

RELATIONSHIP OF MATERNAL SERUM FATTY ACIDS AND BODY MASS INDEX

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## ABSTRACT

Maternal supply of nutrients is critical for the developing fetus during all stages of gestation. The altered lipid metabolism that is often seen in pregnancies complicated by obesity and insulin resistance may negatively impact maternal nutrient supply to the fetus. More women are entering pregnancy overweight or obese. Recently body mass index (BMI) has been found to be a positive predictor for decreased maternal plasma phospholipid concentrations of the long-chain polyunsaturated fatty acids (LCPUFA) docosahexanoic acid (DHA) and arachidonic acid (ARA). These nutrients play important roles in early development and their availability is critical to fetal growth and development. The purpose of the present study was to assess if maternal serum fatty acids in the second trimester of pregnancy are associated with BMI.

Serum samples are frequently stored after blood draws during pregnancy. The availability of these samples provided the opportunity to examine if serum samples collected from non-fasting, pregnant women could provide information similar to what has previously been reported for BMI and fatty acid status in pregnancy.

Sera from 265 women from the Foundation for Blood Research (FBR, Maine) were analyzed for fatty acid content using gas chromatography (GC). The BMIs of each participant at approximately 13-18 weeks of gestation were provided by FBR. Participants were grouped by BMI category and fatty acid concentrations were compared across BMI categories.

There were significant differences for weight percent of the LCPUFA eicosapentanoic acid (EPA) as well as for the total  $\omega$ 3 LCPUFA. In this data set BMI was a negative predictor of maternal serum concentrations of DHA and EPA. These results support previous findings for fatty acids in plasma phospholipids of pregnant women. We conclude that increased BMI may negatively impact maternal LCPUFA concentrations and stored sera may be used to further assess this relationship.

# CHAPTER 1

## INTRODUCTION

Lipids have long been recognized as important dietary substances for growth and development. Recently, there has been considerable interest in the role of dietary lipids, specifically essential fatty acids, in the earliest phases of life. The long chain polyunsaturated fatty acids (LCPUFA) docosahexaenoic acid (DHA, 22:6 $\omega$ 3) and arachidonic acid (ARA, 20:4 $\omega$ 6) are important structural components of membrane phospholipids. The highest concentrations of these LCPUFA, especially DHA, are found in the brain. Exponential fetal accumulation begins during the last trimester of pregnancy and continues until the age of two. During the prenatal period the fetus depends on the maternal supply of preformed DHA and ARA to meet its needs. These fatty acids are transferred across the placenta by placental fatty acid binding proteins (p-FABP).

Recent findings suggest that insulin resistance may result in altered lipid metabolism and thus impact fetal LCPUFA status. Insulin resistance in pregnancy is often characterized by increased maternal body mass index (BMI). Due to the increase in the number of women entering pregnancy as overweight or obese, concern has been raised regarding insulin resistance, which can lead to gestational diabetes mellitus (GDM). These conditions could prove detrimental to the developing fetus in part because of their impact on nutrient supply to the fetus. The purpose of the present study is to assess if maternal serum fatty acids are associated with maternal BMI in the second trimester of pregnancy.

### **Justification**

It is well established that the LCPUFAs DHA and ARA are critical nutrients during early development. They play important roles throughout the lifespan, especially in early infant growth, brain, and retinal development. With more women entering pregnancy as overweight or

obese, it is imperative that there be research directed at determining the impact of these conditions on the developing fetus. Because overweight and obesity are often accompanied by insulin resistance and impaired lipid metabolism there is the possibility that maternal supply of DHA and ARA may be impacted. Inadequate supply of these nutrients may be detrimental to fetal growth. Results from this study may lend support to the growing body of evidence for decreased  $\omega$ 3 LCPUFA status in pregnancies complicated by overweight or obesity.

### **Objectives**

1. To measure fatty acids in sera collected in the second trimester of pregnancy from a large cohort of pregnant women living in Maine.
2. To investigate whether serum fatty acid concentrations are associated with maternal BMI.

### **Research Statement**

It is hypothesized that women with higher BMIs will have lower concentrations of serum DHA and ARA than women with lower BMIs.

### **Limitations**

1. This is a relational study so we cannot infer causality.
2. The results of the present study will only be applicable to women of similar age, race, parity and health status.

### **Definitions**

1. Gestation: the period of fetal development in the uterus from conception to delivery.
2. Body Mass Index (BMI): weight in kilograms divided by height in meters squared ( $\text{kg}/\text{m}^2$ ). The most widely used weight-height measure of adiposity.
3. Essential fatty acids (EFA): those fatty acids that cannot be synthesized by humans and must be obtained from the diet to prevent development of disease. In humans the parent

EFA from which all other fatty acids are derived are linoleic acid (LA, C18:2 $\omega$ 6) and alpha-linolenic acid (ALA, C18:3 $\omega$ 3).

4. Docosahexaenoic acid (DHA, C22:6 $\omega$ 3): A 22-carbon polyunsaturated fatty acid that has its first double bond at the third carbon from the omega or methyl end.
5. Arachidonic Acid (ARA, C20:4 $\omega$ 6): A 20-carbon polyunsaturated fatty acid that has its first double bond at the sixth carbon from the omega or methyl end.
6. Diabetes mellitus: A metabolic disorder characterized by inadequate insulin secretion by the pancreas or the inability of certain cells to use insulin resulting in abnormally high serum glucose levels. Diabetes mellitus can be classified as type 1 diabetes, type 2 diabetes, or GDM.

### **Assumptions**

1. The sample size is adequate to reflect the relationship between the variables under investigation.
2. The Hewlett Packard 5890 gas chromatograph is a reliable instrument for measuring the concentration of fatty acids in serum samples.
3. The BMI of each participant was calculated and recorded correctly.

## CHAPTER 2

### REVIEW OF LITERATURE

#### **Lipids and Fatty Acids**

Lipids have long been recognized as important and essential dietary substances for growth and development. Although these compounds are chemically diverse, they all share a common feature, insolubility in water. These compounds are necessary for a variety of functions in organisms including providing a storage form of energy and providing structural support for biological membranes. The most commonly used fats and oils for stored forms of energy are derivatives of fatty acids.

