INFANT FORMULA WITH LONG CHAIN POLYUNSATURATED FATTY ACID AND CHILDHOOD BLOOD PRESSURE

BY

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Abstract

Background: Breastfeeding of term infants has been associated with decreased blood pressure (BP) in later ages compared to formula feeding. The protective effect of human milk against childhood BP has been linked to its docosahexaenoic acid (DHA) content. It is unclear whether early exposure to DHA supplementation can benefit offspring from high BP in childhood.

Objective: to determine whether the amount of DHA fed to infants during infancy influences their blood pressure at ages 4, 4.5, 5, 5.5 and 6 years.

Methods: BP was measured longitudinally from 4 to 6 years of age at 6-months interval, in a cohort of 77 children who were randomized at birth into four infant formula groups comprise of 0.0%, 0.32%, 0.64%, and 0.96% of total fatty acids (FA) from DHA. Both the control and intervention groups were subsequently dichotomized into 2 groups based on their weight status ($\leq 85^{th}$ and $> 85^{th}$ percentiles), using the Center for Disease Control BMI-for-age growth charts ($\geq$ 2 years) reference. A three-way ANOVA model was run to examine the degree of interaction among BMI-for-age percentiles, formula group (control vs DHA) and age (4, 4.5, 5, 5.5, and 6 years) on systolic or diastolic BP.

Results: Higher BMI-for-age percentiles predicted higher systolic blood pressure (SBP) ($p=0.0054$) but group and age were unrelated to SBP. No variable was related to diastolic blood pressure (DBP) in young children.

Conclusion: Intake of a LCPUFA-supplemented infant formula did not protect against higher BP levels seen in overweight/obese children compared to intake of a non-supplemented formula.

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Chapter 1: Introduction

Docosahexaenoic acid (DHA), an omega-3 long chain polyunsaturated fatty acid (n-3 LCPUFA) and arachidonic acid (ARA), an omega-6 (n-6) LCPUFA are essential fatty acids found in high concentration in neuronal membranes of the brain. DHA is vital for infant neurocognitive development. Thus, having an adequate supply of DHA during late pregnancy and the first years of life is critical for proper child growth and development (1). There is evidence showing that LCPUFA supplementation of infant formula during infancy may lower childhood blood pressure (BP) when compared to standard formula, and may have long-term benefit by reducing risk of hypertension-related diseases in adulthood (2). In addition, there are strong observational and clinical evidence supporting that higher BMI in youth is associated with harmful levels of BP and lipids, putting this population at increased risk of CVDs later in life (3).

In adults, the heart health benefits of consuming a diet rich in omega-3 has been well documented. Epidemiological studies on dietary omega-3 consumption of different populations have demonstrated that in countries where consumption of fish is high, people tend to have higher levels of red blood cell (RBC) DHA, and lower risk of cardiovascular disease (CVD). Fish oil contains both DHA and EPA (eicosapentanoic acid), another omega-3 FA, found mainly in cold-water fatty fish (e.g., salmon, mackerel, and sardines). The cardiovascular benefits of fish oil supplementation have also been supported by numerous randomized controls trials (RCTs). A recent meta-analysis of RCTs concluded that supplementation with DHA and EPA, lowers systolic blood pressure (SBP), in both hypertensive and normotensive groups compared to the placebo group (4) .

In infants, breastfeeding is the recommended feeding practice (5). Breastfeeding of term infants has been associated with decreased BP in later ages (6, 7). A systematic review by Horta

et al. (8), showed that breastfeeding was inversely associated with overweight/obesity (pooled OR: 0.87; 95% CI: 0.76; 0.99) and breastfed infants tended to have lower BP than formula-fed infants (8, 9). The lower BP seen in breastfed infants has been associated with LCPUFA and, more specifically, with the DHA content of human milk (1). However, DHA levels in breastmilk depends partly on maternal diet, and U.S. pregnant and lactating women have very low DHA levels compared to other parts of the world (10); this is mainly due to the consumption of a "western diet" that lacks adequate amounts of DHA. In this respect, for infants who are not breastfed, early supplementation of the infant diet with LCPUFA may be beneficial in protecting children from developing chronic diseases such as hypertension later in adulthood.

