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Maternal fatty acid and inflammatory status during pregnancy are related to infant heart rate and heart rate variability

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MATERNAL FATTY ACID AND INFLAMMATORY STATUS DURING PREGNANCY ARE RELATED TO INFANT HEART RATE AND HEART RATE VARIABILITY

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It is with honor that I dedicate this dissertation to the women of my family – my mother, Shelli Edwards, and grandmothers, Marsha LaFour and Becky Wilder. Although starkly different in personality, each of you continually inspire me and have uniquely contributed to the person I have become. I aspire to be a positive, influential figure in somebody's life, as each of you have been in mine. This is for you, ladies.

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ABSTRACT

Early life heart rate (HR) and heart rate variability (HRV) reflect autonomic maturation. Intervention with n-3 long chain polyunsaturated fatty acids (LCPUFAs) during pregnancy favorably affects fetal HR and HRV; similar observations have been reported with infant n-3 LCPUFA intake. Infant HR and HRV have not been assessed in relation to maternal fatty acid status during pregnancy. Further, exposure to intrauterine inflammation may underlie these observations, although this hypothesis has not been tested. The aim of this observational study was to explore associations between maternal fatty acid and inflammatory status during pregnancy and infant HR and HRV. Simple linear and multiple regression were used to describe relationships for infant HR and HRV at 2 weeks, 4 months, and 6 months of age and: 1) maternal erythrocyte n-6 and n-3 fatty acids, 2) maternal plasma n-6 and n-3 endocannabinoids, and 3) maternal serum cytokines (interleukin-6, tumor necrosis factor-α), adipokine (adiponectin), and acute phase reactant (C-reactive protein) at 20, 24, 32, and 36 gestational weeks. Higher maternal n-3 fatty acid status, especially DHA, during pregnancy was inversely related to infant HR and positively related to HRV; the inverse was observed for n-6 fatty acids. Maternal n-3 endocannabinoids during pregnancy were inversely related with infant HR and positively related to infant HRV. Conversely, when the n-6:n-3 endocannabinoid ratio more heavily favored the n-6 endocannabinoid series, there was a positive and inverse association with infant HR and HRV, respectively. Limited associations between the other inflammatory biomarkers (interleukin-6, tumor necrosis factor-α, adiponectin, C-reactive protein) and infant HR/HRV were observed. As such, we cannot definitively conclude there is a link between intrauterine exposure to these biomarkers and infant autonomic development. These data build on existing literature evidencing a role for n-3 fatty acids in accelerating fetal and infant autonomic development and may

indicate an anti-inflammatory role for n-3 endocannabinoids. This study is the first to examine potential relationships between maternal fatty acid status, maternal inflammation, and infant autonomic development. Further, this study is the first to examine endocannabinoids in relation to HR and HRV in any population.

CHAPTER 1. LITERATURE REVIEW

1.1 Biological significance of essential fatty acids

1.1.1 Fatty acid characteristics

Fatty acids are hydrocarbon chains varying in length from 2 to 30-plus carbons with a methyl group at one end of the chain and a carboxyl group at the other. Structurally, fatty acids differ in number, type, and position of double bonds, as well as chain length. The molecular structure of various fatty acid classes is depicted in Figure 1.1.

Stearic acid, an example of a saturated fatty acid, contains 18 carbons and no double bonds. The term "saturated" refers to the hydrogen molecules, as all carbons in the chain, excepting that in the carboxyl group, are linked to as many hydrogen atoms as possible. Fatty acids with at least one double bond between adjacent carbon atoms are "unsaturated". Degree of unsaturation varies; fatty acids with only one double bond are monounsaturated fatty acids, while fatty acids with two or more double bonds are polyunsaturated fatty acids (PUFA). Double bonds can exist in *cis*- or *trans*- conformation and may occur in different positions within the hydrocarbon chain, as demonstrated in Figure 1.1.

Double bonds (=) are counted from the methyl end (CH_3) of the molecule and denoted by n-x or ω-x, where n- or ω- denotes the terminal carbon and x is the position of the first double bond.

Stearic acid (18:0)
CH_3-$(CH_2)_{16}$-COOH

Oleic acid (18:1n-9)
CH_3-$(CH_2)_7$-CH=CH-$(CH_2)_7$-COOH

Linoleic acid (18:2n-6)
CH_3-$(CH_2)_4$-CH=CH-CH_2-CH=CH-$(CH_2)_7$-COOH

Linolenic acid (18:3n-3)
CH_3-CH_2-CH=CH-CH_2-CH=CH-CH_2-CH=CH-$(CH_2)_7$-COOH

Figure 1.1 Molecular structure and nomenclature of fatty acids

1.1.2 Fatty acid nomenclature

In naming unsaturated fatty acids as a biologist, double bonds are counted from the methyl end of the molecule and are denoted by “n-x” or “ω-x”, where “x” is the position of the double bond and “n-” or “ω-” represents the terminal carbon, or the methyl end. For example, stearic acid, a saturated fatty acid, is designated as 18:0, as it contains 18 carbons and no double bonds. Oleic acid, a monounsaturated fatty acid containing 18 carbons, is denoted as 18:1n-9 or 18:1ω-9, with “1” indicating the presence of a single double bond and “n-9” or “ω-9” indicating where the double bond occurs, between carbon 9 and 10 from the terminal methyl, in this case.

A fatty acid containing 18 carbons and 3 double bonds with the first double bond occurring between the third and fourth carbons from the terminal methyl would be written as 18:3n-3 or 18:3ω-3 and termed an “n-3” fatty acid. Conversely, a fatty acid with two double bonds, the first of which occurs between the sixth and seventh carbons from the methyl end, would be written as 18:2n-6, and termed an “n-6” fatty acid.

1.1.3 Essential fatty acids

Most fatty acids can be synthesized de novo; however, human and mammalian cells lack enzymes required to synthesize linoleic acid (LA), the n-6 series precursor, and α-linolenic acid (α-LA), the n-3 series precursor (Simopoulos, 2002a). Therefore, these fatty acids must be obtained through the diet and are, therefore, “essential fatty acids” for humans.

Essential fatty acids are ubiquitous components of cell membranes and dictate membrane fluidity and membrane-bound enzyme/receptor activity. Essential fatty acids have various functions in the body; most of these functions require conversion to eicosanoids or other products but the fatty acids themselves can be functionally active (ie., platelet aggregation, leukocyte stimulation) and directly influence membrane fluidity (Simopoulos, 2002a).

1.1.4 Metabolism of essential fatty acids

The n-3 and n-6 fatty acid families share an enzymatic pathway and are metabolically competitive (reviewed by Simopoulos, 2002a). LA (18:2n-6) is converted to γ-LA (18:3n-6) by Δ^6 desaturase, then elongated to form dihomo-γ-LA (DGLA; 20:3n-6), which can be converted to arachidonic acid (ARA, 20:4n-6) via Δ^5 desaturase. In the n-3 series, α-LA (18:3n-3) is converted to eicosapentaenoic acid (EPA, 20:5n-3) by a series of Δ^{5-} and Δ^{6-}desaturases and elongase. An additional desaturase (Δ^4) and elongase converts EPA to docosahexaenoic acid (DHA, 22:6n-3). Retroconversion of DHA to EPA can occur with DHA supplementation, albeit at low basal rates (Brossard et al., 1996). The preferred substrate for Δ^{5-} and Δ^{6-}desaturases and elongases is α-LA; however, in the case of excess dietary LA, shared enzymes are saturated, preventing significant conversion of α-LA to longer chain n-3 metabolites (Kris-Etherton et al., 2000). Clinically, less than 8% of dietary α-LA is metabolized to EPA while conversion rate of α-LA to DHA is even lower, 0.02 – 4% (Burdge et al., 2002).

Dietary essential fatty acid deficiency is uncommon in developed countries. Optimal LA to α-LA intake is estimated to be 4:1, but dietary intake in Western countries approaches or exceeds 15:1, reflecting consumption of LA-rich vegetable oils (Wall et al., 2010) and a negative net effect on α-LA conversion. Therefore, intake of preformed α-LA and the longer chain metabolites EPA and DHA, known as n-3 long chain polyunsaturated fatty acids (LCPUFA), EPA and DHA, is recommended.

1.1.5 Dietary sources of n-3 LCPUFA

The primary dietary sources of EPA and DHA, along with their respective n-3 LCPUFA content per serving, are listed in Table 1.1. In general, deep water fish from colder temperatures (tuna, salmon, mackerel) have the highest content of EPA and DHA. As demonstrated in Table

1.1, fatty acid content and profile varies considerably according to fish species, geographical location, and method of harvesting/farming. As such, reported fatty acid content should be taken as rough estimates with the understanding that content also varies with cooking method.

Table 1.1 DHA and EPA content of major dietary sources of n-3 LCPUFA[1,2]

	DHA, mg/4 oz.	EPA, mg/4 oz.	# 4 oz. servings to provide 250 mg n-3 LCPUFA[3]	Oz. to provide 250 mg n-3 LCPUFA
Bass				
Sea	492	183	0.37	1.48
Striped	663	192	0.29	1.17
Catfish				
Farmed	64	19	3.02	12.10
Wild	265	147	0.61	2.43
Cod				
Atlantic	136	72	1.20	4.81
Pacific	109	39	1.69	6.76
Herring				
Atlantic	977	804	0.14	0.56
Pacific	781	1099	0.13	0.53
Flounder	123	155	0.90	3.61
Salmon				
Atlantic, farmed	1251	977	0.11	0.45
Atlantic, wild	1264	364	0.15	0.61
Pink	377	207	0.43	1.71
Sockeye	1797	395	0.11	0.46
Tilapia	97	5	2.44	9.74
Trout	599	229	0.30	1.21
Tuna				
Bluefin	1009	321	0.19	0.75
Light, canned in water	223	32	0.98	3.93
Yellowfin	100	13	2.21	8.82
White, canned in water	713	264	0.26	1.02

[1]Adapted from the USDA National Nutrient Database for Standard Reference, Release 28 and Drewery et al., 2016; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; LCPUFA = long chain polyunsaturated fatty acid
[2]Nutrient values are estimates and depend on species of fish, total fat content of fish, geographical location, method of raising/harvesting, and cooking. All values are for raw portions.
[3]# of servings (4 oz.) were calculated to meet 250 mg of n-3 LCPUFA per day, as recommended for pregnant women by the Dietary Guidelines for Americans (2015-2020).

1.1.6 PUFA status and inflammation

Inflammation is an immunological response of tissue to injury or infection. The inflammatory response is a normal, protective mechanism to remove harmful stimuli and initiate the healing process. However, persistent or excessive inflammation can lead to development of acute and chronic diseases characterized by production of cytokines, eicosanoids, and other inflammatory factors.

Inflammation and PUFA status are biologically interrelated. Essential fatty acids are precursors of eicosanoids, and these modulate the intensity and duration of an inflammatory response, dictating overall pathophysiological outcome (reviewed by Calder, 2006). Eicosanoids derived from 20-carbon PUFAs (DGLA, ARA, EPA) include prostaglandins, thromboxanes, leukotrienes, hydroperoxyeicosatetraenoic acids (HPETE), and hydroxyeicosatetraenoic acids (HETE).

In response to inflammation, phospholipase A_2 releases a 20-carbon fatty acid from the lipid pool within the cell membrane. The fatty acid is converted to an eicosanoid by cyclooxygenase (COX), lipoxygenase (LOX), or cytochrome P450 enzymes; COX enzymes produce prostaglandins and thromboxanes, LOX enzymes produce leukotrienes, and cytochrome P450 enzymes produce HPETE and HETE (reviewed by Calder, 2013). There are two isoforms of the COX enzyme; COX-1 is a constitutive enzyme and COX-2 is specific to immune cells. While the same COX and LOX pathways metabolize each 20-carbon PUFA, the eicosanoids produced from each PUFA precursor vary in potency and differ structurally and physiologically (Lee et al., 1984a).

1.1.7 Inflammatory events directed by PUFA

Pro-inflammatory events are initiated and exacerbated by eicosanoids derived from ARA, whereas equivalent products from EPA tend to nullify and reverse the inflammatory response. The 2 series of prostaglandins and thromboxanes (PGE_2, PGF_2, PGD_2, and TXA_2) are synthesized from ARA by the COX pathway. These prostaglandins have various physiological effects, including increased vascular permeability, vascular dilation, neutrophil chemotaxis, and stimulation of smooth muscle cell migration and proliferation (Richard et al., 2000). The 2-series of thromboxanes are involved in platelet aggregation and blood vessel constriction (Sellers and Stallone, 2008). Metabolism of ARA by 5-LOX produces hydroxy- and hydroperoxy-derivatives (5-HETE and 5-HPETE) and the 4-series of leukotrienes (LTC_4, LTB_4, LTD_4, and LTE_4). These leukotrienes are involved in induction of smooth-muscle contraction and act as chemoattractants of neutrophils (Peters-Golden et al., 2005).

The n-3 derived prostaglandins (PGD_3, PGF_3, and PGE_3), thromboxanes (TXA_3), and leukotrienes (LTB_5, LTC_5, LTD_5, and LTE_5) have weaker bioactivity than their respective n-6 derived metabolites (reviewed by Calder, 2006). For example, LTB_5 is 10- to 100-fold less potent as a neutrophil chemoattractant than LTB4 (Goldman et al., 1983; Lee et al., 1984b). Similarly, PGE_2 stimulates COX-2 in fibroblasts, up-regulating its own production and inducing pro-inflammatory cytokine production by macrophages; EPA-derived PGE_3 is a less potent inducer of these pathways (Bagga et al., 2003).

Biological activity and potency is related to affinity of cellular eicosanoid receptors. These receptors have greater affinity for n-6 derived eicosanoids than those derived from n-3. Thus, collective actions of n-3 produced eicosanoids are weak (Wada et al., 2007).

The type and amount of PUFA released in response to inflammatory stimuli depends on fatty acid content of the cell membrane phospholipid pool. When cell membranes contain large amounts of ARA relative to DGLA or EPA, which characterizes a typical Western diet, ARA is the primary substrate for eicosanoid synthesis (reviewed by Calder, 2006). Eicosanoids from n-6 are biologically active in small quantities and play an important modulatory role in the immune response by stimulating leukocytes (reviewed by Simopoulos, 2002b). However, if ARA-derived eicosanoids are present in large quantities, damage to host tissues can ensue and contribute to inflammatory disorders (Kinsella et al., 1990).

Supplementation with fish oil, a source of EPA and DHA, displaces ARA from cell membrane phospholipid pools, modulating the inflammatory response. This occurs especially in membranes of erythrocytes, neutrophils, monocytes, and liver cells (reviewed by Simopoulos, 2003; reviewed by Calder, 2006). In addition to competing for metabolic enzymes, EPA also competes with ARA for active sites of COX- and LOX-enzymes, suppressing generation of n-6 derived eicosanoids, further dampening the ARA-mediated pro-inflammatory response (James et al., 2000; reviewed by Calder, 2006).

Increased fish oil intake and subsequent EPA-induced suppression of n-6 derived eicosanoids is reflected in an elevation of n-3 derived eicosanoids. Fish oil supplementation decreases inflammatory cell production of PGE_2, LTB_4, TXB_2, LTE_4, and 5-HETE while increasing production of the less inflammatory LTB_4, LTE_5, and 5-HEPE (Sperling et al., 1993; von Schacky et al., 1993; Caughey et al., 1996).

Potent anti-inflammatory mediators, resolvins and protectins, are also generated from EPA and DHA (Serhan et al., 2008). The E-series resolvins are generated from EPA and the D-series resolvins, docosatrienes and neuroprotectins, are generated from DHA by COX-2 and

LOX-initiated pathways (Serhan et al., 2002). Both the D- and E-series of resolvins exert anti-inflammatory and immunoregulatory actions, including suppression of neutrophil chemotaxis, regulation of cytokines, and elimination of endothelial production of reactive oxygen species (Serhan et al., 2000; Serhan et al., 2002; Hong et al., 2003; Mukherjee et al., 2004).

Anti-inflammatory actions of n-3 LCPUFA are not strictly eicosanoid-dependent. Fish oil supplementation also reduces generation of pro-inflammatory cytokines (Lo et al., 1999; Zhao et al., 2004; Bhattacharya et al., 2006) by altering inflammatory gene expression via transcription factors, nuclear factor kappa B (NFκB) and peroxisome proliferator-activated receptors (PPARs) (reviewed by Calder, 2002; reviewed by Calder, 2006). NFκB is involved in up-regulation of several cytokines and enzymes implicated in the pathogenesis of chronic inflammatory diseases. In its inactive form, NFκB is a cytosolic trimer. Once activated by extracellular inflammatory stimuli, the inhibitory subunit (IκB) is phosphorylated and dissociates, allowing the remaining NFκB dimer to translocate to the nucleus and bind motifs in the promoter regions of pro-inflammatory genes (Hayden et al., 2006; Perkins, 2007).

There are several mechanisms by which n-3 LCPUFA interfere with NFκB activation: i) induction and activation of PPARγ, which physically blocks NFκB nuclear translocation; ii) disruption of membrane lipid rafts that initiate inflammatory signaling and activate NFκB; and iii) promotion of a cell surface G-protein coupled receptor which initiates an anti-inflammatory signaling cascade and inhibits signaling of NFκB activation (Lee et al., 2001; Van den Berghe et al., 2003; Oh et al., 2010).

Although infection resolution depends on cytokines, over-production has implications in pathological responses which can lead to inflammation. Specifically, cytokines induce fever, activate B- and T-lymphocytes, and regulate the acute phase response (reviewed by Simopoulos,

2002b). Cytokines with relevancy in inflammation are the interleukins (IL), especially IL-1, IL-6, IL-1β, and tumor necrosis factor (TNF)-α.

Clinically, DHA and EPA supplementation has been documented to decrease production of pro-inflammatory cytokines (Meydani et al., 1991; Endres et al., 1993; Gallai et al., 1993; Caughey et al., 1996), while null effects have also been reported (Yaqoob et al., 2000; Schmidt et al., 2010). Decreased production of pro-inflammatory cytokines translates to interest in n-3 LCPUFA as therapeutic agents in chronic inflammatory diseases, including rheumatoid arthritis, asthma, and inflammatory bowel disease. Evidence for the effectiveness of n-3 LCPUFA in improving clinical conditions and biochemical factors of these diseases has been reviewed (Simopoulos et al., 2002b). Overall, significant benefits are associated with n-3 LCPUFA supplementation, including decreased disease activity and lowered use of anti-inflammatory drugs (Simopoulos et al., 2002b). These observations underline the importance of incorporating DHA and EPA in the diet and shifting essential fatty acid intake (LA:α-LA) to favor the n-3 series.

1.2 Metabolic status and pregnancy as a state of inflammation

1.2.1 Inflammation as a natural state during pregnancy

Pregnancy is a natural state of inflammation characterized by distinct physiological changes (Sacks et al., 2004). In early pregnancy, maternal pancreatic β-cells undergo hyperplasia, resulting in increased insulin secretion and heightened insulin sensitivity (reviewed by Butte, 2000). As gestational age advances, secretion of diabetogenic hormones, including human placental lactogen, growth hormone, cortisol, and progesterone, markedly reduce insulin sensitivity by interfering with insulin receptor signaling at peripheral tissues (Newbern and Freemark, 2011). As insulin becomes less effective at suppressing lipid catabolism, there is a

shift from lipogenesis, which promotes accumulation of maternal fat stores in early and mid-pregnancy, to lipolysis in late pregnancy (Elliot, 1975; reviewed by Butte, 2000).

Cytokine production further stimulates lipolysis and inhibits lipoprotein lipase activity, with a net result of adipose tissue mobilization (Feingold and Grunfield, 1992). Maternal hyperlipidemia occurs and basal circulating triglyceride and cholesterol concentrations increase 2- to 3-fold (Knopp et al., 1980). Together, these hormonal alterations and hyperlipidemia result in a state of insulin resistance that peaks during the third trimester (Catalano et al., 1991).

Women with normal glucose tolerance prior to pregnancy have 50 – 70% decreased insulin sensitivity by the third trimester (Catalono et al., 1991; reviewed by Butte, 2000). Further, during the third trimester, lipoprotein lipase activity is redistributed from maternal tissues to the placenta, increasing placental capacity for fatty acid uptake (Herrera et al., 1988; reviewed by Haggarty, 2004). The progression of a normal pregnancy depends on these processes as they: i) provide an energy source for maternal needs in late gestation, ii) support enhanced nutrient transport across the placenta to the fetus, and iii) ensure a continuous supply of nutrients to the fetus despite intermittent maternal food intake (Swislocki and Kraemer, 1989; reviewed by Butte, 2000). The mother is able to preferentially use fat for fuel, preserving available glucose and amino acids for the fetus and minimizing protein catabolism (reviewed by Butte, 2000; Soma-Pillay et al., 2016). These physiological responses coincide with a time of significant energy demands from the mother and fetus. Maternal energy needs are greatest during the third trimester (reviewed by Catalano, 2010) and 90% of fetal fat deposition occurs after 30 weeks of gestation (reviewed by Haggarty, 2002).

Beyond the effects on maternal metabolism, physiological changes during pregnancy also prevent rejection of the placenta/fetus and are necessary for delivery (reviewed by Challis et al.,

2009). With advancing gestational age, inflammatory responsiveness increases. Pro-inflammatory cytokines actively remodel the cervix which weakens until fetal membranes rupture, activating uterine contractions (Young et al., 2002; Osman et al., 2003; King et al., 2007). T lymphocytes regulate this immunological response by producing cytokines. T lymphocytes can be divided into two functional groups that produce different cytokines: i) Th1, which promote cell-mediated immune responses, and ii) Th2, which are involved in humoral immunity (reviewed by Challis et al., 2009). The major sites of Th2 cytokine production are non-lymphoid tissues, including placental and decidual tissues (Chaouat, 1999). During normal pregnancy, the balance of Th1/Th2 activity is shifted to Th2, which plays a protective role in the feto-maternal relationship (reviewed by Challis et al., 2009).

1.2.2 Exaggerated response to excess maternal weight

In the United States, over two-thirds of women of reproductive ages have a body mass index (BMI) classified as overweight or obese ($\geq$25.0 kg/m^2; Flegal et al., 2010). Maternal overweight and obesity are associated with increased risk of adverse obstetric outcome(s); antenatal risks include gestational diabetes and hypertension (Sebire et al., 2001; Bhattacharya et al., 2007; Denison et al., 2008) while peripartal risks include induction of labor and unplanned operative delivery (Sebire et al., 2001; Denison et al., 2008; Galán et al., 2011).

Heightened inflammation, which accompanies excess maternal weight, alters the Th1/Th2 balance, causing a shift to Th1 predominance (reviewed by Challis et al., 2009). This shift initiates and intensifies the cascade of pro-inflammatory cytokine production implicated in spontaneous abortion, preterm delivery, and gestational diabetes mellitus (reviewed by Challis et al., 2009). Compared to normal weight pregnant women, obese pregnant women have elevated circulating levels of pro-inflammatory cytokines (Challier et al., 2008; reviewed by Catalano et

al., 2009; Basu et al., 2011; Roberts et al., 2011; Aye et al., 2014), including TNF-α and IL-6, both of which are implicated in insulin resistance (Borst, 2004; Kershaw and Flier, 2004). The source of these cytokines are likely T lymphocytes (reviewed by Challis et al., 2009), maternal adipose tissue (Denison et al., 2010; Aye et al., 2014), and the placenta (Denison et al., 2010).

Adipose tissue is an endocrine organ and a significant source of cytokines (Kershaw and Flier, 2004). Table 1.2 outlines pro-inflammatory cytokines that are expressed and secreted by adipose tissue and the effects of obesity on that expression. Obesity is defined by massive expansion of adipose tissue. mRNA expression of monocyte chemoattractant protein (MCP)-1 and TNF-α, pro-inflammatory molecules, is increased 2- to 5-fold in adipose tissue and placenta of obese pregnant women as compared to normal weight pregnant women (Basu et al, 2011; Roberts et al., 2011). Further, macrophages infiltrate adipose tissue of obese pregnant women (Denison et al., 2010).

The placenta can be described as an inflammatory organ. Obese pregnant women have a 2- to 3-fold increase in placental macrophages compared to normal weight or overweight women (Challier et al., 2008). Further, these macrophages are associated with increased expression of pro-inflammatory cytokines (Challier et al., 2008). These findings are consistent with animal studies which highlight a difference in placental production of inflammatory markers (carboxypeptidase E and resistin) between obese and non-obese pregnant populations (Singh et al., 2006; Zhou et al., 2006). It has also been reported that obese pregnant women have increased placental mRNA expression of IL-1β, IL-8, MCP-1, and CXCR2 (IL-8 receptor) versus normal weight pregnant women (Roberts et al., 2011). Overall, these findings support a role of the placenta in inflammation and indicate an up-regulation of placental inflammatory activities associated with maternal overweight and/or obesity.

Table 1.2 Pro-inflammatory factors expressed and secreted by adipose tissue			
Factor[1]	Obesity-induced changes	Functional effect(s)	Reference
TNF-α	Increased circulating	Downregulates anti-inflammatory pathway Stimulates lipolysis Increases insulin resistance	Kern et al., 2001 Cartier et al., 2008
MCP-1	Increased expression	Promotes monocyte recruitment	Christiansen et al., 2005 Di Gregorio et al., 2005
IL-6	Increased circulating	Increases hepatic insulin resistance	Kern et al., 2001
IL-8	Increased circulating	Acts as a potent chemoattractant	Straczkowski et al., 2002
IL-1β	Increased expression	Impairs insulin signaling	Juge-Aubry et al., 2003
[1]TNF-α: tumor necrosis factor α; MCP-1: monocyte chemoattractant protein; IL: interleukin			

Obesity, especially when visceral in nature, is associated with glucose intolerance and insulin resistance (Ramsay et al., 2002). Greater abdominal visceral adiposity in the first trimester is associated with a positive glucose challenge test between 24 and 28 gestational weeks (Martin et al., 2009). Thus, women entering pregnancy with excess weight or gain significant weight during pregnancy have increased susceptibility to developing gestational diabetes mellitus compared to women who enter pregnancy with a normal BMI (Denison et al., 2010).

While increased susceptibility to gestational diabetes mellitus could be attributed to exaggerated pro-inflammatory cytokine production, it has been estimated that C-reactive protein (CRP) concentrations, an acute phase protein synthesized in response to pro-inflammatory cytokines (Sheldon et al., 1993), and circulating triglycerides explain only 30% of the risk of increased BMI on gestational hypertension (Bodnar et al., 2005). Colomiere et al. (2009) reported defects in the insulin signaling cascade in adipose tissue and skeletal muscle of pregnant obese women with normal glucose tolerance. This report points to another factor which may contribute to susceptibility of obese women to gestational diabetes mellitus. These observations (Challier et al., 2008; Denison et al., 2010; Basu et al., 2011) suggest that T lymphocytes,

maternal adipose tissue, and the placenta contribute to local inflammation which is exaggerated by pregavid overweight/obesity. Thus, exaggerated inflammation during pregnancy may be a factor in increased risk of adverse obstetric outcome(s) in overweight and obese pregnant women.

