# University of Cincinnati

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**I, Lindsay E Greiner B.S., hereby submit this original work as part of the requirements for the degree of Master of Science in Nutrition.**

It is entitled:

**Markers of Maternal Metabolism and Maternal Glucose Responsiveness Following Supplementation with Docosahexaenoic Acid**

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# Markers of Maternal Metabolism and Maternal Glucose Responsiveness Following Supplementation with Docosahexaenoic Acid

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**Master of Science**

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**Abstract**

Background: To examine the effects of 10 weeks of daily supplementation with algal oil (800 mg docosahexaenoic acid {DHA}) on maternal glucose metabolism during the third trimester in healthy, obese pregnant women.

Design: Randomized, double-blinded, placebo-controlled trial

Participants/Setting: Sixty healthy gravidas between the ages of 18-40 years who were English speaking with a singleton pregnancy, BMI $\geq$25-$\leq$60kg/m$^2$ and who had complete data for analysis were included in this sample.

Intervention: Women were supplemented beginning at the 26$^{th}$ week until the 35$^{th}$-37$^{th}$ week of their pregnancy.

Main Outcome Measures: Effect of docosahexaenoic acid supplementation on maternal insulin sensitivity.

Statistical Analysis Performed: Pearson or Spearman correlation coefficients were used to identify the strength and direction of the linear relationship between erythrocyte DHA and factors affecting maternal glucose metabolism (insulin, A1c, leptin, adiponectin, TNF-α, and IL-6) and the indices of insulin sensitivity (HOMA-IR, ISI {comp}). One-way analysis of variance (ANOVA) was used to compare the mean differences in the outcome variables between the two

groups. Statistical significance was set as a p value <0.05; trends (p> .05 and <.10) were also noted.

Results: The DHA group had higher erythrocyte DHA at the end of the study (p<.0001). No relationship was seen between glucose, insulin, A1c, leptin, adiponectin, IL-6 and erythrocyte DHA. There were no differences between groups in the indices of insulin sensitivity. There was a significant difference between mean values of TNF-α (p=.025) and the change in TNF-α (p=.03) between groups; 8.4% of the variance in TNF-α could be explained by erythrocyte DHA. There was no relationship between the change in factors that affect maternal glucose metabolism and the change in erythrocyte DHA after supplementation.

Conclusion: DHA supplementation decreased plasma TNF-a concentrations in the third trimester of pregnancy, but did not affect insulin sensitivity or other markers of glucose metabolism. Further research is needed to see if this improvement in TNF-a could reduce the risk of fetal overgrowth in healthy, obese, pregnant women.

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## Introduction

During pregnancy, insulin resistance is the natural physiological state. As gestation progresses to term, the mother becomes increasingly insulin resistant as a way to shuttle the most crucial substrate, glucose, to her developing fetus. The physiological state of increased adiposity is also associated with the presence of insulin resistance.[1] When an obese woman enters gestation, the already existing low-grade inflammation in her body is coupled with the physiological inflammation of pregnancy, resulting in the potential for serious and adverse outcomes for both mother and child.

Worldwide, obesity is increasing at an alarming weight and carries with it numerous adverse consequences for both mothers and their children. Data from the Pregnancy Risk Assessment Monitoring System (PRAMS) between 1993-2003 was used to identify trends in maternal obesity, defined as a body mass index (BMI) $\geq$29.0 kg/m$^2$ based upon pregnancy-specific BMI ranges set forth by the Institute of Medicine, for 66,221 live births occurring in 9 of the fifty states in the United States of America. According to this data, the prevalence of obesity rose among all races, education levels, parity, and ages; women who had live births in 2002-2003 had a 70% increased risk of pre-pregnancy obesity compared to their counterparts in 1993-1994.[2] Aside from the known health risks of obesity, the metabolic dysregulations that occur during an obese pregnancy are correlated with gestational diabetes, hyperinsulinemia, cesarean delivery, gestational hypertension, preeclampsia in the mother and macrosomia, stillbirth, and congenital anomalies in the infant.[3],[2] Therefore, it is critical to identify ways to prevent these adverse outcomes to improve the health of mothers and their infants.

Docosahexaenoic acid (DHA), an omega-3 fatty acid, has been shown to mediate insulin resistance in animal models and in obese, non-pregnant humans. It is hypothesized that omega-3

fatty acids improve insulin sensitivity by modifying the composition of skeletal muscle phospholipids.[4] Supplementing the diets of populations with characteristically low intakes of DHA could assist in improving the ratio of omega-3 fatty acids in the phospholipid membrane. Therefore, by maintaining an optimal amount of omega-3 fatty acids in the diet, membrane fluidity, and the number of insulin receptors significantly increase, thereby increasing insulin action.[4] When obese pregnant women are supplemented with DHA, the phospholipid membranes should change and result in a more favorable ratio.

## Literature Review

### Glucose Metabolism in Pregnant Women

Pregnancy is a state of physiological inflammation and both anabolism and catabolism. Throughout gestation, mothers experience increases in insulin resistance and blood concentrations of triglyceride, tumor necrosis factor- alpha (TNF-α), and leptin. During early pregnancy, increases occur in maternal insulin sensitivity and fat stores to support the maternal and fetal needs of later gestation.[5] In the third trimester, insulin sensitivity decreases which leads to subsequent increases in free fatty acids and maternal glucose which are transported to the fetus for the accumulation of fetal fat mass.[5]

Maternal glucose metabolism changes significantly throughout pregnancy due to the variation in fetal needs and maternal demands, with maternal glucose levels decreasing as gestation progresses.[5] Although the mechanism is poorly understood, it is speculated that the increases in plasma volume and substrate utilization play significant roles in the contribution to the fall in glucose levels.[5] As gestation progresses, hepatic glucose production begins to increase along with fasting insulin concentrations. Normally, hepatic glucose output is suppressed by the presence of insulin.[5] However, due to hormonal changes in pregnancy women exhibit maternal hepatic insulin resistance through which hepatic glucose production continues to rise even in the presence of insulin.[5] The purpose behind this phenomenon is to limit maternal glucose uptake in order to provide adequate substrates for the growing fetus, as it requires 80% of its available energy to come from glucose.[6]

As pregnancy progresses, skeletal muscle and adipose tissue become severely insulin resistant, leading to a significant increase in whole-body insulin resistance.[7] Growth hormones produced by the placenta are counterregulatory hormones to insulin; therefore, by shifting to a

more insulin-resistant environment, the transfer of energy substrates to the fetus are increased.[7] Similar to growth hormone, human placental lactogen (HPL) promotes insulin resistance in animals. In a human study, 12-hours of continuous infusions of HPL were given to 15 men and 8 women; glucose tolerance was impaired despite an increase in plasma insulin response.[8] Human placental growth hormone (hPGH) has been speculated to play a significant role in this response as well. Transgenic mice with an over-expression of the hPGH gene were used to evaluate the in vivo effects on insulin sensitivity and were compared to their wild-type littermates.[6] Transgenic mice exhibited fasting insulin levels that were 4 times higher than the control mice ($1.57 \pm 0.22$ ng/mL vs $0.38 \pm 0.07$ ng/mL; $p < .001$); after stimulation with glucose, fasting insulin levels were 7 times higher in the transgenic mice 30 minutes after administration ($4.17 \pm 0.54$ ng/mL vs $0.62 \pm 0.10$ ng/mL; $p < .0001$).[6] The transgenic mice also expressed marked reductions in insulin sensitivity, when upon being injected with insulin they exhibited an insignificant decrease in glucose while the control mice had a >65% decrease in the level of blood glucose ($P<.001$).[6] These data suggest that hPGH could potentially play a significant role in the pathogenesis of insulin resistance during pregnancy.

In the third trimester, energy metabolism shifts from the primary use of carbohydrates as a substrate to that of lipid oxidation to further shuttle glucose to the fetus. As this occurs, free fatty acid levels rise in the maternal circulation due to an increase in lipolytic gestational hormones in plasma.[9] Aside from other mediators of insulin resistance, elevated plasma levels of free fatty acids cause peripheral and hepatic insulin resistance in human and animal models. Free fatty acids affect glucose metabolism by inhibiting insulin-stimulated glucose transport within 2-4 hours due to the accumulation of diaclyglycerol which activates protein kinase C.[9] This enzyme decreases the phosphorylation of tyrosine in the insulin receptor substrate-1, which

inhibits insulin signaling.[9] Consequently, glucose cannot enter the cell and be utilized. In response to the concentration of glucose in the maternal circulation, the fetus produces its own insulin, resulting in the accumulation of fetal adipose tissue. However, it is important to note that in a normal, healthy pregnancy these mechanisms are crucial as the increasing insulin resistance shunts glucose to the fetus as previously discussed, who is accruing lean and fat mass in preparation for birth.

In fetal metabolism, glucose is the main energy source for development.[10] The placenta continuously transfers glucose to the fetus; therefore, fetal glucose levels are in a close equilibrium to those of the mother.[10] Glucose is transferred from the mother's circulation to the fetus using the GLUT 1 transporter; this is a dependent process as the fetus does not produce its own glucose.[11],[12] However, insulin is unable to cross from maternal circulation to the fetus due to the presence of the placental barrier. Therefore, the infant secretes its own insulin in response to maternal glucose levels.[13] The fetal synthesis of glycogen begins in the second trimester and slowly increases until 36 weeks of gestation.[10] All of the enzymes required for gluconeogenesis are present at 8 weeks of gestation, but this process is inhibited because of high circulating levels of insulin.[10] The presence of insulin also enhances the uptake of glucose in the adipose tissue and fatty acid synthesis in the liver, leading to the synthesis of triglycerides.[10] When glucose levels are high, the infant must produce excess insulin leading to an increased accretion of fat mass, leading to infants born with insulin resistance.

