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Polyunsaturated fatty acid content of breast milk from women with and without gestational diabetes mellitus

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**POLYUNSATURATED FATTY ACID CONTENT OF BREAST MILK FROM WOMEN
WITH AND WITHOUT GESTATIONAL DIABETES MELLITUS**

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The School of Human Ecology

by

Julissa Marisel Salguero
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LIST OF ABBREVIATIONS

ALA	Linolenic Acid
ARA	Arachidonic Acid
DHA	Docosahexaenoic Acid
EFA	Essential Fatty Acids
EPA	Eicosapentaenoic Acid
ESPGAN	European Society of Paediatric Gastroenterology and Nutrition
FAME	Fatty Acid Methyl Ester
GC	Gas Chromatography
GDM	Gestational Diabetes Mellitus
HPL	Human Placental Lactogen
IDDM	Insulin Dependent Diabetes Mellitus
IR	Insulin Resistance
LA	Linoleic Acid
LC-PUFA	Long Chain Polyunsaturated Fatty Acids
MCFA	Medium Chain Fatty Acid
PS/DHA	Prenatal supplement with DHA
PUFA	Polyunsaturated Fatty Acids

ABSTRACT

Reduced insulin sensitivity and abnormal nutrient metabolism in gestational diabetes mellitus (GDM) may compromise polyunsaturated fatty acid (PUFA) content of breast milk. The purpose of the present study was to evaluate and compare PUFA milk composition of women with and without GDM. Breast milk collections were performed in GDM (n=6) and controls (n=12) at weeks 2, 6, 10 and 12 postpartum using a hospital grade breast pump. Estimated dietary intake of PUFA and use of prenatal supplements containing DHA (PS/DHA) was determined by 24-hour dietary recalls conducted after each milk collection.

Based on these preliminary data, milk docosahexaenoic acid (DHA; C22:6n-3) concentration in milk of women with GDM not consuming PS/DHA had a tendency to be lower compared to controls at all weeks, and the n-3 to n-6 long chain fatty acid ratio was lower at 6 and 10 weeks. There were no differences between groups for other milk PUFAs. Further, women with GDM and controls who consumed PS/DHA during lactation had higher milk DHA compared to women not consuming PS/DHA at weeks 2 and 6 for GDM and week 12 for controls. Milk eicosapentaenoic acid (EPA; C20:5n-3) was higher at weeks 2, 6, and 10 in women with GDM consuming PS/DHA and weeks 10 and 12 for controls.

Dietary + supplement linoleic acid (LA; C18:2n-6), linolenic acid (ALA; C18:3n-3), DHA, EPA, and n-3/n-6 were correlated with breast milk content of these fatty acids. Dietary ALA and LA did not correlate with milk DHA and arachidonic acid (ARA; C20:4n-6). Interestingly, there was an inverse association between 1 hour postprandial glycemia during pregnancy and average milk DHA over the four time points.

The current investigation points to lower milk DHA concentration in women with GDM who did not consume PS/DHA compared to controls not consuming PS/DHA. Based on these

findings, and an earlier report of low DHA concentration in infants born to women with GDM, the importance of DHA supplementation during breast feeding for women with GDM is underscored.

CHAPTER 1

INTRODUCTION

Breast milk from healthy lactating women provides the neonate with the necessary fats for early infant growth and development. The lipids supplied through breast milk provide the infant with energy necessary for early rapid growth, lipid soluble vitamins and long chain fatty acids that are essential for infant growth and visual and neurological development. However, several factors can affect lipid content of breast milk, including lactation period, gestational age, maternal diet, and maternal metabolic diseases, such as diabetes (1).

Diabetes, specifically gestational diabetes mellitus (GDM), is an increasing medical problem that affects between 4 to 12 % of all pregnancies depending of the population studied (2). Its prevalence rate in the US are steadily increasing from 1.9% in 1989-1990 to 4.2% in 2003-2004, a 2.2 fold increase (3). Annually, 200,000 cases are diagnosed representing approximately 7 % of all pregnancies (4). GDM is defined as glucose intolerance with first diagnosis during pregnancy. Even though the exact cause of GDM remains unclear, decreased maternal insulin sensitivity before pregnancy coupled with insufficient insulin response during pregnancy appear to be the principal pathophysiological mechanisms that explain the development of GDM (5). Previous studies report that conditions characterized by insulin deficiency, such as insulin dependent diabetes mellitus (IDDM), may impact breast milk content of the long chain polyunsaturated fatty acids (LC-PUFAs), including docosahexaenoic acid (DHA, C22:6n-3) (6). DHA is an n-3 fatty acid important to the development of infants, particularly visual and brain development. It comprises approximately 40 % of the polyunsaturated fatty acids (PUFA) of the brain and 60 % of the PUFA in the retina. After birth, exclusively breast fed infants rely on breast milk as their only source of DHA and other fatty

acids. Therefore, alterations in LC-PUFA content of breast milk in fully breast fed infants would significantly impact infant accretion of these essential fatty acids needed for optimal visual and neurological development.

Justification

DHA and arachidonic acid (ARA C20:4n-6) are the LC-PUFA found in highest concentrations in human milk, and are necessary for early visual and neurological development and growth of the infant. ARA, an n-6 LC-PUFA, generally does not pose a problem in terms of adequacy since it is widely found in the maternal diet. However, the intake of DHA and other n-3 long chain fatty acids may be compromised in populations with low marine food intake such as witnessed in western countries.

Previous studies report that IDDM may impact breast milk content of LC-PUFAs such as DHA (6). Bitman et al (7) found abnormalities in mammary lipid metabolism of a mother with IDDM. They found increased amounts of free fatty acids, lower levels of cholesterol and medium chain fatty acids, and an increase in the 18:3n-6, 18:3n3, 22:5n6, and 22:5n3 LC-PUFAs of breast milk of a diabetic mother. On the other hand, Jackson et al (6) reported lower concentrations of the LC-PUFAs in both the n-3 and n-6 series in breast milk of women with IDDM from days 14 to 84 postpartum, suggesting that insulin deficiency may reduce metabolism of the shorter chain fatty acids to their longer chain derivatives, including DHA and ARA.

The importance of breast milk fatty acid concentration with respect to LC-PUFAs is highlighted by the report that infants of women with GDM are born with DHA concentrations that are approximately one-half of those of infants of women without GDM (8). Based on these studies, insulin resistance coupled with perturbation in placental transfer of fatty acids in GDM underlines the importance of establishing if breast milk of women with GDM is a reliable source

of adequate LC-PUFA for the newborn of these women. Although there are studies that report on the n-3 and n-6 fatty acid content of breast milk of healthy women and women with IDDM, research on the fatty acid content of breast milk of women with GDM is lacking. Based on the foregoing, it is hypothesized that the breast milk of women with GDM will have fatty acid concentrations that differ from the content of milk of women without GDM.

Research Question

Is the PUFA content of breast milk of women with GDM lower than the PUFA content of breast milk of women without GDM?

Objectives

1. To determine the PUFA content of breast milk of women with and without GDM
2. To compare the PUFA content of breast milk of women with GDM to the PUFA content of breast milk of women without GDM

Limitations

1. Dietary intakes were assessed using 24 hour recall these rely on the memory of participants and are subject to reporting errors.
2. Limited number of women with GDM at this point in the study.
3. Limited number of women with GDM during lactation taking a prenatal supplement that contained DHA.

CHAPTER 2

REVIEW OF LITERATURE

Lipids

Lipids are a group of compounds that share the property of insolubility in water but solubility in organic solvents. They differ appreciably in size and polarity and vary from hydrophobic triglycerides to more water-soluble lipids such as phospholipids (9). Chemically, fatty acids are carboxylic acids with generally unbranched hydrocarbon tails. Lipids' hydrocarbon tails have an even number of carbons atoms and differ in the number and arrangement of double bonds along the hydrocarbon chain. Usually, fatty acids are identified by the position of the first carbon of a double bond with respect to the methyl terminus. The term "n" or "omega (ω)" is used to indicate distance of the first bond from the methyl end of the hydrocarbon chain (9). For instance, DHA is a 22 carbon n-3 fatty acid with six double bonds in its hydrocarbon tail, which has its first double bond at the third carbon with respect to the methyl terminus. Furthermore, fatty acids are classified by the number of double bonds within their hydrocarbon chain. Fatty acids without a single double bond in their skeleton are called saturated fatty acids, the ones containing one single double bond or more than one double bond are called monounsaturated and polyunsaturated, respectively. A fatty acid must be at least 12 carbons long to contain a single double bond. A maximum of 6 double bonds occurs in the dietary fatty acids. Double bonds naturally occurring in the foods we consume exist primarily in the *cis* configuration (9).

Essential Fatty Acids

Burr in 1929 showed the nutritional importance of a specific fatty acid in the diet. He proved that the impaired growth, scaly skin, tail necrosis, and increased mortality that were a

consequence of feeding weanling rats on a fat-free diet could be reversed by feeding them linoleic acid (LA, C18:2n-6), an n-6 fatty acid (10). Essential fatty acids (EFA), LA and linolenic acid (ALA, C18:3n-3), cannot be synthesized by mammals and must be supplied through the diet. These fatty acids are considered indispensable because humans are not able to synthesize them. Humans can only insert double bonds at the n-9 position or higher from the methyl end. Therefore, n-6 and n-3 fatty acids cannot be synthesized by humans and must be obtained from the diet to prevent deficiencies (9).

After EFAs are consumed a portion of these fatty acids is used to provide energy and the rest is metabolized to LC-PUFA, which are later incorporated into structural lipids, such as phospholipids. LA is metabolized to ARA and ALA to DHA, and eicosapentanoic acid (EPA, C20:5n-3) as a product of a sequence of desaturation and elongation steps that occur mainly in the liver (11). Interestingly, these two EFAs (LA and ALA) share the same set of enzymes for their metabolism to the longer chain derivatives, competing for the same enzymatic pathway. As a consequence, higher intakes of n-6 PUFA compromise the metabolic synthesis of n-3 PUFA. It appears that the conversion efficiency among EFA differ considerably among species and seems to be relatively inefficient in humans, especially in early life. It has been estimated that 23% of plasma ARA in term infants derives from LA and that infant microsomes are capable of desaturating LA and ALA (12). However, the endogenous synthesis may not be sufficient to meet the infant's DHA high demands for growth and maturation of central nervous system. Humans, including newborn infants, convert less than 1% of dietary ALA to DHA, and increasing maternal dietary ALA has not resulted in an increase in the transfer of maternal DHA to the infant, either before birth or through increasing secretion in milk (13). Dietary LC-PUFAs are incorporated into structural lipids 20 times more efficiently than after its endogenous

synthesis from their 18-carbon precursors (9). Therefore, it appears that an adequate maternal DHA intake is necessary to ensure an optimal DHA supply to the fetus and infant.