1.2.3 Implications for fetal exposure to inflammation

Maternal overweight and obesity also pose significant health risks to the infant. Increased risk for adverse neonatal outcome, including congenital anomalies, asphyxia, death, and hypoglycemia are associated with pre-pregnancy overweight and obesity (Kalk et al., 2009; Stothard et al., 2009; Galán et al., 2012; Cresswell et al., 2012). Infants born to overweight and obese mothers are more likely to be large for gestational age, macrosomic, and insulin resistant (Catalano et al., 2009; Sewell et al., 2006; Yu et al., 2013). There is also an association between maternal obesity and increased disease risk in later life for the infant, including impaired neurodevelopment, cardiovascular, and/or metabolic diseases (Whitaker, 2004; Boney et al., 2005; Drake and Reynolds, 2010; Van Lieshout et al. 2011; Khandaker et al., 2012).

While maternal insulin resistance ensures redirection of nutrients for fetal growth and development, the degree of insulin sensitivity is a governing factor determining optimal fetal growth versus adiposity (Boney et al., 2005). The natural state of insulin resistance during pregnancy is exaggerated by pregavid obesity (Ramsay et al., 2002; Martin et al., 2009; Denison et al., 2010). Further, decreased maternal insulin sensitivity is associated with an increase in infant adiposity (Jansson and Powell, 2007) which is likely a result of fetal exposure to over-nutrition in the form of hyperglycemia and hyperlipidemia.

Infants born to overweight or obese women with normal glucose tolerance have a 2% increase in body fat compared to those born to normal weight women with normal glucose

tolerance (Sewell et al., 2006). Thus, inflammation accompanying excess maternal weight contributes to infant adiposity independently of maternal insulin resistance. The observation that maternal obesity activates placental inflammatory signaling without affecting cytokine levels in fetal circulation (Aye et al., 2014) indicates that obesity-related inflammation impacts the fetus by altering placental function rather than by direct fetal exposure to elevated concentrations of pro-inflammatory cytokines. However, increased IL-6 has been found in the umbilical cord plasma of fetuses born to obese versus lean women (Catalano et al., 2009).

Regardless of the pathway by which it exerts its effects, obesity-related inflammation can be mitigated, in part, by altering maternal nutrition. Supplementation of n-3 LCPUFA reduces inflammation associated with excess weight (Makhoul et al., 2011), although this has not been studied during pregnancy in relation to improving adverse obstetric, fetal, or infant outcome associated with pregavid overweight and/or obesity, to the authors' knowledge. The role of n-3 LCPUFA in reducing inflammation is covered in Section 1.1, benefits of maternal and/or infant n-3 LCPUFA intake during the perinatal period are covered in Section 1.3, and a more in-depth discussion of the implications of fetal exposure to inflammation, with specificity to the autonomic nervous system, are covered in Section 1.4.

1.3 n-3 and n-6 LCPUFA in development

1.3.1 The developing brain

Development of the human brain begins shortly after conception with neural tube formation. During the embryonic phase, before 7 weeks of gestation, brain structure is defined. Functional development occurs during the fetal phase, after 8 weeks of gestation (Fitzgerald and Folan-Curran, 2002; Larsen et al., 2011).

Neural proliferation and apoptosis occur during the first half of pregnancy. Once neurons are formed, they migrate to their final destination (Graaf-Peters and Hadders-Algra, 2006). This migratory period is accompanied by neuronal differentiation, encompassing processes including dendritic formation, axonal formation, and production of neurotransmitters and synapses. The number of cortical neurons peaks at 28 weeks of gestation, then declines by 70% to reach a stable number by birth (Rabinowicz et al., 1996).

Beginning during the third trimester and extending to 18 months of age, the brain undergoes exponential growth, increasing 10-fold in size (Dobbing and Sands, 1973). This period is characterized by synaptogenesis, myelination, axonal and dendritic differentiation, axon elimination, and glial cell proliferation, providing a neuronal base for the fetus/neonate to learn (Reiss, 1996; reviewed by Georgieff, 2007). At birth, the brain is only 25% of its final volume with 70% of cell division complete, but reaches 80% of final adult weight by the end of the "growth spurt", in the first year of life (Crawford, 1993; Helland et al., 2003).

1.3.2 Importance of n-3 and n-6 LCPUFA to the developing brain

N-3 and n-6 LCPUFAs are important components of tissue lipids. Specifically, their structural and functional roles are centered in cell membranes where they are integrated into phospholipids (Neuringer et al., 1988; reviewed by Kidd, 2007). While ARA, the primary n-6 LCPUFA, is present in all biological membranes and comprises 5 – 15% of total fatty acids in most tissue phospholipids, DHA, the primary n-3 LCPUFA, is highly concentrated in the cerebral cortex and retina (Neuringer et al., 1988). Specifically, DHA is enriched in brain gray matter as 10 – 16% of total fatty acids and rod and cone outer segment membranes of the retina as 35% of total fatty acids (Sastry, 1985; Guisto et al., 2002; reviewed by Wall et al., 2010). Overall, approximately 64% of total lipids in the adult brain are LCPUFAs, virtually all of which

are structural and unavailable for energy metabolism (Yehuda et al., 1999). These concentrations are consistent across mammalian species, despite disparities in dietary intake, suggesting a functional role for ARA and DHA in brain tissue (Crawford et al., 1976).

At term, the infant brain contains relatively more ARA than DHA, but higher DHA accretion persists after birth, resulting in DHA as the predominant LCPUFA in the adult brain (Farquharson et al., 1992; Martinez, 1992a; Martinez and Mougan, 1998). Accrual of brain ARA and DHA increases 3 – 5 times in the last trimester of pregnancy and continues throughout the first 18 months of life, though at a slower rate (Neuringer et al., 1988; reviewed by Colombo, 2011). The rate at which ARA and DHA are incorporated into the brain during early postnatal life is 10 times faster than that of their respective precursors, LA and α-LA (Greiner et al., 1997).

The developing brain is sensitive to environmental insults, including nutritional deficits. Critical periods of vulnerability occur during the perinatal period, when development of underlying neuronal circuitry is more susceptible to perturbations than at any other time (Anand and Scalzo, 2000). This critical period corresponds to peak rates of brain growth, myelination, and accelerated production of synaptic sites (Rakic, 1998).

Neuronal cell growth and development are dependent on all nutrients, but are especially responsive to LCPUFA. Deficiency of these fatty acids during fetal or neonatal life can result in detrimental global- and/or circuit-specific effects, based on timing and degree of the deficit (reviewed by Georgieff, 2007). The developing brain is plastic and amenable to repair after nutrient repletion. However, vulnerability to insult likely outweighs plasticity as early nutritional deficits manifest themselves as brain dysfunction during the deficit with continued dysfunction after nutrient repletion (reviewed by Georgieff, 2007).

ARA is involved in cell signaling pathways and is a precursor to eicosanoids that participate in cellular processes; however, the specific functional role(s) of DHA in the brain are not as well defined (Bhattacharya et al., 1989). Several roles for DHA in the excitable membranes of the nervous system and retina have been proposed: i) high polyunsaturation of DHA influences biophysical properties of membranes, such as molecule shape and fluidity; ii) DHA modulates aspects of lipid-protein interactions, such as activities of membrane-bound enzymes and receptors; and iii) DHA is a precursor for functionally important eicosanoid products (Spector and Yorek, 1985; Neuringer et al., 1988).

1.3.3 Accretion of fetal ARA and DHA, as related to the developing brain

The fetus accumulates approximately 212 mg/kg ARA and 45 mg/kg DHA per day during the last trimester of pregnancy (Lapillone and Jensen, 2009). The primary determinant of availability and delivery of a fatty acid to the fetus is the concentration of that fatty acid in maternal circulation, which is closely related to maternal intake (Haggarty et al., 1999).

Tracer studies indicate rate of fetal ARA synthesis is significantly greater than that of DHA, suggesting the fetus has a greater ability to regulate ARA supply by de novo synthesis from LA or placental re-uptake. Thus, it has been proposed that exogenously derived DHA may be more critical than ARA during fetal life (reviewed by Haggarty, 2004). However, Western diets are notoriously low in DHA. Therefore, maternal stores may be insufficient to meet fetal DHA demands for optimal development. This hypothesis remains untested, however, as data on DHA accumulation in human placenta and other pregnancy-related tissues, or losses in turnover, are not available, making it difficult to quantify DHA requirements of the fetus (reviewed by Innis, 2009).

Unique biological processes, termed “progressive biomagnification” direct DHA and ARA from maternal liver to the placenta, fetal liver and, ultimately, fetal brain. Clinically, biomagnification is evident by comparing circulating maternal fatty acid concentrations with cord venous concentrations, which represent fetal circulation, at delivery. ARA and DHA are enriched in cord erythrocyte phospholipids relative to maternal erythrocyte phospholipids (30 – 35% increase, by weight), although precursor fatty acids are greater in maternal circulation (Wijendran et al., 2000). These processes ensure LCPUFAs are selectively supplied to the fetus and subsequently incorporated into the brain (Neuringer et al., 1988).

1.3.4 Accretion of postnatal ARA and DHA, as related to the developing brain

The importance of DHA extends to postnatal life, as the brain is still developing. Postnatally, accretion of infant ARA and DHA depend on the diet and, to a limited extent, endogenous synthesis (Birch et al., 2007).

Brenna et al. (2007) estimated that, worldwide, human breast milk contains an average of 0.32% of fatty acids as DHA. In United States populations, however, human breast milk contains an average of 0.15 – 0.19% of fatty acids as DHA (Auestad et al., 2001; Jensen et al., 2000; Yuhas et al., 2006). Breast milk fatty acids are derived from maternal body stores and diet (Demmelmair et al., 1998). Thus, lower n-3 LCPUFAs in breast milk from United States populations reflects consumption of the n-6 PUFA-saturated Western Diet.

Infants fed formula without DHA have lower brain cortex and erythrocyte DHA concentrations than breast-fed infants (Makrides et al., 1994). Similarly, unsupplemented formula-fed infants have 11 – 40% lower DHA concentrations in brain gray matter than breast-fed infants (Farquharson et al., 1992; Jamieson et al., 1999). It is important to note these studies

were conducted when commercial infant formulas were not fortified with DHA. Thus, these observations illustrate that diet can modify DHA composition of the developing human brain.

As in fetal life, neonatal synthesis of ARA is significantly greater than that of DHA (reviewed by Haggarty, 2002). It has been estimated that DHA requirements are not addressed by endogenous synthesis in a significant capacity until 16 weeks after term delivery (Uauy et al., 2000; Carnielli et al., 2007); this time frame would presumably be greater in a preterm infant. After birth, acylated DHA in liver and adipose tissue decrease, mirroring an increase of acylated DHA in the brain and retina. This finding suggests that the liver and adipose tissues sequester DHA in utero to provide a reservoir during early neonatal life (Martinez, 1992b).

1.3.5 Deficits and benefits of ARA and DHA in preterm infants

Preterm infants (<37 gestational weeks) are more vulnerable to DHA deficits than term infants as they are not exposed to maternal fatty acid stores for the entirety of the third trimester and have an extremely limited ability to synthesize DHA from the precursor fatty acid (reviewed by Kidd, 2007). Consequences for these deficits include increased risk for compromised cognitive ability and behavioral impairment at school age. Degree of risk is directly proportional to level of immaturity at birth (Bhutta et al., 2002).

Preterm (and term) infants fed formulas enriched with DHA have similar erythrocyte DHA concentrations as breast-fed infants (Carlson et al., 1987; Makrides et al., 1994). By improving DHA status, visual acuity of term and preterm infants improves, with greater improvements in preterm infants due to less mature visual acuity at study onset (Carlson et al., 1993).

1.3.6 Maternal DHA supplementation and term infant outcome

Studies evaluating the effect of maternal DHA supplementation on infant outcome, as presented in Table 1.3, largely support the "fetal origins hypothesis", the theory that fetal exposure to in utero conditions, including nutrition deficits or abundances, is related to risk for chronic diseases in later life (Barker, 1992). The "fetal origins hypothesis" is discussed in greater depth in Section 1.4.

Table 1.3 Effects of DHA supplementation during pregnancy on infant outcome

Observations vs placebo	Dose per day	Duration of suppl.	Reference
Cognitive/central nervous system maturity[1]			
Improved performance on Mental Processing Composite of KABC at 4 y	0.118 g DHA	GW 18 – 3 mo	Helland et al., 2003
Less distractibility at 12 – 24 mo	0.135 g DHA	GW 24/28 - delivery	Colombo et al., 2004
Improved problem solving at 9 mo	0.214 g DHA	GW 24 – delivery	Judge et al., 2007a
Improved hand eye coordination on GMDS at 2.5 y	2.200 g DHA	GW 20 – delivery	Dunstan et al., 2008
Improved sleep organization at 1 and 2 d	0.214 g DHA	GW 24 – delivery	Judge et al., 2012
Visual acuity			
Improved visual acuity at 4 mo	0.214 g DHA	GW 24 – delivery	Judge et al., 2007b
Improved visual acuity at 2 mo	0.400 g DHA	GW 16 – delivery	Innis and Friesen, 2008
Body composition			
Decreased BMI at 21 mo	0.200 g DHA	GW 21 – 3 mo	Bergmann et al., 2007
Decreased ponderal index at birth	0.214 g DHA	GW 21 – delivery	Courville et al., 2011

[1]DHA: docosahexaenoic acid; GW: gestational week; KABC: Kaufman Assessment Battery for Children; GMDS: Griffiths Mental Developmental Scales

Supplemental DHA during pregnancy improves infant central nervous system maturity, assessed as cognitive performance, as early as 9 months and out to 4 years of age (Helland et al., 2003; Colombo et al., 2004; Dunstan et al., 2006; Judge et al., 2007a). These observations have

been reported with a range of DHA intakes (120 – 220 mg per day), at different intervention times (16 – 24 weeks of gestation throughout term delivery), and with various validated, age-appropriate assessment tools (Kaufman Assessment Battery for Children, Griffiths Mental Developmental Scales, problem solving tasks). Using aggregation analysis, Cohen et al. (2005) estimated that for every increase in maternal intake of 100 mg DHA per day during pregnancy, child IQ increases by 0.13 points, with an average maximum benefit of 1.3 IQ points.

Infants whose mothers received 210 – 400 mg DHA per day during pregnancy have improved visual acuity at 2- and 4-months of age as compared to infants born to women receiving no supplemental DHA (Judge et al., 2007b; Innis and Friesen, 2008). A similar dose of DHA (approximately 200 mg per day) consumed from 21 weeks of gestation to delivery has also been linked to improved infant body composition (lower ponderal index and BMI) at delivery (Courville et al., 2011) and 21 months of age (Bergmann et al., 2007).

1.3.7 Postnatal DHA supplementation and term infant outcome

Significant work, focused on the effects of postnatal DHA supplementation, has been conducted and is presented in Table 1.4. Generally, infants receiving supplemental DHA from birth to 3 – 4 months of age perform similarly to breast-fed infants on cognitive assessments, with both groups performing better than infants not receiving supplemental DHA (Agostoni et al., 1995; Birch et al., 2000; Jensen et al., 2005; Jensen et al., 2010; Colombo et al., 2011). Cognitive improvements are apparent as early as 4 months of age, extend to 2.5 years of age, and have been documented using age-appropriate assessments, including the Brunet-Lézine Psychomotor Development Test and Bailey Scales of Infant Development.

Table 1.4 Effects of postnatal DHA supplementation on infant outcome			
Observations vs placebo	Dose	Duration of suppl.	Reference
Cognitive/central nervous system maturity[1]			
Better performance on BLPDT for breast-fed and formula + DHA at 4 mo	Breast-fed, formula (no DHA), formula + 0.30% FA as DHA	Birth – 4 mo	Agostoni et al., 1995
Improved performance on mental developmental index of BSID-II at 18 mo	Formula (no DHA), formula + 0.35% FA as DHA	Birth – 4.25 mo	Birch et al., 2000
Improved performance on psychomotor development index of BSID at 30 mo	Breast-feeding mothers supplemented 0 or 200 mg DHA/d	Birth – 4 mo	Jensen et al., 2005
Improved performance on the sustained attention subscale of the LIPS	Breast-feeding mothers supplemented 0 or 200 mg DHA/d	Birth – 4 mo	Jensen et al., 2010
Increased sustained attention for 0.32 and 0.64% at 4, 6, and 9 mo	0.0, 0.32, 0.64, 0.92% FA in formula as DHA	Birth – 3 mo	Colombo et al., 2011
Improved information processing at 6 y	Formula (no DHA), formula + 0.21% FA as DHA	Birth – 4 mo	Willatts et al., 2013
Visual acuity[2]			
Higher grating acuity on TACP at 2 mo for formula + DHA and breast-milk	Formula (no DHA), formula + 0.10% FA as DHA, breast-milk	Birth - 2 mo	Carlson et al., 1996
Better sweep VEP acuity at 6, 17, and 52 wks for formula + DHA	Formula (no DHA), formula + 0.35% FA as DHA	Birth – 17 wks	Birch et al., 1998
Higher VEP acuity at 1 y	Formula (no DHA), formula + 0.36% FA as DHA	5 – 12 mo	Hoffman et al., 2003
Improved visual acuity at 4 y	Formula (no DHA), formula + 0.35% FA as DHA	Birth – 17 wks	Birch et al., 2007

Table 1.4, continued Effects of postnatal DHA supplementation on infant outcome			
Observations vs placebo	Dose	Duration of suppl.	Reference
Autonomic nervous system[3]			
Decreased mean- and diastolic-BP for formula + DHA at 6 y	Formula (no DHA), formula + 0.20% FA as DHA	Birth – 4 mo	Forsyth et al., 2003
Decreased systolic BP; increased plasma total- and LDL-cholesterol for formula + DHA at 12 mo	Formula (no DHA), formula + DHA (0.37 g DHA/d)	9 – 12 mo	Damsgaard et al., 2006

[1]DHA: docosahexaenoic acid; FA: fatty acid; BLPDT: Brunet-Lézine Psychomotor Development Test; BSID: Bayley Scales of Infant Development; LIPS: Leiter International Performance Scale
[2]TACP: Teller Acuity Card Procedure; VEP: visual evoked potential
[3]Studies assessing heart rate and heart rate variability, autonomic indices, are covered in Section 1.4 and not included here; BP: blood pressure

Although the amount of DHA consumed in the aforementioned studies varied and, in some cases, was impossible to calculate due to non-reporting of volume consumed, the concentration commonly supplemented (0.30% of total fatty acids) is similar to the worldwide average DHA concentration in human breast milk (0.32% of total fatty acids; Brenna et al., 2007). Cohen et al. (2005) estimated that for every 1% increase in breast milk or formula DHA phospholipids, child IQ increases by 4.6 points. This impact on child IQ was greater than that when the same amount of DHA was consumed by the mother during pregnancy. Complementing observations from maternal DHA supplementation during pregnancy, it has been reported that infants receiving DHA as formula or breast-milk have better visual acuity than those fed standard formula not enriched with DHA (Carlson et al., 1996; Birch et al., 1998). These group differences manifested themselves by 6 weeks and remained present at 52 weeks of age.

Postnatal DHA plays a role in autonomic development. Forsyth et al. (2003) observed a decrease in mean- and diastolic-blood pressure for children at 6 years of age who were supplemented with DHA (0.20% of fatty acids) from delivery to 4 months of age. Studies

focused on the relationship between postnatal DHA and infant HR and HRV, autonomic indices, are covered in depth in Section 1.4.

1.3.8 Association between DHA status and term infant outcome

Observational studies also point to a relationship between indices of DHA status and positive infant outcomes, as presented in Table 1.5. There is a positive association between cord plasma phospholipid DHA concentrations and newborn cerebral maturation (Helland et al., 2001), infant novelty preference at 6 months (Jacobson et al., 2008), performance on mental- and psychomotor-development indices at 11 months (Jacobson et al., 2008), and improved motor function at 7 years of age (Bakker et al., 2009). Higher DHA concentrations in maternal plasma phospholipids at delivery are positively related to more mature sleep patterning, a measure of functional central nervous system integrity, at 2 days of age (Cheruku et al., 2002). In terms of

Table 1.5 Associations between DHA status and infant outcome[1]

Associated with increased DHA status	Index of DHA status	Reference
Cognitive/central nervous system maturity[2]		
More mature EEG function at 2 d	Cord plasma PL	Helland et al., 2001
More mature sleep patterning at 2 d	Maternal plasma PL at delivery	Cheruku et al., 2002
Greater novelty preference on FTII at 6 mo; improved performance on mental- and psychomotor-development indices of BSID-II at 11 mo	Cord plasma PL	Jacobson et al., 2008
Improved motor function at 7 y, assessed by MMT	Cord plasma PL	Bakker et al., 2009
Visual acuity[3]		
Improved visual acuity at 2- and 12-mo	Infant erythrocyte phosphatidylethanolamine	Innis et al., 2001
More mature retinal development at 1 wk, assessed by scotopic ERG	Cord erythrocyte	Malcolm et al., 2003
Improved visual acuity at 6 mo	Cord plasma PL	Jacobson et al., 2008

[1]DHA: docosahexaenoic acid
[2]PL: phospholipid; EEG: electroencephalography; FTII: Fagan Test of Infant Intelligence; BSID: Bayley Scales of Infant Development; MMT: Maastricht's Motor Test
[3]ERG: electroretinogram

visual acuity and retinal development, improved measures are positively associated with DHA in cord and infant blood and are clinically apparent from 1 week to 12 months of age (Innis et al., 2001; Malcolm et al., 2003; Jacobson et al., 2008).

1.3.9 Recommended ARA and DHA intake during pregnancy and infancy

A committee tasked with determining optimal fat intake during pregnancy and lactation concluded that the only fatty acid requiring alteration, in terms of intake, in the diet of pregnant women was DHA (Koletzko et al., 2007). The committee recommended pregnant women consume 200 mg DHA per day, but recognized intakes up to 1000 mg DHA per day are without adverse outcome. Taken together with amounts supplemented and corresponding observations cited in the studies discussed above (Tables 1.3, 1.4, and 1.5), 200 mg DHA per day is conservative and will likely fulfill absolute maternal and fetal/infant requirements, but not result in "optimal" outcome (ie., infant IQ would continue to increase with > 200 mg DHA; Janssen and Kiliaan, 2014).

Recommendations for preterm and term infants differ, as preterm infants are not exposed to maternal fatty acid stores during critical periods of development and, thus, require more LCPUFA to accelerate maturation of underdeveloped systems. Recommended DHA and ARA concentrations for preterm infants are 0.35 – 1% and 0.40 – 0.80% of total formula fatty acids, respectively (Koletzko et al., 2001; Koletzko et al., 2007; Hadders-Algra, 2011; Simmer et al., 2011; Lapillone et al., 2013). For term infants, it is advised that DHA concentrations are 0.20 – 0.32% and ARA concentrations are 0.35% of total formula fatty acids. Again, these recommendations may be conservative and are estimated to fulfill absolute infant requirement, as opposed to "optimize" outcome. However, the recommended DHA concentration clearly exceeds that of breast milk from the United States (Auestad et al., 2001; Jensen et al., 2001; Yuhas et al.,

2006) and is more similar to that of the worldwide average (0.32%) as estimated by Brenna et al (2007).

1.4 Mechanism underlying n-3 LCPUFA effects on heart rate and heart rate variability

1.4.1 The autonomic nervous system

The autonomic nervous system contains two antagonistic divisions, the sympathetic and parasympathetic (vagal) nervous systems (reviewed by Berntson et al., 1997). Autonomic output is mediated through preganglionic sympathetic and parasympathetic neurons which innervate the heart via the stellate ganglia and vagus nerve, respectively (reviewed by Benarroch, 1993). The interaction between these inputs and the sinoatrial (SA) node reflect spontaneous changes in autonomic activity which are apparent in HR and HRV measures (Massin and von Bernuth, 1997).

1.4.2 Autonomic innervation of the sinoatrial node

Autonomic innervation lowers resting HR by 30% of its intrinsic value (reviewed by Thomas, 2011). Greater parasympathetic influence further reduces HR, with the opposite occurring for greater sympathetic influence (reviewed by Thomas, 2011). Sympathetic stimulation, via β-adrenergic receptors and release of norepinephrine, increases SA node automaticity and atrioventricular (AV) node conduction, increasing HR (reviewed by Berntson et al., 1997; reviewed by Sztajzel, 2004; reviewed by Thomas, 2011). Sympathetic stimulation increases the probability pacemaker channels will be open, allowing greater ion flow, increasing the steepness of the slope required for depolarization (reviewed by Lilly, 2007; reviewed by Thomas, 2011). As a result, nodal cells reach threshold and spontaneously depolarize earlier than normal. With greater sympathetic influence, there is also a greater probability that Ca channels will be open; this shifts the threshold required for an action potential to more negative voltages,

resulting in a lower threshold potential for diastolic depolarization (Trautwein and Kameyama, 1986; reviewed by Lilly, 2007).

Parasympathetic stimulation, via muscarinic cholinergic receptors and release of acetylcholine (ACh), the primary parasympathetic neurotransmitter, reduces intrinsic firing rate of the SA node and slows conduction in the AV node, reducing intrinsic HR (reviewed by Berntson et al., 1997; reviewed by Sztajzel, 2004; reviewed by Thomas, 2011). Parasympathetic influence decreases the probability pacemaker channels will be open, reducing flow and the slope of depolarization. Furthermore, the probability of Ca channels being open decreases in the face of parasympathetic stimulation, increasing the action potential threshold to a more positive voltage (Noma and Trautwein, 1978). Parasympathetic stimulation also increases the probability transmembrane ACh-sensitive K channels will be open at rest, resulting in a more negative maximum diastolic potential (Sakmann et al., 1983; reviewed by Lilly, 2007).

1.4.3 Autonomic influence on circulating cytokines and catecholamines

Innervation of the SA node is a direct mechanism by which the autonomic nervous system controls HR and HRV. A separate, indirect autonomic mechanism also exists. By modulating circulating inflammatory factors (i.e., cytokines) and catecholamines, the autonomic nervous system indirectly affects HR and HRV.

Electrical or mechanical stimulation of the vagus nerve and the resulting increase in parasympathetic tone reduces cytokine production (Borovikova et al., 2000). Further, peripheral release of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-18), but not anti-inflammatory cytokines (IL-10), is attenuated by ACh (Borovikova et al., 2000). The interaction between ACh and the α-7 nicotinic ACh receptor subunit, expressed on cytokine-producing cells, is the molecular basis for this anti-inflammatory circuit (Wang et al., 2002).