## Glucose Metabolism in Obese Pregnant Women

Obesity, defined as a BMI $\geq$30kg/m$^2$, is a state of low grade, chronic inflammation associated with numerous co-morbidities. Abdominal adiposity, an excess of visceral fat in the

abdomen is the most pathogenic, as it causes a decrease in the insulin signaling pathways resulting in insulin resistance.[1] Insulin resistance occurs when a lower than normal response occurs to a given dose of insulin, causing dysfunction of normal glucose metabolism.[14] As insulin resistance occurs as a natural phenomenon during pregnancy, it becomes critical to understand the mechanisms of insulin resistance during obesity as they are coupled together during an obese pregnancy to produce a pathogenic state.

In obesity, the excess visceral tissue causes adipocytes to release free fatty acids, inflammatory cytokines, resistin and plasminogen activator inhibitor-1.[1] The presence of free fatty acids is a double-edged sword, as they simultaneously enhance peripheral insulin resistance while decreasing peripheral glucose utilization, leading to inefficient glucose metabolism.[1] The inflammatory cytokines, TNF-α and IL-6, indicate an increased state of inflammation, while resistin induces insulin resistance.[1]

Adipose tissue also produces adipokines, such as leptin and adiponectin, which play key roles in insulin sensitivity.[1] In healthy individuals, adiponectin enhances the action of insulin, lowers the level of blood glucose, and reduces the amount of lipid that is stored in the muscle and liver.[1] Leptin not only increases satiety, but functions as a cytokine during inflammatory processes.[1] In the presence of obesity, leptin levels are increased while adiponectin levels are decreased, thereby further exacerbating insulin resistance.[1] As insulin resistance worsens, there is an increased need for more insulin to be secreted by the pancreas, which can lead to type 2 diabetes.[15] For obese women of childbearing age, the normal physiological inflammation of pregnancy includes changes in glucose metabolism, due to variations in the placental production of cytokines, and adipokines. However, when this effect is coupled with the presence of obesity these processes become dysregulated leading to increased inflammation, higher risks for

complications, disruptions in fetal metabolic programming, and lasting health consequences for both mother and child. This is in part due to that fact that the increase in maternal insulin resistance is paramount in obese women, who have shown to have even higher increases in maternal hepatic insulin resistance even when their pre-gravid blood glucose measurements were of normal tolerance.[5]

Women of child bearing are not an exception to the elevated and continuous trends of overweight and obesity continue throughout the United States. In the United States alone, the prevalence of obesity is high with prevalence of greater than 30% in most age and gender groups.[16] A BMI of $\geq 25$ signifies overweight, while $\geq 30$ is the cut-point for obesity. In the National Health and Examination Survey (NHANES 1999-2008) data, 59.5% (54.5 – 64.5, 95% CI) of women in the United States, between the ages of 20-39 years, would be classified as overweight or obese.[16] For obesity, the prevalence is 31% for Non-Hispanic White women, 38% for Hispanic women, 40% for Mexican- American women, and 47% for Non-Hispanic Black women .[16]

As obesity itself is a chronic state of low-grade inflammation, the presence of inflammation in the body only escalates when combined with the normal physiological inflammation of pregnancy. This results in an increased production of inflammatory cytokines by the adipose tissue and placenta, leading to changes in both the maternal and fetal environment.[17] Aside from the risks to the mother of being obese during gestation, it is thought that changes in maternal metabolism cause subsequent dysfunction in the metabolism of the infant due to the intrauterine exposure during gestation.[18] This dysregulation results in a variety of adverse side effects, as obesity in pregnancy is correlated with gestational diabetes, chronic hypertension, preeclampsia, stillbirth and congenital anomalies in the infant.[3]

Intrauterine exposure to high blood glucose has been shown to increase the risk for the development of obesity and diabetes later in life.[19] When compared to lean mothers, obese mothers have higher fasting insulin and glucose.[18] As glucose is transferred from the mother to the fetus through the GLUT 1 transporter during gestation, it is thought that high levels of glucose can cause disrupt infant metabolic programming. When examining the presence of insulin resistance at birth among infants and obese mothers at birth using the HOMA-IR model, both were shown to be insulin resistant.[18] Infants born to obese mothers have been shown to have increased fat mass, a greater percent body fat and higher levels of HOMA-IR, cord leptin and IL-6 than those infants born to normal weight women.[18] Also, cord blood insulin concentrations directly correlate with weight-for-gestational age; with larger infants having higher levels of fasting insulin in cord blood.[20] As insulin is an anabolic hormone, it follows that high levels of insulin would increase adiposity in the developing fetus. Infants who are born large-for-gestational age have an increased risk of insulin resistance and cardiovascular disease in adulthood.[21] Therefore, a mechanism to ameliorate impaired glucose metabolism in obese, pregnant women becomes crucial to the health of future generations.

Obese women with normal glucose tolerance tests performed during the third trimester were examined to determine changes in insulin sensitivity during and after pregnancy.[22] In the third trimester, the stimulation of peripheral glucose uptake due to physiological hyperinsulinemia was reduced by 40% ($P<0.05$) when compared to postpartum.[22] Negligible changes were seen between the second trimester when compared to the postpartum state, indicating an increase in insulin resistance occurring during the third trimester of pregnancy.[22]

An observational study (N= 23,316 pregnant women with a mean BMI of 27.7±5.1) examined the effects of hyperglycemia on adverse pregnancy outcomes. [23] The women

participating in the study were given a standard 75g oral glucose tolerance test between the 24th and 32nd week of pregnancy.[23] The data was unblinded if the woman's fasting plasma glucose level exceeded 105mg/dL, her 2-hour plasma glucose level was >200mg/dL, her random plasma glucose level was ≥160 mg/dL or if her glucose level was <45mg/dL as these were considered unsafe and in need of treatment.[23] Upon delivery, cord-blood was collected to examine levels of serum C peptide and plasma glucose. There was a strong and continuous association with maternal glucose levels below the value for diagnosis of gestational diabetes with cord blood serum C peptide and infant birth weight. Therefore, the effects of dysregulated insulin resistance during an obese pregnancy can lead to adverse outcomes for the mother and child.

Factors that Affect Glucose Metabolism in Pregnancy

There are many factors that affect glucose metabolism during pregnancy. Insulin, hemoglobin A1c (A1c), HOMA-IR and ISI (comp) can be used as markers of glucose metabolism as they provide information regarding glycemia, insulinemia, long-term blood glucose control, insulin resistance, and insulin sensitivity. As several other metabolic factors have been demonstrated to play significant roles in glucose metabolism during obesity and pregnancy, they must be examined as well. These factors, adiponectin, TNF- α, leptin and IL-6, have various functions in their influence on glucose metabolism and response. Adiponectin tends to be insulin sensitizing while TNF- α increases insulin resistance. Leptin levels have been shown to be dysfunctional in the presence of insulin resistance while IL-6 has been shown to be up-regulated in the presence of hyperglycemia. Therefore, it becomes necessary to critically examine these metabolic markers as they play noteworthy roles in glucose response.

## Glucose and Insulin

Glucagon and insulin are two primary hormones that regulate physiological glucose metabolism. Through the process of carbohydrate metabolism and the regulation of normal physiologic blood glucose control, glucagon and insulin continuously play interacting roles. While glucose can be taken up by the liver, brain and red blood cells independent of insulin, muscle and adipose tissue require insulin to be present for the transport of glucose into these cells by way of the GLUT transporters.[1]

Under normal physiological conditions, insulin serves as an anabolic hormone. During pregnancy, the role of insulin in anabolism is expanded, as it becomes crucial for adequate fetal development.[13] In pregnancy, the GLUT 1 transporter regulates the influx of glucose into the placenta and fetal tissues; as it serves to facilitate glucose uptake across blood-tissue barriers.[1] As the third trimester of pregnancy is crucial for the growth of the fetus, glycogen stores accumulate and fat is stored in the adipose tissue in preparation of birth.[12]

## Hemoglobin A1c

Hemoglobin A1c (A1c) is a measure of long-term glycemic control.[24] In the blood, glucose links to the terminal amino acid of the beta chains of hemoglobin A.[24] The amount of glucose that is bound to hemoglobin depends on the concentration of glucose in the blood over the past 3 months. This measure is useful as it gives a measure of blood glucose control over time. Under normal physiological conditions, the goal for A1c is <7%, which corresponds to an average blood glucose level of <170 mg/dL.

In pregnancy, A1c levels are typically decreased in early pregnancy due to the increase in insulin sensitivity that occurs at this time. When examining A1c levels among pregnant women

with a normal oral glucose tolerance test (OGTT), those with a BMI $\leq$25 kg/m$^2$ were shown to have an A1c level of 4.5-5.7% in early pregnancy and 4.4-5.6% during the 3rd trimester.[25] This phenomenon can be explained by the decrease in fasting glucose that occurs nataurally during gestation.

