was significantly directly associated with cortisol at 36 wk gestation; however, it is possible that other characteristics not measured in this trial, such as psychosocial stress (66) or amount of sleep deprivation (67), differed between the younger and older women that could explain the differing cortisol response to LNSs between these 2 age groups.

There are several differences between LNSs and MMNs that might explain the lower cortisol concentrations in younger women who received LNSs. In terms of micronutrients, the LNSs and MMNs used in this study were similar, except that the LNS contained an additional 4 micronutrients: calcium, phosphorus, potassium, and magnesium. Of these, it is biologically plausible that magnesium affects cortisol. Magnesium deficiency triggers a proinflammatory state by promoting endothelial cell dysfunction, which is reversible upon magnesium supplementation (68). Inflammation leads to higher concentrations of circulating cytokines, which stimulate the production of cortisol by acting directly on the hypothalamus-pituitary-adrenal axis and indirectly by, for example, activating neural

TABLE 4 Mean salivary cortisol in pregnant women in the iLiNS-DYAD trial in Ghana at 28 and 36 wk gestation by age and supplementation group: IFA, MMN, or LNS[1]

	Results by study group				Comparison between groups					
					IFA − LNS		MMN − LNS		IFA − MMN	
Outcome	IFA (n = 252)[2]	MMN (n = 256)[3]	LNS (n = 250)[4]	P[5]	Difference in means (95% CI)	P[5]	Difference in means (95% CI)	P[5]	Difference in means (95% CI)	P[5]
Women ≤26 y (n = 386)										
Cortisol at 28 wk gestation, nmol/L										
Unadjusted	7.13 ± 4.14	6.79 ± 3.86	6.65 ± 2.88	0.58	0.48 (−0.37, 1.3)	0.79	0.14 (−0.73, 1.0)	0.92	0.34 (−0.52, 1.2)	0.56
Adjusted model 1[6]	6.85 ± 0.320	6.48 ± 0.320	6.43 ± 0.333	0.39	0.42 (−0.47, 1.3)	0.71	0.052 (−0.84, 0.95)	0.84	0.37 (−0.51, 1.3)	0.36
Adjusted model 2[7]	6.71 ± 0.321	6.31 ± 0.321	6.20 ± 0.334	0.35	0.51 (−0.38, 1.4)	0.62	0.11 (−0.78, 1.0)	0.88	0.40 (−0.47, 1.3)	0.32
Cortisol at 36 wk gestation, nmol/L										
Unadjusted	8.41 ± 3.28	8.32 ± 2.65	7.52 ± 2.93	0.030	0.90 (−0.074, 1.7)	0.07	0.81 (0.011, 1.6)	0.048	0.091 (−0.72, 0.90)	1.00
Adjusted model 1[6]	8.23 ± 0.284	8.20 ± 0.274	7.44 ± 0.284	0.029	0.79 (−0.10, 1.6)	0.08	0.76 (0.040, 1.5)	0.038	0.034 (−0.73, 0.80)	0.97
Adjusted model 2[7]	8.06 ± 0.293	8.08 ± 0.275	7.21 ± 0.293	0.015	0.85 (−0.060, 1.6)	0.06	0.87 (0.10, 1.6)	0.018	−0.012 (−0.79, 0.74)	0.92
Women >26 y (n = 372)										
Cortisol at 28 wk gestation, nmol/L										
Unadjusted	5.99 ± 2.75	5.99 ± 3.35	6.16 ± 3.42	0.98	0.18 (−1.1, 0.70)	1.00	−0.18 (−1.0, 0.69)	0.97	0.00 (−0.87, 0.86)	0.99
Adjusted model 1[6]	5.85 ± 0.319	5.88 ± 0.311	6.31 ± 0.319	0.79	−0.46 (−1.3, 0.41)	0.83	−0.43 (−1.3, 0.43)	0.81	−0.027 (−0.89, 0.83)	1.00
Adjusted model 2[7]	5.92 ± 0.321	6.01 ± 0.313	6.49 ± 0.321	0.61	−0.57 (−1.5, 0.31)	0.63	−0.48 (−1.3, 0.40)	0.70	−0.092 (−0.96, 0.77)	0.99
Cortisol at 36 wk gestation, nmol/L										
Unadjusted	7.59 ± 2.90	7.26 ± 3.36	8.03 ± 2.99	0.07	−0.44 (−1.3, 0.39)	0.58	−0.77 (−1.6, 0.044)	0.052	0.33 (−0.72, 0.90)	0.39
Adjusted model 1[6]	7.71 ± 0.281	7.50 ± 0.274	8.08 ± 0.281	0.13	−0.37 (−1.1, 0.41)	0.64	−0.58 (−1.3, 0.18)	0.11	0.21 (−0.55, 0.98)	0.53
Adjusted model 2[7]	7.80 ± 0.293	7.65 ± 0.278	8.11 ± 0.278	0.22	−0.32 (−1.1, 0.47)	0.74	−0.46 (−1.3, 0.29)	0.19	0.15 (−0.60, 0.94)	0.59