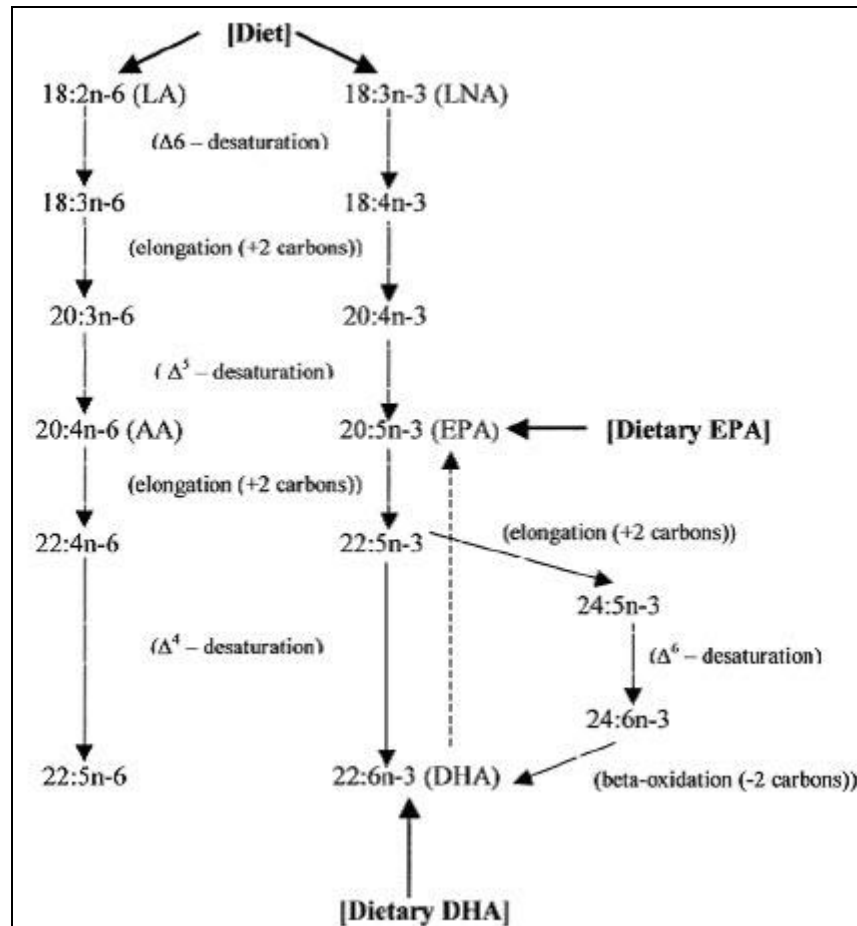


Figure 1. Metabolism of Omega-6 and Omega-3 Fatty Acids. Source: DHA/EPA Omega-3 Institute (14).

Fats in Human Milk

In 1977 the European Society of Paediatric Gastroenterology and Nutrition (ESPGAN) reported that breast milk from healthy and well-nourished women is the ideal method of feeding for healthy infants for the first 6 months after birth (15). This recommendation is based on the acknowledged benefits of breast feeding to infant health and nutrition: gastrointestinal function;

immunological defense; neurodevelopment; and psychological, economic, emotional, and environmental well-being (16). This human fluid provides to the infant a diverse group of substances, including specific proteins, easily digested fats, lactose, minerals, vitamins, antimicrobial and anti-inflammatory factors, growth factors, hormones, digestive enzymes and other protective agents that promote normal growth and development of the infant. Furthermore, breast milk composition changes during lactation to meet the higher needs of the growing infant (17). Colostrum is the milk produced during the first several days following delivery. This yellowish fluid is high in protein, fat soluble vitamins, minerals, and electrolytes and provides the infant with immunoglobulins that protect against environmental agents. Transitional milk begins from approximately 7 to 14 days postpartum. Its composition is lower in immunoglobulins and total proteins compared to colostrum but higher in the amounts of lactose and fat that provide the infant with the necessary energy for rapid growth. Finally, mature milk begins at about 2 weeks postpartum and continues throughout lactation until about 7 or 8 months. The main mature milk component is water (87%) followed by fat; it contains lactose as the main carbohydrates and protein constitute 0.9 % of mature milk including casein, serum albumin, immunoglobulins, and other glycoproteins. Mature milk's average energy content is 75 kcal/dL and it also provides vitamins, minerals, hormones, growth factors and protective agents (17).

The fat content of breast milk ranges from 2.9% in colostrum to 3.8% in mature milk and accounts for 40-50% of the total energy consumed by the infant (18). Lipids in breast milk are present as lipid globules with an average diameter of 3-5 μm (19). The fat globule core is coated with phospholipids, cholesterol, proteins and enzymes as a bipolar layer that prevent milk from separating into two phases, liquid and fat. Triacylglycerols account for 98% of the lipid content in breast milk and are present in the core of the fat globule. The fatty acids attached to the

glycerol backbone of the triacylglycerols usually give the triacylglycerol its composition and are easily modified by diet (19).

Factors Affecting Human Milk Lipid Content and Fatty Acid Composition

Several factors influence the fat content and fatty acid composition of human milk, including stage of lactation, diurnal variation, maternal parity, gestational age at delivery, and maternal diet (19).

Stage of Lactation

Milk composition changes considerably during the first day after birth. Milk secretion changes from colostrum to mature milk in the course of lactation to ensure an adequate provision of nutrients to the developing infant. The amounts of lactose and fat increase during the first weeks after birth, while the protein content decreases as lactation progresses (19). Furthermore, the EFA content in human milk increases, whereas the percentages of LC-PUFAs decrease markedly during the first month of lactation. It has been estimated that ARA decreases by about 38% and that of DHA by about 50% during the first month of lactation (1). The high content of LC-PUFAs in colostrum might benefit the newborn infant immediately after birth when the infants' requirements for LC-PUFA are high for continued visual and neurological development.

Diurnal Variation

Fat is the most variable nutrient in human milk. Its concentration varies substantially during a feed and throughout the day. Typically, lipid content rises during a nursing and has been found to be 2-3 times higher in hindmilk than in foremilk with a complete expression of the breast (1). Furthermore, the lipid content changes throughout the day with a rise from early morning and plateau about mid day (20). Lammi-Keefe et al (21) reported that from a group of lactating women with breast milk collection performed every 4 hours (0600, 1000, 1400, 1800,

and 2200 h) lipid content tended to be higher in the 1000– hour samples. These diurnal changes can be attributed in part to the amount of time elapsed between nursings, the degree of emptying of the breast within each nursing, which if not emptied completely may allow for a carryover of the high-fat hindmilk to the next nursing, and the interval of sampling (19).

Maternal Parity

The composition of human milk seems to be affected by both parity and the age of the lactating mothers. Primiparous young women tend to have higher milk concentration of certain nutrients, such as protein and fat, than mothers of parity 9 or greater (19). Prentice et al (22) found that parity had a marked influence on breast milk fat concentration from Gambian mothers. They reported that primiparous mothers had milk fat concentrations that were 23% higher than milk fat of mothers with parity greater than 3. Further, infants from primiparous mothers seemed to consume 40% more fat per day than infants born to mothers of parity 4-9 and 80 % more than infants born to mothers of parity 10 or higher (22). It is hypothesized that the mammary gland secretory function may deteriorate with increasing parity and that a reduction in the availability of essential nutrients in women with several parities may limit the lactation performance (22).

Gestational Stage at Delivery

Milk from mothers giving birth prematurely has been reported to have a greater concentration of nitrogen, immune proteins, lipid, medium-chain fatty acids (MCFA), energy, vitamins and some minerals when compared to term milk at similar lactation stages (19). Bitman et al (23) found that breast milk from women delivering prematurely (31-36 weeks) had a higher content of the medium-chain fatty acids, cholesterol, and phospholipids compared to women delivering at term, and the colostrum LC-PUFAs content was higher in mothers of preterm

infants. However, these compositional differences between preterm milk and term milk are not consistent in all studies. Genzel-Boroviczény and Koletzko (24) concluded that preterm milk contained a significantly higher amount of the MCFA compared to term milk, but there was no difference in DHA and AA content at days 5 and 10 postpartum. These findings raised concern since preterm have a higher demand for nutrients, including LC-PUFAs, compared to term infants, suggesting that the milk of mothers of preterm infants may not be better suited to meet the high LC-PUFA demands of their infants during the first weeks after birth (24). The lack of consistency among investigators evaluating nutrient content of breast milk of mothers of preterm infants has been attributed to differences in sample collection procedure, wide ranges of gestational stage, and a high degree of interindividual variability in milk composition in preterm compared to term milk (19).

Maternal Diet

Diet has the greatest effect on human milk composition. Maternal diet high in carbohydrates increases mammary gland synthesis of C8:0-C14:0 resulting in a 10 to 20% increase of these fatty acids in milk. However, high-fat or fasting maternal diets result in decreased breast milk content of the C8:0 to C14:0 probably due to the inhibitory effect that the free fatty acids exert over acetyl Co-A carboxylase (12). The polyunsaturated fatty acid content of human milk can be modified by altering the type of fats in the maternal diet. A study in which the diets of lactating women were supplemented with six different test fats (herring oil, Menhaden oil, safflower oil, canola oil, coconut oil, and cocoa butter), and each one of the fats were consumed once at a 2 week interval, resulted in an acute change in milk fatty acid composition within 6 to 24 hours after ingestion and remained significantly elevated for 1- 3 days (25). Populations whose diets are rich in marine foods show higher DHA and EPA breast

milk concentrations compared to western populations with low seafood intake (12, 26). The mean DHA concentration in milk of Chinese women living on the island of Zhangzi is 2.78 wt%, much higher than the 0.45% mean content reported for women from western populations who usually have a low marine food intake (Table 1) (12, 27) Furthermore, supplementation of 200 mg of DHA for 2 weeks in lactating women, from weeks 4 to 6 postpartum, has been shown to be effective to increase DHA content in breast milk by almost 2 fold compared to a placebo group (0.37% vs. 0.21%) (27).

Table 1 Fatty acid composition (wt%) of milks from women consuming western and non-western diets.

FA	Western		Non-Western	
	Milk (wt %)	Diet (g/day)	Milk (wt %)	Diet (g/day)
10:0	1.42	0.06-2.39	1	0.10-0.59
12:0	5.67	1.70-12.32	6.14	2.40-16.51
14:0	6.58	1.98-11.78	7.22	5.30-15.90
16:0	21.58	19.25-25.10	19.60	14.10-25.77
18:0	6.04	5.83-9.70	5.90	0.80-8.20
18:1	31.08	22.63-38.70	27.10	17.93-47.00
18:2	11.73	9.57-16.80	18.14	8.84-23.80
20:4n-6	0.42	0.36-0.68	0.92	0.09-0.70
18:3n-3	1.08	0.31-1.85	2.07	0.10-0.98
20:5n-3	0.09	0.00-0.16	0.52	0.05-1.10
22:6n-3	0.45	0.10-0.56	0.88	0.10-1.40

Adapted from Jensen et al (12, 28) Table 31 in reference 10. Table 35-37 in reference 26. Milk from Canadian, French, Spanish, Dutch, German, Australian women for western diets. Milk from Japanese, Nigerian, Israeli, Sudanese and Chinese mothers for non western diets.