Elevated A1c levels during the 3rd trimester of pregnancy have been associated with numerous adverse outcomes for both mother and child. It is thought that suboptimal levels of blood glucose may perhaps predispose the fetus to hyperinsulinemia, fetal distress and hyperlactacidemia.[26] Preeclampsia risk has been shown to be higher among mothers with an A1c level greater than >8% when compared to mothers with levels <8% at or before the 20th week of gestation.[27] Macrosomia risk has been associated with a two to threefold increase when A1c levels are greater than 6.5% during the third trimester.[28] Third trimester A1c levels greater than 8% have been correlated with a significantly higher preterm delivery rate (<37th week of gestation).[29] An audit of pregnancies resulting in stillbirth illustrated that suboptimal glucose control, indicated by an A1c, >7.5% was present in 67% of cases.[26]

Adiponectin

Adiponectin, an inflammatory cytokine, is produced by adipose tissue and is part of a negative feedback mechanism in which higher levels of adipose tissue result in a decrease in the amount of adiponectin being produced.[30] While TNF-α, IL-6 and resistin are negative regulators of insulin action, adiponectin, in contrast is an insulin-sensitizing adipokine.[31] High levels of adiponectin serve to reduce the amount of lipid stored in muscle and liver tissue through enhancing the action of insulin and lowering circulating glucose levels.[1]

Adiponectin functions in metabolism to reduce hepatic glucose production by way of AMP-activated protein kinase, while also stimulating the uptake of glucose in skeletal muscle.[32] In the liver, adiponectin also plays a role in substrate cycling as it reduces the activity of two rate-limiting enzymes in gluconeogenesis - phosphoenolypyruvate carboxykinase and glucose-6 phosphatase.[32] Adiponectin is therefore thought to be insulin sensitizing and an inverse relationship has been illustrated between insulin resistance and this adipokine.[30]

During pregnancy, adiponectin levels have been shown to be negatively correlated with gestational age, therefore as insulin resistance increases throughout the course of pregnancy, adiponectin levels decrease.[30] Along the course of gestation, hypoadiponectinaemia occurs, with plasma adiponectin levels in the third trimester decreasing below pre-gravid levels. In pregnancy, this has been associated with a 2.5 decrease in white adipose tissue adiponectin mRNA, regardless of maternal adiposity, during the third trimester.[31]

Leptin

Leptin, a product the *ob* gene, plays a key role in the regulation of metabolism in terms of body weight, adipose tissue, appetite suppression and energy balance.[33] In animals, the amount of leptin synthesized by adipose tissue is proportionate to the amount of adipose tissue. In humans, the majority of circulating leptin is produced by white adipose tissue.[34] Leptin metabolism is dysfunctional in chronic diseases that are correlated with the presence of insulin resistance, such as type 2 diabetes and obesity.

As the placenta functions as an endocrine organ throughout the course of pregnancy producing hormones and encouraging fetal growth, it synthesizes and secretes leptin, leading to a transfer of the primary leptin production from white adipose tissue to the placental tissue.[33,34]

The placental unit has been shown to express significant quantities of mRNA for leptin during all stages of pregnancy.[34] During the first trimester of pregnancy, leptin levels soar to 30% higher than pre-gravid concentrations with levels stabilizing to their pre-gravid concentrations shortly following delivery.[34] It is suggested that the increase in leptin shown during pregnancy is due to the need for the transplacental transfer or lipids and the mobilization of maternal fat stores, as the final trimester of pregnancy is crucial for fetal fat accumulation.[34] Placental leptin receptors are found on the syncytiotrophoblast cells, the outermost layer of the placenta, thus causing them to be available to the maternal circulation, but not fetal circulation .[34] In pathogenic states of pregnancy, leptin production has been shown to be dysregulated with subsequent alterations in fetal growth.[34]

Tumor Necrosis Factor Alpha

Tumor necrosis factor alpha (TNF-α) is an inflammatory cytokine produced by monocytes and macrophages that plays a significant role in the pathophysiology of insulin resistance. The expression of TNF-α has been shown to be higher in the adipose tissue of obese animal models.[35] The physiological effect of TNF-α is broad, as it has documented roles in lipid metabolism, endothelial function, insulin resistance and coagulation.[35] During gestation, TNF- α is produced by both the placenta and adipose tissue.[36] It has been suggested that TNF-α plays a significant role in the development of insulin resistance throughout pregnancy.

During the normal mechanism of insulin action, insulin binds to the insulin receptor thereby increasing receptor tyrosine kinase activity of the β subunits, leading to the autophosphorylation of tyrosyl residues. This autophosphorylation results in the activation of β subunit tyrosine kinase and the phosphorylation of insulin receptor substrate-1.[1] However, this

mechanism is altered through the presence of high circulating levels of TNF- α. When circulating levels of TNF- α are high, they interfere with insulin receptor autophosphorylation, thereby inhibiting tyrosine kinase activity and interfering with the phosphorylation of insulin receptor substrate-1. [36,1, 14]

TNF- α binds to the glycoproteins p55 TNFR and p75 TNFR; it has been suggested through in vitro studies that sphingomyelinase and C6 ceramide, which are formed through the activation of p55 TNFR, inhibit insulin receptor substrate-1 tryosine phosphorylation and lead to conversion into inhibitors of tyrosine kinase.[14] This implies that these compounds not only have the ability to inhibit the insulin receptor and the phosphorylation of insulin receptor substrate-1, but also to translate insulin receptor substrate-1 into an insulin receptor inhibitor.[14]

TNF- α has been shown to be a predictor of insulin sensitivity; among women with normal glucose tolerance but also women with gestational diabetes mellitus.[36] It has been suggested that the increase in levels of TNF- α in late gestation, cause the increase in insulin resistance demonstrated in pregnancy through the mitigation of insulin signaling.[36]

Interleukin-6

IL-6 is a proinflammatory cytokine that is secreted from a variety of cells such as phagocytes, B cells, osteoblasts, T cells and endothelial cells; IL- 6 is also synthesized by adipose tissue.[37,38] Aside from its role in immune function and inflammation, IL-6 also affects the nervous system, endocrine system, hematopoiesis and bone metabolism.[38] This cytokine has a variety of physiologic functions, for example it mediates the acute phase response and enhances B cell production and the delineation to plasma cells.[37] Apart from these functions, IL-6 plays significant roles in cell survival and the phenomenon of apoptosis.[38]

In the immune response, IL-6 interacts with T-cells and B-cells. The interaction of IL-6 with B cells leads to an increase in immunoglobin production resulting in higher levels of immunoglobin A (IgA), immunoglobin G (IgG) and immunoglobin M (IgM).[38] IL-6 interacts with T-cells to support the proliferation and survival of both the type-1 helper T cell (Th 1) and type-2 helper T cell (Th 2) responses by preventing T-cell apoptosis.[38]. During the acute phase response, IL-6 upregulates the expression of acute phase respondent mRNA by the hepatocytes; through this mechanism pathogens are further prevented from invasion and recovery is promoted.[38] Recently, aside from the pro-inflammatory aspects, IL-6 has been shown to exhibit not only anti-inflammatory effects as well depending upon the environment, although this area needs further research.[38]

In pregnancy, IL-6 is released into both maternal and fetal circulation and is found in both the trophoblast and endothelial cells.[17] Therefore, IL-6 has the ability to express endocrine function at other sites throughout the body.[17] Upregulation of IL-6 has been illustrated in patients with gestational diabetes mellitus. Compared to non-pregnant women and pregnant women with normal blood glucose control, mothers with gestational diabetes mellitus have been shown to have significantly higher levels of IL-6.[39]

Indices of Insulin Sensitivity

In the body, glucose homeostasis is maintained by the β-cells of the pancreas, the peripheral tissues and the liver. As obese pregnant women have higher levels of insulin resistance, it is crucial to examine maternal insulin sensitivity using methods that are reliable and valid in pregnant women. The euglycemic insulin clamp is the gold standard for measuring whole-body insulin sensitivity.[40] The clamp method can also be combined with a glucose tracer

to quantify the differences in the liver and peripheral tissues to that of whole-body insulin sensitivity. However, when the use of the euglycemic insulin clamp and tracer method are not possible, there are indices that can be used to assess changes in β cell function and whole-body insulin sensitivity.

## Homeostasis Model Assessment

Homeostasis Model Assessment (HOMA-IR), the homeostasis model assessment, is a method for measuring insulin resistance.[41] HOMA-IR uses basal plasma insulin and glucose concentrations to determine the degree of insulin resistance and deficient β islet cell function.[42],[43] It is calculated by the following equation assuming mg/dL units: (fasting glucose x fasting insulin)/405; as it uses fasting measurements it primarily gives information about hepatic insulin resistance. In 1985, when HOMA-IR was first studied, patients with diabetes where shown to have a HOMA-IR of 2.89 while healthy patients and a HOMA-IR of 1.21.[42] A recent study suggested that HOMA-IR is a reliable way to measure insulin resistance in patients with type 2 diabetes who are being seen for follow up when compared to a euglycemic clamp.[41]

As HOMA-IR can be used to estimate insulin resistance in healthy patients and those with type 2 diabetes, it was questioned if it could be validated for use in pregnant women as they exhibit insulin resistance. Catalano et al. examined the use of the HOMA-IR in early and late pregnancy and compared this method to a 2-hour euglycemic-hyperinsulinemic clamp, using women with normal glucose tolerance and gestational diabetes mellitus.[40] Significant correlations were found between the HOMA-IR and the clamp method with an $r^2$=+0.52 (P=.002) in early pregnancy and $r^2$=+0.61 (P=.0003) in late pregnancy.[40] In 2006, Cohen et al. validated the use of the HOMA-IR in healthy obese pregnant women with normal glucose

tolerance during the second and third trimesters of pregnancy.[43] Significant correlations were found between the rate of glucose disappearance and the HOMA-IR metabolic parameters of insulin sensitivity ($R^2$=.43, P=0.003), indicating that HOMA accurate estimates insulin resistance in the second and third trimester of an obese pregnancy.[43]

