Improving Maternal and Child Health Through Prenatal Fatty Acid Supplementation: A Randomized Controlled Study in African American Women Living in Low-income Urban Environments

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SIGNIFICANCE

Evidence from multiple studies strongly supports the hypothesis that the mother's level of psychosocial stress during pregnancy is significantly associated with suboptimal developmental outcomes in the offspring. In controlled animal studies, maternal stress during pregnancy has been shown to permanently compromise neurodevelopment in the offspring (Chapillon et al., 2002; Weinstock, 2005). Prenatal stress has been linked with a range of adverse outcomes in the offspring including disturbances in attention (Schneider et al., 2002; Keenan et al., 2013, impaired learning and disruption in neurogenesis (Chapillon et al., 2002; Coe, Lulbach, Schneider, 2002) and increased anxiety-like behaviors (Schneider et al., 2002). The strength of the causal claim that maternal stress affects the development of the offspring is based on rigorous controlled experiments in which the prenatal effect is distinguished from postnatal effects by using methods such as cross-fostering or nursery rearing. Furthermore, there is now good evidence that at least some aspects of the underlying mechanisms have been identified. The strongest candidate is the maternal hypothalamic-pituitary-adrenal (HPA) axis, although other systems are likely to be involved. Prenatal stress causes long-term alterations in the functioning of the offspring's HPA axis (Henry et al., 1994; Weinstock, 1997), and each of the phenotypic outcomes identified above can be linked with disruptions in the HPA axis.

Several non-experimental, but prospective, studies of humans have shown that maternal stress during pregnancy is associated with pregnancy outcomes and psychological processes tat confer risk for later physical and mental health including obstetric complications (Lou et al., 1994), shorter gestational length (Dayan et al., 2006), smaller size infant at birth (Wadhwa et al., 1993), individual differences in the diurnal rhythm and reactivity of the offspring's HPA-axis (Keenan et al., 2007; O'Connor et al., 2005), and temperamental problems (Huizink et al., 2002). The pattern of findings in humans closely mirrors those from controlled animal studies (Talge et al., 2007).

Given this strong evidence for the negative impact of maternal stress during pregnancy on the offspring, and the viable hypothesis that prenatal stress disrupts the programming of the systems involved in stress response, including the HPA axis, it is important to initiate studies of the prevention of the negative effects of prenatal stress in humans. This is especially relevant for sociodemographically vulnerable populations of women, who are at higher risk for adverse birth outcomes (Giscombé & Lobel, 2005) and whose offspring evidence less optimal neurodevelopmental outcomes (Noble, McCandliss & Farah, 2007; Reiss, 2013). Women living in poverty, for example, have higher rates of preterm birth and their infants have lower birth weights (Ruth et al., 2012). African American women living in dense, urban poverty appear to have the highest risk for adverse birth outcomes (Kent et al., 2013).

Changes in consumption of polyunsaturated fatty acids (PUFAs)

Polyunsaturated fatty acids (PUFAs) are comprised of two families: linoleic acid, and its omega 6-derivative arachadonic acid (AA) and α-linoleic acid, and its omega-3 derivatives, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Omega-6 fatty acids are mostly found in plant oils, whereas omega-3 fatty acids are found mostly in marine oils. The most important and abundant PUFA in the brain is DHA, which serves a critical role in neural functioning (Assisi et al., 2006; English et al., 2013). PUFAs are not synthesized endogenously and consequently diet determines the level of fatty acid available to the central nervous system. Because omega-6 and omega-3 fatty acids compete for the same enzymes required for desaturation and elongation, a higher ratio of omega-6 to omega-3 PUFAs in the diet reduces the availability of DHA. Over the past several decades, the intake of omega-3 PUFAs has decreased in the U.S. whereas dietary intake of omega-6 PUFAs has held relatively steady (Ailhaud et al., 2006; Blasbalg et al., 2011). In a recent review, average intake of seafood omega-3 fatty acids in the United States was less than 150 mg/day, far less than the optimal consumption of 250mg/day (Micha et al., 2014). Among pregnant women living in low-income environments omega-3 fatty acid intake and blood levels of DHA are approximately a third of the recommended levels for pregnant women (Stark et al., 2005; Nochera et al., 2011). Importantly, fish oil supplementation during pregnancy has been shown to be as effective as consuming more sea fish in increasing DHA levels in maternal and cord plasma and erythrocyte phospholipids (Escolano-Margarit et al., 2013).

PUFAs as modulators of stress hormones

In animal studies, DHA supplementation is associated with a decreased stress response to controlled stimuli. For example, Takeuchi and colleagues (2003) reported that DHA supplementation reduced stress behaviors in rodents (e.g., rearing, smelling, freezing) that were manifest in response to a corticotropin releasing hormone (CRH) infusion and to a conditioned fear response. In a study designed to test the effects of DHA supplementation on cardiac responses to

psychosocial stress, stressed Wistar rats receiving DHA supplements demonstrated lower heart rate and blood pressure than stressed rats whose diets were not supplemented (Rousseau et al., 1998).

In humans, fatty acid supplementation is also associated with reductions in stress reactivity in controlled studies. Maes and colleagues (2000) found an association between university students' responses of inflammatory cytokines to oral exams and serum omega-3 PUFA levels, with students above the mean in their serum omega-3 PUFA levels prior to the stressor demonstrating significantly lower levels in several proinflammatory cytokines, including interleukin-6. Results of a randomized controlled trial of 12 weeks of omega-3 supplementation in healthy men who were exposed repeatedly to a social stressor, revealed that omega-3 supplementation modulated the cortisol stress response, especially among individuals reporting high levels of chronic stress (Helhammer et al., 2012). Yehuda and colleagues (2005) reported on the efficacy of a daily dose of 225 mg omega-3 fatty acid taken for 1 month in reducing test anxiety among students. Morning cortisol levels were assessed at the beginning of the study and then a month later among 126 participants divided into three groups: students without test anxiety receiving no supplement, students with test anxiety receiving placebo, and students with test anxiety receiving 225mg/day of omega-3. Morning cortisol levels decreased significantly from pre- to post- in the supplement group; their morning cortisol levels were similar to the students without test anxiety. In contrast, morning cortisol levels did not change from pre- to post- in the placebo group. Delarue and colleagues (2003) reported a blunting of the cortisol response to stress in the context of an open trial in seven subjects. All subjects received 7.2g/day of fish oil and completed a mental arithmetic challenge and the Stroop Test at two time points: prior to, and three weeks after supplementation. The magnitude of change in cortisol in response to the stressors significantly decreased from pre to post omega-3 supplementation.

Thus, there is growing evidence that biobehavioral responses to stressors can be modified with supplementation of omega-3 fatty acids. In the controlled experiments described above, DHA supplementation was associated with a decrease in the magnitude of the response of systems involved in the regulation of stress, including the HPA-axis.