This relationship has been observed in vivo; CRP levels are inversely related to surrogate measures of vagus nerve activity in healthy individuals (Sloan et al., 2007) and individuals with coronary heart disease (Frasure-Smith et al., 2009). Further, IL-6 levels in individuals with coronary heart disease are inversely related to HRV measures before covariate adjustment (Frasure-Smith et al., 2009).

The sympathetic nervous system also indirectly modulates HR by increased catecholamine release and pro-inflammatory cytokine production (reviewed by Lilly, 2007; Nance and Sanders, 2007). Stimulation of β-adrenoreceptor (a target of sympathetic-derived catecholamines) increases gene expression and protein production of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) in myocardial cells (Murray et al., 2000) and β-blockade reduces plasma IL-6 in patients with congestive heart failure (Mayer et al., 2005). Further, stress-induced activation of NFκB, a mediator of TNF-α responses (Beg and Baltimore, 1996), is dependent on the primary sympathetic neurotransmitter, norepinephrine, and this activation can be abrogated by α1-adrenoreceptor blockade (Bierhaus et al., 2003).

1.4.4 Autonomic maturation and age-dependence of heart rate and variability

Autonomic control is affected by age, especially during the perinatal period. These age-related autonomic changes are characterized by differences in the sympathetic:parasympathetic (sympathovagal) balance (Chatow et al., 1995; Massin and von Bernuth, 1997).

Heart rate decreases with increasing age (Finley and Nugent, 1995; Umetani et al., 1998); however, HRV changes through life are not linear or as clear. The sympathetic nervous system matures earlier than the parasympathetic nervous system and is predominant in early fetal life (Assali et al., 1978; Siimes et al., 1990; Chatow et al., 1995). At approximately 35 – 36 weeks of gestation, there is a pronounced increase in parasympathetic tone (Ramet et al., 1991). This

becomes clinically apparent during the third trimester. Fetal HRV differences among groups can be observed as early as 32 – 34 weeks of gestation (Groome et al.,1999; Gustafson et al., 2013) and remain age-dependent throughout the lifespan (Finley and Nugent, 1995).

Infants exhibit the most profound changes in HR and HRV of any life stage (Finley and Nugent, 1995), and this is at least partially attributable to the significant degree of autonomic maturation that occurs during this time (Massin and von Bernuth, 1997). Individual differences in HRV originate in utero and persist postnatally (DiPietro et al., 2007; van Leeuwen et al., 2013), suggesting HRV is established during development and related to postnatal values.

In general, low HR and high HRV are hallmarks of health (Stein et al., 2005), although there is a threshold beyond which greater decreases in HR and/or increases in HRV would indicate abnormal functioning (Simopoulos, 1991). Heart rate and HRV are easily assessed in populations across the age continuum (Massin and von Bernuth, 1997), and these measures may indirectly reflect cardiac health and cardiac-autonomic integration.

1.4.5 Relationship between n-3 LCPUFA, heart rate, and variability in different populations

Dietary fats are ubiquitous components of cell membranes, including those of the cardiovascular system (reviewed by Simopoulos, 1991). Fatty acid composition of cell membranes reflects that of the diet (reviewed by Simopoulos, 1991), implying that dietary interventions may affect cardiac health, reflected as alterations in HR and HRV. Further, HR and HRV are modulated by the autonomic nervous system, which may also be impacted by dietary fat intake.

The LCPUFA of the n-3 family, EPA and DHA, have been studied extensively with regard to human health. There is interest in exploring if these fatty acids affect HR and HRV. Similar to the age-dependence of HR and HRV (Finley and Nugent, 1995), which is largely

dictated by autonomic control, the effect of n-3 LCPUFA supplementation depends on the population.

1.4.6 Effect of n-3 LCPUFA on heart rate and variability: fetuses and infants

The perinatal period is a sensitive time during which nutrition may have programming effects on later infant health and outcome (Barker et al., 1993; reviewed by Das, 2004). In fetuses and infants, HRV is considered a developmental expression of maturation, thought to be linked to parasympathetic activity and the integrity of the developing autonomic nervous system (Richards and Cameron, 1989; Massin and von Bernuth, 1997; Longin et al., 2005; Gustafson et al., 2013). The autonomic nervous system significantly matures during perinatal life (Massin and von Bernuth, 1997); therefore, provision or deficits of nutrients during this time may exert long-term effects (reviewed by Christensen, 2011).

Colombo et al. (2011) administered 3 levels of DHA (0.32, 0.64, and 0.92% of fatty acids as DHA) to term infants from birth to 12 months of age and measured HR at 4, 6, and 9 months. Groups receiving supplemental DHA had lower HR than the control group receiving no DHA; this effect was not dose-dependent. Similarly, term infants who were breast-fed, fed DHA-enriched milk formula, or fed DHA-enriched soy formula had lower HR and higher HRV than infants fed a DHA-deficient soy formula (Pivik et al., 2009). These effects were documented from 4 to 12 months of age. In another study (Lauritzen et al., 2008), male infants receiving 924 mg fish oil, a source of n-3 LCPUFA, per day from 9 to 12 months of age had lower HR than those receiving no supplemental fish oil; no effect was observed in female infants. There was a positive association between the changes in HR and erythrocyte n-3 PUFA content, regardless of gender (Lauritzen et al., 2008)

Gustafson et al. (2013) explored if maternal supplementation of n-3 LCPUFA during pregnancy affected the developing fetus. Pregnant women were supplemented with 600 mg DHA per day or a placebo from gestational week 14.4 to term delivery. Fetal HR and HRV were assessed at 24, 32, and 36 weeks of gestation with magnetocardiography. There was a trend for lower fetal HR and higher indices of HRV, assessed as time-domain metrics.

1.4.7 Effect of n-3 LCPUFA on heart rate and variability: healthy adults

In healthy adults, HR and HRV are prognostic markers for later cardiovascular morbidity and mortality (Tibblin et al., 1974; Mølgaard et al., 1991; Gillman et al., 1993; Dekker et al., 1997; Hjalmarson, 1998; Curtis and O'Keefe, Jr., 2002). Low HRV indicates the autonomic nervous system has been strained by chronic excessive sympathetic tone and/or diminishment of parasympathetic tone (Curtis and O'Keefe, Jr., 2002). Interventions that shift autonomic balance to favor parasympathetic dominance and minimize sympathetic regulation improve disease prognosis (Curtis and O'Keefe, Jr., 2002).

In a meta-analysis of intervention trials including healthy adult populations (n = 16) and populations of adults with at least 1 chronic condition (n = 22), n-3 LCPUFA intake significantly reduced HR by 1.6 beats per minute (bpm) versus a placebo, with no effect of intake amount (Mozaffarian et al., 2005). When examining only those trials conducted in healthy adults, there was still a reduction in HR with n-3 LCPUFA intake, albeit a smaller effect, 1.4 bpm (Mozaffarian et al., 2005). Although the meta-analysis evidenced an overall HR-lowering effect in accordance with n-3 LCPUFA intervention in healthy adults (Mills et al., 1989; Vandogen et al., 1993; Conquer and Holub, 1998; Christensen et al., 1999; Dyerberg et al., 2004; Stark and Holub, 2004; Mozaffarian et al., 2005; Ninio et al., 2008), a HR-raising effect (Mills et al., 1990;

Geelen et al., 2003; Monahan et al., 2004), and mixed effects (Desylpere, 1992; Grimsgaard et al., 1998) have also been reported.

The relationship between n-3 LCPUFA and HRV in healthy populations is inconsistent. A cross-sectional study in Inuit adults, a population known for significant fish and marine mammal consumption, indicates a positive association between n-3 LCPUFA levels in erythrocyte membranes and HRV indices for females, but not males (Valera et al., 2011). In contrast, a positive association between blood n-3 LCPUFA levels and HRV has been observed in males (Christensen et al., 1999; Brouwer et al., 2002; Dallongeville et al., 2003).

In intervention trials, n-3 LCPUFA supplementation has been found to increase (Ninio et al., 2008) and decrease (Geelen et al., 2003; Dyerberg et al., 2004) HRV in healthy adults, while others have found no effect of intervention (Christensen et al., 1999). Using spectral analysis to assess HRV, Sjoberg et al. (2010) observed a shift in the low to high frequency ratio with increasing doses of fish oil, indicating a shift toward parasympathetic regulation.

Inconsistent findings could be due to heterogeneous populations and differences in sample design (intervention time, duration, and/or dosage), but could also be attributed to the analysis and interpretation of HR and HRV. Heart rate and HRV data can be derived from recordings ranging from 5 minutes to 24 hours. Further, HRV can be analyzed using time- or frequency (spectral)-domain metrics. Thus, it is difficult to conclude with certainty if, and to what extent, n-3 LCPUFA intake affects HR and HRV in healthy adults.

1.4.8 Effect of n-3 LCPUFA on heart rate and variability: cardiovascular diseases

The extent to which n-3 LCPUFA intake decreases HR is more dramatic in diseased populations than healthy adults. N-3 LCPUFA intervention has a HR-lowering effect of 2.7 bpm

in adults with coronary artery disease versus a reduction of 1.3 bpm in healthy adults (Mozaffarian et al., 2005).

Individuals at risk for a subsequent cardiac event commonly have blunted HRV (Zipes and Wellens, 1998). There is a positive association between n-3 LCPUFA consumption and HRV in adults who have previously suffered a myocardial infarction and have left ventricular dysfunction (Christensen et al., 1997). A similar association has been observed in individuals who are suspected to have ischemic heart disease (Christensen et al., 2001). In comparison with a placebo, n-3 LCPUFA increase indices of HRV in adults with cardiovascular disease (Christensen et al., 1996; O'Keefe, Jr. et al., 2006; Nodari et al., 2009; Carney et al., 2010). In contrast, Hammad et al. (2006) noted a HRV-lowering effect of n-3 LCPUFA in adults with coronary artery disease.

1.4.9 Potential mechanisms by which n-3 LCPUFA modulate heart rate and variability

In summary, n-3 LCPUFA intake is associated with decreased HR and increased HRV indices across the lifespan and for different health conditions (Christensen et al., 1999; Holguin et al., 2005; Mozaffarian et al., 2005; Mozaffarian et al., 2008; Sjoberg et al., 2010), although contradictory findings have also been reported for HR (Mills et al., 1990; Desylpere, 1992; Grimsgaard et al., 1998; Geelen et al., 2003; Monahan et al., 2004) and HRV (Christensen et al., 1999; Geelen et al., 2003; Dyerberg et al., 2004). The mechanism behind this association remains a source of controversy. Common hypotheses are that n-3 LCPUFA intake affect HR and HRV by: i) autonomic modulation (Hibbeln et al., 2006; Gustafson et al., 2008), ii) changes in cardiac electrophysiology (Harris et al., 2006; Mozaffarian et al., 2006; Kang, 2012), and/or iii) autonomic modulation of circulating cytokines and catecholamines (Berntson et al., 1997). These potential mechanisms, along with existing evidence for each, are presented here.

1.4.10 n-3 LCPUFA affect heart rate and variability by autonomic modulation: evidence

The mechanism linking n-3 LCPUFA intake and direct autonomic effects on HR and HRV is possibly two-fold and rooted in ACh, the primary vagal neurotransmitter. Firstly, in animal models, dietary n-3 LCPUFA modulates hippocampal and cerebral ACh levels (Minami et al., 1997; Favreliere et al., 2003; Aïd et al., 2005) without affecting ACh-esterase activity (Aïd et al., 2005; Shahdat et al., 2004), leading to an overall increase in ACh. Secondly, α-LA, the precursor of EPA and DHA, induces a long-term enhancement of ACh receptors (Nishizaki et al., 1997). This is mediated by activation followed by phosphorylation of the receptor's protein kinase (Nishizaki et al., 1997) and would presumably occur with the longer chain n-3 derivatives of α-LA, as well, although this interaction has not been explored, to the authors' knowledge.

It is noteworthy that, in the study by Nishizaki et al. (1997) the n-6 PUFA precursor, LA, had the same effect on ACh receptors as α-LA. Further, these findings were reported using nicotinic receptors in *Xenopus* oocytes, which may not be consistent with neuronal nicotinic ACh receptors in vivo. Nevertheless, an increase in brain ACh levels with n-3 LCPUFA intake has been reported in several animal models (Minami et al., 1997; Favreliere et al., 2003; Aïd et al., 2005), although it remains unclear if this is at least partially due to an enhancement in ACh receptors. An increase in brain ACh increases parasympathetic tone with corresponding decreases and increases in HR and HRV, respectively.

1.4.11 n-3 LCPUFA affect heart rate and variability by modulating cardiac electrophysiology: evidence

Another mechanism by which n-3 LCPUFA potentially alter HR is by directly influencing myocardial voltage-gated ion channels. Harris et al. (2006) investigated the effects of n-3 LCPUFA on HR in heart transplant recipients, the hearts of whom were functionally denervated and, thus, void of direct autonomic control. The reduction in HR after 4 – 6 months

of n-3 LCPUFA supplementation was similar for denervated and innervated individuals. The authors asserted the observed effects involve electrophysiological alterations to the myocardium and suggested the autonomic nervous system is not essential to lower HR.

Using cell culture, it was demonstrated that n-3 LCPUFA directly affect cardiac cell membrane electrical excitability (Kang and Leaf, 1994; Kang et al., 1995; Xiao et al., 1995; Kang and Leaf, 1996; Leaf, 2001). These findings, along with those of Harris et al. (2006) prompted the hypothesis that n-3 LCPUFAs lower HR by altering intrinsic HR at the level of the myocardium.

Using isolated neonatal rat cardiac myocytes, which beat independently of neural or hormonal inputs, Kang and Leaf (1994) observed that treatment with n-3 LCPUFA reduced myocyte contraction rate without affecting amplitude. To further demonstrate that n-3 LCPUFA can suppress the automaticity of cardiac contraction, the researchers investigated the response of cardiac myocytes to electrical pacing by pairing a series of stimulating impulses with addition of n-3 LCPUFA to cell media (Kang and Leaf, 1996). Exposure of cardiac myocytes to n-3 LCPUFA slowed, then maintained a reduced contraction rate, which returned to its previous value after treatment removal.

Using a patch-clamp technique in cardiac myocytes treated with n-3 LCPUFA, Kang et al. (1995) induced an action potential and measured the required strength of the current. In the presence of n-3 LCPUFA, a greater depolarizing current was necessary to induce an action potential as the result of a threshold increase and more negative resting membrane potential.

Finally, the effects of n-3 LCPUFA on single ion channel activity was observed. Using the neonatal rat cardiac myocyte model, addition of n-3 LCPUFA to cell media inhibited Na currents (Xiao et al., 1995), which is in line with similar work demonstrating an effect on Na and

Ca channels (Leaf, 2001). The depolarizing stimulus required to induce an action potential was increased by 40 – 50% and the refractory period was prolonged three-fold (Leaf, 2001).

Thus, by modulating conductance of the myocardial Na and Ca channels, n-3 LCPUFA increase the depolarizing current required to elicit an action potential and prolong the refractory period, resulting in an overall reduction in HR. Together, these studies provide a basis for the hypothesis that the mechanism by which n-3 LCPUFA reduce HR is electrophysiological. There is no evidence exploring this mechanism with specificity to HRV, to the authors' knowledge.

1.4.12 n-3 LCPUFA affect heart rate and variability by modulating circulating factors: evidence

Section 1.4.3 documents evidence of autonomic-directed alterations in circulating cytokines and catecholamines. As n-3 LCPUFA shift sympathovagal balance to favor increased parasympathetic tone (Pluess et al., 2007; Sjoberg et al., 2010) and are documented in their ability to alter circulating cytokines (Meydani et al., 1991; Gallai et al., 1993; Caughey et al., 1996), these fatty acids can presumably indirectly affect HR and HRV via modulation of circulating factors.

In individuals exposed to an endotoxin challenge, those receiving an intravenous fish oil emulsion before exposure had enhanced parasympathetic activity (assessed with frequency-domain HRV), suppressed plasma norepinephrine, and reduced circulating TNF-α versus the untreated group (Pluess et al., 2007). Supplementation with n-3 LCPUFA shifts plasma catecholamine concentrations to a more favorable epinephrine to norepinephrine ratio (Pluess et al., 2007; Hamazaki et al., 2005), indicating a suppression of sympathetic tone.

N-3 LCPUFA directly affect cytokine production and release by various methods, including competition with n-6 fatty acids for shared enzymatic pathways and altered expression of inflammatory genes via transcription factors (reviewed by Calder, 2002; reviewed by Calder,

2006), covered in full in Section 1.1. This suggests n-3 LCPUFA can also affect circulating inflammatory factors independent of autonomic modulation which, in turn, may affect HR and HRV.

1.4.13 Hypothesis

The mechanism by which n-3 LCPUFA affect HR and HRV is not fully elucidated. However, there are three potential mechanisms, presented above. These are not the only mechanisms proposed, however, they are commonly cited and have recently received attention.

While n-3 LCPUFA can directly influence the automaticity of cardiac myocytes, when PUFA are consumed, they bind albumin and other proteins and are unlikely to directly affect the myocardium unless they are incorporated into the phospholipid fraction of the myocardial cell membrane pool (reviewed by Das, 2000). Thus, those studies utilizing cardiac myocytes (Kang and Leaf, 1994; Kang et al., 1995; Xiao et al., 1995; Kang and Leaf, 1996; Leaf, 2001) may not be applicable for, or have limited relevance to, an in vivo model.

Harris et al. (2006) noted a reduction in HR in cardiac transplant patients given capsules containing n-3 LCPUFA for 4 – 6 months and attributed their findings to modification of myocardial electrophysiological properties by n-3 LCPUFA. However, in denervated individuals, the sympathetic and parasympathetic nervous systems can modulate HR and HRV indirectly by release of catecholamines and cytokines (Berntson et al., 1997). There is also potential for n-3 LCPUFA to affect HR and HRV via cytokine production and release, independent of autonomic modulation. These circulating factors would impart their effects despite cardiac denervation (Lilly, 2007).

Harris et al. (2006) noted significant effects on the QRS complex but not the QTc-interval in cardiac transplant patients after n-3 LCPUFA treatment. The QT interval and QRS

complex are both affected by the autonomic nervous system but are also influenced by circulating catecholamines (Bexton et al., 1986; Nakagawa et al., 2000), which can be altered by n-3 LCPUFA (Hamazaki et al., 2005; Pluess et al., 2007). Similar research indicates that, following cardiac denervation, a reduction in HR characteristic of sleep occurs, albeit not as great of a reduction as would be observed in normal subjects (Baust and Bohnert, 1969). This effect may possibly be attributed to the circadian rhythm of endogenously derived circulating catecholamines (Baust and Bohnert, 1969; Barnes et al., 1980; Bexton et al., 1986; Nakagawa et al., 2000). Thus, the n-3 LCPUFA effect on the QRS complex reported by Harris et al. (2006) may be explained, in part, by alterations in circulating catecholamines as regulated by the sympathetic nervous system.

In conclusion, there is evidence n-3 LCPUFA affect HR and HRV: 1) directly by increasing brain ACh and, thereby, parasympathetic tone, and 2) indirectly by modulating circulating factors, dependently and independently of the autonomic branches. Modulation of HR and HRV is complicated and could involve other processes, but these mechanisms appear to be major contributors to the observed effects, based on evidence presented in the literature. Although the hypothesis that n-3 LCPUFA affect HR and HRV via alterations in cardiac electrophysiology should not be dismissed, current evidence needs strengthening. Future studies assessing the relationship between dietary n-3 LCPUFA, HR, and HRV should also consider circulating factors, such as catecholamines and cytokines.

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CHAPTER 2. MATERNAL FATTY ACID STATUS DURING PREGNANCY IS RELATED TO INFANT HEART RATE AND HEART RATE VARIABILITY

2.1 Introduction

Heart rate (HR) and heart rate variability (HRV) are early life developmental expressions of autonomic maturation, reflecting autonomic integrity and cardiac-autonomic integration (Massin and von Bernuth, 1997; Richards and Cameron, 1989; Longin et al., 2005; Gustafson et al., 2013). Further, individual differences in HRV originate in utero and persist postnatally (DiPietro et al., 2007; van Leeuwen et al., 2013), suggesting HRV is established during fetal life and related to postnatal measures.

Consumption of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), n-3 long chain polyunsaturated fatty acids (LCPUFAs), impacts HRV across the lifespan and in healthy and diseased states, as reviewed by Christensen (2011). There is a positive association between cellular n-3 LCPUFA content and HRV (Brouwer et al., 2002; Christensen et al., 1997; Christensen et al., 1999; Christensen et al., 2001). Further, n-3 LCPUFA supplementation reduces HR (Ninio et al., 2008; Carney et al., 2010) and increases HRV (Christensen et al., 1996; Holguin et al., 2005; Ninio et al., 2008; Carney et al., 2010). Autonomic modulation by n-3 LCPUFAs is hypothesized to be the mechanism underlying these observations (Hibbeln et al., 2006; Gustafson et al., 2008). The relationship between n-3 LCPUFAs, HR, and HRV during early life mirrors that of adults (Christensen, 2011).

Male infants receiving 924 mg fish oil, a concentrated source of n-3 LCPUFA, per day from 9 to 12 months of age had lower HR than those receiving no supplemental fish oil (Lauritzen et al., 2008). Further, there was a positive association between changes in HR and erythrocyte n-3 PUFA content for male and females (Lauritzen et al., 2008). Building on these observations, Pivik et al. (2009) demonstrated that term infants who were breast-fed, fed DHA-

enriched milk formula, or fed DHA-enriched soy formula had lower HR and higher HRV from 4 to 6 months of age than infants fed a DHA-deficient soy formula. Further, term infants supplemented with DHA (0.32, 0.64, and 0.92% of fatty acids) from birth to 12 months of age had lower HR at 4, 6, and 9 months of age compared to infants who were not supplemented with DHA (Colombo et al., 2011). In a randomized clinical trial, Gustafson et al. (2013) demonstrated that fetuses of pregnant women supplemented with 600 mg of DHA per day beginning at 14.4 gestational weeks trended towards lower HR and had higher HRV at 24, 32, and 36 gestational weeks than fetuses of mothers receiving a placebo.

The autonomic nervous system, especially the parasympathetic (vagal) nervous system, matures significantly during the third trimester (Porges and Furman, 2011). Therefore, provision or deficits of nutrients during this vulnerable developmental period may exert long-term programming effects (Christensen, 2011). The fetus inefficiently converts DHA from precursors (Carnielli et al., 1996; Salem et al., 1996), thus, the primary determinant of fetal availability and delivery of n-3 LCPUFAs is the concentration in maternal circulation (Haggarty, 1999).

The studies outlined above examine the effects of maternal n-3 LCPUFA supplementation on fetal HR and HRV (Gustafson et al., 2013) and of postnatal n-3 LCPUFA supplementation on infant HR and HRV (Lauritzen et al., 2008; Pivik et al., 2009; Colombo et al., 2011). However, to the best of our knowledge, the relationship between maternal fatty acid status during pregnancy and infant HR and HRV have not previously been explored.

The aim of the current trial was to assess the relationship between maternal fatty acid status during pregnancy and infant HR and HRV during the first 6 months of life. Fatty acids of specific interest were LCPUFA of the n-3 series, DHA and EPA, and of the n-6 series,

arachidonic acid (ARA), along with their respective precursors, as these fatty acid families share an enzymatic pathway and are, therefore, metabolically competitive.

2.2 Methods

All procedures involving human subjects were approved by the Louisiana State University AgCenter, Woman's Hospital, and Pennington Biomedical Research Center Institutional Review Boards.

2.2.1 Participants

Participants enrolled in a larger study (LA Moms and Babies Study [LAMBS] for Nutrition and Growth) were invited to enroll in the current study. For the larger study, women were recruited from an obstetrics and gynecology clinic, Associates in Women's Health, Woman's Hospital in Baton Rouge, LA. Women were invited to participate in the study if they: were 18 – 35 years of age, had a singleton pregnancy, were between 17 and 20 gestational weeks, and had a pre-pregnancy body mass index of 25.0 – 29.9. Exclusion criteria included: history or current diagnosis of high blood pressure, high blood lipids, kidney disease, liver disease, polycystic ovarian syndrome, HIV, or diabetes mellitus (type 1, type 2, or gestational); a first degree relative diagnosed with diabetes mellitus (type 1 or type 2); uncontrolled thyroid disorder; smoking in the past 6 months; parity > 5; pre-term birth (≤ 37 gestational weeks); positive test for group B streptococcus, syphilis, or Hepatitis B; and pregnant or lactating within the previous 6 months.

From the LAMBS study, 17 women were approached between 37 gestational weeks and 1 week after delivery and invited to participate in the current study. Overall, 13 mother-infant pairs completed informed consent; one mother-infant pair was excluded from analysis due to infant diagnosis of congenital heart defects at 6 months of age and one mother-infant pair was

excluded from the study for failure to comply with study protocol. Maternal and infant characteristics (n = 11) are presented in Table 2.1.

Table 2.1 Maternal and infant characteristics, n = 11[1]	
Maternal age (y)	26.7 ± 3.7[2]
Pregavid BMI (kg/m^2)	26.9 ± 1.4
Race (n)	
White	4
African American	7
Parity	
0	4
1	5
3	2
Length of gestation (wk)	39.9 ± 1.0
Birth type (n)	
Vaginal	9
Cesarean	2
Infant birth weight (kg)	3.4 ± 0.4
Infant sex (n)	
Male	2
Female	9
Infant birth length (cm)	7.9 ± 0.3
Infant head circumference (cm)	5.4 ± 0.3
1-min Apgar score[3]	7.8 ± 0.6
5-min Apgar score[3]	7.9 ± 0.3
Breastfed (n)	
2 wk of age	9
4 mo of age	5
6 mo of age	3

[1]Total number of subjects included in analyses unless otherwise noted
[2]$\bar{x}$ ± SD
[3]n = 10

2.2.2 Blood collection and analysis

Blood collections occurred prior to informed consent for the current study. However, in the LAMBS study, informed consent contained an optional clause for collected blood samples to

be used in future, ancillary studies; all women approached for the current study consented to the clause. Maternal blood samples were collected at 17-20, 24, 32, and 36 gestational weeks. Blood (~10 mL) was sampled from the antecubital vein and collected in EDTA-containing tubes. Erythrocytes were separated from plasma by centrifugation (2600 × *g* at 4°C for 10 min), washed in 0.9% saline, portioned, and immediately stored at -80°C until analysis.