Matsuda Composite Index for Measurement of Insulin Sensitivity

The Composite Index for Measurement of Insulin Sensitivity (ISI {comp}) is a method developed by Matsuda and Defronzo, through which whole-body insulin sensitivity is estimated using available data from an OGTT using the area under the curve.[44] The ISI (comp) gives a composite index of hepatic and peripheral tissue sensitivity to insulin after consumption of a 75g glucose load.[44] The equation for this calculation is as follows: (10,000/√(fasting glucose x fasting insulin) x (geometric mean glucose x geometric mean insulin during OGTT). The geometric mean glucose is calculated as $g_m$= (15 x $g_0$+ 30 x $g_{30}$ + 30 x $g_{60}$ + 30 x $g_{90}$ + 15 x $g_{120}$)/120.[45] The geometric mean insulin is calculated as $i_m$= (15 x $i_0$+ 30 x $i_{30}$ + 30 x $i_{60}$ + 30 x $i_{90}$ + 15 x $i_{120}$)/120.[45] This method was highly correlated to the euglycemic insulin clamp r=+0.73, P<0.0001.[44]

Catalano et al. examined the use of the ISI (comp) in pregnant women, both with normal glucose tolerance and gestational diabetes mellitus, and compared this method to a 2-hour euglycemic-hyperinsulinemic clamp.[40] Significant correlations were found between the ISI (comp) and euglycemic clamp method with an $r^2$=+0.74, P<.0001, indicating that this method is a valid indicator of maternal insulin sensitivity.[40] When compared to the HOMA and QUICKI methods, which use fasting glucose and insulin, the ISI (comp) gave a significantly improved correlation to the hyperinsulinemic-euglycemic clamp.[40]

Matsuda Reduced Time Point Composite Index for Measurement of Insulin Sensitivity

The original Matsuda Composite Index uses all available time points during the OGTT. However, a simplified equation was published in 2010 that uses baseline time points and those at 120 minutes to determine whole-body insulin sensitivity.[46] The calculation for this index is as follows: 10,000/√(fasting glucose x fasting insulin x glucose 120 minutes x insulin 120 minutes). To validate the index, authors used data from the original publication and compared the new equation to the rate of both insulin-stimulated glucose disposal and hepatic insulin sensitivity during the euglycemic insulin clamp; the shortened ISI was significantly correlated to the rate of glucose disposal (r=0.772) and hepatic insulin sensitivity (r=0.651).[46]

Docosahexaenoic Acid as a Mediator of Insulin Resistance in Obese Pregnancy

Docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and α-linolenic acid (ALA) are polyunsaturated fatty acids that are commonly referred to as the omega-3 fatty acids. Omega-3 fatty acids are similar in their synthesis to the omega-6 fatty acids, as both pathways compete for the same desaturase enzyme. This desaturase enzyme is used in the conversion of the omega-3 fatty acid ALA to EPA or DHA. Omega-3 fatty acids are predominantly metabolized to the anti-inflammatory mediators PGE3 and LTB5.[47] They have the unique ability to displace omega-6 fatty acid stores in the body, thereby decreasing the production of inflammatory mediators[47] It is hypothesized that omega-3 fatty acids influence inflammation through the alteration of membrane lipids, metabolism into eicosanoid inflammatory mediators, inhibition of nuclear receptor activation and metabolism into resolvins and protectins.[48] Omega-3 fatty acids are thought to improve insulin sensitivity by changing the composition of skeletal muscle phospholipids.[4] Various studies have linked the fatty acid composition of skeletal muscle

to peripheral insulin sensitivity. By increasing the amount of omega-3 fatty acids in the diet, there is a significant increase in membrane fluidity, the number of insulin receptors and insulin action.[4]

When mothers are supplemented with DHA during pregnancy, the proportion of omega-6 fatty acids to omega-3 fatty acids in phospholipid membranes changes; thus resulting in a more favorable ratio. As previously discussed, obese women who are insulin resistant during pregnancy give birth to insulin-resistant infants. It has been demonstrated that mothers supplemented with DHA had lower insulin cord blood concentrations, suggesting improved insulin sensitivity in the infant.[13] Limited amounts of studies have been done examining the effects of DHA on insulin resistance during pregnancy, although there is evidence illustrating this response in the non-pregnant state.

Mouse and rat model studies have examined the effects of DHA, EPA and ALA on insulin sensitivity, although none of these experiments took place in pregnant animals. These studies provide evidence to support the ability of omega-3 fatty acids to mediate insulin resistance.

A randomized trial of normoinsulinemic rats examined the effects of supplementation with ALA, EPA, DHA or a mixture of EPA and DHA on insulin sensitivity over the course of 8 weeks in forty animals. After randomization, each group received standard chow supplemented with either ALA methylester, EPA methylester, DHA methylester or a mixture of DHA and EPA methylester, in the dose of 0.5g per kilogram body weight.[49] HOMA-IR was used as an approximation of insulin resistance. Upon analysis, both fasting plasma glucose and insulin levels had significantly decreased in the EPA, DHA and EPA/DHA groups when compared to the ALA group ($p<0.05$).[49] Insulin sensitivity was notably improved, as evidenced by a

significant decrease in HOMA-IR among the EPA, DHA and EPA/DHA groups when compared to the ALA group ($P<0.01$).[49] Significant differences among glucose, insulin and HOMA-IR were not seen between the DHA, EPA or DHA and EPA groups.[49] This particular study gives evidence to support DHA, EPA or a combination of DHA and EPA as the ideal omega-3 fatty acids to mediate insulin resistance.

The effects of EPA on diet-induced obesity and glucose homeostasis were examined using 4 groups of 9-10 mice who were fed varying diets throughout the course of 11 weeks.[50] The diets included low-fat, high-fat and high-saturated fat EPA; the final group was fed a high-fat diet for 6 weeks and then fed the high-saturated fat EPA diet. Upon analysis, the high-fat group developed glucose intolerance and gained more weight than any of the other groups. The high-fat groups also showed a higher area under the curve (AUC) at 6 weeks and 10 weeks when compared to the low-fat groups.[50] The high-saturated fat EPA group maintained their glucose tolerance, with an AUC similar to that of the low-fat group. It is important to note that while AUC was similar between the high-saturated fat EPA group and low-fat group, the EPA group had considerably higher body weights and fat pad weights when compared to the low-fat group.[50] This effect was not seen with group that received both the high-fat and EPA diet. However, when examining HOMA-IR, insulinemia and glycemia the group receiving both the high-fat and EPA diet showed similar results to the EPA diet alone and low fat groups.[50] This infers that EPA helped to recover the insulin sensitivity that was lost following the feeding of the high-fat diet. Aside from this, adiponectin levels were also found to be lower in the high-fat group but comparable in the low-fat and high-fat EPA groups.[50] When 3T3-L1 cells were treated with arachidonic acid (AA) and EPA, cells treated with AA secreted less adiponectin than those treated with EPA.[50] When cells were co-treated with EPA the reduction in adiponectin synthesis

did not occur.[50] This suggests that adiponectin serves as a factor in the mediation of insulin resistance.

A quasi-experimental study in humans examined twenty-one obese patients to determine if a diet restriction would result in changes to their muscle membrane phospholipid fatty acid composition.[51] Participants were required to follow a hypocaloric, low-fat diet (total fat 30% of calories; 10% monounsaturated FA, 10% saturated FA and 10% PUFA).[51] Subjects were encouraged to replace fatty fish with lean fish; thereby decreasing their consumption of n-3 PUFA's from fish in an effort to determine if an enhancement in elongation and desaturation of other n-3 dietary sources was occurring.[51] Upon analysis, n-3 PUFA's increased by 51% ($P=0.0001$) and DHA by 75%($P<0.0001$). These changes in n-3 PUFA's correlated significantly with changes in HOMA-IR ($R= -0.57$, $P<0.01$).[51] This indicates that when more long-chain n-3 PUFA's are incorporated into the membrane phospholipid there is a significant impact on insulin action.

While no study to date has examined the effects of supplementation with DHA on insulin sensitivity during pregnancy, DHA is needed in pregnancy for optimal fetal development. Limited studies have shown health benefits for both mother and child associated with the dietary consumption or supplementation of omega-3 fatty acids during pregnancy.[52] For infants, relationships between maternal omega-3 fatty acid consumption during pregnancy and improved neurodevelopmental outcomes, higher IQ's and reduced preterm births due to longer length of gestation have been reported.[52] Epidemiological studies have also shown that mothers consuming high amounts of omega-3 fatty acids have a decreased risk of developing postpartum depression.[52] As omega-3 fatty acids have crucial physiological functions such as oxygen transport, roles in cell membrane function and regulation of cell proliferation and inflammation,

they play significant roles in fetal development during the third trimester.[52] DHA has been shown to be safe for consumption during pregnancy and leads to healthier babies as it is crucial for growth and development.

While there is currently not a Dietary Reference Intake (DRI) for consumption of the omega-3 fatty acids during pregnancy, the Institute of Medicine states that women who are pregnant may benefit from consuming seafood high in DHA/EPA and recommends consumption of between 6 and 12 ounces per week.[53] The United States Food and Drug Administration and Environmental Protection agency states that pregnant women can safely consume up to 12 oz of fish and shellfish that are lower in methylmercury.[54] It has been recommended by various organizations that pregnant and lactating women consume 200-300mg per day of DHA and EPA which equates to 12oz fish per week, but the majority of women in the United States fail to meet this goal.[55-57]

An observational study of 2,109 women enrolled in Project Viva examined the associations between marine n-3 PUFA consumption during pregnancy and infant birth outcomes.[58] Diet was assessed using a semi-quantitative food frequency questionnaire validated for the Nurses' Health Study that had been modified for use in pregnancy. Upon analysis, birth weight was 94g lower (95%CI, 23-166) lower in mothers who were in the highest quartile of n-3 PUFA intake during their first trimester of pregnancy.[58] Infants whose mothers consumed high amounts of n-3 PUFAs during the first trimester of pregnancy also had a lower birth weight for gestational age z value of 0.19 units (95% CI, 0.08-0.31).[58] No differences were seen between consumption levels in relation to length of gestation or preterm birth.[58] A limitation of this study is that EPA/DHA intake was unable to be controlled and the particular food frequency questionnaire used was not validated for use in pregnancy. Also, using weight and birth weight

for length may not be the most accurate measure to assess adiposity in newborn infants. More recent studies have suggested supplementation with DHA during pregnancy leads to infants with lower adiposity, so it is possible this was a factor in these outcomes.