Evidence for the association between DHA levels during pregnancy and offspring outcomes

Tests of the association between DHA levels during pregnancy and offspring outcomes provide further evidence for the hypothesis that DHA modulates the effects of prenatal stress. Using experimental rodent models, deficient levels of DHA during pregnancy result in offspring deficits in learning, such as habituation to novelty and latency to reach learning criteria (Moriguchi, Sheaff-Griener & Salem, 2000), prolonged corticosterone response to restraint stress and longer periods of immobility during the Porsolt forced swim test (Chen & Su, 2012), and less exploration of environmental stimuli (Palsdottir, et al., 2012). Takeuchi and colleagues (2003) demonstrated that pups of DHA deficient dams spent less time in the open arms of a plus maze (indicative of higher stress levels) than the offspring of the normally fed animals. A one-week period of DHA supplementation to the diet of the pups, however, resulted in significant increases in time spent in the open arms. In one of the most compelling experimental studies to date, Feng and colleagues (2012) demonstrated in a rodent model that DHA administration during pregnancy attenuated the effects of prenatal stress on offspring hippocampal functioning including observed learning and memory, apoptosis and mitochondrial metabolism. In another rodent model, offspring of dams who received DHA and EPA supplementation beginning in pre-conception and continuing through lactation showed an attenuation of the behavioral and cognitive deficits typically elicited by olfactory bulbectomy, a manipulation that results in a phenotype consistent with depression in the human (Pudell et al., 2014).

To date, studies on DHA levels in pregnancy and the impact on the human offspring have been largely focused on immediate birth outcomes including birth weight, infant head circumference, and length of gestation. In a recently completed RCT of DHA supplementation during the second half of pregnancy of approximately 300 women, DHA supplementation resulted in significantly longer gestation, heavier and longer newborns, and larger head circumferences (Carlson et al., 2013). A few investigators have observed the association between DHA consumption during pregnancy and later child developmental functioning. Using a population-based sample of close to 12,000 women, Hibbeln and colleagues (2007) tested the association between maternal report of seafood consumption during pregnancy and children's development. Women who reported greater psychosocial adversity, including lower education and overcrowding in the home, were less likely to consume three or more servings of fish per week. After controlling for these psychosocial factors, however, low or no fish consumption during pregnancy was significantly associated with suboptimal communication skills, verbal IQ, fine motor skills, and social communication in the child, with findings extending out to age 8. In another observational study, a test of the association between levels of fatty acids in cord blood and maternal report of behavioral and emotional problems at age 10 revealed significant negative associations: higher levels of fatty acids were associated with lower levels of behavior problems (Kohlboeck et al., 2011).

Among the few double-blinded, randomized controlled studies of DHA supplementation during pregnancy in humans the results on offspring neurodevelopment have been mixed. Helland and colleagues (2003) assigned 341 women to receive either 10 mL/day of cod liver oil (1183 mg DHA) or corn oil beginning at 18 weeks of gestation through 3 months post-partum. All the mothers breast-fed their infant during this period. At age 4, cognitive development was assessed. Children whose mothers received cod liver oil during pregnancy had IQ scores that were significantly higher then children whose mothers received corn oil during pregnancy (106.4 versus 102.3, $p < .05$). Even after controlling for birth outcomes, cod liver oil intake during infancy, and other potential confounding variables, maternal intake of cod liver oil during pregnancy was the only significant predictor of IQ at age 4. These effects, however, were not maintained at age 7 years (Helland et al., 2008). In the DHA to Optimize Mother Infant Outcome (DOMInO) study, a large RCT of DHA supplementation during pregnancy in a community sample of Australian women, supplementation resulted in lower risk of preterm birth, but there were no effects on later cognitive functioning assessed in a subsample of children at 18 months (Makrides, et al., 2010).

One possible reason for the lack of consistent findings on human neurodevelopment is that DHA effects may be most evident among vulnerable populations in terms high levels of stress exposure and limited resources and/or in terms of offspring functioning under conditions of stress. In the majority of experimental animal studies, effects of DHA on offspring functioning was observed under conditions of manipulated prenatal stress and/or manipulated stress exposure in the offspring as opposed to typical functioning. Even in the DOMInO study there was evidence of differential efficacy among subpopulations: although mean cognitive scores did not differ between the two groups, toddlers in the control groups were more likely than toddlers in the DHA group to have scores in the delayed range (Makrides, et al., 2010). Thus, the protective effects of DHA may be most salient for individuals exposed to high levels of stress.

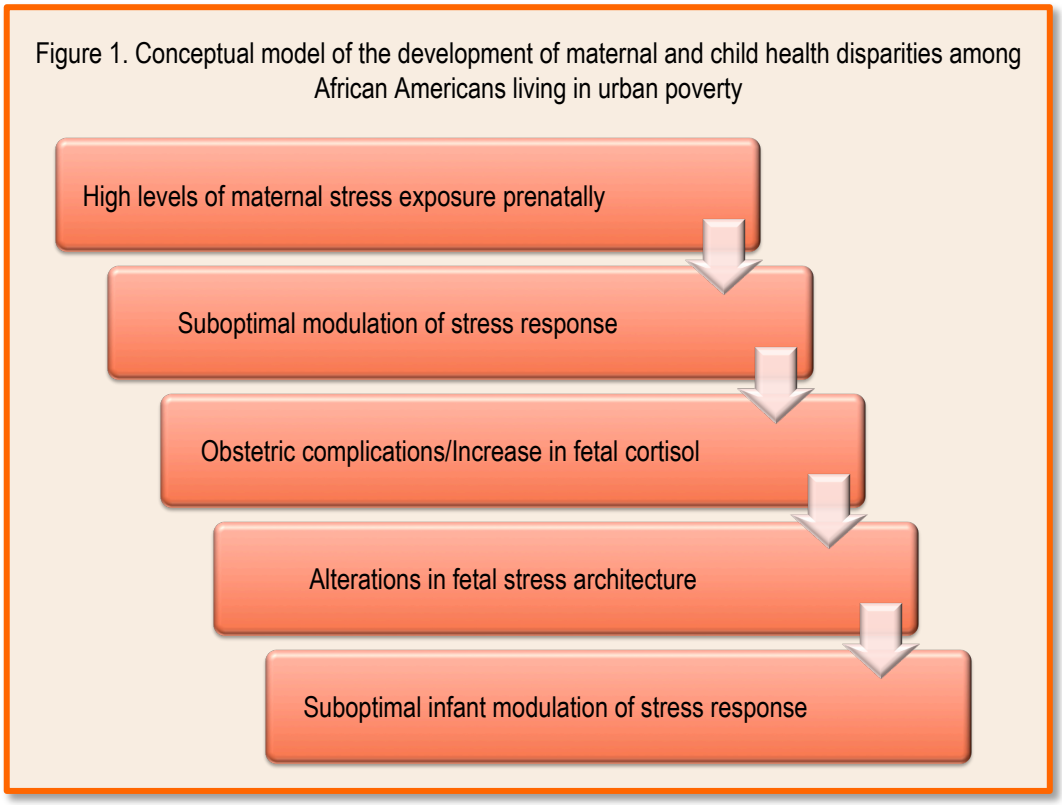
INNOVATION

Potential for addressing health disparities via fatty acid supplementation during pregnancy

The extant data support the hypotheses that prenatal stress confers neurodevelopmental risks in the offspring; that DHA supports a more modulated response to stress; that DHA levels during pregnancy are associated with neurodevelopment in the offspring; and that supplementing the diet of mothers with DHA during pregnancy can lead to more optimal child outcomes. Importantly, research using animal models show that DHA administration during pregnancy attenuates the effects of prenatal stress on offspring neurodevelopment. Although both the animal and human literature supports the hypothesis that fatty acid supplementation may be most beneficial for pregnancy women exposed to high levels of stress, to date no investigator has tested whether supplementing the diets of women who are experiencing acute and/or chronic stress will improve maternal health and reduce the suboptimal development outcomes in humans.