Maternal Metabolic Disorders

Maternal metabolic diseases, such as IDDM, alter fatty acid metabolism, resulting in an altered milk fatty acid composition (1). Higher levels of MCFAs and lower levels of the total milk LC-PUFAs in both the n-3 and n-6 class have been observed in women with IDDM at 14 to 84 days postpartum compared to a reference group. At 14 days postpartum IDDM women had

lower levels of 20:2n-6, 20:3n-6, 20:4n-6, 20:5n-3, 22:5n-3 and 22:6n-3. The lower LC-PUFA content of breast milk of IDDM women may reflect an altered fatty acid metabolism due to a reduced activity of the Δ -9, Δ -6, and Δ -5 desaturases needed for the metabolism of the EFA to these longer chain derivatives (6).

Human Milk Fatty Acids

Human milk fat contains a variety of saturated, monosaturated and PUFAs. Contrary to the saturated and monosaturated fatty acids, which can be endogenously synthesized from carbohydrates and comprise far more than 80 % of the milk fat, EFA must be supplied through the diet (1). Fatty acids in breast milk triacylglycerols can be obtained from different sources: de novo synthesis in the mammary gland, maternal diet, maternal fat body stores, and de novo synthesis in sites other than the mammary gland. Mammary epithelial cells contain enzymes necessary for the synthesis of MCFA (C8:0 to C12:0) but lack Δ -12 and Δ -15 desaturase enzymes for EFA synthesis (13). As a result, milk EFA reflects maternal dietary intake or mobilization of body fat stores.

Following consumption of preformed DHA and other fatty acids, dietary fatty acids are re-esterified into triacylglycerols in the enterocyte and packaged into secretory units called chylomicrons. These secretory units are later released to the lymphatic system and reach the superior vena cava via the thoracic duct. Chylomicrons travel throughout the circulatory system and lipoprotein lipase, an enzyme located on the endothelial side of vessel tissue, breaks down the triacylglycerols from the chylomicrons releasing fatty acids (9). The mammary gland obtains free fatty acid from the circulation and resynthesizes triglycerides, assembling them into fat globules that are secreted with breast milk (1). Thus, fatty acids supplied with the diet occur to some extent in breast milk. Studies with isotope labeled fatty acids have reported a 30% direct

transfer of dietary LA to milk (29). Milk LC-PUFA composition is markedly influenced by maternal dietary intake of these fatty acids. Studies evaluating the effect of DHA supplementation during lactation have shown that human milk DHA content is very susceptible to maternal diet and increases in a linear dose dependent manner (11). Mobilization of maternal body fat stores has been described as the greater contributor to the fatty acids secreted in breast milk (1). During lactation fatty acids are constantly secreted by adipose tissue as products of cellular triacylglycerol hydrolysis and are transported to the mammary gland bound to albumin. LA is the predominant n-6 PUFA present in adipose tissue accounting for 12-16% of the fatty acids while ALA is the most abundant of the n-3 PUFA and represents 1% of the fatty acids. Interestingly, only very small amounts of DHA and EPA are stored in adipose tissue; pointing to the need of a continuous dietary supply of these fatty acids (11). Hachey et al (30) using isotopically labeled dietary fatty acids during late lactation estimated that 60% of breast milk fatty acids are originated from mobilized maternal fat stores. Furthermore, a relationship between LA content of colostrum and mature milk and white adipose tissue has been reported. However, this relationship was not found for ALA (31). The absence of association between ALA content of breast milk and white adipose tissue may reflect a preference of the mammary gland in taking up the ALA over LA from the circulation. As mentioned earlier, humans are capable of synthesizing LC-PUFA from EFA with the elongation and desaturation steps occurring primarily in the liver. However, studies have consistently shown that only a small proportion of ALA is metabolized to DHA (11). Interestingly, the conversion rate seems to be more efficient in women than in men. Up to 9% of ALA is converted to DHA in women and < 4% in men (9). Further, it has been reported that a significant correlation exists between LCPUFA content of the n-6 series in mature milk and LA content, but this did not hold for colostrum. The difference might be due

to a decrease in the pool size of liver LC-PUFA that occurs as lactation progresses which probably accounts for the high concentration of LCPUFA found in colostrum (31). It seems that the amount of LC-PUFA in human milk may be subjected to some metabolic regulation that is not yet entirely understood.

Breast milk contains a full range of LC-PUFA of the n-3 and n-6 series, such as DHA, EPA and ARA. Human milk contains preformed ARA and DHA in amounts that range from 0.05 to 1.12 wt % and 0 to 2.78 wt % of the total fatty acids, respectively (12). The mean amount of DHA is 0.45 wt % in breast milk for western women and 0.88 wt % for non-western women (Table 1) (12). These amounts vary widely and DHA content in human milk is strongly associated with DHA maternal consumption (12). Several studies have examined the effect of fish oil supplementation on lactating women. Jensen et al (32) reported that increasing through supplementation the maternal intake of DHA in lactating women (230, 170 to 260 mg of DHA/d) resulted in an increased secretion of DHA in milk in the three supplemented groups. Further, breast fed infants born to those supplemented lactating women had higher phospholipid DHA (11-42%) compared to a placebo group. Henderson et al (33) showed that after supplementing 5 lactating women with 6 g of fish oil daily for 21 days, milk n-3 fatty acid content increased within 8 hours and the n-6 fatty acid content decreased. Further, maternal erythrocyte DHA increased from 1.4% to 2.2 % and infant erythrocyte DHA from 0.30% to 0.78%.

Requirements of n-6 and n-3 Long-Chain Polyunsaturated Fatty Acid for the Term Infant

ARA and DHA availability to the growing fetus and infant is important because this is the period of most rapid brain growth and development. Human brain is at its maximum growth rate during the last three months of pregnancy and first 18 months of life; therefore, the third

trimester fetus and neonates are particularly vulnerable to developmental deficit if DHA accretion in these particular stages is limited (13). These two LC-PUFAs, ARA and DHA, are selectively incorporated, retained and highly concentrated in brain and retinal neural membranes. Their incorporation into the cell membrane affects the cell properties such as fluidity, permeability for metabolite exchange, activity of membrane bound enzymes and receptors, and electrical and humoral signal transduction necessary for normal cell and tissue functioning (1). Moreover, LC-PUFAs are precursors of eicosanoids, important modulators of a variety of essential biological processes, and they act as ligands for membrane receptors and transcription factors that regulate gene expression (13).

The brain is considered the organ with the highest lipid content in the body and is 60% lipid by dry weight. On average, DHA accounts for 40% of the PUFAs in the brain, 60% of the PUFAs in the retina, and almost 50% of the weight of the neuronal membrane reflecting the importance of an adequate DHA accretion early in life necessary for normal visual and neurological development of infants (34). *In utero*, the fetus ARA and DHA demands are met by transfer of the LC-PUFAs through the placenta, relying completely on maternal supply of DHA and ARA from lipid stores, maternal diet and nutritional supplements. After birth, the high demand of the LC-PUFAs in fully breast fed infants is met through breast milk. Therefore, an adequate content of LC-PUFA in breast milk is necessary to ensure a sufficient provision of DHA and ARA to the growing infant. ARA content in breast milk usually does not pose a problem in terms of adequacy since it is widely distributed in the maternal diet. Usual ARA intakes for adults consuming a western diet, which are high in animal products such as eggs, organ meat, and other meat, have been estimated to be between 50 and 300 mg/d (35). On the other hand, DHA content in breast milk may be compromised especially in populations with low

marine food consumption. In North America, at least 50% of pregnant and lactating women consume <100 mg DHA/d (13). Glew et al (36) reported an average daily intake from food of 47 mg among lactating women in New Mexico. This insufficient intake of DHA during pregnancy and lactation may compromise the DHA accretion for the infant early in life. Furthermore, a desirable ratio between LA and ALA of 5:1 to 15:1 in breast milk has been established (12).

Gestational Diabetes Mellitus

Gestational diabetes mellitus (GDM) is defined as “glucose intolerance with onset or first recognition during pregnancy”(37). Its prevalence rate in the US is steadily increasing from 1.9% in 1989-1990 to 4.2% in 2003-2004, a 2.2 fold increase (3). Annually, 200,000 cases are diagnosed representing approximately 7 % of all pregnancies (4). This condition affects African Americans, Hispanic/Latino Americans, and American Indians more frequently, and obesity and family history of diabetes increase its risk. GDM usually resolves after pregnancy in 90 % of women. Nevertheless, women with a history GDM have a 20 to 50 % chance of developing type 2 diabetes in the next five to 10 years following pregnancy (2).

Pathophysiology

The exact cause of GDM remains unclear; however, it seems that an under recognized insulin resistance previous to pregnancy coupled with insufficient insulin response are the main pathophysiological mechanisms for the development of this condition (5). Pregnancy is characterized by insulin resistance (IR) that begins near mid-pregnancy and is accentuated in the third trimester. It has been estimated that insulin sensitivity decreases ~50 - 70 % in late pregnancy. IR during normal pregnancy results in changes in carbohydrate and lipid metabolism that ensure a continuous supply of nutrients to the growing fetus. Increased adipose depot and high levels of pregnancy related hormones, such as human placental lactogen (HPL), estrogen,

and progesterone, are thought to be responsible for the changes in lipid and carbohydrate metabolism seen in late pregnancy. HPL inhibits peripheral uptake of glucose in the pregnant women and if the pancreas is not able to compensate, by increasing insulin secretion, GDM is likely to result (5, 38).

Diagnosis

Currently, screening for GDM is routinely practiced at the first prenatal visit in all pregnancies and a second evaluation is undertaken at weeks 24 to 28 of gestation. Women at higher risk for GDM, including those with marked obesity, previous history of GDM, family history of diabetes, previous macrosomia, polycystic ovarian syndrome, and glycosuria should be tested as soon as possible. Women of average risk for GDM are required to complete an oral glucose challenge test (OGCT) at weeks 24-28 of gestation. A 50 g glucose load is given orally and serum glucose concentration is evaluated 1 hour after the ingestion. If glycemia is ≥ 140 mg/dL, an oral glucose tolerance test (OGTT) is administered after 3 days of an unrestricted carbohydrate diet. During the OGTT, women are asked to fast for at least 8 hours before the test and the fasting glucose level is evaluated initially. A 100 g oral glucose drink is given and blood is drawn at 1, 2, and 3 hours later to evaluate serum glucose concentration after the glucose oral load. GDM is diagnosed if at least 2 of the glycemia values are $\geq 180, 155, 140$ mg/dl at 1, 2, 3 h, respectively (39). Women at high risk for GDM that present symptoms of hyperglycemia may be diagnosed with GDM with a random blood glucose level ≥ 200 mg/dL or a fasting plasma glucose level > 126 mg/dl. The diagnosis must be confirmed on a subsequent day (40, 41).

Complications

GDM may be accompanied by chronic hyperglycemia and hyperinsulinemia that have serious health implications for the mother and developing fetus. The maternal complications

usually associated with GDM include hypertensive disorders (preeclampsia), polydramnios (excess amniotic fluid in the amniotic sac), difficult and long birth, preterm delivery (before 38 weeks gestation), and a higher rate of cesarean sections. The latter complication may result from excessive fetal growth usually seen in mothers who developed GDM. Furthermore, women with GDM are at increased risk for the development of diabetes, usually type 2, after pregnancy. The risk is particular high in women with persistent hyperglycemia, obesity, and an early diagnosis, earlier than 24 weeks of gestation, of GDM (40, 42).