Erythrocytes were prepared for fatty acid analysis using a direct methylation procedure. Prior to methylation, heptadecanoic acid (17:0) was added as an internal standard for calculation of relative weight percentages (wt%) of erythrocyte fatty acids. Fatty acid methyl esters (FAME) were separated with a Hewlett-Packard 6890 series gas chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a flame ionization detector (FID) and an Omegawax® 250 fused silica capillary column (30 m × 0.25 mm × 0.25 µm; Sigma-Aldrich, St. Louis, MO). The oven temperature was programmed from 180 – 210°C at a rate of 2°C/min with a final hold of 33 min. The FID temperature was set at 280°C. Helium was used as the carrier gas and flow was maintained at 1.2 mL/min. External standards (FIM-FAME; Matreya, LLC, State College, PA) were run with each set of samples. Samples were injected in duplicate. Erythrocyte FAMEs were identified by comparison with external standards and expressed as relative wt%.

2.2.3 Infant heart rate and heart rate variability analysis

Continuous ambulatory electrocardiograph monitoring was conducted in infants for a 24 h period at 2 weeks, 4 months, and 6 months of age with Holter monitors (DigiTrak XT; Philips, Amsterdam, NL). Recordings were conducted in the participant's home. Mothers were encouraged to allow infants to engage in normal activities, advised to dress infants in a tight-fitting onesie to minimize choking hazard, and cautioned against immersing infants in water during the recording period. Recordings were analyzed by a certified technician with aid of a

diagnostic software program (Philips Zymed Holter 1810 Series, Version 2.9.4) to identify and label each QRS complex as normal or abnormal based on morphology and timing. Only QRS complexes with normal morphology were used in calculating HR and HRV measures. All recordings contained ≥ 23.5 h of analyzable data.

Mean HR was calculated as the average of all filtered RR intervals over the 24 h recording period. Three time-domain measures reflecting HRV were calculated: 1) SDNN, the standard deviation of filtered RR intervals over the 24 h recording period; 2) SDANN, the standard deviation of the means of all filtered RR intervals for all 5 min segments of the analysis; and 3) ASDNN (also known as SDNN-index), the mean of the standard deviations of all filtered RR intervals for all 5 min segments of the analysis.

Interpretation and application of each time-domain HRV index are outlined in Task Force, 1996; Stein and Kleiger, 1999; and Sztajzel, 2004. Each time-domain index is highly correlated ($r^2 \geq 0.89$) to a frequency-domain metric (Bigger Jr. et al., 1992). Time-domain analysis quantitates variability while frequency-domain analysis addresses the underlying rhythms responsible for that variability (Stein et al., 1994).

When assessed over a 24 h period, SDNN is an overall metric of HRV which is correlated to total power ($r^2 = 0.96$) and reflects all cyclic components contributing to variation, including short-term high frequency variations and long-term low frequency components (Task Force, 1996; Sztajzel, 2004). Total power, the total variance in the signal, is calculated as the summation of high frequency, low frequency, very-low frequency, and ultra-low frequency metrics (Stein and Kleiger, 1999). As such, SDNN encompasses parasympathetic- and sympathetically-modulated variations in HR. SDANN, which is strongly correlated to the ultra-low frequency metric ($r^2 = 0.96$), is an estimate of long-term components of HRV and represents

variation due to low frequency activities, such as physical activity, postural changes, and circadian rhythm (Bigger Jr. et al., 1992; Task Force, 1996; Sztajzel, 2004). ASDNN is correlated to very-low frequency ($r^2 = 0.90$) and low frequency metrics ($r^2 = 0.89$) and, thus, represents variations related to the thermoregulatory, peripheral vasomotor, and/or renin angiotensin systems, as well as oscillatory rhythms of the baroreceptor system (Bigger Jr. et al., 1992; Stein and Kleiger, 1999).

2.2.4 Statistical analyses and calculations

Statistical analyses were performed using SAS, by SAS Institute, Inc., version 9.4 (Cary, NC). For all measures, level of significance was set at ≤ 0.05 and trends were ≤ 0.10. Repeated measures of HR and HRV at 2 weeks, 4 months, and 6 months of infant age were analyzed using a randomized block design with factors infant (blocking factor) and time. Individual one-tailed t-tests were used to describe changes in HR and HRV between timepoints (2 weeks to 4 months; 4 months to 6 months; 2 weeks to 6 months).

Simple linear and multiple backward stepwise regression analyses were performed to explore associations between maternal erythrocyte fatty acids (independent or predictor variables) during pregnancy and infant HR and HRV (dependent variables). In most cases, it was impossible to assess HR/HRV at exactly 2 weeks, 4 months, or 6 months of infant age. This, combined with HR and HRV changes that are a natural reflection of advancing age during the first 6 months of life (Finley and Nugent, 1995), prompted the inclusion of exact infant age at HR/HRV assessment as an independent variable in regression analyses to explore if timing of assessment factored into our findings.

Maternal n-6 and n-3 families were included as independent variables. Fatty acids included in the n-6 series were γ-linolenic acid (GLA), dihomo-γ-linolenic acid (DGLA), linoleic

acid (LA), and ARA. Fatty acids included in the n-3 series were α-linolenic acid (α-LA), EPA, and DHA. Percent change in HR and HRV from 2 weeks to 4 and 6 months of infant age was calculated and included in analyses as a dependent variable. Example calculation: [((HR at 6 months of age – HR at 2 weeks of age)/HR at 2 weeks of age)*100].

2.3 Results

2.3.1 Fatty acid analysis

Table 2.2 provides relative wt% of n-6 and n-3 fatty acids in maternal erythrocytes at each timepoint during pregnancy (20, 24, 32, and 36 gestational weeks).

Table 2.2 Relative wt% of n-6 and n-3 fatty acids in maternal erythrocytes during pregnancy[1]

	20 GW[2]	24 GW	32 GW	36 GW
n-6 fatty acids[3]				
GLA	0.17 ± 0.03	0.17 ± 0.04	0.14 ± 0.02	0.15 ± 0.02
DGLA	1.61 ± 0.22	1.58 ± 0.23	1.59 ± 0.27	1.60 ± 0.26
LA	11.20 ± 1.78	10.83 ± 1.16	10.72 ± 0.87	10.06 ± 1.00
ARA	15.43 ± 1.28	14.98 ± 0.91	14.66 ± 0.72	14.11 ± 1.27
n-3 fatty acids				
α-LA	0.14 ± 0.05	0.14 ± 0.04	0.17 ± 0.04	0.17 ± 0.03
EPA	0.21 ± 0.10	0.29 ± 0.17	0.33 ± 0.25	0.31 ± 0.23
DHA	8.57 ± 1.12	8.53 ± 1.22	9.46 ± 1.51	9.51 ± 1.27

[1]Fatty acids expressed as relative weight percentage (wt%); values are $\bar{x}$ ± SD (n = 11)
[2]GW: gestational weeks
[3]GLA: γ-linolenic acid; DGLA: dihomo-γ-linolenic acid; LA: linoleic acid; ARA: arachidonic acid; α-LA: α-linolenic acid: EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid

2.3.2 Heart rate and heart rate variability changes over time

Infant HR and HRV during the first 6 months of life and changes between assessments (2 weeks to 4 months, 4 months to 6 months, and 2 weeks to 6 months) are presented in Table 2.3. There was a significant decrease in HR from 2 weeks to 4 month ($P \leq 0.01$), 4 month to 6 month ($P \leq 0.01$), and 2 week to 6 month assessment ($P \leq 0.01$) for an overall reduction of 17 bpm. The increase in SDNN was significant from 2 week to 4 month ($P \leq 0.01$), trended from 4 month to 6

month ($P = 0.08$), and was significant from 2 week to 6 month assessment ($P \leq 0.01$) for an overall increase of 16 ms. The increase in ASDNN from 2 week to 4 month ($P = 0.18$) and 4 month to 6 month assessment ($P = 0.19$) was not significant, but the overall increase (4 ms) from 2 week to 6 month assessment reached statistical significance ($P = 0.01$). The increase in SDANN was significant from 2 week to 4 month ($P \leq 0.01$), trended from 4 month to 6 month ($P = 0.08$), and was significant from 2 week to 6 month assessment ($P \leq 0.01$) for an overall increase of 18 ms.

Table 2.3 Infant heart rate and heart rate variability in the first 6 months of life[1,2,3]

	HR, bpm	SDNN, ms	ASDNN, ms	SDANN, ms
2 weeks	146 ± 8	45 ± 11	25 ± 6	35 ± 10
4 months	135 ± 5	56 ± 7	27 ± 4	47 ± 6
6 months	128 ± 7	61 ± 10	29 ± 4	53 ±10
	------ *Difference between timepoints* ------			
2 weeks to 4 months	-10 ± 2[4]	10 ± 3[4]	2 ± 2	12 ± 3[4]
4 months to 6 months	-7 ± 2[4]	6 ± 3	2 ± 2	6 ± 3
2 weeks to 6 months	-17 ± 2[4]	16 ± 3[4]	4 ± 2[4]	18 ± 3[4]

[1]Values are $\bar{x}$ ± SD ($n = 11$)

[2]HR: heart rate; bpm: beats per min; SDNN: standard deviation of filtered RR intervals over 24-h period; ms: milliseconds; ASDNN: mean of standard deviations of filtered RR intervals for all 5 min segments of analysis; SDANN: standard deviation of means of filtered RR intervals for all 5 min segments of analysis

[3]HR, SDNN, ASDNN, and SDANN were significantly different ($P \leq 0.05$) among participants

[4]Significant difference ($P \leq 0.05$) between timepoints

2.3.3 Maternal fatty acid status and infant heart rate: simple linear regression

Significant and trending simple linear regression models describing the relationship between individual maternal n-6 and n-3 fatty acids during pregnancy and infant HR at the 2 week, 4 month, and 6 month assessment are presented in Table 2.4.

Maternal DGLA at 20, 24, 32, and 36 gestational weeks was a positive predictor of infant HR at the 2 weeks assessment ($r^2 \geq 0.42$, $P \leq 0.03$). Infant age at 2 week HR assessment was also significantly related to infant HR at 2 weeks ($P = 0.04$). At 24, 32, and 36 gestational

Table 2.4 Relationships for individual maternal erythrocyte n-6 and n-3 fatty acids and infant heart rate: simple linear regression, $n = 11$[1,2,3]

	Infant heart rate, beats per min 2 week assessment		
Gestational week	β ± SE	r^2	*P*
20 weeks			
DGLA	23.8 ± 9.3	0.43	0.03
24 weeks			
DGLA	22.4 ± 8.9	0.42	0.03
32 weeks			
DGLA	20.5 ± 7.5	0.45	0.02
36 weeks			
DGLA	22.5 ± 7.2	0.52	≤ 0.01
Infant age[4]	1.4 ± 0.6	0.39	0.04
	4 month assessment		
20 weeks			
DHA	-2.7 ± 1.2	0.37	0.05
24 weeks			
ARA	3.5 ± 1.4	0.42	0.03
EPA	-20.4 ± 6.6	0.51	≤ 0.01
DHA	-2.1 ± 1.2	0.28	0.09
32 weeks			
ARA	5.3 ± 1.5	0.59	≤ 0.01
EPA	-14.7 ± 4.6	0.53	≤ 0.01
DHA	-2.2 ± 0.8	0.45	0.02
36 weeks			
DGLA	11.9 ± 4.9	0.40	0.04
ARA	2.8 ± 0.9	0.45	0.02
EPA	-17.4 ± 4.0	0.68	≤ 0.01
DHA	-2.8 ± 0.9	0.51	≤ 0.01
	6 month assessment		
20 weeks			
DGLA	18.8 ± 8.2	0.37	0.05
24 weeks			
DGLA	17.6 ± 7.8	0.36	0.05
EPA	-24.0 ± 10.4	0.37	0.05
32 weeks			
DGLA	18.7 ± 5.8	0.53	≤ 0.01
36 weeks			
GLA	186.2 ± 79.0	0.38	0.04
DGLA	20.3 ± 5.5	0.60	≤ 0.01
Infant age	-0.6 ± 0.3	0.31	0.08

[1]Only observations reaching statistical significance ($P \leq 0.05$) or trend ($P \leq 0.10$) reported
[2]DGLA: dihomo-γ-linolenic acid; DHA: docosahexaenoic acid; ARA: arachidonic acid; EPA: eicosapentaenoic acid; GLA: γ-linolenic acid
[3]DGLA, GLA, ARA: n-6 fatty acids; EPA, DHA: n-3 fatty acids
[4]Exact infant age (days) at heart rate assessment

weeks, maternal ARA was a positive predictor of infant HR at the 4 month assessment ($r^2 \geq 0.42$, $P \leq 0.03$). There was an inverse relationship between maternal EPA at 24, 32, and 36 weeks and infant HR at the 4 month assessment ($r^2 \geq 0.51$, $P \leq 0.01$). Maternal DHA at each timepoint (20, 24, 32, 36 gestational weeks) was inversely related to infant HR at the 4 month assessment ($r^2 \geq 0.28$); this relationship was trending at 24 weeks ($P = 0.09$) and significant at 20, 32, and 36 weeks ($P \leq 0.05$). Maternal DGLA at 36 gestational weeks was also positively related to 4 month infant HR ($r^2 = 0.40$, $P \leq 0.04$). Significant predictors of infant HR at 6 months mirrored those related to HR at 2 weeks. At every timepoint (20, 24, 32, and 36 gestational weeks), maternal DGLA was positively related to infant HR at 6 months ($r^2 \geq 0.36$, $P \leq 0.05$). At 36 gestational weeks, maternal GLA was positively related to infant HR at 6 months ($r^2 = 0.38$, $P = 0.04$). Maternal EPA at 24 gestational weeks was an inverse predictor of 6 month infant HR ($r^2 = 0.37$, $P = 0.05$). Infant age at 6 month assessment was also related to infant HR at 6 months ($P = 0.08$).

2.3.4 Maternal fatty acid families, ratios, and infant heart rate: simple linear regression

Significant and trending simple linear regression models describing the relationship between maternal n-6 and n-3 fatty acid families, fatty acid ratios, and infant HR at 4 months of life are presented in Table 2.5. Maternal n-3 family at 20, 24, 32, and 36 gestational weeks (inverse; $r^2 \geq 0.35$, $P \leq 0.01$), and maternal n-6:n-3 ratio (positive; $r^2 \geq 0.27$, $P \leq 0.10$) were related to infant HR at the 4 month assessment. For both relationships, the greatest significance was observed at the 36 week timepoint ($P \leq 0.03$). Maternal ratio of ARA:DHA at 24, 32, and 36 gestational weeks was positively related to infant HR at the 4 month assessment ($r^2 \geq 0.38$, $P \leq 0.05$).

Table 2.5 Relationships for maternal erythrocyte n-6 and n-3 fatty acid families, fatty acid ratios, and infant heart rate: simple linear regression, $n = 11$[1]			
	Infant heart rate, beats per min 4 month assessment		
Gestational week	β ± SE	r^2	*P*
20 weeks			
n-3 family[2]	-2.6 ± 1.1	0.37	0.05
n-6:n-3 families	4.6 ± 2.5	0.28	0.09
24 weeks			
n-3 family	-2.3 ± 1.0	0.35	0.06
n-6:n-3 families	4.5 ± 2.5	0.27	0.10
ARA:DHA	8.2 ± 3.6	0.38	0.05
32 weeks			
n-3 family	-2.1 ± 0.7	0.49	0.02
n-6:n-3 family	5.7 ± 2.2	0.41	0.03
ARA:DHA	10.7 ± 3.5	0.51	≤ 0.01
36 weeks			
n-3 family	-2.5 ± 0.8	0.54	≤ 0.01
n-6:n-3 families	6.7 ± 2.6	0.42	0.03
ARA:DHA	11.1 ± 3.7	0.50	0.02
[1]Only observations reaching statistical significance ($P \leq 0.05$) or trend ($P \leq 0.10$) are reported [2]n-3 family = α-linolenic acid + eicosapentaenoic acid + docosahexaenoic acid (DHA); n-6 family = γ-linolenic acid + dihomo-γ-linolenic acid + linoleic acid + arachidonic acid (ARA)			

2.3.5 Maternal fatty acid status and infant heart rate: multiple regression

Significant and trending multiple regression models describing the relationship between individual maternal n-6 and n-3 fatty acids during pregnancy and infant HR at 2 weeks, 4 months, and 6 months of life are presented in Table 2.6. Infant age at assessment was included in all multiple regression models for infant HR at 2 weeks and 6 months as it was a significant or trending variable ($P = 0.04$ and $P = 0.08$, respectively) in simple linear regression.

Maternal LA and DGLA at 24 gestational weeks and infant age were positively related to infant HR at the 2 week assessment ($r^2 \geq 0.73$, $P = 0.02$). Maternal DGLA and ARA at 24, 32, and 36 gestational weeks were inverse predictors of infant HR at the 4 month assessment ($r^2 \geq 0.59$, $P \leq 0.03$). Maternal EPA and ARA at 24 gestational weeks were significantly related to infant HR at 4 months ($r^2 \geq 0.81$, $P \leq 0.01$) such that EPA was an inverse and ARA was a

Table 2.6 Relationships for individual maternal erythrocyte n-6 and n-3 fatty acids and infant heart rate: multiple regression, $n = 11$[1]

	Infant heart rate, beats per min				
	2 week assessment				
	Parameters[2]	Parameter estimates		Model estimates	
Gestational week		β ± SE	*P*	r^2	*P*
24 weeks	LA	3.1 ± 1.5	0.07	0.73	0.02
	DGLA	19.7 ± 8.1	0.04		
	Infant age[3]	1.0 ± 0.5	0.09		
	4 month assessment				
24 weeks	ARA	3.0 ± 0.9	≤ 0.01	0.81	≤ 0.01
	EPA	-18.0 ± 4.5	≤ 0.01		
	DGLA	8.7 ± 4.9	0.10	0.59	0.03
	ARA	3.3 ± 1.3	0.03		
32 weeks	DGLA	7.3 ± 3.3	0.06	0.75	≤ 0.01
	ARA	5.3 ± 1.2	≤ 0.01		
36 weeks	DGLA	11.5 ± 2.5	≤ 0.01	0.87	≤ 0.01
	ARA	2.7 ± 0.5	≤ 0.01		
	6 month assessment				
20 weeks	DGLA	16.5 ± 7.1	0.05	0.59	0.03
	Infant age	-0.5 ± 0.3	0.07		
24 weeks	DGLA	14.9 ± 4.5	≤ 0.01	0.84	≤ 0.01
	ARA	4.3 ± 1.5	0.02		
	Infant age	-0.1 ± 0.2	≤ 0.01		
32 weeks	DGLA	15.8 ± 2.6	≤ 0.01	0.93	≤ 0.01
	ARA	4.6 ± 1.0	≤ 0.01		
	Infant age	-0.7 ± 0.1	≤ 0.01		
36 weeks	DGLA	17. 6 ± 3.5	≤ 0.01	0.88	≤ 0.01
	ARA	1.7 ± 0.7	0.05		
	Infant age	-0.6 ± 0.2	≤ 0.01		
	EPA	-15.9 ± 6.5	0.04	0.61	0.02
	Infant age	-0.66 ± 0.3	0.03		

[1]Only observations reaching statistical significance ($P \leq 0.05$) or trend ($P \leq 0.10$) are reported
[2]LA: linoleic acid; DGLA: dihomo-γ-linolenic acid; ARA: arachidonic acid; EPA: eicosapentaenoic acid
[3]LA, DGLA, ARA: n-6 fatty acids; EPA: n-3 fatty acid
[4]Exact infant age (days) at assessment was included in all multiple regression models for infant heart rate at 2 weeks
and 6 months as it was a significant or trending variable ($P = 0.04$ and $P = 0.08$, respectively) in simple linear regression

positive predictor. Maternal DGLA and ARA at 24, 32, and 36 gestational weeks and infant age at assessment were associated with infant HR at the 6 month assessment ($r^2 \geq 0.59$, $P \leq 0.01$); the association was positive for DGLA and ARA and inverse for age. Maternal EPA at 36 gestational weeks and infant age at assessment were inversely related to infant HR at the 6 month assessment ($r^2 = 0.61$, $P = 0.02$).

2.3.6 Maternal fatty acid status and infant heart rate variability: simple linear regression

Significant and trending simple linear regression models describing the relationship between individual n-6 and n-3 maternal fatty acids and infant HRV during the first 6 months of life are presented in Tables 2.7 and 2.8, respectively.

Table 2.7 Relationships for individual maternal erythrocyte n-6 and n-3 fatty acids and infant SDNN: simple linear regression, $n = 11$[1,2,3]

	Infant SDNN, ms		
	2 week assessment		
Gestational week	β ± SE	r^2	*P*
32 weeks			
DGLA	-26.1 ± 11.0	0.38	0.04
36 weeks			
DGLA	-24.9 ± 11.7	0.34	0.06
	4 month assessment		
24 weeks			
α-LA	103.4 ± 46.6	0.35	0.05
32 weeks			
GLA	-285.1 ± 126.1	0.36	0.05
α-LA	105.4 ± 42.6	0.41	0.04
36 weeks			
LA	-3.8 ± 1.9	0.30	0.08
	6 month assessment		
20 weeks			
LA	-3.8 ± 1.3	0.49	0.02
32 weeks			
DGLA	-22.9 ± 9.2	0.41	0.03
36 weeks			
DGLA	-21.1 ± 9.9	0.33	0.06

[1]Only observations reaching statistical significance ($P \leq 0.05$) or trend ($P \leq 0.10$) are reported
[2]SDNN: standard deviation of filtered RR intervals over 24-h period; DGLA: dihomo-γ-linolenic acid; GLA: γ-linolenic acid; α-LA: α-linolenic acid; LA: linoleic acid
[3]DGLA, LA: n-6 fatty acids; α-LA: n-3 fatty acid

Table 2.8 Relationships for individual and families of maternal erythrocyte n-6 and n-3 fatty acids and infant ASDNN: simple linear regression, $n = 11$[1,2]

	Infant ASDNN, ms 2 week assessment		
Gestational week	β ± SE	r^2	*P*
24 weeks			
DGLA	-13.6 ± 7.4	0.27	0.09
32 weeks			
DGLA	-13.9 ± 5.5	0.41	0.04
36 weeks			
DGLA	-11.6 ± 6.3	0.27	0.09
	4 month assessment		
32 weeks			
n-6 family[3]	-2.8 ± 1.3	0.34	0.06
36 weeks			
EPA	10.2 ± 4.9	0.32	0.07
DHA	2.0 ± 0.9	0.35	0.05
n-3 family	1.7 ± 0.8	0.35	0.05
n-6 family	-2.1 ± 1.0	0.32	0.07
n-6:n-3 families	-5.2 ± 2.4	0.35	0.06
	6 month assessment		
20 weeks			
α-LA	1.4 ± 0.65	0.35	0.06
24 weeks			
DGLA	-9.8 ± 5.2	0.28	0.09
EPA	14.1 ± 6.8	0.33	0.07
32 weeks			
DGLA	-10.6 ± 4.0	0.43	0.03
36 weeks			
EPA	10.1 ± 5.1	0.30	0.08

[1]Only observations reaching statistical significance ($P \leq 0.05$) or trend ($P \leq 0.10$) are reported

[2]ASDNN: mean of standard deviations of filtered RR intervals for all 5 min segments of analysis; DGLA: dihomo-γ-linolenic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; α-LA: α-linolenic acid

[3]n-6 family = γ-linolenic acid + DGLA + linoleic acid + arachidonic acid; n-3 family = α-LA + EPA + DHA

There was an inverse relationship between maternal DGLA at 32 and 36 gestational weeks and infant SDNN at the 2 week assessment ($r^2 \geq 0.34$, $P \leq 0.06$). Maternal DGLA at 24, 32, and 36 gestational weeks was inversely associated with ASDNN at the 2 week assessment ($r^2 \geq 0.27$, $P \leq 0.09$).

At the 4 month assessment, infant SDNN was positively related to maternal α-LA at 24 and 32 gestational weeks ($r^2 \geq 0.35$, $P \leq 0.05$). Maternal GLA at 32 gestational weeks and LA at 36 gestational weeks were significant ($r^2 = 0.36$, $P = 0.05$) and trending inverse predictors ($r^2 = 0.30$, $P = 0.08$) of infant SDNN at the 4 month assessment, respectively. Maternal n-6 family at 32 and 36 gestational weeks was a trending inverse predictor of infant ASDNN at the 4 month assessment ($r^2 \geq 0.32$, $P \leq 0.07$). Maternal DHA, EPA, and n-3 family at 36 gestational weeks were all positively related to ASDNN at the 4 month assessment ($r^2 \geq 0.32$, $P \leq 0.07$). Maternal n-6:n-3 ratio at 36 gestational weeks predicted infant ASDNN at the 4 month assessment (inverse; $r^2 = 0.35$, $P = 0.06$).

Maternal LA and DGLA were inversely and α-LA was positively related to infant SDNN and ASDNN at the 6 month assessment; these relationships were observed at 20, 24, 32, and 36 gestational weeks. There was a trending, positive relationship for maternal DGLA at 32 and 36 gestational weeks and infant SDANN at the 6 month assessment (data not shown; $r^2 \geq 0.29$, $P \leq 0.08$). Maternal EPA at 24 and 36 gestational weeks was inversely associated with infant ASDNN at the 6 month assessment ($r^2 \geq 0.28$, $P \leq 0.07$).

2.3.7 Maternal fatty acid status and infant heart rate variability: multiple regression

Significant and trending multiple regression models describing the relationship between individual maternal n-6 and n-3 fatty acids during pregnancy and infant HRV during the first 6 months of life are presented in Table 2.9.

A model including maternal LA and DGLA consistently had predictive value for infant HRV ($r^2 \geq 0.49$, $P \leq 0.04$). This was observed at different timepoints (32 and 36 gestational weeks), at various HRV assessments (2 week and 6 month), and across HRV indices (SDNN, ASDNN, and SDANN). In each instance, LA and DGLA were both inversely related to the

respective HRV index. α-LA and DHA at 20 gestational weeks were also related to infant ASDNN at 6 months (r^2 = 0.57, P = 0.04); both variables in the model were positive predictors.