A recent cross-sectional study examined the consumption of DHA and EPA among pregnant and lactating low-income women in Michigan. Sixty-eight women were enrolled using a convenience sample of the Maternal and Infant Health Program administered by the Kent County Health Department. DHA and EPA intake was assessed using the N-3 Fatty Acid Food Frequency Questionnaire component of the Diet Habit Survey.[56] The mean consumption of DHA+EPA was found to be 1.18g + 0.41 over the course of one month among all ethnicities, with no woman meeting the recommended intake of 300mg per day.[56] It can be concluded from this study that low-income women are less likely to be consuming sufficient amounts of DHA+EPA during pregnancy, which could have negative consequences on the growth and development of their infant.

In the United Kingdom, a randomized single-blinded study examined the effects of consumption of ≥300 grams of salmon per week on maternal and umbilical n-3 fatty acid content in pregnant women.[59] One hundred and seven women completed the study, which began in 20$^{th}$ week of gestation and lasted until delivery. Women in the intervention group were given salmon fillets containing 0.57g EPA and 1.16 g DHA per serving. Upon analysis, the control groups percentages of DHA in maternal plasma phosphatidylcholine decreased throughout pregnancy ($P=0.008$) while they increased among intervention participants ($P<.0001$).[59] Maternal plasma phosphatidylcholine DHA percentages were also found to be higher at both weeks 34 and 38 ($P<0.001$) in the intervention group. Higher levels of umbilical cord plasma phosphatidylcholine DHA were also seen in the intervention group; this is indicative of enhanced transfer of DHA

and EPA to the developing fetus (mean 7.4±0.2, P<0.001).[59] Gestation length was reported to be an average of 5 days longer in the intervention group, although this did not reach statistical significance. This study demonstrates that women are able to increase both their own DHA status and that of their infant by consuming 300g of salmon per week during pregnancy.

Several studies have investigated the relationship between supplementation with DHA and the resulting effects on duration of gestation and weight at birth. A randomized double-blinded controlled clinical trial used a population-based sample to explore the effects of supplementation with DHA enhanced eggs in 291 women during the 3rd trimester of pregnancy.[60] The supplemented eggs supplied in the study contained 133mg of DHA, while the control eggs contained 33mg of DHA per egg. Gestational age was determined by ultrasound at 15-20 weeks, this number was used to calculate the expected delivery date using Naegele's rule.[60] Upon analysis, participants in the DHA group were found to have a mean gestational length increase of 6.0±2.3 (P=.009) days after controlling for the confounding variables of maternal BMI and number of prior pregnancies.[60] While birth weight (P=.184), length (P=.061) and head circumference increased (P=.081) none of these levels reached statistical significance although some were indicative of trends. A statistically significant relationship was also seen in the DHA group between infant DHA and gestation (r=0.227, P=.022); among all infants each additional percent of DHA in RBC cord blood phospholipid was shown to be correlated with an increase in gestation of 2 days (P=.011).[60] Mothers experiencing one or more adverse events during pregnancy was significantly higher in the traditional egg group than the supplemented group, 38% versus 25% respectively (P=.01).[60] It is thought that these differences in gestational age could be related to the synthesis of prostagladins that are synthesized for labor and delivery, as they are derived from AA, it is hypothesized that DHA may be displacing AA in the cell

membrane.[60] A second randomized double-blinded placebo controlled study of 1,094 pregnant women examined the effects of supplementation with DHA on gestational age and birth size. Women in the intervention group received 400mg/day of algal DHA from 18-22 weeks until delivery. Upon analysis, no differences were seen in gestational age, weight, length, or head circumference. However, differences were seen in primigravid women receiving DHA as their infants were heavier (99.4g, 95% CI 5.5-193.4) and had larger head circumferences (0.5cm, 95% CI .1-.9).[61] It is imperative to note that previous studies have controlled for previous number of pregnancies, as this can be a confounding variable in relation to infant birth variables. As this study did not mention controlling for previous pregnancies, this could be a significant limitation. DHA concentrations in maternal plasma and cord blood were found to be significantly higher in the intervention group ($p<.05$).[61] The DHA group also showed lower ratio of omega-6 to omega-3 fatty acids when compared to the control ($p<.05$) which suggests the possible displacement of omega-6's with omega-3 fatty acids.[61]

Therefore, it is possible that supplementation with DHA during pregnancy could improve the n-3 PUFA content in the phospholipid membrane, thereby improving insulin sensitivity in obese pregnant women. As an association between an increase in omega-3 fatty acids in the membrane phospholipid and insulin sensitivity has been seen in animal models and humans, it is plausible this same result would be seen in the pregnant woman.

**Purpose**

The purpose of this sub-study was to investigate the relationship between erythrocyte DHA and markers of maternal glucose metabolism at the $35^{th}$-$37^{th}$ week of gestation in healthy, overweight/obese pregnant women after 10 weeks of supplementation with 800 mg purified algae docosahexaenoic acid or 530 mg corn/soybean oil blend.

**Null Hypotheses ($H_0$)**

1. In overweight and obese women, there is no relationship between the absolute factors of maternal glucose metabolism and erythrocyte docosahexaenoic acid after supplementation with 800 mg purified algae docosahexaenoic acid in the $3^{rd}$ trimester of pregnancy.
2. In overweight and obese pregnant women, there is no relationship between the indices used to assess insulin sensitivity/insulin resistance and absolute erythrocyte docosahexaenoic acid in the $3^{rd}$ trimester of pregnancy.
3. In overweight and obese pregnant women, there is no relationship between the change in markers of maternal glucose metabolism and change in erythrocyte docosahexaenoic acid in the $3^{rd}$ trimester of pregnancy.

**Methods**

This study is a sub-study of the randomized placebo controlled trial "DHA, Inflammation, and Insulin Sensitivity in Obese Pregnant Women," (5R21HL093532-02) which was granted to the Debra Krummel, PhD, RD who is the principal investigator. The Omega-3 Pregnancy Study was a double-blinded, randomized placebo-controlled trial focusing on the

effects of 10 weeks of supplementation with DHA on metabolism and inflammation in overweight and obese pregnant women. The study protocol was approved by both the University of Cincinnati Institutional Review Board and the Cincinnati Children's Hospital Medical Center Institutional Review Board.

Subjects

Subjects used in this sub-study were part of the larger Omega-3 Pregnancy study. Healthy gravidas between the ages of 18-40 years who were English speaking with a singleton pregnancy, BMI $\geq$25-<60kg/m$^2$ and who had complete data were included for analysis in this sample.

Study Visits

All study visits occurred at the Clinical Translational Research Center (CTRC) at Cincinnati Children's Hospital Medical Center (CCHMC). The study visits began at the initiation of the third trimester; the first during the 26$^{th}$ week of pregnancy, the second during the 29$^{th}$-32$^{nd}$ week and the third during the 35$^{th}$-37$^{th}$ week. Analyses of the blood samples were then performed at the CTRC core laboratory, the CCHMC clinical lab, and the Genome Research Institute.

Before the initiation of the first study visit, participants received an informed consent and Health Insurance Portability and Accountability Act (HIPAA) form from IRB approved study personnel. After signing and dating both forms, participants were then enrolled in the study. The nursing staff completed height, weight and a 10mL blood sample for analysis of fasting insulin, fasting glucose, leptin, adiponectin, A1c, and erythrocyte DHA. Participants then

received a 45-day supply of their supplement, DHA or placebo, a supplement tracking tool and a pill dispenser.

At the second study visit, a non-fasting 10 mL blood sample was drawn by the CTRC nursing staff for analysis of erythrocyte DHA to monitor supplement adherence. Participants also returned their original supplement tracking tool. Following this, they received a 45-day supply of their supplement and a second supplement tracking tool.

At the third study visit, a 10 mL fasting blood sample was drawn by the CTRC nursing staff for analysis of fasting insulin, fasting glucose, leptin, adiponectin, A1c and erythrocyte DHA. A meal glucose tolerance test was then performed using a meal that consisted of 500 kilocalories and 75 grams of carbohydrate. Venous blood samples were drawn by the CTRC nursing staff at baseline, 30, 60, 90 and 120 minutes after ingestion of the meal. Glucose measurements were obtained using a bedside glucometer while insulin was determined through venous blood samples.

## Laboratory Methods

### Gas Chromatography

The University of Cincinnati Metabolic Diseases Institute measured erythrocyte DHA content. Whole blood was collected at 3 time points for each study subject. Venous whole blood, 2 ml, was collected into an EDTA-coated vacutainer tube and then placed on ice. After being centrifuged for 20 min (3000 x g @4), the plasma was removed and erythrocytes were washed three times in NaCl. Fatty acid compositions were determined using methylation and saponification methods. Capillary gas chromatograph Shimadzu-GC201 and helium carrier gas

were used to perform this analysis. The identification of fatty acids was performed using the retention times of authenticated fatty acid standards.

Radioimmunoassay

Plasma insulin and leptin levels were obtained by the use of radioimmunoassay (Linco Research, City, State). For this assessment, a labeled tracer antigen is incubated with a constant dilution of the antiserum. Then, the unlabeled antigen is added to the system, where the unlabeled antigen competes with the labeled tracer antigen for binding sites on the antibody. As the concentration of the unlabeled antigen increases, the amount of tracer that is bound to the antibody decreases. Next, the amount of tracer antigen and bound antibody is calculated. To determine the amount of unlabeled antigen in the unknown samples, a standard curve is created. The leptin assay uses $^{125}$I-labeled human leptin and a human leptin antiserum to determine the level of leptin in the serum. This analysis was conducted in the Clinical Laboratory at CCHMC.