Fetal and neonatal complications of GDM include macrosomia, hypoglycemia, respiratory distress syndrome, hypertrophic cardiomyopathy, hypocalcemia, hyperbilirubinemia, and polycythemia. Macrosomia is the most common sign of persistent hyperglycemia in GDM. The persistent fetal exposure to hyperglycemia during pregnancy results in an excessive glucose delivery to the fetus, and the fetus responds by increasing insulin secretion which stimulates fetal body growth. As a consequence of the increased body size, macrosomia increases the risk of birth injuries (shoulder dystocia) and asphyxia during labor (40). Macrosomia occurs in 15–45% of infants exposed to hyperglycemia during pregnancy and large for age infants are defined as those with a birth weight greater than the 90th percentile for gestational age and sex or a birth weight >4,000 – 4,500 g at birth. Moreover, infants born to women with GDM have an increased risk of diabetes and obesity in childhood and adulthood (42). A study conducted among Pima Indians suggests that fetal exposure to a persistent high glucose environment in *utero* may be associated with the shift of onset of diabetes to a younger age in this ethnic group (43).

Possible Association between DHA Content in Breast Milk and Gestational Diabetes Mellitus

Lactation is a physiological process with high nutrient demands. These high nutrient requirements are met mostly by increasing food intake and by some metabolic adaptation that

facilitates the availability of extra nutrients required for lactogenesis (44). Interestingly, during lactation there is an increased number of insulin receptors in the mammary gland and insulin seems to act as a physiological regulator of lipogenesis (44). Burnol *et al* (45) reported that insulin deficiency in lactating rats reduces the lipogenic activity in the mammary gland by decreasing the activity of the enzyme phosphofructokinase, which is necessary for glucose metabolism; or by reducing the activity of acetyl-CoA carboxylase, the regulatory enzyme in fatty acid synthesis. Furthermore, insulin sensitivity apparently influences the activity of enzymes necessary for essential fatty acid metabolism, such as elongases and desaturases. Studies of insulin deficiency indicate that insulin has an important effect on fatty acid desaturase activity. Faas *et al* (46) reported that streptozotocin diabetic rats had a lower $\Delta 6$ and $\Delta 9$ desaturase activity evidenced by liver microsomal fatty acid composition with a decreased proportion of palmitoleic, oleic, and arachidonic acids and an increased proportion of linoleic acid. These altered fatty acid levels were returned to normal when insulin treatment was initiated. It seems that when insulin treatment was initiated the $\Delta 6$ and $\Delta 9$ desaturase activity was restored. It has been recognized that pathologies characterized by IR and hyperglycemia, such as obesity and cardiovascular disease, impair $\Delta 5$ and $\Delta 6$ desaturase and elongase activity. Borkman *et al* (47) reported that patients with coronary artery disease undergoing an elective coronary artery bypass surgery had a negative association between fasting serum insulin level and the percentage of LC-PUFA in the phospholipid fraction of muscle, specifically ARA. Furthermore, Wijendran *et al* (48) found that cord vein erythrocyte phospholipid content of ARA and DHA were significantly lower in women with GDM compared to healthy pregnant women. In addition, maternal plasma phospholipid DHA was negatively associated with the mean fasting plasma insulin levels, probably as a consequence of an impaired desaturase activity which has

been documented for situations of insulin resistance, such as GDM. To summarize, based on the foregoing, conditions characterized by insulin deficiency or resistance result in an impaired activity of desaturases and elongases necessary for the metabolism of the EFA to their longer chain derivatives (46, 48, 49).

CHAPTER 3

EXPERIMENTAL DESIGN AND METHODS

Study Design

A longitudinal study from weeks 2 to 12 of lactation among women with and without GDM was conducted to determine if differences exist in the breast milk fatty acid content between groups. Breast milk samples were collected at 4 different times (weeks 2, 6, 10, and 12) postpartum for analysis of fatty acid composition. A 24-hour recall was conducted at the end of each visit and information relating to blood glucose control during pregnancy, pregravid body mass index, maternal weight and height, parity number, gestational length, medications, pregnancy complications and infant statistics was obtained from each subject's medical chart by contacting the subjects' physicians. A completed health history form (Appendix A) with the participant's medical information was completed by each subject nurse and faxed from the physician office to the laboratory.

Subject Recruitment

Pregnant women planning to breast feed for at least 12 weeks were recruited from the Louisiana State University (LSU) campus and local physician offices by public announcement (flyers and brochure advertising the study; Appendix B), word of mouth, or through a Woman's Hospital reference list for pregnant women who indicated an interest in being contacted for research projects. Each subject on the list was contacted by phone to inform them about the study and, if they indicated interest, they were sent an informational packet via e-mail or regular mail. Women interested in participating were contacted one week before the expected delivery date to schedule their first breast milk collection appointment. On site and in home visits were offered to all subjects.

Subject Description

Pregnant women with and without GDM, between the ages of 18 and 38 and who were planning to breast feed for a minimum of 12 weeks were recruited in their last trimester of pregnancy. GDM diagnosis was confirmed by review of the medical information obtained from each subject's physician. Pregnant women with complications, such as preeclampsia or eclampsia; treatment with insulin; premature delivery; or those who had been pregnant or breast feeding in the last year, were excluded from the study. Women who met the inclusion criteria and agreed to participate signed an informed consent form which had been approved by the Louisiana State University AgCenter Institutional Review Board (IRB). By consenting, women agreed to provide breast milk samples using a hospital grade breast pump (Medela Lactina Select) at weeks 2, 6, 10, and 12 postpartum, to complete a health history by interview, and provide permission to contact their physician for access to their medical chart for the purpose of retrieving information about diabetes control, gestational length, laboratory values, medications, infant statistics, and any pregnancy complications.

Sample Collection

The subjects were visited on 4 occasions by members of the research team to collect breast milk samples at 2, 6, 10, and 12 weeks postpartum. Appointments were made in advance and collections took place at the subject's home or at the Human Ecology Nutritional Assessment Laboratory, Knapp Hall at LSU. The researchers traveled with all the necessary equipment (hospital grade breast pump, breast pump accessories, collection bottles, wash cloths, and latex gloves) to successfully perform milk collections in the morning or mid day. At each visit breast milk samples were collected by obtaining a complete emptying of one breast. A complete breast emptying was achieved when milk no longer flowed in a steady stream

according to Ferris et al (50). Breast milk samples were transported to the laboratory in an ice box filled with ice packs. At the laboratory, samples were warmed to 37 °C in a water bath and dispensed into aliquots in small glass vials with teflon lined caps. Each vial was labeled with a code number, collection date, and visit number. Samples were immediately stored at -80 °C until analysis. On the day of milk collection a 24 hour dietary recall was conducted by telephone using the University of Minnesota Nutrition Data System for Research (NDSR, 2008 version) and the use of dietary supplements was evaluated at each visit.

Sample Analysis

Breast milk samples were swirled gently in a water bath (~37 °C) to mix completely. Total lipids were transesterified with a direct method (51). In brief, 100 µl of milk was placed in a screw top glass tube fitted with a teflon lined top. Two ml of methanol:benzene (4:1) containing 120 µg/ml of 17:0 as an internal standard was added. Fatty acid methyl esters (FAMES) were prepared by adding 200 µl of acetyl chloride slowly over a 1 minute period. Tubes were capped tightly, mixed thoroughly and heated at 100 °C for 1 hour in a heating block. After allowing for cooling, the addition and mixing of 5 ml of a 6% potassium carbonate solution neutralized the mixture and stopped the reaction. The reaction tubes were centrifuged at 4000 rpm for 10 minutes. The FAMES were injected into a Hewlett Packard 5890 gas chromatograph for analysis. The gas chromatograph was equipped with a 30-m fused silica capillary column with an internal standard diameter of 0.25 mm. Helium was used as a carrier gas and the GC was equipped with a flame-ionization detector. The chromatographic conditions were set as follows: oven temperature was programmed from 150 °C to a final temperature of 210 °C at a rate of 2 °C/minute with a final hold of 20 minutes. The injector and flame ionization detector temperatures were maintained at 200 °C and 250 °C, respectively. The inlet split ratio was 1:50.

Fatty acids were identified by retention times and are expressed as a relative weight percent (wt%).

Statistical Analysis

Data were analyzed using Statistical Analysis Software (SAS, version 9.1). Differences at weeks 2, 6, 10 and 12 postpartum between GDM and control groups were determined using the least significance difference (LSD). A two-factor repeated-measures analysis of variance (ANOVA) using PROC GLM was used to evaluate group differences in the fatty acid composition of milk of women with GDM and control over time. The repeated-measures analysis of covariance (ANCOVA) using PROC MIX was used to evaluate the effect of covariates, such as LC-PUFA intake (dietary and supplement). The Relationship between milk LC-PUFA and dietary LC-PUFA was evaluated by calculating Pearson correlation coefficient.

CHAPTER 4

WOMEN WITH GESTATIONAL DIABETES MELLITUS TEND TO HAVE BREAST MILK WITH LOWER CONCENTRATION OF DOCOSAHEXAENOIC ACID COMPARED TO WOMEN WITHOUT GESTATIONAL DIABETES MELLITUS

Introduction

Long chain polyunsaturated fatty acid (LC-PUFA) accretion early in life, specifically docosahexaenoic acid (DHA, 22:6n-3), is essential for a normal visual and neurological development of the infant. DHA, an n-3 LC-PUFA, is highly concentrated in brain and retinal neural membranes and its incorporation in neural tissue modulates its functional activity (1). As a result, adequate supply and delivery of this fatty acid is indispensable in the last trimester of pregnancy and the first two years of life when brain growth is at its maximum rate (52). DHA supply to the fetus and infant is achieved through maternal transfer via the placenta at an accelerated rate in the last trimester of pregnancy and breast milk during the first months of life.

Conditions shown to affect the delivery of DHA to the developing fetus during pregnancy and to infants after birth, include gestational diabetes mellitus (GDM) (48) and insulin dependent diabetes (IDDM) (6, 48). Wijendran et al (48) demonstrated decreased DHA and arachidonic acid (ARA, 20:4n-6) in cord vein erythrocyte phospholipids in women with GDM compared to controls, suggesting an inadequate accretion of these fatty acids in the fetus in the last trimester of pregnancy. Jackson et al (6) reported lower concentrations of both n-3 and n-6 series of LC-PUFAs in breast milk of women with IDDM from days 14 to 84 postpartum. At 14 day postpartum, IDDM women had lower levels of 20:2n-6, 20:3n-6, 20:4n-6, 20:5n-3, 22:5n-3, 22:6n-3 compared to controls concluding that a possible altered fatty acid metabolism in women with IDDM results in decreased milk LC-PUFA. GDM is a metabolic condition diagnosed first

during pregnancy and characterized by abnormal glucose and lipid metabolism (38). Despite the current recommendation given to women with GDM to breast feed their infants, studies to evaluate the fatty acid composition of breast milk of women with GDM are lacking. The purpose of the present study was to evaluate the fatty acid composition of breast milk of women with and without GDM.