Table 2.9 Relationships for maternal erythrocyte n-6 and n-3 fatty acids and infant heart rate variability: multiple regression, $n = 11$[1]

	Heart rate variability, ms				
	Infant SDNN[2], 2 week assessment				
	Parameters[3,4]	Parameter estimates		Model estimates	
Gestational week		β ± SE	*P*	r^2	*P*
36 weeks	LA	-6.5 ± 2.6	0.04	0.63	0.02
	DGLA	-33.9 ± 10.0	≤ 0.01		
	Infant SDNN, 6 month assessment				
36 weeks	LA	-5.8 ± 2.1	0.03	0.65	≤ 0.01
	DGLA	-29.1 ± 8.2	≤ 0.01		
	Infant ASDNN, 2 week assessment				
32 weeks	LA	-3.7 ± 1.3	0.02	0.72	≤ 0.01
	DGLA	-16.3 ± 4.2	≤ 0.01		
36 weeks	LA	-4.9 ± 0.8	≤ 0.01	0.89	≤ 0.01
	DGLA	-18.3 ± 2.9	≤ 0.01		
	Infant ASDNN, 6 month assessment				
20 weeks	α-LA	82.9 ± 25.7	≤ 0.01	0.57	0.04
	DHA	2.1 ± 1.1	0.10		
36 weeks	LA	-2.1 ± 1.2	0.10	0.49	0.07
	DGLA	-11.5 ± 4.5	0.03		
	Infant SDANN, 6 month assessment				
36 weeks	LA	-6.1 ± 2.2	0.03	0.63	0.02
	DGLA	-28.3 ± 8.5	0.01		

[1]Only observations reaching statistical significance ($P \leq 0.05$) or trend ($P \leq 0.10$) are reported
[2]SDNN: standard deviation of filtered RR intervals over 24-h period; ASDNN: mean of standard deviations of filtered RR intervals for all 5 min segments of analysis; SDANN: standard deviation of means of filtered RR intervals for all 5 min segments of analysis
[3]LA: linoleic acid; DGLA: dihomo-γ-linolenic acid; α-LA: α-linolenic acid
[4]LA, DGLA: n-6 fatty acids; α-LA, DHA: n-3 fatty acids

2.3.8 Maternal fatty acid status and percent change in infant heart rate and heart rate variability: simple linear regression

Significant and trending simple linear regression models describing the relationship between individual n-6 and n-3 fatty acids, families, and ratios and percent change in HR and HRV (SDANN) from the 2 week to the 4 and 6 month assessment are presented in Table 2.10.

Table 2.10 Relationships for individual and families of maternal erythrocyte n-6 and n-3 fatty acids and percent change in infant heart rate and heart rate variability: simple linear regression, $n = 11$[1,2]

	Infant heart rate, bpm		
	% change from 2 weeks to 4 month assessment		
Gestational week	β ± SE	r^2	*P*
20 weeks			
n-6:n-3 families[3]	4.9 ± 2.6	0.28	0.09
24 weeks			
ARA	4.5 ± 1.3	0.58	≤ 0.01
32 weeks			
ARA	5.1 ± 1.8	0.48	0.02
36 weeks			
ARA	2.6 ± 1.1	0.38	0.04
ARA:DHA	9.5 ± 4.7	0.32	0.07
	% change from 2 weeks to 6 month assessment		
24 weeks			
ARA	2.2 ± 1.0	0.36	0.05
32 weeks			
ARA	3.4 ± 1.1	0.52	≤ 0.01
36 weeks			
ARA	1.4 ± 0.8	0.28	0.09
	SDANN, ms[4]		
	% change from 2 weeks to 4 month assessment		
32 weeks			
ARA	-31.5 ± 15.0	0.33	0.06
	% change from 2 weeks to 6 month assessment		
20 weeks			
DHA	13.0 ± 6.6	0.31	0.08
n-3 family	12.4 ± 6.4	0.30	0.08
ARA:DHA	-75.3 ± 25.3	0.50	0.02
24 weeks			
ARA	-15.3 ± 8.3	0.28	0.09
32 weeks			
ARA	-28.4 ± 7.9	0.59	≤ 0.01
n-6 family	-16.9 ± 8.4	0.31	0.07
36 weeks			
ARA	-12.0 ± 5.7	0.33	0.07
DHA	11.3 ± 5.9	0.29	0.09
ARA:DHA	-47.3 ± 23.1	0.32	0.07
n-6 family	-15.2 ± 5.9	0.42	0.03
n-3 family	9.8 ± 5.0	0.30	0.08
n-6:n-3 families	-32.4 ± 14.9	0.34	0.06

[1]Only observations reaching statistical significance ($P \leq 0.05$) or trend ($P \leq 0.10$) are reported

[2]Example percent change calculation: [((6 month heart rate - 2 week heart rate)/2 week heart rate)*100].

[3]n-6 family = γ-linolenic acid + dihomo-γ-linolenic acid + linoleic acid + arachidonic acid (ARA); n-3 family = α-linolenic acid + eicosapentaenoic acid + docosahexaenoic acid (DHA)

[4]SDANN: standard deviation of means of filtered RR intervals for all 5 min segments of analysis

Percent change in HR from the 2 week to the 4 month assessment was positively related to maternal n-6:n-3 ratio at 20 gestational weeks ($r^2 = 0.28$, $P = 0.09$). Maternal ARA at 24, 32, and 36 gestational weeks was a significant predictor of change in percent HR from the 2 week to the 4 month assessment ($r^2 \geq 0.38$, $P \leq 0.04$); the relationship was positive at all timepoints. Maternal ARA:DHA ratio at 36 gestational weeks was a positive predictor of percent change in HR at the 4 month assessment ($r^2 \geq 0.32$, $P = 0.07$).

In examining percent change in HR from the 2 week to the 6 month assessment, maternal ARA at 24, 32, and 36 gestational weeks was a trending or significant predictor ($r^2 \geq 0.36$, $P \leq 0.09$). This relationship was positive and most significant at 32 gestational weeks ($P = 0.02$). Percent change in SDANN from the 2 week to the 4 month assessment was inversely associated with maternal ARA at 32 gestational weeks ($r^2 = 0.33$, $P = 0.06$). There was a positive relationship between maternal n-6 family at 20 gestational weeks (data not shown; $r^2 = 0.31$, $P = 0.08$) and percent change in SDNN at the 4 month assessment. Maternal LA and GLA at 20 gestational weeks were independent, inverse predictors of percent change in ASDNN at the 4 month assessment (data not shown; $r^2 = 0.35$, $P = 0.05$ and $r^2 = 0.30$, $P = 0.08$, respectively).

Maternal DHA and n-3 family at 20 gestational weeks were independent, positive predictors of percent change in infant SDANN from the 2 week to the 6 month assessment ($r^2 = 0.30 – 0.31$, $P = 0.08$). Maternal ARA:DHA at 20 gestational weeks was inversely associated with percent change in infant SDANN at 6 months ($r^2 = 0.50$, $P = 0.02$). Percent change in infant SDANN at the 6 month assessment was inversely associated with maternal ARA at 24 ($r^2 = 0.28$, $P = 0.09$) and 32 gestational weeks ($r^2 = 0.59$, $P \leq 0.01$) and maternal n-6 family at 32 gestational weeks ($r^2 = 0.31$, $P = 0.07$). At 36 gestational weeks, maternal ARA, n-6 family, ARA:DHA ratio, and n-6:n-3 family ratio were all inversely related to percent change in infant

SDANN from the 2 week to the 6 month assessment (r^2 = 0.32 – 0.42, $P \leq 0.07$). Maternal DHA and the n-3 family were positively related to percent change in SDANN at the 6 month assessment (r^2 = 0.29 – 0.30, $P \leq 0.09$).

2.4. Discussion

2.4.1 Infant heart rate and heart rate variability are age-dependent

In the current study, HR decreased and HRV increased with advancing infant age, a finding which is consistent with previous reports (Finley and Nugent 1995; Massin and von Bernuth, 1997). Decreased HR and increased HRV are hallmarks of health, although there are certain populations for whom this interpretation is inappropriate (Stein et al., 2005). For the current population, decreased HR and increased HRV are healthful attributes. Thus, independent variables that are inversely related to HR and/or positively related to HRV may have a role in accelerating autonomic maturation.

2.4.2 Cautions for data interpretation

Caution should be used when interpreting parameter and model estimates for the current data. For example, parameter estimates for maternal DGLA were consistently larger than those for maternal ARA. This is likely an artifact of low maternal DGLA relative to ARA. This example is also applicable to parameter estimates associated with GLA, α-LA, and EPA. Thus, the nature of the relationship (inverse or positive) should be the focus of the current data rather than the parameter or model estimate.

The percent change calculation required the assumption that the 2 week HR and HRV assessment was similar among participants, although this assumption was not met. As such, this calculation may not accurately represent HR and HRV maturation from 2 weeks to 6 months of infant age and should be interpreted with care.

2.4.3 Maternal fatty acid status during pregnancy is related to infant autonomic development

This observational study is the first to link maternal fatty acid status during pregnancy to infant HR and HRV in the first 6 months of life. Previous research examined the effects of maternal n-3 LCPUFA supplementation on fetal HR and HRV (Gustafson et al., 2013) and of postnatal n-3 LCPUFA supplementation on infant HR and HRV (Lauritzen et al., 2008; Pivik et al., 2009; Colombo et al., 2011). Current findings are in line with these reports, which evidenced a HR-lowering and HRV-increasing effect of n-3 LCPUFA, especially DHA. Higher maternal n-3 fatty acid status, especially DHA, was associated with reduced infant HR and increased HRV, characteristics of a mature, robust autonomic nervous system. Conversely, higher maternal n-6 fatty acids and/or n-6:n-3 ratio were related to increased infant HR and reduced HRV. By calculating percent change in HR and HRV, we also observed evidence for maternal n-3 fatty acid status to accelerate postnatal autonomic development, although there were limitations to this calculation, as noted above.

Interestingly, we observed an unfavorable association between maternal n-6 fatty acids infant HR and HRV more often than we observed a beneficial association for n-3 fatty acids. Thus, it is likely n-3 LCPUFA intake improves HR and HRV: 1) indirectly, by n-6 fatty acid displacement, and 2) directly, by biological actions of n-3 fatty acids in vivo, such as autonomic modulation.

Infant ASDNN, also known as SDNN-index, was the most sensitive time-domain HRV index to maternal fatty acids during pregnancy in the current study. Lauritzen et al. (2008) did not observe changes in infant HRV, including ASDNN, after postnatal intervention with fish oil. However, Gustafson et al. (2013) reported fetuses whose mothers were supplemented with DHA during pregnancy had higher metrics of very-low frequency and low frequency power at 24, 32,

and 36 gestational weeks than those whose mothers received a placebo. These frequency-domain metrics of HRV are highly, independently correlated ($r^2 \geq 0.89$) with ASDNN (Bigger Jr. et al., 1992) and, as such, are consistent with our data.

Our data suggests the developing autonomic nervous system is sensitive to maternal fatty acid status as early as 20 gestational weeks, although fatty acids at 32 and 36 gestational weeks were the most significant predictors of infant HR and HRV. The number of myelinated vagal (parasympathetic) fibers rapidly increases around 24 gestational weeks and throughout the first postnatal year (Pereyra et al., 1992) with the greatest increases occurring from 30 – 32 gestational weeks to 6 months of age (Sachis et al., 1982). Thus, significant or trending predictors observed at earlier timepoints (20 and 24 gestational weeks) were likely interacting with the fetal sympathetic nervous system while those observed at 32 and 36 gestational weeks were also a reflection of interactions with the developing parasympathetic nervous system.

It has previously been proposed that 3 – 5 months of infant age is an appropriate time to assess if n-3 LCPUFAs have a programming effect on cardiovascular outcomes, including HR and HRV (Pivik et al., 2009). However, maternally-influenced group differences in HR and HRV, including differences resulting from maternal n-3 LCPUFA intervention, have been observed in utero (May et al., 2010; Gustafson et al., 2013). In the current study, relationships between maternal erythrocyte fatty acids and infant HR and HRV were clinically apparent as early as 2 weeks and persisted until 6 months of infant age, indicating programming effects of the autonomic nervous system may be observed in neonatal life.

2.5 Limitations

This study was primarily limited by sample size ($n = 11$). Much of the presented data existed as statistical trends. However, patterns and themes were consistently observed and are

expected to be stronger in larger samples with more statistical power. Further, sample size limited inclusion of factors other than maternal fatty acid concentrations and exact infant age at HR/HRV assessment in multiple regression models due to loss of statistical power. However, for study enrollment, participants were required to meet strict inclusion and exclusion criteria, defined above. These criteria ensured the study participants (mothers) had similar characteristics to minimize the influence of external factors on outcomes.

2.6 Conclusion

We have demonstrated that maternal fatty acid status during pregnancy is related to infant HR and HRV, reflecting autonomic development and cardiac-autonomic integration. These data confirm that the developing autonomic nervous system is sensitive to nutritional programing and build upon existing literature evidencing a role for n-3 fatty acids, especially DHA, in accelerating autonomic development.

HR and frequency-domain metrics of HRV at 4 months of age are reliable markers of their respective trajectories throughout life, up to 4 years of age (Bar-Haim et al., 2000). Accordingly, we speculate that maternal nutrition during pregnancy affects autonomic development, which is apparent in infancy and extends to later life measures. Specifically, maternal intake of n-3 fatty acids and a lower n-6:n-3 ratio accelerates autonomic maturation. While this hypothesis is a bold extrapolation of previous and current data, it has scientific merit and is worth testing in future studies.

2.7 Literature cited

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CHAPTER 3. MATERNAL INFLAMMATORY STATUS DURING PREGNANCY IS RELATED TO INFANT HEART RATE AND HEART RATE VARIABILITY

3.1 Introduction

The fetal origins hypothesis is the well-accepted observation that the intrauterine environment, especially nutritional status (Rinaudo and Lamb, 2008), can program neonatal outcome with lifetime health and developmental consequences (Barker, 1992). Alterations in the fetal environment, including a stimulus or an insult during a critical developmental period, can result in adaptations, including structural, physiological, and metabolic changes, potentially predisposing the fetus to adverse outcomes, including chronic diseases, in later life (Barker, 1995; Muhlhausler and Ong, 2011). Adults who were prenatally exposed to famine have less DNA methylation of certain genes than their unexposed, same-sex siblings (Heijmans et al., 2008). This study was among the first to provide empirical support for the fetal origins hypothesis.

The fetal autonomic nervous system is sensitive to programming by maternal factors, including nutrition (Gustafson et al., 2013) and exercise (May et al., 2010) during pregnancy. It has also been suggested that this developing system can be programmed by inflammatory stress, although this is largely speculative (Karrow, 2006). It follows then that fetal autonomic sensitivity to inflammation may be a common factor underlying those previous observations.

Central nervous system function depends on pro- and anti-inflammatory reactions during development (Pousset et al., 1997; Zou et al., 1998; Dziegielewska et al., 2000; Vela et al., 2002; Farkas et al., 2003). However, some pro-inflammatory molecules can be neurotoxic, depending on their concentrations and the developmental stage at which the fetus is exposed (Nelson and Willoughby, 2002). Thus, it is plausible that high circulating concentrations of these molecules during critical developmental periods could alter the autonomic nervous system (Karrow, 2006).

Endocannabinoids, endogenous fatty acid derivatives, are ligands for cannabinoid receptors which are densely expressed in the brain (Glass et al., 1997). Endocannabinoids have a role in modulating inflammation (De Petrocellis et al., 2000) and autonomic function (reviewed by Pertwee, 1997). The relationship between circulating endocannabinoids, the placenta, and the fetus prompted the recent hypothesis that endocannabinoids function as a long-distance signaling system underlying fetal programming (Keimpema et al., 2013).

Autonomic activity is readily assessed through analysis of heart rate (HR) and heart rate variability (HRV). Currently, there is a body of literature exploring the effects of inflammation, assessed as circulating cytokines, on HRV, reviewed by Haensel et al. (2008). The authors determined that, in healthy adults and adults with cardiovascular diseases, HRV was inversely correlated with inflammatory biomarkers, including interleukin (IL)-6 and C-reactive protein (CRP). Studies examining cannabis use (Δ^9-tetrahydrocannabinol, THC) evidence a HR-lowering and HRV-increasing effect (Benowitz and Jones 1981; Kunos et al., 2000; Pacher et al., 2008; Schmid et al., 2010). Endocannabinoids are endogenously-derived THC counterparts that activate cannabinoid receptor type 1 (CB1) and 2 (CB2). HRV effects have not been assessed as they relate to endocannabinoid concentrations, to the best of our knowledge.

The effect of intrauterine exposure to inflammatory mediators on infant HR and HRV has not been previously explored. Accordingly, the aim of this observational study was to examine the relationship between maternal inflammatory status during pregnancy and infant HR and HRV during the first 6 months of life. Inflammatory status was assessed as endocannabinoids, cytokines (IL-6, tumor necrosis factor-α [TNF-α]), adipokines (adiponectin), and acute phase reactants (CRP).

3.2 Methods

All procedures involving human subjects were approved by the Louisiana State University AgCenter, Woman's Hospital, and Pennington Biomedical Research Center Institutional Review Boards.

3.2.1 Participants

Participants enrolled in a larger study (LA Moms and Babies Study [LAMBS] for Nutrition and Growth) were invited to enroll in the current study. For the larger study, women were recruited from an obstetrics and gynecology clinic, Associates in Women's Health, at Woman's Hospital in Baton Rouge, LA. Women were invited to participate in the study if they: were 18 – 35 years of age, had a singleton pregnancy, were between 17 and 20 gestational weeks, and had a pre-pregnancy body mass index of 25.0 – 29.9. Exclusion criteria included: history or current diagnosis of high blood pressure, high blood lipids, kidney disease, liver disease, polycystic ovarian syndrome, HIV, or diabetes mellitus (type 1, type 2, or gestational); a first degree relative diagnosed with diabetes mellitus (type 1 or type 2); uncontrolled thyroid disorder; smoking in the past 6 months; parity > 5; pre-term birth (≤ 37 gestational weeks); positive test for group B streptococcus, syphilis, or Hepatitis B; or pregnant or lactating within the previous 6 months.

From the LAMBS study, seventeen women were approached between 37 gestational weeks and 1 week after delivery and invited to participate in the current study. Overall, thirteen mother-infant pairs completed informed consent; one mother-infant pair was excluded from analysis due to infant diagnosis of congenital heart defects at 6 months of age and one mother-infant pair was excluded from the study for failure to comply with study protocol. The final sample size was $n = 11$. Maternal and infant characteristics are presented in Table 2.1.

3.2.2 Blood collection

Blood collections occurred prior to informed consent for the current study. In the LAMBS study, informed consent contained an optional clause for collected blood samples to be used in future, ancillary studies. All women consented for the current study had consented to the clause. Maternal blood samples were collected at 17-20, 24, 32, and 36 gestational weeks. Maternal blood (~10 mL) was sampled from the antecubital vein and collected in EDTA-containing tubes. Umbilical cord blood (~5-10 mL) was sampled from the venous vein into EDTA-containing tubes at delivery. Plasma and serum were individually separated from erythrocytes by centrifugation (2600 × *g* at 4°C for 10 min), portioned, and immediately stored at -80°C until analysis.

3.2.3 Endocannabinoid analysis

As previously described (Williams et al., 2007; Wood et al., 2008), a known amount of deuterated internal standard mixture was added to thawed plasma. Plasma proteins were precipitated with chilled acetone and PBS (3:1) and internal standard then centrifuged (14,000 × *g*, 5 min, 4°C). Acetone was evaporated from the recovered supernatant under nitrogen. Liquid-liquid phase extraction was performed on the remaining supernatant with PBS, methanol, and chloroform (1:1:2, by volume). The two phases were separated by centrifugation (14,000 × *g*, 5 min, 4°C), and the lower organic layer was quantitatively recovered and evaporated to dryness under nitrogen. Dried lipid extracts were reconstituted in ethanol, vortexed, sonicated, and centrifuged (14,000 × *g*, 5 min, 4°C).

Multiple reaction monitoring (MRM) of the endocannabinoid metabolome and the corresponding deuterated internal standards was performed as previously described (Williams et al., 2006; Williams et al., 2007) using a TSQ Quantum Ultra triple quadrupole mass spectrometer

(Thermo Electron, San Jose, CA) with an Agilent 1100 HPLC on the front end (Agilent Technologies, Wilmington, DE). Chromatographic separation was achieved using an Agilent Zorbax SB-CN column (2.1 × 50 mm, 5 μm) with gradient elution using 10 mM ammonium acetate and 100% methanol. Eluted peaks were ionized via atmospheric pressure chemical ionization in MRM mode. Deuterated internal standards were used for each analyte's standard curve and their concentrations per mL of plasma were determined.

3.2.4 Cytokine, adipokine, and acute phase reactant analysis

Serum was utilized for analysis of cytokines, adipokines, and acute phase reactants. High sensitivity CRP was determined by chemiluminescent immunoassay (Immulite 2000^{TM}, Siemens Healthcare Diagnostics, Deerfield, IL, USA), TNF-α and IL-6 by immunoassay (Luminex 100^{TM}, Luminex Corp., Austin, TX, USA), and adiponectin by radioimmunoassay (Linco Research Inc., St Charles, MO, USA).

3.2.5 Infant heart rate and heart rate variability analysis

Continuous ambulatory electrocardiograph monitoring was conducted in infants for a 24 h period at 2 weeks, 4 months, and 6 months of age with Holter monitors (DigiTrak XT; Philips, Amsterdam, NL). Recordings were conducted in the participant's home. Mothers were encouraged to allow infants to engage in normal activities, advised to dress infants in a tight-fitting onesie to minimize choking hazard, and cautioned against immersing infants in water during the recording periods. Recordings were analyzed by a certified technician with aid of a diagnostic software program (Philips Zymed Holter 1810 Series, Version 2.9.4) to identify and label each QRS complex as normal or abnormal based on morphology and timing. Only QRS complexes with normal morphology were used in calculating HR and HRV measures. All recordings contained ≥ 23.5 h of analyzable data.

Mean HR was calculated as the average of all filtered RR intervals over the 24 h recording period. Three time-domain measures reflecting HRV were calculated: 1) SDNN, the standard deviation of filtered RR intervals over the 24 h recording period; 2) SDANN, the standard deviation of the means of all filtered RR intervals for all 5 min segments of the analysis; and 3) ASDNN (also known as SDNN-index), the mean of the standard deviations of all filtered RR intervals for all 5 min segments of the analysis.

Interpretation and application of each time-domain HRV index are outlined in Task Force, 1996; Stein and Kleiger, 1999; and Sztajzel, 2004. Each time-domain index is highly correlated ($r^2 \geq 0.89$) to a frequency-domain metric (Bigger Jr. et al., 1992). Time-domain analysis quantitates variability while frequency-domain analysis addresses the underlying rhythms responsible for that variability (Stein et al., 1994).

When assessed over a 24 h period, SDNN is an overall metric of HRV which is correlated to total power ($r^2 = 0.96$) and reflects all cyclic components contributing to variation, including short-term high frequency variations and long-term low frequency components (Task Force, 1996; Sztajzel, 2004). Total power, the total variance in the signal, is calculated as the summation of high frequency, low frequency, very-low frequency, and ultra-low frequency metrics (Stein and Kleiger, 1999). As such, SDNN encompasses parasympathetic- and sympathetically-modulated variations in HR. SDANN, which is strongly correlated to the ultra-low frequency metric ($r^2 = 0.96$), is an estimate of long-term components of HRV and represents variation due to low frequency activities, such as physical activity, postural changes, and circadian rhythm (Bigger Jr. et al., 1992; Task Force, 1996; Sztajzel, 2004). ASDNN is correlated to very-low frequency ($r^2 = 0.90$) and low frequency metrics ($r^2 = 0.89$) and, thus, represents variations related to the thermoregulatory, peripheral vasomotor, and/or renin

angiotensin systems, as well as oscillatory rhythms of the baroreceptor system (Bigger Jr. et al., 1992; Stein and Kleiger, 1999).

3.2.6 Statistical analyses and calculations

Statistical analyses were performed using SAS, by SAS Institute, Inc., version 9.4 (Cary, NC). For all measures, level of significance was set at ≤ 0.05 and trends were ≤ 0.10. Repeated measures of HR and HRV at the 2 week, 4 month, and 6 month assessment were analyzed using a randomized block design with factors infant (blocking factor) and time. Individual one-tailed t-tests were used to describe changes in HR and HRV between timepoints (2 weeks to 4 months; 4 months to 6 months; 2 weeks to 6 months).

Simple linear and multiple (backward stepwise) regression analyses were performed to explore associations between maternal erythrocyte fatty acids (independent or predictor variables) during pregnancy and infant HR and HRV (dependent variables). In most cases, it was impossible to assess infant HR/HRV at exactly 2 weeks, 4 months, or 6 months of age. This, combined with HR and HRV changes that are a natural reflection of advancing age during the first 6 months of life (Finley and Nugent, 1995), prompted the inclusion of exact infant age at HR/HRV assessment as an independent variable in regression analyses to explore if timing of assessment factored into our findings.

Endocannabinoid data are limited to those related to the n-6 (AEA) and n-3 (EEA, EPEA, and DHEA) fatty acids. Endocannabinoids related to the n-3 fatty acid family were combined and included as an independent variable; this variable is referred to as “n-3 family” and includes eicosanoyl ethanolamine (EEA), eicosapentaenoyl ethanolamine (EPEA), and docosahexaenoyl ethanolamine (DHEA).

3.3 Results

3.3.1 Endocannabinoid analysis

Maternal and venous cord plasma n-6 and n-3 endocannabinoids are presented in Table 3.1. Eleven *N*-acyl-ethanolamine and acyl-glycerol species recognized as constituents of the endocannabinoid metabolome were simultaneously analyzed by the LC-MS/MS-based metabolomics approach (Williams et al., 2007; Wood et al., 2008). Standard curves for each analyte were linear with regression values ≥ 0.98. Extraction efficiencies, determined by comparing area ratios of each extracted BSA analyte to the un-extracted standards in ethanol (Williams et al., 2007), were ≥ 83% except for DHEA and acyl-glycerol species. Of the eleven endocannabinoid-related metabolites studied, AEA, PEA, OEA, and DHEA were readily detected. EPEA and EEA were moderately detected and the acyl-glycerols were not detected.

3.3.2 Cytokine, adipokine, and acute phase reactant analysis

Maternal and venous cord serum cytokines, adipokine, and acute phase reactant concentrations are presented in Table 3.1. Adiponectin and CRP were readily detected and TNF-α was moderately detected (≥ 82%) in maternal serum at 20, 24, 32, and 36 gestational weeks. Maternal serum IL-6 was marginally detected (≤ 28%) at 20, 24, 32, and 36 gestational weeks and excluded from statistical analyses. In venous cord serum, adiponectin, TNF-α, and IL-6 were moderately detected (≥ 64%). Venous cord serum CRP was marginally detected (≤ 28%) in venous cord serum and excluded from statistical analysis.