[1] Values are means ± SDs for unadjusted analyses and means ± SEs for adjusted analyses; n = 758. IFA, iron-folic acid; iLiNS-DYAD, International Lipid-Based Nutrient Supplement Project; LNS, lipid-based nutrient supplement; MMN, multiple micronutrient.
[2] Missing data for 28 wk cortisol (n = 4), 28 wk time of waking and time between waking and saliva collection (n = 5), 36 wk time of waking (n = 43), 36 wk time between waking and saliva collection (n = 44), 36 wk cortisol (n = 46), and BMI (n = 7).
[3] Missing data for 28 wk cortisol (n = 14), 28 wk time of waking and time between waking and saliva collection (n = 12), 36 wk time of waking and time between waking and saliva collection (n = 32), 36 wk cortisol (n = 34), and BMI (n = 6).
[4] Missing data for 28 wk cortisol (n = 8), 28 wk time of waking and time between waking and saliva collection (n = 10), 36 wk time of waking and time between waking and saliva collection (n = 45), 36 wk cortisol (n = 42), and BMI (n = 5).
[5] P values were obtained from linear regression and adjusted for multiple comparisons by using the Tukey adjustment.
[6] Adjusted for baseline cortisol, time of waking, and time between waking and saliva collection.
[7] Adjusted for baseline cortisol, time of waking, time between waking and saliva collection, BMI, and education.

mechanisms (69). Low magnesium may lead to increased inflammation, thus increasing circulating cortisol. Magnesium supplementation studies in which cortisol was measured have only been performed in male athletes and showed cortisol decreasing (70, 71), increasing (72), or exhibiting no response (73) after supplementation. However, as noted above, the MMNs used in the Nepal study in pregnant women included magnesium and was associated with lower serum cortisol, offering support for the idea that magnesium is a contributory factor.

In addition, LNSs provide 118 kcal, 2.6 g protein, and 10 g fat [4.59 g linoleic acid (18:2n−6), 0.59 g α-linolenic acid (18:3n−3)]. Male athletes supplemented with carbohydrates (74) or protein/amino acids (75–79) typically showed a decrease in cortisol, whereas ω-3 FA supplementation trials have shown mixed results (33, 34, 80–83). In rats, protein deficiency stimulates adrenocorticotropic hormone synthesis and secretion (84), which is an upstream regulator of cortisol (85). ω-3 FAs have anti-inflammatory properties, so the α-linolenic acid provided in the LNS might decrease cortisol concentrations by decreasing inflammation. We think it is unlikely that the energy contributed by LNSs was a major factor, given the small amount of energy provided (118 kcal) and the high mean BMI and prevalence of overweight, even among the younger women.

Lower cortisol in younger women who received LNSs may have important implications for birth outcomes. In recent preliminary analyses of this same study population, we showed that higher cortisol at 36 wk gestation is associated with a shorter duration of gestation and a smaller newborn head circumference z score. Marginal associations were evident with newborn weight and height z scores (86).

Several strengths of this study should be noted. Saliva was used to determine cortisol concentration, which is currently deemed the most accurate measurement of bioavailable cortisol (87). Saliva contains primarily free, unbound cortisol, whereas plasma cortisol is mostly bound to binding proteins and not biologically active (87). Plasma cortisol can also be affected by the stress of a blood draw, whereas saliva collection is relatively noninvasive. Salivary cortisol and plasma cortisol tend to be highly correlated (88, 89). Cortisol was measured at 3 time points during pregnancy, allowing us to track the pattern of cortisol during pregnancy and adjust for baseline variability, whereas the study in Nepal had only a single measurement of serum cortisol taken in the third trimester.

There are several limitations to this study. First, saliva was collected just once at each time point. Some studies advocate for multiple saliva samples throughout the day, beginning with

waking (44). However, for large epidemiologic studies such as this one, such methods are often impractical. By using just a single saliva sample, our results may be biased toward the null. Second, participants were not fully blinded to the intervention, because participants who received the LNS could see that it was different from the capsules given to the women in the other 2 arms of the trial. We had losses to follow-up; however, the reasons and numbers of losses to follow-up did not differ significantly between groups, and there were no significant differences in baseline characteristics when compared with those included in the analyses. In addition, we examined several potential interactions, so it is possible that the interaction with age is due to chance.

We conclude that supplementation with an LNS or MMNs during pregnancy did not affect cortisol concentration in the study population as a whole, in comparison with IFA, but that LNS consumption among younger women may lead to lower cortisol at 36 wk gestation. Further research is needed to explore mechanisms by which improved maternal nutrition may reduce cortisol concentrations, such as the potential anti-inflammatory effects of magnesium and ω-3 FAs.

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