Justification for Further Investigation

Data on LCPUFA supplementation during infancy and its effect on BP in childhood is very limited. Currently, there is only a single multi-center RCT that investigated the effect of LCPUFA supplemented infant formula during infancy and childhood BP as a primary outcome. This study demonstrated a positive association between DHA supplementation during infancy and lower BP at 6 years of age (2). Another RCT looked at the impact of LCPUFA supplementation in infancy (only from birth to 2 months of age) on cardiovascular health and anthropometric measures, including childhood BP at 9 years of age. They found no difference in BP between the LCPUFA supplemented bottle-fed and breast-fed babies (11). Two other studies have looked at fish oil supplementation and BP in childhood and had different outcomes. One showing positive effect of fish oil supplementation (12), and one showing no effect (13). Overall, RCTs on LCPUFA intake and childhood BP have resulted in mixed findings with some showing either positive or no effect.

The limited body of literature on DHA supplementation of term babies and BP later in childhood needs further investigation because of inconsistent results surrounding this topic. The dose of DHA supplementation during infancy, as well as duration of intake, age and method of analysis remain very different among studies, making their comparison challenging.

The present secondary data analysis of this RCT comprised of a sample of healthy term infants who were fed one of four different amounts of DHA throughout infancy gave us the prospect to address some of the existing gaps found in the literature, including DHA dose and duration of intake and its effect on childhood BP, which is a risk factor for CVD. We examined the dose-response relationship between DHA-supplemented intake at three different doses (0.32%; 0.64%; and 96%) of total fatty acid intake as DHA and 0.64% ARA and also the combined groups that were provided DHA and ARA. These were compared to 0% DHA 0% ARA, the control formula, for childhood SBP and DBP by BMI-for-age ($\leq 85^{th}$ and $> 85^{th}$ percentiles). This is also the first study to look at a longer period of DHA intake from birth to 12 months of age.

Statement of purpose

The purpose of this project was to determine whether the amount of DHA fed to infants during infancy influences their blood pressure at ages 4, 4.5, 5, 5.5 and 6 years.

Research question

Does the amount of DHA in formula fed to infants throughout infancy influence their blood pressure at 4, 4.5, 5, 5.5 and 6 years of age in relation to their weight status (BMI $\leq 85^{th}$ vs $>85^{th}$ percentile)?

Chapter 2: Review of Literature

Childhood Obesity and High Blood Pressure

According to NHANES 2011-2014 data, the prevalence of obesity in US children and adolescents, ages 2 to 19 years, remains high at approximately 17% and severe obesity was on the rise at 5.8% (15). There are strong observational and clinical evidence supporting that higher BMI in youth is associated with harmful levels of BP and lipids, putting this population at increased risk of CVDs later in life (3).

Compelling evidence indicates that hypertension in childhood tracks into adulthood (18). The prevalence of high BP (HBP), defined as having a SBP/DBP of >140/ 90 mm Hg, is estimated at 29% among U.S. adults (≥ 18 years old) (19) . Hypertension in children and adolescents is defined as "the average SBP and /or DBP ≥ 95^{th} percentile for age, gender, and height, on 3 or more occasions" (20), and remains one of the leading causes of CVDs (21) . Amongst U.S. youths, 1 in 10, aged between 8 and 17 years, has either borderline or HBP (20). Uncontrolled HBP can cause damage to the artery wall and lead to the development of atherosclerosis (22), which is a risk factor for myocardial infarction. Other complications include risk of stroke, kidney disease, and death, if left untreated (23) . In children, the increased rate of obesity and type 2 diabetes is concerning because obesity-associated insulin resistance is a risk factor for hypertension and other CVDs (2) .

Polyunsaturated Fatty Acids

Dietary FAs are classified based on the number of double bonds and saturation levels. Saturated FAs (SFAs) have no double bonds, monounsaturated FAs (MUFAs) have only one double bond, and polyunsaturated FAs (PUFAs) have two or more double bonds. The class of PUFAs can be separated into 12 families based on their configurations. The most important

prevalent dietary PUFAs are from the omega-6 and omega-3 families (24). The essential PUFAs, linoleic acid (LA) (18:2n6) and α-linolenic acid (ALA) (18:3n-3), cannot be synthesized by humans and need to be taken exogenously. Once consumed, these PUFAs can form long chain (LC) PUFAs, omega-3 (n-3) and omega-6 (n-6) FAs (24).