In the U.S., higher levels of acute and chronic stress are found among families living in low-income environments than among families living in other income environments: neighborhood disorder, lack of safety and exposure to violence are all significantly higher in areas with lower per capita income (Evans, 2003; Ewart & Shuchdat, 2002). African Americans live in poverty at a disproportionately high rate, with more than a quarter of African Americans living in poverty (DeNavas-Walt & Proctor, 2014). Pregnant women living in poverty are at higher risk for poor nutrition during pregnancy (Noble et al., 2007; Fowles & Gabrielson, 2005) and are more likely to experience pregnancy complications (Giscombé & Lobel, 2005). This is especially the case for African American women living in urban low-income environments (Kent et al., 2013). Greater exposure to psychosocial stress during the prenatal period has been hypothesized be a primary mechanism by which poverty confers risk for physical and mental health disorders to the offspring (Reynolds et al., 2013; Robinson et al., 2008).

This is a viable hypothesis given the data on the impact of race and poverty on the functioning of the HPA-axis. Higher levels of cortisol in the afternoon and evening found among individuals living in lower SES environments than among individuals living in higher SES environments (Chen & Paterson, 2006). Within a sample of post-menopausal women caring for a disabled family member, African American women were more likely to demonstrate a significant increase in cortisol in response to a psychosocial stressor than were European American women (Wilcox et al., 2005). Furthermore, data from studies using exposure to a controlled stressor provide evidence for racial differences in inflammatory response (i.e., interleukin-6) to stress, with African American pregnant and non-pregnant women show higher responses that European American women (Christian et al., 2013). In a study in which both cortisol and pro-inflammatory cytokines were measured during pregnancy, minority race and low-income status was characterized by high levels of cortisol without a compensatory decrease in cytokines, suggesting impaired feedback between the neuroendocrine and immune systems (Corwin et al.,



2013). Thus, race and SES appear to impact both the diurnal rhythm and feedback loop of the stress response system, and the interface between HPA-axis and other systems critical for maintaining health such as immune functioning.

As shown in Figure 1, we posit that a primary cause of maternal and child health disparities among African Americans in the U.S. begins with maternal psychosocial stress during pregnancy. This high level of chronic exposure to stress leads to suboptimal modulation of stress response and consequently, exposure of the fetus to high levels of glucocorticoids released by the mother. This exposure, in turn, affects the pregnancy and the development of the fetal stress architecture in part by adjusting the set point for mounting a stress response and interfering with the feedback mechanisms for maintaining homeostasis. Although the postpartum environment will continue to affect brain development, for a substantial number of children this initial insult sets the stage for a developmental trajectory that begins with a poorly modulated response to stress in infancy.

Identifying factors that can potentially interrupt this cycle has significant implications for public health. Growing evidence indicates that DHA supplementation improves maternal stress reactivity during pregnancy and protects neurodevelopment of the offspring, especially in the context of high levels of stress exposure. We propose a program of research that aims to translate such findings to address the significant health disparities for African American women and children. In the following section we present preliminary evidence for the efficacy of prenatal fatty acid supplementation on maternal perceived stress and stress reactivity in this high-risk population.

Preliminary support for hypotheses

We recently completed the first randomized controlled trial of DHA supplementation among pregnant African American women living in stressful, low-income, urban environments (for a full report see Keenan et al., in press, *Obstetrics & Gynecology*). Sixty-four pregnant women were recruited from the Obstetrics Clinics at the University of Pittsburgh. Inclusion criteria were: African American race, age between 20 and 30 years, 16-21 weeks of gestation, Medicaid insurance or Medicaid eligible, and low levels of DHA consumption as defined as less than two fish servings per week. Exclusion criteria were medical complications (e.g., gestational diabetes, pre-eclampsia), regular use of steroid medications, use of blood thinners or anti-coagulants, use of psychotropic medications, current BMI >40, and allergy to iodine and/or soy. Participants were reimbursed on an accelerated schedule with $40 for their first visit and an increase in payments of $10 for each subsequent visit. The Institutional Review Boards of the Universities of Chicago and Pittsburgh approved all study procedures. Once enrolled, women were randomly assigned to receive the omega-3 nutritional supplement (n = 43) or a corn and soybean oil placebo (n = 21) beginning at enrollment and up through the end of pregnancy. Women received supplement via two gel capsules providing: 450 mg of DHA; 40 mg of docospentaenoic acid (DPA) and eicosatetranoic acid

(ETA); 90 mg of eicosapentaenoic acid (EPA); and 15 IU of Vitamin E. Randomization was conducted by the pharmacy at the University of Pittsburgh, thus ensuring that participants and investigators were blinded to the group to which the participants were assigned.

Cortisol response to a social evaluative stressor was measured at baseline, 24 and 30 weeks of gestation. Saliva was collected at three time points at the baseline, 24 and 30 week assessments: 20 minutes following arrival to the lab, and 20 and 45 minutes post-TSST. Participants completed questionnaires assessing perceived stress, stressful life events, and symptoms of depression at baseline, 24, and 30 weeks of gestation. Research assistants contacted participants by phone 3 times per week to ask the time of day that supplement was taken, and collected data on perception of taste, and possible gastrointestinal side effects.

Of the 64 participants who completed the baseline assessment, 4 (6.3%) withdrew from the study. Two participants randomized to placebo withdrew: one miscarried and the other withdrew due to mood changes, and two participants randomized to active supplement withdrew: one reported headaches and the other upset stomach. Forty-seven participants (73.4%) attended all three sessions. Thus, acceptability and feasibility were high, and retention was very good.

The first goal was to examine group differences in level of depression, negative life events, and perceived stress by conducting analyses of variance for each measure, controlling for the other two measures. At baseline and 24 weeks of gestation, there were no group differences in any of the three measures. By 30 weeks, perceived stress was significantly lower among the participants receiving supplementation compared to those receiving placebo, controlling for negative life events and depression scores at 30 weeks (F [1,47] = 5.06, p = .029, Figure 2).