Methods

Subjects and Methods

Six lactating women with GDM and twelve lactating women without GDM (controls) between the ages of 21 and 38 and who were planning to breast feed for a minimum of 12 weeks were recruited from Louisiana State University (LSU) campus and local physician offices by public announcement, word of mouth, or through a Woman's Hospital reference list for women who indicated an interest in being contacted for research projects. Each subject on the list was contacted by phone to inform them about the study and, if they indicated interest, they were sent informational packet via e-mail or regular mail. Women with complications during pregnancy, such as preeclampsia or eclampsia, treatment with insulin, premature delivery, or those who had been pregnant or breast feeding in the last year, were excluded from the study. Women who met the inclusion criteria and agreed to participate signed an informed consent form which had been approved by the Louisiana State University AgCenter Institutional Review Board (IRB). By consenting, women agreed to provide breast milk samples using a hospital grade breast pump (Medela Lactina Select) at weeks 2, 6, 10, and 12 postpartum, complete a health history form by interview, and provide permission to contact their physicians for access to medical chart information for the purpose of retrieving information about diabetes control, gestational length, possible pregnancy complications, and infant statistics.

Sample Collection

Subjects were visited on 4 occasions by members of the research team to collect breast milk samples at 2, 6, 10, and 12 weeks postpartum. Appointments were made in advance and collections took place at the subject's home or at the Human Ecology Nutritional Assessment Laboratory, Knapp Hall at LSU. At each visit, breast milk samples were collected using the breast pump and a complete emptying of one breast was obtained. A complete breast emptying was achieved when milk no longer came out in an even intermittent stream according to Ferris et al (50). Breast milk samples were transported to the laboratory in a lunch box with ice packs. At the laboratory, the samples were warmed to 37 °C in a water bath, swirled to achieve homogeneous mixing, and dispensed into aliquots for storage in scintillation vials with teflon lined caps. All vials were labeled with subject's code, time postpartum and date of collection. Storage was at -80 °C until analysis. On the days of milk collection 24 hour dietary recalls were performed by telephone interview using the University of Minnesota Nutrition Data System for Research (NDSR, 2008 version).

Sample Analysis

Frozen milk samples were swirled gently in a 37 °C water bath to thaw. Total milk lipids were transesterified with a direct method (51). In brief, 100 µl of milk was added to screw top tubes fitted with teflon lined tops. Two ml of methanol:benzene (4:1) containing 120 µg/ml of 17:0 as an internal standard was added. Fatty acid methyl esters (FAMES) were prepared by adding 200 µl of acetyl chloride slowly over a 1 minute period. Reaction tubes were capped tightly, mixed thoroughly and heated at 100 °C for 1 hour in a heating block. After cooling, 5 ml of a 6% potassium carbonate solution was added and mixed thoroughly to neutralize the mixture and stopped the reaction. The tubes were centrifuged at 4000 rpm for 10 minutes. The FAMES

were injected into a Hewlett Packard 5890 gas chromatograph for analysis. The gas chromatograph was equipped with a 30-m fused silica capillary column with an internal standard diameter of 0.25 mm. Helium was used as a carrier gas and the GC was equipped with a flame-ionization detector. The chromatographic conditions were set as follows: oven temperature was programmed from 150 °C to a final temperature of 210 °C at a rate of 2 °C/minute with a final hold of 20 minutes. The injector and flame ionization detector (FID) temperatures were maintained at 200 °C and 250 °C, respectively. The inlet split ratio was 1:100. Fatty acids were identified by retention times and are expressed as relative weight percent (wt %).

Statistical Analysis

Statistical analysis was performed using Statistical Analysis Software (SAS, version 9.1). Differences at weeks 2, 6, 10 and 12 postpartum between GDM and control groups were determined using the least significant difference (LSD). A two-factor repeated-measures analysis of variance (ANOVA) using PROC GLM was used to evaluate group differences in the fatty acid composition of women with GDM and control over time. The repeated-measures analysis of covariance (ANCOVA) was used to adjust for the effect of covariates, such as LC-PUFA intake (dietary and supplement). The relationship between milk LC-PUFA and dietary LC-PUFA was evaluated using Pearson correlation coefficients.

Results

Subject Description

Milk collections at the four time points (1, 2, 6 and 10 weeks postpartum) were made for five GDM and eight controls. Two controls are still in the process of completing the last two collections and one control withdrew from the study, citing insufficient milk production. Women with GDM and controls were comparable in age, gestational length, parity, and pre-pregnancy

BMI. Women with GDM gained less weight during pregnancy compared to controls and had higher 1 hour postprandial glycemia during pregnancy (Table 2). With regard to dietary intake, women with GDM not consuming a prenatal supplement that contained DHA (PS/DHA) consumed 22% fewer calories than controls without PS/DHA but had similar macronutrient distributions (Table 3). LA and ALA accounted for 5.3% and 0.6% of total energy intakes, respectively in non supplementing subjects in both groups. Two of the six GDM women and five of the twelve controls reported taking PS/DHA during the time of the study. Supplementation increased dietary levels of DHA to 560 ± 545 mg for controls and to 196 ± 104 mg for GDM. The n-3 fatty acid content in the prenatal supplements ranged from 100 mg to 470 mg of DHA and from 40 mg to 580 mg of EPA (Table 4).

Table 2 Characteristics of women with and without gestational diabetes mellitus

	Controls (n=12)	GDM (n=6)
Ethnicity (n)		
Asian	0	1
White	8	3
Black	4	2
Age (y)	30 ± 5 [12]	30 ± 4 [6]
Gestational length (weeks)	39 ± 1 [12]	38.7 ± 1 [6]
Parity (n)	2.5 ± 1 [12]	2.3 ± 2 [6]
Pregravid BMI (kg/m^2)	24.7 ± 5 [12]	25.1 ± 7 [6]
Weight gain (kg)	13.9 ± 6 [12] ^a	8.3 ± 5 [6] ^b
1 hour postprandial glycemia (mg/dL)	108 ± 13 [9] ^a	194 ± 12 [6] ^b

Mean \pm SD. Number of subjects in brackets. Means within a row with different superscript letter indicate significant difference, $p \leq 0.07$. GDM, gestational diabetes mellitus; BMI, body mass index.

Breast Milk Polyunsaturated Fatty Acid (PUFA) Composition for Women with GDM and Control

PUFA content of breast milk (wt%) was similar for GDM and controls without PS/DHA, with the exception of DHA (Table 5). Women with GDM not consuming PS/DHA during lactation had lower milk DHA at weeks 6 and 10 compared to controls. However, after

controlling for dietary DHA no significant differences were observed between groups at each time point but milk DHA of women with GDM remained lower compared to controls. n3/n-6 was lower at 6 and 10 weeks. LC-PUFA milk content decreased over the four weeks of study, with a marked decrease from week 2 to 6 postpartum. The mean LA: ALA ratio in breast milk of nonsupplementing GDM and controls was 16 and 14, respectively.

Table 3 Dietary intake of lactating women with and without gestational diabetes mellitus not consuming a prenatal supplement with DHA

	Control (n=7)	GDM (n=4)
Energy (kcal)	2265 ± 745 ^a	1771 ± 474 ^b
Protein (%)	15 ± 4	16 ± 4
Carbohydrate (%)	51 ± 10	53 ± 9
Total fat (%)	33 ± 8	32 ± 7
Saturated fat (g)	30 ± 16	24 ± 9
Linoleic acid (g)	15 ± 8	10 ± 7
Linolenic acid (g)	1.55 ± 1.5	1.13 ± 0.5
Arachidonic acid (g)	0.14 ± 0.09	0.09 ± 0.06
Eicosapentaenoic acid (g)	0.008 ± 0.01 ^a	0.031 ± 0.04 ^b
Docosahexaenoic acid (g)	0.034 ± 0.03	0.04 ± 0.04

Mean ± SD. Means within a row with different superscript letter indicate significant difference, $p \leq 0.05$. GDM, gestational diabetes mellitus.

Table 4 DHA content of prenatal supplements being taken by women with and without gestational diabetes mellitus

DHA (mg)	Control (n)	GDM (n)
< 200 mg	0	1
200-400 mg	5	1
≥ 400 mg	1	0

Women with GDM consuming PS/DHA during lactation had higher breast milk DHA compared to nonsupplementing GDM at weeks 2 and 6 (Table 6). Similarly, controls supplementing with DHA had higher milk DHA levels compared to controls not consuming PS/DHA ($p \leq 0.06$). However, milk DHA was significantly higher only at week 12 compared to

their nonsupplemented counterpart. Milk EPA was also higher in GDM consuming PS/DHA at weeks 2, 6, and 10 compared to GDM without PS/DHA, and this was for controls with PS/DHA at weeks 10 and 12. Milk n-3/n-6 was higher in GDM with PS/DHA compared to GDM without PS/DHA at weeks 2, 6, and 10. Controls taking PS/DHA during lactation also showed higher milk n-3/n-6 at weeks 10 and 12 compared to the nonsupplementing controls.

Table 5 Polyunsaturated fatty acid content (wt %) of breast milk of women with and without gestational diabetes mellitus not consuming a prenatal supplement with DHA.

	Control	GDM
Week 2		
C18:2n-6	17.28 ± 1.1 [7]	18.3 ± 1.4 [4]
C20:2n-6	0.57 ± 0.02 [7]	0.54 ± 0.03 [4]
C20:3n-6	0.62 ± 0.05 [7]	0.59 ± 0.06 [4]
C20:4n-6	0.75 ± 0.03 [7]	0.73 ± 0.03 [4]
C22:2n-6	0.1 ± 0.01 [7]	0.09 ± 0.01 [4]
Total LC-PUFA n-6	2.04 ± 0.07 [7]	1.95 ± 0.09 [4]
C18:3n3	1.28 ± 0.1 [7]	1.15 ± 0.2 [4]
C20:5n-3	0.18 ± 0.01 [7]	0.17 ± 0.01 [4]
C22:6n-3	0.35 ± 0.02 [7]	0.29 ± 0.03 [4]
Total LC-PUFA n-3	0.53 ± 0.03 [7]	0.45 ± 0.03 [4]
n-3/n-6	0.26 ± 0.02 [7]	0.22 ± 0.02 [4]
Week 6		
C18:2n-6	24.59 ± 1.4 [5]	21.64 ± 1.4 [4]
C20:2n-6	0.46 ± 0.03 [5]	0.46 ± 0.03 [4]
C20:3n-6	0.53 ± 0.06 [5]	0.51 ± 0.06 [4]
C20:4n-6	0.71 ± 0.03 [5]	0.62 ± 0.03 [4]
C22:2n-6	0.05 ± 0.01 [5]	0.05 ± 0.01 [4]
Total LC-PUFA n-6	1.74 ± 0.09 [5]	1.63 ± 0.09 [4]
C18:3n3	1.82 ± 0.2 [5]	1.46 ± 0.2 [4]
C20:5n-3	0.19 ± 0.01 [5]	0.18 ± 0.01 [4]
C22:6n-3	0.27 ± 0.03 [5] ^a	0.16 ± 0.03 [4] ^b
Total LC-PUFA n-3	0.45 ± 0.03 [5] ^a	0.34 ± 0.03 [4] ^b
n-3/n-6	0.27 ± 0.02 [5] ^a	0.20 ± 0.02 [4] ^b