3.3.3 Heart rate and heart rate variability changes over time

Infant HR and HRV during the first 6 months of life and changes between assessments (2 weeks to 4 months, 4 months to 6 months, and 2 weeks to 6 months) are presented in Table 2.3. There was a significant decrease in HR from the 2 week to 4 month ($P \leq 0.01$), 4 month to 6 month

Table 3.1 Maternal and venous cord plasma n-6 and n-3 endocannabinoids and serum cytokines, adipokines, and acute phase reactants[1]

	20 GW[2]	24 GW	32 GW	36 GW	Delivery
Endocannabinoids, ng/mL[3]					
AEA	0.24 ± 0.07[6]	0.17 ± 0.09[6]	0.16 ± 0.08	0.30 ± 0.40[5]	0.42 ± 0.19[6]
EEA	0.07 ± 0.03	0.07 ± 0.03	0.10 ± 0.08	0.11 ± 0.14	0.06 ± 0.02[6]
EPEA	0.30 ± 0.20[5]	0.27 ± 0.05[7]	0.35 ± 0.33[6]	0.31 ± 0.18[6]	0.33 ± 0.08[6]
DHEA	0.41 ± 0.15	0.50 ± 0.28[7]	0.59 ± 0.38	0.66 ± 0.59	0.78 ± 0.22[6]
Cytokines, pg/mL					
IL-6	n/a[8]	n/a	n/a	n/a	13.8 ± 10.1[5]
TNF-α	9.8 ± 4.8[6]	9.4 ± 3.5[6]	8.9 ± 3.5[6]	9.1 ± 3.1[6]	28.3 ± 7.6[5]
Adipokine, μg/mL					
Adiponectin	7.8 ± 3.5	6.5 ± 2.4	5.9 ± 2.6	5.9 ± 3.6	28.3 ± 8.5[4]
Acute phase reactant, mg/L					
CRP	10.2 ± 6.4	17.9 ± 27.2	9.6 ± 6.7	7.6 ± 4.9	n/a

[1]Values are $\bar{x}$ ± SD; n = 11, unless otherwise noted

[2]GW: gestational weeks; Delivery indicates venous cord plasma/serum collected at birth

[3]AEA: anandamide (n-6 related); EEA: eicosanoyl ethanolamine (n-3 related); EPEA: eicosapentaenoyl ethanolamine (n-3 related); DHEA: docosahexaenoyl ethanolamine (n-3 related); IL-6: interleukin-6; TNF-α: tumor necrosis factor-α; CRP: C-reactive protein

[4]n = 7; [5]n = 8; [6]n = 9; [7]n = 10; [8]n/a indicates the analyte was marginally detected (≤ 28%) and excluded from statistical analyses

($P \leq 0.01$), and 2 week to 6 month assessment ($P \leq 0.01$) for an overall reduction of 17 bpm. The increase in SDNN was significant from the 2 week to 4 month ($P \leq 0.01$), trended from the 4 month to 6 month ($P = 0.08$), and was significant from the 2 week to 6 month assessment ($P \leq 0.01$) for an overall increase of 16 ms. The increase in ASDNN from the 2 week to 4 month ($P = 0.18$) and 4 month to 6 month assessment ($P = 0.19$) was not significant, but the overall increase (4 ms) from the 2 week to 6 month assessment reached statistical significance ($P = 0.01$). The increase in SDANN was significant from the 2 week to 4 month ($P \leq 0.01$), trended from 4 month to 6 month ($P = 0.08$), and was significant from the 2 week to 6 month assessment ($P \leq 0.01$) for an overall increase of 18 ms.

3.3.4 Maternal endocannabinoids and infant heart rate: simple linear regression

Significant ($P \leq 0.05$) and trending ($P \leq 0.10$) simple linear regression models describing relationships for individual, families, and ratios of maternal endocannabinoids during pregnancy and infant HR in the first 6 months of life are presented in Table 3.2.

Maternal EPEA at 24 gestational weeks was related (inverse; $r^2 = 0.32$; $P = 0.09$) to infant HR at the 2 week assessment. Maternal n-3 endocannabinoid family at 24 gestational weeks was also inversely related to 2 week infant HR ($r^2 = 0.38$; $P = 0.05$). Maternal AEA:n-3 family ratio at 24 gestational weeks was positively related to infant HR at the 2 week assessment ($r^2 = 0.38$; $P = 0.08$). There was a significant relationship between infant age at assessment and 2 week HR (positive; $r^2 = 0.39$; $P = 0.04$).

At several timepoints in pregnancy (24, 32, 36 gestational weeks) and in cord plasma at delivery, EPEA was inversely related to infant HR at 4 months of age; this relationship was trending at 36 weeks ($r^2 = 0.38$; $P = 0.08$) but significant at 24 and 32 gestational weeks and delivery ($r^2 \geq 0.46$; $P \leq 0.03$). Maternal DHEA and maternal n-3 endocannabinoid family at 24 and 32 gestational weeks and at delivery were also inversely related to 4 month infant HR ($r^2 \geq 0.33$; $P \leq 0.06$ and $r^2 \geq 0.33$; $P \leq 0.07$, respectively).

Maternal AEA at 20 gestational weeks (r^2: 0.41; $P = 0.06$) and maternal AEA:DHEA ($r^2 = 0.38$; $P = 0.08$) at delivery were positive predictors of infant HR at the 6 month assessment. There were inverse relationships for maternal n-3 family at 24, 36 gestational weeks and 6 month infant HR ($r^2 \geq 0.30$; $P \leq 0.08$). Maternal EEA and EPEA at 24 and 32 gestational weeks were

Table 3.2 Relationships for individual, families, and ratios of maternal n-6 and n-3 endocannabinoids and infant heart rate: simple linear regression[1,2]

	Infant heart rate, beats per min			
	2 week assessment			
Gestational week	*n*	β ± SE	r^2	*P*
24 weeks				
EPEA	10	-84.8 ± 43.6	0.32	0.09
n-3 family	11	-13.7 ± 5.9	0.38	0.05
AEA:n-3 family	9	53.4 ± 25.9	0.38	0.08
Infant age[3]	11	1.4 ± 0.6	0.39	0.04
	4 month assessment			
24 weeks				
EPEA	10	-66.4 ± 25.5	0.46	0.03
DHEA	11	-10.1 ± 4.8	0.33	0.06
n-3 family	11	-7.8 ± 3.7	0.33	0.07
32 weeks				
EPEA	9	-11.6 ± 3.6	0.60	0.02
DHEA	10	-11.1 ± 2.7	0.67	≤ 0.01
n-3 family	11	-5.3 ± 1.3	0.64	≤ 0.01
36 weeks				
EPEA	9	-17.8 ± 8.5	0.38	0.08
Delivery[4]				
EPEA	9	-44.9 ± 14.4	0.58	0.02
DHEA	9	-18.2 ± 4.7	0.68	≤ 0.01
n-3 family	9	-14.0 ± 3.2	0.73	≤ 0.01
	6 month assessment			
20 weeks				
AEA	9	63.7 ± 28.7	0.41	0.06
24 weeks				
EEA	11	-136.1 ± 69.0	0.30	0.08
EPEA	10	-92.5 ± 32.0	0.51	0.02
n-3 family	11	-10.7 ± 5.1	0.33	0.07
32 weeks				
EEA	11	-47.9 ± 21.6	0.35	0.05
EPEA	9	-12.4 ± 5.8	0.39	0.07
36 weeks				
n-3 family	11	-4.9 ± 2.5	0.30	0.08
Delivery				
AEA:DHEA	9	14.9 ± 7.3	0.38	0.08
Infant age	11	-0.6 ± 0.3	0.31	0.08

[1]Only observations reaching statistical significance ($P \leq 0.05$) or trend ($P \leq 0.10$) are reported
[2]EPEA: eicosapentaenoyl ethanolamine; n-3 family: eicosanoyl ethanolamine (EEA) + EPEA + docosahexaenoyl ethanolamine (DHEA); AEA: anandamide (n-6 related)
[3]Exact infant age (days) at heart rate assessment
[4]Venous cord plasma collected at delivery

both independent, inverse predictors of infant HR at the 6 month assessment ($r^2 \geq 0.30$; $P \leq 0.08$ and $r^2 \geq 0.39$; $P \leq 0.07$, respectively). Infant age at assessment was an inverse predictor ($r^2 = 0.31$; $P = 0.08$) of 6 month infant HR.

3.3.5 Maternal endocannabinoids and infant heart rate: multiple regression

Significant ($P \leq 0.05$) and trending ($P \leq 0.10$) multiple regression models describing relationships between individual, families, and ratios of maternal endocannabinoids during pregnancy and infant HR in the first 6 months of life are presented in Table 3.3. Infant age at assessment was included in models at 2 weeks and 6 months as it was a significant or trending independent variable in simple linear regression.

Infant HR at 2 weeks was positively related to maternal AEA:DHEA at 24 gestational weeks and infant age at assessment ($r^2 = 0.89$; $P \leq 0.01$). Both variables in the model were positively related to HR. Maternal n-3 family and AEA at 36 gestational weeks predicted infant HR at the 4 month assessment ($r^2 = 0.62$; $P = 0.09$). There was an inverse relationship for the n-3 family and a positive relationship was observed for AEA; the variables in the model were both independently significant ($P \leq 0.05$). Maternal EEA at 20 gestational weeks and infant age at assessment were both inversely related to 6 month infant HR ($r^2 = 0.52$; $P = 0.05$). A model including infant age at assessment, maternal EEA, and EPEA at 24 gestational weeks was inversely related to infant HR at the 6 month assessment ($r^2 = 0.82$; $P \leq 0.01$). Further, maternal n-3 family at 32 gestational weeks and infant age at assessment were inversely related to 6 month infant HR ($r^2 = 0.74$; $P \leq 0.01$). Venous cord EPEA and AEA, with infant age at assessment, were related to infant HR at 6 months; all variables in the model were inverse ($r^2 = 0.87$; $P \leq 0.01$).

Table 3.3 Relationships for maternal n-6 and n-3 endocannabinoids and infant heart rate: multiple regression[1]

	Heart rate, beats per min				
	2 week assessment				
	Parameters[2]	Parameter estimates		Model estimates	
Gestational week		β ± SE	*P*	*n*	r^2	*P*
24 weeks	AEA:DHEA	20.9 ± 9.6	0.07	9	0.89	≤ 0.01
	Infant age[3]	1.6 ± 0.3	≤ 0.01			
	4 month assessment					
36 weeks	n-3 family	-10.8 ± 4.2	0.05	8	0.62	0.09
	AEA	24.5 ± 8.6	0.04			
	6 month assessment					
20 weeks	EEA	-104.6 ± 54.8	0.09	11	0.52	0.05
	Infant age	-0.8 ± 0.3	0.03			
24 weeks	EEA	-61.7 ± 24.8	0.05	10	0.82	≤ 0.01
	EPEA	-141.5 ± 49.4	0.03			
	Infant age	-0.5 ± 0.2	0.06			
32 weeks	n-3 family	-6.2 ± 1.7	≤ 0.01	11	0.74	≤ 0.01
	Infant age	-0.8 ± 0.2	≤ 0.01			
Delivery[4]	EPEA	-76.1 ± 14.8	≤ 0.01	11	0.87	≤ 0.01
	AEA	-20.5 ± 7.4	0.04			
	Infant age	-0.9 ± 0.2	≤ 0.01			

[1]Only observations reaching statistical significance ($P \leq 0.05$) or trend ($P \leq 0.10$) are reported
[2]AEA: ananamide (n-6 related); DHEA: docosahexaenoyl ethanolamine; n-3 family: eicosanoyl ethanolamine (EEA) + EPEA: eicosapentaenoyl ethanolamine + DHEA
[3]Exact infant age (days) at heart rate variability assessment
[4]Venous cord plasma collected at delivery

3.3.6 Maternal endocannabinoids and infant heart rate variability: simple linear regression

Significant ($P \leq 0.05$) simple linear regression models describing relationships between individual, families, and ratios of maternal endocannabinoids during pregnancy and infant HRV at the 2 week and 6 month assessments are presented in Tables 3.4 and 3.5, respectively.

At 24 gestational weeks, maternal AEA:n-3 family was significantly, inversely related to infant SDNN at the 2 week assessment ($r^2 = 0.44$; $P = 0.05$). Similarly, there was an inverse

relationship between maternal AEA:DHEA at 24 gestational weeks and SDNN at the 2 week assessment (data not shown; $r^2 = 0.36$; $P = 0.09$). Maternal DHEA and n-3 family at 36 gestational weeks were significant, positive predictors of 2 week infant SDNN ($r^2 = 0.37 – 0.38$; $P \leq 0.05$) while there was also a positive association for maternal EEA at 36 gestational weeks (data not shown; $r^2 = 0.30$; $P = 0.08$).

Table 3.4 Relationships for individual, families, and ratios of maternal n-6 and n-3 endocannabinoids and infant heart rate variability at 2 weeks: simple linear regression[1]

	Infant heart rate variability[2,3] 2 week SDNN, ms			
Gestational week	*N*	β ± SE	r^2	*P*
24 weeks				
AEA:n-3 family	9	-86.2 ± 37.1	0.44	0.05
36 weeks				
DHEA	11	11.7 ± 5.0	0.37	0.05
n-3 family	11	9.2 ± 3.9	0.38	0.04
	2 week ASDNN, ms			
24 weeks				
AEA:n-3 family	9	-47.6 ± 15.4	0.58	0.02
36 weeks				
EEA	11	27.1 ± 10.3	0.43	0.02
DHEA	11	7.2 ± 2.2	0.54	≤ 0.01
n-3 family	11	5.8 ± 1.7	0.57	≤ 0.01

[1]Only observations reaching statistical significance ($P \leq 0.05$) are reported
[2]SDNN: standard deviation of filtered RR intervals over 24-h period; ASDNN: mean of standard deviations of filtered RR intervals for all 5 min segments of analysis; SDANN: standard deviation of means of filtered RR intervals for all 5 min segments of analysis
[3]AEA: anandamide (n-6 related); n-3 family: eicosanoyl ethanolamine (EEA) + (eicosapentaenoyl ethanolamine) EPEA + docosahexaenoyl ethanolamine (DHEA)

Predictors of ASDNN at the 2 week assessment were largely similar to those of SDNN. Maternal AEA:n-3 family at 24 gestational weeks was inversely related to infant ASDNN at the 2 week assessment ($r^2 = 0.58$; $P = 0.02$) and maternal AEA:DHEA was an inverse predictor (data not shown; $r^2 = 0.42$; $P = 0.06$). Maternal EEA and DHEA at 36 gestational weeks were positively related to ASDNN at the 2 week assessment ($r^2 \geq 0.43$; $P \leq 0.02$), as was the maternal n-3 family ($r^2 = 0.57$; $P \leq 0.01$). Further, AEA at 36 gestational weeks was inversely related to

infant ASDNN at the 2 week assessment (data not shown; $r^2 = 0.46$; $P = 0.07$). There were no significant relationships between maternal individual, families, or ratios of n-6 or n-3 endocannabinoids and infant SDANN at the 2 week assessment.

There were few significant ($P \leq 0.05$) or trending ($P \leq 0.10$) n-6 or n-3 endocannabinoid predictors of infant HRV (SDNN, ASDNN, and/or SDANN) at the 4 month assessment. For all three HRV indices, cord venous EPEA was positively related to 4 month HRV (data not shown; $r^2 \geq 0.36$; $P \leq 0.09$), although significant only for SDNN ($P = 0.04$). For 4 month ASDNN, maternal EPEA at 36 gestational weeks was also positively related (data not shown; $r^2 \geq 0.38$; $P = 0.08$).

At 24 gestational weeks, maternal AEA, AEA:DHEA, and AEA:n-3 family were all inversely related to infant SDNN at the 6 month assessment ($r^2 \geq 0.45$; $P \leq 0.05$). At 32 gestational weeks, maternal EPEA was the only predictor of 6 month infant SDNN (positive; $r^2 = 0.52$; $P \leq 0.03$). Maternal EEA, EPEA, and DHEA at 36 gestational weeks were independently related to infant SDNN ($r^2 \geq 0.43$; $P \leq 0.05$), as was the combined n-3 family ($r^2 \geq 0.72$; $P \leq 0.01$); all predictors were positive.

Infant ASDNN at the 6 month assessment was inversely related to maternal AEA:n-3 family at 24 gestational weeks ($r^2 \geq 0.48$; $P = 0.04$). At 32 gestational weeks, maternal EEA and the n-3 endocannabinoid family were trending, positive predictors of 6 month ASDNN (data not shown; $r^2 \geq 0.28$; $P = 0.09$) and maternal EPEA was significant (positive; $r^2 = 0.77$; $P \leq 0.01$). Maternal EEA, EPEA, DHEA, and the combined n-3 family at 36 gestational weeks were positive predictors of infant ASDNN at the 6 month assessment ($r^2 \geq 0.53$; $P \leq 0.08$). There were positive, independent associations for venous cord EEA, EPEA, DHEA, and n-3 family with infant ASDNN at the 6 month assessment ($r^2 \geq 0.51$; $P \leq 0.03$).

Table 3.5 Relationships between individual, families, and ratios of maternal n-6 and n-3 endocannabinoids and infant heart rate variability at 6 months: simple linear regression[1]

	Infant heart rate variability[2,3]			
	6 month SDNN, ms			
Gestational week	*N*	β ± SE	r^2	*P*
24 weeks				
AEA	9	-66.0 ± 27.8	0.45	0.05
AEA:DHEA	9	-65.5 ±17.4	0.67	≤ 0.01
AEA:n-3 family	9	-88.8 ± 20.1	0.74	≤ 0.01
32 weeks				
EPEA	9	18.0 ± 6.6	0.52	0.03
36 weeks				
EEA	11	50.6 ± 15.0	0.56	≤ 0.01
EPEA	9	30.6 ± 13.3	0.43	0.05
DHEA	11	13.4 ± 3.0	0.68	≤ 0.01
n-3 family	11	10.7 ± 2.3	0.71	≤ 0.01
	6 month ASDNN, ms			
24 weeks				
AEA:n-3 family	9	-28.1 ± 11.1	0.48	0.04
32 weeks				
EPEA	9	10.4 ± 2.2	0.77	≤ 0.01
36 weeks				
EEA	11	22.2 ± 7.0	0.53	≤ 0.01
EPEA	11	19.1 ± 3.8	0.78	≤ 0.01
DHEA	11	6.0 ± 1.4	0.68	≤ 0.01
n-3 family	11	5.1 ± 0.8	0.81	≤ 0.01
Delivery[4]				
EEA	9	142.1 ± 43.8	0.60	≤ 0.01
EPEA	9	44.6 ± 6.6	0.87	≤ 0.01
DHEA	9	12.8 ± 4.7	0.51	0.03
n-3 family	9	11.3 ± 2.7	0.72	≤ 0.01
	6 month SDANN, ms			
24 weeks				
AEA	9	-72.8 ± 27.5	0.50	0.03
AEA:DHEA	9	-68.7 ± 18.0	0.68	≤ 0.01
AEA:n-3 family	9	-89.4 ± 22.9	0.69	≤ 0.01
36 weeks				
DHEA	11	12.9 ± 3.4	0.62	≤ 0.01
n-3 family	11	10.1 ± 2.6	0.62	≤ 0.01

[1]Only observations reaching statistical significance ($P \leq 0.05$) are reported
[2]SDNN: standard deviation of filtered RR intervals over 24-h period; ASDNN: mean of standard deviations of filtered RR intervals for all 5 min segments of analysis; SDANN: standard deviation of means of filtered RR intervals for all 5 min segments of analysis
[3]AEA: anandamide (n-6 related); EPEA: eicosapentaenoyl ethanolamine; DHEA: docosahexaenoyl ethanolamine; n-3 family: eicosanoyl ethanolamine (EEA) + EPEA + DHEA
[4]Venous cord plasma collected at delivery

Maternal AEA, AEA:DHEA, and AEA:n-3 family at 24 gestational weeks were independently, inversely associated with infant SDANN at the 6 month assessment ($r^2 \geq 0.50$; $P \leq 0.03$). Maternal EPEA at 32 gestational weeks was a positive predictor of 6 month SDANN (data not shown; $r^2 = 0.38$; $P = 0.08$). At 36 gestational weeks, maternal DHEA and n-3 family were positive, independent predictors of 6 month SDANN ($r^2 = 0.62$; $P \leq 0.01$). There was a trend for a positive relationship between maternal AEA:n-3 family at 36 gestational weeks and infant SDANN at the 6 month assessment (data not shown; $r^2 = 0.43$; $P = 0.08$).

3.3.7 Maternal cytokines, adipokine, and acute phase reactant and infant heart rate and heart rate variability: multiple regression

Significant ($P \leq 0.05$) multiple regression models describing relationships between maternal adiponectin, CRP, and TNF-α during pregnancy and infant HR and HRV are presented in Table 3.6. Simple linear regression models are not presented as significant or trending variables were also significant in and strengthened by multiple regression modeling.

Maternal TNF-α at 24 gestational weeks and exact infant age at assessment were positively related to infant HR at the 2 week assessment ($r^2 = 0.82$; $P \leq 0.01$); both variables in the model were independent predictors ($P \leq 0.05$). Infant HR at the 6 month assessment was predicted by maternal adiponectin at 20 gestational weeks and infant age at assessment ($r^2 = 0.65$; $P = 0.02$). Adiponectin was a positive, significant variable in the model ($P = 0.02$) while age was inverse and trending ($P = 0.09$). A model including maternal adiponectin, CRP, and TNF-α at 20 gestational weeks was related to infant SDNN and SDANN at the 6 month assessment ($r^2 \geq 0.70$; $P \leq 0.03$); all variables in the model were inversely related to both HRV indices and independently significant or trending ($P \leq 0.07$).

Table 3.6 Relationships for maternal inflammatory biomarkers, infant heart rate, and heart rate variability: multiple regression[1]

Gestational week	Parameters[2]	Parameter estimates		Model estimates		
		β ± SE	*P*	*n*	r^2	*P*
		Infant heart rate, beats per min				
		2 week assessment				
24 weeks	TNF-α	0.9 ± 0.4	0.05	9	0.82	≤ 0.01
	Infant age[3]	2.1 ± 0.4	≤ 0.01			
		Infant heart rate, beats per min				
		6 month assessment				
20 weeks	Adiponectin	1.2 ± 0.4	0.02	11	0.65	0.02
	Infant age	-0.5 ± 0.2	0.09			
		Infant SDNN, ms				
		6 month assessment				
20 weeks	Adiponectin	-1.5 ± 0.5	0.03	9	0.85	0.02
	CRP	-1.1 ± 0.3	0.01			
	TNF-α	-1.3 ± 0.4	0.02			
		Infant SDANN, ms				
		6 month assessment				
20 weeks	Adiponectin	-1.3 ± 0.6	0.07	9	0.70	0.03
	CRP	-1.0 ± 0.3	0.02			
	TNF-α	-1.4 ± 0.4	0.02			

[1]Only observations reaching statistical significance ($P \leq 0.05$) or trend ($P \leq 0.10$) are reported
[2]TNF-α: tumor necrosis factor-α; CRP: C-reactive protein
[3]Exact infant age (days) at heart rate/heart rate variability assessment

3.4 Discussion

3.4.1 Infant heart rate and heart rate variability are age-dependent

With advancing age, infant HR decreased and HRV increased. This was predicted, given developmental stage of the population, and is consistent with previous reports (Finley and Nugent 1995; Massin and von Bernuth, 1997). In healthy term infants, low HR and high HRV indicate accelerated autonomic maturation (Massin and von Bernuth, 1997). Thus, in interpreting the current data, maternal circulating factors that are inversely related to HR and/or positively related to HRV may have a role in accelerating autonomic development.

3.4.2 Maternal endocannabinoids are associated with infant heart rate and heart rate variability

To the best of our knowledge, this study is the first to explore and link maternal inflammation during pregnancy to infant HR and HRV. Further, this study is the first to assess endocannabinoids in relation to HR and HRV in any population. The endocannabinoids related to n-6 (AEA) and n-3 (EEA, EPEA, DHEA) fatty acids were of specific interest as the n-6 and n-3 fatty acid families are metabolically competitive and have contrasting, potent inflammatory effects (Calder, 2002; Calder, 2006). Further, the n-6 and n-3 related endocannabinoids themselves have divergent roles in modulating inflammation (Yang et al., 2011; Meijerink et al., 2011) and autonomic function (reviewed by Pertwee, 1997), as discussed below.

Inflammation-related diseases (i.e., rheumatoid arthritis, coronary heart disease, and metabolic syndrome) are characterized by decreased parasympathetic tone and/or HRV (Brunner et al., 2002; Hamaad et al., 2005; Goldstein et al., 2007; Bruchfeld et al., 2010). Similarly, in otherwise healthy adults, HR increases and HRV reductions are associated with subclinical inflammation (Sajadieh et al., 2004).

Fetal HR and HRV are responsive to maternal nutrition (Gustafson et al., 2013) and exercise (May et al., 2010). Maternal inflammation may, in part, underlie these observations. Indeed, dietary fatty acid intake (Wood et al., 2010; Balvers et al., 2012; Hansen, 2013; Meijerink et al., 2013) and exercise (reviewed by Tantimonaco et al., 2014) modulate the endocannabinoid system, supporting this hypothesis.

We cannot, with certainty, state that anti- and pro-inflammatory properties of endocannabinoids underlie our observations. Endocannabinoids have several biological roles in vivo, making it difficult to pinpoint causality. Further, endocannabinoid concentrations reflect those of their respective fatty acid precursors. Dietary n-3 LCPUFA shifts n-3/n-6 fatty acid

balance of membrane lipids, resulting in compensatory increases in n-3 endocannabinoids, EPEA and DHEA, and reduced production of AEA (Wood et al., 2010). Beneficial cardiovascular effects of n-3 LCUPFA are well-documented, although the mechanism(s) of action have not been elucidated (reviewed in Jung et al., 2008; Adkins and Kelly, 2010). Thus, our observations may be a consequence of effects mediated by fatty acids from which the endocannabinoids were derived. However, given the central role of inflammation in chronic diseases, especially those of cardiovascular significance, and similar influences of n-3 LCPUFA and n-3 related endocannabinoids on inflammatory processes, it is plausible that endocannabinoids may partially: 1) explain underlying mechanisms and 2) be responsible for benefits associated with n-3 LCPUFA and inflammation (Wainwright and Michel, 2013).

Endocannabinoids bind CB1 and CB2 receptors (Yang et al., 2011; Anagnostopoulos et al., 2010) with AEA preferentially binding CB1 and DHEA having a greater affinity for CB2 receptors in human inflammatory cells (Yang et al., 2011). CB1 receptors have been implicated in pro-inflammatory chemokine secretion (Gaffal et al., 2013) while CB2 receptor activation inhibits release of inflammatory mediators associated with pain (Ibrahim et al., 2003). Further, DHEA exerts potent anti-inflammatory effects in LPS-stimulated RAW246.7 macrophages (Meijerink et al., 2011).

AEA downregulates autonomic function (reviewed by Pertwee, 1997). Activation of prejunctional or presynaptic CB1 receptors by AEA inhibits acetylcholine release (ACh) by the parasympathetic nervous system (Coutts and Pertwee, 1996; Gifford and Ashby, 1996) and norepinephrine release by the sympathetic nervous system (Ishac et al., 1996). Inhibition of ACh release suppresses parasympathetic tone and, thus, HRV.