Through elongations and saturations, PUFAs are transformed to LCPUFAs. ALA forms EPA and DHA, and LA is transformed to arachidonic acid (ARA) (1) . A sizeable proportion of LCPUFAs found in the brain and the retina are DHA and ARA. DHA and ARA have been extensively researched for their role in neural development of the fetus and offspring (1). Breastmilk contains DHA and ARA but the amount of DHA in human milk depends on genetics (25), maternal diet (largely DHA intake), smoking, socio-economic status and gestational age (26).

LCPUFAs dietary sources

The best sources of the n-3 LCPUFA, EPA and DHA are marine sources namely fatty fish, seafood, and fish oil (27). Other non-marine sources of omega-3 FAs are vegetable oils such as walnut oil, linseed oil, flaxseed oil, and canola oil but the conversion rate of ALA from vegetable sources to EPA and DHA is estimated to be low at 0.2% and 0.05%, respectively (24). Therefore, marine sources and fish oil supplements are considered superior sources of n-3 LCPUFAs (27) .

LCPUFAs in infant diet

The FA composition of human milk is a mixture of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and PUFAs. The main SFA is palmitic acid (C16:0), and the major MUFA is oleic acid (18:1). PUFA is a combination of essential FAs, LA and ALA. A study examining the FA composition of 440 samples of human milk from nine countries showed

that the highest variability in the FA composition of breastmilk among countries was seen in the ARA: DHA ratio followed by the LA: ALA ratio. The United States and Canada were the two countries with the lowest DHA levels (0.17%wt of total FA) and Japan had the highest levels (0.99%wt of total FA) (28) .

Breastmilk naturally contains DHA and ARA and is considered the optimum nutrition for infants (29). The World Health Organization recommends breastfeeding infants during the first six months of life to promote healthy growth (5). The Food and Agriculture Organization of the United Nation/WHO joint commission recommend an intake of 0.3 g/day (or a minimum of 0.2g/day) of EPA and DHA during pregnancy and lactation to ensure proper supply of these essential FAs to the fetus and offspring (30).

A recent review of DHA and ARA content of breastmilk by geographic region, including 41 countries and 4,163 samples of breastmilk, revealed that worldwide mean DHA and ARA levels were 0.37% (SD ±0.11%) and 0.55% (SD ±0.14%), respectively (10) . High income countries, including the USA, had the lowest breastmilk DHA levels compared to middle- or low-income countries ($p<0.05$) (10).

LCPUFAs in infant formula

Beginning in 2002, some US formula companies began adding the LCPUFAs, ARA and DHA, to infant formulas in an effort to mimic the average worldwide profile of human milk (1). For infants 0-6 months old, the FAO recommends an AI for DHA and ARA at 0.1-0.18 and 0.2-0.3% of total energy, respectively (30). The AI increases to 10-12mg/kg weight for babies 6-24 months old, assuming 50% of total energy is provided through breastfeeding (30).

LCPUFAs role in fetus and infant development

LCPUFAs play an important role in cognitive and visual development of infants (1). During the last trimester of pregnancy and first 18 months of life, the rate of DHA and ARA accretion in the brain tissue and the retina of the fetus and offspring increases rapidly (1). DHA is transferred through the placenta from the mother to the fetus and through breastfeeding to the infant. Therefore, it is important for pregnant women, both pre- and postnatally, to maintain an adequate level of DHA status for the development of their infants (1).

LCPUFAs role in blood pressure

A meta-analysis of 70 randomized controlled trials (RCTs) on n-3 LCPUFAs supplementation and BP, conducted with adults involving mostly hyper- and normotensive individuals, reported that EPA and DHA supplementation had a lowering effect on SBP (-1.52 mm Hg; 95% CI: -2.25 to -0.79 mm Hg;) and DBP (-0.99 mm Hg; 95% CI: -1.54 to -0.44), all studies combined (4) . In infants, similar differences in BP were found between formula-fed and breast-fed babies. In the U.K., Wilson et al. (9) compared BP levels between formula-fed versus exclusively breastfed infants (n=301). Formula-fed infants had significantly higher mean SBP (94.2 mm Hg) than exclusively breastfed (90.3 mm Hg) babies. A systematic review and meta-analysis of 15 studies, with a total number of 17,503 subjects, showed a lower SBP (95% CI, pooled difference of -1.4 mm Hg) in breastfed versus formula-fed babies (6) . However, another systematic review conducted by Owen et al. (7) showed that smaller studies (<300 subjects) tended to report more pooled mean differences in SBP (-2.05 mm Hg) between exclusively breastfed babies and exclusively formula-fed babies than larger studies (>1000 subjects) (-0.16mm Hg). The authors concluded that breastfeeding tends to have a small protective effect on BP (7) .