(Table 5 to be continued)

Week 10

C18:2n-6	23.09 ± 1.4 [5]	21.25 ± 1.4 [4]
C20:2n-6	0.39 ± 0.03 [5]	0.42 ± 0.03 [4]
C20:3n-6	0.39 ± 0.06 [5]	0.50 ± 0.06 [4]
C20:4n6	0.70 ± 0.03 [5]	0.65 ± 0.03 [4]
C22:2n-6	0.04 ± 0.01 [5]	0.05 ± 0.01 [4]
Total LC-PUFA n-6	1.51 ± 0.09 [5]	1.62 ± 0.09 [4]
C18:3n3	1.75 ± 0.2 [5]	1.62 ± 0.4 [4]
C20:5n-3	0.19 ± 0.01 [5]	0.20 ± 0.01 [4]
C22:6n-3	0.29 ± 0.03 [5] ^a	0.17 ± 0.03 [4] ^b
Total LC-PUFA n-3	0.47 ± 0.03 [5] ^a	0.37 ± 0.03 [4] ^b
n-3/n-6	0.31 ± 0.02 [5] ^a	0.23 ± 0.02 [4] ^b

Week 12

C18:2n-6	21.39 ± 1.45 [5]	22.40 ± 1.2 [5]
C20:2n-6	0.33 ± 0.03 [5] ^a	0.42 ± 0.02 [5] ^b
C20:3n-6	0.46 ± 0.06 [5]	0.54 ± 0.05 [5]
C20:4n-6	0.68 ± 0.03 [5]	0.66 ± 0.03 [5]
C22:2n-6	0.04 ± 0.01 [5]	0.05 ± 0.01 [5]
Total LC-PUFA n-6	1.52 ± 0.09 [5] ^a	1.76 ± 0.08 [5] ^b
C18:3n3	1.61 ± 0.2 [5]	1.38 ± 0.1 [5]
C20:5n-3	0.17 ± 0.01 [5]	0.18 ± 0.01 [5]
C22:6n-3	0.22 ± 0.03 [5]	0.18 ± 0.03 [5]
Total LC-PUFA n-6	0.39 ± 0.03 [5]	0.38 ± 0.03 [5]
n-3/n-6	0.26 ± 0.02 [5]	0.22 ± 0.02 [5]

LSM ± SEM; number of subjects in brackets. Means within a row with different superscript letter indicate significant difference before controlling for dietary DHA, $p \leq 0.05$. n-3/n-6, Σ 20:5n-3, 22:6n-3/ Σ 20:2n-6, 20:3n-6, 20:4n-6, 22:2n-6; GDM, gestational diabetes mellitus; LC-PUFA, long chain polyunsaturated fatty acid.

There was a significant correlation between the dietary and supplement intake of LA, ALA, DHA, EPA and n-3/n-6 and the content of these FA in milk: LA ($r = 0.49$, $p \leq 0.01$), ALA ($r = 0.61$, $p \leq 0.01$), DHA ($r = 0.77$, $p \leq 0.01$), EPA ($r = 0.90$, $p \leq 0.01$), n-3/n-6 ($r = 0.69$, $p \leq 0.01$). However, there was no significant correlation between dietary ALA and LA and DHA and ARA content of milk. For a subset of GDM and controls (6 GDM and 9 controls) for whom 1

hour postprandial glycemia during pregnancy were reported from their physicians' offices, there was a significant negative correlation between the four week mean milk DHA content and the maternal 1 hour postprandial glycemia during pregnancy ($r = -0.75$, $p \leq 0.01$).

Table 6 Effect of DHA supplementation during lactation on long chain polyunsaturated fatty acid content (wt%) of breast milk of women with and without gestational diabetes mellitus

	Control		GDM	
	with PS/DHA	without PS/DHA	with PS/DHA	without PS/DHA
Week 2				
C20:4n-6	0.82 ± 0.04 [4]	0.72 ± 0.03 [7]	0.86 ± 0.06 [1]	0.74 ± 0.03 [4]
C20:5n-3	0.24 ± 0.03 [4]	0.19 ± 0.02 [7]	0.32 ± 0.02 [1] ^a	0.18 ± 0.01 [4] ^b
C22:6n-3	0.46 ± 0.07 [4]	0.42 ± 0.06 [7]	0.52 ± 0.05 [1] ^a	0.29 ± 0.03 [4] ^b
n-3/n-6	0.32 ± 0.05 [4]	0.27 ± 0.04 [7]	0.37 ± 0.03 [1] ^a	0.23 ± 0.01 [4] ^b
Week 6				
C20:4n-6	0.70 ± 0.03 [5]	0.68 ± 0.04 [5]	0.59 ± 0.04 [2]	0.62 ± 0.03 [4]
C20:5n-3	0.26 ± 0.02 [5]	0.19 ± 0.03 [5]	0.23 ± 0.01 [2] ^a	0.18 ± 0.01 [4] ^b
C22:6n-3	0.43 ± 0.07 [5]	0.35 ± 0.07 [5]	0.27 ± 0.06 [2] ^a	0.17 ± 0.03 [4] ^b
n-3/n-6	0.38 ± 0.04 [5]	0.28 ± 0.05 [5]	0.32 ± 0.02 [2] ^a	0.21 ± 0.01 [4] ^b
Week 10				
C20:4n-6	0.75 ± 0.06 [2]	0.68 ± 0.04 [5]	0.64 ± 0.04 [2]	0.65 ± 0.03 [4]
C20:5n-3	0.52 ± 0.04 [2] ^a	0.19 ± 0.02 [5] ^b	0.24 ± 0.01 [2] ^a	0.20 ± 0.01 [4] ^b
C22:6n-3	0.46 ± 0.09 [2]	0.38 ± 0.06 [5]	0.24 ± 0.05 [2]	0.18 ± 0.03 [4]
n-3/n-6	0.73 ± 0.07 [2] ^a	0.32 ± 0.05 [5] ^b	0.30 ± 0.02 [2] ^a	0.23 ± 0.01 [4] ^b
Week 12				
C20:4n-6	0.76 ± 0.04 [3]	0.65 ± 0.04 [5]	0.50 ± 0.06 [1] ^a	0.66 ± 0.02 [5] ^b
C20:5n-3	0.33 ± 0.03 [3] ^a	0.18 ± 0.03 [5] ^b	0.15 ± 0.02 [1]	0.18 ± 0.01 [5]
C22:6n-3	0.53 ± 0.07 [3] ^a	0.31 ± 0.06 [5] ^b	0.18 ± 0.05 [1]	0.20 ± 0.03 [5]
n-3/n-6	0.44 ± 0.06 [3] ^a	0.28 ± 0.05 [5] ^b	0.25 ± 0.03 [1]	0.22 ± 0.01 [5]

LSM ± SEM; number of subjects in brackets. Means within a row with different superscript letters indicate significant difference after controlling for dietary DHA, $p \leq 0.05$. Σ 20:5n-3, 22:6n-3 / Σ 20:2n-6, 20:3n-6, 20:4n-6, 22:2n-6. GDM, gestational diabetes mellitus; PS/DHA, prenatal supplement with DHA

Discussion

To our knowledge, this is the first study reporting on the fatty acid composition of breast milk of women with GDM. Compared to women without GDM, milk of women with GDM tended to have lower concentration of DHA at four weeks postpartum if the women were not supplementing with a prenatal supplement that contained DHA. However, likely due to the limited number of GDM with these exploratory preliminary data there was no significant difference between groups at each time point. Other fatty acids were not different and were within the ranges reported by others for other populations of women (6, 53, 54). This finding is relevant for women with GDM who exclusively breast feed their infants as breast milk would be the sole source of DHA for the developing infant in the early months after birth. The significance of this finding is underscored by the earlier report of Wijendran et al (48) who observed that infants of mothers with GDM had DHA concentrations at birth that were approximately one-half those for infants born to women without GDM. Additionally, in line with other studies (1, 31) LC-PUFA milk content decreased over the four weeks of study if the subjects were not supplementing with LC-PUFA during lactation, with a marked decrease from week 2 to 6 postpartum.

Humans have the elongase and desaturase enzymes necessary for the synthesis of LC-PUFA from the 18-carbon precursors. However, the conversion rate efficiency is very limited and varies among different species (11). Previous studies have reported that pathologies characterized by insulin resistance and hyperglycemia are associated with an impaired $\Delta 5$ and $\Delta 6$ desaturase and elongase activity that compromise phospholipid LC-PUFA content (46, 55). Borkman et al (47) reported that subjects with coronary artery disease undergoing a coronary bypass had a significant inverse relationship between serum insulin concentration and the

percentages LC-PUFA in muscle phospholipids, particularly ARA and the C20 to C22 PUFA, reflecting impaired EFA metabolism when insulin action is deficient. This association is consistent with the inverse association between the 1 hour postprandial glycemia during pregnancy and milk DHA concentration reported in this study. Hyperglycemia in GDM suggests an abnormal insulin sensitivity or insulin deficiency that may compromise EFA metabolism to their longer chain derivatives. However, despite the effect that insulin exerts on desaturase and elongase activity, the amount of milk DHA and ARA that originates from the endogenous conversion of dietary LA and ALA is very low, 1.2 % of ARA and 1% of DHA, and another mechanism that explains for the low milk DHA in women with GDM needs to be elucidated (13, 29).

Mobilization of maternal fat stores have been described as the most significant contributor to breast milk fatty acids (1). Fatty acids during lactation are constantly secreted by adipose tissue and transported to the mammary gland bound to albumin. The mammary gland obtains free fatty acids from the circulation and assembles them into fat globules that are secreted with breast milk (1). It has been estimated that 60% of the breast milk fatty acids derive from maternal fat stores (30). Based on the foregoing, one may speculate that insulin resistance, characteristic in GDM, compromises the delivery of PUFA from adipose tissue to the mammary gland or may compromise the EFA metabolism to LC-PUFA in peripheral tissue. However, more detailed studies using isotope labeled fatty acid methodology that would allow for a better description of fatty acid absorption, distribution and metabolism in insulin resistance conditions are needed. With regard to diet analysis, GDM and C had a saturated fat intake above the current dietary guideline of 10% of less the total energy and had a mean dietary intake of DHA of 13% for GDM vs. 11 % for controls of the 300 mg recommended for lactating women by the National

Institutes of Health (NIH) workshop (39). Although no formal recommendation for n-3 fatty acids intake exists in the United States a recommendation of 650 mg/day of EPA and DHA combined has also been suggested (56). The low DHA dietary intakes reported in this study are similar to the data previously described for a subset of pregnant and lactating women by the USDA Continuing Survey of Food Intakes by Individuals (1994-1996), with a median DHA intake of ~ 44 mg/day (57). A recent study reported a mean DHA intake for lactating women in New Mexico to be 47 mg/day (36). In the current study, women with GDM and controls had a similar low DHA intake. Further, the dietary LA: ALA ratio in controls without PS/DHA was higher than the 5-10:1 recommended by the World Health Organization (56). Due to Louisiana's proximity to the coast and the inclusion of seafood in many typical dishes of this area, the low n-3 dietary intake in this population of lactating women is surprising. However, the mean DHA and EPA intakes reported in this study are congruent with previous data reporting n-3 fatty acid intake in the U.S. (36).

