CHAPTER 1

INTRODUCTION

1.1 Background

Long-chain polyunsaturated fatty acids (LCPUFA) are essential components of cell membranes in the central nervous system and retina. Docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (ARA, 22:4n-6) are the most abundant LCPUFA in the developing human brain (Rojas, Greiner et al. 2002). LCPUFA occur naturally in breast milk and US infant formula manufacturers began including DHA and ARA in 2002. However, levels of LCPUFA in commercial formula vary and the amount of DHA and ARA required for optimal growth and neurodevelopment has not yet been established.

The goal of this thesis is to evaluate safety and efficacy of formula ARA and moderate and high levels of DHA in baboon neonates. This investigation focuses on tissue fatty acid composition and hematological measurements using a comparative model to advance our understanding of human infant LCPUFA requirements.

1.2 Metabolism of n-3 and n-6 Fatty Acids

Essential fatty acids (EFA) are nutrients that humans need to obtain from the diet and cannot be synthesized endogenously. Fatty acids (FA) are hydrocarbon chains of various lengths and number of double bonds. The n-6 and n-3 FA are two separate but closely related families of FA, named according to the position of the first double bond at either the third or sixth carbon from the methyl end of the molecule. Mammals lack the enzymes to insert double bonds at either the n-3 or n-6 positions and must supply these FA from dietary sources.

The importance of the EFA, α-linolenic acid (ALA,18:3n-3) and linoleic acid (LA, 18:2n-6), were discovered over 70 years ago, in classic studies demonstrating

scaly skin, growth retardation and infertility when fat was absent from the diet of rats (Burr and Burr 1930). Felines fed EFA-deficient diets for 6 months after weaning developed general lethargy, rough dry coats, skin lesions and exhibited poor growth (Sinclair, Slattery et al. 1981). The essentiality of these FA in human diets was discovered in infants fed skim milk during the 1940s and 50s (Holman 1998). Skim milk was a common substitute for breast milk and infant eczema, due to EFA deficiency, was a prevalent medical problem (Holman 1998; Lauritzen, Hansen et al. 2001). Lard, which contains both LA and ARA, ameliorated the skin conditions in these infants (Lauritzen, Hansen et al. 2001). The first total parenteral nutrition preparations were completely devoid of fat and induced rapid and severe EFA deficiency in both human infants and elderly adults (Holman 1998).

LCPUFA are FA containing 20 or more carbon atoms and may be obtained from the diet or synthesized from shorter chain fatty acid precursors and their metabolites. Common dietary sources of EFA and LCPUFA are shown in Table 1.1. The liver is the primary site for FA metabolism and LCPUFA production occurs through a series of desaturation and elongation steps outlined in Figure 1.1. The n-3 and n-6 FA compete for the same enzymes but do not interconvert with one another. The final conversion to docosapentaenoic acid (DPA, 22:5n-6) and DHA has not been completely elucidated and biosynthesis occurs either through a direct Δ^4-desaturation or an elongation, followed by a Δ^6-desaturation and then partial β-oxidation to form DPAn-6 and DHA (Sprecher, Luthria et al. 1995; Infante and Huszagh 1998; Leonard, Pereira et al. 2004). Figure 1.2 depicts the synthesis of LA to ARA, which takes place on the carboxyl end of the molecule and does not alter the methyl end of the molecule (Lauritzen, Hansen et al. 2001).

Table 1.1 Common dietary sources of n-6 and n-3 fatty acids.

n-6 LA	n-3 ALA	n-3 LCPUFA
Corn oil	Flaxseed oil	Mackerel
Safflower oil	Canola oil	Herring
Sunflower oil	Soy bean oil	Salmon
Peanut oil	Walnut oil	Trout
		Halibut
		Tuna

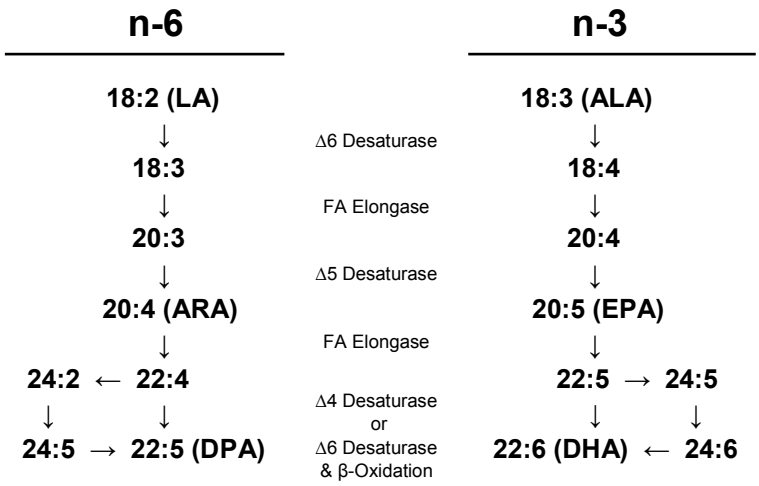


Figure 1.1 Metabolic pathways for the conversion of linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3) into longer-chain polyunsaturated fatty acids.

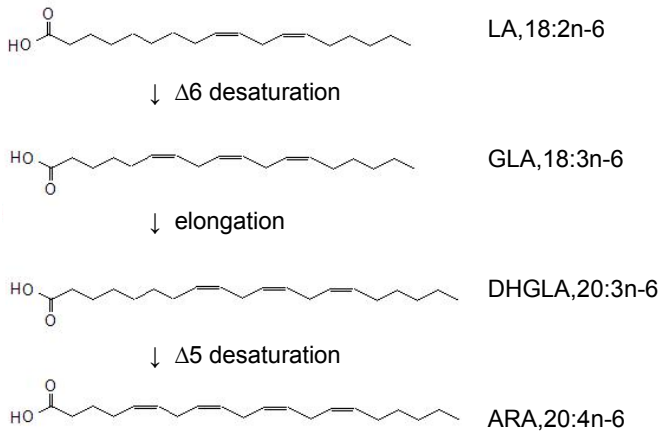


Figure 1.2 Conversion of linoleic acid (LA, 18:2n-6) to arachidonic acid (ARA, 20:4n-6).

Despite their structural similarities, concentrations of n-6 and n-3 LCPUFA in tissues vary greatly. During EFA deficiency, amounts of n-6 and n-3 LCPUFA in tissues decrease and 20:3n-9 (Mead acid) levels in tissues increase. Mead acid can be synthesized endogenously by the same enzymes that convert EFA to n-6 or n-3 LCPUFA and replaces the n-3 and n-6 LCPUFA during EFA-deficiency (Holman 1998). The triene/tetraene ratio (20:3n-9/ARA) is often used as an index of EFA status and to diagnose deficiency (Holman 1998). Due to competition between LA and ALA for desaturation, the ratio of LA:ALA in the diet can also affect biosynthesis of n-6 and n-3 LCPUFA. In experiments holding levels of dietary LA constant, increasing dietary ALA suppressed n-6 FA products (Mohrhauer and Holman 1963). Similar studies have demonstrated that the opposite is also true, when dietary ALA was held constant and LA levels in the diet increased, n-3 FA products were

suppressed (Rahm and Holman 1964). The competitive nature of n-6 and n-3 pathways can be seen in models of n-3 deficiency, where DPAn-6 (Osbond acid) increases nearly reciprocally to the loss of DHA in rats (Mohrhauer and Holman 1963; Salem, Loewke et al. 2005). Δ^6-desaturase, the first enzyme in the conversion process, has a higher affinity for ALA and LA compared to 18:1n-9, which is why 20:3n-9 is formed in excess only during an EFA deficiency. The presence of n-6 or n-3 LCPUFA can also inhibit the conversion of parent EFA to their respective long chain metabolites and optimal synthesis of ALA to n-3 PUFA occur when the diet is low in n-6 FA and n-3 LCPUFA.

The major physiologically important metabolites of LA and ALA are dihomo-γ-linolenic acid (DHGLA, 20:3n-6), ARA, eicosapentaenoic (EPA, 20:5n-3) and DHA. DHGLA, ARA and EPA are precursors to a series of biologically active compounds known as eicosanoids, which include prostaglandins, thromboxanes, and leukotrienes (Das 2005). These molecules mediate a number of cell processes including inflammation, platelet aggregation, and chemotaxis. ARA is the most abundant n-6 LCPUFA in the brain and retina and also present in high concentration in other tissues (Rojas, Greiner et al. 2002). ARA serves as a precursor for series 2 prostaglandins and thromboxanes and series 4 leukotrienes (Das 2005). DHGLA is the elongation product of dietary γ–linolenic acid (GLA, 18:3n-6) which is found in evening primrose, blackcurrant and borage oil. DHGLA is present in human milk and possesses anti-inflammatory and anti-proliferative properties (Fan and Chapkin 1998). EPA, a n-3 FA, is also the precursor to series 3 prostaglandins, series 5 leukotrienes and lipoxins (Das 2005). ARA, EPA and DHA also form precursors for lipoxins, ecoisanoids with anti-inflammatory actions (Das 2005). DHA is the most abundant n-3 LCPUFA in the brain, retina and testes of many species primarily in the form of phospholipids (Lauritzen, Hansen et al. 2001).

Recent evidence has demonstrated that EPA and DHA are also precursors to bioactive mediators known as resolvins, docosatrienes and protectins (Bazan, Marcheselli et al. 2005; Serhan 2005). Docosatrienes are generated from DHA and have been shown to reduce neutrophils activity and alter the magnitude of an inflammatory response (Serhan 2005). Docosatrienes and resolvins are both neuroprotectins and prevented tissue damage in a mouse model of brain ischemia and oxidative stress mediated injury in transformed human retinal pigmented epithelial cells (Marcheselli, Hong et al. 2003; Mukherjee, Marcheselli et al. 2004).

1.3 Essential Fatty Acid Deficiency

Traditional EFA deficiency studies involve dietary deprivation of either total fat or both LA and ALA. n-6 FA deficiency has only been examined in models where ALA is also absent from the diet and requirements for LA have not been defined (Crawford, Golfetto et al. 2003). Low levels of dietary ALA exacerbates LA deficiency, but classic acute clinical signs of EFA deficiency can be completely cured by either LA or ALA alone (Lauritzen, Hansen et al. 2001; Crawford, Golfetto et al. 2003). Functions of the n-6 FA include structural components of skin ceramides and precursors of eicosanoids (Lauritzen, Hansen et al. 2001). DHA, an n-3 LCPUFA, is involved in membrane function and highly concentrated in the retina and neural tissues (Lauritzen, Hansen et al. 2001). Here, discussion focuses on the nutritional status of n-3 FA during neurodevelopment.

Animal models of n-3 FA deficiency during the perinatal period have produced evidence of biochemical, behavioral, visual and cognitive consequences in rats, guinea pigs and rhesus monkeys. Second generation adult male rats with decreased levels of frontal cortex DHA induced by dietary n-3 FA deprivation displayed learning deficits in olfactory- based discrimination tasks and the Morris water maze compared to

animals maintained on n-3 adequate diets (Rojas, Greiner et al. 2002). Similar studies in the developing rat have supported the assertion that adequate levels of n-3 FA, particularly DHA, in the CNS are required for optimal spatial and higher order learning (Catalan, Moriguchi et al. 2002; Niu, Mitchell et al. 2004). Newly weaned guinea pigs fed safflower oil, a poor source of n-3 FA, exhibited reduced electroretinogram (ERG) responses at 6, 11 and 16 weeks (Weisinger, Vingrys et al. 1996). In the same study, repletion with canola oil, a sufficient source of n-3 FA, for either 5 or 10 weeks, recovery of retinal function occurred only in animals fed n-3 FA for 10 weeks.

In rhesus monkeys, deficiency of n-3 FA during fetal life and infancy resulted in alterations in visual acuity and retinal function, significantly lower levels of retina and visual cortex DHA and polydipsia (Neuringer, Connor et al. 1986; Reisbick, Neuringer et al. 1990). Another rhesus study assessing deprivation of n-3 FA throughout pregnancy, began feeding high ALA (n-3 FA) diets at birth (Anderson, Neuringer et al. 2005). Although brain DHA reached normal levels, DHA recovery in the retina was not complete in repleted animals, and visual assessments revealed lower ERG amplitudes in the monkeys of n-3 deficient mothers compared to controls at 3 years of age (Anderson, Neuringer et al. 2005). Functional consequences due to nutritional perturbations during development may not be completely reversed despite corrections in the diet. Variables such as duration, severity of dietary deprivation, degree of repletion, specific period of development and appropriate functional outcomes contribute to the complexity of these investigations and gaps in our knowledge remain.

1.4 Essential Fatty Acid Conversion to LCPUFA

Dietary ALA is primarily oxidized to carbon dioxide, partially oxidized to form acetate, or synthesized to LCPUFA (Brenna 2002). Synthesis of DHA from ALA in the diet appears to be limited, and the best estimates from stable isotope tracer studies are less than 5% for normal adults (Brenna 2002). Conversion efficiency in a population of adult males with and without retinal pigmentosa was estimated around 5% (Hoffman, Theuer et al. 2004). In a compartmental model study using adult men and women, 0.2% of plasma ALA was converted to EPA (20:5n-3) and only 63% of the EPA was available for further conversion to DPA (22:5n-3) (Pawlosky, Hibbeln et al. 2001). Of that n-3 DPA, 37% was available for the biosynthesis of DHA.

Other researchers using compartmental modeling to examine ALA conversion in healthy adults, estimated that approximately 7% of dietary ALA was converted to LCPUFA, primarily as EPA (Goyens, Spilker et al. 2005). Plasma data from these studies suggest that as dietary ALA is converted to LCPUFA, relative amounts of the precursor pool and efficacy decreases and simply increasing the content of precursors in the diet may supply EPA but not DHA. Interestingly, two studies found significantly greater synthesis of EPA and DHA from dietary ALA in females compared to age matched males, hinting at a possible role during pregnancy and lactation (Burdge and Calder 2005).

1.5 LCPUFA Synthesis in Neonates

The ability of very-low birth weight preterm infants to synthesize LCPUFA from LA and ALA precursors at 1 month of age was demonstrated using stable isotopes (Carnielli, Wattimena et al. 1996). Another human infant study employed deuterated LA and ALA to estimate conversion of ARA and DHA in the plasma of term and preterm infants (Salem, Wegher et al. 1996). Evidence of n-6 LCPUFA

synthesis from ^{13}C-labeled LA was found in breastfed, term neonates in the first week of life (Szitanyi, Koletzko et al. 1999). Preterm infants born as early as 26 weeks gestation were capable of forming ARA and DHA from deuterated LA and ALA (Rojas, Greiner et al. 2002). Infants were given an enteral dose by day 4 of life and plasma was collected at 24, 48 and 96 hours after dosing. ARA and DHA levels in plasma were consistently higher in preterm infants compared to term and an intrauterine growth restricted (IUGR) group, suggesting that LCPUFA synthesis was more active at earlier gestational ages.

A separate study in term neonates consuming formulas with different ALA content, estimated the conversion of LA to ARA and ALA to DHA at 3 weeks of age using plasma FA concentrations (Sauerwald, Hachey et al. 1996). More recent data from preterm infants consuming formula containing DHA and ARA (0.4%/0.6%) using ^{13}C-labeled ALA showed a large range in absorption, oxidation and the ability to convert ALA to DHA (Mayes, Burdge et al. 2006). A 2 to 3-fold reduction in DHA formation from ALA was found in the plasma of human infants with IUGR compared to birth weight and gestational age matched controls (Llanos, Lin et al. 2005). Collectively, experimental evidence in human term and preterm neonates indicates that endogenous synthesis of LCPUFA occurs. However, rates of production are extremely variable, especially in premature infants and the magnitude of conversion may not be sufficient to match the demands of developing tissues.

1.6 LCPUFA during the Perinatal Period

LCPUFA play a critical role in infant central nervous system (CNS) and retina development. LCPUFA are structural components of cell membranes and DHA is highly concentrated in brain grey matter and retina rod photoreceptors (Lauritzen, Hansen et al. 2001; Heird and Lapillonne 2005). The retina membrane phospholipids

are comprised of over ~47% DHA and approximately 14% of brain DHA is contained in grey matter (Diau, Hsieh et al. 2005; Heird and Lapillonne 2005). The human brain undergoes a period of intense growth during the 3rd trimester of pregnancy that continues postnatally until about 2 years of age (Dobbing and Sands 1979). This "brain-growth-spurt" is a time of accelerated development and LCPUFA accretion, particularly DHA and ARA (Rojas, Greiner et al. 2002). During prenatal development, acquisition of LCPUFA occurs by placental transfer from the mother or from shorter chain FA precursors that can be converted to LCPUFA. Preformed DHA and ARA are preferentially transferred to the fetus in a phenomenon known as "biomagnification"(Lauritzen, Hansen et al. 2001)

The initial phase of the brain growth spurt for preterm infants occurs postnatally instead of prenatally. These infants are born with low fat stores and lack a maternal source of LCPUFA, requiring an alternative dietary source. Incorporation of LCPUFA into neural tissues is of particular importance to preterm infants. Since these infants are born before the normal accretion of FA into the brain and retina, they must rely on dietary sources of n-3 FA to supply the CNS with adequate DHA. The absolute amount of DHA needed for optimal development of the human brain and retina has not been determined. The CNS of these infants are thought to be especially susceptible to any interruptions in the nutritional supply of FA and it is not yet known whether prematurity affects the intensity of the brain growth spurt.

Maturation of the CNS is most dynamic in early life. During the first year, human brain weight increases from 350 grams to about 1000 grams, representing approximately 10% of total body weight. In humans, basic brain formation is completed around 6 weeks of gestation. Timing of neural development follows a distinct ontogeny in the mammalian brain (Finlay, Hersman et al. 1998). During brain growth and maturation, formation of brain structures first occurs in the "older"

regions, progressing from caudal to rostral structures (Erecinska, Cherian et al. 2004). Maturation begins in the inner layers of the cerebral cortex, progresses to the outer more superficial layers, and is followed by the development of sensory, motor and associative functions (Finlay, Hersman et al. 1998; Erecinska, Cherian et al. 2004). The major phases of mammalian brain development include induction of the neural plate, neural cell genesis and migration, proliferation of the axons and dendrites, synaptogenesis, the appearance of electrical activity and myelination of the axons (Erecinska, Cherian et al. 2004). Axonal myelination peaks around the 3rd trimester, beginning in the primary motor, somatosensory, visual and auditory cortical areas and continues throughout the life cycle (Guillery 2005). Different regions of the brain mature at various rates, and not all sub-regions reach their peak concurrently. The brain growth spurt, originally defined as the change in total brain weight over time, may not reflect more subtle differences that occur in different areas of the brain. The LCPUFA requirements during the brain growth spurt may correlate with the disproportionate development occurring in distinct regions of the brain.

The amount of DHA and ARA incorporated into neural tissues during the perinatal period is still unknown. Estimates of rate and substrate demand for LCPUFA synthesis and incorporation are limited to animal models of development and stable isotope tracer experiments in non-human primates have provided some insight. One such study found that both ALA and DHA are transferred from baboon mothers to their fetuses (Rojas, Greiner et al. 2002). Another experiment documented the kinetics and conversion of LA to ARA in the plasma of pregnant baboons, and traced the fate of ^{13}C-labeled LA to major organs in the fetus, showing that LA is transported across the placenta and elongated to ARA (Su, Corso et al. 1999). Fetal conversion of ALA to DHA has also been demonstrated in baboons and preformed DHA was found to be more effective than ALA as a substrate for accretion of brain

DHA by 8-fold (Su, Huang et al. 2001). In a study examining the bioequivalence of ALA and DHA as substrates for DHA accretion, 4 week old term baboons received an oral dose of ^{13}C-labeled fatty acids, ALA and DHA. At 6 weeks, tissue analyses revealed that preformed DHA was preferred to ALA-derived DHA for accretion in the brain and retina by 7 and 15 fold, respectively (Su, Bernardo et al. 1999). These findings indicate that fetal and infant primates incorporate preformed DHA preferentially compared to ALA in the brain and retina, and provide some of the best estimates for the dietary levels of LCPUFA required during infant development.

A different stable isotope tracer study assessed the influence of breast milk and formula fatty acid composition on DHA and ARA biosynthesis in term and preterm baboons. At 2 weeks adjusted age, neonates received ^{13}C- labeled LA and ALA. 2 weeks post dose, accretion of DHA and ARA was significantly higher in formula fed baboons. However, brain and retina DHA levels were significantly decreased compared to breastfed animals despite supplementation at 0.61% DHA and 1.21% ARA. Dietary DHA and ARA augmented DHA synthesis in preterm animals and restored LCPUFA supplemented preterm DHA levels to that of term baboons consuming LCPUFA free formula (Sarkadi-Nagy, Wijendran et al. 2004).

1.7 NHP Models for Human Infant Development and FA Metabolism

Experimental animal studies are essential for the comprehension and interpretation of human clinical findings. Non-human primate (NHP) models in biomedical research are especially important when ethical and practical constraints preclude mechanistic information and tissue sampling. Baboons are a common NHP model and possess specific characteristics resembling human physiology and behavior including anatomy, visual and nervous system, reproduction, lipid metabolism, omnivorous diet and an extended developmental period (Watts 1985). Baboons have

been used to examine the influence of LCPUFA supplementation, prematurity, central nervous system development and retinal function (Sarkadi-Nagy, Wijendran et al. 2004; Diau, Hsieh et al. 2005; Gubhaju and Black 2005; Tambunting, Beharry et al. 2005).

The comparable brain-weight-to-body ratio, unique timing of brain-growth spurt and similar omnivorous diet make baboons an especially appropriate & accurate model for studying human infant nutrition and development. While the brain growth spurt occurs in most mammalian species, the timing and intensity of the growth spurt with respect to birth varies greatly from species to species and needs to be considered when experimental outcomes are being extrapolated to human development (Dobbing and Sands 1979). In the rat, the brain growth spurt begins around birth and reaches its maximum at 10 days of age and ends around 30 days after birth. The porcine brain growth spurt is similar in timing to that of the human, but only half the intensity. Accelerated brain growth in rhesus monkeys occurs primarily *in utero* and at birth, the brain is much more developed relative to humans. The baboon brain growth spurt closely follows that of the human, beginning before birth and continuing after birth (Dobbing and Sands 1979).

1.8 Influence of LCPUFA on Infant Neurodevelopment and Growth

Effects of dietary LCPUFA on human infant development have focused on assessments probing functions related to the brain and retina, tissues highly concentrated in DHA. Functional outcomes examining vision typically measure acuity using preferential looking or visual evoked potential (VEP) and electroretinography (ERG) (SanGiovanni, Parra-Cabrera et al. 2000; Heird and Lapillonne 2005). Teller acuity cards are a forced preferential looking procedure that use black and white striped patterned cards. The stripes are at different widths and

observers score infants based on the finest grating where patterns are distinguishable. VEP or sweep VEP measures visual cortex responses to electrical potentials and estimates visual acuity by using relationships between the size of the response and elements of the stimulus presentation (Klein 2002; Heird and Lapillonne 2005). ERG assesses retina activity and response to a light flash. Components of the wave-shaped response are used to calculate specific parameters to determine maturity of the retina.