Cortisol response to the TSST was examined as a function of group status (supplementation versus placebo) at baseline, 24 and 30 weeks of gestation by repeated measures analysis of variance, using a Greenhouse-Geisser correction to account for lack of sphericity. Time of day was significantly associated with initial cortisol levels at baseline, but not at 24 and 30 weeks, thus time of day was controlled in all analyses involving cortisol levels at baseline. Cortisol levels before and after the TSST did not vary as a function of supplementation at baseline or at 24 weeks. At 30 weeks, cortisol levels over time significantly differed as a function of supplementation (F [1.78, 83.85] = 6.24, p = .004, *cohen's d* = .76). As shown in Figure 3, women who received placebo had higher levels of cortisol upon arrival to the lab compared to women receiving

Figure 2. Effect of omega-3 supplementation on perceived stress controlling for negative life events and depression scores

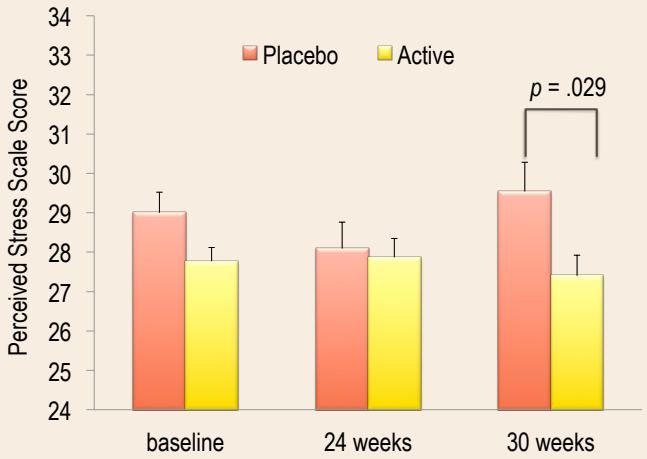
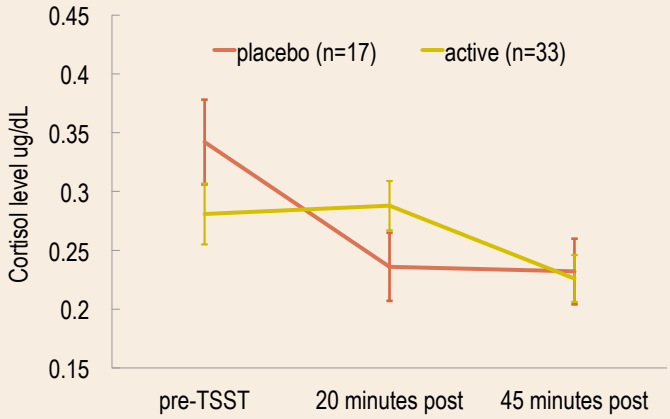
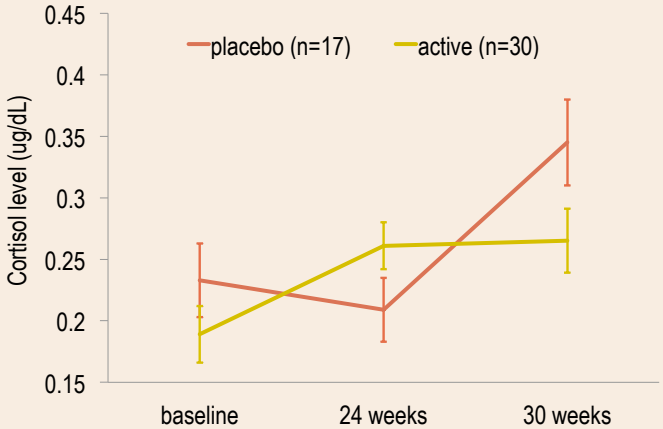


Figure 3. Cortisol levels before and after the Trier Social Stress Test (TSST) at 30 weeks of gestation



F (1.78, 83.85) = 6.24, p = .004, *cohen's d* = .76

Figure 4. Cortisol levels 20 minutes following arrival to laboratory at baseline, 24, and 30 weeks of gestation



F (1.74, 74.63) = 3.51, p = .041, *cohen's d* = .56

omega-3 supplementation. Levels for women receiving placebo were characterized by a relatively steep decline, whereas levels for women receiving omega 3 supplement evidenced a slight increase in response to the stressor, on average, followed by decline during the period of recovery. To further probe the differences in levels upon arrival to the laboratory, the two groups were compared at baseline, 24 weeks, and 30 weeks of gestation. As shown in Figure 4, cortisol levels upon arrival to the lab were similar for the two groups at baseline, but diverged over time such that by 30 weeks of gestation women who received placebo had levels that were on average 20% higher than women receiving supplement (F [1.74, 74.63] = 3.51, p = .041, *cohen's d* = .56).

Results from the present study provide preliminary evidence that changes in prenatal nutrition may be one way to interrupt the suboptimal prenatal programming of the fetus developing in the context of high levels of maternal stress. Pregnant women living in high stress, low-income, urban environment, receiving a fatty acid supplement reported lower levels of perceived stress than women who received placebo, despite a lack of change in exposure to stressors. Moreover, the reduction of perceived stress was independent of depression, which was not associated with fatty acid supplementation. In fact, evidence from the present study suggests that DHA supplementation does affect the functioning of the HPA-axis by modulating response to a social stressor. This effect was demonstrated primarily via a lower cortisol response to arriving at the laboratory. By 30 weeks of gestation, participants who received DHA supplementation had lower levels of cortisol upon arrival to the laboratory, and on average demonstrated a slight increase in cortisol in response to the stressor, a pattern typically observed late in pregnancy (Nierop et al., 2006; de Weerth et al., 2005). In contrast, participants who had received placebo evidenced high levels of cortisol upon arrival to the laboratory, followed by a steep decrease in cortisol levels during and after exposure to the TSST. One interpretation of this pattern is that an exaggerated response to anticipatory stress leads to a less flexible response to the stressor. The high levels of cortisol upon arrival to the lab followed by a lack of responsiveness to the stressor has been described as a pattern of dysregulated HPA axis functioning associated with depression both in adults (Burke et al., 2005) and children (Suzuki et al., 2013). Lack of cortisol response to a social stressor also has been observed among adults reporting high levels of early life adversity, especially females (Lovallo, 2013). DHA supplementation appears to protect pregnant women living in low-income environments from manifesting this type of dysregulation.

In addition to associations between DHA supplementation and prenatal stress, we observed significantly higher birth weights among neonates born to women receiving DHA compared to placebo controlling for gestational age (3,174 grams versus 2,889 grams, p = .018) and greater likelihood of having a 1-minute Apgar score of 9 (odds ratio = 5.99 [*95% CI* = 1.25 – 28.75], p = .025, controlling for birth weight and gestational age). Finally, 13% of neonates of mother receiving DHA supplementation were born prior to 37 weeks, compared to 22% of mothers received placebo; a difference that did not reach the level of statistical significance in the pilot study, but is clinically meaningful.

In summary, the data from our preliminary study are compelling in terms of the potential impact on health disparities in maternal and infant health, a significant public health problem that is poorly understood. We now seek to expand on these preliminary findings by conducting a more rigorous assessment of prenatal DHA supplementation on maternal and infant health by increasing the number of participants to 162, beginning supplementation earlier in pregnancy, adding additional measures of maternal health and stress response during pregnancy, increasing the frequency of assessment, assessing DHA levels in red blood cells at each visit, and including multiple postnatal assessments of infant neurodevelopment. The proposed study offers an opportunity for replication and to test the impact of supplementation on infant functioning and, if effects are observed, whether they are mediated by changes in prenatal stress.