Some studies in full-term bottle-fed babies with LCPUFA supplemented formula achieved the same results as breastfed babies in lowering BP levels later in childhood (2, 12) but not all studies (13, 31) . Research in this area is limited and results from studies are inconclusive.

LCPUFA intake and blood pressure in Children

A prospective study using data from a Dutch population-based birth cohort (n=1432), compared two subsamples of full-term healthy children who were followed from birth and had medical examinations done at ages 4, 8 and 12 years (32). This study investigated the association between FA composition of breastmilk and BP at 12 years of age. They analyzed the RBC FA composition (DHA, EPA, and total n-3 LCPUFAs) of human milk consumed in a subgroup of breastfed babies (n =109) and compared it to a reference subgroup of infants (n= 205) who were never breastfed. At 12 years of age, children who consumed human milk containing higher amount of n-3 LCPUFA had a 4.79 mm Hg lower SBP (95% CI, -7.64 to -1.94) and a 2.47mm Hg lower DBP (95% CI, -4.45 to -0.49), compared to the reference group. However, n-3 LCPUFA status was not associated with BP at 12 years of age (32).

These findings are supported by a cross-sectional study using NHANES (2003-2005) data but with a different population of low birth-weight children (n=354; $<10^{th}$ %centile; mean birth-weight: 2483g) (33) . In this study, Skilton et al. (33) investigated the hypothesis that EPA and DHA dietary intake in childhood lowers BP at 8 to15 years of age. As reported by these authors, low-birth weight was associated with increased risk of hypertension in later life (33). They used two 24-hour dietary recalls and categorized the EPA and DHA consumption by tertiles (low, medium, and high). Results showed an inverse association between the group in the highest tertile of EPA and DHA intake and SBP (-4.9 mm Hg; 95% CI: -9.7 to -0.1) compared to

the group in the lowest tertile. The low-birth weight group with the highest EPA and DHA intake had the same BP levels as normal weight children (33).

Both studies have some limitations. To assess LCPUFA consumption, the NHANES cross-sectional study used two 24-hour dietary recalls. This dietary assessment method might not be representative of the usual or long-term dietary habits of the individual. It is also a self-reported dietary method that relies on the subject's memory and can under- or overestimate portion sizes. The Dutch study used only one sample of human milk which may or may have not been representative of the FA composition of human milk during the lactation period, and other food sources of FA intake throughout infancy were not collected (32) . As a biomarker of EPA and DHA intake, RBC FA composition is best used for short-term LCPUFA intake and considered less valid for long-term intake (32). Finally, the initial study was designed to examine incidence of asthma and allergy in this cohort and not BP, thus making its observations weak in comparison to trials that are designed with the specific aim of evaluating BP as the main outcome.

Both observational studies showed an inverse association between LCPUFA intake and BP levels, but major differences between baseline characteristics of subjects and dietary assessment methodologies, make the comparison of these two studies complex. Overall, cross-sectional designs are useful to investigate associations between n-3 PUFA and BP but they cannot confirm causality. On the other hand, RCTs can determine the effect of LCPUFAs on cardiovascular risk markers.

Forsyth et al. (2) showed that children who were bottle-fed with LCPUFA supplemented formula during the first four months of life had lower mean DBP (n=65; 95% CI: -6.5 mm Hg to -0.6 mm Hg; $p = 0.0018$) and mean BP (95% CI: -5.4 mm Hg to -0.5 mm Hg; $p = 0.02$) at six

years of age compared to standard formula-fed children. However, breastfed infants and DHA supplemented formula-fed infants had comparable BP levels. They linked the lowering BP effect of human milk to its DHA content. This trial lacked data on dietary n-3 LCPUFA intake between the end of the trial period (four months old) and BP levels at six years of age, thus making it difficult to draw a cause-and-effect conclusion between these two variables.