The most common standardized assessments used to examine effects of dietary LCPUFA are the Bayley Scales of Infant Development and the Fagan Test of Infant Intelligence (Rojas, Greiner et al. 2002; Fleith and Clandinin 2005). The Bayley Scales of Infant Development is a global assessment designed to identify deficits during development and comprised of two indices, the Mental Development Index (MDI) and Psychomotor Development Index (PDI). The Fagan Test of Infant Intelligence examines the novelty preference paradigm, using visual stimuli. A single stimulus is presented and then the same stimulus is shown with an unfamiliar one. When infants "recognize" the familiar stimulus there is a preference for the novel stimulus (Klein 2002; Heird and Lapillonne 2005). Numerous neurobehavioral measures have demonstrated differences in LCPUFA supplementation using specific assessments including visual attention, problem-solving, language, and temperament ratings (Simmer and Patole 2004; Heird and Lapillonne 2005).

Randomized, controlled clinical trials of DHA and ARA supplementation in term and preterm infants have shown improvements in both visual function and neurobehavioral outcomes (Koletzko, Agostoni et al. 2001; Fleith and Clandinin 2005). Preterm infants are more vulnerable to inadequate LCPUFA and require a postnatal supply of DHA and ARA. Preterm infants generally benefit from dietary LCPUFA and large scale clinical trials have been summarized in recent reviews (SanGiovanni, Parra-Cabrera et al. 2000; Gibson, Chen et al. 2001; Simmer and Patole

2004; Fleith and Clandinin 2005). Experimental evidence for term infants is less consistent with both neutral and positive effects of LCPUFA (Koletzko, Agostoni et al. 2001; Rojas, Greiner et al. 2002; Fleith and Clandinin 2005). Importantly, no negative effects of formulas supplementation with both DHA and ARA have been reported on visual and cognitive function (Simmer and Patole 2004; Fleith and Clandinin 2005) and this discussion concentrates on transient changes during neurodevelopment with an emphasis on potential long-term advantages suggested by accelerated maturation in early infancy.

An LCPUFA study assigned term infants to either a DHA and ARA formula or an unsupplemented control formula for 52 weeks after birth (Birch, Castaneda et al. 2005). VEP acuity was assessed at 6, 17, 39 and 52 weeks and was significantly improved for infants consuming LCPUFA at all time points. The difference in VEP acuity at 39 weeks of age was equivalent to one line on a standard eye chart, for example 20/37 instead of 20/52 (Birch, Castaneda et al. 2005). Stereoacuity was tested at 17, 39 and 52 weeks of age and better acuity was seen in the DHA and ARA formula group at 17 weeks of age. However, differences were no longer detectable at the later time points (Birch, Castaneda et al. 2005). Term infants fed formula containing DHA and ARA for 4 months after birth exhibited advanced multi-level problem solving skills at 10 months of age compared to unsupplemented controls (Willatts, Forsyth et al. 1998; Forsyth, Willatts et al. 2003). A follow-up study of these same infants found significantly lowered mean blood pressure and diastolic blood pressure at 6 years of age in the group that had consumed LCPUFA formula (Forsyth, Willatts et al. 2003). These subtle or transient functional effects may have long-term benefits that persist beyond infancy and point to nutritional requirements during sensitive periods of central nervous system maturation.

Transient advantages during development were evident in several studies examining breastfed infants. While the data are not from randomized trials, some of the confounding variables present in breastfed and formula fed infant comparisons of neurodevelopment have been minimized (Rojas, Greiner et al. 2002). In exclusively breastfed term infants, visual acuity at 2 and 12 months, but not at 4 or 6 months of age was associated with RBC DHA content (Innis, Gilley et al. 2001). Language development at 9 months was also significantly correlated to plasma and RBC DHA levels at 2 months at age. Infants with higher DHA levels had superior speech perception performance and could discriminate unfamiliar syllables better than breastfed infants with lower RBC DHA concentrations (Innis, Gilley et al. 2001). Another maternal supplementation study using 1.3g/day fish oil, containing n-3 LCPUFA and 60% DHA, enhanced the problem solving abilities of breastfed term infants at 9 months of age. However, passive vocabulary was lower at 1 year in the LCPUFA group but differences were no longer apparent at 2 years of age (Lauritzen, Hansen et al. 2001). In the same study, infant visual acuity was not different at 2 and 4 months, but there was a positive association between visual acuity and RBC DHA at 4 months of age (Lauritzen, Hansen et al. 2001). At 2.5 years of age, body mass index of children in the LCPUFA supplemented group were significantly higher than the unsupplemented breastfed group (Lauritzen, Hansen et al. 2001). A different study examining breastfeeding mothers randomized to either a DHA (200mg/day) or placebo group for 4 months after term delivery resulted in higher infant Bayley Psychomotor Development Index scores at 30 months (Sauerwald, Hachey et al. 1996). Earlier, however, at 12 months of age no differences were detected in either the Bayley Mental Development or Psychomotor score. At 4 and 8 months, no differences were observed between the DHA group and unsupplemented controls in visual acuity.

Studies examining the influence of maternal LCPUFA status during pregnancy and birth have shown improvements in term infants as early as 1 day of life. DHA levels in maternal plasma were associated with maturity of infant sleep patterns 1 and 2 days after birth (Cheruku, Montgomery-Downs et al. 2002). Positive correlations between levels of breast milk DHA and term infant scores on the Brazelton Neonatal Behavioral Assessment Scale were evident at 9 days of life (Carnielli, Wattimena et al. 1996). Infants consuming breast milk with higher amounts of DHA could maintain their state of arousal far better than infants with lower levels of DHA. Connections between early improvements in neonatal behavior and prenatal LCPUFA supply may indicate developmental advantages that may not be apparent until later ages. However, beneficial effects of LCPUFA during the perinatal period have also been associated with long term neurobehavioral advantages in older children. In a study examining visual acuity in preterm and term formula fed infants and a breastfed reference group, stereo acuity at 3 years of age was associated with LCPUFA status at 4 months of age (Birch, Birch et al. 1993). Population based evidence linked maternal consumption of oily fish during pregnancy and accelerated stereo acuity maturity in children at 3.5 years (Williams, Birch et al. 2001) and intelligence scores at 4 years of age have been significantly correlated with maternal cod liver oil supplementation during pregnancy, which contain both DHA and EPA (Helland, Smith et al. 2003).

In a number of randomized controlled LCPUFA supplementation trials during pregnancy, differences in term infant retinal maturation and anthropometric parameters were also reported (Decsi and Koletzko 2005). A supplementation study assessing high DHA (1.3g/day) with low DHA (0.3g/day) eggs during the third trimester of pregnancy reported longer body lengths at birth and a 6 day increase in the duration of gestation of infants in the high maternal DHA group (Smuts, Huang et al. 2003). A clinical trial examining the effects of ALA (2.8g/day) + LA (9.0g/day) with

supplements of LA (10.9g/day) alone beginning the 14th week of pregnancy reported higher birth weights in infants of mothers supplemented with higher amounts of LA, a difference that disappeared when corrections for potential confounders were made (de Groot, Hornstra et al. 2004). Another study found positive correlations between DHA concentrations in umbilical cord plasma and retinal maturity at 3 weeks of age, body length at term birth, and increased gestational age (Malcolm, Hamilton et al. 2003). These data support existing epidemiological data suggesting an additional benefit of LCPUFA during pregnancy for populations at risk for preterm delivery and low birth weight. Whether the effects of LCPUFA supplementation during pregnancy confer to neurodevelopmental or physiological advantages beyond 4 years of age awaits results from future studies.

1.9 LCPUFA in Human Milk and Infant Formula

Human breast milk LCPUFA concentrations are widely variable and reflective of maternal diet. DHA levels in breast milk ranges from 0.06% for urban women in the US to 1.33% of total fatty acids in Japanese women with high fish consumption (Nettleson 1995; Sauerwald, Hachey et al. 1996). Worldwide breast milk DHA averages approximately 0.34% and between 0.4-0.67% for ARA (Nettleson 1995; Sauerwald, Hachey et al. 1996). ARA levels in human milk are relatively consistent and depends less on dietary intake (Klein 2002). Lipids provide about 50-60% of the calories in human milk (Sauerwald, Hachey et al. 1996). While the total lipid content of breast milk remains relatively constant, subtle changes occur during lactation. FA composition increases during the duration of a feed and hind milk fractions of human milk samples contain 2 to 3-fold more fat compared to foremilk in the first 6 months of lactation (Saarela, Kokkonen et al. 2005). FA content varies with the stage of lactation, the EFA LA and ALA increase with maturation while n-6 and n-3 LCPUFA

decrease during the first month (Sauerwald, Hachey et al. 1996; Koletzko, Rodriguez-Palmero et al. 2001). Breastmilk lipids also decrease with parity (Sauerwald, Hachey et al. 1996).

Higher human milk levels of DHA and ARA have been identified in samples of mothers with very low birth weight (VLBW) preterm infants compared to full-term infants during the first month of lactation (Kovacs, Funke et al. 2005). An earlier comparison of lipid profiles in the breast milk of mothers with term and preterm infants, showed highest LCPUFA levels in colostrum and preterm milk (Bitman, Wood et al. 1984). Overall, highest levels were in mothers of preterm infants born 26-30 weeks gestation compared to milk from mothers of preterm infants born at later ages (31-36 weeks) and breast milk fat content increased during the duration of lactation. Another study examining changes in FA composition of preterm and term milk at 1, 2, 4, 12, and 26 weeks after birth reported highest LCPUFA levels at one week, which later decreased in both types of breast milk (Luukkainen, Salo et al. 1994). However, in term milk samples, LCPUFA content dropped from 1 to 6 months while preterm milk fat changed more moderately. During the 6^{th} month of lactation, ARA and DHA levels were 1.5 and 2-fold higher, respectively, in the breast milk of mothers with preterm infants (Luukkainen, Salo et al. 1994). Infants born prematurely have special nutritional needs and the observed changes in breast milk LCPUFA composition may impart special benefits to these infants.

Breast milk is considered the ideal source of nutrition for neonates and the model for human infant formulas. Formula-feeding is commonly used to supplement or replace breast feeding throughout the world. The LCPUFA content of experimental infant formulas vary widely in both animal and human clinical studies. Rhesus monkey neonates fed formula supplemented with 1.0%ARA and 1.0%DHA demonstrated accelerated neuromotor development compared to an unsupplemented

formula group and a breastfed mother-reared group (0.2%ARA, 0.4%DHA) (Champoux, Hibbeln et al. 2002). Formula levels of DHA and ARA also varied in a study examining effects of LCPUFA on CNS FA profiles in term and preterm baboons (Diau, Hsieh et al. 2005). LCPUFA concentrations of supplemented formulas and milk from a randomized breastfed group ranged from 0.62-1.21%ARA and 0.30-0.68%DHA with ARA to DHA ratios between 0.67 and 2.0 (Diau, Hsieh et al. 2005).

A Cochrane review of LCPUFA studies in preterm human infants included experimental formulas containing combinations of EPA/DHA, ARA/EPA/DHA, DHA alone, or ARA/DHA (Simmer and Patole 2004). Formula EPA/DHA levels were 0.6 or 0.3%EPA and 0.2%DHA. Concentrations of ARA/EPA/DHA were 0.31%, 0.04%, and 0.17%, respectively. Two of the clinical trials included DHA alone at 0.15% and 0.34%. The majority of preterm studies included varying concentrations of ARA/DHA ranging from 0.23-1.1% for ARA and 0.14-0.76% DHA with ARA to DHA ratios between 1 and 2 (Simmer and Patole 2004). More recently, a different Cochrane review of term and preterm infant LCPUFA trials was published with additional formulas varying in LCPUFA concentrations (Fleith and Clandinin 2005). Preterm formulas that were not included in the previous review contained mixtures of ARA/GLA/EPA/DHA and GLA/EPA/DHA. The term infant formulas also contained different amounts and mixtures of LCPUFA including, EPA/DHA, GLA/DHA, ARA/EPA/DHA, GLA/EPA/DHA, ARA/DHA and DHA alone (Fleith and Clandinin 2005). The levels for DHA formula were 0.15% and 0.35% while ranges for ARA/DHA formulas were 0.03-0.72%ARA and 0.10-0.36%DHA (Fleith and Clandinin 2005). Published clinical trial results from 1990-2002 included DHA levels from 0.2-0.76% DHA for preterm infants and 0.10-0.36% DHA in term neonates, with ARA to DHA ratios between 0.3-4.0 and 1.3-2.0 for preterm and term studies, respectively (Simmer and Patole 2004; Fleith and Clandinin 2005).

LCPUFA have been added to infant formulas in Europe, South America, North America, Australia, Japan, Thailand and other Asian countries beginning in 1995 (Fleith and Clandinin 2005). US and Canadian infant formulas have included DHA and ARA since 2002. Concentrations of DHA and ARA vary in term infant formulas sold throughout the United States. Table 1.2 summarizes the LCPUFA content in commercially available infant formula, with levels of DHA ranging from 8-19 mg/100 cal, and ARA between 21-34 mg/100 cal. ARA is included to insure normal growth, and for this outcome present levels in formula of about 0.64% ARA appear to be effective (Fleith and Clandinin 2005). ARA may also enhance the function of DHA (Hoffman, DeMar et al. 2001) though data on this point are fewer than for DHA alone. Requirements for LCPUFA during the perinatal period have not been determined. Conclusive evidence for establishing LCPUFA requirements during the perinatal period have not been determined and intake of DHA above 0.36% may impart benefits to term infants that have not been detected at present formula levels.

Table 1.2 LCPUFA concentrations in US commercial term infant formulas (mg/100cal).

Product and Brand	DHA	ARA
Enfamil® LIPIL® with Iron Mead-Johnson Nutritionals	17	34
Ultra Bright Beginings™ PBM Products	19	34
Good Start® SUPREME Nestle	17	32
Similac® Advance® with Iron Ross Products	8	21

1.10 Summary

In summary, this thesis evaluates the safety and efficacy of formula ARA and moderate and high levels of DHA in term baboon neonates from 2 to 12 weeks of age. Chapter 2 focuses on the influence of formula DHA and ARA levels on tissue fatty acid composition and anthropometric parameters. Chapter 3 describes effects of dietary LCPUFA on hematological measurements with an emphasis on erythrocytes. Here, we present complete blood count data and speculate on the role of DHA and ARA in hematopoiesis during early postnatal development. Chapter 4 assesses safety using clinical chemistry parameters and documents the ontogeny of white cell measurements. Finally, Chapter 5 provides a brief summary of results and outlines future studies necessary to address unanswered questions regarding infant LCPUFA requirements during postnatal neurodevelopment.

1.11 References

Anderson, G. J., M. Neuringer, et al. (2005). "Can prenatal N-3 fatty acid deficiency be completely reversed after birth? Effects on retinal and brain biochemistry and visual function in rhesus monkeys." Pediatr Res **58**(5): 865-72.

Bazan, N. G., V. L. Marcheselli, et al. (2005). "Brain response to injury and neurodegeneration: endogenous neuroprotective signaling." Ann N Y Acad Sci **1053**: 137-47.

Birch, E., D. Birch, et al. (1993). "Breast-feeding and optimal visual development." J Pediatr Ophthalmol Strabismus **30**(1): 33-8.

Bitman, J., D. L. Wood, et al. (1984). "Comparison of the phospholipid composition of breast milk from mothers of term and preterm infants during lactation." Am J Clin Nutr **40**(5): 1103-19.

Brenna, J. T. (2002). "Efficiency of conversion of alpha-linolenic acid to long chain n-3 fatty acids in man." Curr Opin Clin Nutr Metab Care **5**(2): 127-32.

Burdge, G. C. and P. C. Calder (2005). "Conversion of alpha-linolenic acid to longer-chain polyunsaturated fatty acids in human adults." Reprod Nutr Dev **45**(5): 581-97.

Burr, G. O. and M. M. Burr (1930). "On the nature and role of the fatty acids essential in nutrition." Journal of Biological Chemistry **86**(2): 587-620.

Carnielli, V. P., D. J. Wattimena, et al. (1996). "The very low birth weight premature infant is capable of synthesizing arachidonic and docosahexaenoic acids from linoleic and linolenic acids." Pediatr Res **40**(1): 169-74.

Catalan, J., T. Moriguchi, et al. (2002). "Cognitive deficits in docosahexaenoic acid-deficient rats." Behav Neurosci **116**(6): 1022-31.

Champoux, M., J. R. Hibbeln, et al. (2002). "Fatty acid formula supplementation and neuromotor development in rhesus monkey neonates." Pediatr Res **51**(3): 273-81.

Cheruku, S. R., H. E. Montgomery-Downs, et al. (2002). "Higher maternal plasma docosahexaenoic acid during pregnancy is associated with more mature neonatal sleep-state patterning." Am J Clin Nutr **76**(3): 608-13.

Cunnane, S. C. (2000). "The conditional nature of the dietary need for polyunsaturates: a proposal to reclassify 'essential fatty acids' as 'conditionally-

indispensable' or 'conditionally-dispensable' fatty acids." Br J Nutr **84**(6): 803-12.

Das, U. N. (2005). "COX-2 inhibitors and metabolism of essential fatty acids." Med Sci Monit **11**(7): RA233-7.

de Groot, R. H., G. Hornstra, et al. (2004). "Effect of alpha-linolenic acid supplementation during pregnancy on maternal and neonatal polyunsaturated fatty acid status and pregnancy outcome." Am J Clin Nutr **79**(2): 251-60.

Decsi, T. and B. Koletzko (2005). "N-3 fatty acids and pregnancy outcomes." Curr Opin Clin Nutr Metab Care **8**(2): 161-6.

Diau, G. Y., A. T. Hsieh, et al. (2005). "The influence of long chain polyunsaturate supplementation on docosahexaenoic acid and arachidonic acid in baboon neonate central nervous system." BMC Med **3**(11): 11.

Dobbing, J. and J. Sands (1973). "Quantitative growth and development of human brain." Arch Dis Child **48**(10): 757-67.

Dobbing, J. and J. Sands (1979). "Comparative aspects of the brain growth spurt." Early Hum Dev **3**(1): 79-83.

Erecinska, M., S. Cherian, et al. (2004). "Energy metabolism in mammalian brain during development." Prog Neurobiol **73**(6): 397-445.

Fan, Y. Y. and R. S. Chapkin (1998). "Importance of dietary gamma-linolenic acid in human health and nutrition." J Nutr **128**(9): 1411-4.

Finlay, B. L., M. N. Hersman, et al. (1998). "Patterns of vertebrate neurogenesis and the paths of vertebrate evolution." Brain Behav Evol **52**(4-5): 232-42.

Fleith, M. and M. T. Clandinin (2005). "Dietary PUFA for preterm and term infants: review of clinical studies." Crit Rev Food Sci Nutr **45**(3): 205-29.

Forsyth, J. S., P. Willatts, et al. (2003). "Long chain polyunsaturated fatty acid supplementation in infant formula and blood pressure in later childhood: follow up of a randomised controlled trial." Bmj **326**(7396): 953.

Gibson, R. A., W. Chen, et al. (2001). "Randomized trials with polyunsaturated fatty acid interventions in preterm and term infants: functional and clinical outcomes." Lipids **36**(9): 873-83.

Goyens, P. L., M. E. Spilker, et al. (2005). "Compartmental modeling to quantify alpha-linolenic acid conversion after longer term intake of multiple tracer boluses." J Lipid Res **46**(7): 1474-83.

Greiner, R. C., J. Winter, et al. (1997). "Brain docosahexaenoate accretion in fetal baboons: bioequivalence of dietary alpha-linolenic and docosahexaenoic acids." Pediatr Res **42**(6): 826-34.

Greiner, R. S., T. Moriguchi, et al. (1999). "Rats with low levels of brain docosahexaenoic acid show impaired performance in olfactory-based and spatial learning tasks." Lipids **34 Suppl**(43): S239-43.

Gubhaju, L. and M. J. Black (2005). "The baboon as a good model for studies of human kidney development." Pediatr Res **58**(3): 505-9.

Guillery, R. W. (2005). "Is postnatal neocortical maturation hierarchical?" Trends Neurosci **28**(10): 512-7.

Hart, S. L., L. M. Boylan, et al. (2006). "Brief report: newborn behavior differs with decosahexaenoic acid levels in breast milk." J Pediatr Psychol **31**(2): 221-6.

Heird, W. C. and A. Lapillonne (2005). "The role of essential fatty acids in development." Annu Rev Nutr **25**: 549-71.

Helland, I. B., L. Smith, et al. (2003). "Maternal supplementation with very-long-chain n-3 fatty acids during pregnancy and lactation augments children's IQ at 4 years of age." Pediatrics **111**(1): e39-44.

Hoffman, D. R., J. C. DeMar, et al. (2001). "Impaired synthesis of DHA in patients with X-linked retinitis pigmentosa." J Lipid Res **42**(9): 1395-401.

Holman, R. T. (1998). "The slow discovery of the importance of omega 3 essential fatty acids in human health." J Nutr **128**(2 Suppl): 427S-433S.

Infante, J. P. and V. A. Huszagh (1998). "Analysis of the putative role of 24-carbon polyunsaturated fatty acids in the biosynthesis of docosapentaenoic (22:5n-6) and docosahexaenoic (22:6n-3) acids." FEBS Lett **431**(1): 1-6.

Innis, S. M., J. Gilley, et al. (2001). "Are human milk long-chain polyunsaturated fatty acids related to visual and neural development in breast-fed term infants?" J Pediatr **139**(4): 532-8.

Jensen, C. L. (1999). "Lipids in human milk." Lipids **34**(12): 1243-71.

Jensen, C. L., R. G. Voigt, et al. (2005). "Effects of maternal docosahexaenoic acid intake on visual function and neurodevelopment in breastfed term infants." Am J Clin Nutr **82**(1): 125-32.

Klein, C. J., Editor (2002). "Nutrient Requirements for Preterm Infant Formulas: A report from the American Society for Nutritional Sciences, Life Sciences Research Office." Journal of Nutrition **132**(6S-I).

Koletzko, B., C. Agostoni, et al. (2001). "Long chain polyunsaturated fatty acids (LC-PUFA) and perinatal development." Acta Paediatr **90**(4): 460-4.

Koletzko, B., M. Rodriguez-Palmero, et al. (2001). "Physiological aspects of human milk lipids." Early Hum Dev **65 Suppl**: S3-S18.

Kovacs, A., S. Funke, et al. (2005). "Fatty acids in early human milk after preterm and full-term delivery." J Pediatr Gastroenterol Nutr **41**(4): 454-9.