Thirty nine percent of the randomly recruited lactating women in this study reported consuming PS/DHA during lactation. Breast milk of women with GDM and controls taking a PS/DHA had higher DHA concentrations compared to non-supplemented women at weeks 2 and 6 for GDM and week 12 for controls. Similarly, milk EPA concentration and n-3/n-6 were higher in GDM and controls with PS/DHA compared to their nonsupplemented counterparts. These findings are in accordance with previous studies evaluating the effect of fish oil supplementation on breast milk lipid composition. Jensen et al (32) reported that by increasing the maternal intake of DHA in lactating women through supplementation of DHA in 3 groups of lactating women with different DHA doses (230, 170 to 260 mg/day) the milk DHA in the three supplemented groups was increased compared to placebo. Further, breast fed infants born to

supplemented lactating women had higher phospholipid DHA (11-42%) compared to a placebo group. Henderson et al (33) showed that after supplementing 5 lactating women with 6 g of fish oil daily for 21 days, milk n-3 fatty acid content increased within 8 hours and the n-6 fatty acid content decreased.

The relationship between PUFA intake and their concentration in the breast milk was evaluated in the present study. A significant positive correlation was found between dietary (food or supplement) LA, ALA, DHA, EPA, and n-3/n-6 ratio intake and their breast milk concentration. Women reporting to consume PS/DHA the day prior to milk collection had higher DHA, EPA, and n-3/n-6 (wt%) in breast milk. These results reflect the dose dependent effect that FA intake from diet or supplement has on milk fatty acid composition. Similar to previous studies, no significant association was found between the LA and ALA content in breast milk and the ARA and DHA milk concentration.

Conclusion

Although women with GDM are encouraged to breast feed their infants, these exploratory preliminary data suggest that exclusively breast fed infants born to women with GDM without PS/DHA during lactation may be at risk for inadequate DHA intake. However, it seems that supplementation with DHA by women with GDM can increase breast milk DHA concentrations. A limitation of these findings is the low number of women with GDM at this point of the study and the even low number of women with GDM who were supplementing with DHA during the lactation period. Therefore, further studies that evaluate the effectiveness of DHA supplementation in women with GDM to increase milk DHA concentration to levels comparable to controls are necessary. Furthermore, this study revealed that DHA and EPA dietary intake in this population was remarkably below the recommendation for lactating and

pregnant women. Therefore, lactating women should be encouraged to increase their intake of DHA, either by supplementation or through food sources, to ensure an adequate DHA supply to infant.

In summary, women with GDM tended to have lower DHA concentrations in breast milk compared to control women. Further, it seems that DHA supplementation of women with GDM during lactation may be an effective mean to increase the milk content of DHA, helping to insure an adequate supply for the developing infant.

CHAPTER 5

SUMMARY

The objective of this study was to evaluate the PUFA content of milk of women with and without GDM in a longitudinal design. These preliminary data point to lower DHA in milk of women with GDM not supplementing with DHA during lactation compared to controls. The significance of this finding is highlighted by the earlier report of Wijendran et al (48) who observed that infants of mothers with GDM had DHA concentrations at birth that were approximately one-half those of infants born to women without GDM.

It is well established that breast milk LC-PUFA can be obtained from de novo synthesis in the mammary gland, maternal diet, maternal fat body stores or de novo synthesis in sites other than the mammary gland. Humans have the enzymes necessary for the synthesis of MCFA (C8:0 to C12:0) and for the elongation and desaturation of the 18-carbon fatty acids to their long chain derivatives, but lack the Δ -12 and Δ -15 desaturase enzymes necessary for EFA synthesis (9, 13). LA is metabolized to ARA and ALA to EPA and DHA as products of a sequence of desaturation and elongation steps that occur mainly in the liver (11).

Milk LC-PUFAs can be derived from endogenous synthesis from their 18 carbon precursors in the mammary gland and other peripheral tissues. However, insulin seems to have a permissive effect on the desaturase and elongase enzymes necessary for EFA metabolism. Previous studies have reported that pathologies characterized by insulin resistance or deficiency and hyperglycemia are accompanied by impaired Δ 5 and Δ 6 desaturase and elongase activities that compromise phospholipid LC-PUFA content (46, 55). Borkman et al (47) reported that subjects with coronary artery disease undergoing a coronary bypass had a significant inverse relationship between serum insulin concentration and the percentages LC-PUFA in muscle

phospholipids, reflecting impaired EFA metabolism as a result of insulin deficiency when insulin action is deficient. This association is consistent with the findings of a negative association between the 1 hour postprandial glycemia during pregnancy and milk DHA concentration reported here. Hyperglycemia in GDM suggests an abnormal insulin sensitivity or insulin deficiency during pregnancy that could also reflect an unrecognized pregravid insulin resistance that was exacerbated during pregnancy. However, aside from the effect that insulin exerts on desaturase and elongase activities, the amount of milk DHA and ARA that originates from endogenous conversion of dietary LA and ALA is very low, 1.2 % of ARA and 1% of DHA (13, 29). Thus another mechanism likely explains the tendency for women with GDM to have lower milk DHA, supported by the preliminary data reported herein.

Mobilized maternal fat body stores have been described as the most significant contributor to fatty acids secreted in breast milk (1). During lactation fatty acids are constantly secreted by adipose tissue as products of cellular triacylglycerols hydrolysis and they are transported to the mammary gland bound to albumin. LA is the predominant n-6 PUFA present in adipose tissue, accounting for 12-16% of the fatty acids while, ALA is the most abundant of the n-3 PUFA and represents 1% of the fatty acids. Interestingly, only very small amounts of DHA and EPA are stored in adipose tissue and this translates to the need for a continuous supply of these fatty acids, e.g., the diet (11). Hachey et al (30), using isotopically labeled dietary fatty acids during late lactation, estimated that 60% of breast milk fatty acids originate from mobilized maternal fat stores. Additionally, an association between LA content of colostrums and mature milk and white adipose tissue has been reported. This relationship does not hold for ALA (31). The absence of a relationship of milk ALA and white adipose tissue ALA may suggest a selective advantage of the mammary gland in taking up the ALA over LA from the

circulation. It may be concluded that human milk LC-PUFA are subjected to some metabolic regulation that is not yet understood. Based on the contribution of maternal fat stores to breast milk fatty acid composition one may speculate that the insulin resistance, found in GDM, may compromise the delivery of PUFA from adipose tissue to the mammary gland in some way or that it compromises the metabolic synthesis of LC-PUFAs from their 18-carbon precursors in peripheral tissue.

In the study reported here women with GDM and controls consuming a PS/DHA had higher milk DHA. This finding suggests that women with GDM can benefit from DHA supplementation during lactation. However, subjects' number needs to be increased to be able to definitively conclude that this holds. Interestingly, because the PS/DHA consumed by the breast feeding women in this study generally contained EPA, milk EPA was higher in milk of women with GDM consuming the supplements.

The most remarkable feature of the estimated dietary intake for women in this study was a persistent low DHA and EPA intake. Mean dietary intakes of DHA and EPA were 40 and 31 mg/d respectively for women with GDM and 34 and 8 mg/d for women without GDM. These DHA values represent one-eighth of the recommended DHA intake for pregnant and lactating women (39). Lactating women should be encouraged to increase their intake of DHA, either by supplementation or food sources, or a combination of the two, to ensure an adequate DHA supply for the breast feeding infant.

Finally, we investigated the relationship between dietary PUFAs intake and the corresponding milk contents. There was a significant correlation between the dietary + supplement intake of LA, ALA, DHA, EPA, and n-3/n-6 and breast milk content of these. There were no relationships between dietary ALA and LA and milk DHA and ARA. Thus, while there

was a dose dependent effect of PUFA intake on milk respective fatty acids, higher intakes of LA and ALA were not associated with milk DHA and ARA concentrations.

These preliminary data suggest a tendency of women with GDM not consuming PS/DHA during lactation to have lower milk DHA compared to controls. Further, DHA supplementation during lactation seems to be effective in increasing milk DHA in women with and without GDM. Based on these exploratory data, women with GDM who breast feed should be encouraged to increase their intake of DHA, either by supplementation or through food sources, ensuring an adequate DHA supply for infant visual and neurological development.

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APPENDIX A **HEALTH HISTORY FORM**

Code #: _____

Age:_____ Ethnicity: _____ Date:_____

Physican's name: _____

Gestational Age: _____ Method of Delivery: _____

Sex of the Infant: _____ Number of pregnancy: _____ Birth Date:_____

Current weight: _____kg. Height: _____feet. Pre-pregnancy weight: _____kg.

Pre-pregnancy BMI: _____ Weight gain during pregnancy: _____kg.

Infant birth weight:_____ kg. Infant length: _____ cm. Head circumference: _____cm

Prior lactation experience: _____

Previous Medical History: _____

Gestational age at which GDM diagnosis was made, if applies:_____

Medications during pregnancy:_____

Medical complication during pregnancy: _____

Laboratory values

Date			
HbA1c			
Fasting blood glucose levels			
Post-pandrial blood glucose levels			

APPENDIX B
ADVERTISING FLYER



ARE YOU PREGNANT?

ARE YOU
PLANNING TO
BREAST FEED
YOUR BABY?

If you answered yes to these questions you may be eligible to participate in a research study evaluating the fat content in breast milk of women with gestational diabetes.

You may be eligible to participate if you:

- Are pregnant
- Plan to breast feed
- You do or do not have Gestational Diabetes
- This is your first pregnancy in the last 2 years

Qualified study participants will receive:

- Nutritional analysis, if desired
- **An \$ 80.00 check at the end of the study**
- In home or on site visits with flexible scheduling are available

To learn more about
this study, contact:

- Julissa or Emily
- 225 578-7160
- jsalgu1@lsu.edu
- 202B Knapp Hall,
Louisiana State
University
- Se habla español

APPENDIX C

RESULTS SUMMARY FIGURES

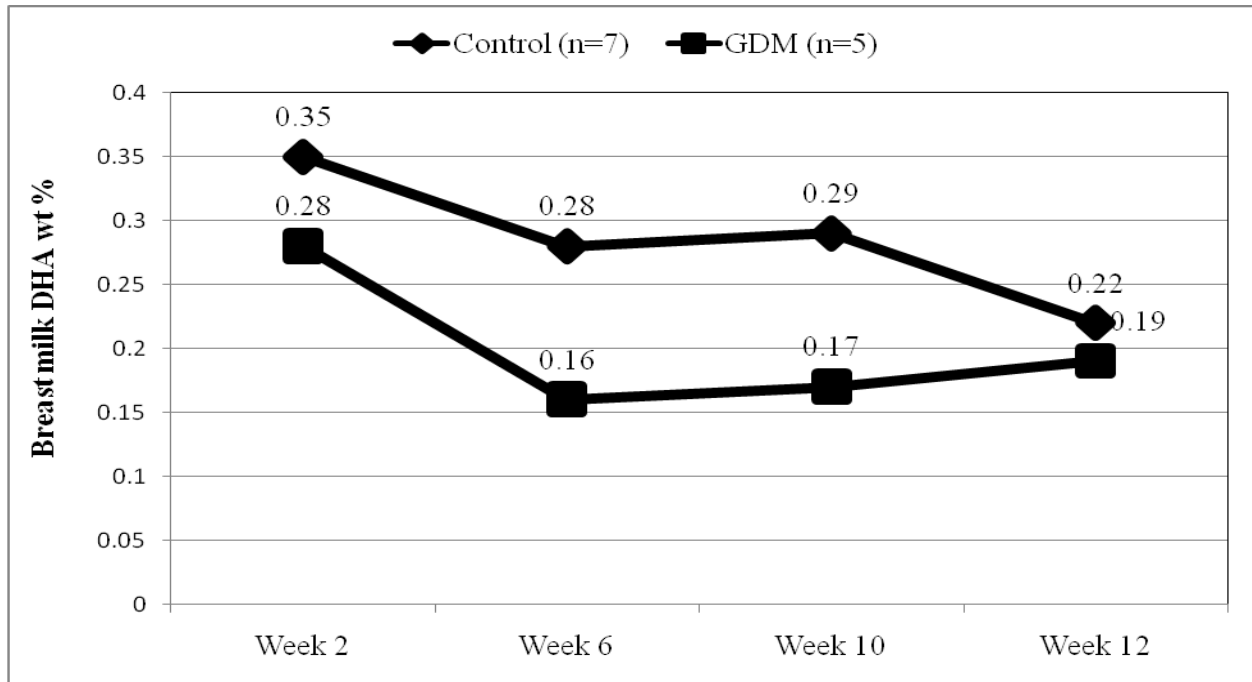


Figure 1. Milk DHA concentrations in women with and without GDM not supplementing with DHA during lactation after controlling for dietary DHA.