Fatty acids are the simplest class of lipid whose basic structure is that of a chain of carbon atoms linked together and flanked by hydrogen atoms. One end of the fatty acid chain is designated the acid end and contains a carboxyl group, while the other end is designated the omega ( $\omega$ ) end and contains a methyl group. A large proportion of the fatty acids found in nature contain an even number of carbon atoms in an unbranched chain.

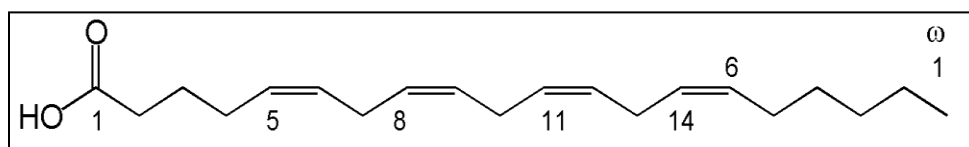
Fatty acids are subdivided based on their chain length, either short (less than six carbons), medium (six to ten carbons) or long (12 or more carbons). Saturated fatty acids are those that have single bonds between carbons and have the maximum amount of possible hydrogen atoms linked to all the carbon atoms. Therefore, monounsaturated fatty acids contain one carbon-carbon double bond (site of unsaturation) and are missing two hydrogen atoms from the carbon chain. Carbon chains with two or more double bonds are thus termed polyunsaturated fatty acids (PUFA) and contain two fewer hydrogen atoms per carbon-carbon double bond or site of unsaturation. The physical properties of fatty acids and also of the compounds that contain them are determined by the length and degree of unsaturation of the carbon chain.

Fatty acids are further classified based on the location of the first carbon-carbon double bond. An omega-3 ( $\omega$ 3 or n-3) fatty acid is one which has its first double bond at the third carbon from the methyl group (omega) end while an omega-6 ( $\omega$ 6 or n-6) fatty acid has its first double bond at the sixth carbon from the methyl group end. These compounds are most often identified by their chain length, number of double bonds, and  $\omega$ 3 or  $\omega$ 6 family.

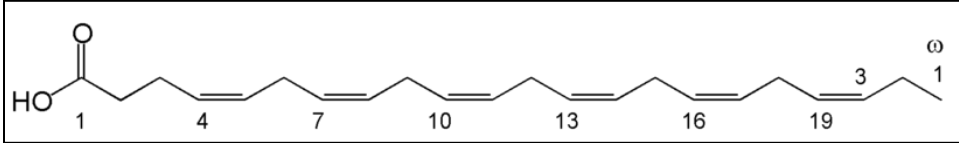
Human cells lack the enzymes necessary to synthesize some long-chain polyunsaturated fatty acids (LCPUFA) and, therefore, depend on the diet to obtain the precursor  $\omega$ 3 and  $\omega$ 6 fatty acids. These fatty acids are thus termed essential fatty acids (EFA). Alpha-linolenic acid (C18:3 $\omega$ 3, ALA) is the  $\omega$ 3 EFA and linoleic acid (C18:2 $\omega$ 6, LA) is the  $\omega$ 6 EFA. Humans can desaturate and elongate the parent EFAs to form several important LCPUFAs.

### Synthesis of Long-Chain Polyunsaturated Fatty Acids (LCPUFA)

The conversion pathway of LA and ALA to LCPUFA as described by Voss (1991) involves a series of elongation and desaturation enzymes and includes several intermediate compounds (1). These chain elongation and desaturation systems occur mainly in the cytosol of cells, but can also be found in the mitochondria of brain cells (2-6). One of the products of LA conversion is the 20 carbon LCPUFA arachidonic acid (C20:4 $\omega$ 6, ARA) which can be seen in Figure 1. It is formed by adding two carbon atoms of acetate to the carboxyl group of LA and removing four hydrogen atoms (7). ALA is converted to the LCPUFAs eicosapentaenoic acid (C20:5 $\omega$ 3, EPA) and docosahexaenoic acid (C22:6 $\omega$ 3, DHA) (Figure 2).

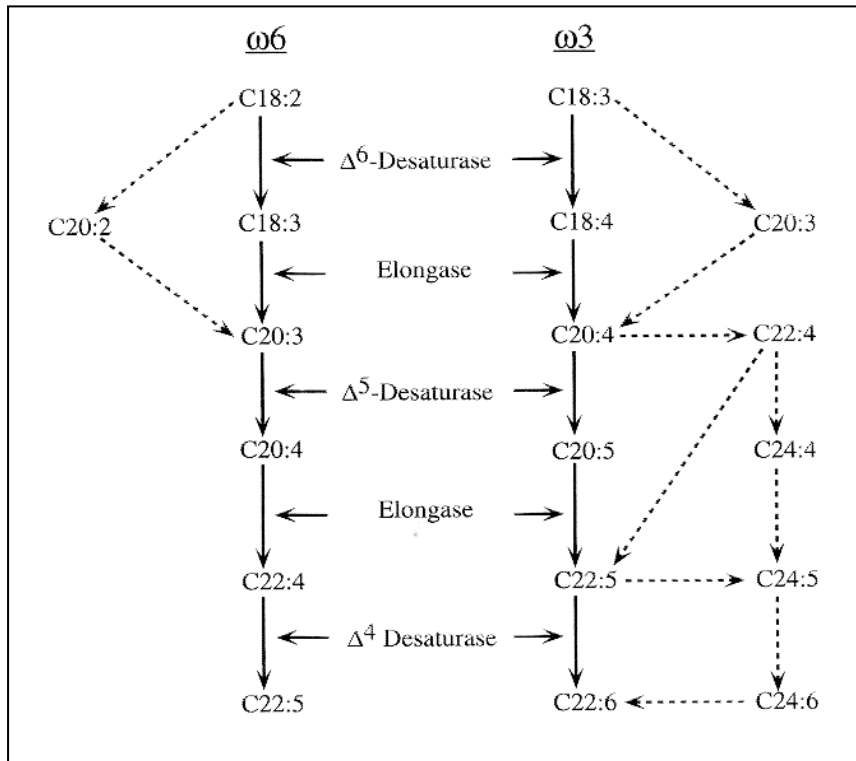


**Figure 1.** A schematic of the structure of arachidonic acid. Upper numbers refer to the numbering system from the methyl carbon and lower numbers refer to the number system from the acid carbon end, which is known as the delta system.