Similar to Forsyth et al. (2), another RCT investigated the effect of three months' fish oil (FO) supplementation on BP of infants from 9 to 12 months (12). This trial was a 2x2 factorial design in which four groups were formed (n=83): milk or formula-fed group without FO (-FO) and milk and formula-fed group with FO (+FO). The results showed that at 12 months adjusted for age and all confounding factors, SBP was lower in the + FO group (+ FO: 100.4 mm Hg (±2.4) vs -FO: 106.7 mm Hg (±2.2), p-value < 0.02). In addition to dietary intake, they measured plasma erythrocyte (RBC) FA concentration as a biomarker of fish oil intake. RBC EPA was positively associated with fish intake. They found a weak inverse association between RBC DHA and SBP (β= -1.81, $p < 0.05$) and no association between RBC EPA and BP (12).

In contrast to Forsyth et al. (2), de Jong et al. (31) did a follow-up study on children who were previously part of a double-blind RCT ("The Groningen LCPUFA Study"). They investigated the effect of LCPUFA supplementation during the first two months of life on BP at nine years of age. The authors concluded that short-term LCPUFA supplementation had no influence on BP and other markers of CVDs (31). While short-term RCTs can be valuable, long-term RCTs allow better tracking of outcomes overtime. In that regard, Ayer et al. (13) conducted a longitudinal trial to examine the effect of fish oil supplementation in children, from the time of weaning or introduction of solids to five years of age. Children were assessed at 18 months, three years, and five years of age (the end of the trial period) for BP and plasma FA composition. The

intervention group (n= 304) received a daily supplement of 500 mL capsule of tuna oil /day with goal of increasing the n-3 to the n-6 ratio, and the control group (n= 304) received a daily supplement of a vegetable capsule with a ratio n-3: n-6 ranging from 1:15 to 1:20, representative of the Australian diet (13). Similar to de Jong et al. (31) findings, no differences in BP were found between the diet intervention and the control groups, at 8 years of age (13).

Overall, RCTs on LCPUFA intake and BP in children have resulted in mixed findings. The dose and duration of LCPUFA intake, study participants' characteristics, and methodologies used to assess the association between LCPUFA status and BP are different in each trial. These design elements make the comparison of these investigations difficult.

LCPUFA intake and blood pressure in adolescents

In a cross-sectional study within the prospective Raine Study, O'Sullivan et al. (34) investigated the relationship between dietary FA intake and BP in 14-year-old adolescents (n=814). They used a 3-day food record to evaluate PUFA consumption. Their results showed an inverse association between intake of LA, ALA, and n-3 PUFA and SBP in boys (34). Similarly, another cross-sectional study examined the association between LCPUFA intake and incidence of metabolic syndrome in a cohort of Danish children at 17 years of age (n=109) (35). They used a 7-day food record, pre-coded by category, and erythrocyte (RBC) FA composition from fasting blood samples to evaluate omega-3 FA consumption and DHA status. In contrast to the Raine Study, they found a positive association between RBC-DHA status and SBP (35). As noted by the authors, these contrasting and unexpected results could possibly be due to dietary habits or lifestyle factors commonly seen in teens that were not captured in their data analysis, such as high intake of food or lack of physical activity (35).

Both studies found gender specific differences between LCPUFA intake and BP (34, 35). The proposed explanation was that sex hormones may play a role in modulating either BP (34) or the effect of PUFA on tissue lipid composition (36). The correlation between DHA ($r=0.2$; $p<0.001$) and LA ($r=0.1$; $p<0.001$) dietary intake against RBC-FA status was significant in the Raine study (34) and a positive association was found between fish intake and RBC-DHA status in the Danish cohort (36). Erythrocyte FA content is a good biomarker of LCPUFA intake but it only indicates short-term dietary intake (32).

Pederson et al. (37) conducted a 16-week RCT with 13-15-year-old (n=78) slightly overweight Danish boys to examine the effect of fish oil supplementation on CVD risk factors during their pubertal growth. They administered 1.5g/day supplement of n-3 LCPUFA to the intervention group and vegetable supplement to the control group. At the end of the intervention, both SBP and DBP were lower (3.8 ± 1.4 mm Hg, $p < .006$); 2.6 ± 1.1 mm Hg $p<.01$) in the FO group compared with the control arm. They also found a significant inverse correlation between changes in RBC EPA and BP when adjusted for baseline values and other confounders (37).

Only a limited number of studies have looked at the influence of n-3 LCPUFA consumption in adolescence and BP levels, with conflicting results. These studies are different in methodology, in subject characteristics, and in duration of exposure to LCPUFA.