Lauritzen, L., H. S. Hansen, et al. (2001). "The essentiality of long chain n-3 fatty acids in relation to development and function of the brain and retina." Prog Lipid Res **40**(1-2): 1-94.

Lauritzen, L., M. H. Jorgensen, et al. (2004). "Maternal fish oil supplementation in lactation: effect on visual acuity and n-3 fatty acid content of infant erythrocytes." Lipids **39**(3): 195-206.

Lauritzen, L., M. H. Jorgensen, et al. (2005). "Maternal fish oil supplementation in lactation: effect on developmental outcome in breast-fed infants." Reprod Nutr Dev **45**(5): 535-47.

Leonard, A. E., S. L. Pereira, et al. (2004). "Elongation of long-chain fatty acids." Prog Lipid Res **43**(1): 36-54.

Lim, S. Y., J. Hoshiba, et al. (2005). "N-3 fatty acid deficiency induced by a modified artificial rearing method leads to poorer performance in spatial learning tasks." Pediatr Res **58**(4): 741-8.

Llanos, A., Y. Lin, et al. (2005). "Infants with intrauterine growth restriction have impaired formation of docosahexaenoic acid in early neonatal life: a stable isotope study." Pediatr Res **58**(4): 735-40.

Luukkainen, P., M. K. Salo, et al. (1994). "Changes in the fatty acid composition of preterm and term human milk from 1 week to 6 months of lactation." J Pediatr Gastroenterol Nutr **18**(3): 355-60.

Malcolm, C. A., R. Hamilton, et al. (2003). "Scotopic electroretinogram in term infants born of mothers supplemented with docosahexaenoic acid during pregnancy." Invest Ophthalmol Vis Sci **44**(8): 3685-91.

Marcheselli, V. L., S. Hong, et al. (2003). "Novel docosanoids inhibit brain ischemia-reperfusion-mediated leukocyte infiltration and pro-inflammatory gene expression." J Biol Chem **278**(44): 43807-17.

Martinez, M. (1992). "Tissue levels of polyunsaturated fatty acids during early human development." J Pediatr **120**(4 Pt 2): S129-38.

Mayes, C., G. C. Burdge, et al. (2006). "Variation in [U-13C] alpha linolenic acid absorption, beta-oxidation and conversion to docosahexaenoic acid in the pre-term infant fed a DHA-enriched formula." Pediatr Res **59**(2): 271-5.

Mohrhauer, H. and R. T. Holman (1963). "Effect of Linolenic Acid Upon the Metabolism of Linoleic Acid." J Nutr **81**: 67-74.

Mukherjee, P. K., V. L. Marcheselli, et al. (2004). "Neuroprotectin D1: a docosahexaenoic acid-derived docosatriene protects human retinal pigment epithelial cells from oxidative stress." Proc Natl Acad Sci U S A **101**(22): 8491-6.

Nettleson, J. A. (1995). Omega-3 Fatty Acids and Health. New York, Chapman and Hall.

Neuringer, M., W. E. Connor, et al. (1986). "Biochemical and functional effects of prenatal and postnatal omega 3 fatty acid deficiency on retina and brain in rhesus monkeys." Proc Natl Acad Sci U S A **83**(11): 4021-5.

Niu, S. L., D. C. Mitchell, et al. (2004). "Reduced G protein-coupled signaling efficiency in retinal rod outer segments in response to n-3 fatty acid deficiency." J Biol Chem **279**(30): 31098-104.

Pawlosky, R. J., J. R. Hibbeln, et al. (2001). "Physiological compartmental analysis of alpha-linolenic acid metabolism in adult humans." J Lipid Res **42**(8): 1257-65.

Rahm, J. J. and R. T. Holman (1964). "Effect of Linoleic Acid Upon the Metabolism of Linolenic Acid." J Nutr **84**: 15-9.

Reisbick, S., M. Neuringer, et al. (1990). "Polydipsia in rhesus monkeys deficient in omega-3 fatty acids." Physiol Behav **47**(2): 315-23.

Rojas, C. V., R. S. Greiner, et al. (2002). "Long-term n-3 FA deficiency modifies peroxisome proliferator-activated receptor beta mRNA abundance in rat ocular tissues." Lipids **37**(4): 367-74.

Saarela, T., J. Kokkonen, et al. (2005). "Macronutrient and energy contents of human milk fractions during the first six months of lactation." Acta Paediatr **94**(9): 1176-81.

Salem, N., Jr., J. Loewke, et al. (2005). "Incomplete replacement of docosahexaenoic acid by n-6 docosapentaenoic acid in the rat retina after an n-3 fatty acid deficient diet." Exp Eye Res **81**(6): 655-63.

Salem, N., Jr., B. Wegher, et al. (1996). "Arachidonic and docosahexaenoic acids are biosynthesized from their 18-carbon precursors in human infants." Proc Natl Acad Sci U S A **93**(1): 49-54.

SanGiovanni, J. P., S. Parra-Cabrera, et al. (2000). "Meta-analysis of dietary essential fatty acids and long-chain polyunsaturated fatty acids as they relate to visual resolution acuity in healthy preterm infants." Pediatrics **105**(6): 1292-8.

Sarkadi-Nagy, E., V. Wijendran, et al. (2004). "Formula feeding potentiates docosahexaenoic and arachidonic acid biosynthesis in term and preterm baboon neonates." J Lipid Res **45**(1): 71-80.

Sauerwald, T. U., D. L. Hachey, et al. (1996). "Effect of dietary alpha-linolenic acid intake on incorporation of docosahexaenoic and arachidonic acids into plasma phospholipids of term infants." Lipids **31 Suppl**(5): S131-5.

Serhan, C. N. (2005). "Novel eicosanoid and docosanoid mediators: resolvins, docosatrienes, and neuroprotectins." Curr Opin Clin Nutr Metab Care **8**(2): 115-21.

Simmer, K. and S. Patole (2004). "Longchain polyunsaturated fatty acid supplementation in preterm infants." Cochrane Database Syst Rev **1**(1): CD000375.

Sinclair, A. J., W. Slattery, et al. (1981). "Essential fatty acid deficiency and evidence for arachidonate synthesis in the cat." Br J Nutr **46**(1): 93-6.

Smuts, C. M., M. Huang, et al. (2003). "A randomized trial of docosahexaenoic acid supplementation during the third trimester of pregnancy." Obstet Gynecol **101**(3): 469-79.

Sprecher, H., D. L. Luthria, et al. (1995). "Reevaluation of the pathways for the biosynthesis of polyunsaturated fatty acids." J Lipid Res **36**(12): 2471-7.

Su, H. M., L. Bernardo, et al. (1999). "Bioequivalence of dietary alpha-linolenic and docosahexaenoic acids as sources of docosahexaenoate accretion in brain and associated organs of neonatal baboons." Pediatr Res **45**(1): 87-93.

Su, H. M., T. N. Corso, et al. (1999). "Linoleic acid kinetics and conversion to arachidonic acid in the pregnant and fetal baboon." J Lipid Res **40**(7): 1304-12.

Su, H. M., M. C. Huang, et al. (2001). "Fetal baboons convert 18:3n-3 to 22:6n-3 in vivo. A stable isotope tracer study." J Lipid Res **42**(4): 581-6.

Szitanyi, P., B. Koletzko, et al. (1999). "Metabolism of 13C-labeled linoleic acid in newborn infants during the first week of life." Pediatr Res **45**(5 Pt 1): 669-73.

Tambunting, F., K. D. Beharry, et al. (2005). "Impaired lung vascular endothelial growth factor in extremely premature baboons developing bronchopulmonary dysplasia/chronic lung disease." J Investig Med **53**(5): 253-62.

Uauy, R., D. R. Hoffman, et al. (2003). "Term infant studies of DHA and ARA supplementation on neurodevelopment: results of randomized controlled trials." J Pediatr **143**(4 Suppl): S17-25.

Uauy, R., P. Mena, et al. (2000). "Long chain polyunsaturated fatty acid formation in neonates: effect of gestational age and intrauterine growth." Pediatr Res **47**(1): 127-35.

Watts, E. S. (1985). Nonhuman Primate Models for Human Growth and Development. New York, Alan R. Liss, Inc.

Weisinger, H. S., A. J. Vingrys, et al. (1996). "Effect of dietary n-3 deficiency on the electroretinogram in the guinea pig." Ann Nutr Metab **40**(2): 91-8.

Willatts, P., J. S. Forsyth, et al. (1998). "Effect of long-chain polyunsaturated fatty acids in infant formula on problem solving at 10 months of age." Lancet **352**(9129): 688-91.

Williams, C., E. E. Birch, et al. (2001). "Stereoacuity at age 3.5 y in children born full-term is associated with prenatal and postnatal dietary factors: a report from a population-based cohort study." Am J Clin Nutr **73**(2): 316-22.

CHAPTER 2

THE INFLUENCE OF MODERATE AND HIGH LEVELS OF LONG CHAIN POLYUNSATURATED FATTY AID SUPPLEMENTATION ON BABOON NEONATE TISSUE FATTY ACIDS

2.1 Abstract

Docosahexaenoic acid (DHA) and arachidonic acid (ARA) are now common ingredients in commercial infant formulas. However the optimal amount has not been established. Our previous data showed that the current amount of DHA in U.S. term formulas, 0.3%wt, is insufficient to normalize cerebral cortex DHA to levels in breastfed controls (Diau, Hsieh et al. 2005). Here we report on the influence of higher formula DHA levels on 12 week old term baboon CNS and visceral organs. Fourteen nursery reared baboons were randomized to one of three diets: Control (C, no DHA-ARA, n=5); 1× LCPUFA (L, 0.32%DHA-0.64%ARA, n=4); 3× LCPUFA (L3, 0.96%DHA-0.64%ARA, n=5). At 12 weeks, tissues were collected and analyzed for fatty acid profiles using gas chromatography (GC) and GC/mass spectrometry. Supplementation increased DHA in all tissues but significantly altered ARA concentrations in fewer tissues. DHA increased significantly ($p<0.05$) in liver (C<L<L3), heart (C<L<L3), plasma (C<L<L3), RBC (C<L,L3) and CNS regions: precentral gyrus (C<L<L3), and frontal lobe (C<L,L3) of the cerebral cortex, inferior and superior colliculi (C<L,L3), globus pallidus (C<L,L3), and caudate (C<L,L3); in putamen DHA differences were marginally significant (C<L.L3), the DHA trend in amygdala and retina were consistent but non-significant. These data extend and confirm previous observations indicating that 1) tissue DHA is more sensitive to dietary manipulations than ARA, and 2) cerebral cortex DHA increases with higher levels of DHA than are included in present commercial formulas, 3) basal ganglia and limbic system DHA appears to saturate with levels of DHA currently available in

formulas. These results imply that higher levels of DHA are necessary to normalize cortex DHA to those found in breastfed animals.

2.2 Introduction

Docosahexaenoic acid (DHA, DHA) and arachidonic acid (ARA) are ingredients specifically added to many infant formulas worldwide. Studies of DHA and ARA as formula components have focused on infant functions closely associated with neural tissue normally high in DHA, specifically visual acuity (Hoffman, Theuer et al. 2004; Birch, Castaneda et al. 2005), and retinal (Jeffrey, Weisinger et al. 2001; Sarkadi-Nagy, Wijendran et al. 2003) and cognitive function (Willatts and Forsyth 2000). ARA's importance is associated with insuring normal growth, and for this specific outcome present levels in formula of about 0.64% (w/w) of fatty acids appear to be effective (Fleith and Clandinin 2005). ARA may also enhance the function of DHA (Birch, Garfield et al. 2000) though data on this point are fewer than for DHA alone.

Prior to 1995, infant formulas worldwide were devoid of LCPUFA. US infant formulas have contained DHA and ARA since 2002, and about 80% of the formulas consumed in the US contain DHA/ARA at this writing. However, the amount of DHA and ARA required for optimal development is not well characterized. Experimental infant formulas range over at least 6-fold in human and primate studies (Gibson, Chen et al. 2001; Champoux, Hibbeln et al. 2002), and presently varies by more than two-fold in commercial US infant formulas (Hsieh 2006). In 2001, a group of clinical researchers recommended a minimum of 0.35%(w/w) DHA and 0.40% (w/w) ARA (Koletzko, Agostoni et al. 2001), corresponding roughly to about 19 mg DHA/100kcal and 21 mg ARA/100kcal. However, 11 studies of healthy term infants considered in a comprehensive systematic review in 2000 used levels from 0.10-0.36%(w/w)

(SanGiovanni, Parra-Cabrera et al. 2000). Benefits to term infants of higher DHA levels cannot be ruled out because they have not been investigated.

Intake of DHA above 0.36% may impart benefits not observed at present levels. Worldwide breastmilk has a median level of about 0.32%(w/w), but ranges to at least 1% (Lauritzen, Hansen et al. 2001); for instance, it averaged 1.4% for the Inuit women of northern Canada, who have a diet high in marine mammals (Innis, Gilley et al. 2001). These levels of n-3 LCPUFA are well known to have bioactivities associated with cardiovascular function that are not observed at moderate intakes. Beyond these human compositional data, one primate study of 1.0% DHA formula showed improved neuromotor development in one month old rhesus monkey neonates (Champoux, Hibbeln et al. 2002).

The central nervous system (CNS) has long been known to be rich in DHA and ARA, and for this reason has been a major target for DHA studies. Work in our laboratory has demonstrated that DHA (0.30%) and ARA (0.55%) supplementation to four weeks of age in neonatal baboons increases DHA throughout the CNS, but ARA is largely unaffected (Diau, Hsieh et al. 2005). Those data further showed that DHA levels of supplemented neonates were similar in most CNS regions (basal ganglia, limbic system, hippocampus, amygdala, thalamus, midbrain) to those of a randomized breastfed group. Importantly, all sampled regions of gray matter of the cerebral cortex and cerebellum were lower in supplemented animals than in cortex of breastfed animals. The cerebral cortex and cerebellum contain more than half the DHA in the CNS, and thus it is reasonable to expect that smaller regions such as the basal ganglia might be less susceptible to DHA insufficiency.

Our purpose here is to report on the changes in tissue fatty acid composition that accompany increased levels of DHA, with emphasis on the CNS. This study was conducted in neonatal baboons, who in the wild are omnivorous primates with lipid

metabolism similar to that of humans (Kushwaha and McGill 1998). An LCPUFA-free formula control was compared to LCPUFA formulas supplemented at two different levels in a randomized controlled study.

2.3 Materials and Methods

2.3.1 Animals

All animal work took place at the Southwest Foundation for Biomedical Research (SFBR) located in San Antonio, TX. Animal protocols were approved by the SFBR and Cornell University Institutional Animal Care and Use Committee (IACUC). Animal characteristics are summarized in Table 2.1. Fourteen pregnant baboons delivered spontaneously around 182 days gestation. Neonates were transferred to the nursery within 24 hours of birth and randomized to one of three diet groups. Animals were housed in enclosed incubators until 2 weeks of age and then moved to individual stainless steel cages in a controlled access nursery. Room temperatures were maintained at 76ºF -82ºF, with a 12 hour light/dark cycle.

2.3.2 Diets

Animals were assigned one of the following formulas, C: Control, LCPUFA-free; L: 0.32% DHA/0.64% ARA; L3: 0.96% DHA/0.64% ARA. C and L are the commercially available human infant formulas Enfamil® and Enfamil® LIPIL®, respectively.

Formula nutrient information for Enfamil® LIPIL® is summarized in Table 2.2. Formulas were kindly provided by Mead-Johnson Nutritionals (Evansville, IN) in ready-to-feed form, in color-coded cans. Each diet was color-coded to two different colors to further mask investigators. Animals were offered 1 ounce of formula four times daily at 07:00, 10:00, 13:00 and 16:00 with an additional feed during the first 2 nights. On day 3 and beyond, neonates were offered 4 ounces total; when they

consumed the entire amount, the amount offered was increased in daily 2 ounce increments. Neonates were hand fed for the first 7-10 days until independent feeding was established. Formula consumption was recorded throughout the 12 weeks of life.

Table 2.1 Characteristics of baboon neonate groups.

Number of animals (n)	14
Gender	10F, 4M
Conceptional age at delivery (d)	181.8 ± 6.2
Birth weight (g)	860.3 ± 150.8
Weight at 12 weeks (g)	1519.1 ± 280.7
Weight gain (g)	658.8 ± 190.4

Data expressed as mean ± SD.

2.3.3 Growth

Neonatal growth was assessed using body weight measurements, recorded two or three times weekly. Head circumference and crown-rump length data were obtained weekly for each animal. Organ weights were recorded at necropsy at 12 weeks.

2.3.4 Sampling

Animals were anesthetized, and euthanized by exsanguination at 84.57 ± 1.09 days. Blood was collected in EDTA-containing Vacutainer tubes, and RBCs and plasma were separated by centrifugation. Eyes and one hemisphere of the brain were removed and immediately dissected. Retinas were placed in sterile saline. Organs were weighed and all samples were flash frozen in liquid nitrogen. Samples were

transferred and stored in a -80ºC freezer until they were shipped to Cornell University (Ithaca, NY) for analysis.

Table 2.2 Nutrient composition of Enfamil® LIPIL® with iron (per 100 calories).

Nutrient	Unit	Amount
Protein	g	2.1
Fat	g	5.3
Carbohydrate	g	10.9
Water	g	134
Linoleic acid	mg	860
Vitamins		
A	IU	300
D	IU	60
E	IU	2
K	µg	8
Thiamin (Vitamin B1)	µg	80
Riboflavin (Vitamin B2)	µg	140
B6	µg	60
B12	µg	0.3
Niacin	µg	1000
Folic acid (Folacin)	µg	16
Pantothenic acid	µg	500
Biotin	µg	3
C (Asorbic acid)	mg	12
Choline	mg	12
Inositol	mg	6
Minerals		
Calcium	mg	78
Phosphorus	mg	53
Magnesium	mg	8
Iron	mg	1.8
Zinc	mg	1
Manganese	µg	15
Copper	µg	75
Iodine	µg	10
Selenium	µg	2.8
Sodium	mg	27
Potassium	mg	108
Chloride	mg	63

2.3.5 Fatty Acid Analyses

Total lipids were extracted from tissue homogenates using the Bligh and Dyer method (Bligh and Dyer 1959), modified to ensure a single phase solvent extraction mixture. Fatty acid methyl esters (FAME) were prepared using sodium hydroxide and 14% boron-trifluoride (BF_3) in methanol. FAME were analyzed by gas chromatography (Hewlett Packard 5890 GC-flame ionization detector; BPX 70 column, SGE, Austin, TX), using H_2 carrier gas as described in detail previously (Sarkadi-Nagy, Wijendran et al. 2003). Fatty acid (FA) identities were determined by covalent adduct chemical ionization tandem mass spectrometry (Van Pelt and Brenna 1999) and then quantified using methyl heptadecanoate as an internal standard and response factors derived from an equal weight FAME mixture. FA concentrations are expressed as percent weight of total fatty acids from 14 to 24 carbons. Saturates and monounsaturates are presented in summary form in the Tables.

2.3.6 Statistics

Data are expressed as mean ± SD. Statistical analysis was conducted using analysis of variance (ANOVA) to test the hypothesis of equivalent means for measures taken at 12 weeks, and Tukey's correction was used to control for multiple comparisons. Formula consumption, body weight, head circumference, and crown-rump length changes over time were tested with a random coefficient regression model to compare LCPUFA groups (L, L3) to control (C). Analyses were performed using SAS for Windows 9.1 (SAS Institute, Cary, NC) with significance declared at $p<0.05$.

2.4 Results

2.4.1 Formula Consumption and Growth

Figure 2.1A shows formula consumption throughout the 12 weeks; there were no significant differences between LCPUFA groups and the C group over time ($p=0.64$). Similarly, no significant changes over time were found for body weight ($p=0.47$), head circumference ($p=0.68$) or crown-rump length ($p=0.38$) (data not shown). Figure 2.1B shows that there were no significant differences in the 12 week data for these anthropometry measures. Figure 2.1C shows that there were no significant differences and no trends in the 12 week organ weights, expressed as a percent of body weight (BW), for brain, liver, thymus, spleen, heart, lungs, the right kidney, or the pancreas.

2.4.2 RBC and plasma fatty acids

Table 2.3 and Figure 2.1 summarize FA profiles for RBC and whole plasma. Supplementation significantly elevated RBC DHA for L and L3 groups by 3.8- and 4.6-fold, compared to controls. A similar trend was observed in plasma, DHA increased by 4.6- and 7.5- fold for the LCPUFA supplemented groups, L and L3. While ARA significantly increased from C to L for RBC, ARA levels declined from the L to the L3 group. A consistent but non-significant trend is present for ARA plasma concentrations, with a moderate increase from C (5.36 ± 1.00) to L (10.06 ± 0.99) and an intermediate level in L3 (7.79 ± 0.84). The ARA elongation product adrenic acid (22:4n-6) is a minor component but did respond to diets in both RBC and plasma, where it decreased significantly in the L3 group compared to the C and L groups. 22:5n-6 concentrations were significantly higher in RBC of controls. 22:5n-3 levels were higher in the C group compared to the L and L3 groups in both RBC and plasma measurements. The 22:5n-6/DHA ratio was significantly greater for control and L animals compared to the L3 group, approximately by 4- and 10-fold.

2.4.3 Liver and Heart Fatty Acids

FA composition of whole liver is presented in Table 3, and results for DHA, ARA, and the ratio of 22:5n-6/DHA are shown graphically in Figure 2.1. Increasing formula DHA significantly elevated liver DHA concentrations; the L and L3 groups had 2.2 and 3.6-fold more DHA than the unsupplemented C group, respectively. In contrast to DHA, dietary ARA increased liver levels in L group while significantly lowering ARA 14.3% from the L to L3 group. Adrenic acid concentrations were significantly higher in the C group (0.99 ± 0.13%) relative to L and L3. A similar, but non-significant trend was observed for 22:5n-6, levels were highest in unsupplemented animals, followed by the L and L3 groups. 22:5n-3 concentrations dropped 2-fold for LCPUFA supplemented animals compared to controls. The pentaene to hexaene ratio, 22:5n-6/DHA, a measure of n-3 fatty acid sufficiency, was significantly elevated for the C and L groups compared to L3 by 4.6 and 14 fold. Increases in LCPUFA were compensated by decreases in total monounsaturates (MUFA) and 18:2n-6, but not total saturated fatty acids (SFA).

As with the liver, heart DHA increased in the L and L3 groups, 2.8 and 3.9 fold, respectively, while 22:5n-3 dropped significantly. The increases in DHA appear to be at the expense of SFA, though the decrease in SFA from C to L to L3 did not reach statistical significance. 18:2n-6 decreased from C to L but L and L3 were not different.