**Figure 2.** A schematic of the structure of docosahexaenoic acid.

A schematic of the conversion pathway of fatty acids can be seen in Figure 3. It is important to note that the synthesis pathways of ARA and DHA share enzymes which compete for substrates. Also of importance is the fact that synthesis of  $\omega 3$  and  $\omega 6$  fatty acids is not interconvertible, meaning an LCPUFA of the  $\omega 3$  series cannot be synthesized from an LCPUFA of the  $\omega 6$  series and vice versa. Humans obtain ARA and DHA either from synthesis from their parent EFAs or from the consumption of animal tissues, with fatty fish being the richest source of DHA. Because humans have a low capacity for de novo lipogenesis and are unable to synthesize the EFA, the dietary fatty acid supply is critical for the incorporation of fatty acids into membrane bilayers.



**Figure 3.** A schematic of the conversion pathway of LA and ALA to the LCPUFA C20:4 $\omega 6$ , C20:5 $\omega n$  and C22:6 $\omega 3$ .

## **Importance of $\omega$ 3 and $\omega$ 6 Fatty Acids**

The  $\omega$ 3 and  $\omega$ 6 class of fatty acids are important components of storage lipids, cell membrane phospholipids, intracellular cholesterol esters and plasma lipids (8). Animal studies have provided evidence that EFAs and their longer chain derivatives have a structural role in cell membranes, a role in the transport of other lipids and both direct and indirect roles in the activity of enzyme systems (7).

The long-chain fatty acids have also been found to play a role in regulation of gene expression. These LCPUFA enhance the DNA binding affinity of peroxisome proliferator-activated receptors (PPARs), which regulate the genes involved in fatty acid and glucose oxidation, fatty acid uptake, fatty acid activation, triacylglycerol biosynthesis, and lipoprotein metabolism. There is also some evidence that EPA enhances the transcriptional activity of this family of transcription factors; however, these findings need to be substantiated by live animal studies. Synthesis and activation of the sterol regulatory element binding protein (SREBP)-1 is also influenced by unsaturated fatty acids. The SREBP transcription factors regulate transcription of several of the genes involved in lipid, cholesterol, bile, and lipoprotein biosynthesis. There is evidence that dietary  $\omega$ 3 fatty acids suppress hepatic lipogenesis by inhibition of SREBP-1 gene transcription and by increasing SREBP-1 mRNA decay (9). The activity and abundance of hepatic nuclear factor 4 $\alpha$  (HNF4 $\alpha$ ), a steroid receptor that enhances expression of genes involved in hepatic lipogenesis and carbohydrate metabolism, is modulated by the  $\omega$ 3 class of fatty acids. Taken together, the genomic effects of  $\omega$ 3 fatty acids on hepatic metabolism involve a shift from triacylglycerol synthesis, storage, and apolipoprotein secretion towards hepatic oxidation of lipids. This shift may influence the risk of developing several chronic diseases including coronary heart disease and obesity.

ARA and DHA make up the majority of the lipid content of the nervous system (10, 11). Fetal brain accumulation of LCPUFA rapidly increases during the third trimester of gestation and continues into the first two years after birth. These LCPUFA are incorporated into highly specialized membrane phospholipids found in the retina and synapses. ARA is found in relatively large amounts in most tissues, while DHA is distributed preferentially to specific tissues. Compared to ARA, DHA is found in higher amounts in the cerebral cortex, retina and testis (12). Within each of these tissues, DHA is especially abundant in rod photoreceptors and synaptic membranes. These findings suggest an important role for the LCPUFA in visual and cognitive development.

There is ample evidence that these LCPUFA are important for normal brain development, visual acuity and cognitive function. Studies show that deficiency of the LCPUFAs is related to degenerative diseases of the retina (13-15), poor performance on a test of retinal function and tests of learning capabilities (10), memory loss, and diminished cognitive function (16). Infant supplementation studies point to relationships between LCPUFAs and better visual acuity (17-25), and mental development (21, 25-29).

### **Importance of LCPUFAs during Pregnancy**

Substrate transfer from the mother to the fetus is critical for successful conception and proper fetal growth. The accretion of LCPUFA is especially important during the prenatal period for both the mother and the growing fetus. There is a growing body of evidence that these nutrients are necessary for a variety of processes during gestation. For the mother, these nutrients have been shown to play a role in the proper development of the placenta, in the initiation of labor and delivery, and possibly development of the mammary gland (30). Studies of increased dietary LCPUFA and supplementation have shown positive effects in the prevention of pregnancy-induced hypertension and prolonging gestation in those women at risk of

delivering preterm (31). Recent work found that low maternal plasma  $\omega$ 3 fatty acids and high ARA during early pregnancy were associated with a 40-50% increase in risk of small for gestational age (SFA) infants (32). For the fetus these nutrients are important for proper growth (33, 34), are significantly related to anthropometric measurements at birth (35) and also influence neurological conditions at birth (36). There is also a large pool of evidence that suggests the LCPUFA are especially important for postnatal growth and development of preterm infants (35, 37).

### **Placental Transfer of $\omega$ 3 and $\omega$ 6 Fatty Acids**

The elongation and desaturation enzymes necessary for EFA conversion to DHA and ARA are present in the fetal liver during the early stages of gestation; however, their activity is very low before birth (38). For this reason, the  $\omega$ 3 and  $\omega$ 6 LCPUFA that the fetus accumulates come from placental transfer from the mother. To meet the additional needs of the growing fetus, the maternal diet should contain sufficient EFAs and their long chain derivatives. If intake is too low to meet the physiological needs, maternal body stores may be mobilized to supply the required fatty acids (39). These stores are usually made available during the third trimester of pregnancy when fetal growth reaches its maximum rate and fetal requirements for fatty acids, especially DHA and ARA, are greatly increased (40). Evidence suggests that the accumulation of brain DHA and ARA is far more efficient from preformed, dietary DHA and ARA rather than their EFA precursors, LA and ALA. Additionally, recent evidence points to an inefficiency in the conversion of DHA from ALA. This raises the question of whether DHA may be considered conditionally essential during pregnancy when the requirement is increased.