Conclusion

In light of this review, the current evidence of an association between LCPUFA supplementation in early life on BP in later life is inconsistent and no conclusion can be drawn in the healthy term pediatric population. Future RCTs should examine the effects of different doses of DHA supplementation on metabolic health outcomes. There is also a need for long-term RCTs with the ability to assess LCPUFA intake and its tracking effect on BP at various stages in life.

Chapter 3: Methods

Subject

The aim of this study was to establish whether DHA supplementation throughout infancy results in lower blood pressure in children between 4 and 6 years of age who become overweight or obese. A detailed description of the "DHA Intake And Measurement Of Neural Development" (DIAMOND) study has previously been published (38). Briefly, this was a double-blinded, placebo controlled, randomized, parallel-group trial, investigating the maturation of infant visual acuity in relation to four different levels of DHA supplemented formula-fed infants at one year of age (38). The original cohort included 343 infants (1 to 9 days old), who were recruited and enrolled from two sites located in Dallas, TX and Kansas City, KS. The inclusion criteria included full-term, healthy, singleton-birth infants (37-42 weeks gestation), and all subjects able to complete the 12-months trial. All infants born with pre-existing chronic illnesses were excluded from the study. Consented participants were randomized into one of the four groups of cow's milk-based infant formulas composed of matching nutrients except for its DHA amount: control (0% DHA), 0.32% DHA (17 mg/100 kcal), 0.64% DHA (34 mg/100 kcal), or 0.96% DHA (51 mg/100kcal) of total fatty acid. The ARA content (0.64% of total FA) of all DHA-supplemented formulas were similar, except for the control formula that had no ARA. A subset of 77 infants from the Kansas City cohort had their BP and BMI measured at 6-months interval, from 4 to 6 years of age. All four formulas had the same concentrations of linoleic acid (16.9-17.5% fatty acids) and alpha-linolenic acid (1.61-1.98% fatty acids). The DHA and ARA sources were single-cell algal and fungal oils respectively, (Martek Biosciences in Columbia, MD).

Subjects were randomized using a sealed envelope method that allocated subjects to one of 4 groups with 2 colors per group; and were followed for 12 months. Infants were to be fed

exclusively with the assigned formula until 4 months of age and could be introduced to other food sources subsequently, based on the physician's recommendation. But parents were advised not to give any additional DHA supplement until the end of the 12-month trial period.

Ethics

The DIAMOND study protocol was approved by the Institutional Review Boards at the participating hospitals in Dallas, TX and Kansas City, KS as well as the University of Missouri Kansas City, MO. The written informed consent was presented to infant's parents or guardians prior the study.

Design and data evaluation

This is an evaluation of secondary data obtained from children enrolled in the Kansas City cohort of the DIAMOND study (38). Data are measures of SBP and DBP taken at 5 different time points. Each subject (n=77) had their SBP and DBP taken 3 times at each visit starting at 4 years of age, and at 6-months intervals until 6 years of age. Of the triplicate measures of SBP and SDP, average of 2 points was used as the average SBP and DBP. Other data collected were sex, DHA content of infant formulas and BMI-for-age percentiles. The database also included other covariates related to child's intake and maternal characteristics that were disregarded for our analysis.

Blood pressure measurements were taken at 6 months intervals from 4 to 6 years of age. Blood pressure was measured using "Philips SureSigns VS3". The appropriate child and toddler cuff sizes were used to run BP measurements by using the index line as an indicator of size. The cuff was placed around the child's arm and aligned with the brachial artery. Each BP measurements were performed in triplicate while the child remained seated. The method used to take the average SBPs and DBPs is described as follows: first, the Coefficient of Variance (CV)

was calculated with the two last measures of BP. If CV was greater than 0.095, then two out of the three closest measures were selected and averaged. If all measures were equidistant, then the average of all 3 measures were calculated. If only two measures were taken, then these two measures were calculated. Finally, if only one measurement was taken, this data point was discarded due to lack of verifiability. All SBP and DBP averages were further sorted by age group and DHA concentrations. Weight risk was assessed using the US 2000 CDC sex-specific BMI-for-age growth charts. Average BMI-for-age percentiles were dichotomized into two groups of underweight/normal weight ($\leq 85^{th}$ percentile) and overweight/obese ($>85^{th}$ percentiles).