2.4.4 Retina Fatty Acids

Table 2.4 and Figure 2.1 depict retinal FA composition. Changes in retinal DHA due to dietary LCPUFA did not reach significance, though the L and L3 group means were greater than the C group by amounts similar to our previous report (Sarkadi-Nagy, Wijendran et al. 2003). ARA concentrations were not influenced by formula composition. Non-significant changes in adrenic acid were observed, with

highest levels in the C group, followed by L and L3. 22:5n-6 concentrations were significantly higher in controls compared to the highest supplemented group, L3. Levels of 22:5n-3 increased with dietary LCPUFA, with L3 significantly elevated compared to the C group. The 22:5n-6/DHA index for C and L groups were 3.6-fold higher than the high DHA formula group, L3.

2.4.5 CNS Fatty Acids

CNS FA concentrations are summarized in Tables 2.4-2.6 and Figure 2.1. DHA concentrations significantly increased with higher levels of formula DHA in the cerebral cortex precentral gyrus, the primary motor cortex region. Supplementation improved DHA levels by 24% and 43% compared to controls in the L and L3 groups, respectively, and the difference between L and L3 was statistically significant. LCPUFA supplementation also significantly increased DHA in frontal cortex by 30% and 41% in the L and L3 groups, respectively, compared to controls, however the difference between L and L3 was borderline significant ($p=0.10$).

Formula DHA increased DHA in the basal ganglia regions globus pallidus and caudate, and in the midbrain regions superior colliculus and inferior colliculus, however there were no detectable differences between L and L3 groups. The non-significant trends in putamen and amygdala were consistent with this pattern. In only the globus pallidus was the non-significant difference in L and L3 DHA of potential biological importance (11%); in the other tissues, DHA increased by less than 4% or decreased slightly. 22:5n-6 decreased significantly and consistently from C to L to L3 in all CNS regions.

With the exception of two CNS regions, dietary manipulation had little influence on ARA levels. Levels of ARA in globus pallidus and superior colliculus were highest in the L formula group, but significantly declined 10% with additional formula DHA.

Adrenic acid (22:4n-6) levels were significantly elevated in the C group compared to the L3 group in the precentral and frontal lobes, globus pallidus, caudate, and superior colliculus, and the L group consistently was of intermediate level and not significantly different from the other groups. Putamen and inferior colliculus showed a similar but non-significant trend, and amygdala showed no trend.

Significant changes in 22:5n-3 concentrations were evident in the frontal cortex, globus pallidus, caudate, putamen, superior colliculus, inferior colliculus and amygdala. For all regions except the superior and inferior colliculi, 22:5n-3 decreased from C to L, but improved slightly with higher amounts of formula DHA. A similar but non-significant trend was observed in the precentral gyrus. 22:5n-3 levels in the superior and inferior colliculi significantly increased in the L3 group compared to C and L.

Similar results for n-3 sufficiency indices were obtained in all brain regions. The 22:5n-6/DHA ratio was significantly elevated for C and L groups compared to the high formula DHA group, L3, in all CNS regions. C and L groups were consistently elevated by 2- to 5-fold, respectively, compared with the L3 group.

Table 2.3 Liver, RBC, plasma and heart fatty acid composition (%w/w; mean ± SD).

Liver

	C	L	L3
Σ SFA	41.46 ± 0.65	42.38 ± 0.93	41.96 ± 0.53
Σ MUFA	18.04 ± 1.37	16.51 ± 2.13	14.83 ± 2.09
18:2n-6	15.56 ± 0.20[a]	13.43 ± 0.54[b]	12.83 ± 0.78[b]
ARA	13.28 ± 1.12[a,b]	14.52 ± 1.02[a]	12.45 ± 0.88[b]
22:4n-6	0.99 ± 0.13[a]	0.68 ± 0.08[b]	0.32 ± 0.09[c]
22:5n-6	0.45 ± 0.37	0.29 ± 0.21	0.10 ± 0.04
Σ n-6	33.72 ± 0.75[a]	30.77 ± 1.90[b]	27.55 ± 1.43[c]
18:3n-3	0.43 ± 0.09	0.44 ± 0.07	0.58 ± 0.14
22:5n-3	1.32 ± 0.10[a]	0.64 ± 0.17[b]	0.61 ± 0.14[b]
DHA	3.81 ± 0.85[a]	8.28 ± 0.78[b]	13.53 ± 1.21[c]
Σ n-3	6.22 ± 0.96[a]	9.85 ± 1.05[b]	15.16 ± 1.09[c]
22:5 n-6/DHA	0.14 ± 0.13	0.04 ± 0.03	0.01 ± 0.00

RBC

	C	L	L3
Σ SFA	42.74 ± 1.93	42.59 ± 1.45	43.70 ± 3.69
Σ MUFA	19.65 ± 0.88	18.05 ± 1.43	18.35 ± 1.41
18:2n-6	15.67 ± 0.53[a]	11.15 ± 0.98[b]	12.35 ± 0.77[b]
ARA	13.82 ± 1.72[a]	17.55 ± 2.05[b]	15.41 ± 1.40[a,b]
22:4n-6	2.06 ± 0.34[a]	2.08 ± 0.33[a,b]	1.14 ± 0.24[c]
22:5n-6	0.64 ± 0.20[a]	0.49 ± 0.09[a,b]	0.31 ± 0.10[b]
Σ n-6	34.69 ± 2.60[a]	33.32 ± 2.39[a,b]	30.84 ± 1.40[b]
18:3n-3	0.27 ± 0.05	0.31 ± 0.14	0.21 ± 0.05
22:5n-3	1.16 ± 0.17[a]	0.81 ± 0.12[b]	0.66 ± 0.10[b,c]
DHA	1.59 ± 0.49[a]	6.17 ± 0.82[b]	7.31 ± 1.92[b]
Σ n-3	2.74 ± 0.59[a]	6.98 ± 0.85[b]	7.96 ± 1.89[b]
22:5 n-6/DHA	0.35 ± 0.05[a]	0.08 ± 0.01[b]	0.04 ± 0.01[b]

Table 2.3 (continued)

Plasma

	C			L			L3		
Σ SFA	50.67	±	2.28	50.21	±	2.68	50.47	±	2.71
Σ MUFA	20.01	±	2.22[a]	14.32	±	1.16[b]	15.00	±	1.66[b]
18:2n-6	17.95	±	0.86[a]	16.10	±	1.37[a,b]	15.82	±	0.98[a]
ARA	5.36	±	1.00[a]	10.06	±	0.99[b]	7.79	±	0.84[c]
22:4n-6	0.40	±	0.14[a]	0.38	±	0.14[a]	0.14	±	0.09[b]
22:5n-6	0.18	±	0.03	0.14	±	0.03	0.14	±	0.02
Σ n-6	26.88	±	1.65	28.71	±	1.77	25.59	±	1.88
18:3n-3	0.41	±	0.08	0.31	±	0.04	0.39	±	0.06
22:5n-3	0.74	±	0.10[a]	0.39	±	0.05[b]	0.37	±	0.05[b]
DHA	0.83	±	0.17[a]	3.78	±	0.36[b]	6.24	±	0.84[c]
Σ n-3	1.57	±	0.25[a]	4.18	±	0.40[b]	6.61	±	0.86[c]
22:5n-6/DHA	0.22	±	0.05[a]	0.04	±	0.01[b]	0.02	±	0.00[b]

Heart

	C			L			L3		
Σ SFA	63.88	±	1.11	59.35	±	2.55	53.44	±	1.55
Σ MUFA	30.59	±	3.64	31.74	±	2.04	42.10	±	3.91
18:2n-6	19.12	±	3.08[a]	12.64	±	1.63[b]	13.01	±	0.85[b]
ARA	16.57	±	3.42	18.96	±	2.84	17.96	±	3.07
22:4n-6	0.52	±	0.41	0.54	±	0.29	0.38	±	0.11
22:5n-6	0.65	±	0.32	0.45	±	0.17	0.34	±	0.21
Σ n-6	37.75	±	2.51[a]	33.29	±	1.48[b]	32.44	±	2.46[b]
18:3n-3	0.39	±	0.11	0.40	±	0.13	0.50	±	0.15
22:5n-3	1.48	±	0.22[a]	0.59	±	0.23[b]	0.49	±	0.14[b]
DHA	2.12	±	0.40[a]	5.91	±	0.91[b]	8.28	±	1.12[c]
Σ n-3	38.21	±	0.78[a]	41.98	±	0.81[b]	41.56	±	1.08[c]
22:5n-6/DHA	0.31	±	0.14[a]	0.08	±	0.04[b]	0.04	±	0.03[b]

Table 2.4 Cerebral cortex precentral gyrus and frontal lobes and retina fatty acid compositions (%w/w; mean ± SD).

Cerebral cortex, precentral gyrus

	C			L			L3		
Σ SFA	45.83	±	1.05	46.09	±	1.00	45.03	±	0.32
Σ MUFA	17.90	±	0.52	17.22	±	0.46	17.71	±	0.45
18:2n-6	1.30	±	0.07[a]	1.09	±	0.06[b]	1.20	±	0.10[a,b]
ARA	11.32	±	0.52	11.49	±	0.53	11.19	±	0.18
22:4n-6	6.15	±	0.51[a]	6.02	±	0.21[a,b]	5.39	±	0.37[b]
22:5n-6	3.21	±	0.52[a]	1.64	±	0.24[b]	0.88	±	0.07[c]
Σ n-6	23.60	±	1.42[a]	21.50	±	0.83[b]	20.01	±	0.45[b,c]
22:5n-3	0.55	±	0.08	0.42	±	0.18	0.46	±	0.02
DHA	11.27	±	0.78[a]	13.97	±	0.66[b]	16.11	±	0.73[c]
Σ n-3	11.82	±	0.73[a]	14.39	±	0.50[b]	16.57	±	0.72[c]
22:5n-6/DHA	0.29	±	0.06[a]	0.12	±	0.01[b]	0.05	±	0.01[b]

Cerebral cortex, frontal lobe

	C			L			L3		
Σ SFA	44.33	±	0.74	44.30	±	0.67	42.96	±	2.98
Σ MUFA	15.48	±	1.31	16.04	±	0.62	16.38	±	1.06
18:2n-6	1.25	±	0.06	1.08	±	0.17	1.93	±	1.84
ARA	11.57	±	0.62	11.19	±	0.77	10.67	±	0.64
22:4n-6	6.51	±	0.59[a]	6.11	±	0.52[a,b]	5.58	±	0.14[b]
22:5n-6	3.22	±	0.40[a]	1.75	±	0.16[b]	0.93	±	0.11[c]
Σ n-6	14.14	±	0.61[a]	11.15	±	0.23[b]	10.96	±	1.75[b,c]
22:5n-3	0.53	±	0.04[a]	0.32	±	0.01[b]	0.42	±	0.04[c]
DHA	9.86	±	0.42[a]	12.80	±	1.30[b]	13.89	±	0.95[b]
Σ n-3	10.39	±	0.40[a]	13.12	±	1.30[b]	14.31	±	0.96[b]
22:5 n-6/DHA	0.33	±	0.05[a]	0.14	±	0.02[b]	0.07	±	0.01[c]

Retina

	C			L			L3		
Σ SFA	45.61	±	0.81	46.41	±	3.08	46.68	±	3.37
Σ MUFA	16.63	±	0.49	15.57	±	0.50	15.87	±	0.88
18:2n-6	2.78	±	0.21	2.63	±	0.29	2.39	±	0.20
ARA	9.99	±	0.76	9.76	±	0.88	9.09	±	1.37
22:4n-6	1.89	±	0.49	1.34	±	0.17	1.25	±	0.38
22:5n-6	1.82	±	0.96[a]	0.51	±	0.03[a,b]	0.55	±	0.48[b]
Σ n-6	18.27	±	2.19	15.78	±	1.30	14.74	±	2.20
18:3n-3	0.22	±	0.10	0.26	±	0.08	0.25	±	0.11
22:5n-3	0.86	±	0.09[a]	0.98	±	0.10[a,b]	1.27	±	0.25[b]
DHA	17.44	±	2.28	20.04	±	1.50	20.12	±	2.23
Σ n-3	18.30	±	2.37	21.02	±	1.53	21.39	±	2.33
22:5 n-6/DHA	0.11	±	0.06[a]	0.03	±	0.00[b]	0.03	±	0.03[b]

Table 2.5 Basal ganglia (globus pallidus, putamen, caudate) and amygdala (%, w/w; mean ± SD).

Globus pallidus

	C			L			L3		
Σ SFA	40.83	±	0.42	41.89	±	0.95	40.74	±	0.71
Σ MUFA	18.38	±	1.11	17.46	±	0.56	18.65	±	0.82
18:2n-6	1.09	±	0.05[a]	0.97	±	0.03[b]	1.06	±	0.07[a,b]
ARA	9.84	±	0.61[a,b]	10.26	±	0.63[a]	9.14	±	0.19[b]
22:4n-6	5.90	±	0.29[a]	5.64	±	0.25[a,b]	5.18	±	0.24[b]
22:5n-6	2.71	±	0.27[a]	1.44	±	0.21[b]	0.70	±	0.07[c]
Σ n-6	21.07	±	0.28[a]	19.48	±	0.82[b]	17.29	±	0.43[c]
22:5n-3	0.42	±	0.09[a,c]	0.27	±	0.02[b]	0.39	±	0.05[c]
DHA	11.19	±	1.41[a]	13.39	±	1.26[b]	14.82	±	0.83[b]
Σ n-3	11.61	±	1.32[a]	13.66	±	1.24[a,b]	15.21	±	0.86[b]
22:5n-6/DHA	0.25	±	0.04[a]	0.11	±	0.02[b]	0.05	±	0.01[c]

Putamen

	C			L			L3		
Σ SFA	44.38	±	1.59	43.71	±	2.29	43.96	±	2.83
Σ MUFA	17.60	±	1.36	17.23	±	0.78	18.31	±	3.39
18:2n-6	1.11	±	0.04[a]	1.00	±	0.06[b]	0.98	±	0.07[b]
ARA	10.87	±	0.93	10.30	±	0.77	9.99	±	1.52
22:4n-6	6.26	±	0.47	5.94	±	0.23	5.66	±	0.51
22:5n-6	2.85	±	0.35[a]	1.51	±	0.28[b]	0.81	±	0.18[c]
Σ n-6	22.59	±	1.89[a]	19.97	±	1.07[b]	18.87	±	0.99[b]
22:5n-3	0.44	±	0.06[a]	0.28	±	0.03[b]	0.42	±	0.07[a,c]
DHA*	10.03	±	1.71	12.84	±	1.55	12.19	±	2.88
Σ n-3	10.47	±	1.68	13.12	±	1.52	12.61	±	2.83
22:5n-6/DHA	0.29	±	0.08[a]	0.12	±	0.02[b]	0.07	±	0.00[b]

*Multiple comparison p=0.08; L vs. C, p<0.04.

Caudate

	C			L			L3		
Σ SFA	42.95	±	0.87	43.18	±	1.50	43.10	±	0.61
Σ MUFA	15.70	±	0.42	15.79	±	0.83	17.02	±	1.13
18:2n-6	1.11	±	0.08	1.00	±	0.10	1.03	±	0.06
ARA	11.64	±	0.30	11.40	±	0.79	10.77	±	0.38
22:4n-6	6.41	±	0.32[a]	6.14	±	0.39[a,b]	5.74	±	0.32[b]
22:5n-6	3.17	±	0.36[a]	1.74	±	0.12[b]	0.90	±	0.05[c]
Σ n-6	23.69	±	0.86[a]	21.27	±	1.04[b]	19.54	±	0.66[c]
22:5n-3	0.38	±	0.04[a]	0.25	±	0.04[b]	0.36	±	0.05[a,c]
DHA	11.46	±	0.95[a]	13.86	±	0.68[b]	14.41	±	1.48[b]
Σ n-3	11.85	±	0.92[a]	14.11	±	0.68[b]	14.77	±	1.45[b]
22:5n-6/DHA	0.28	±	0.05[a]	0.13	±	0.01[b]	0.06	±	0.01[c]

Table 2.5 (continued)

Amygdala

	C			L			L3		
Σ SFA	46.38	±	2.15	44.90	±	3.90	45.96	±	3.28
Σ MUFA	18.20	±	0.77	17.83	±	1.02	18.68	±	0.90
18:2n-6	1.00	±	0.09	0.86	±	0.06	0.97	±	0.16
ARA	10.67	±	1.36	11.23	±	0.52	10.01	±	0.35
22:4n-6	5.60	±	0.79	5.92	±	0.84	5.07	±	1.10
22:5n-6	2.08	±	0.21[a]	1.41	±	0.22[b]	0.85	±	0.08[c]
Σ n-6	20.82	±	1.93	20.59	±	1.44	18.28	±	1.35
22:5n-3	0.35	±	0.03[a]	0.28	±	0.01[b]	0.41	±	0.03[c]
DHA	7.36	±	1.59	9.55	±	1.57	9.09	±	0.93
Σ n-3	7.71	±	1.61	9.84	±	1.57	9.50	±	0.95
22:5n-6/DHA	0.29	±	0.06[a]	0.15	±	0.01[b]	0.09	±	0.02[b]

Table 2.6 Superior and inferior colliculi fatty acid composition (%, w/w; mean ± SD).

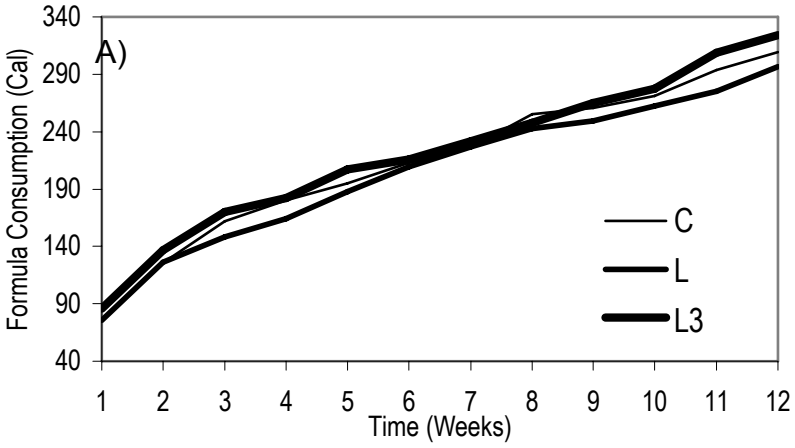
Superior Colliculus

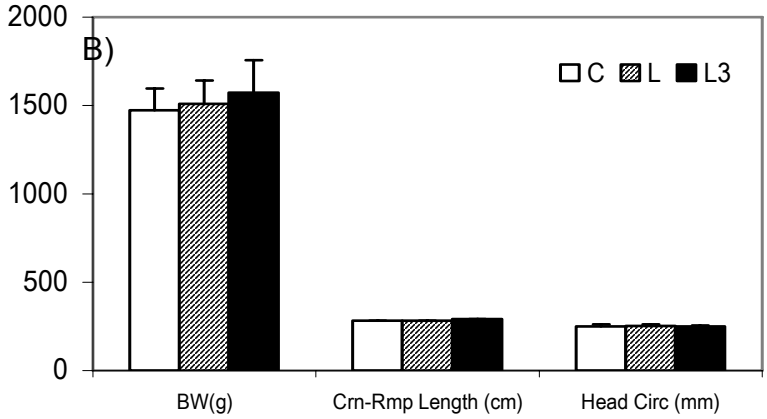
	C			L			L3		
Σ SFA	39.17	±	3.01	39.91	±	3.72	37.88	±	1.08
Σ MUFA	22.56	±	0.72	22.40	±	0.80	23.39	±	0.35
18:2n-6	1.02	±	0.08	0.93	±	0.03	1.02	±	0.08
ARA	8.95	±	0.28^{a}	8.96	±	0.19^{a}	8.22	±	0.22^{b}
22:4n-6	6.28	±	0.17^{a}	6.01	±	$0.28^{a,b}$	5.70	±	0.36^{b}
22:5n-6	1.65	±	0.34^{a}	0.84	±	0.11^{b}	0.48	±	$0.02^{b,c}$
Σ n-6	19.51	±	0.71^{a}	18.03	±	0.38^{b}	16.89	±	0.46^{c}
22:5n-3	0.36	±	0.06^{a}	0.35	±	0.07^{a}	0.50	±	0.05^{b}
DHA	10.01	±	0.56^{a}	11.36	±	0.51^{b}	11.27	±	0.34^{b}
Σ n-3	10.38	±	0.54^{a}	11.71	±	0.54^{b}	11.77	±	0.35^{b}
22:5n-6/DHA	0.16	±	0.03^{a}	0.07	±	0.01^{b}	0.04	±	0.00^{b}

Inferior Colliculus

	C			L			L3		
Σ SFA	39.52	±	3.49	40.52	±	3.07	37.85	±	1.49
Σ MUFA	23.20	±	$0.91^{a,b}$	22.70	±	0.79^{a}	24.07	±	0.47^{b}
18:2n-6	1.12	±	0.12	0.94	±	0.05	1.11	±	0.17
ARA	8.50	±	0.77	8.76	±	0.35	7.87	±	0.32
22:4n-6	6.13	±	0.15	5.98	±	0.25	5.61	±	0.43
22:5n-6	1.47	±	0.23^{a}	0.81	±	0.14^{b}	0.47	±	0.04^{c}
Σ n-6	18.98	±	0.67^{a}	17.86	±	0.28^{b}	16.60	±	0.59^{c}
22:5n-3	0.46	±	$0.13^{a,b}$	0.35	±	0.05^{a}	0.54	±	0.07^{b}
DHA	9.81	±	1.16^{a}	11.38	±	0.52^{b}	11.49	±	0.48^{b}
Σ n-3	10.26	±	1.09^{a}	11.72	±	0.53^{b}	12.03	±	0.53^{b}
22:5n-6/DHA	0.15	±	0.02^{a}	0.07	±	0.01^{b}	0.04	±	0.00^{c}

Figure 2.1. A) Formula consumption, expressed as calories consumed per day, B) 12 week anthropometry measurements and C) organ weights as a percent of body weights at 12 weeks of age for baboon neonates on the 3 experimental formulas. There were no significant differences among the groups in consumption over time and no differences in 12 week growth measurements or organ weights.