Extensive research has investigated the amount of LCPUFA that is transferred to the fetus in relation to the amount available in maternal circulation. Results of these studies show that the amounts in cord blood (plasma phospholipids) are strongly correlated with maternal

plasma phospholipids (41, 42) and are influenced by the maternal diet (43). This evidence supports previous findings of Otto et al. who showed that in an international population, the  $\omega$ 3 LCPUFA concentrations in umbilical plasma, vein, and artery phospholipids were the highest in those countries that had the highest maternal plasma  $\omega$ 3 LCPUFA concentrations (44). In the same international population,  $\omega$ 6 LCPUFA concentrations in umbilical material seemed less dependent on the maternal  $\omega$ 6 LCPUFA concentrations in blood phospholipids, which researchers suggested could be due to some type of fetal autonomy with respect to establishing its ARA supply (44).

In the very early stages of pregnancy, maternal plasma and erythrocyte DHA concentrations begin to increase without any concomitant change in dietary intake. The increase is most probably due to the release of fatty acids from the maternal stores to provide the requirements for highly proliferating and differentiating tissues during this stage (45).

Compounding the issue of insufficient maternal dietary DHA are the findings by Al et al (41). In a prospective, longitudinal study the total concentration (mg/L) of fatty acids in maternal plasma phospholipids increased by ~51% throughout the course of pregnancy. The absolute amount of ARA increased by 23% while DHA increased by 52%. It was thought that the significant increase in DHA was due to an increase in fish intake; however, the dietary information for this population showed no increase in fat intake during pregnancy.

Taken together, the body of evidence regarding DHA points to reduced maternal status during pregnancy with suboptimal supply for the fetus (46). For this reason, the LCPUFA are often thought of as conditionally essential during pregnancy. Further, the reduction in maternal EFA status over the course of pregnancy appears to be independent of cultural differences in dietary intake and ethnic origin (44).

The concentrations of DHA and ARA in fetal plasma phospholipids are much higher compared to the concentration in maternal phospholipids; however, the LA and ALA precursors are lower. Research supports the notion that higher LCPUFA concentrations in fetal plasma reflect selective placental transfer, synthesis in placental or fetal tissues, or selective fetal retention. Most evidence points to the selective transfer of ARA and DHA by membrane associated proteins with higher affinity and binding capacity for these LCPUFA than for other fatty acids. Findings by Ghebremeskel et al. (2000) suggest the possible role of a physiological mechanism that works to maintain an appropriate balance between ARA and DHA (47).

Early research suggested the existence of a mechanism within the placenta that allowed for fetal uptake of ARA by selective incorporation of ARA into phosphoglycerides and export to the fetal circulation (48). These findings were followed by the purification and characterization of a fatty acid-binding protein (FABP) from human placentas (49). This protein was isolated from aborted and term placentas and was found to have at least three distinct fractions: one that is nearly lipid-free, one that binds LCPUFA nonspecifically and one that binds mainly ARA. Further studies suggested a saturability of fatty acid binding and the occurrence of binding sites specific to different fatty acids (50). Later research revealed that the FABP is located exclusively in the maternal facing microvillous membranes which may result in the unidirectional flow of maternal free fatty acids to the fetus (51).

It was recently established that placental FABP preferentially binds both ARA and DHA (52). These findings are further supported by the work of Haggerty (1997) and Dutta-Roy (2000) (53, 54). Most recently, the involvement of two fatty acid transport proteins has been suggested to play a role in the placental transfer of LCPUFA. The supply of DHA during pregnancy affects the expression of fatty acid transport proteins in the placenta and therefore plays a role in placental transfer (55, 56). Taken together, these findings clearly indicate that the

fetus is dependent upon the maternal supply of substrates and also on adequate placental transport.

### **Obesity, BMI, and Insulin Resistance**

Obesity is a condition marked by excess body fat. Measures of height and body weight are widely used to identify the overweight and obese because it is difficult to obtain accurate measures of body fatness in populations. Body Mass Index (BMI) is a measure used to assess body weight relative to height. The number, obtained by dividing weight in kilograms (kg) by height in meters squared ( $m^2$ ), has a relatively high correlation with estimates of body fatness. As BMI increases above  $25 \text{ kg}/m^2$ , there is a concomitant, gradual increase in risk of morbidity from conditions such as type 2 diabetes mellitus, hypertension, coronary heart disease, stroke, gallbladder disease, osteoarthritis, sleep apnea and some forms of cancer (prostate, colon, endometrial and breast). See Table 1 for classification of BMI.

**Table 1.** Classification of Overweight and Obesity by Body Mass Index.

	<b>BMI (<math>\text{kg}/m^2</math>)</b>
Underweight	< 18.5
Normal	18.5-24.9
Overweight	25.0-29.9
Obese	$\geq 30.0$

Adapted from: Nutritional Assessment, 3<sup>rd</sup> Edition.

### **Obesity and Pregnancy**

Current estimates, based on data from the Pregnancy Risk Assessment Monitoring System, show a 69% increase in pre-pregnancy obesity between 1993 and 2003 (57). These data are supported by the finding that nearly 30% of women of childbearing age (20-39 years) were obese between 1999 and 2002. The highest prevalence of obesity occurred in non-Hispanic black women (49%) followed by Mexican-Americans (38.9%) and non-Hispanic white women

(31.3%) (58). Pre-pregnancy BMI is an important measure that is used by the Institute of Medicine to make weight gain recommendations for pregnancy (59). Excess weight during pregnancy poses a risk for both the mother and the developing fetus. Several adverse pregnancy outcomes including miscarriage, neural tube defects, preeclampsia, GDM, macrosomia, shoulder dystocia, and fetal death have been linked to high maternal BMI.