ELISA

Plasma samples using enzyme-linked immunosorbent assays (ELISA) were used to measure adiponectin. According to the manufacturer's protocol, Milliplex$^{TM}$ Multiplex kits (Millipore, Billerica, MA) were used for these assays. Using a 96-well plate, 40 samples were run in duplicate and the plates were then read using luminex technology on the Bio-Plex$^{TM}$ (Bio-Rad, Hercules, CA). Standard curves using recombinant proteins were used to calculate the concentrations. This cytokine analysis was conducted by the Cytokine and Mediator Measurement Core Laboratory run at CCHMC.

## Blood Glucose

Fasting blood glucose was determined at the time of the blood draw during the study visits by the nursing staff at the CTRC of CCHMC. A bedside glucometer was used to analyze blood from venous puncture.

## Hemoglobin A1c

Hemoglobin A1c was measured using a modification of a high-performance-liquid-chromatography (HPLC). In the Hemoglobinopathy Lab, an Alliance 2690/2695 HPLC (Waters Corporation) and a PolyCAT A (PolyLC, Inc.) column were used to separate the hemoglobin fractions by cation-exchange chromatography. A1c was then quantified using a dual wavelength detector (model 2487, Waters Corp.) and Empower Software (Water Corp.). Results were reported as percent (normal range 3.5-6.3%). The preferred specimen is taken through venipuncture or fingerstick; a minimum of 500 μl of EDTA whole blood is collected. The sample was then refrigerated (2-8ºC) after collection. The coefficient of variation based on the normal control <3.2% and <1.3% for the elevated control. The HPLC method is sensitive to 0.05%. This analysis was conducted in the Clinical Laboratory at Cincinnati Children's Hospital.

## Insulin Resistance

Insulin resistance was assessed using the HOMA-IR, ISI (comp), ISI comp 2010 and 120 minute glucose and insulin.

HOMA-IR was calculated using the following equation was employed, (glucose x insulin)/405.

ISI (comp) was calculated using the following equation: ($\kappa/\sqrt{}$(fasting glucose x fasting insulin) x (mean glucose x mean insulin during OGTT), where $\kappa = 10{,}000$.[44] Mean glucose was calculated as $g_m = (15 \times g_0 + 30 \times g_{30} + 30 \times g_{60} + 30 \times g_{90} + 15 \times g_{120})/120$. Mean insulin was calculated as $i_m = (15 \times i_0 + 30 \times i_{30} + 30 \times i_{60} + 30 \times i_{90} + 15 \times i_{120})/120$.

ISI (comp) was calculated using the following equation: $\kappa/\sqrt{}\ (g_0 \times i_0 \times g_{120} \times i_{120})$.[46] All versions of the ISI composite assume glucose in mg/dL units and insulin in μU/ml units.[45]

**Statistical Analysis**

For this analysis, statistical significance was set as a p value of $<0.05$. The relationship between supplementation with 800 mg purified algal docosahexaenoic acid in the 3rd trimester of pregnancy and the factors affecting maternal glucose response were examined. The factors used to assess glucose response were examined in relation to erythrocyte docosahexaenoic acid. These factors were as follows: glucose, insulin, A1c, HOMA-IR, ISOGTT, adiponectin, leptin, IL-6 and TNF-α.

There were no significant differences between groups in erythrocyte DHA at baseline. The distributions of the absolute variables were tested for skewness and kurtosis. A1c, HOMA-IR, ISOGTT, adiponectin and IL-6 were normally distributed. Glucose, insulin, and leptin required log transformation in order to approximate a normal distribution. As TNF-α was skewed it was analyzed using Spearman's nonparametric correlation. In ANOVA, absolute TNF-α was log transformed to assume a normal distribution. Change variables were also assessed for these factors and were defined as the absolute measurement at SV3 minus the measurement at SV1; no log transformation was necessary.

Pearson's correlations and Spearman's nonparametric correlations were used to identify the strength and direction of the linear relationship between absolute red blood cell DHA and the factors affecting maternal glucose response. Pearson's partial correlation was used to identify the strength and direction of the linear relationship after controlling for pre-pregnancy BMI. One-way analysis of variance (ANOVA) was used to compare the mean in outcome variables by group.

Intention to treat analyzes participants in the groups they were originally randomized to when the study began.[62] Using intention to treat analysis allows compensation for any missing data that occurs throughout the course of the study.[62] For this particular analysis, intention to treat was only used for participants who completed the study that were missing DHA data from the third study visit, as the change in erythrocyte DHA is negligible between the second and third study visit, erythrocyte DHA data available from the second study visit was carried forward.

Data analysis was performed though the use of the statistical software Statistical Package for the Social Sciences (version 18.0, 2010, SPSS, Inc, Chicago. IL).

**Results**

The demographics of the women included in the sample are reported in Table 1 and 2. Pre-pregnancy BMI was calculated using self-reported height and pre-pregnancy weight. Age, race, parity, education level and marital status were assessed using self-reported data. Table 3 shows the mean values of factors related to maternal glucose metabolism by treatment group. The mean values for maternal adipokines are reported in table 4. Table 5 reports the maternal indices of insulin sensitivity by treatment group. Table 6 illustrates the change in the factors related to maternal metabolism; while table 7 reports the change values for maternal adipokines. Table 8 reports the differences in erythrocyte DHA and change in erythrocyte DHA by treatment group.

Table 1. Demographic Characteristics

| Variable | N | Mean ±SD |
|---|---|---|
| Age | 60 | 27.17±4.8 |
| Education level (years) | 43 | 14.802±2.8 |
| Parity | 44 | 1.48±1.5 |
| Pre-pregnancy BMI | 60 | 34.55±8.1 |

Table 2. Demographic Characteristics of Mothers Included in Sample

| Variable | N | % of Sample |
|---|---|---|
| Race and Ethnicity | | |
| Black, not Hispanic | 27 | 45.0 |
| White, not Hispanic | 28 | 46.7 |
| Chicano, Latino, Hispanic | 1 | 1.7 |
| Native American, Native Alaskan, Indian | 1 | 1.7 |
| Asian or Pacific Islander | 1 | 1.7 |
| Other, describe | 2 | 3.3 |
| | | |
| Education | | |
| Some high school | 4 | 9.1 |
| High school diploma, GED, or equivalent | 4 | 9.1 |
| Some college credit | 18 | 81.8 |
| College degree | 15 | 34.1 |
| Post-college coursework | 3 | 6.8 |
| | | |
| Marital Status | | |
| Single, never married | 17 | 37 |
| Married, not single | 25 | 54.3 |
| Living together | 4 | 8.7 |

Table 3. Markers of Maternal Metabolism at 35th-37th Week of Gestation

| Variable | | DHA | Placebo | Significance |
|---|---|---|---|---|
| Glucose (mg/dL) | Mean | 86.34 (N=32) | 87.39 (N=28) | 0.681 |
| | SD | 10.78 | 8.60 | |
| Insulin (uU/mL) | Mean | 30.39 (N=32) | 25.64 (N=28) | 0.605 |
| | SD | 17.50 | 8.67 | |
| A1c (%) | Mean | 4.78 (N=31) | 4.91 (N=28) | 0.343 |
| | SD | 0.49 | 0.52 | |

Table 4. Maternal Adipokines at 35th-37th Week of Gestation

| Variable | | DHA | Placebo | Significance |
|---|---|---|---|---|
| Adiponectin (ng/mL) | Mean | 3778.58 (N=32) | 4012.9965 (N=28) | 0.40 |
| | SD | 1917.58 | 1943.24 | |
| Leptin (ng/mL) | Mean | 34.90 (N=32) | 44.58 (N=28) | 0.212 |
| | SD | 18.14 | 31.20 | |
| TNF-α (pg/mL) | Mean | 4.96 (N=32) | 6.52 (N=28) | 0.025 |
| | SD | 3.016 | 2.75 | |
| IL-6 (pg/mL) | Mean | 8.02 (N=32) | 9.56 (N=28) | 0.446 |
| | SD | 5.14 | 9.90 | |

Table 5. Maternal Indices of Insulin Sensitivity at 35-37th Week of Gestation

| Variable | | DHA | Placebo | Significance |
|---|---|---|---|---|
| HOMA-IR | Mean | 6.746 (N=32) | 5.57 (N=28) | 0.205 |
| | SD | 4.44 | 2.08 | |
| ISI Composite 99 | Mean | 1.61 (N=32) | 1.66 (N=28) | 0.772 |
| | SD | 0.82 | 0.55 | |
| ISI Composite 2010 | Mean | 1.93 (N=32) | 2.08 (N=28) | 0.584 |
| | SD | 1.14 | 0.89 | |

Table 6. Change in Markers of Maternal Metabolism between 26th and 35-37th Week of Gestation

| | | DHA Total Δ | Placebo Total Δ | P value |
|---|---|---|---|---|
| Glucose (mg/dL) | Mean | 1.09 (N=32) | 3.07 (N=28) | 0.390 |
| | SD | 8.24 | 9.44 | |
| Insulin (uU/mL) | Mean | 4.89 (N=32) | 2.92 (N=28) | 0.488 |
| | SD | 13.51 | 6.81 | |
| A1c (%) | Mean | 0.31 (N=31) | 0.39 (N=27) | 0.436 |
| | SD | 0.36 | 0.42 | |