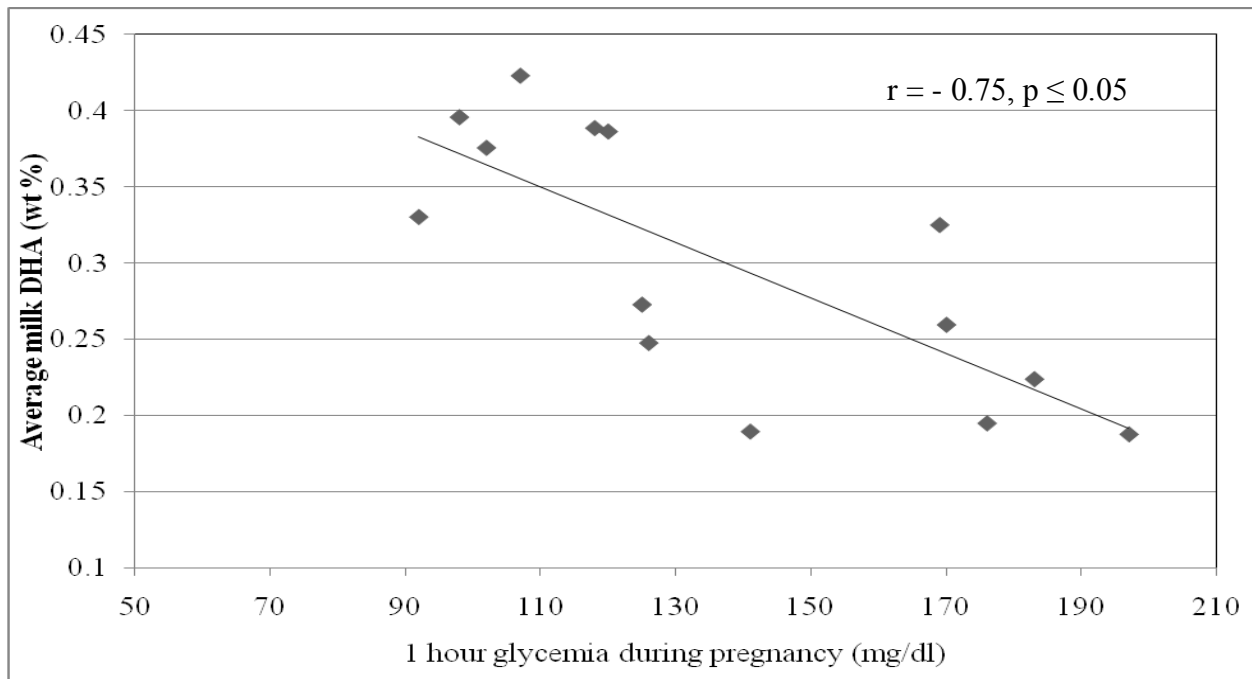


Figure 2. Inverse association between glycemia levels during pregnancy and average milk DHA concentration in women with and without GDM.

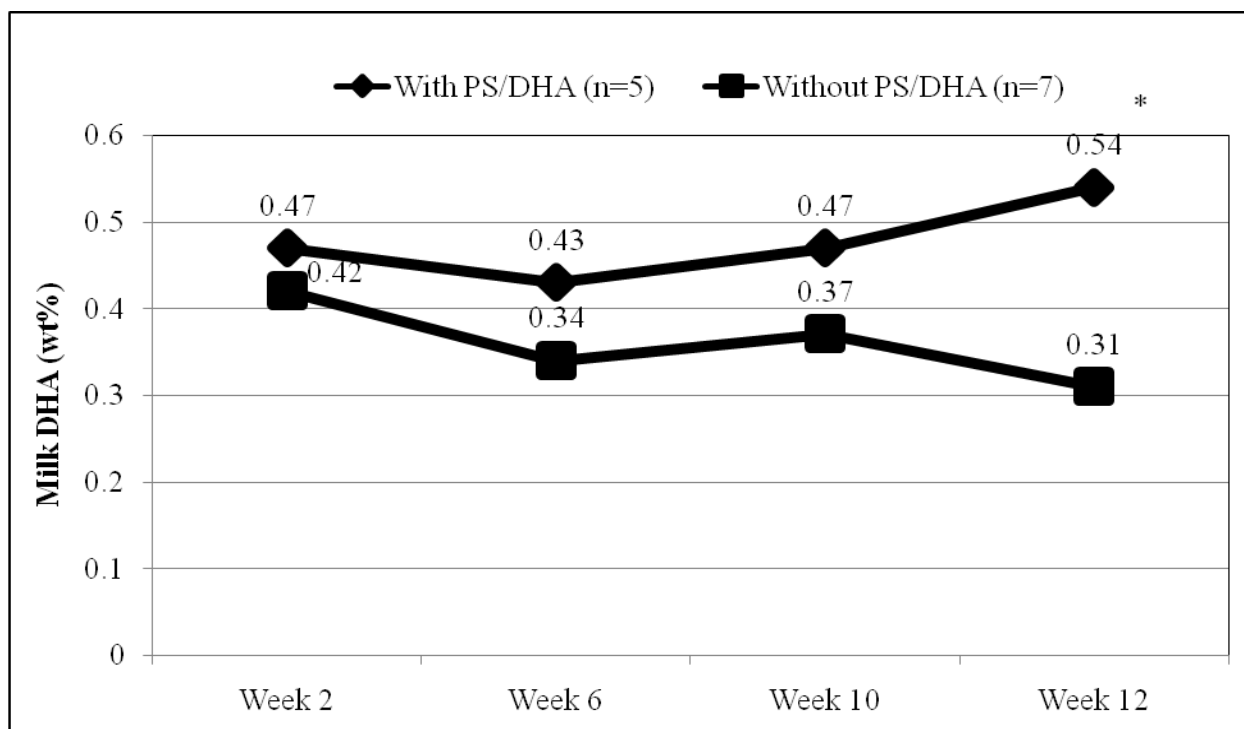


Figure 3. Effect of DHA supplementation during lactation on milk DHA concentrations in women without GDM. * Significant difference, $p \leq 0.05$.

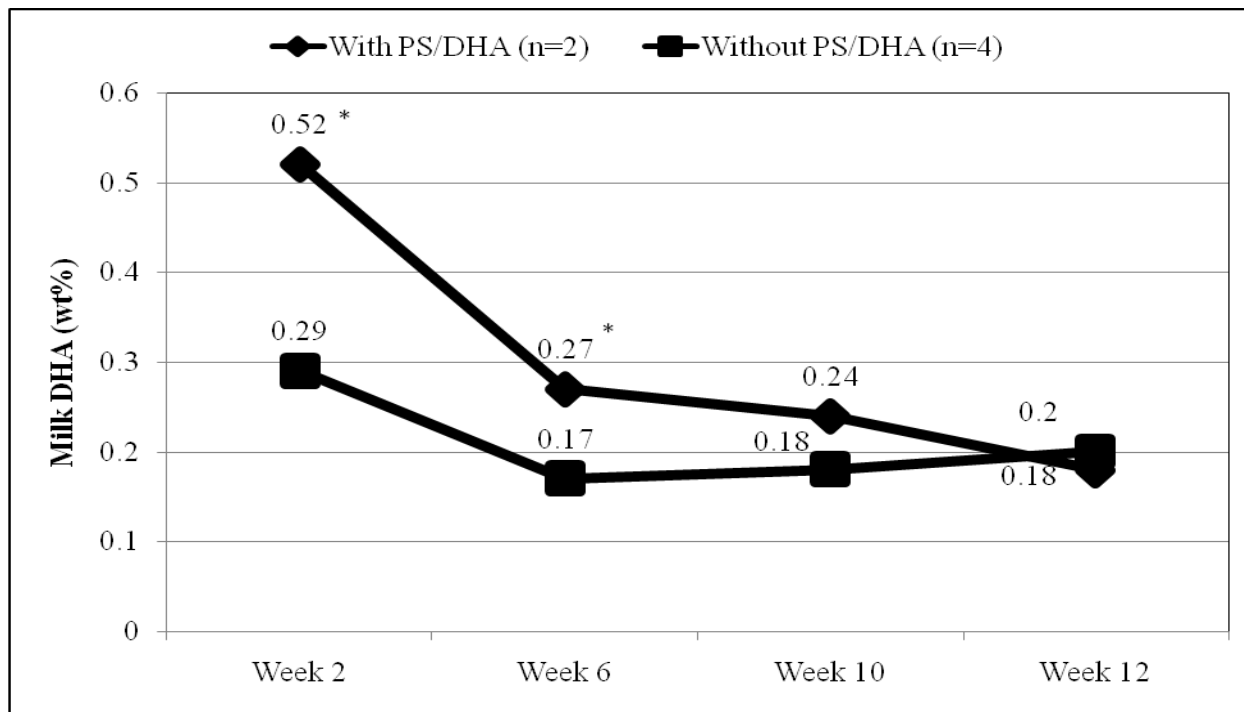


Figure 4. Effect of DHA supplementation during lactation on milk DHA concentrations in women with GDM. * Significant difference, $p \leq 0.05$.

VITA

Julissa Marisel Salguero Salguero was born in Guatemala City, Guatemala, in November, 1982. Julissa daughter of Leonel Salguero and Maria del Carmen Salguero is the middle girl of 3 children. She received her Bachelor of Science Degree in nutrition science in September, 2005 from Francisco Marroquin University in Guatemala City. She began her master's program in Fall 2007 in the Louisiana State University School of Human Ecology with a concentration in human nutrition. She is a member of the American Dietetic Association and American Oil Chemistry Society.