### **Influence of BMI and Insulin Resistance on LCPUFA Transfer**

Little is known about the maternal factors that may influence supply and transfer of substrates to the fetus during gestation. There is evidence of altered lipid metabolism in pregnancies complicated by type 2 diabetes mellitus (NIDDM) and GDM (60). In the animal model, increasing maternal glycemia has been associated with a decrease in the unidirectional transfer of both EFA and non-essential fatty acids. Holman et al (1983) found that maternal diabetes was associated with an overall decrease in PUFA status in the whole animal (61). Decreased insulin sensitivity, seen in conditions of diabetes and obesity, has been associated with decreased concentration of PUFA in skeletal muscle and plasma phospholipids (62), (63). While little is known about the underlying mechanisms of these phenomena, it is suggested that there is a possible impairment in the enzymes that regulate EFA metabolism (64). In a study of Pima Indians, a negative correlation was reported between high percentage of body fat and impaired activity of an enzyme involved in the EFA to LCPUFA conversion pathway. Both pregnancy and obesity are often accompanied by decreased insulin sensitivity. Any impairment in the enzyme system that results in decreased ARA or DHA would mean a smaller pool of these essential nutrients available for transfer to the fetus and, thus, could have severe, deleterious effects on fetal growth. Increased insulin resistance is a phenomenon associated with normal pregnancies and to a greater extent in those complicated by diabetes (65).

Abnormalities in placental transfer may also be a consequence of pregnancies complicated by insulin resistance and obesity. Bitsanis et al (2006) found that women diagnosed with GDM had higher placental concentrations of both ARA and DHA when compared to control women (66). These results could not be explained by differences in ethnicity or enhanced placental synthesis of the LCPUFAs. Because neonates born to women with GDM tend to have low blood levels of AA and DHA, the authors hypothesize that GDM enhances uptake of the two fatty acids from maternal circulation and causes them to be retained instead of transferred to the fetus. These results are supported by the findings of increased placental incorporation of ARA in pregnancies complicated by type 1 diabetes mellitus (IDDM) (67) and enhanced expression of placental FABP in pregnancies complicated by GDM (68).

In a cohort of pregnant women, BMI appears to be a significant predictor of maternal concentrations of plasma phospholipid ARA and DHA (69), as well as fetal erythrocyte phospholipid DHA (70). More recently, both IDDM and NIDDM have been shown to compromise maternal red blood cell (RBC) DHA, as well as cord plasma and RBC ARA and DHA (71). These effects seem to be independent of ethnicity or diet and related more to the disease itself (72).

## CHAPTER 3

### METHODS

#### **Participants and Sampling**

Sera from pregnant women living in Maine were collected in the decade between 1996 and 2006. The fatty acids of sera from 265 women who delivered term infants who were appropriate for gestational age were analyzed. The sample population was selected from a larger population of 400 women who participated in a similar study. Samples were collected during the second trimester of pregnancy from non-fasting women and stored in blood banks at FBR in Maine. The 265 samples were shipped from FBR and upon arrival at the Louisiana State University nutrition lab were immediately placed in storage at -80°C. Data for participants' ages and BMIs, which were calculated at 13-18 weeks gestation, were also provided by FBR. Assessment of samples that had been in storage for five to ten years provided assurance that fatty acids were detectable and quantifiable.

#### **Procedures**

##### **Sample Collection**

Samples were shipped from FBR to the Knapp Hall laboratory overnight on dry ice. When samples arrived they were immediately transferred to a freezer and stored at -80°C until analysis for fatty acids began.

##### **Sample Methylation**

Sera fatty acids were methylated according to Lepage (73) using an internal standard. In brief, 100 µl of the stored sera were transferred to 9 ml glass tubes with Teflon lined caps. Two ml of methanol:benzene (4:1, v/v) containing 40 µg/ml of internal standard (heptadecanoic acid, C17:0) was added to the sample. Working under a ventilated hood, 200 µl of acetyl chloride was added slowly over a period of one minute. After the addition of acetyl chloride, samples were

capped tightly and mixed thoroughly. Samples were heated for one hour at 100 °C in an analog heat block (VWR). After one hour the samples were removed from the heat block and allowed to cool. When samples were cool to the touch, 5 ml of 6% (w/v) potassium carbonate (K<sub>2</sub>CO<sub>3</sub>) was added to neutralize the mixture and stop the reaction. Samples were then centrifuged in a Beckman refrigerated centrifuge for 10 minutes at ~3500 rpm. After centrifuging, the methyl esters in the solvent were pipetted off as the top layer and placed in a glass vial labeled with the sample number. Fatty acids were separated by gas chromatography, identified based on retention times and quantified against the internal standard. Fatty acids were expressed as relative weight percent (wt%).

### **Sample Analysis**

Analysis was performed using a Hewlett Packard 5890 series gas chromatograph (GC) equipped with flame ionization detection (FID). Samples were injected into a 30 m fused silica column with an internal diameter of 0.32 mm (Omegawax 320). The initial oven temperature was programmed at 190°C and increased to 210°C in increments of 2°/minute with a final hold of 20 minutes. Helium was used as the carrier gas with a flow set at 1.0 ml/minute and hydrogen was used as the make-up gas. The split ratio was set at 1:50, which means one part in fifty entered the column. The injection port and detector temperatures were set to 250°C and 280°C, respectively. Each sample was injected into the chromatograph three times. Peak identification of serum fatty acids was based on comparison with the relative retention times of single fatty acid methyl esters (FAME). Relative weight percents were calculated using the following formula:

$$(\text{area \% of single FAME} * 100)/(\text{total area \% of fatty acids} - \text{area \% of C17:0})$$

## **Statistical Analysis**

The data set was analyzed using JMP® 8 from Statistical Analysis Software (SAS). Differences in relative weight percents of individual fatty acids and the ratios of fatty acids were determined using one-way analysis of variance (ANOVA). Tukey's Post-Hoc analyses were performed to determine where significant differences occurred between BMI groups.