A)
Formula Consumption (Cal)
340
290
240
190
140
90
40
1
2
3
4
5
6
7
8
9
10
11
12
Time (Weeks)
C
L
L3


B)
2000
1500
1000
500
0
C
L
L3
BW(g)
Crn-Rmp Length (cm)
Head Circ (mm)

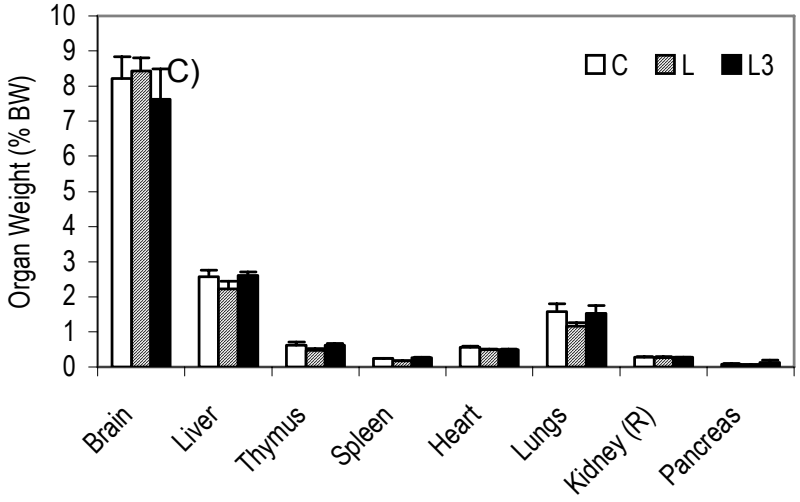
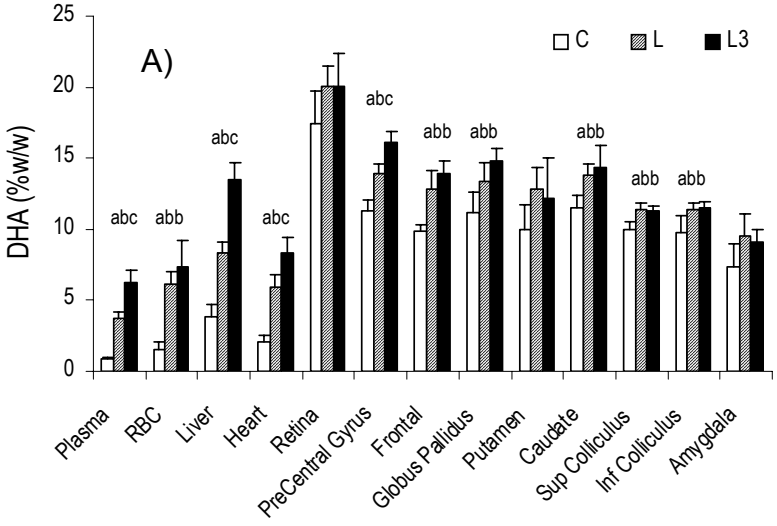
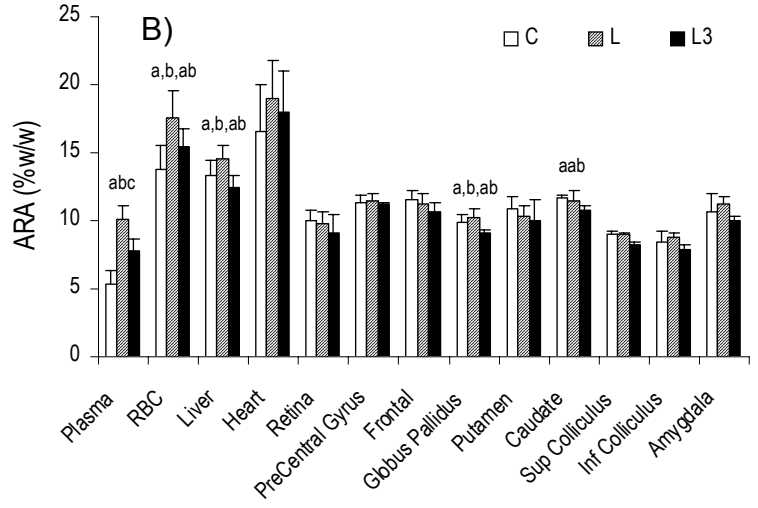
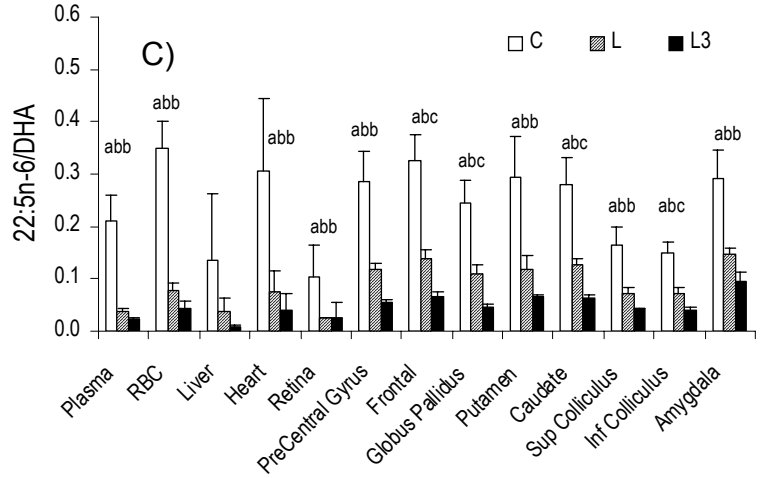

C)
Organ Weight (% BW)
10
9
8
7
6
5
4
3
2
1
0
C
L
L3
Brain
Liver
Thymus
Spleen
Heart
Lungs
Kidney (R)
Pancreas

Figure 2.2. Baboon neonate FA concentrations at 12 weeks of age for A) DHA, B) ARA and C) [22:5n-6]/[DHA].


A)
C
L
L3
DHA (%w/w)
25
20
15
10
5
0
abc
abb
abc
abc
abc
abb
abb
abb
abb
Plasma
RBC
Liver
Heart
Retina
PreCentral Gyrus
Frontal
Globus Pallidus
Putamen
Caudate
Sup Colliculus
Inf Colliculus
Amygdala


B)
C
L
L3
ARA (%w/w)
25
20
15
10
5
0
abc
a,b,ab
a,b,ab
a,b,ab
aab
Plasma
RBC
Liver
Heart
Retina
PreCentral Gyrus
Frontal
Globus Pallidus
Putamen
Caudate
Sup Colliculus
Inf Colliculus
Amygdala


C)
C
L
L3
22:5n-6/DHA
0.6
0.5
0.4
0.3
0.2
0.1
0.0
abb
abb
abb
abb
abb
abc
abc
abb
abc
abb
abc
abb
Plasma
RBC
Liver
Heart
Retina
PreCentral Gyrus
Frontal
Globus Pallidus
Putamen
Caudate
Sup Colliculus
Inf Colliculus
Amygdala

2.5 Discussion

We have published several studies on tissue compositional changes that accompany DHA and ARA dietary supplementation in perinatal baboons (Su, Bernardo et al. 1999; Su, Corso et al. 1999; Su, Huang et al. 2001; Sarkadi-Nagy, Wijendran et al. 2003; Diau, Hsieh et al. 2005). The present data are in most cases consistent with our previous findings and extend them to formulas with higher levels of DHA. Increasing DHA from 0 (C) to 0.32% (L) increases DHA in all tissues studied, consistent with previous observations in 4 week old baboons (Diau, Hsieh et al. 2005), though the increases in retina, putamen, and amygdala did not reach statistical significance in the present study.

We previously showed that the response of individual CNS regions to dietary DHA was dichotomous. Dietary DHA at 0.3%,w/w normalized tissue DHA to levels found in breastfed neonates for all regions of the CNS except for the lobes of the cerebral cortex, where DHA increased compared to controls but was 87% to 90% of breastfed levels (Diau, Hsieh et al. 2005). A reasonable hypothesis is that higher DHA levels might further increase cortex DHA to breastfed levels. Our present data show that precentral gyrus DHA increased by 24% from C to L, and 43% from C to L3. The additional increase from L to L3 of 19% was statistically significant, indicating that the greater DHA in the L3 formula was effective at increasing precentral gyrus DHA. Although we did not have a breastfed control group in this study, the magnitude of the increase is similar to the enhancement associated with the breastfed vs. term comparison. We note also that the magnitude of the precentral gyrus DHA increase was less than two-fold, while the amount of DHA in the diet is triple between L and L3. This observation indicates that the leveling off of tissue fatty acid concentrations in response to increases in dietary fatty acids, demonstrated in rats

(Lands, Morris et al. 1990), is achieved in primate brain at dietary DHA levels similar to the highest reported breastmilk levels.

Human (Lauritzen, Hansen et al. 2001) and baboon breastmilks (Sarkadi-Nagy, Wijendran et al. 2004) contain the n-3 LCPUFA EPA and DPA at concentrations that are a substantive fraction of the DHA concentration. In adults, these LCPUFA are much more efficiently converted to DHA than 18:3n-3 (Pawlosky, Hibbeln et al. 2001). U.S. infant formulas contain negligible amounts of EPA and n-3 DPA because the source of n-3 LCPUFA, oil from the marine algae *Crypthecodinium cohnii*, does not contain these LCPUFA. Higher DHA levels than currently in formulas, and more similar to our L3 formula, may be indicated to make up for these minor n-3 LCPUFA. Indeed, we find that n-3 DPA drops in most tissue in response to moderate DHA but rebounds at the L3 DHA level. The exception is retina in which n-3 DPA increases as DHA increases. EPA is at trace levels in the CNS.

The consensus that ARA supplementation is necessary whenever DHA is supplemented was based on early studies showing that premature infants grow more slowly on formula (Colombo, Kannass et al. 2004), and a report indicating that dietary DHA is more effective when accompanied by ARA (Birch, Garfield et al. 2000). In liver, RBC and plasma, ARA rises significantly in the L group and then achieves an intermediate value in the L3 group; an equivalent but non-significant pattern was found for heart. Our present results are consistent with previous data (Diau, Hsieh et al. 2005) indicating that tissue ARA concentrations, particularly in the CNS are more refractory to formula ARA than DHA. No changes were found in the cerebral cortex, retina, putamen, caudate, and amygdala. However, L3 group ARA was reduced compared to control in the superior colliculus and compared to L in the globus pallidus. When a difference in ARA concentrations was evident, our previous data

usually showed that tissue ARA was lower in groups consuming no ARA compared to a breastfed group (Diau, Hsieh et al. 2005).

n-6 DPA (Osbond acid) is an elongation and 4-5 desaturated product of ARA that consistently rises in experimental n-3 fatty acid deficiency (Lim, Hoshiba et al. 2005), and also drops in response to DHA supplementation in otherwise normal primates (Sarkadi-Nagy, Wijendran et al. 2003). n-6 DPA dropped in all tissues with increasing DHA, and in some tissues such as the cerebral cortex L3 n-3 DPA values were a fraction of the C values. This decrease and the accompanying increase in DHA drove the DPA/DHA ratio decrease from the L to L3 groups. We have previously shown that DPA replacement with DHA results in improved retinal function directly in premature neonate baboons (Sarkadi-Nagy, Wijendran et al. 2003), and dietary supply of DHA has been linked to improve retinal function in numerous human infant studies (Hoffman, Theuer et al. 2004). Cognition also improves in response to DHA and ARA supplementation (Willatts and Forsyth 2000). Whether increased DHA results in enhancement of function sufficient to measure awaits studies directly in humans.

Adrenic acid (22:4n-6) is the immediate elongation product of ARA, and is considered to be an intermediate in the pathway to 22:5n-6. Human cortex 22:4n-6 has recently been shown to be more tightly correlated (r=-0.89) to DHA decline than n-6 DPA (Pamplona, Dalfo et al. 2005). A substantial fraction, about 17%, of 22:4n-6 recovered in 4 week old baboon cortex was from dietary ARA (Wijendran, Lawrence et al. 2002). The order of 22:4n-6 concentrations in all but one tissue was C>L>L3, and in all but two tissues the C group was significantly elevated compared to the L3 group. These changes were therefore parallel but apparently milder than 22:5n-6, the desaturation product of 22:4n-6. Both these fatty acids are usually viewed in the context of DHA sufficiency or as substrates for oxidation, and neither are well studied

as substrates for conversion to bioactive products, as are the C20 LCPUFA (ARA and 20:5n-3) or DHA in recent studies (e.g., (Serhan, Gotlinger et al. 2006)). Currently, there is no known molecular role for 22:4n-6 or 22:5n-6 apart from bulk biophysical properties in membranes and in association with membrane proteins (Feller and Gawrisch 2005; Grossfield, Feller et al. 2006), that may be associated with structural similarities to DHA.

2.6 Conclusion

These results confirm previous observations indicating that DHA is more sensitive to dietary manipulations than ARA in most tissues. They extend our previous conclusions by showing that cerebral cortex DHA increases with higher concentrations of DHA than are included in present commercial formulas, while not increasing the levels of DHA in basal ganglia and limbic system. These data provide support for the hypothesis that formula DHA at concentrations higher than presently used in formulas, but nevertheless well within the known range of human breastmilk, normalizes CNS tissue composition closer to that of breastfeeding. Changes in tissue composition by themselves do not justify alteration of diet composition, and should be coupled to demonstrations of efficacy associated with improvements in functional outcomes. The enhanced DHA in the primary motor cortex (precentral gyrus) may help explain enhanced motor maturity seen in one month old rhesus neonates fed on a similar level of DHA (Champoux, Hibbeln et al. 2002), and a correlation of plasma phospholipid DHA with gross motor developmental quotient in four month old human infants (Jensen, Voigt et al. 2005). Studies of enhanced motor function linked to DHA consumption in humans are warranted.

2.7 Acknowledgments

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2.8 References

Bligh, E. G. and W. J. Dyer (1959). "A rapid method for total lipid extraction and purification." Can J Biochem Physiol **37**(8): 911-7.

Carlson, S. E., S. H. Werkman, et al. (1993). "Arachidonic acid status correlates with first year growth in preterm infants." Proc Natl Acad Sci U S A **90**(3): 1073-7.

Champoux, M., J. R. Hibbeln, et al. (2002). "Fatty acid formula supplementation and neuromotor development in rhesus monkey neonates." Pediatr Res **51**(3): 273-81.

Clandinin, M. T., J. E. Van Aerde, et al. (2005). "Growth and development of preterm infants fed infant formulas containing docosahexaenoic acid and arachidonic acid." J Pediatr **146**(4): 461-8.

Diau, G.-Y., A. T. Hsieh, et al. (2005). "The influence of long chain polyunsaturate supplementation on docosahexaenoic acid and arachidonic acid in baboon neonate central nervous system." BMC Med **3**: 11.

Diau, G. Y., E. R. Loew, et al. (2003). "Docosahexaenoic and arachidonic acid influence on preterm baboon retinal composition and function." Invest Ophthalmol Vis Sci **44**(10): 4559-66.

Feller, S. E. and K. Gawrisch (2005). "Properties of docosahexaenoic-acid-containing lipids and their influence on the function of rhodopsin." Curr Opin Struct Biol **15**(4): 416-22.

Gibson, R. A., W. Chen, et al. (2001). "Randomized trials with polyunsaturated fatty acid interventions in preterm and term infants: functional and clinical outcomes." Lipids **36**(9): 873-83.

Grossfield, A., S. E. Feller, et al. (2006). "A role for direct interactions in the modulation of rhodopsin by {omega}-3 polyunsaturated lipids." Proc Natl Acad Sci U S A **103**(13): 4888-93.

Hoffman, D. R., R. C. Theuer, et al. (2004). "Maturation of visual acuity is accelerated in breast-fed term infants fed baby food containing DHA-enriched egg yolk." J Nutr **134**(9): 2307-13.

Hsieh, A. T. (2006). From Consumer Helpline, February.

Innis, S. M. (2004). "Polyunsaturated fatty acids in human milk: an essential role in infant development." Adv Exp Med Biol **554**: 27-43.

Jeffrey, B. G., H. S. Weisinger, et al. (2001). "The role of docosahexaenoic acid in retinal function." Lipids **36**(9): 859-71.

Jensen, C. L., R. G. Voigt, et al. (2005). "Effects of maternal docosahexaenoic acid intake on visual function and neurodevelopment in breastfed term infants." Am J Clin Nutr **82**(1): 125-32.

Khedr, E. M. H., W. M. A. Farghaly, et al. (2004). "Neural maturation of breastfed and formula-fed infants." Acta Paediatr **93**(6): 734-8.

Koletzko, B., C. Agostoni, et al. (2001). "Long chain polyunsaturated fatty acids (LC-PUFA) and perinatal development." Acta Paediatr **90**(4): 460-4.

Kushwaha, R. S. and H. C. McGill, Jr. (1998). "Diet, plasma lipoproteins and experimental atherosclerosis in baboons (Papio sp.)." Hum Reprod Update **4**(4): 420-9.

Lands, W. E., A. Morris, et al. (1990). "Quantitative effects of dietary polyunsaturated fats on the composition of fatty acids in rat tissues." Lipids **25**(9): 505-16.

Lauritzen, L., H. S. Hansen, et al. (2001). "The essentiality of long chain n-3 fatty acids in relation to development and function of the brain and retina." Prog Lipid Res **40**(1-2): 1-94.

Pamplona, R., E. Dalfo, et al. (2005). "Proteins in human brain cortex are modified by oxidation, glycoxidation, and lipoxidation. Effects of Alzheimer disease and identification of lipoxidation targets." J Biol Chem **280**(22): 21522-30.

Pawlosky, R. J., J. R. Hibbeln, et al. (2001). "Physiological compartmental analysis of alpha-linolenic acid metabolism in adult humans." J Lipid Res **42**(8): 1257-65.

Salem, N., Jr., J. Loewke, et al. (2005). "Incomplete replacement of docosahexaenoic acid by n-6 docosapentaenoic acid in the rat retina after an n-3 fatty acid deficient diet." Exp Eye Res **81**(6): 655-63.

SanGiovanni, J. P., C. S. Berkey, et al. (2000). "Dietary essential fatty acids, long-chain polyunsaturated fatty acids, and visual resolution acuity in healthy fullterm infants: a systematic review." Early Hum Dev **57**(3): 165-88.

Sarkadi-Nagy, E., V. Wijendran, et al. (2004). "Formula feeding potentiates docosahexaenoic and arachidonic acid biosynthesis in term and preterm baboon neonates." J Lipid Res **45**(1): 71-80.

Sarkadi-Nagy, E., V. Wijendran, et al. (2003). "The influence of prematurity and long chain polyunsaturate supplementation in 4-week adjusted age baboon neonate brain and related tissues." Pediatr Res **54**(2): 244-52.

Serhan, C. N., K. Gotlinger, et al. (2006). "Anti-inflammatory actions of neuroprotectin D1/protectin D1 and its natural stereoisomers: assignments of dihydroxy-containing docosatrienes." J Immunol **176**(3): 1848-59.

Su, H. M., L. Bernardo, et al. (1999). "Bioequivalence of dietary alpha-linolenic and docosahexaenoic acids as sources of docosahexaenoate accretion in brain and associated organs of neonatal baboons." Pediatr Res **45**(1): 87-93.

Su, H. M., T. N. Corso, et al. (1999). "Linoleic acid kinetics and conversion to arachidonic acid in the pregnant and fetal baboon." J Lipid Res **40**(7): 1304-12.

Su, H. M., M. C. Huang, et al. (2001). "Fetal baboons convert 18:3n-3 to 22:6n-3 in vivo. A stable isotope tracer study." J Lipid Res **42**(4): 581-6.

Van Pelt, C. K. and J. T. Brenna (1999). "Acetonitrile chemical ionization tandem mass spectrometry to locate double bonds in polyunsaturated fatty acid methyl esters." Anal Chem **71**(10): 1981-9.

Wijendran, V., P. Lawrence, et al. (2002). "Significant utilization of dietary arachidonic acid is for brain adrenic acid in baboon neonates." J Lipid Res **43**(5): 762-7.

Willatts, P. and J. S. Forsyth (2000). "The role of long-chain polyunsaturated fatty acids in infant cognitive development." Prostaglandins Leukot Essent Fatty Acids **63**(1-2): 95-100.

CHAPTER 3

FORMULA DOCOSAHEXAENOIC ACID AND ARACHIDONIC ACID IMPROVES POSTNATAL HEMOGLOBIN AND RELATED INDICES IN TERM BABOON NEONATES

3.1 Abstract

Neonatal anemia is a physiologic condition characterized by a postnatal reduction in hemoglobin concentration. Adequate red blood cell (RBC) production and nutrition are special concerns for both term and preterm infants during this period of rapid growth and development. We evaluated the effects of medium and high levels of formula docosahexaenoic (DHA) and arachidonic acid (ARA) on hematological values in 14 term baboon (Papio cynocephalus) neonates. Methods: Term animals were randomized to 3 formula groups: C, Control, no DHA/ARA; L, containing 0.32% DHA/0.64% ARA; L3, containing with 0.96% DHA/0.64% ARA. All formulas had iron (1.8mg/100cal). Clinical hematology parameters were assessed at 2, 4, 8, 10 and 12 weeks of age. Results: All measured values were within accepted normal ranges for infant baboons. DHA/ARA consumption significantly elevated RBC, hematocrit, hemoglobin, and red blood cell distribution width (RDW) measurements at two weeks of life compared to the C group consuming no LCPUFA. The rate of decline of these parameters was greatest in the L3 and L groups compared to the C group, and by 12 weeks significant effects of DHA/ARA consumption were no longer detectable. Conclusion: These results provide the first indication that dietary DHA/ARA may enhance the oxygen carrying capacity of neonatal blood, and that dietary LCPUFA may alleviate the severity of neonatal anemia by buffering the precipitous decline in red cell indices during the first weeks of life.

3.2 Introduction

Docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (ARA, 20:4n-6) are long-chain polyunsaturated fatty acids (LCPUFA) that play a critical role in neurodevelopment during the perinatal period (Lauritzen, Hansen et al. 2001; Heird and Lapillonne 2005). LCPUFA are structural components of cell membranes and DHA is highly concentrated in brain grey matter and retina rod photoreceptors (Lauritzen, Hansen et al. 2001; Diau, Hsieh et al. 2005). The brain-growth spurt is a period of intense brain growth and rapid LCPUFA accretion that occurs during the last trimester of pregnancy and first year of life (Dobbing and Sands 1979; Rojas, Greiner et al. 2002). Both preterm and term infants have the ability to synthesize LCPUFA from shorter chain precursors, linoleic acid (LA, 18:2n-6) and α-linolenic acid (LNA, 18:3n-3), obtained in the diet (Carnielli, Wattimena et al. 1996; Salem, Wegher et al. 1996; Sauerwald, Hachey et al. 1996). However, endogenous conversion efficiency has been estimated at <1 % (Brenna 2002), indicating that preformed sources of LCPUFA may be necessary to meet the demands of developing neural tissues.

DHA and ARA occur naturally in breast milk, and about 80% of US infant formula now contains these LCPUFA since first permitted in 2002. Human breast milk LCPUFA concentrations are widely variable and reflective of maternal diet. Worldwide DHA breast milk content ranges from a low of 0.06% in urban populations to over 1% in predominantly fish eating populations, and approximately 0.4% to 0.6% total fatty acids for ARA (Nettleton 1995; Heird and Lapillonne 2005). US commercial infant formula LCPUFA concentrations also vary (DHA: 8-19 mg/100 kcal, ARA: 21-34 mg/100 kcal) and currently, insufficient evidence exists to firmly establish optimum levels required during neonatal development.