Table 7. Change Values for Markers of Maternal Adipokines between 26th and 35-37th Week of Gestation

| | | DHA Total Δ | Placebo Total Δ | P value |
|---|---|---|---|---|
| Adiponectin **Δ** (ng/mL) | Mean | -680.09 (N=32) | -919.45 (N=28) | 0.656 |
| | SD | 1678.12 | 2432.21 | |
| Leptin **Δ** (ng/mL) | Mean | 6.55 (N=32) | 4.10 (N=28) | 0.647 |
| | SD | 18.68 | 22.56 | |
| TNF-α **Δ** (pg/mL) | Mean | 0.22 (N=32) | 1.07 (N=28) | 0.030 |
| | SD | 1.12 | 1.80 | |
| IL-6 **Δ** (pg/mL) | Mean | -1.97 (N=32) | 1.29 (N=28) | 0.132 |
| | SD | 10.63 | 4.11 | |

Table 8: Value for Absolute Erythrocyte DHA at the 35th-37th Week of Gestation and Change in Erythrocyte DHA between the 26th and 35th-27th Week of Gestation

| | | DHA Total Δ | Placebo Total Δ | P value |
|---|---|---|---|---|
| Erythrocyte DHA | Mean | 6.13 (N=32) | 4.17 (N=28) | 0.001 |
| | SD | 2.60 | 1.55 | |
| Erythrocyte DHA **Δ** (%) | Mean | 1.73 (N=32) | -.22 (N=28) | 0.007 |
| | SD | 2.95 | 2.36 | |

## Markers of Maternal Metabolism

### Fasting Glucose

There was no significant correlation between erythrocyte DHA and fasting glucose after controlling for pre-pregnancy BMI. There was no significant difference in mean fasting glucose between women receiving DHA or placebo (p=.611). This supports the null hypothesis, as there is no relationship between absolute fasting glucose and supplementation with docosahexaenoic acid in the 3rd trimester of pregnancy.

There was no significant difference in mean fasting glucose Δ between women receiving DHA or placebo (p=.390). This supports the null hypothesis, which states there is no relationship between mean fasting glucose Δ and supplementation with docosahexaenoic acid in the 3rd trimester of pregnancy.

### Fasting Insulin

There was no significant correlation between erythrocyte DHA and fasting insulin after controlling for pre-pregnancy BMI. There was no significant difference in mean fasting insulin between women receiving DHA or placebo (p=.605). This supports the null hypothesis, as there is no relationship between absolute fasting insulin and supplementation with docosahexaenoic acid in the third trimester of pregnancy.

There was no significant difference in mean fasting insulin Δ between women receiving DHA or placebo (p=.488). This supports the null hypothesis, which states there is no relationship between mean fasting insulin Δ and Δ in erythrocyte DHA following supplementation with docosahexaenoic acid in the third trimester of pregnancy.

A1c

Although a significant negative correlation between erythrocyte DHA and A1c was not expressed, a trend was shown between these two variables before ($p<0.066$) and after ($p<0.086$) controlling for pre-pregnancy BMI. This trend indicates that higher erythrocyte DHA levels were associated with lower A1c levels. There was no significant difference in mean absolute A1c between women receiving DHA or placebo ($p=.343$). This supports the null hypothesis, which states there is no significant relationship between absolute A1c and supplementation with docosahexaenoic acid in the third trimester of pregnancy.

There was no significant difference in mean A1c Δ between women receiving DHA or placebo ($p=.436$). This supports the null hypothesis, as there is no relationship between mean A1c Δ and Δ in erythrocyte DHA after supplementation with docosahexaenoic acid in the third trimester of pregnancy.

Markers of Maternal Adipokines

TNF-α

A significant difference was also seen in mean absolute TNF-α between women receiving DHA or placebo ($p=.025$); the difference between groups is illustrated in *figure 1*. A significant difference was also seen between groups, $F(1,58)=5.329$, $p=.025$; 8.4% of the variance in TNF-α could be explained by DHA supplementation ($R^2=.084$). This disproves the null hypothesis, which states that there is no relationship between absolute TNF-α and supplementation with docosahexaenoic acid.

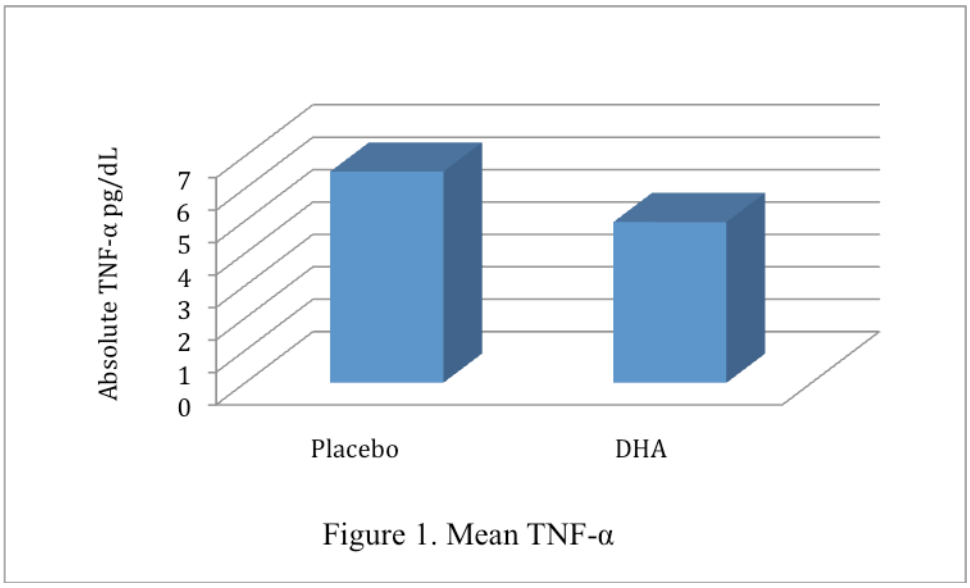


Figure 1. Mean TNF-α

A significant difference was seen in the mean change in TNF-α between women receiving DHA or placebo (p=.030); this relationship is expressed in *figure 2*. This disproves the null hypothesis, which states that there is no relationship between Δ in TNF-α and Δ in erythrocyte DHA after supplementation with docosahexaenoic acid in the third trimester of pregnancy.

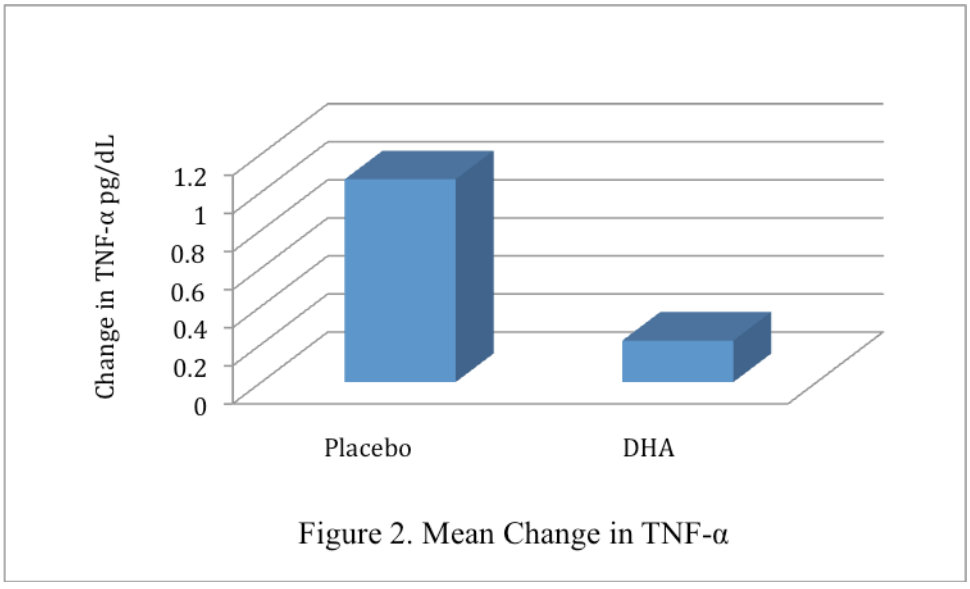


Figure 2. Mean Change in TNF-α

Adiponectin

There was no significant correlation between erythrocyte DHA and adiponectin after controlling for pre-pregnancy BMI. There was no significant difference in mean absolute adiponectin between women receiving DHA or placebo (p=.640). This supports the null hypothesis, as there is no relationship between absolute adiponectin and supplementation with docosahexaenoic acid in the third trimester of pregnancy.

There was no significant difference in mean adiponectin Δ between women receiving DHA or placebo (p=.656). This supports the null hypothesis, as there is no relationship between mean adiponectin Δ and Δ in erythrocyte DHA after supplementation with docosahexaenoic acid in the third trimester of pregnancy.

Leptin

There was no significant correlation between leptin and erythrocyte DHA after controlling for pre-pregnancy BMI. There was no significant difference in mean absolute leptin between women receiving DHA or placebo (p=.212). This supports the null hypothesis, as there is no relationship between absolute leptin and supplementation with docosahexaenoic acid in the third trimester of pregnancy.

There was no significant difference in mean leptin Δ between women receiving DHA or placebo (p=.647). This supports the null hypothesis, as there is no relationship between mean leptin Δ and Δ in erythrocyte DHA after supplementation with docosahexaenoic acid in the third trimester of pregnancy.

IL-6

There was no significant correlation between IL-6 and erythrocyte DHA after controlling for pre-pregnancy BMI. There was no significant difference in mean absolute IL-6 between women receiving DHA or placebo (p=.446). This supports the null hypothesis, as there is no relationship between absolute IL-6 and supplementation with docosahexaenoic acid in the third trimester of pregnancy.

There was no significant difference in mean IL-6 Δ between women receiving DHA or placebo (p=.132). This supports the null hypothesis, which states there is no relationship between mean IL-6 Δ and Δ in erythrocyte DHA after supplementation with docosahexaenoic acid in the third trimester of pregnancy.