## CHAPTER 4

### RELATIONSHIP OF MATERNAL SERUM FATTY ACIDS AND BODY MASS INDEX

#### Introduction

The polyunsaturated fatty acids docosahexaenoic acid (DHA, C22:6 $\omega$ 3) and arachidonic acid (ARA, C20:4 $\omega$ 6) are important components of the nervous system and have critical roles in fetal growth and in brain and retinal development and function. Exponential fetal accretion of these fatty acids begins during the third trimester of gestation and continues into the first two years after birth. Fetal conversion of the  $\omega$ 3 and  $\omega$ 6 fatty acids is low; therefore, fetal accumulation depends on the maternal supply. The placenta plays an important role in transferring these fatty acids from maternal diet or body stores to the growing fetus. Because the fetus depends on the maternal supply, the importance of identifying factors that influence fatty acid transfer is underscored.

During pregnancy there is a natural decrease in maternal LCPUFA status, in particular, DHA. A suboptimal supply of EFAs as well as their long chain derivatives could be detrimental to the development of the fetus. In addition to this, some maternal conditions may impact placental transfer of nutrients to the fetus. One such condition is insulin resistance that is seen in diabetes mellitus. Several studies support an overall decrease in EFA and LCPUFA status in these conditions (60-65, 69, 70, 74).

During pregnancy it is normal to experience some insulin resistance and this resistance is exacerbated by excess weight. Data document that the number of women entering pregnancy as overweight or obese is increasing. Together, these conditions, insulin resistance and excess weight, may negatively influence the efficiency of nutrient transfer. Recently, maternal BMI has been shown to be negatively related to plasma phospholipid concentrations of ARA and DHA (69) as well as fetal erythrocyte phospholipid DHA (70). The objective of the current study was

to determine if there is a similar relationship between maternal serum fatty acids and BMI. If these results mimic the results of studies of phospholipid fatty acids, more studies using total lipids in stored sera may be feasible as a more convenient and relatively rapid way to investigate the maternal factors that influence substrate transfer to the fetus as well as fetal outcomes.

## **Subjects and Methods**

### **Subjects**

Sera from pregnant women living in Maine were collected in the decade between 1996 and 2006. Samples were collected during the second trimester of pregnancy from non-fasting women. These samples were shipped from the Foundation for Blood Research in Maine and upon arrival at the Louisiana State University nutrition lab were immediately placed in storage at  $-80^{\circ}\text{C}$ . Data for women's ages and BMIs, which were determined at approximately 13-18 weeks gestation, were provided by FBR. Prior assessment of samples that had been in storage for five to ten years provided assurance that length of storage would not be a significant factor. The fatty acids in sera from 265 women who delivered term infants who were appropriate for gestational age were analyzed for this study.

### **Sample Analysis**

Samples were directly methylated following the LePage method (73). In brief, 100  $\mu\text{l}$  of the stored sera were transferred to 9 ml glass tubes with Teflon lined caps. Two ml of methanol:benzene (4:1, v/v) containing 40  $\mu\text{g}/\text{ml}$  of internal standard (heptadecanoic acid, C17:0) was added to each tube. Working under a ventilated hood, 200  $\mu\text{l}$  of acetyl chloride was added slowly over a period of one minute. After the addition of acetyl chloride, samples were capped tightly and mixed thoroughly. Samples were heated for one hour at  $100^{\circ}\text{C}$  in an analog heat block (VWR). After one hour the samples were removed from the heat block and allowed to cool. Once cooled, 5 ml of 6% (w/v) potassium carbonate ( $\text{K}_2\text{CO}_3$ ) was added to neutralize

the mixture and stop the reaction. Samples were centrifuged in a Beckman refrigerated centrifuge for 10 minutes at ~3500 rpm. After centrifuging, the methyl esters in the solvent were pipetted off as the top layer and placed in a glass vial labeled with the sample number.

Fatty acid analysis was performed using a Hewlett Packard 5890 series gas chromatograph equipped with flame ionization detection. Samples were injected into a 30 m fused silica column with an internal diameter of 0.32 mm (Omegawax 320). The initial oven temperature was programmed at 190°C and increased to 210°C in increments of 2°/minute with a final hold of 20 minutes. Helium was used as the carrier gas with a flow set at 1.0 ml/minute and hydrogen was used as the make-up gas. The split ratio was set at 1:50. The injection port and detector temperatures were set to 250°C and 280°C, respectively. Each sample was injected into the chromatograph three times. Peak identification of serum fatty acids was based on comparison with the relative retention times of single fatty acid methyl esters (FAME). Relative weight percents were calculated using the following formula:

$$(\text{area \% of single FAME} * 100) / (\text{total area \% of fatty acids} - \text{area \% of C17:0})$$

### **Statistical Analysis**

The data were analyzed using JMP® 8 from Statistical Analysis Software (SAS). Differences between BMI categories in relative weight percents of individual fatty acids and the ratios of fatty acids were determined using one-way analysis of variance (ANOVA). Tukey's Post-Hoc analyses were performed to determine where significant differences occurred between BMI groups.

### **Results**

#### **Sample Characteristics**

The 265 participants had an average age of 27.7 years and an average BMI of 27.5 kg/m<sup>2</sup>. Participants were grouped according to BMI category (Table 2; < 18.5, underweight; 18.5-24.9,

normal; 25.0-29.9, overweight;  $\geq 30.0$ , obese). For statistical analysis, participants under age 18 were excluded as well as six participants in the obese class III category with outlying BMIs and two participants with outlying weight percents for several fatty acids.