APPROACH

Participants

One hundred-sixty-eight pregnant, African American, women will be recruited from the University of Chicago obstetric clinic. Inclusion criteria will include: age between 18 and 34 years, household receipt of public assistance (e.g., Medicaid insurance) due to low-income, and low levels of DHA consumption as defined as less than two fish servings per week. Exclusion criteria are reports of known medical complications, regular use of steroid medications, alcohol, cigarettes, or illegal substances (by maternal report), use of blood thinners or anti-coagulants, use of psychotropic medications, BMI >40, and allergy to iodine and/or soy. Participants may be excluded if medical complications arise after enrollment. Neonates with significant medical complications will be maintained in the study.

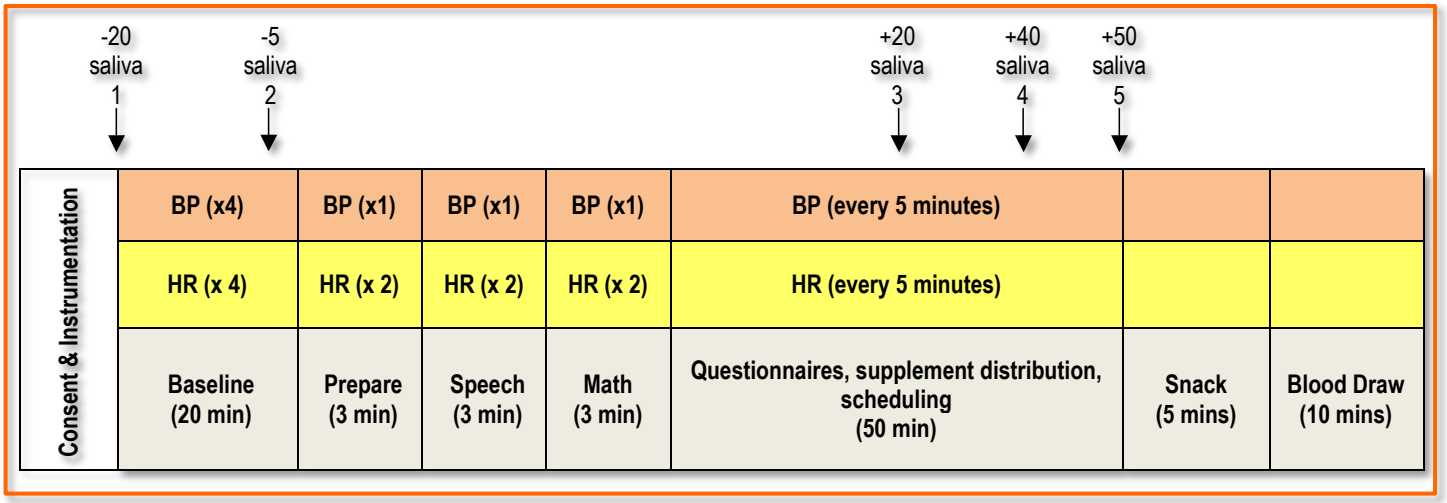
There are two reasons for our focus on African American women. First, as described above, African American women are disproportionately represented among families living in inner-city poverty and have the highest rates of suboptimal birth outcomes in the U.S. (Giscombé & Lobel, 2005). Second, there are racial differences in cortisol reactivity to

stress and pregnancy (De Santis et al., 2007; Glynn et al., 2007; Wilcox et al., 2005) and in the genes regulating cortisol (Baerwald et al., 1999). Given the scope of the proposed study and the potential to generate data that can be translated into reductions in health disparities, we chose to study African Americans only, but acknowledge that it will be important to test racial and ethnic differences in maternal and infant response to DHA supplementation during pregnancy.

Women will be randomly assigned to receive the omega-3 nutritional supplement (n = 112) or a corn and soybean oil placebo (n = 56) beginning at enrollment and up through the end of pregnancy. In the proposed design we allocate the number of participants to groups to optimize power to test the hypotheses. We expect greater variability in the independent measure (i.e. DHA level) and dependent measures (e.g., stress reactivity) in participants in the experimental group than those in the control group given anticipated differences in uptake and metabolism. Thus, we plan to enroll a higher number of participants in the experimental group than in the control group to adequately capture that variability (Cox, 1958). Participants and investigators will be blinded to the group to which they have been assigned. Treating clinicians will identify demographically eligible participants who will be contacted by research staff for further screening. Participants who meet the inclusion and exclusion criteria will then be scheduled for the baseline visit.

Participants will be reimbursed on an accelerated schedule with $100 for their first visit and an increase in payments by $25 for each subsequent visit, ending with a $250 payment for the last post-partum visit. Participants will receive incentives in between visits including water bottles, tote bags, newborn t-shirts, etc. They also will be reimbursed with $10 to cover travel costs for each visit.

Figure 5. Protocol for prenatal laboratory visits



Methods

All visits will take place in the Department of Psychiatry and Behavioral Neuroscience at the University of Chicago. All prenatal visits will follow the same protocol (see Figure 5). Participants will provide a saliva sample upon arrival to the laboratory and an occluding cuff will be placed on the non-dominant arm for automated measurement of BP. A 3-lead electrocardiogram (ECG) will be attached to the shoulders and xyphoid process for continual measurement of heart rate. Following instrumentation, there will be a period of acclimation during which they will rest quietly while viewing a non-stressful video at the end of which a second saliva sample will be obtained. This will be followed by administration of the Trier Social Stress Test (TSST). Participants will complete psychosocial stress questionnaires following the TSST. These questionnaires will cover perceived stress, stressful life events, and symptoms of anxiety and depression. Following the final saliva sample, participants will be provided with a snack and then will be accompanied to the Clinical Research Center (which is located two floors above our laboratory) for the blood draw which will be used to assess DHA levels and inflammatory markers (C-reactive protein (CRP); IL-6; TNF-α).

Offspring stress reactivity will be measured via behavioral and cortisol response to the Brazelton Neonatal Behavioral Assessment Scale (NBAS) at 1 month and in response to the Face-to-Face Still-Face (FFSF) paradigm at 4 and 9 months. Infant cognitive development and behavior and emotion regulation during developmental testing also will be assessed.

Nutritional Supplement. As in the pilot study, the nutritional supplement we propose to use is ProDHA. The same dose will be used, with 2 gel capsules providing: 450 mg of DHA; 40 mg of DPA and ETA; 90 mg EPA; and 15 IU Vitamin E.

Nordic Naturals adheres to high standards on safety and purity by setting low levels of allowances for peroxides, heavy metals, dioxins, furans, and PCBs. A certificate of analysis is provided for every batch of ProDHA based on both an internal analysis and an analysis conducted by an independent laboratory.

The investigational pharmacy at the University of Chicago will use computer generated random assignment of ID numbers to active supplement or placebo in blocks of 18: 12 ID numbers will be randomized to active supplement and six to placebo. In order to maintain the double-blind, the pharmacist will divide each randomization block of 18 ID numbers into groups of three: six ID numbers were assigned to group A (placebo) and the remaining 12 will be assigned to either group B or C, both of which will receive identical doses of active supplement. This approach will allow the pharmacist to randomize on a 2:1 ratio without having the unbalanced design break the blind. Once the study is complete and the blind broken, the two groups receiving identical doses of active supplement will be combined for analyses.