Indices of Insulin Sensitivity

HOMA-IR

There was no significant correlation between erythrocyte DHA and HOMA-IR after controlling for pre-pregnancy BMI. There was no significant difference in mean HOMA-IR between women receiving DHA or placebo (p=.205). This supports the null hypothesis, as there is no relationship between HOMA-IR and absolute erythrocyte DHA following supplementation with docosahexaenoic acid.

ISI (comp)

There was no significant correlation between erythrocyte DHA and ISI (comp) after controlling for pre-pregnancy BMI. There was no significant difference in mean ISI (comp) between women receiving DHA or placebo (p=.772). This supports the null hypothesis, as there

is no relationship between ISI (comp and absolute erythrocyte DHA following supplementation with docosahexaenoic acid.

ISI (comp) 2010

There was no significant correlation between erythrocyte DHA and ISI (comp) 2010 after controlling for pre-pregnancy BMI. There was no significant difference in mean ISI (comp) between women receiving DHA or placebo (p=.584). This supports the null hypothesis, as there is no relationship between ISI (comp) 2010 and absolute erythrocyte DHA following supplementation with docosahexaenoic acid.

**Discussion**

DHA supplementation with 800 mg of purified DHA did not have a significant effect on maternal insulin sensitivity in the third trimester in healthy obese pregnant women. There were no significant effects of DHA on glucose, insulin, adiponectin, IL-6 or leptin. However, significant differences were seen in TNF-α, an adipokine related to maternal glucose metabolism, between treatment groups. The data used in this study is comparable to studies published by other researches examining overweight and obese pregnant women and those with gestational diabetes mellitus (GDM). This study has several limitations in that our sample size of 60 may not be enough to give high statistical power to the relationship between docosahexaenoic acid and insulin sensitivity. A possible dose response relationship could have also occurred in that 800mg of algal docosahexaenoic acid was simply not enough to overcome the insulin resistance of obesity and pregnancy. However, it is possible that a favorable response could be seen at higher levels of DHA supplementation and is an area for future exploration. As infant data was not available for use in this study, it is also unable to be determined if the positive impacts of docosahexaenoic acid on TNF-α had any effect on infant birth outcomes.

Our data illustrates that during the third trimester, our women exhibited significant indications that their glucose metabolism was impaired. The values of glucose for women included in this sample were similar to those of Endo et al., Catalano et al., Caudana et al. and Lapolla et al. who examined women who were overweight, diagnosed with GDM or a combination of the two.[40, 63-65] Our values for insulin were also similar to those shown by Catalano et al. who examined women with GDM during late pregnancy.[40] Among both treatment groups, levels for A1c were comparable to those shown by Lapolla et al. for lean pregnant women with a normal OGTT and those with a false positive OGTT.[63] Although our

women demonstrated impaired glucose tolerance and insulin response during the third trimester, none of them were diagnosed with GDM. As the diagnostic test for GDM occurs in the second trimester and A1c identifies a time period of 3 months, this could be due to the increase in insulin sensitivity that occurs during early pregnancy. However, our corresponding data for glucose and insulin indicate that the women in our sample exhibited strong levels of insulin resistance during the third trimester of pregnancy.

This relationship was further illustrated when comparing the indices of insulin sensitivity to those seen in other published research studies examining insulin sensitivity and resistance. When examining maternal insulin resistance, women in this sample were similar to glucose intolerant women in other studies. Das et al. examined women with GDM during the third trimester, their values for HOMA-IR were comparable to those values seen in both the placebo and DHA groups.[66] When compared to Elkind et al.'s overall data, our HOMA-IR was much higher for placebo and DHA groups.[67] Elkind et al. further stratified their sample by race due to the higher proportion of Caucasian women and illustrated that both African American women with GDM and impaired glucose tolerance had much higher HOMA-IR levels then Caucasian women.[67] The HOMA-IR levels in our sample are comparable to Elkind et al.'s African American women with GDM and those with impaired glucose tolerance, this relationship is crucial in our study as 45% of our sample is African American.[67] When examining our values for insulin sensitivity, they were once again quite comparable to those found by Elkind et al. for African American women with GDM and impaired glucose tolerance.[67] This extreme level of insulin resistance and impaired insulin sensitivity further illustrates the impairment in glucose metabolism in healthy obese pregnant women during the third trimester of pregnancy.

Although there was no significant effect of DHA on adiponectin, values for the women in this sample were similar to those found in lean healthy pregnant women in the second trimester by Fasshauer et al.[68] However, Fasshauer et al.'s healthy lean women exhibited higher levels of adiponectin in the third trimester when compared to our healthy overweight and obese women.[68] This is to be expected as leaner individuals often have higher levels of adiponectin than obese individuals. Although there was no significant difference in mean leptin between groups, our leptin levels for the placebo group correspond closely to those seen by Radelli et al. for women diagnosed with GDM while our DHA group was not comparable.[69] However, differences in the diagnostic criteria for GDM among physicians could explain this phenomenon. In a separate study, Radaelli et al. divided mothers into three tertiles based upon infant adipose tissue at birth.[70] Leptin values for women in the placebo group were comparable to women in Radelli's study who gave birth to infants who were included in the first two tertiles; leptin values for the DHA group were lower than those seen by Radaelli et al. and therefore not comparable.[70]

The vast majority of studies involving pregnant women and IL-6 focus on maternal preeclampsia, which causes significant elevations compared to a healthy pregnancy, or use less sensitive methods for estimation. Waisberg et al. examined IL-6 in non-pregnant overweight African women; our levels were higher as expected due to pregnancy.[71] Marantos et al. examined obese non-pregnant women, whose levels of IL-6 were higher than those shown by Waisberg et al.[72] While these women had levels of IL-6 that were less than those seen in this study, the values were comparable to what we would expect our overweight and obese women to express in a non-pregnant state. Studies examining IL-6 in pregnant women where performed by Madazli et al. and Kocyigit et al. who examined differences between normotensive and preeclamptic pregnancies. Our results for both placebo and DHA groups were comparable to

Madazli et al.'s normotensive women during the third trimester.[73] Kocyigit et al. examined lean normotensive pregnant women; as expected these women exhibited IL-6 values that were slightly lower than those seen in this study due to their healthy pre-pregnancy BMI.[74] Kuzmicki et al. found that mothers with GDM had higher levels of IL-6 when compared to mothers with normal glucose tolerance; their results were not comparable to ours due to variations in the assessment method.[39] Ramsay et al. examined differences in IL-6 in the third trimester of pregnancy between healthy lean and obese women; obese mothers were shown to have higher elevations in IL-6 when compared to lean controls.[75] Although their findings were unable to be compared to the findings in this study due to variations in assessment methods and units, they provide evidence to support that higher levels of IL-6 in the obese mother contribute to metabolic disruption during pregnancy.

Melczer et al. found a positive linear relationship between BMI and TNF-α in the 3rd trimester of pregnancy and attributed this to the significant role of TNF-α in mediating insulin resistance during pregnancy.[76] In analyses for this study, no correlations were seen between TNF-α and insulin sensitivity despite significant improvements in TNF-α for women receiving DHA. However, Kirwan et al found TNF-α during late pregnancy to be inversely correlated to insulin sensitivity.[36] In a multi-regression analysis, Kirwan et al found that TNF-α was a primary predictor of insulin sensitivity.[36] Numerous studies described the effects of TNF-α on insulin resistance, although despite improvements in TNF-α no improvements were seen in insulin sensitivity in our obese pregnant women.

Differences were seen between the values for TNF-α between placebo and DHA groups when compared to other studies. Winkler et al. examined differences in TNF-α among women with GDM and pregnant women with normal glucose tolerance to examine the pathological role

of TNF-α during the third trimester of pregnancy.[77] Women in our placebo group had levels of TNF-α that corresponded almost identically and were just slightly higher than those in Winkler et al.'s GDM group. However, women in the DHA group had levels that corresponded to Winkler et al.'s healthy population of pregnant women with normal glucose tolerance.[77] It is crucial to remember that TNF-α is not the only adipokine that contributes to insulin resistance during pregnancy, as leptin and IL-6 both contribute to this occurance. Numerous other factors, such as the placental hormones HPL and hPGH, play significant roles in the pathogenesis of insulin resistance. It is likely that supplementation with DHA at the level of 800mg was unable to be prophylactic during pregnancy, when compounded against these all of these additional causes of insulin resistance.

While no effect of DHA on insulin sensitivity or resistance was seen in this study, effects have been seen in animal models, obese adults and healthy older adults.[49, 50, 78] Haugaard et al. gave evidence to support that when concentrations of omega-3 polyunsaturated fatty acids are incorporated into the membrane phospholipid, there is a significant positive effect on insulin sensitivity.[51] Perhaps, the effects of 800mg of DHA are not enough to overcome the insulin resistance of both pregnancy and obesity. It is possible that while DHA did not affect insulin sensitivity, it could have affected the intrauterine environment. While no study to date has examined the effects of DHA supplementation on maternal insulin sensitivity, studies have examined infant growth. As the fetus secretes its own insulin, high levels of maternal glucose lead to increased accumulation of adipose tissue in the fetus. Oken et al. illustrated that mothers with high consumption of omega-3 fatty acids during pregnancy gave birth to infants with lower birth weight for gestational age, suggestive of decreased adiposity.[58] However, as infant data

was not used as an outcome in this particular study, we are unable to determine the effects of DHA on fetal glucose metabolism and birth outcomes.

## Conclusions

There is no improvement in insulin sensitivity when supplementing healthy obese pregnant women with 800 mg DHA in the third trimester of pregnancy. DHA supplementation did result in significant improvements in TNF-α; the potential impact of lower circulating levels of TNF-α in the fetal intrauterine environment could result in improved fetal outcomes.

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