**Table 2.** Characteristics of sample population

<b>BMI Category</b>	<b>Samples (n)</b>	<b>% of Population</b>
Normal weight	110	44.2
Overweight	74	29.7
Obese	65	26.1

### **Serum Fatty Acid Analysis**

Saturated,  $\omega 3$ , and  $\omega 6$  fatty acids are presented in Tables 3, 4, and 5, respectively. There were significant differences for the saturated fatty acid lignoceric acid (C24:0) in normal versus obese participants (Table 3,  $p = 0.03$ ). Weight percent of the  $\omega 3$  fatty acid eicosapentanoic acid (EPA, C20:5n3) was significantly different between the overweight and obese participants (Table 4,  $p = 0.02$ ) while the total relative weight percent of DHA trended toward significant difference ( $p=.08$ ) (Table 4). In the overall model there was a significant difference for the total  $\omega 3$  LCPUFA ( $p = .04$ ); however, Tukey’s Post-Hoc analysis failed to determine significant differences between BMI categories. In general, participants with higher BMIs tended to have lower total  $\omega 3$  LCPUFA ( $\sum$  C20:5 $\omega 3$  and C22:6 $\omega 3$ ).

**Table 3.** Average relative weight percent (wt %) of saturated fatty acids by BMI category<sup>1</sup>

	<b>Normal</b>	<b>Overweight</b>	<b>Obese</b>
C16:0	27.37 $\pm$ 0.32 [110]	28.13 $\pm$ 0.39 [74]	28.33 $\pm$ 0.42 [65]
C18:0	5.52 $\pm$ 0.13 [110]	5.42 $\pm$ 0.17 [72]	5.42 $\pm$ 0.17 [65]
C20:0	2.06 $\pm$ 0.14 [109]	1.98 $\pm$ 0.17 [73]	2.01 $\pm$ 0.18 [63]
C22:0	1.27 $\pm$ 0.05 [106]	1.18 $\pm$ 0.06 [71]	1.19 $\pm$ 0.07 [62]
C24:0	0.95 $\pm$ 0.06 [110] <sup>a</sup>	1.09 $\pm$ 0.07 [74] <sup>a</sup>	0.81 $\pm$ 0.08 [65] <sup>b</sup>

<sup>1</sup>Mean  $\pm$  standard error. Means within a row with different superscript letters indicate significant differences among BMI categories,  $p \leq 0.05$ . Sample number in brackets.

**Table 4.** Average relative weight percent (wt %) for the  $\omega$ 3 fatty acids by BMI category<sup>1</sup>

	Normal	Overweight	Obese
C18:3 $\omega$ 3	1.70 $\pm$ 0.16 [100]	1.75 $\pm$ 0.20 [67]	1.99 $\pm$ 0.21 [62]
C20:5 $\omega$ 3	1.38 $\pm$ 0.04 [110] <sup>a</sup>	1.27 $\pm$ 0.05 [74] <sup>a</sup>	1.17 $\pm$ 0.06 [65] <sup>b</sup>
C22:6 $\omega$ 3	1.59 $\pm$ 0.04 [110]	1.73 $\pm$ 0.05 [74]	1.57 $\pm$ 0.06 [65]

<sup>1</sup>Mean  $\pm$  standard error. Means within a row with different superscript letters indicate significant differences among BMI categories,  $p \leq 0.05$ . Sample number in brackets.

**Table 5.** Average relative weight percent (wt %) for the  $\omega$ 6 fatty acids by BMI category<sup>1</sup>

	Normal	Overweight	Obese
C18:2 $\omega$ 6	24.07 $\pm$ 0.37 [110]	23.71 $\pm$ 0.45 [74]	23.42 $\pm$ 0.48 [65]
C18:3 $\omega$ 6	1.59 $\pm$ 0.19 [26]	1.25 $\pm$ 0.22 [19]	1.37 $\pm$ 0.28 [12]
C20:2 $\omega$ 6	1.14 $\pm$ 0.14 [100]	0.84 $\pm$ 0.16 [72]	0.89 $\pm$ 0.17 [65]
C20:3 $\omega$ 6	1.32 $\pm$ 0.06 [110]	1.37 $\pm$ 0.07 [74]	1.29 $\pm$ 0.08 [64]
C20:4 $\omega$ 6	2.88 $\pm$ 0.09 [110]	2.99 $\pm$ 0.11 [74]	2.96 $\pm$ 0.12 [65]
C22:2 $\omega$ 6	0.53 $\pm$ 0.09 [10]	0.59 $\pm$ 0.11 [6]	0.38 $\pm$ 0.20 [2]

<sup>1</sup>Mean  $\pm$  standard error. Sample number in brackets.

**Table 6.** Sums and ratios of serum fatty acid content (wt %)<sup>1</sup>

	Normal	Overweight	Obese
Total $\omega$ 3 PUFA	4.48 $\pm$ 0.16 [110]	4.53 $\pm$ 0.19 [74]	4.58 $\pm$ 0.20 [65]
Total $\omega$ 6 PUFA	29.73 $\pm$ 0.37 [110]	29.26 $\pm$ 0.45 [74]	28.81 $\pm$ 0.48 [65]
SFA: $\omega$ 3 PUFA	9.22 $\pm$ 0.30 [110]	9.18 $\pm$ 0.36 [74]	9.30 $\pm$ 0.38 [65]
SFA: $\omega$ 6 PUFA	1.27 $\pm$ 0.02 [110]	1.32 $\pm$ 0.03 [74]	1.33 $\pm$ 0.03 [65]
$\omega$ 3: $\omega$ 6	0.15 $\pm$ 0.01 [110]	0.16 $\pm$ 0.01 [74]	0.16 $\pm$ 0.01 [65]
Total $\omega$ 3 LCPUFA	2.97 $\pm$ 0.06 [110]	3.00 $\pm$ 0.08 [74]	2.74 $\pm$ 0.08 [65]
Total $\omega$ 6 LCPUFA	5.28 $\pm$ 0.22 [110]	5.23 $\pm$ 0.27 [74]	5.14 $\pm$ 0.29 [65]
$\omega$ 3 LCPUFA: $\omega$ 6 LCPUFA	0.65 $\pm$ 0.02 [110]	0.64 $\pm$ 0.03 [74]	0.58 $\pm$ 0.03 [65]
DHA:ARA	0.60 $\pm$ 0.02 [110]	0.63 $\pm$ 0.03 [74]	0.56 $\pm$ 0.03 [65]
Total SFA	37.14 $\pm$ 0.33 [110]	37.59 $\pm$ 0.41 [74]	37.64 $\pm$ 0.43 [65]
Total PUFA	34.19 $\pm$ 0.42 [110]	33.76 $\pm$ 0.51 [74]	33.38 $\pm$ 0.54 [65]
SFA:PUFA	1.11 $\pm$ 0.02 [110]	1.15 $\pm$ 0.03 [74]	1.15 $\pm$ 0.03 [65]

<sup>1</sup>Mean  $\pm$  standard error. PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; LCPUFA, long-chain polyunsaturated fatty acid; DHA, docosahexaenoic acid; ARA, arachidonic acid. Sample number in brackets.