Our data support conclusions that can be drawn from the above discussion, that n-3 endocannabinoids displace their n-6 counterparts and dampen pro-inflammatory processes, reducing HR and increasing HRV. Using simple linear and multiple regression analysis, we consistently observed that individual n-3 endocannabinoids and the combined n-3 endocannabinoid family (EEA + EPEA + DHEA) in maternal and/or venous cord plasma are associated with reduced infant HR and increased HRV. Conversely, our data indicate an association between maternal AEA, increased infant HR, and reduced HRV. The ratio of maternal AEA:n-3 family and AEA:DHEA also had predictive value such that shifts favoring AEA were related to increased infant HR and reduced HRV. We observed an inverse relationship between venous cord AEA and infant HR at the 6 month assessment; this was the only beneficial association for AEA. Venous cord EPEA was also a significant, inverse predictor of 6 month infant HR, however, to a much greater extent. Compared AEA, an increase of equal amounts of venous cord plasma EPEA was predicted to result in an approximate 3.7-fold reduction in HR. Thus, while these data indicate cord AEA may be associated with accelerated HR development, they also point to EPEA as a more potent agent.

3.4.3 Maternal cytokines, adipokines, and acute phase reactants in relation to infant heart rate and heart rate variability: further investigation needed

In the current study, we observed few significant relationships between maternal cytokine (TNF-α, IL-6), adipokine (adiponectin), or acute phase reactant (CRP) concentrations and infant HR and HRV during the first 6 months of life. We hypothesize this is dually due to sample size and low- to moderate-detection of biomarkers in maternal and venous cord serum. IL-6 was not included in statistical analyses for any timepoint excepting delivery (venous cord serum) due to marginal detectability. As such, many regression models in the current study were limited to <11 women-infant pairs.

In the current study, significant multiple regression models were specific to inflammatory biomarkers at earlier timepoints during pregnancy (20 and 24 gestational weeks). The driving force behind this observation is unclear, but may reflect direct interactions between inflammatory biomarkers and the sympathetic nervous system as parasympathetic influence emerges around 30 – 32 gestational weeks (Sachis et al., 1982). This observation may also be coincidental and a result of sample size limitations.

Parasympathetic activity and/or HRV are consistently, inversely associated with CRP (Kon et al., 2006; Lanza et al., 2006; Psychari et al. 2007) and TNF-α (Malave et al., 2003; Marsland, et al., 2007). Our data are in line with these observations, as CRP and TNF-α were both inverse predictors of HRV in multiple regression models.

Associations between adiponectin, HR, and HRV have been explored (Wakabayashi and Aso, 2004; Takahashi et al., 2007; Piestrzeniewicz et al., 2008), albeit, not as extensively as for CRP and TNF-α. There were no associations between adiponectin and frequency- or time-domain HRV indices in males with acute myocardial infarction (Piestrzeniewicz et al., 2008). However, low serum adiponectin in type 2 diabetes was associated with sympathetic hyperactivity (Wakabayashi and Aso, 2004; Takahashi et al., 2007), which would be clinically apparent as increased HR and reduced HRV. In the current study, maternal adiponectin was a positive and inverse predictor of infant HR and time-domain indices of HRV, respectively. The disconnect between our observations and those of previous studies may reflect the population assessed. Pregnancy is characterized by a physiological increase in insulin resistance that ensures nutrient delivery to the developing fetus. In the non-pregnant state, adiponectin concentrations negatively correlate with insulin sensitivity (Hotta et al., 2000; Weyer et al., 2001). However, in a population similar to the current study (pregnant women free of gestational, type 1, or type 2

diabetes), adiponectin concentrations did not differ across trimesters (Mazaki-Tovi et al., 2007) despite assumed progressive decreases in insulin sensitivity. These observations suggest adiponectin regulation may be altered during pregnancy and may explain the inconsistencies between previous observations (Wakabayashi and Aso, 2004; Takahashi et al., 2007; Piestrzeniewicz et al., 2008) and findings of the current study.

3.5 Limitations

This study was limited primarily by sample size (n = 11). Much of the presented data existed as statistical trends However, many relationships were highly significant and consistently observed, especially with regard to n-3 related endocannabinoids. Further, sample size limited inclusion of factors other than maternal inflammatory biomarkers and infant age at HR/HRV assessment in multiple regression models due to loss of statistical power. To address this, participants were required to meet strict inclusion and exclusion criteria, defined above, for study enrollment. These criteria ensured the study participants (mothers) had similar characteristics to minimize the influence of external factors on study outcomes.

3.6 Conclusion

Alterations in maternal inflammatory status may be the common mechanism underlying the previously observed effects of maternal nutrition and exercise on fetal HR and HRV (May et al., 2010; Gustafson et al., 2013), as supported by the current study. We have provided compelling data evidencing an association between maternal endocannabinoids during pregnancy with infant HR and HRV. Although we are unable to definitively conclude these observations are a direct consequence of anti-inflammatory actions of n-3 endocannabinoids, we provide a discussion supporting this hypothesis and call for future studies to include assessments of these novel bioactive fatty acid derivatives. Due to study limitations, we cannot conclusively

provide a link between maternal concentrations of adiponectin, CRP, TNF-α, and IL-6 during pregnancy and infant HR and HRV. However, these inflammatory biomarkers are worthy of future consideration in larger, more robust studies, as supported by our discussion.

3.7 Literature cited

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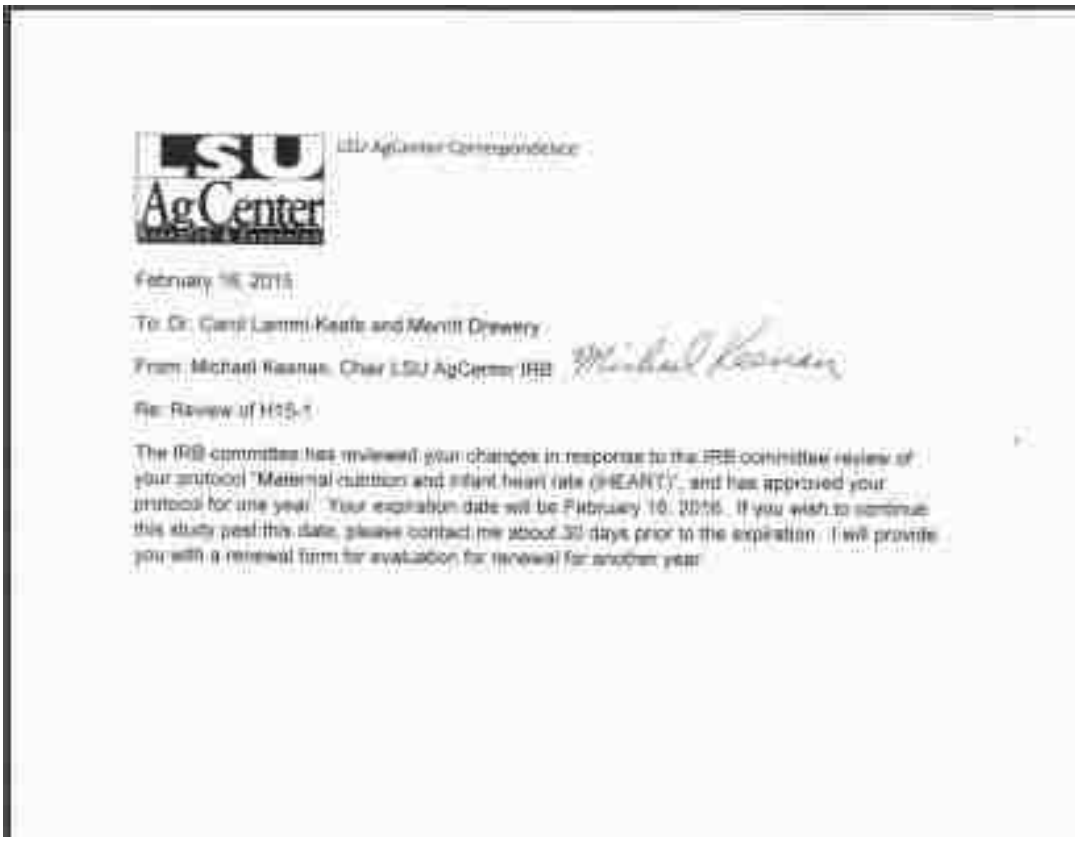
APPENDIX A. LSU AGCENTER IRB APPROVAL FOR CURRENT STUDY



LSU AgCenter Correspondence

February 16, 2015

To: Dr. Carol Lammi-Keefe and Merritt Drewery

From: Michael Keenan, Chair LSU AgCenter IRB

Re: Review of H15-1

The IRB committee has reviewed your changes in response to the IRB committee review of your protocol "Maternal nutrition and infant heart rate (HEART)", and has approved your protocol for one year. Your expiration date will be February 16, 2016. If you wish to continue this study past this date, please contact me about 30 days prior to the expiration. I will provide you with a renewal form for evaluation for renewal for another year.

APPENDIX B. CONTINUING IRB APPROVAL FOR THE LAMBS STUDY AT PENNINGTON BIOMEDICAL RESEARCH CENTER

PENNINGTON BIOMEDICAL RESEARCH CENTER

IRB Certificate of Approval

FWA # 00006218

Date of Approval: **September 21, 2016**
Study Expiration Date: **September 20, 2017**
Submission Type: **Continuing Review**
Review Frequency: **12 months**
Number of Subjects Approved: **60**
Review Type: Full Board
Approval Status: **Approved**

Principal Investigator: **Carol Lammi-Keefe, Ph.D., RD**
IRB # **11031-PBRC LAMBS**
Title: **LA Moms and Babies Study (LAMBS) for Nutrition and Growth**
Sponsor: **USDA**

Approval Includes: Study and Investigator(s) for an additional continuing review period. This approval expires on the date noted above.

Investigators and study staff must comply with the Human Research Protection Program policies and procedures that apply to IRB members and staff, which can be found at www.pbrc.edu/hRPP

Signed Thursday, September 22, 2016 2:14:01 PM ET by Gadde, Kishore MD

APPENDIX C. CONTINUING IRB APPROVAL FOR THE LAMBS STUDY AT WOMAN'S HOSPITAL

WOMAN'S HOSPITAL FOUNDATION
INSTITUTIONAL REVIEW BOARD
100 Woman's Way
Baton Rouge, Louisiana 70817

Peggy Dean, RPh, MBA, Chair (225) 231-5339

October 11, 2016

Carol Lammi- Keefe, PhD
Louisiana State University
297B Knapp Hall
Baton Rouge, LA 70803

Dear Dr. Lammi-Keefe:

This letter is to inform you that at the Woman's Hospital Foundation Institutional Review Board meeting of October 10, 2016, the protocol, informed consent form, authorization forms, screening form, case report forms, physician letter, patient information cards, food amounts booklet, protocol deviation, and advertisements for **RP-11-007, LA Moms and Babies Study (LAMBS) for Nutrition and Growth,** were reviewed for continuing review.

Approval has been granted for one year. The study is next subject to continuing review on or before October 10, 2017. We recommend that it be presented two months prior to this date to avoid a delay in enrollment in the case of unforeseen circumstances.

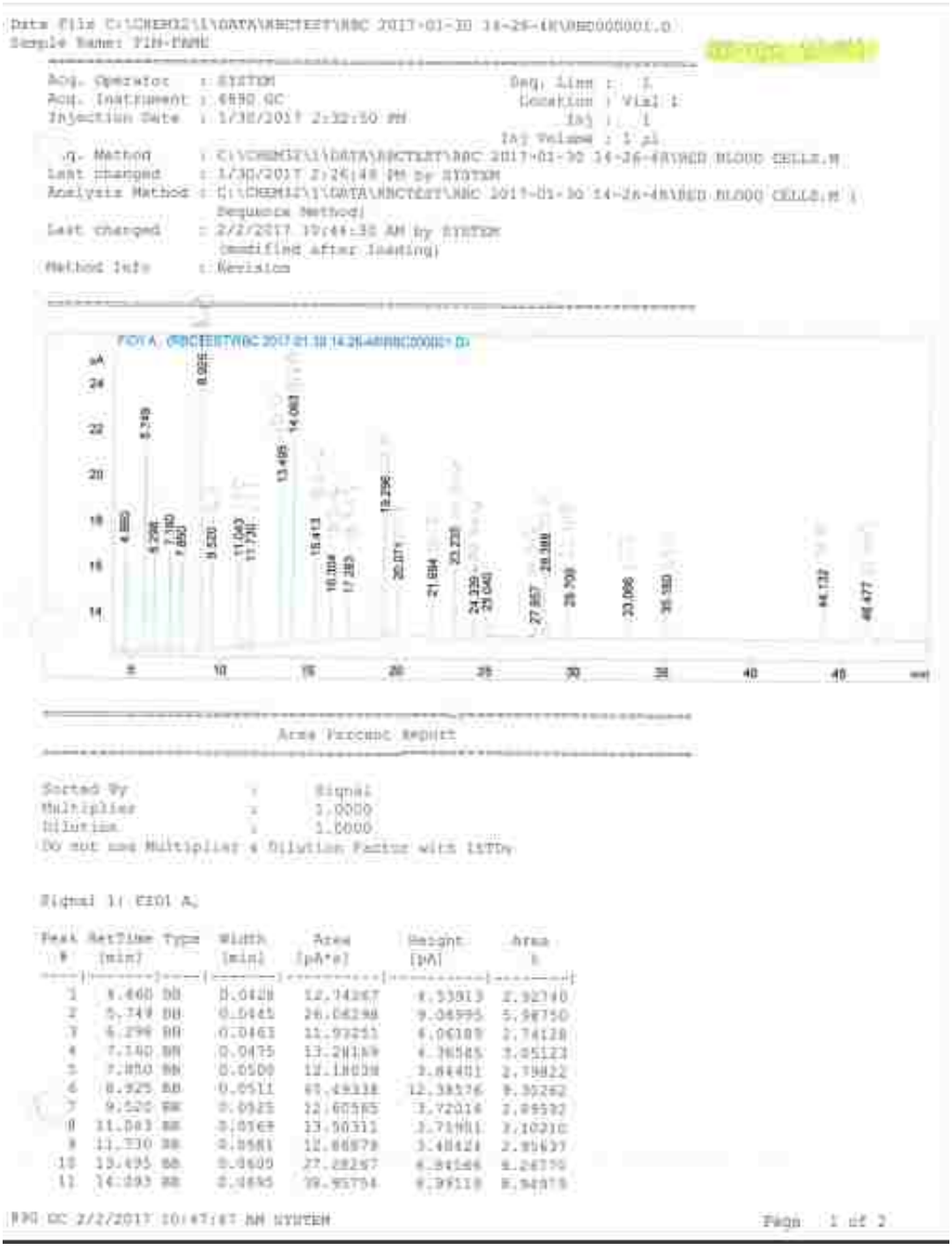
Attached are the informed consent form and authorization forms with the IRB stamp of approval in the lower right hand corners. Please note that these documents are the official ones and copies for future participants must be reproduced from these originals.

Changes to the study must be promptly reported and approved. Contact Ericka Seidemann, Human Protections Administrator, at (225) 231-5296 if you have any questions or require further information.

Sincerely,

Peggy Dean, RPh., MBA
IRB Chair

APPENDIX D. CHROMATOGRAM USED FOR FATTY ACID IDENTIFICATION



Data File C:\CHEM32\1\DATA\RBCTEST\RBC 2017-01-30 14-26-48\RBD000001.D
Sample Name: FISH-FRANC

Acq. Operator : SYSTEM
Acq. Instrument : 6890 GC
Injection Date : 1/30/2017 2:32:50 PM

Seq. Line : 1
Location : Vial 1
Inj : 1
Inj Volume : 1 µl

Q. Method : C:\CHEM32\1\DATA\RBCTEST\RBC 2017-01-30 14-26-48\RED BLOOD CELLS.M
Last changed : 1/30/2017 2:26:48 PM by SYSTEM
Analysis Method : C:\CHEM32\1\DATA\RBCTEST\RBC 2017-01-30 14-26-48\RED BLOOD CELLS.M (
Sequence Method)
Last changed : 2/2/2017 10:44:30 AM by SYSTEM
(modified after loading)
Method Info : Revision

FID1 A, (RBCTEST\RBC 2017-01-30 14-26-48\RBD000001.D)

pA

Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Do not use Multiplier & Dilution Factor with ISTDs

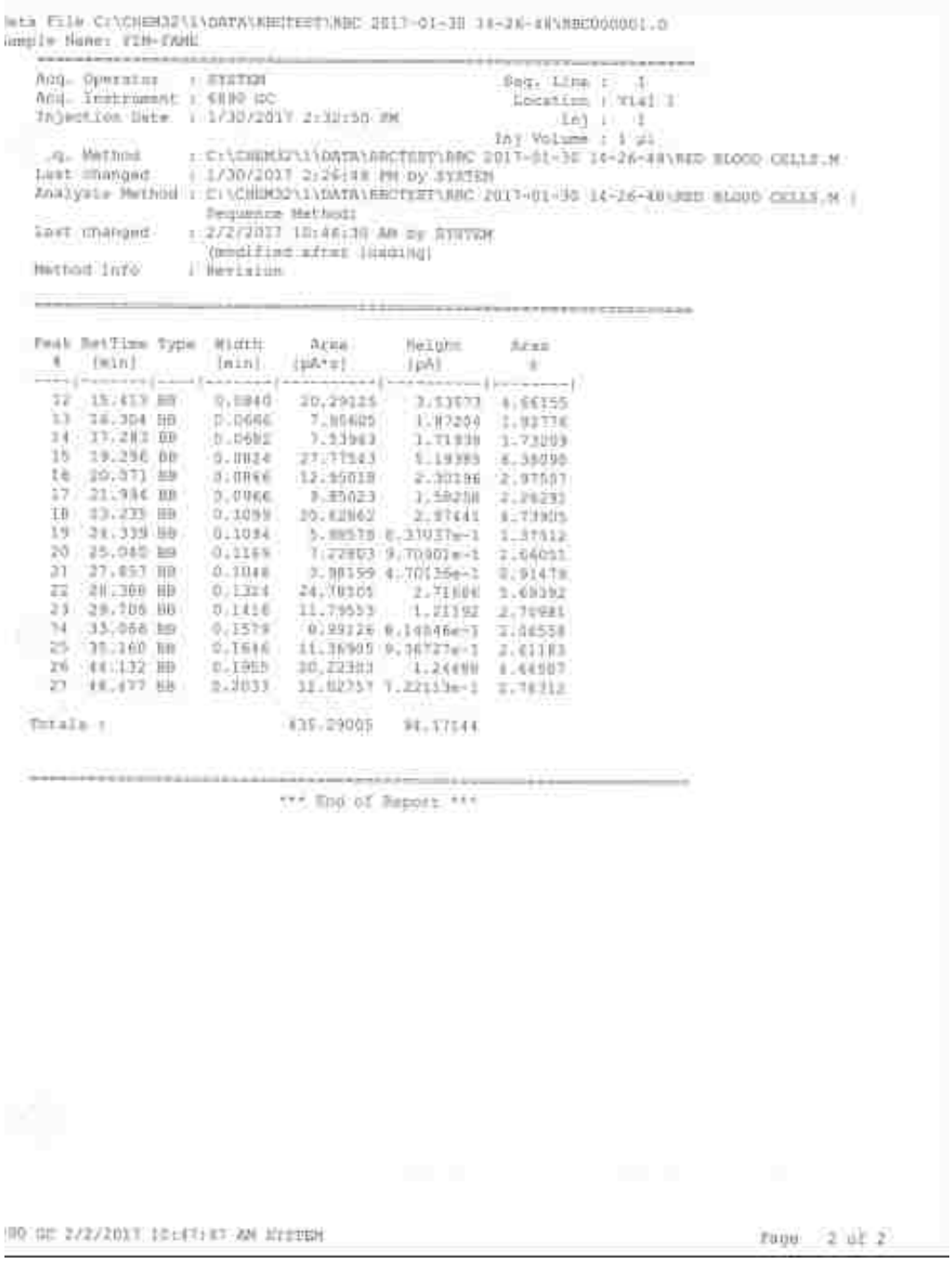
Signal 1: FID1 A,

Peak #	RetTime [min]	Type	Width [min]	Area [pA*s]	Height [pA]	Area %
1	4.880	BB	0.0428	12.74367	4.53813	2.92740
2	5.749	BB	0.0445	26.04298	9.04995	5.98750
3	6.296	BB	0.0461	11.93251	4.06189	2.74128
4	7.140	BB	0.0475	13.28169	4.36585	3.05123
5	7.850	BB	0.0500	12.18038	3.84401	2.79822
6	8.925	BB	0.0511	65.49338	12.38576	9.30262
7	9.520	BB	0.0525	12.60585	3.72014	2.89592
8	11.043	BB	0.0569	13.50311	3.71981	3.10210
9	11.730	BB	0.0581	12.86878	3.48424	2.95637
10	13.495	BB	0.0609	27.28257	6.84586	6.26770
11	14.083	BB	0.0695	39.95754	8.99119	8.94975

RBC GC 2/2/2017 10:47:47 AM SYSTEM

Page 1 of 2

APPENDIX D. CHROMATOGRAM USED FOR FATTY ACID IDENTIFICATION CONTINUED



Data File C:\CHEM32\1\DATA\RBCTEST\RBC 2017-01-30 14-26-48\RBC000001.D
Sample Name: FIN-FAME

Acq. Operator : SYSTEM
Acq. Instrument : 6890 GC
Injection Date : 1/30/2017 2:32:50 PM

Seq. Line : 1
Location : Vial 1
In) : 1
Inj Volume : 1 µl

Acq. Method : C:\CHEM32\1\DATA\RBCTEST\RBC 2017-01-30 14-26-48\RED BLOOD CELLS.M
Last Changed : 1/30/2017 2:26:48 PM by SYSTEM
Analysis Method : C:\CHEM32\1\DATA\RBCTEST\RBC 2017-01-30 14-26-48\RED BLOOD CELLS.M (
Sequence Method
Last Changed : 2/2/2017 10:46:30 AM by SYSTEM
(modified after loading)
Method Info : Revision

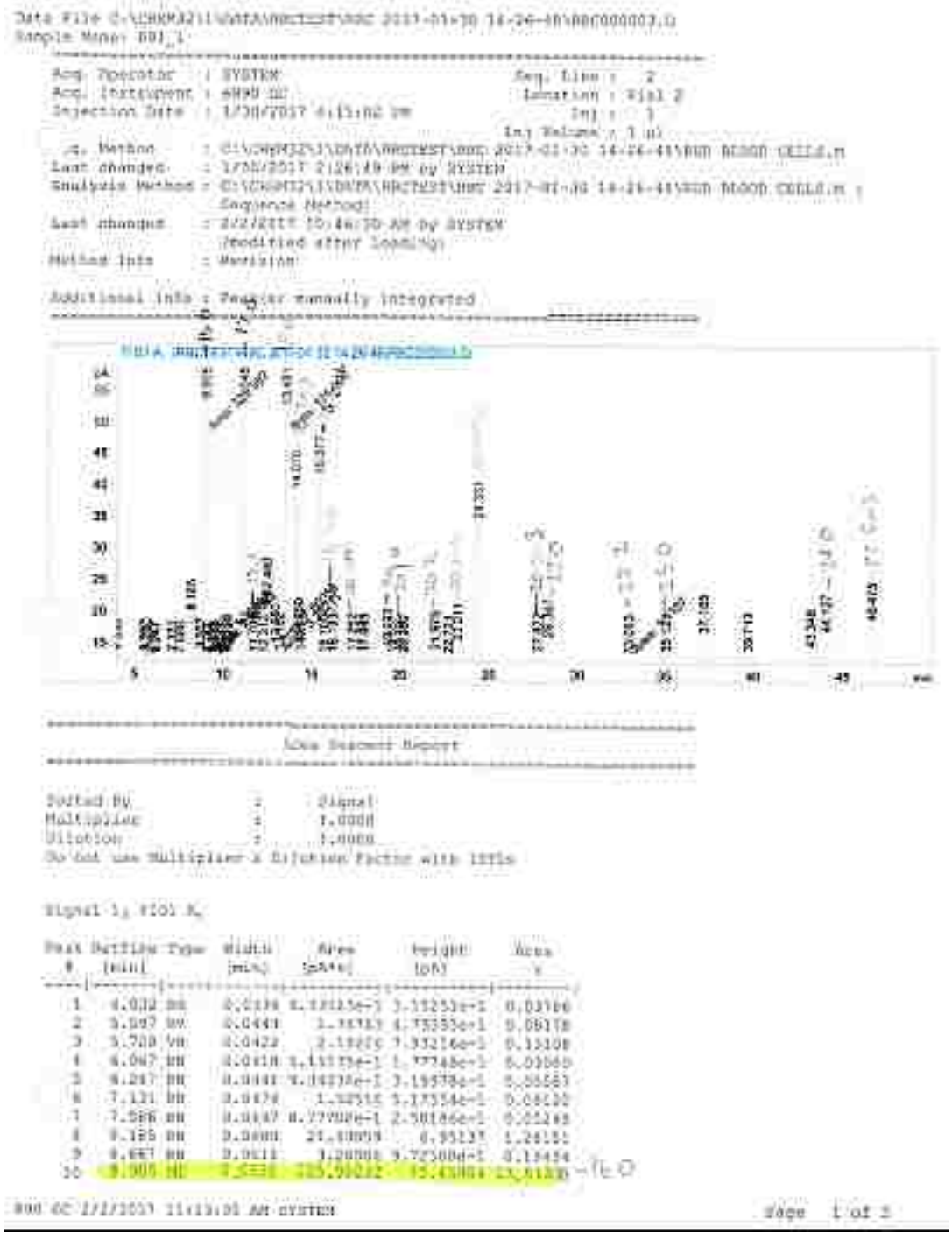
Peak #	RetTime [min]	Type	Width [min]	Area [pA*s]	Height [pA]	Area %
12	15.413	BB	0.0840	20.29125	3.53573	4.66155
13	16.304	BB	0.0686	7.80605	1.87209	1.92776
14	17.283	BB	0.0682	7.53963	1.71839	1.73209
15	19.256	BB	0.0824	27.77543	5.19385	6.38090
16	20.571	BB	0.0866	12.95018	2.30196	2.97607
17	21.986	BB	0.0966	9.85023	1.59208	2.26283
18	23.235	BB	0.1059	30.62862	2.97441	6.73805
19	24.339	BB	0.1034	5.88578	8.31031e-1	1.37512
20	25.040	BB	0.1169	7.72893	9.70901e-1	2.64051
21	27.857	BB	0.1048	3.98159	4.70135e-1	0.91478
22	28.308	BB	0.1324	24.78305	2.71606	5.69392
23	29.706	BB	0.1416	11.79553	1.21152	2.70981
24	33.068	BB	0.1579	0.99126	8.14046e-1	2.06558
25	35.160	BB	0.1646	11.36905	9.36727e-1	2.61183
26	44.132	BB	0.1955	30.22303	1.24498	6.44507
27	48.477	BB	0.3033	11.82757	7.22113e-1	2.78312