Most studies of n-3 nutriture during the perinatal period investigate visual and cognitive outcomes, and many studies have revealed functional deficits that may have

long term consequences (Neuringer, Connor et al. 1986; Yamamoto, Hashimoto et al. 1988; Niu, Mitchell et al. 2004; Anderson, Neuringer et al. 2005). Dietary DHA and ARA significantly alter fatty acid profiles in most tissues, with plasma and erythrocyte membranes generally used as indices of DHA/ARA status in human studies (Fleith and Clandinin 2005; Heird and Lapillonne 2005). In randomized controlled trials of term and preterm neonates consuming LCPUFA, improvements in visual acuity, neurobehavioral outcomes and language development were seen compared to controls consuming formula without LCPUFA (Gibson, Chen et al. 2001; Innis, Gilley et al. 2001; Fleith and Clandinin 2005; Heird and Lapillonne 2005). Long-term effects of LCPUFA have been observed in children with accelerated visual development at 1 and 3.5 years and improved cognitive abilities at 4 years of age (Williams, Birch et al. 2001; Helland, Smith et al. 2003; Hoffman, Theuer et al. 2004).

Anemia is a common problem during infancy and childhood worldwide. It is typically defined as a reduction in red cell mass or hemoglobin concentration. Clinical signs and symptoms include poor feeding, dyspnea, tachycardia, tachypnea, diminished activity, and pallor as infants struggle to compensate for inadequate oxygenation (Stockman 1986). Anemia is caused by blood loss, decreased RBC production, or increased red cell destruction (Nathan, Ginsburg et al. 2003). The "physiologic anemia of infancy" is a specific postnatal concern during early infancy where neonates tolerate remarkably low levels of hemoglobin without any other abnormalities. This temporary and expected fall in hemoglobin is not fully understood and results from a decrease in hematopoietic activity, red cell mass, and shortened RBC survival as infants adapt to complex changes in environmental oxygen tension and oxygen transport initiated at birth (Ohls 1998; Nathan, Ginsburg et al. 2003). Infants born with widely varying hemoglobin values reach similarly low values before

the natural onset of active erythropoiesis (Stockman 1986; Strauss 1995; Nathan, Ginsburg et al. 2003).

In the context of a study of high DHA formulas in neonate baboons (Hsieh, Anthony et al. 2006), we examined a wide array of hematological and clinical parameters to assess safety. We report here on significant and consistent findings that reveal ontogeny of hematological responses to increasing levels of formula DHA and ARA from 2-12 weeks of age. We also briefly discuss possible mechanisms for these unexpected findings with respect to neonatal erythropoiesis.

3.3 Materials and Methods

3.3.1 Animals and Diets

Animal characteristics are summarized in Table 3.1. Animal work took place at the Southwest Foundation for Biomedical Research (SFBR) located in San Antonio, Texas and protocols were approved by the SFBR and Cornell University Institutional Animal Care and Use Committees. Details of the experimental protocol are available elsewhere (Hsieh, Anthony et al. 2006) and will be summarized here. Fourteen pregnant baboons delivered spontaneously around 182 days gestation. Neonates were transferred to the nursery within 24 hours of birth and randomized to one of three diet groups. Animals were assigned to one of the following formulas: C, control formula with no LCPUFA; L, control formula plus 0.32% DHA/0.64% ARA; L3, control formula with 0.96% DHA/0.64% ARA. All diets provided 1.8 mg iron/100 cal. C and L are the commercially available human infant formulas Enfamil®, and Enfamil® LIPIL®, respectively, provided by Mead-Johnson Nutritionals (Evansville, IN).

Table 3.1 Characteristics of baboon neonates.

Number of animals (n)	14
Gender	10F, 4M
Conceptional age at delivery (d)	181.8 ± 6.2
Birth weight (g)	860.3 ± 150.8
Weight at 12 weeks (g)	1519.1 ± 280.7
Weight gain (g)	658.8 ± 190.4

Data expressed as mean ± SD.

3.3.2 Blood sampling

Blood was obtained via femoral venipuncture in unsedated fasted animals between 07:00 and 08:30. Hematological measurements were made on whole blood collected in potassium EDTA microtainer tubes at 2, 4, 8, 10, and 12 weeks of age.

3.3.3 Hematology

Samples were analyzed at the Clinical Pathology Laboratory at the Southwest Foundation for Biomedical Research. Complete blood count (CBC) measurements were assessed for each animal at each of the sampling time points. CBC parameters evaluated included white blood cell (WBC) counts, red blood cell (RBC) counts, hemoglobin concentrations, hematocrit, mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentrations (MCHC), and red blood cell distribution width (RDW). Red cell indices MCV, MCH, MCHC and RDW are calculations based on the relationship between RBC, hemoglobin and hematocrit. Data from parameters not significantly affected by the diets are the subject of a separate report (Hsieh, Anthony et al. 2006). Measurements were determined using a Coulter MAXM autoloader instrument (Beckman Coulter, Inc., Fullerton, CA).

Details associated with the determination have been reported previously (Havill, Snider et al. 2003; Schlabritz-Loutsevitch, Hubbard et al. 2005).

3.3.4 Statistics

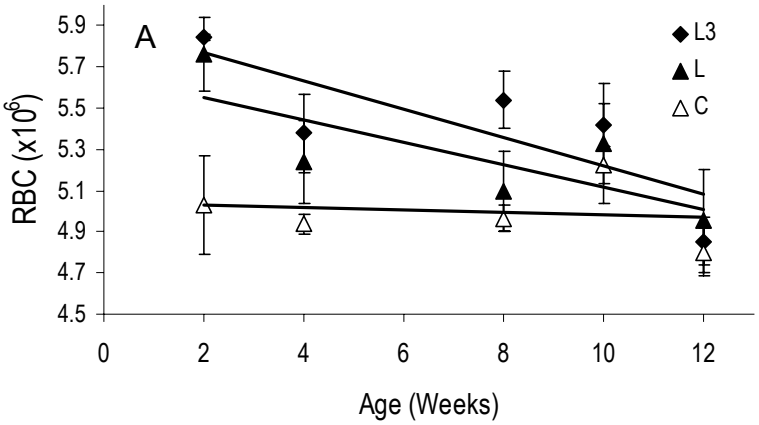
Data are expressed as mean ± SD. Hematological values were evaluated using a random coefficient regression model to detect effects of dietary LCPUFA. Fixed effects included diet treatment, age and the age*diet interaction and the random effect was subject. Statistical analyses were performed using the proc mixed procedure in SAS for Windows 9.1 (SAS Institute, Cary, NC), with significance declared at $p<0.05$.

3.4 Results and Discussion

Several hematological values related to red cells decreased during the first 12 weeks of life. Figure 3.1 shows that dietary LCPUFA resulted in elevated values for RBC, hematocrit, hemoglobin, and RDW compared to controls. The consistent, significant trend was for highest values in the L3 group followed by L, and lowest values in C. At 2 weeks of age, RBC, hemoglobin and hematocrit measurements were highest in the L3 group ($5.8 \pm 0.03 \times 10^6$, 16.3 ± 0.5 g/dl, $50.5 \pm 0.2\%$) while C was nearly 15% lower at $5.0 \pm 0.5 \times 10^6$, 14.1 ± 0.9 g/dl, $42.6 \pm 3.7\%$, respectively.

Figure 3.1 also shows that the rate of decline in these parameters was significantly influenced by LCPUFA. DHA/ARA animals in the L3 and L groups declined more sharply than the C group, and by 12 weeks differences between groups were no longer present. RBC and hemoglobin values fell from $5.5 \pm 0.5 \times 10^6$ and 15.34 ± 1.26 g/dl to $4.9 \pm 0.3 \times 10^6$ and 12.04 ± 0.67 g/dl at 12 weeks, respectively. Regression analysis revealed consistent trends, with significantly higher initial values for L3 and L compared to the C group. This pattern points to an influence of LCPUFA on hematological factors starting at birth and moderating in the first postnatal weeks.

Figure 3.1. Regression analysis calculated with initial values at 2 weeks of age, the first sampling timepoint. Means ± SD. (A) RBC ($\times 10^6$/ml); Regression equations for the ontogeny (a: age) within each treatment: RBC_C=-0.006a+5.0, RBC_L=-0.07a+5.6, RBC_{L3}=-0.08a+5.8. (B) Hemoglobin (g/dl); Hb_C=-0.23a+14.1, Hb_L=-0.37a+15.7, Hb_{L3}=-0.38a+16.4. (C) Hematocrit (%), Ht_C=-0.56a+41.8, Ht_L=-1.17a+46.9, Ht_{L3}=-1.17a+49.3. (D) RDW (%), RDW_C=-0.04a+11.9, RDW_L=-0.15a+12.9, RDW_{L3}=-0.17a+13.4. At two weeks of age, all parameters are significantly lower in the control (C) group compared to the LCPUFA groups (L, L3); L and L3 are not significantly different.


A
RBC (x10^6)
5.9
5.7
5.5
5.3
5.1
4.9
4.7
4.5
0
2
4
6
8
10
12
Age (Weeks)
L3
L
C

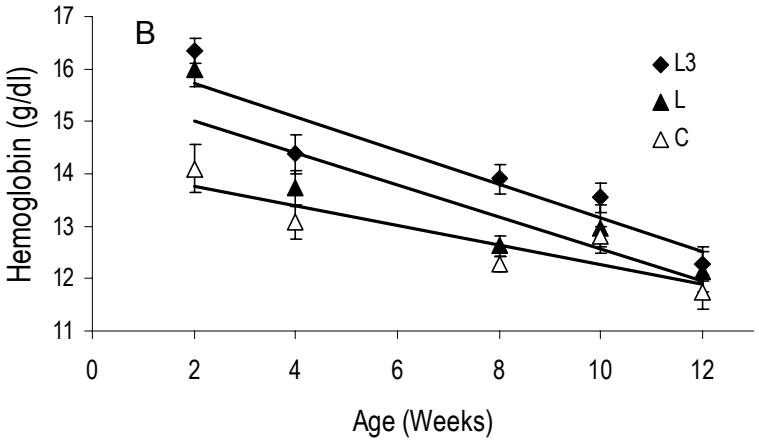

B
Hemoglobin (g/dl)
17
16
15
14
13
12
11
0
2
4
6
8
10
12
Age (Weeks)
L3
L
C

Figure 3.1 (continued)

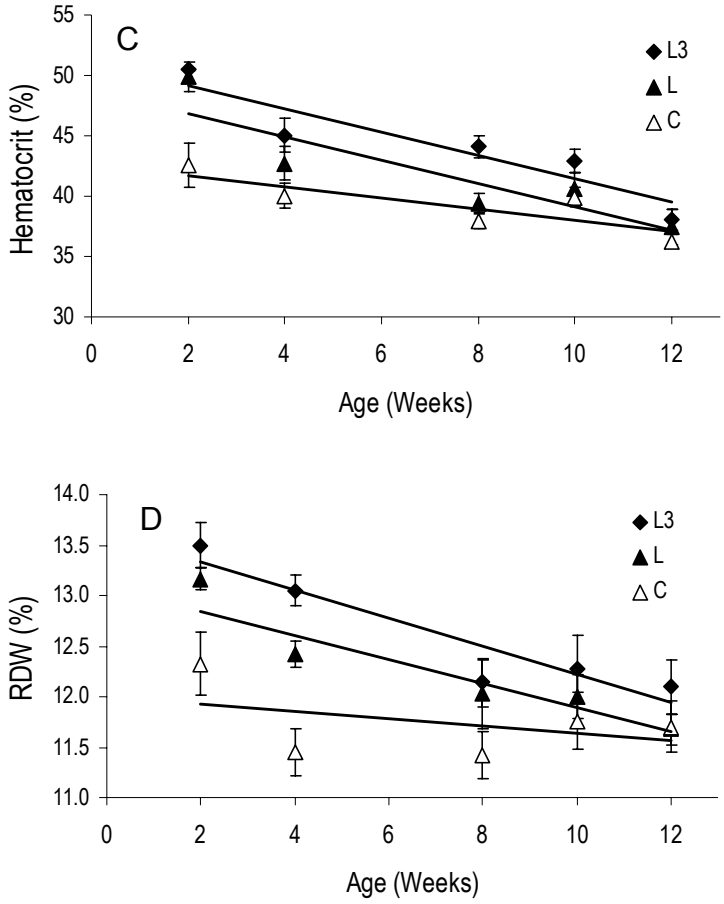


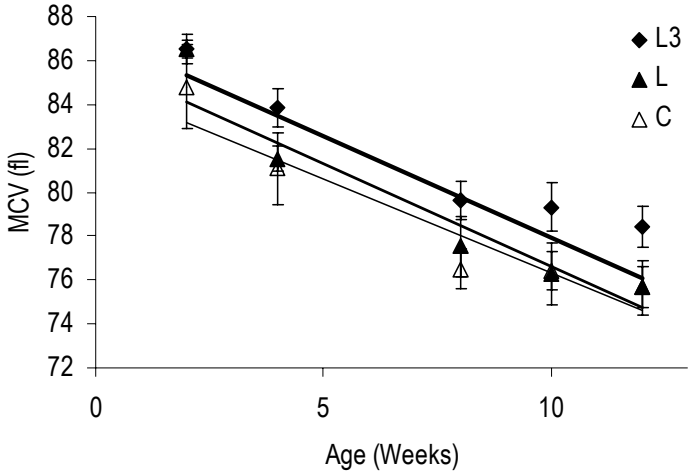
Figure 3.2 presents data for MCV, MCH, and MCHC. MCV and MCH both drop over time, consistent with the other hematological factors. None of the three parameters show any statistically significant relationship to LCPUFA consumption.

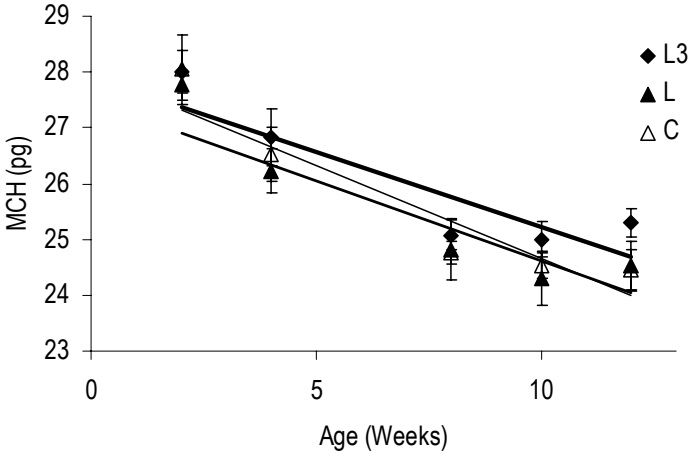
Infant baboon hematology reference ranges are available for selected CBC parameters and our data falls within those normal ranges (Havill, Snider et al. 2003).

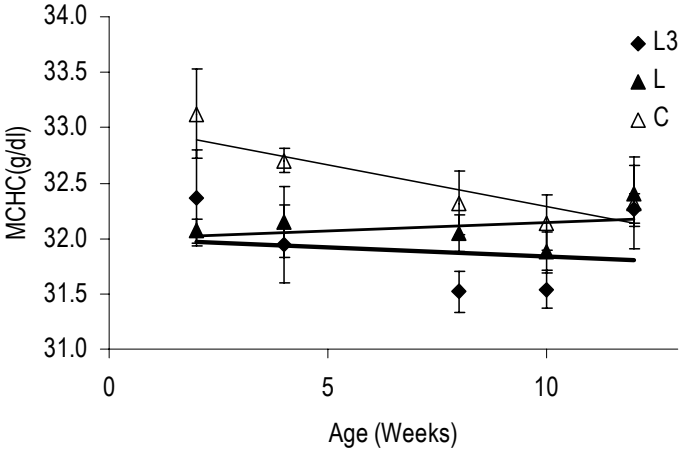
Declining red cell measurements during the first postnatal months are consistent with other published normal baboon values (Berchelmann and Kalter 1973). Baboon hematological development follows trends documented in healthy human term infants (Matoth, Zaizov et al. 1971; Saarinen and Siimes 1978; Newland and Evans 1997; Geaghan 1999; Nathan, Ginsburg et al. 2003; Wasiluk 2005). Postnatally, human infants reach a physiological nadir in RBC, hemoglobin and hematocrit at approximately 2 months (Matoth, Zaizov et al. 1971; Saarinen and Siimes 1978; Geaghan 1999). At 3 months, baboon hemoglobin concentrations decreased to 12.04 ± 0.67 g/dl and are expected to attain lowest values around 4 months of age (Berchelmann and Kalter 1973). Besides species variability, blood count values depend on collection site and differences may have been magnified due to sampling sites (Berchelmann and Kalter 1973; Ohls 1998; Geaghan 1999; Nathan, Ginsburg et al. 2003).

Red cell indices during the first day of life change rapidly in humans (Matoth, Zaizov et al. 1971; Ohls 1998; Geaghan 1999; Nathan, Ginsburg et al. 2003). The neonates reported here were born after spontaneous labor overnight, and cord blood could not be collected, nor was blood collected until the two week time point. It is therefore not possible for us to determine whether DHA/ARA moderated a decline, or enhanced, RBC and related parameters in the first two weeks of life. Older reference values for baboon cord blood are on the low side of our two week RBC, hematocrit, and hemoglobin values (Berchelmann and Kalter 1973), and if applied to our data imply that DHA/ARA increased hematological parameters, albeit all within the normal range.

Figure 3.2 Regression analysis calculated with initial values at 2 weeks of age, the first sampling time point. Means ± SD. (A) Mean Cell Volume (fl); Regression equations for the ontogeny (a: age) within each treatment: MCV_C=-0.86a+84.9, MCV_L=-0.94a+86.0, MCV_{L3}=-0.92a+87.2. (B) Mean Cell Hemoglobin (pg); MCH_C=-0.33a+28.0, MCH_L=-0.29a+27.5, MCH_{L3}=-0.27a+27.9. (C) Mean Cell Hemoglobin Concentration (g/dl, $MCHC_C$=-0.07a+33.0, $MCHC_L$=0.01a+32.0, $MCHC_{L3}$=-0.02a+32.0. No statistical differences between the control (C) group compared to the LCPUFA groups were detected for MCV, MCH and MCHC.


88
86
84
82
80
78
76
74
72
MCV (fl)
0
5
10
Age (Weeks)
L3
L
C


29
28
27
26
25
24
23
MCH (pg)
0
5
10
Age (Weeks)
L3
L
C


34.0
33.5
33.0
32.5
32.0
31.5
31.0
MCHC(g/dl)
0
5
10
Age (Weeks)
L3
L
C

From our present data, we cannot determine whether DHA or ARA alone are active in enhancing hematological parameters relative to the control group. However, comparison of the L3 and L groups enables an assessment of whether high DHA at a constant ARA level has an influence on red cell parameters. For all time points from 2 to 12 weeks, the L3 parameters were greater than the L parameters and the best fit regression lines were also greater. Values calculated for the two week time point were statistically different for hemoglobin and hematocrit, though a simple t-test of the L and L3 experimental values at two weeks is not significant. Given that the L3 formula contained 3-fold more DHA than the L formula, the magnitude of the difference at two weeks is small compared to the difference between the L and C groups. We conclude that most of the effect is obtained with the L formula in these healthy term neonates.

Our results suggest a protective mechanism for baboons consuming LCPUFA during the early phase of the physiological anemia of infancy. Elevated RBC and hemoglobin levels enhance oxygenation of body tissues, and while these effects were not significant at 12 weeks of age, the transient benefits of formula DHA and ARA on postnatal erythropoiesis may impart later advantages.

The association between dietary DHA/ARA and hematological parameters, found in this randomized controlled trial, is unexpected. A mechanism purely based on enhanced intestinal absorption of iron from the gut in the presence of DHA/ARA, thereby providing more iron for hemoglobin synthesis, cannot be excluded but would not be consistent with currently available evidence. Adult human and rats studies show that iron absorption is enhanced in the presence of stearic acid compared to linoleic acid (Johnson, Lukaski et al. 1992; Lukaski, Bolonchuk et al. 2001; Pabon and Lonnerdal 2001); we are not aware of comparable studies with LCPUFA.

Dietary LCPUFA are known to alter RBC and tissue fatty acid profiles in animal and human neonates (Sarkadi-Nagy, Wijendran et al. 2004; Fleith and

Clandinin 2005). Lipid composition of erythrocyte membranes are ~50% by weight, predominately in the form of phospholipids. A potential explanation for the elevated red cell parameters of LCPUFA animals may be increased RBC survival. The normal life span of adult red cells is approximately 120 days and RBCs created during last months of fetal life range between 45-70 days (Nathan, Ginsburg et al. 2003). Erythrocytes from term infants survive around 60-80 days, while those of premature infants are considerably shorter (Stockman 1986; Strauss 1995; Geaghan 1999; Nathan, Ginsburg et al. 2003). Alterations in membrane function are thought to be responsible for the decreased survival of fetal RBC. Normal neonatal red cells tend to be less flexible and more resistant to lysis, but more susceptible to oxidant induced injury than adult cells (Geaghan 1999; Nathan, Ginsburg et al. 2003). Incorporation of LCPUFA into blood cell membranes may have provided protection against oxidative damage or improved flexibility and integrity to enhance survival in circulation (Crawford, Golfetto et al. 2003).

Elevated RBC count and hematocrit may be due to increased production of new cells. RDW, a measure of the variation in red cell size, was significantly different for LCPUFA neonates compared to controls. Although RDW values were within appropriate ranges, animals consuming dietary LCPUFA had slightly greater variation in cell size. Reticulocytes, RBC precursors, are larger in size than mature red cells (Nathan, Ginsburg et al. 2003). If RBCs were elevated due to increased production of cells, the newly released reticulocytes would have influenced RDW measures as we observe. We were not able to confirm this hypothesis because the shape of the RBC size distribution was not available for estimation of reticulocyte abundance, and blood smears were not available for reticulocyte counts.