## Discussion

In this study we have established that sera collected from pregnant women and stored for a decade or so may be used to study relationships of fatty acid status, maternal risk factors, and pregnancy outcomes. In previous research, positive relationships were found between higher

BMI and lower  $\omega$ 3 fatty acid concentrations in plasma phospholipids (69, 70). Similar results for total serum lipids were found in this study with participants in the highest BMI category tending to have lower total  $\omega$ 3 LCPUFA, and statistically, significantly lower EPA.

The findings of this study support the theory that BMI is a predictor of maternal LCPUFA status and suggest that stored serum may be a feasible medium to further explore the relationships between maternal factors that influence pregnancy and fetal outcomes. These results are limited by the lack of dietary intake information in this group as well as the absence of pre-pregnancy BMI. The nature of the sample is also a limitation because of the influence of recent dietary intake. The serum contains fatty acids bound to triglycerides, which would reflect recent intake, as well as fatty acids contained in phospholipid membranes.

In this study, phospholipids were not extracted from the sera. The samples were analyzed for total fatty acid content as a convenient and relatively quick way of investigating the relationship between maternal BMI and fatty acid content of sera. It is interesting to note that a similar relationship was found using the sera as was found in previous studies using plasma phospholipids. This suggests the plausibility of analyzing serum in this population to investigate maternal factors influencing LCPUFA status. Sera is often stored after routine blood collections and archived over many years and demographic areas. Using stored sera to explore fatty acid status during pregnancy provides a unique opportunity to find relationships in a large number of samples that include many different populations. In addition to the availability of a large number of samples, the methodology for total serum fatty acid analysis is less laborious than the methodology for plasma phospholipids. To further investigate these relationships, future studies should include a more diverse sample population and control for dietary intake, supplementation, and diagnosis of diabetes.

## CHAPTER 5

### SUMMARY

The purpose of the present study was to investigate the relationship between maternal serum fatty acids and BMI. The fatty acid content of serum was analyzed in 265 women in their second trimester of pregnancy. Serum samples were provided by the Foundation for Blood Research in Maine along with maternal age and BMI. Sera were collected between 1996 and 2006 and maternal BMIs were calculated at 13-18 weeks gestation. These samples provided a unique opportunity to examine a relationship that has previously been reported in studies of plasma phospholipids. Participants were grouped by BMI category and after analysis using gas chromatography, fatty acid concentrations were compared across BMI categories.

In the sample population, women with the highest BMIs tended to have lower total weight percent of the  $\omega$ 3 LCPUFA DHA and statistically, significantly lower EPA. These findings are in line with recent reports that maternal BMI negatively influences plasma phospholipid concentrations of DHA and ARA in the third trimester of pregnancy (69).

DHA is especially important for fetal growth and development. During the last trimester of pregnancy, brain accumulation rapidly increases and continues until the age of two. Maternal supply of preformed LCPUFA is critical for the growing fetus. Activity of the elongation and desaturation enzymes needed for production of LCPUFAs from EFAs is low in the fetus; therefore, the  $\omega$ 3 and  $\omega$ 6 LCPUFAs that the fetus accumulates must be transferred from the mother across the placenta. Maternal dietary intake and body stores provide sources of the LCPUFAs. Because tissue accumulation of the fatty acids is more efficient from preformed DHA and ARA than from their 18-carbon parent fatty acids, it is important that the mother provide sufficient amounts of these LCPUFAs. There is strong evidence that the amount of

LCPUFAs transferred to the fetus is strongly related to maternal plasma phospholipid concentrations and dietary intake (41-44).

Because the fetus relies on maternal supply of LCPUFA, there are many maternal factors that may negatively impact the amount that is transferred. Animal studies investigating the influence of maternal insulin resistance on substrate transfer found an overall decrease in PUFA status in the presence of diabetes (61). In human models, decreased insulin sensitivity has been associated with decreased PUFA concentrations in skeletal muscle (62) and plasma phospholipids (63). Insulin resistance is often seen in conditions of diabetes and obesity and it is normal to see decreased insulin sensitivity during pregnancy. There is great concern considering the 69% increase in prepregnancy obesity reported between 1993 and 2003 (57). If maternal LCPUFA supply is inadequate, the consequences could be deleterious since these nutrients are important for proper fetal growth (34, 75) and are significantly related to anthropometric measures (35) and neurological conditions (36) at birth.

This study lends support to findings that BMI is a predictor of maternal  $\omega$ 3 LCPUFA concentrations. However, these data are limited by several factors. Serum is not the preferred sample for assessing fatty acid status as it is a reflection of both dietary intake and fatty acids of cell membranes rather than fatty acids of the membrane phospholipids that are known to reflect fatty acids of tissues cells, i.e. fatty acid status (76). In addition, ideally, blood should have been drawn after a fast. Also, the maternal BMIs provided were calculated well into pregnancy at approximately 13-18 weeks gestation. This BMI is likely to represent baseline BMI plus the amount of weight the mother gained up to that point in pregnancy. Perhaps a more accurate BMI would have been calculated before pregnancy, which would represent baseline BMI. Finally, these results are only applicable to women of similar age, race, parity, and health status. Based on these findings we conclude that women with increased BMI may be at risk for inadequate

PUFA supply to the fetus. More studies are needed to determine the impact of maternal BMI and insulin resistance on placental transfer of LCPUFAs as well as the physiologic mechanisms causing these results.

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## VITA

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