The Tukey Fences method was used to remove outliers from the database to compare the average SBP and DBP for individual DHA groups. Values below Q1-1.5 IQR or above Q3+1.5 IQR were excluded (where Q1 = 1^{st} or lowest quartile, IQR = Interquartile range, and Q3 = 3^{rd} or highest quartile from the median). Data were then used to calculate mean SBP or DBP, BMI-for-age percentiles, and DHA concentrations at ages 4, 4.5, 5, 5.5, and 6 years against the reference group.

Statistical Analysis

The statistical software SAS version 9.4 was used to run a GLM Procedure effect model to evaluate the association between BMI-for-age percentiles and DHA supplementation on SBP and DBP at 4, 4.5, 5.0, 5.5, and 6 years of age. All DHA-supplemented groups were combined into one group, then stratified by BMI-for-age ($\leq 85^{th}$ and $> 85^{th}$ percentiles), and compared to the control group by weight status (BMI $\leq 85^{th}$ and $> 85^{th}$ percentiles). Outliers were included in the statistical analysis.

Chapter 4: Results

The original Kansas City cohort of the DIAMOND study enrolled 158 infants. A subset of 77 subjects had their blood pressure taken at 4, 4.5, 5, 5.5, and 6 years of age. Subject's characteristics are provided in Table 1. The subset studied for blood pressure was 64% female subjects versus 53% in the original sample, but other parameters were comparable. The number of observations differed at each visit. The total number (n) of participants at each visit considers no shows and outliers and is depicted in table 2.

Mean SBP and DBP measures at different DHA concentrations 0%, 0.32%, 0.64%, and 0.96% of total FA) by age group (4, 4.5,5,5.5, and 6 years) dichotomized by weight status (BMI ≤ 85%percentile for underweight/normal weight, and BMI >85% percentile for overweight/obese) are presented in tables 3 and 4. The average SBP and DBP (mm Hg) of the three DHA randomized groups and control are shown in figures 1 and 2 and Table 5 by age and BMI category. These tables and figures represented a preliminary look at BP and weight status by age. They were not used for the final statistical analysis and conclusions of my thesis.

Results from GLM PROC repeated measures of ANOVA are shown in Table 6 to 9 and Figures 3 and 4. There was a highly significant effect of BMI on SBP ($p=0.0002$) but no effect of age or group assignment (combined DHA groups vs control) (Table 6). Mean SBP (mm Hg) for each age, BMI category and group are shown in Table 7. There was no effect of group assignment, age or weight status on DBP (Table 8). Mean DBP (mm Hg) for each age, BMI category and group are shown in Table 9.

TABLE 1. Characteristics of the subset cohort in comparison with the original study population

Characteristics	**Subset study population (n = 77)**	**Original study population (n = 158)**
Child characteristics		
Female [n (%)]	49 (64%)	84 (53%)
Male [n (%)]	28 (36%)	74 (47%)
Weight at birth (g)	3401.8 ± 350.4	3392.2 ± 377.2
Length at birth (cm)	50.0 ± 1.6	50.2 ± 1.9
Mother Ethnicity		
African American [n (%)]	51 (66%)	97 (62%)
Caucasian [n (%)]	19 (24%)	50 (31%)
Hispanic [n (%)]	5 (7%)	9 (6%)
Other [n (%)]	2 (3%)	2 (1%)

TABLE 2. Number of subjects at each visit by age group, and systolic and diastolic blood pressure measures including missing data and outliers (total n = 77).

Age groups (years)	**4.0**	**4.5**	**5.0**	**5.5**	**6.0**
SBP[1] (n)	57	63	64	63	65
SBP Missing data (n)	16	10	11	11	11
SBP outliers (n) [2]	4	5	3	4	2
DBP[3] (n)	58	60	62	59	64
DBP missing data (n)	15	9	11	13	10
DBP outliers (n)	4	8	4	5	3

[1]SBP= systolic blood pressure
[2]Outliers are shown here but were included in GLM PROC analysis
[3]DBP=diastolic blood pressure

TABLE 3. Average systolic blood pressure (mm Hg) by age group, control/DHA groups and weight status (BMI ≤ 85th and >85th percentiles).