Accelerated maturation due to formula DHA/ARA should be considered as a mechanism for enhanced red cell indices. A key growth factor for erythroid

production is erythropoietin (EPO) (Ohls 1998; Nathan, Ginsburg et al. 2003). Production of EPO switches from the fetal liver to the kidneys in the first postnatal months (Strauss 1995; Dame and Juul 2000). The adult kidney produces EPO in response to hypoxia and is more sensitive to fluctuations in oxygen than the liver (Strauss 1995; Ohls 1998; Semba and Juul 2002). At birth, the sudden increase in oxygen tension initiates several changes that include decreased hematopoiesis, reticulocyte count, and EPO suppression (Ohls 1998; Geaghan 1999). EPO production declines for 4-6 weeks until adult concentrations are attained around 10-12 weeks of age (Dame and Juul 2000). EPO receptors have been identified in the gastrointestinal tract (GI), endothelial cells, spleen, liver, kidney, lung, spinal cord, and brain (Dame and Juul 2000; Semba and Juul 2002). EPO is thought to interact with other growth factors and promote maturation of crypt cells in the villi (Dame and Juul 2000; Philpott 2002). In the developing neonatal rat intestine, EPO increases small bowel length and villus surface area (Juul, Ledbetter et al. 2001). A retrospective study examining very low birth weight human infants reported lower incidence of necrotizing enterocolitis (NEC) when recombinant EPO was administered (Ledbetter and Juul 2000; Crawford, Golfetto et al. 2003). A randomized trial in preterm infants treated with recombinant EPO and iron had higher hematocrit and reticulocyte counts and fewer blood transfusions compared to infants treated with EPO alone (Carnielli, Da Riol et al. 1998). These considerations hint at an association between accelerated maturation by dietary LCPUFA, mediated by EPO, which would provide a connection to enhanced erythroid production.

Iron homeostasis is a complex and tightly regulated process, controlled at the level of absorption in the small intestine (Frazer and Anderson 2005). Regulation of iron absorption is poorly regulated in young infants and improves with development (Pabon and Lonnerdal 2001), and adequate iron is required for hemoglobin synthesis.

While all baboon neonates consumed formula containing 1.8 mg iron/100 cal (as ferrous sulfate), absorption would have depended on GI tract maturity. Iron status is thought to influence the signaling for production of EPO. As iron regulatory mechanisms developed and iron became more available for RBC production, erythropoiesis may have been slightly accelerated in LCPUFA groups. A human clinical study found less severe NEC in preterm infants consuming formulas supplemented with DHA and ARA (Colombo, Kannass et al. 2004), possibly indicating a role for LCPUFA in GI tract maturation. EPO may have simultaneously stimulated red cell production and maturation of the intestinal mucosa.

Improvements in red cell indices of LCPUFA animals may provide transient physiological advantages and hint at accelerated erythropoiesis during early development. Our findings capture specific changes during a dynamic period that have not been reported in previous infant LCPUFA studies, most of which had more limited blood collection. Similar studies examining LCPUFA consumption and cognitive function in human infants have also shown initial developmental advantages that dissipate at later ages (Colombo, Kannass et al. 2004) and those principles are likely to extend to physiological maturation as well. Future studies will be necessary to investigate the origins of these observations focusing on developmental changes in iron absorption, assessments of iron status via ferritin, transferrin receptor, and EPO levels.

3.5 Conclusion

We assessed the influence of dietary LCPUFA on ontogeny of hematological profiles in term baboon neonates. Hematological values were similar to established infant baboon reference ranges and consistent with increasing maturity documented during human neonatal development. During the first postnatal weeks, formula with 0.32% DHA/0.64% ARA and 0.96% DHA/0.64% ARA increased RBC, hemoglobin

and hematocrit values by 12% and 15%, respectively when compared to a control group consuming formula with no LCPUFA. Infant formulas containing LCPUFA may promote accelerated erythropoiesis and GI maturation to mitigate neonatal anemia.

3.6 Acknowledgments

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3.7 References

Anderson, G. J., M. Neuringer, et al. (2005). "Can prenatal N-3 fatty acid deficiency be completely reversed after birth? Effects on retinal and brain biochemistry and visual function in rhesus monkeys." Pediatr Res **58**(5): 865-72.

Berchelmann, M. L. and S. S. Kalter (1973). "The baboon. Hematology." Primates Med **8**: 51-64.

Berchelmann, M. L., T. E. Vice, et al. (1971). "The hemogram of the maternally-reared neonatal and infant baboon (Papio cynocephalus)." Lab Anim Sci **21**(4): 564-71.

Brenna, J. T. (2002). "Efficiency of conversion of alpha-linolenic acid to long chain n-3 fatty acids in man." Curr Opin Clin Nutr Metab Care **5**(2): 127-32.

Carlson, S. E., M. B. Montalto, et al. (1998). "Lower incidence of necrotizing enterocolitis in infants fed a preterm formula with egg phospholipids." Pediatr Res **44**(4): 491-8.

Carnielli, V. P., R. Da Riol, et al. (1998). "Iron supplementation enhances response to high doses of recombinant human erythropoietin in preterm infants." Arch Dis Child Fetal Neonatal Ed **79**(1): F44-8.

Carnielli, V. P., D. J. Wattimena, et al. (1996). "The very low birth weight premature infant is capable of synthesizing arachidonic and docosahexaenoic acids from linoleic and linolenic acids." Pediatr Res **40**(1): 169-74.

Colombo, J., K. N. Kannass, et al. (2004). "Maternal DHA and the development of attention in infancy and toddlerhood." Child Dev **75**(4): 1254-67.

Crawford, M. A., I. Golfetto, et al. (2003). "The potential role for arachidonic and docosahexaenoic acids in protection against some central nervous system injuries in preterm infants." Lipids **38**(4): 303-15.

Dame, C. and S. E. Juul (2000). "The switch from fetal to adult erythropoiesis." Clin Perinatol **27**(3): 507-26.

Diau, G. Y., A. T. Hsieh, et al. (2005). "The influence of long chain polyunsaturate supplementation on docosahexaenoic acid and arachidonic acid in baboon neonate central nervous system." BMC Med **3**(11): 11.

Dobbing, J. and J. Sands (1979). "Comparative aspects of the brain growth spurt." Early Hum Dev **3**(1): 79-83.

Fleith, M. and M. T. Clandinin (2005). "Dietary PUFA for preterm and term infants: review of clinical studies." Crit Rev Food Sci Nutr **45**(3): 205-29.

Frazer, D. M. and G. J. Anderson (2005). "Iron imports. I. Intestinal iron absorption and its regulation." Am J Physiol Gastrointest Liver Physiol **289**(4): G631-5.

Geaghan, S. M. (1999). "Hematologic values and appearances in the healthy fetus, neonate, and child." Clin Lab Med **19**(1): 1-37, v.

Gibson, R. A., W. Chen, et al. (2001). "Randomized trials with polyunsaturated fatty acid interventions in preterm and term infants: functional and clinical outcomes." Lipids **36**(9): 873-83.

Havill, L. M., C. L. Snider, et al. (2003). "Hematology and blood biochemistry in infant baboons (Papio hamadryas)." J Med Primatol **32**(3): 131-8.

Heird, W. C. and A. Lapillonne (2005). "The role of essential fatty acids in development." Annu Rev Nutr **25**: 549-71.

Helland, I. B., L. Smith, et al. (2003). "Maternal supplementation with very-long-chain n-3 fatty acids during pregnancy and lactation augments children's IQ at 4 years of age." Pediatrics **111**(1): e39-44.

Hoffman, D. R., R. C. Theuer, et al. (2004). "Maturation of visual acuity is accelerated in breast-fed term infants fed baby food containing DHA-enriched egg yolk." J Nutr **134**(9): 2307-13.

Hsieh, A., J. Anthony, et al. (2006). "The Influence of Moderate and High Levels of Long Chain Polyunsaturated Fatty Acid (LCPUFA) Supplementation on Baboon Neonate Tissue Fatty Acids." Submitted to Ped Res.

Hsieh, A., J. Anthony, et al. (2006). "Ontogeny of Clinical and Hematological Profiles of Baboon Neonates Through Twelve Weeks." Submitted to J Med Primatol.

Innis, S. M., J. Gilley, et al. (2001). "Are human milk long-chain polyunsaturated fatty acids related to visual and neural development in breast-fed term infants?" J Pediatr **139**(4): 532-8.

Johnson, P. E., H. C. Lukaski, et al. (1992). "Effects of stearic acid and beef tallow on iron utilization by the rat." Proc Soc Exp Biol Med **200**(4): 480-6.

Juul, S. E., D. J. Ledbetter, et al. (2001). "Erythropoietin acts as a trophic factor in neonatal rat intestine." Gut **49**(2): 182-9.

Lauritzen, L., H. S. Hansen, et al. (2001). "The essentiality of long chain n-3 fatty acids in relation to development and function of the brain and retina." Prog Lipid Res **40**(1-2): 1-94.

Ledbetter, D. J. and S. E. Juul (2000). "Erythropoietin and the incidence of necrotizing enterocolitis in infants with very low birth weight." J Pediatr Surg **35**(2): 178-81; discussion 182.

Lim, S. Y., J. Hoshiba, et al. (2005). "N-3 fatty acid deficiency induced by a modified artificial rearing method leads to poorer performance in spatial learning tasks." Pediatr Res **58**(4): 741-8.

Lukaski, H. C., W. W. Bolonchuk, et al. (2001). "Interactions among dietary fat, mineral status, and performance of endurance athletes: a case study." Int J Sport Nutr Exerc Metab **11**(2): 186-98.

Martinez, M. (1992). "Tissue levels of polyunsaturated fatty acids during early human development." J Pediatr **120**(4 Pt 2): S129-38.

Matoth, Y., R. Zaizov, et al. (1971). "Postnatal changes in some red cell parameters." Acta Paediatr Scand **60**(3): 317-23.

Nathan, D. G., S. H. Orkin, et al. (2003). Hematology of Infancy and Childhood. Philadelphia, W.B. Saunders Company.

Nettleton, J. (1995). Omega-3 Fatty Acids and Health. New York, Chapman and Hall.

Neuringer, M., W. E. Connor, et al. (1986). "Biochemical and functional effects of prenatal and postnatal omega 3 fatty acid deficiency on retina and brain in rhesus monkeys." Proc Natl Acad Sci U S A **83**(11): 4021-5.

Newland, A. C. and T. G. Evans (1997). "ABC of clinical haematology. Haematological disorders at the extremes of life." Bmj **314**(7089): 1262-5.

Ohls, R. (1998). Developmental Erythropoiesis. Fetal and Neonatal Physiology. R. Polin and W. Fox. Phildelphia, W.B. Saunders Company.

Pabon, M. L. and B. Lonnerdal (2001). "Effects of type of fat in the diet on iron bioavailability assessed in suckling and weanling rats." J Trace Elem Med Biol **15**(1): 18-23.

Philpott, C. C. (2002). "Molecular aspects of iron absorption: Insights into the role of HFE in hemochromatosis." Hepatology **35**(5): 993-1001.

Saarinen, U. M. and M. A. Siimes (1978). "Developmental changes in red blood cell counts and indices of infants after exclusion of iron deficiency by laboratory criteria and continuous iron supplementation." J Pediatr **92**(3): 412-6.

Salem, N., Jr., B. Wegher, et al. (1996). "Arachidonic and docosahexaenoic acids are biosynthesized from their 18-carbon precursors in human infants." Proc Natl Acad Sci U S A **93**(1): 49-54.

Sarkadi-Nagy, E., V. Wijendran, et al. (2004). "Formula feeding potentiates docosahexaenoic and arachidonic acid biosynthesis in term and preterm baboon neonates." J Lipid Res **45**(1): 71-80.

Sauerwald, T. U., D. L. Hachey, et al. (1996). "Effect of dietary alpha-linolenic acid intake on incorporation of docosahexaenoic and arachidonic acids into plasma phospholipids of term infants." Lipids **31 Suppl**(5): S131-5.

Schlabritz-Loutsevitch, N. E., G. B. Hubbard, et al. (2005). "Ontogeny of hematological cell and biochemical profiles in maternal and fetal baboons (Papio species)." J Med Primatol **34**(4): 193-200.

Semba, R. D. and S. E. Juul (2002). "Erythropoietin in human milk: physiology and role in infant health." J Hum Lact **18**(3): 252-61.

Stockman, J. A., 3rd (1986). "Anemia of prematurity. Current concepts in the issue of when to transfuse." Pediatr Clin North Am **33**(1): 111-28.

Strauss, R. G. (1995). "Neonatal anemia: pathophysiology and treatment." Immunol Invest **24**(1-2): 341-51.

Wasiluk, A. (2005). "Thrombocytopoiesis in healthy term newborns." J Perinat Med **33**(3): 252-4.

Williams, C., E. E. Birch, et al. (2001). "Stereoacuity at age 3.5 y in children born full-term is associated with prenatal and postnatal dietary factors: a report from a population-based cohort study." Am J Clin Nutr **73**(2): 316-22.

Yamamoto, N., A. Hashimoto, et al. (1988). "Effect of the dietary alpha-linolenate/linoleate balance on lipid compositions and learning ability of rats. II. Discrimination process, extinction process, and glycolipid compositions." J Lipid Res **29**(8): 1013-21.

CHAPTER 4

BIOCHEMICAL AND WHITE BLOOD CELL PROFILES OF BABOON NEONATES CONSUMING FORMULAS WITH MODERATE AND HIGH DIETARY LONG-CHAIN POLYUNSATURATED FATTY

4.1 Abstract

Clinical chemistry and complete blood count (CBC) values were established in 14 term baboons (*Papio hamadryas*) consuming formula with moderate and high levels of dietary long-chain polyunsaturated fatty acids (LCPUFA) from 2 to 12 weeks of age. Methods: Baboon neonates were randomized to 3 formula groups: C: Control, no LCPUFA; L: 0.32%DHA / 0.64%ARA; L3: 0.96%DHA/0.64%ARA. Blood chemistries were assessed at 6 and 12 weeks of age and CBC parameters were measured at 2, 4, 8, 10 and 12 weeks of age. Data were evaluated for evidence of treatment effects and ontogeny. Results: Significant effects of dietary LCPUFA were detected in serum triglyceride (C>L, L3) and calcium (L>C, L3). DHA/ARA consumption influenced RBC, hemoglobin, hematocrit, and RDW levels; details have been described elsewhere (Hsieh, Anthony et al. 2006). No other significant effects of diet were detected, and pooled values are presented for other parameters. Conclusion: These data provide longitudinal clinical chemistry and white cell data/platelet/immunological data on LCPUFA-fed baboon neonates over the first 12 weeks of life. In many cases these are the first data available for neonatal baboons, and in other cases they are the first available within the first weeks of life. Data ranges are similar to reference data in cases in which literature values exist and hematological changes reflect trends observed during human neonatal development.

4.2 Introduction

Non-human primates (NHP) provide valuable models to study effects of dietary LCPUFA on human maternal and child health (Lin, Connor et al. 1990; Sheaff Greiner, Zhang et al. 1996; Su, Corso et al. 1999; Champoux, Hibbeln et al. 2002). Baboons are a common NHP model for human infant growth and development and have been used to examine LCPUFA supplementation, prematurity, central nervous system development and retinal function (Wijendran, Lawrence et al. 2002; Sarkadi-Nagy, Wijendran et al. 2003; Diau, Hsieh et al. 2005; Gubhaju and Black 2005; Tambunting, Beharry et al. 2005). While reference ranges for biochemistry and white cell parameters have been published for baboon infants, effects of dietary LCPUFA on white cell and clinical chemistry data during the first weeks of the postnatal period have not been established.

Docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (ARA, 20:4n-6) are long-chain polyunsaturated fatty acids (LCPUFAs) found in baboon and human breast milk (Sarkadi-Nagy, Wijendran et al. 2003; Colombo, Kannass et al. 2004; Heird and Lapillonne 2005). Since 2002, DHA and ARA have been added to infant formulas in the United States. Maternal diet greatly influences breast milk fatty acid composition and DHA concentrations vary widely. Breast milk LCPUFA averages from 0.06% to 1.0% for DHA and 0.4% to 0.6% of total fatty acids for ARA (Nettleton 1995; Heird and Lapillonne 2005). Japanese women have some of the highest concentrations of breast milk DHA in the world, due to high fish consumption. LCPUFA concentrations in commercial infant formulas also differ, ranging from 8-19mg/100 cal for DHA and 21-34mg/100cal for ARA. Currently, insufficient evidence exists to determine the optimum levels of LCPUFAs required for developing infants.

Dietary LCPUFAs play a critical role in brain and retina development during the perinatal period (Lauritzen, Hansen et al. 2001; Heird and Lapillonne 2005). DHA and ARA consumption significantly alters plasma, erythrocyte membranes and brain fatty acid profiles of formula infants (Fleith and Clandinin 2005; Heird and Lapillonne 2005). In randomized controlled trials, neonates consuming DHA and ARA have shown accelerated visual maturation and improvements in cognitive ability and neurodevelopment when compared to controls consuming LCPUFA-free formula (Gibson, Chen et al. 2001; Innis, Gilley et al. 2001; Fleith and Clandinin 2005; Heird and Lapillonne 2005). Rhesus monkey neonates consuming formula at levels of 1.0% DHA exhibited significantly stronger visual and motor skills than animals fed formula without LCPUFA (Champoux, Hibbeln et al. 2002).

We investigated the influence of high levels of formula DHA and documented ontogeny of biochemical and white cell profiles in term baboons from 2 to 12 weeks of age. We found a consistent trend in RBC, hemoglobin, hematocrit, and RDW that is the subject of a separate report (Hsieh, Anthony et al. 2006). Here we present results from all clinical chemistry parameters and from CBC parameters other than those associated with red cells.

4.3 Materials and Methods

4.3.1 Animals and diets

Experimental details have been described elsewhere (Hsieh, Anthony et al. 2006) and will be summarized here. Animal characteristics are outlined in Table 4.1. Animal work took place at the Southwest Foundation for Biomedical Research (SFBR) in San Antonio, Texas and protocols were approved by the SFBR and Cornell University Institutional Animal Care and Use Committees. Fourteen pregnant baboons delivered spontaneously around 182 days gestation. Neonates were

transferred to the nursery within 24 hours of birth and randomized to one of the following formulas: C, Control, No DHA/ARA; L, 0.32% DHA/0.64% ARA; L3 0.96% DHA/0.64% ARA. All diets provided 1.8 mg/100 cal of iron; C and L are commercially available human infant formulas (Enfamil and Lipil; Mead-Johnson Nutritionals, Evansville, IN).

4.3.2 Blood sampling

Blood was obtained via femoral venipuncture in fasted animals between 07:00 and 08:30. One mL blood samples were obtained from neonates weighing less than 1 kg; 1.5 mL was drawn from animals weighing between 1 and 1.5 kg. Serum clinical chemistries were assessed at 6 and 12 weeks of age. White cell measurements were made on whole blood collected in potassium EDTA microtainer tubes at 2, 4, 8, 10 and 12 weeks of age.

4.3.2 Clinical chemistry and white cell measurements

All samples were analyzed at the Clinical Pathology Laboratory at the Southwest Foundation for Biomedical Research. Variables evaluated were glucose, blood urea nitrogen (Tambunting, Beharry et al.), creatinine, total protein, albumin, globulin, albumin/globulin ratio (A/G ratio), cholesterol, serum glutamine-pyruvate transaminase (SGPT), serum glutamic-oxaloacetic transaminase (SGOT), alkaline phosphatase, sodium, potassium, chloride, carbon dioxide, anion gap, gamma glutamyl transferase (GGT), lactate dehydrogenase (LDH), creatine phosphokinase (CPK), total bilirubin, direct bilirubin, calcium, phosphorus, and triglycerides. Analyses were preformed using a Beckman Synchron CX5CE (Beckman Coulter, Inc., Fullerton, CA). Determination details have been reported previously (Havill, Snider et al. 2003; Schlabritz-Loutsevitch, Hubbard et al. 2005). CBC parameters were white blood cell (WBC) counts, platelet count, mean platelet volumes (MPV), neutrophils, lymphocytes, monocytes, eosinophils, and basophils. Red cell parameters were

significantly related to DHA/ARA levels and are the subject of a separate report (Hsieh, Anthony et al. 2006). Measurements were determined using a Coulter MAXM autoloader instrument (Beckman Coulter, Inc., Fullerton, CA).

Table 4.1 Characteristics of Baboon Neonates.

Number of animals (n)	14
Gender	10F, 4M
Conceptional age at delivery (d)	181.8 ± 6.2
Birth weight (g)	860.3 ± 150.8
Weight at 12 weeks (g)	1519.1 ± 280.7
Weight gain (g)	658.8 ± 190.4

Data expressed as mean ± SD.

4.3.4 Statistics

Data are expressed as mean ± SD. Statistical analyses for biochemistry values were conducted using repeated measures ANOVA, with diet treatment (C, L, L3) as a between-group factor and age (6, 12) as within group factors. White cell values were evaluated using a random coefficient regression model to examine systematic effects of diet over time. Details of statistical methods have been described previously (Hsieh, Anthony et al. 2006). Analyses were performed using SAS for Windows 9.1 (SAS Institute, Cary, NC) with significance declared at $p<0.05$.

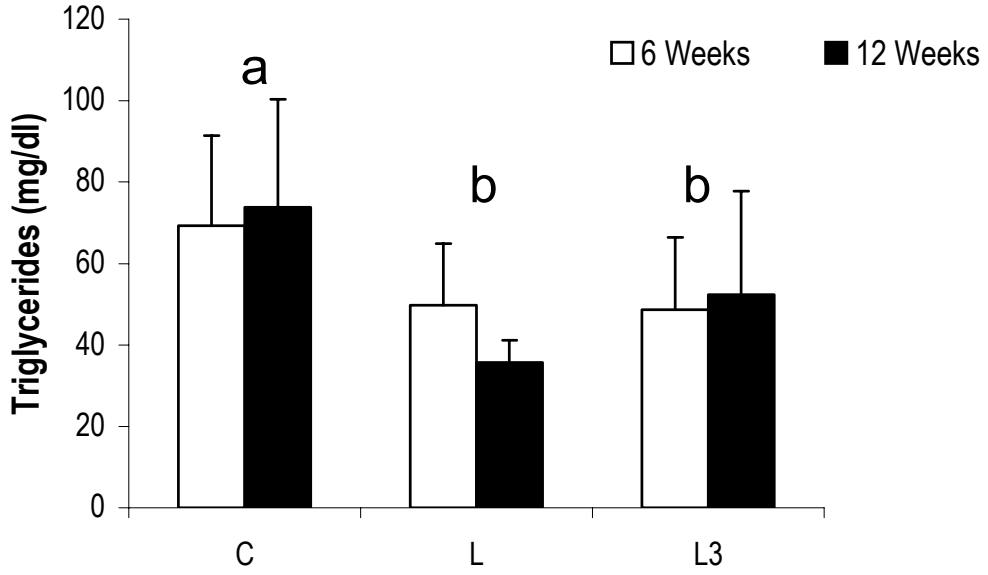
4.4 Results and Discussion

4.4.1 Clinical chemistry

Figure 4.1 presents graphically the results for the two parameters that were significantly influenced by the different infant formulas. Significant differences due to dietary LCPUFAs were seen in serum triglyceride (TG) and calcium measurements. TG measurements were significantly influenced by diet; C levels were higher than both LCPUFA groups ($p=0.03$). Mean TG values were 71.8 ± 23.3 for the Control group and 43.7 ± 13.5(L) and 54.7 ± 20.2 (L3) for LCPUFA animals. Between 6 and 12 weeks of age, no differences were detected and maturation did not affect TG levels. Plasma Ca was also influenced significantly by diet. There were significantly lower levels in C and L3 groups compared to the L group ($p=0.01$, $p=0.03$), as shown in figure 1. Between initial sampling at 6 weeks and serum collection at 12 weeks of age, calcium levels decreased in both control and LCPUFA supplemented animals.