Each participant will receive a 6-week supply. She will bring any unused DHA back to her next visit; pills will be double counted at that time as an additional method for monitoring compliance. At each visit she will receive the next supply. Research assistants will contact participants by phone 3 times per week to ask the time of day that supplement was taken, and data on perception of taste, and possible gastrointestinal side effects. At each laboratory visit research assistants will interview participants about diet over the past 24 hours and past two weeks to determine intake of sea fish or other DHA rich foods (e.g., flax seed).

Maternal Psychosocial Stress. The *Perceived Stress Scale* (PSS; Cohen et al., 1983), a 14-item scale, will be used to assess the degree to which situations in a person's life are appraised as unpredictable, uncontrollable or overloaded. It has been shown to possess test-retest reliability, adequate internal consistency and concurrent and predictive validity (Cohen et al., 1983). The *Difficult Life Circumstances* (DLC; Barnard et al., 1983) is a set of 28 yes-no questions about negative life events. The measure was designed to include items that would be applicable to women living in poverty such as difficulty with finances and housing. We will assess symptoms of depression using the *Edinburgh Depression Scale/Edinburgh Postnatal Depression Scale* (EDS/EPDS; Cox et al., 1987), a 10-item measure designed to assess pre- and postnatal depression without confounding the assessment of depression with somatic symptoms of pregnancy (e.g., weight gain, loss of energy). Anxiety will be assessed via the Spielberger Trait Anxiety Inventory (STAI; Spielberger et al., 1970), a 20-item scale that measures state and trait generalized anxiety.

DHA levels. Blood samples will be taken in EDTA-containing vacuum tubes. Samples will be centrifuged for 10 minutes at 4 degrees C and 3000 times gravity, the plasma will be removed and the red blood cells stored at -80ºC until analyzed. Red blood cell phospholipids will be separated from other lipids by thin layer chromatography on a Hewlett-Packard model 6890 Gas Chromatograph, and then transmethylated with boron triflouride in methanol (Sigma Chemical Company) to yield fatty acid methyl esters. Individual fatty acid methlyl esters will be separated and quantified by gas chromatography. Individual peaks will be identified via comparison to authentic standards (Sigma Aldrich). DHA content will be calculated on a µg/mL basis from the known amount of standard added and determination of response factors. Coefficients of variation for all fatty acid peaks will be measured by analyzing quality control samples randomly distributed throughout the study samples.

Maternal Inflammatory Markers. Blood levels of TNF-α and IL-6 will be determined via enzyme-linked immunoassay (ELISA) from the blood draws at each of the four prenatal visits. The CoV for both cytokines is <5%. CRP will be determined turbidmetrically (CoV, 3.0%). All samples will be assayed in the Heinz Laboratory of the Graduate School of Public Health, which has CLIA certification and is enrolled in the proficiency-testing program organized by the College of American Pathologists.

Maternal Stress Response. Following published recommendations for assessing physiological stress reactivity during pregnancy (de Weerth & Buitelaar, 2005), the *Trier Social Stress Tes*t (TSST; Kirschbaum, 1993) will be used as stressful stimulus in the laboratory. The TSST consists of a three-minute preparation time, followed by a 3-minute speech (job interview), and then a 3-minute mental arithmetic task. The latter two tasks are performed in front of a video camera and an audience. The TSST reliably elicits a cortisol response, even in pregnant women (Nierop et al., 2006a; 2006b).

Saliva will be collected at five time points: 20 minutes and 5 minutes pre-TSST, and 20, 40, and 50 minutes post-TSST. To collect saliva samples, an absorbent, unflavored dental roll is applied to the tongue, cheek, and gums for several minutes. Flow rate will also be timed. The dental roll will then be returned to the plastic salivette and labeled. Samples will be immediately transferred to a freezer and stored at -80° C until assayed. On the day of testing, samples will be thawed, centrifuged at 3,000 rpm for 10 minutes and a clear sample will be pipetted into appropriate test wells. All samples from each subject will be assayed in the same batch to minimize variability, and will be assayed with reagents from the same lot. Samples with sufficient saliva will be assayed in duplicate for cortisol using the Salimetrics protocols. The cortisol assay has

an average between-assay variance of 3.9% and 7.1%, and the average within-assay variance is 6.7% and 6.9% for high and low concentrations, respectively.

In order to control for the circadian rhythm of cortisol and to optimize the ability to detect a response, the lab visit will take place between 2:00 PM and 5:00 PM, when cortisol levels are low. Although there are data to suggest that there may be some habituation to repeated psychosocial stressors like the TSST, a significant cortisol response continues to be elicited even if the magnitude of the response is somewhat attenuated. For example, Schommer et al. (2003) administered the TSST to 65 adults 3 times, separated by 4-week intervals. A significant cortisol response was elicited each time. A significant decrease in the magnitude of the peak response was reported between the first and second administration, but not between the second and third administration. In our own experience in the pilot study we observed increases in cortisol over time in response to arrival at the lab among the placebo group. Although we plan to test for differences in levels upon arrival to the laboratory, we aim to increase the capacity to assess group differences in response to the TSST by enhancing acclimation to the laboratory prior to the administration of the TSST and increasing the number of samples collected pre- and post-TSST.

Because there will be some variability in the time of sample collection, the effect of time of day on the pattern of cortisol response will be tested, and if necessary controlled for in the analyses. Stage of pregnancy also affects cortisol levels and reactivity (de Weeerth & Buitelaar, 2005). Thus, if sufficient variability exists in gestational age and significant effects on cortisol are demonstrated, stage of pregnancy also will be controlled in the analyses.

In addition to changes in cortisol, heart rate and blood pressure (BP) will be assessed in response to the TSST as indirect measures of sympathetic-adrenal activation. Heart rate and BP will be measured throughout the period of acclimation, during the TSST, and during recovery (see Figure 5). Heart rate variability (HRV) will be measured from a continuous time series of inter-beat intervals. Prior to calculating estimates of HRV, the digitalized ECG signals will be examined and artifactual detections of R-wave occurrences will be corrected. The square root of the mean of successive differences (RMSSD) in inter-beat intervals will be used as a time-domain estimate of HRV. Inter-rater reliability for the editing of artifacts will be assessed using 40 x 5 min epochs (10 per participant for 4 randomly selected participants).

Infant outcomes. We include measures of infant outcomes that have been shown to be associated with DHA (e.g., cognition) and maternal stress during pregnancy (e.g., temperament, behavioral and emotional regulation and stress reactivity). In addition, we selected measures that would be comparable to existing studies of infant stress reactivity. We will collect birth outcomes from the medical record including type of delivery, gestational age, weight, length, head circumferene, apgar scores, cord problems, and meconium aspiration, extended hospital stay or NICU admission, etc. The *Neonatal Behavioral Assessment Scale* (NBAS; Brazelton & Nugent 1995) will be administered within the first month to assess the neurodevelopment by sampling a broad range of behaviors including reflexes, state changes, attention, arousal, and regulatory capacities such as intensity and duration of distress, latency to distress and self-soothing.The principal investigator was trained and certified by the Brazelton Institute. In our own work we have demonstrated that the NBAS elicits a cortisol response in neonates (Keenan Gunthorpe, & Grace, 2002; Keenan, Grace & Gunthorpe, 2003), and have observed associations between behavioral and cortisol response to the NBAS and prental stress (Keenan, Gunthorpe & Grace, 2007).