Totals : 435.29005 94.17144

*** End of Report ***

GD GC 2/2/2017 10:47:57 AM SYSTEM Page 2 of 2

APPENDIX E. CHROMATOGRAM LABELED WITH REFERENCE TO STANDARD CHROMATOGRAM, USED IN FATTY ACID ANALYSIS



Data File C:\CHEM32\1\DATA\BRCTEST\BRC 2017-01-30 14-26-48\BRC0000003.D
Sample Name: BRI_1

Acq. Operator : SYSTEM
Acq. Instrument : 6890 GC
Injection Date : 1/30/2017 6:15:02 PM
Acq. Line : 2
Location : Vial 2
Inj : 1
Inj Volume : 1 µl

Q. Method : C:\CHEM32\1\DATA\BRCTEST\BRC 2017-01-30 14-26-48\BRD BLOOD CELLS.M
Last changed : 1/30/2017 2:26:19 PM by SYSTEM
Analysis Method : C:\CHEM32\1\DATA\BRCTEST\BRC 2017-01-30 14-26-48\BRD BLOOD CELLS.M (Sequence Method)
Last changed : 2/2/2017 10:46:30 AM by SYSTEM (modified after loading)
Method Info : Revision

Additional Info : Peaks manually integrated

Area Percent Report

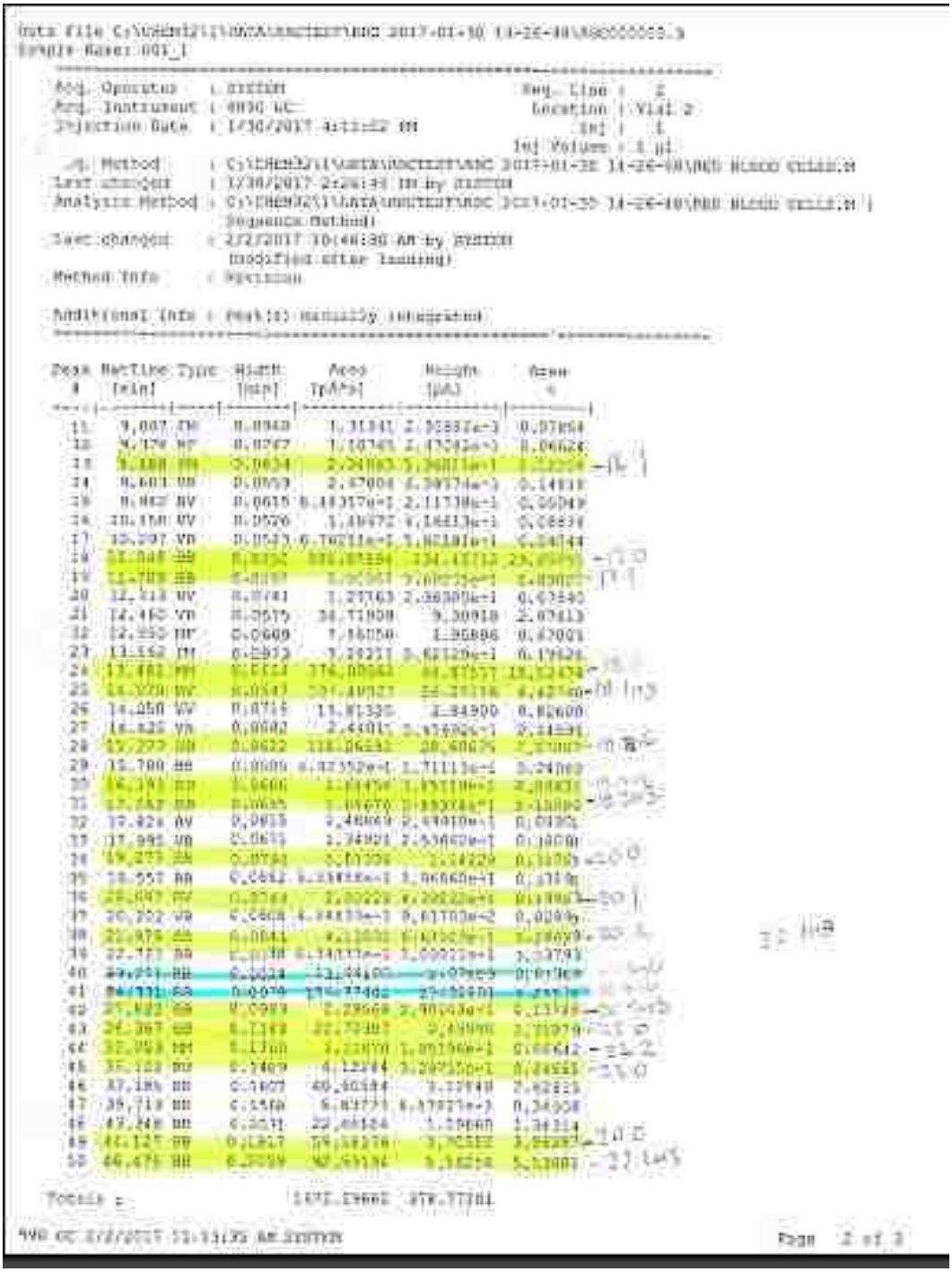
Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Do not use Multiplier & Dilution Factor with ISTDs

Signal 1: FID1 A,

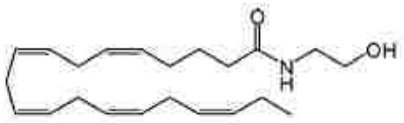
Peak #	RetTime [min]	Type	Width [min]	Area [pA*s]	Height [pA]	Area %
1	4.032	BB	0.0134	6.37135e-1	3.15253e+1	0.03186
2	5.597	BV	0.0441	1.78183	4.75353e+1	0.08178
3	5.720	VB	0.0422	2.19226	7.33216e+1	0.15108
4	6.047	BB	0.0418	1.15179e-1	1.77748e+1	0.03060
5	6.267	BB	0.0441	9.04236e-1	3.19578e+1	0.05583
6	7.121	BB	0.0474	1.52515	5.17554e+1	0.08130
7	7.586	BB	0.0447	8.77902e-1	2.58186e+1	0.05248
8	8.185	BB	0.0688	21.60059	4.95137	1.26151
9	8.667	BB	0.0611	1.20888	3.72508e+1	0.13454
10	8.905	BB	7.5511	225.90032	45.45084	23.91139

BRD GC 2/1/2017 11:13:36 AM SYSTEM Page 1 of 2

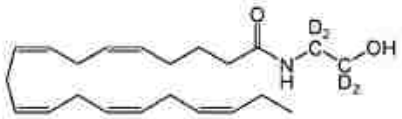
APPENDIX E. CHROMATOGRAM LABELED WITH REFERENCE TO STANDARD CHROMATOGRAM, USED IN FATTY ACID ANALYSIS, CONTINUED



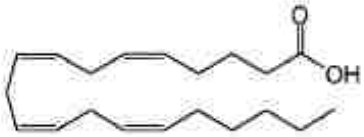
APPENDIX F. STRUCTURES OF ENDOCANNABINOID METABOLITES AND THEIR INTERNAL STANDARDS.



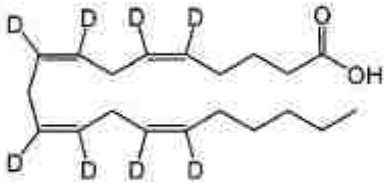
Eicosapentaenoyl ethanolamide (EPEA)
$C_{22}H_{35}NO_2$ mw 345.2668
346.20 → 62.16 [23]



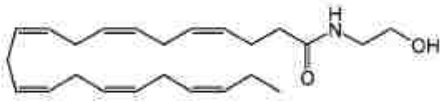
Eicosapentaenoyl ethanolamide d4 (EPEAd4)
$C_{22}H_{31}D_4NO_2$ mw 349.2915
350.22 → 66.06 [23]



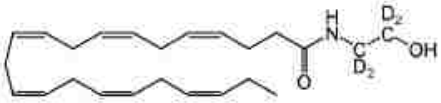
Arachidonic acid (AA)
$C_{20}H_{32}O_2$ mw 304.2402
303.23 → 259.18 [15]



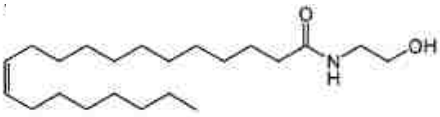
Arachidonic acid d8 (AA d8)
$C_{20}H_{24}D_8O_2$ mw 312.2402
311.23 → 267.18 [15]



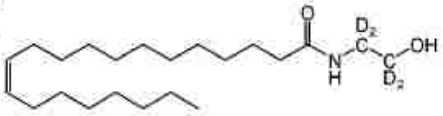
Docosahexaenoyl ethanolamide (DHEA)
$C_{24}H_{37}NO_2$ mw 371.2824
372.24 → 62.16 [17]



Docosahexaenoyl ethanolamide d4 (DHEAd4)
$C_{24}H_{33}D_4NO_2$ mw 375.3071
376.27 → 66.16 [17]



Eicoseneoyl ethanolamide (EEA)
$C_{22}H_{43}NO_2$ mw 353.3294
354.30 → 62.16 [20]

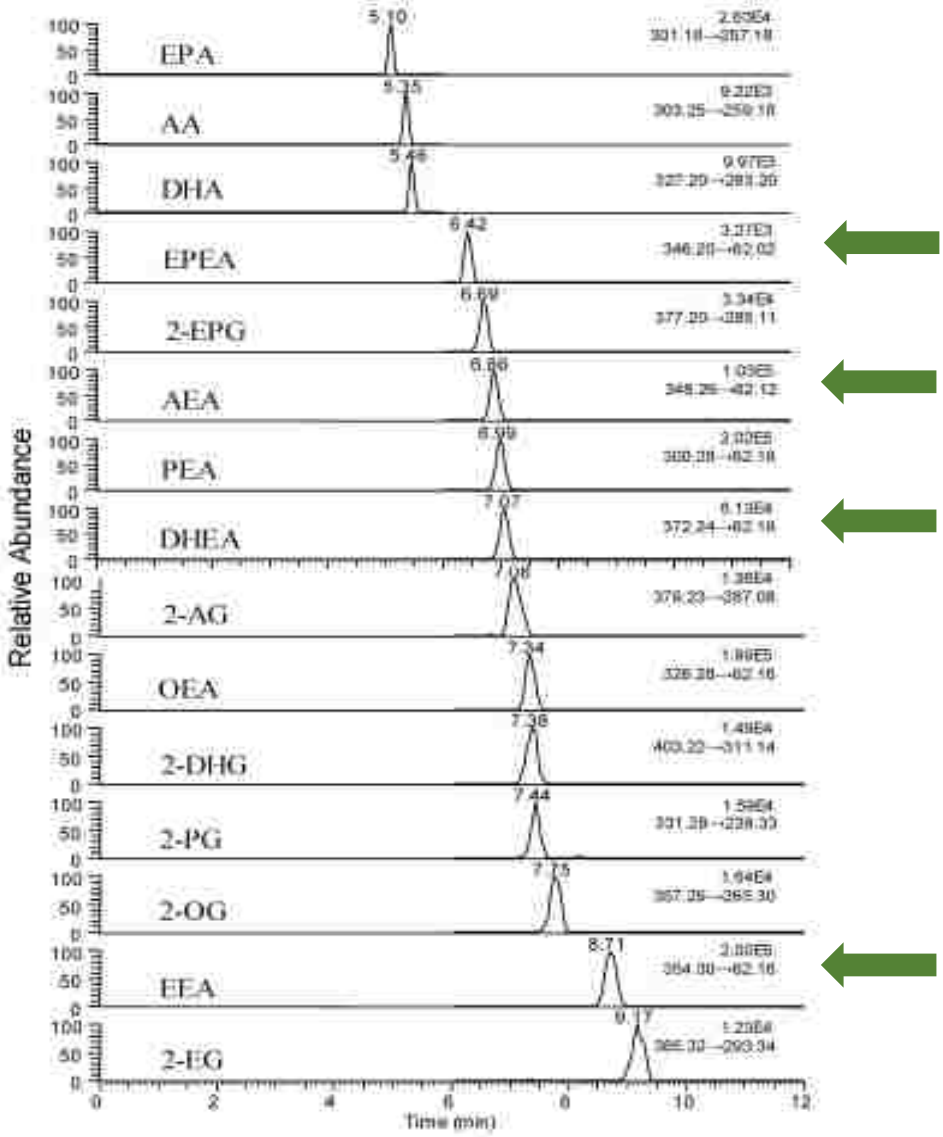


Eicoseneoyl ethanolamide d4 (EEAd4)
$C_{22}H_{39}D_4NO_2$ mw 357.3541
358.36 → 66.16 [20]

Each analyte's single reaction monitoring transition is listed followed by the specific collision energy in brackets. EPEA: eicosapentaenoyl ethanolamide, $C_{22}H_{35}NO_2$; AEA: arachidonyl ethanolamide, $C_{22}H_{32}O_2$; DHEA: docosahexaenoyl ethanolamide, $C_{24}H_{37}NO_2$; EEA: eicoseneoyl ethanolamide, $C_{22}H_{43}NO_2$

Williams J, Wood J, Pandarinathan L, et al. Quantitative method for the profiling of the endocannabinoid metabolome by LC-Atmospheric Pressure Chemical Ionization-MS. Anal Chem 2007;79:5582-5593.

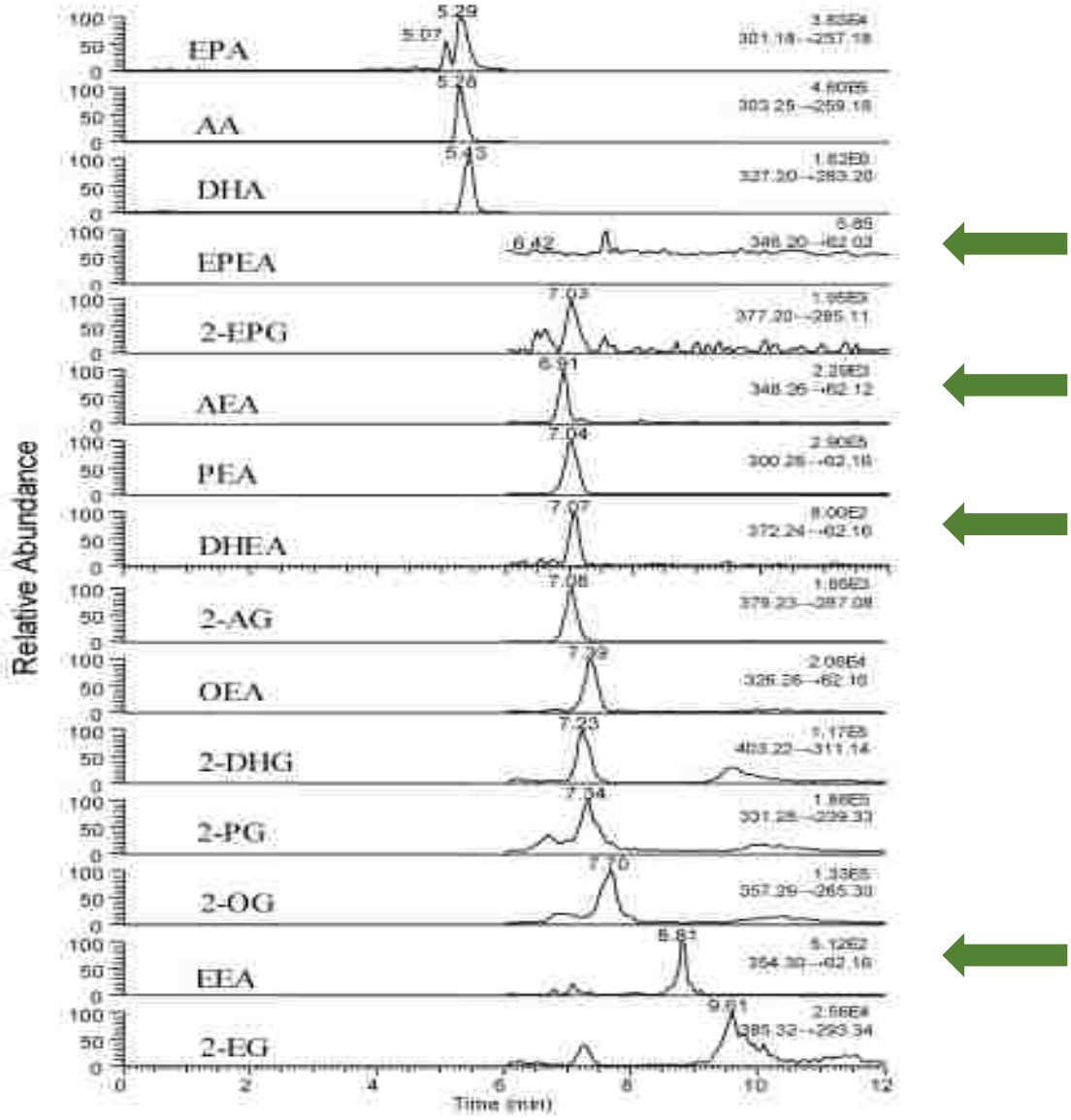
APPENDIX G. CHROMATOGRAMS OF EXTRACTED ENDOCANNABINOID METABOLITES IN THE REFERENCE STANDARD



Arrow indicates analytes analyzed by Northeastern University for the current study. EPEA: eicosapentaenoyl ethanolamide, $C_{22}H_{35}NO_2$; AEA: arachidonyl ethanolamide, $C_{22}H_{32}O_2$; DHEA: docosahexaenoyl ethanolamide, $C_{24}H_{37}NO_2$; EEA: eicoseneoyl ethanolamide, $C_{22}H_{43}NO_2$

Williams J, Wood J, Pandarinathan L, et al. Quantitative method for the profiling of the endocannabinoid metabolome by LC-Atmospheric Pressure Chemical Ionization-MS. Anal Chem 2007;79:5582-5593.

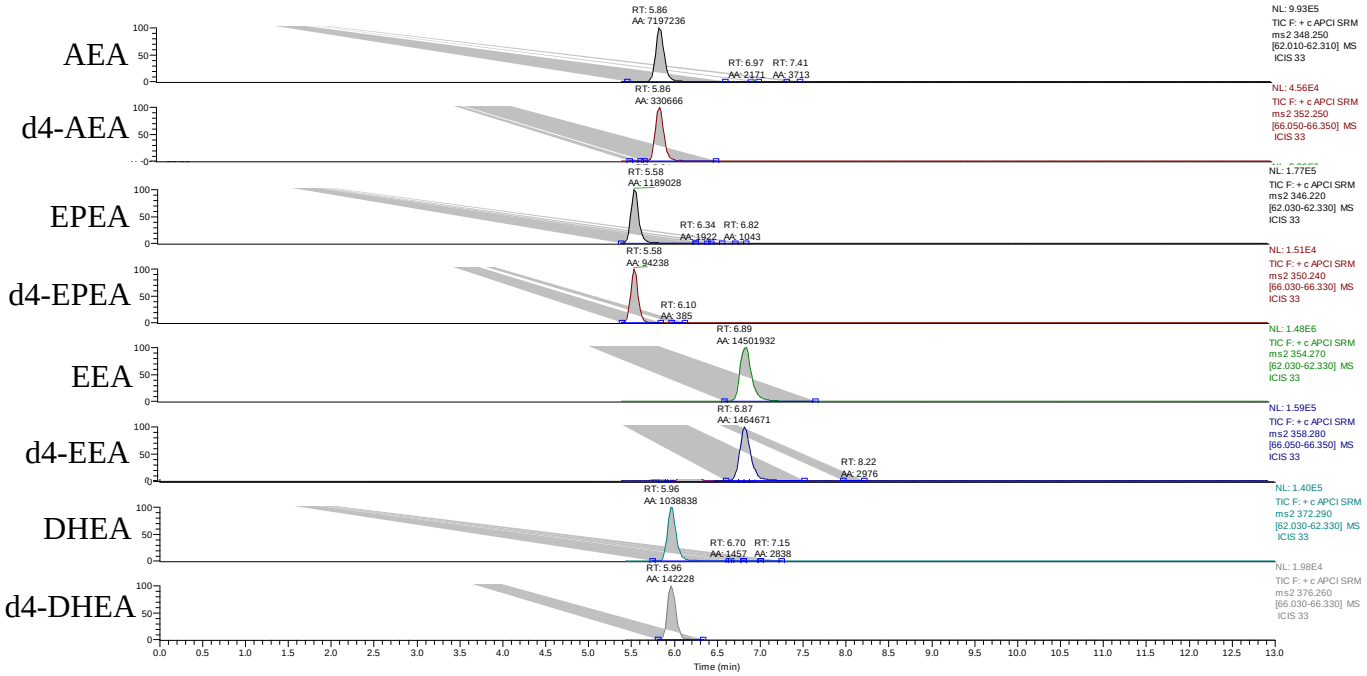
APPENDIX H. CHROMATOGRAMS OF NORMALIZED, EXTRACTED ENDOCANNABINOID METABOLITES IN RAT FRONTAL CORTEX



Arrow indicates analytes analyzed by Northeastern University for the current study. EPEA: eicosapentaenoyl ethanolamide, $C_{22}H_{35}NO_2$; AEA: arachidonyl ethanolamide, $C_{22}H_{32}O_2$; DHEA: docosahexaenoyl ethanolamide, $C_{24}H_{37}NO_2$; EEA: eicoseneoyl ethanolamide, $C_{22}H_{43}NO_2$

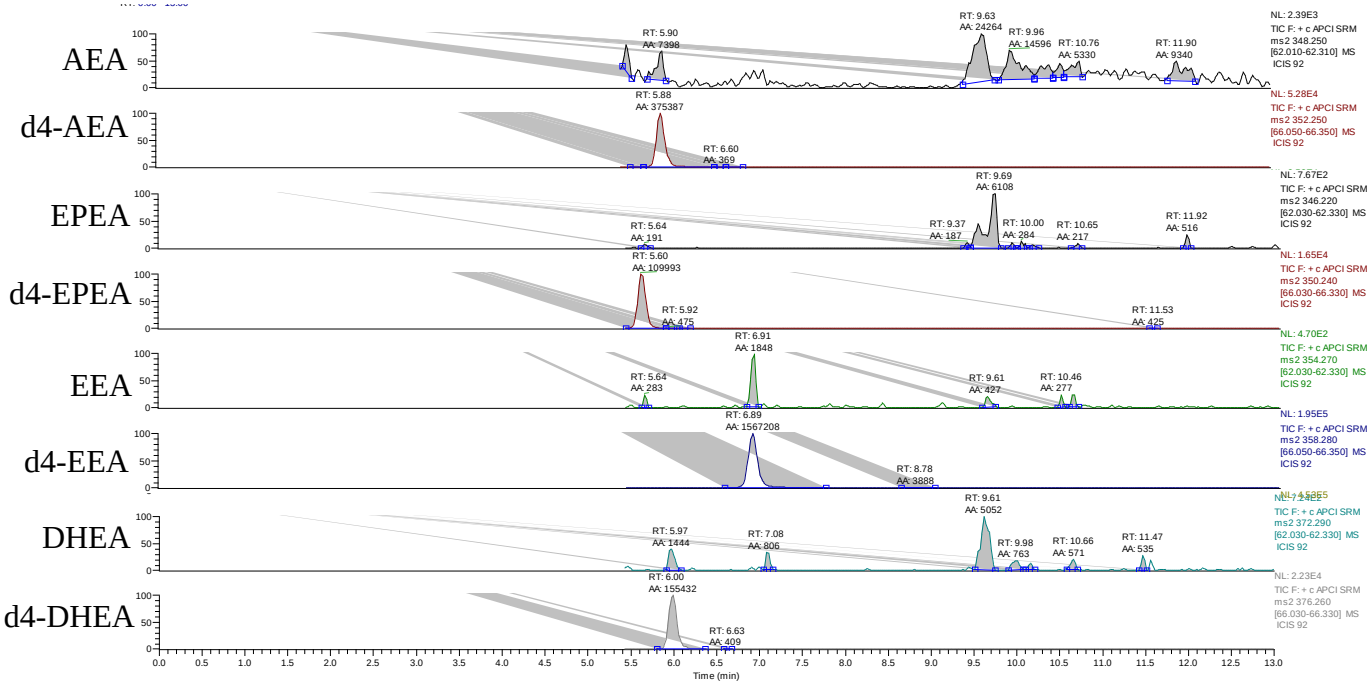
Williams J, Wood J, Pandarinathan L, et al. Quantitative method for the profiling of the endocannabinoid metabolome by LC-Atmospheric Pressure Chemical Ionization-MS. Anal Chem 2007;79:5582-5593.

APPENDIX I. CHROMATOGRAMS OF DEUTERATED INTERNAL STANDARDS AND EXTRACTED ENDOCANNABINOID METABOLITES IN THE REFERENCE STANDARD



EPEA: eicosapentaenoyl ethanolamide, $C_{22}H_{35}NO_2$; AEA: arachidonyl ethanolamide, $C_{22}H_{32}O_2$; DHEA: docosahexaenoyl ethanolamide, $C_{24}H_{37}NO_2$; EEA: eicoseneoyl ethanolamide, $C_{22}H_{43}NO_2$. d4- indicates deuterated analyte.

APPENDIX J. CHROMATOGRAMS OF DEUTERATED INTERNAL STANDARDS AND EXTRACTED ENDOCANNABINOID METABOLITES IN MATERNAL PLASMA DURING PREGNANCY



EPEA: eicosapentaenoyl ethanolamide, $C_{22}H_{35}NO_2$; AEA: arachidonyl ethanolamide, $C_{22}H_{32}O_2$; DHEA: docosahexaenoyl ethanolamide, $C_{24}H_{37}NO_2$; EEA: eicoseneoyl ethanolamide, $C_{22}H_{43}NO_2$. d4- indicates deuterated analyte

VITA

Merritt Drewery is a graduate student pursuing her Doctorate in Philosophy in the School of Nutrition and Food Sciences at Louisiana State University, Baton Rouge, Louisiana. She holds a Bachelors of Science and Masters of Science in Animal Science, with a focus on Ruminant Nutrition, from Texas A&M University, College Station, Texas. Her research interests include the role of perinatal nutrition in optimizing fetal and infant development, with a focus on infants born to women with adverse metabolic conditions. Merritt has been highly involved in the American Oil Chemists' Society throughout her graduate career; she proposes and co-chairs technical sessions at the annual meetings and is the co-chair of the Student Common Interest Group. After her degree is conferred, Merritt hopes to translate evidence-based nutrition science to the public with the ultimate goal of bettering health and wellness in the community.