Table 4.2 summarizes biochemical data obtained at 6 and 12 weeks of age for parameters for which there was no effect of treatment (mean ± SD, range). Neonatal baboon measurements for serum GGT, LDH, total bilirubin, direct bilirubin, CPK, calcium, phosphorus and triglycerides have not been reported previously. Mean values for those parameters for which there are literature values are similar to present values. Mean values for serum glucose, A/G ratio, and carbon dioxide increased from 6 to 12 weeks. Means for creatinine, total protein, globulin, SGOT, potassium, anion gap, LDH and total bilirubin values decreased significantly from 6 to 12 weeks of age. No change was detected between the two time points for BUN, BUN/creatinine ratio, albumin, cholesterol, SGPT, alkaline phosphatase, sodium, chloride, GGT, direct bilirubin, CPK, and phosphorus levels.

Figure 4.1 Clinical chemistry parameters significantly influenced by LCPUFA supplementation for baboon neonates (mean ± SD). Top panel: Serum triglycerides are lower for the L and L3 groups compared to C ($p<0.05$, a,b). No change was detected between 6 and 12 weeks. Bottom panel: Serum calcium is significantly higher for the L group than the C or L3 groups. Serum calcium also significantly decreased from 6 to 12 weeks (*).


120
100
80
60
40
20
0
Triglycerides (mg/dl)
6 Weeks
12 Weeks
a
b
b
C
L
L3

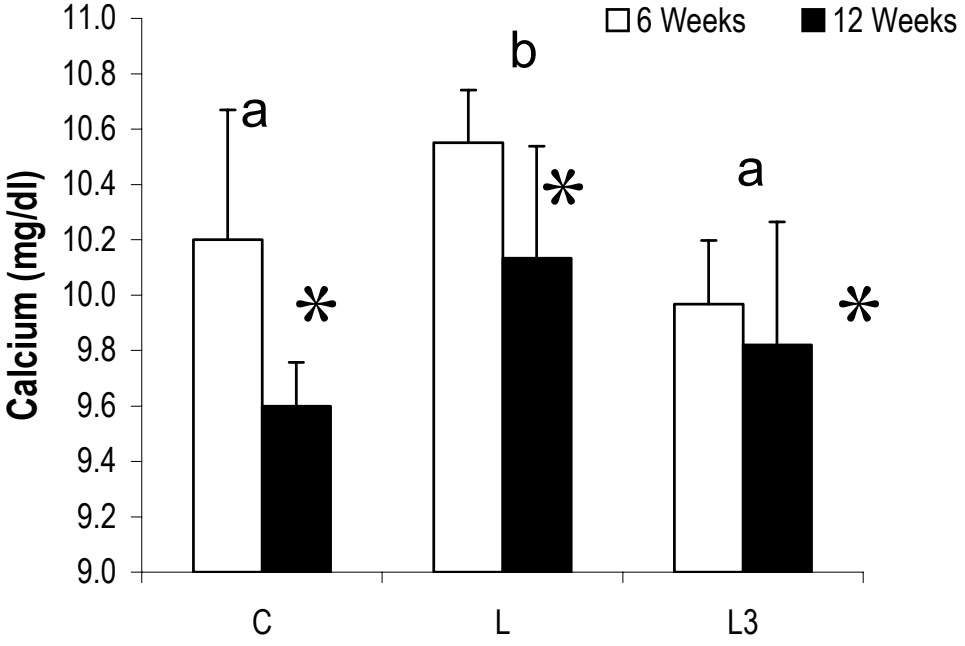

11.0
10.8
10.6
10.4
10.2
10.0
9.8
9.6
9.4
9.2
9.0
Calcium (mg/dl)
6 Weeks
12 Weeks
a
b
a
*
*
*
C
L
L3

Table 4.2 Ontogeny of clinical chemistry parameters for baboon neonates (mean ± SD, range). *Significant changes with age are listed on top ($p<0.05$).

Parameter (unit)	6 Weeks	12 Weeks
Glucose (mg/dl)*	56.4 ± 14.4	76.5 ± 15.6
Range	36-68	63-112
Creatinine (mg/dl)*	0.6 ± 0.1	0.5 ± 0.1
Range	0.3-0.7	0.3-0.7
Total Protein (g/dl)*	6.0 ± 0.5	5.2 ± 0.3
Range	5.6-6.3	4.8-5.2
Globulin (g/dl)*	2.3 ± 0.3	1.7 ± 0.3
Range	1.9-2.6	1.2-1.9
A/G Ratio*	1.5 ± 0.3	2.1 ± 0.4
Range	1.3-1.9	1.7-3.1
SGOT (U/l)*	39.8 ± 9.5	31.7 ± 5.2
Range	34-44	29-43
Potassium (mEq/l)*	4.9 ± 0.4	3.7 ± 0.7
Range	4.3-5	2.9-5.5
Carbon Dioxide (mEq/l)*	18.4 ± 2.5	22.9 ± 2.1
Range	17-22	19-26
Anion Gap (mEq/l)*	18.8 ± 2.3	10.1 ± 2.8
Range	16.3-21.9	7.4-12.5
LDH (U/l)*	288.2 ± 53.3	251.1 ± 39.7
Range	235-390	225-330
Total Bilirubin (mg/dl)*	0.6 ± 0.1	0.4 ± 0.1
Range	0.5-0.7	0.3-0.4

Table 4.2 (continued)

Parameter (unit)	6 Weeks	12 Weeks
BUN (mg/dl)	8.7 ± 2.1	8.6 ± 2.2
Range	6-8	7-10
BUN/Creatinine Ratio	14.7 ± 3.5	19.0 ± 5.1
Range	8.6-14	14-25
Albumin (g/dl)	3.6 ± 0.1	3.5 ± 0.2
Range	3.4-3.7	3.2-3.7
Cholesterol (mg/dl)	94.6 ± 14.7	95.0 ± 15.7
Range	92-123	72-124
SGPT (U/l)	25.5 ± 10.4	27.6 ± 8.9
Range	15-21	17-29
Alkaline phosphatase (U/l)	1304.5 ± 191.3	1264.0 ± 234.6
Range	981-1552	849-1782
Sodium (mEq/l)	144.2 ± 2.0	144.5 ± 1.5
Range	142-146	144-147
Chloride (mEq/l)	111.9 ± 1.9	115.4 ± 1.8
Range	109-112	114-118
GGT (U/l)	70.5 ± 15.7	65.5 ± 14.4
Range	43-99	42-84
Direct Bilirubin (mg/dl)	0.1 ± 0.1	0.1 ± 0.0
Range	0.1-0.2	0.1-0.2
CPK (U/l)	186.8 ± 64.7	445.3 ± 212.2
Range	96-323	273-885
Phosphorus (mg/dl)	7.6 ± 0.7	7.8 ± 0.7
Range	7.8-8	6.4-9.1

4.2.2 White cell measurements

Results for white cell measurements are presented in table 4.3. Reference values for pooled age 0-3 months or 0-12 months are available in one other report, and the present data are within those ranges (Havill, Snider et al. 2003). Although dietary DHA and ARA caused changes in RBC, hemoglobin, hematocrit and RDW (Hsieh, Anthony et al. 2006), no effects of LCPUFA were found for the white cell parameters. Age related changes were seen for platelet count, basophils and monocytes.

Table 4.3 Ontogeny for white cell parameters for baboon neonates from 2-12 weeks of age (mean ± SD).

Parameter (Units)	Age (Weeks) 2	4	8	10	12
Platelet Count ($x10^3$l)[a]	415.67±132.54	349.83±140.76	313.58±87.64	286±77.68	361.71±98.74
Basophil (%)[a]	0.97±2.56	0.48±0.98	0.25±0.44	0.3±0.46	0.09±0.13
Monocyte (%)[b]	1.44±1.10	1.75±0.99	2.39±1.16	1.38±0.59	1.03±0.46
WBC ($x10^3$l)	6.90±1.38	9.02±1.94	8.76±1.99	8.48±2.16	5.24±1.70
MPV (fl)	8.49±0.72	8.73±0.73	8.64±0.81	8.76±0.93	7.65±0.63
Neutrophils (%)	39.12±20.04	40.42±11.63	27.79±9.96	20.4±6.87	34.25±9.46
Lymphocyte (%)	54.36±19.11	53.78±9.96	64.66±9.85	72.56±9.03	59.58±8.97
Eosinophil (%)	2.36±1.56	2.23±1.37	4.56±2.57	4.36±1.78	4.45±1.28

Significantly decreasing values were observed for platelet count and basophils. Monocyte percentages, however, increased in baboon neonates approximately 45% during the sampling period.

We report the first blood biochemistry data for serum GGT, LDH, total bilirubin, direct bilirubin, CPK, calcium, phosphorus and triglycerides measurements in baboon neonates. All other clinical chemistry values were similar to published reference ranges (Havill, Snider et al. 2003). LCPUFA consumption significantly influenced serum triglyceride and calcium measurements in baboon neonates. Triglyceride levels were significantly lower for DHA and ARA neonates compared to controls consuming formula devoid of LCPUFAs. Decreased serum TG are similar to results documented in both rat and pig models of LCPUFA supplementation (Saito, Kubo et al. 1996; Arterburn, Boswell et al. 2000). Lower TG levels are also consistent with evidence from randomized clinical trials in human adults consuming LCPUFA and epidemiological data (Weber and Raederstorff 2000; Hamazaki, Itomura et al. 2003; Maki, Van Elswyk et al. 2003). LCPUFA are well known peroxisome-proliferator activated receptor (PPAR) activators, and potently decrease plasma TG in adults. The present data indicates that they are effective at lowering TG levels in neonates as well.

Calcium was significantly lower in C and L3 groups, compared to L. Calcium levels decreased in both control and LCPUFA supplemented animals from 6 to 12 weeks of age. Calcium is a highly regulated ion, and changes in calcium levels may reflect alterations in bone metabolism (Nakamura, Lane et al. 1998). DHA increases calcium absorption in bone, resulting in less calcium in circulating serum levels (Kruger and Schollum 2005). Plasma calcium concentrations decreased significantly in all treatment groups over time. Decreasing calcium levels suggest modifications in

mechanisms mediating bone turnover and mineralization during a period of rapid growth and development.

Longitudinal changes in serum clinical chemistry parameters; glucose, creatinine, total protein, globulin, A/G ratio, SGOT, potassium, carbon dioxide, and anion gap, were within published ranges (Havill, Snider et al. 2003). Decreasing LDH and total bilirubin values most likely reflect maturation of hepatic and renal processes. Data from health human infants from birth to 12 weeks of age reveal increases with maturity for glucose, potassium and carbon dioxide, and are consistent with our findings (Gomez, Coca et al. 1984). Baboon clinical chemistry parameters that decreased from 6 to 12 weeks, including total protein, LDH and total bilirubin also follow trends reported for developing human infants (Meites and Editor 1981, Gomez, 1984 #78).

Comparisons of our data to reference values reveal trends consistent with patterns seen in normal, healthy term baboon and human hematological development. During the first weeks after birth, significant decreases in platelet count and basophils were observed, while monocytes increased. Ontogeny has been documented for WBC, lymphocyte, monocyte, esinophil, basophil and platelet count measurements in baboon infants (Berchelmann, 1971, Meites, 1981), and our trends for platelet counts and monocytes were opposite to those previously published. However, the Berchelmann report obtained white cell values at birth, 1, 2, 3, 4, 6, 8, 10 and 12 weeks (Berchelmann and Kalter 1973). A close examination of the monocytes data between 6 and 12 weeks reveals a decreasing tend, primarily driven by a low point at 12 weeks of age. If the 12 week value is removed, monocytes percentages actually increase between 6 and 12 weeks, consistent with our results.

Several variables may have contributed to differences in our clinical chemistry and white cell values compared to previous studies. Our animals were nursery-reared,

formula-fed, and fasted prior to blood draws, while values reported by Havill et al (2003) for 0-2.9 or 0-12 months of age were from mother-reared baboons, consuming breast milk, and some were sedated before blood draws. Samples drawn outside the 0-2.9 month age window were also generally taken after the age of 6 months. Data from Berchelmann et al (1971) were also from maternally-reared animals, consuming breast milk. In our study consisting of 11 females and 3 males, statistical differences due to sex were not detected, though with the small number of males the power to detect sex differences is small.

4.5 Conclusion

This is the first study examining the effects of DHA and ARA consumption on biochemical and white cell parameters of baboon neonates. We evaluated the effects of increasing levels of dietary LCPUFA from 2 to 12 weeks of age. Consumption of 0.32% DHA/0.64% ARA and 0.96% DHA/0.64% ARA influenced triglyceride, and calcium levels, when compared to an control group consuming LCPUFA-free formula. These were in addition to red cell indices that were reported elsewhere (Hsieh 2006). Overall, white cell values were similar to established infant baboon reference ranges and consistent with trends observed during human postnatal development. Information on the influence of dietary LCPUFA will provide valuable mechanistic data for interpretation of human clinical nutrition studies.

4.6 Acknowledgments

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4.7 References

Arterburn LM, Boswell KD, Koskelo E, Kassner SL, Kelly C, Kyle DJ. 2000. A combined subchronic (90-day) toxicity and neurotoxicity study of a single-cell source of docosahexaenoic acid triglyceride (DHASCO oil). Food Chem Toxicol 38(1):35-49.

Berchelmann ML, Kalter SS. 1973. The baboon. Hematology. Primates Med 8:51-64.

Berchelmann ML, Vice TE, Kalter SS. 1971. The hemogram of the maternally-reared neonatal and infant baboon (Papio cynocephalus). Lab Anim Sci 21(4):564-71.

Champoux M, Hibbeln JR, Shannon C, Majchrzak S, Suomi SJ, Salem N, Jr., Higley JD. 2002. Fatty acid formula supplementation and neuromotor development in rhesus monkey neonates. Pediatr Res 51(3):273-81.

Diau GY, Hsieh AT, Sarkadi-Nagy EA, Wijendran V, Nathanielsz PW, Brenna JT. 2005. The influence of long chain polyunsaturate supplementation on docosahexaenoic acid and arachidonic acid in baboon neonate central nervous system. BMC Med 3:11.

Fleith M, Clandinin MT. 2005. Dietary PUFA for preterm and term infants: review of clinical studies. Crit Rev Food Sci Nutr 45(3):205-29.

Gibson RA, Chen W, Makrides M. 2001. Randomized trials with polyunsaturated fatty acid interventions in preterm and term infants: functional and clinical outcomes. Lipids 36(9):873-83.

Gubhaju L, Black MJ. 2005. The baboon as a good model for studies of human kidney development. Pediatr Res 58(3):505-9.

Hamazaki K, Itomura M, Huan M, Nishizawa H, Watanabe S, Hamazaki T, Sawazaki S, Terasawa K, Nakajima S, Terano T and others. 2003. n-3 long-chain FA decrease serum levels of TG and remnant-like particle-cholesterol in humans. Lipids 38(4):353-8.

Havill LM, Snider CL, Leland MM, Hubbard GB, Theriot SR, Mahaney MC. 2003. Hematology and blood biochemistry in infant baboons (Papio hamadryas). J Med Primatol 32(3):131-8.

Heird WC, Lapillonne A. 2005. The role of essential fatty acids in development. Annu Rev Nutr 25:549-71.

Hsieh A, Anthony J, Diersen-Schade D, Nathanielsz P, Brenna J. 2006a. Formula Docosahexaenoic Acid And Arachidonic Acid Improves Postnatal Hemoglobin And Related Indices In Term Baboon Neonates. Submitted to Pediatrics.

Hsieh AT, Anthony JC, Diersen-Schade DA, Rumsey SC, Li C, Nathanielsz PW, Brenna JT. 2006b. The influence of moderate and high levels of long chain polyunsaturated fatty acid supplementation on baboon neonate tissue fatty acids. Pediatric Research submitted.

Innis SM, Gilley J, Werker J. 2001. Are human milk long-chain polyunsaturated fatty acids related to visual and neural development in breast-fed term infants? J Pediatr 139(4):532-8.

Kruger MC, Schollum LM. 2005. Is docosahexaenoic acid more effective than eicosapentaenoic acid for increasing calcium bioavailability? Prostaglandins Leukot Essent Fatty Acids 73(5):327-34.

Lauritzen L, Hansen HS, Jorgensen MH, Michaelsen KF. 2001. The essentiality of long chain n-3 fatty acids in relation to development and function of the brain and retina. Prog Lipid Res 40(1-2):1-94.

Lin DS, Connor WE, Anderson GJ, Neuringer M. 1990. Effects of dietary n-3 fatty acids on the phospholipid molecular species of monkey brain. J Neurochem 55(4):1200-7.

Maki KC, Van Elswyk ME, McCarthy D, Seeley MA, Veith PE, Hess SP, Ingram KA, Halvorson JJ, Calaguas EM, Davidson MH. 2003. Lipid responses in mildly hypertriglyceridemic men and women to consumption of docosahexaenoic acid-enriched eggs. Int J Vitam Nutr Res 73(5):357-68.

Meites S, Editor. 1981. Pediatric Clinical Chemistry. Washington D.C.: American Association for Clinical Chemistry.

Nakamura E, Lane MA, Roth GS, Ingram DK. 1998. A strategy for identifying biomarkers of aging: further evaluation of hematology and blood chemistry data from a calorie restriction study in rhesus monkeys. Exp Gerontol 33(5):421-43.

Nathan D, Ginsburg D, Orkin S, Look A, Editors. 2003. Hematology of Infancy and Childhood Philadelphia: W. B. Saunders Company.

Nettleton JA. 1995. Omega-3 Fatty Acids and Health. New York: Chapman and Hall. 266 p.

Saito M, Kubo K, Ikegami S. 1996. An assessment of docosahexaenoic acid (DHA) intake with special reference to lipid metabolism in rats. J Nutr Sci Vitaminol (Tokyo) 42(3):195-207.

Sarkadi-Nagy E, Wijendran V, Diau GY, Chao AC, Hsieh AT, Turpeinen A, Nathanielsz PW, Brenna JT. 2003. The influence of prematurity and long chain polyunsaturate supplementation in 4-week adjusted age baboon neonate brain and related tissues. Pediatr Res 54(2):244-52.

Schlabritz-Loutsevitch NE, Hubbard GB, Jenkins SL, Martin HC, Snider CS, Frost PA, Michelle Leland M, Havill LM, McDonald TJ, Nathanielsz PW. 2005. Ontogeny of hematological cell and biochemical profiles in maternal and fetal baboons (Papio species). J Med Primatol 34(4):193-200.

Sheaff Greiner RC, Zhang Q, Goodman KJ, Giussani DA, Nathanielsz PW, Brenna JT. 1996. Linoleate, alpha-linolenate, and docosahexaenoate recycling into saturated and monounsaturated fatty acids is a major pathway in pregnant or lactating adults and fetal or infant rhesus monkeys. J Lipid Res 37(12):2675-86.

Su HM, Corso TN, Nathanielsz PW, Brenna JT. 1999. Linoleic acid kinetics and conversion to arachidonic acid in the pregnant and fetal baboon. J Lipid Res 40(7):1304-12.

Tambunting F, Beharry KD, Waltzman J, Modanlou HD. 2005. Impaired lung vascular endothelial growth factor in extremely premature baboons developing bronchopulmonary dysplasia/chronic lung disease. J Investig Med 53(5):253-62.

Weber P, Raederstorff D. 2000. Triglyceride-lowering effect of omega-3 LC-polyunsaturated fatty acids--a review. Nutr Metab Cardiovasc Dis 10(1):28-37.

Wijendran V, Lawrence P, Diau GY, Boehm G, Nathanielsz PW, Brenna JT. 2002. Significant utilization of dietary arachidonic acid is for brain adrenic acid in baboon neonates. J Lipid Res 43(5):762-7.

CHAPTER 5

CONCLUSION AND FUTURE STUDIES

We evaluated the safety and efficacy of formula ARA and moderate and high levels of DHA in term baboon neonates from birth to 12 weeks of age. Changes in tissue fatty acid composition were examined at 12 weeks and tissue DHA levels were more sensitive to dietary manipulations than ARA. DHA in the cerebral cortex increased with higher levels of dietary DHA and no differences between L and L3 were detected in the basal ganglia and limbic system. Current levels of LCPUFA in infant formula are not sufficient to optimize DHA levels in the developing cortex.

Dietary LCPUFA elevated hematological parameters RBC, hemoglobin, hematocrit and RDW. We provide evidence for a novel role of formula DHA and ARA in postnatal erythropoiesis. All hematological measurements and clinical chemistry parameters were within normal infant baboon ranges (Berchelmann and Kalter 1973; Havill, Snider et al. 2003)and follow trends described during normal human infant development (Nathan, Ginsburg et al. 2003).

Future studies are necessary to confirm our speculations on LCPUFA and hematopoiesis. The next step would be to monitor iron status from birth through the first months of life, using measures of serum ferritin, iron, total iron binding capacity (TIBC), or transferrin receptor (TfR). It would be essential to perform bone marrow or blood smears to evaluate reticulocytes formation for conclusive evidence on erythrocyte production. Other potential analyses include circulating 2,3-DPG or EPO levels and liver proteins involved in small intestine iron regulation such as hepcidin and divalent metal transporter (DMT1) mRNA expression. Eventually, we hope to define the molecular mechanism responsible for the action of LCPUFA on hematological measurements. Potential explanations for LCPUFA induced changes at the molecular level may include alterations in G-protein signaling, demonstrated in the

rod outer segment membranes of the retina in rats maintained on low n-3 FA diets (Niu, Mitchell et al. 2004) or changes in peroxisomal proliferator-activated receptor (PPAR) mRNA expression shown in rat ocular tissue with n-3 FA deficiency (Rojas, Greiner et al. 2002).

REFERENCES

Berchelmann, M. L. and S. S. Kalter (1973). "The baboon. Hematology." Primates Med **8**: 51-64.

Havill, L. M., C. L. Snider, et al. (2003). "Hematology and blood biochemistry in infant baboons (Papio hamadryas)." J Med Primatol **32**(3): 131-8.

Nathan, D., D. Ginsburg, et al. (2003). Hematology of Infancy and Childhood Philadelphia, W. B. Saunders Company.

Niu, S. L., D. C. Mitchell, et al. (2004). "Reduced G protein-coupled signaling efficiency in retinal rod outer segments in response to n-3 fatty acid deficiency." J Biol Chem **279**(30): 31098-104.

Rojas, C. V., R. S. Greiner, et al. (2002). "Long-term n-3 FA deficiency modifies peroxisome proliferator-activated receptor beta mRNA abundance in rat ocular tissues." Lipids **37**(4): 367-74.