The *Bayley Scales of Infant Development* (Third Edition) (BSID-III; 2006) will be administered as an index of developmental functioning. The Bayley Behavior Ratings will be used to generate continuous scores for observed emotion and behavior regulation during testing. Temperament will be measured using The Infant Characteristics Questionnaire (ICQ; Bates, Freeland, & Lounsbury, 1979), which is designed specifically to assess regulation of negative emotion, and the Infant Behavior Questionnaire-Revised (IBQ-R; Gartstein & Rothbart, 2003), a broader measure of temperament. The ICQ contains 32 seven-point items that assess temperamental characteristics, including fussy-difficult and unadaptable characteristics. The instrument has been shown to have good criterion validity (Bates, Freeland & Lounsbury 1979), and has the additional advantage of being short, and easy to understand. The IBQ-R short form contains 36 items that load on three dimensions: Surgency/Extraversion, Negative Affectivity, and Orienting/Regulation, and has been shown to have adequate reliability and validity (Garstein & Rothbart, 2003)

Cortisol response to NBAS in the first month and to the *Face-to-Face Still-Face* (FFSF; Tronick et al., 1978) at 4 and 9 months will be used to measure stress reactivity. The FFSF is a standard laboratory procedure that is comprised of three 2-minute episodes: mother playing typically with her seated infant, followed by mother maintaining a neutral expression and no vocalization, followed by a return to typical play. The FFSF reliably produces physiological arousal in infants including increases in cortisol (Field, 1994; Hayley & Stansbury, 2003). Collection and assays of saliva samples will be conducted as described above for the mothers. Effects of time of day on the pattern of cortisol response will be tested,

and if necessary controlled for in the analyses. Infants will be required to have not consumed any milk products (human or animal) for 1 hour prior to and during saliva collection. Saliva will be collected at four time points: 5 and 0 minutes pre-FFSF, and 20 and 40 minutes post-FFSF by swabbing the infants' mouths with an unflavored dental roll.

An overview of the main study variables is provided in Table 1. We also will assess maternal weekly sea fish intake at the post-partum visits and type of infant formula to control for possible postnatal omega-3 intake by the infant.

Measure	Variables	9-12 wks	16-20 wks	24-30 wks	34-36 wks	1 month	4 months	9 months
Maternal Psychosocial Stress								
Perceived stress	Total score from PSS	✓	✓	✓	✓	✓	✓	✓
Life events	Total score from DLC	✓	✓	✓	✓	✓	✓	✓
Depressed mood	Total score from EDS	✓	✓	✓	✓	✓	✓	✓
Anxiety	Total score from STAI	✓	✓	✓	✓	✓	✓	✓
Maternal Stress Response								
Reactivity to TSST	Cortisol; HR, BP	✓	✓	✓	✓			
DHA Levels								
Blood draw	DHA levels in red blood cells	✓	✓	✓	✓			
Maternal Inflammatory Markers								
Blood draw	CRP; IL-6; TNF-α	✓	✓	✓	✓			
Infant Outcomes								
Birth	Birth weight, gestational age, Apgar, meconium					✓		
Neurodevelopmental functioning	NBAS, BSID					✓	✓	✓
Stress reactivity	Cortisol response to NBAS, FFSF					✓	✓	✓
Temperament	IBQ; IBQ-R – short form						✓	✓

Table 1. Overview of main study variables

Hypotheses and Analyses

Hypothesis 1. DHA supplementation will improve maternal health during pregnancy among African American women living in urban poverty. Women who receive DHA supplementation will report lower levels of self-reported perceived stress over time, despite a lack of change in exposure to environmental stressors. Inflammatory markers including C-reactive protein (CRP), IL-6, TNF-α will be lower among women receiving DHA supplementation compared to placebo. To test for significant differences in perceived stress and inflammatory marker levels we will use repeated measures ANOVA (rmANOVA) with supplement group as the between-subjects factor and visit as a within-subjects factor. The interaction between group and time will be of particular interest. A Greenhouse-Geisser sphericity adjustment (Geisser & Greenhouse, 1958) will be employed. If any of the dependent measures exhibit a non-normal distribution (e.g. CRP), then a suitable data transformation will be used. Changes over time in prenatal levels of depression and anxiety will also be assessed using rmANOVA. Scatter plots and box plots of the data will be used to further explore these relationships. Plots of the data also will be examined to detect nonlinear time effects in addition to using orthogonal polynomial contrasts to formally test for quadratic and cubic effects. The association between change in DHA levels in the blood from baseline to the last prenatal visit and change in perceived stress will be examined using linear regression models.

Response to a controlled stressor including cortisol, blood pressure, and heart rate will be better modulated among women receiving DHA supplementation than among women who do not, as demonstrated by a lower peak response and more rapid recovery to baseline. Differences in stress reactivity (i.e., cortisol levels, blood pressure, and heart rate) will be tested using two complementary approaches. First, we will use rmANOVA with supplement group as the between-subjects factor and timing of measurement (e.g. 20 minutes and 5 minutes pre-TSST, and 20, 40, and 50 minutes post-TSST for cortisol) as the within-subjects factor for each of the 4 prenatal visits separately. The interaction between group and time will be tested. For further interrogation of these changes, a random intercept, random slope mixed-effects model will be fit and results compared to the rmANOVA results. Second, the area under the curve (AUC) will be calculated for each participant at

each visit. Since primary interest is in assessment of changes over time, the AUC_I (area under the curve with respect to increase) will be used (Pruessner et al., 2003). Differences in these AUCs will be tested using rmANOVA with supplement group as the between-subjects factor and visit as the within-subjects factor. The interaction between group and visit will also be included to explore whether the magnitude of the difference between the groups is affected by the length of DHA supplementation. A Greenhouse-Geisser sphericity adjustment will be employed in both rmANOVA analyses. For further exploration of the patterns over time, a cluster analysis using the AUCs at each visit will be performed, and the association between the "change groups" formed from this cluster analysis and supplement group will be determined using a chi-square or Fisher's exact test. Log-transformed cortisol values will be used if appropriate. Also, time of collection and prenatal depression and anxiety will be controlled for in these analyses if needed.

Power Calculations for Hypothesis 1. In this study with 108 participants in the supplement group and 54 in the placebo group, there will be >90% power to detect a difference in stress levels, at any particular visit, of the same magnitude as that observed in our preliminary study at 30 weeks (27.5+/-3.4 vs. 29.3+/-3.1), using a two-sided test at the 0.05 significance level. Additionally, with 162 participants, there will be 80% power to detect a correlation of 0.22 between changes in DHA levels in the blood versus changes in perceived stress. For the cortisol response to the TSST, there will be >95% power to detect a difference in AUCs at a given visit of the same magnitude as that found in our preliminary study at 30 weeks (-4.5+/-3.9 vs. -8.1+/-6.1; negative values indicating net decreases rather than increases), using a two-sided test at the 0.05 significance level.

Hypothesis 2. DHA supplementation during pregnancy will improve infant birth and developmental outcomes among African American women living in urban poverty.

Infants of mothers who receive DHA supplementation will have longer gestations, higher birth weights, more optimal neurodevelopmental outcomes as measured by the NBAS and Bayley Scales of Infant Development, and more optimal maternal reports of temperament in comparison to infants whose mothers did not receive DHA supplementation. We will use ANOVA (e.g. birth weight) or rmANOVA (e.g. NBAS) to test for significant differences between treatment groups. For categorical infant outcomes, such as pre-term birth or low Apgar scores, logistic regression will be employed. We will also test the association between level of DHA in blood at the last prenatal visit and/or change in DHA levels from baseline and infant outcomes using linear (e.g. birth weight or change in NBAS) or logistic (e.g. pre-term birth) regression. Transformations of the continuous dependent measures will be performed to stabilize the variance across groups if needed.

Infants of mothers who received DHA supplements will demonstrate more modulated cortisol response to the NBAS and still-face paradigm, with a lower peak response and a steeper slope from peak to recovery, in comparison to infants whose mothers did not receive DHA supplementation. Analyses will be performed as described for Hypothesis 1.

Infant gender, mothers' postpartum stress, depression, and anxiety levels will be compared between those received DHA supplements during pregnancy and those who did not using ANOVA. If significant differences are found, then these factors will also be controlled for in the analyses. If time of collection is associated with cortisol values, then this will also be controlled for in the analyses. However, since a circadian rhythm is not firmly established prior to the age of 6 months, we do not expect to have to control for time of day at 1 and 4 months.

Power Calculations for Hypothesis 2. Based on published data on maternal report of difficult temperament in infants using the ICQ (Keenan et al., 1998), the estimated SD for the ICQ in the proposed study is 7.8. With 108 participants in the supplement group and 54 in the placebo group, there will be 80% power to detect a difference in the ICQ of 3.7 between the two groups at a given visit, using a two-sided test at the 0.05 significance level.

The SD for 30–minute post-test cortisol levels in response to the FFSF paradigm was reported as 0.4 µg/dL (Hayley & Stansbury, 2003). If we assume a similar amount of variability in peak responses to the FFSF, with 108 participants in the supplement group and 54 in the placebo group, there will be 80% power to detect a difference of 0.19 µg/dL between the two groups, using a two-sided test at the 0.05 significance level.

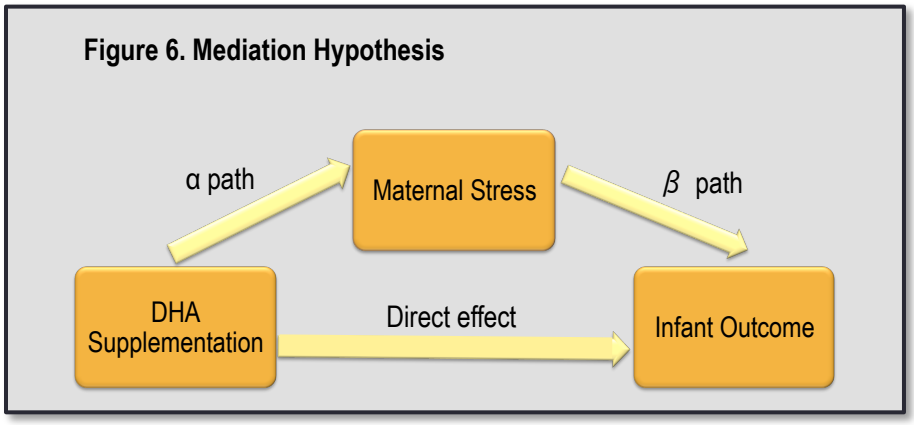
In addition, there will be approximately 90% power to detect a difference in birth weights of the same magnitude as that observed in our preliminary study (3174+/-480 vs. 2889+/-549 g), using a two-sided test at the 0.05 significance level.

Hypothesis 3. The association between DHA supplementation during pregnancy and infant outcomes will be partially mediated by reductions in maternal perceived stress, stress reactivity during pregnancy.

Mediation will be tested using the Baron and Kenny approach (Baron & Kenny, 1986) with the following general model:

$$g(E(Y_i)) = \alpha + \beta M_i + \omega T_i + \boldsymbol{\lambda Z}_i$$

Y_i is an outcome for infant i, g is a link function (e.g. logistic or identity), M_i is a maternal stress measure at the final prenatal visit for the mother of infant i, T_i is treatment group (e.g. 0=placebo vs. 1=active; average DHA level in the blood across prenatal visits), and $\boldsymbol{Z}_i$ is a vector of additional covariates such as infant gender, mothers' postpartum stress, depression, or anxiety. The change in ω due to adding maternal stress to the model provides an estimate of the proportion of the treatment effect due to this factor; if ω is still significant but the magnitude is reduced, this would provide evidence for partial mediation. For infant outcomes collected at multiple visits, the initial approach will be to use data from the last visit, but longitudinal approaches may be considered depending on the results from the initial analyses. To provide greater power for detecting such effects, bootstrapped confidence intervals will be examined. Since multiple measures of maternal stress will be collected, the model may be extended to permit multiple mediators.



Power Calculations for Hypothesis 3. Based on simulation results presented in Fritz et al. and the SAS program provided at http://ripl.faculty.asu.edu/wp-content/uploads/2013/02/baronkenny3.txt, 162 subjects will provide sufficient power (>80%) to detect the following effects based on Cohen's criteria. As shown in Figure 6 and Table 2, the study with 162 subjects will be sufficiently powered (>80%) to detect medium effects for the α and β paths and small effects for the direct effect (scenario 1), or small-medium effects for the α and β paths and medium effects for the direct effect (scenario 2), which is plausible based on the effects found in the preliminary study.

Table 2: Overview of power for mediation hypothesis

	α path	β path	Direct effect
scenario 1	medium	medium	small
scenario 2	small-medium	small-medium	medium

Missing data. Missing data is not expected to be a major issue based on retention rates from the preliminary study. The last observation carried forward (LOCF) approach for missing data will be employed. Sensitivity analyses using complete-cases only will also be performed and the results using the two approaches compared. Related to this, treatment non-compliers will be included in the primary analysis based on the intent-to-treat (ITT) principle.

Timeline for participant visits

Assuming a start date of July 2015, our goal would be to begin recruitment in October 2015. Using an escalating/de-escalating recruitment design, 6 participants would be recruited each month for a period of 8 months, 4 participants per month for 6 months, and then 2 participants per month for 8 months. The recruitment rate would then accelerate again to 4 participants per month for 6 months and then 6 participants per month for 8 months. This design allows us to conduct approximately 21 sessions per month or 5 participants per month on average and complete data collection by January of 2020.