	Control		DHA-0.32%		DHA-0.64%		DHA-0.96%	
	Mean SBP ± SD [n][1]							
Age, years	**BMI ≤ 85%**	**BMI > 85%**	**BMI ≤ 85%**	**BMI > 85%**	**BMI ≤ 85%**	**BMI > 85%**	**BMI ≤ 85%**	**BMI > 85%**
4	95.9 ± 10.1 [10]	97.8 ± 8.1 [8]	99.9 ± 5.4 [8]	102.0 ± 6.1 [10]	100.9 ± 2.9 [6]	101.1 ± 2.2 [4]	98.6 ± 10.0 [11]	102.6 ± 12.8 [6]
4.5	98.3 ± 6.0 [12]	96.0 ± 5.1 [3]	99.9 ± 4.3 [7]	101.9 ± 7.9 [7]	101.4 ± 8.4 [9]	109.9 ± 9.6 [5]	98.5 ± 7.3 [13]	101.8 ± 8.1 [7]
5	98.2 ± 6.5 [12]	106.3 ± 0.4 [2]	97.8 ± 5.9 [8]	102.6 ± 6.6 [8]	98.4 ± 6.8 [9]	102.9 ± 5.5 [6]	97.3 ± 4.2 [10]	107.7 ± 12.8 [9]
5.5	102.4 ± 10.1 [11]	104.3 ± 5.3 [4]	109.4 ± 7.0 [5]	101.2 ± 6.8 [10]	102.5 ± 4.5 [9]	103.8 ± 2.3 [4]	99.1 ± 7.0 [11]	102.4 ± 9.5 [9]
6	100.3 ± 5.6 [10]	108.9 ± 11.2 [4]	98.8 ± 4.7 [8]	104.6 ± 9.3 [7]	102.6 ± 5.7 [10]	102.7 ± 6.4 [5]	98.5 ± 3.8 [13]	103.4 ± 11.3 [8]

[1]SBP ± SD [n] = mean systolic blood pressure (mm Hg) ± standard deviation [total number of observations]

TABLE 4. Average diastolic blood pressure (mm Hg) by age group, control/DHA groups and weight status (BMI ≤ 85th and >85th percentiles).

	Control		**DHA-0.32%**		**DHA-0.64%**		**DHA-0.96%**	
	Mean DBP ± SD [n][1] (mm Hg)							
Age, years	**BMI ≤ 85%**	**BMI > 85%**	**BMI ≤ 85%**	**BMI > 85%**	**BMI ≤ 85%**	**BMI > 85%**	**BMI ≤ 85%**	**BMI > 85%**
4	57.3 ± 5.5 [9]	59.8 ± 6.0 [2]	62.1 ± 7.3 [8]	57.6 ± 9.2 [10]	56.4 ± 6.5 [8]	59.5 ± 5.0 [4]	61.4 ± 7.6 [11]	64.3 ± 11.7 [6]
4.5	62.2 ± 3.1 [10]	61.0 ± 7.0 [3]	59.9 ± 7.6 [7]	60.6 ± 5.5 [7]	58.3 ± 6.5 [8]	66.6 ± 8.9 [7]	61.6 ± 2.3 [12]	63.6 ± 4.9 [8]
5	62.9 ± 7.0 [12]	58.8 ± 3.2 [2]	60.3 ± 6.7 [8]	65.4 ± 2.6 [7]	62.5 ± 6.1 [9]	63.1 ± 3.3 [6]	61.8 ± 5.1 [11]	62.6 ± 4.6 [7]
5.5	64.6 ± 5.4 [10]	67.6 ± 8.1 [4]	64.9 ± 3.4 [4]	59.4 ± 8.6 [9]	69.1 ± 14.0 [7]	69.8 ± 4.8 [5]	63.6 ± 11.8 [11]	62.3 ± 6.8 [9]
6	65.6 ± 3.7 [11]	68.0 ± 10.7 [5]	61.9 ± 5.3 [8]	65.6 ± 9.2 [7]	63.7 ± 7.1 [10]	70.9 ± 2.1 [4]	60.3 ± 4.4 [13]	63.0 ± 4.4 [6]

[1]DBP ± SD (n) = mean diastolic blood pressure (mm Hg) ± standard deviation [total number of observations]

TABLE 5. Relationship between DHA, BMI-for-age percentiles, and blood pressure between 4 and 6 years of age.

Age (years)	Control BMI ≤85%tile	Control BMI >85%tile	DHA BMI ≤85%tile	DHA BMI >85%tile	Group	Weight	Group x Weight
		Mean SBP[1] (mm Hg) (n)					
4	95.9 (10)	97.8 (2)	100.3 (28)	102.5 (21)	NS	NS	NS
4.5	99.7 (13)	96 (3)	99.0 (31)	104.7 (20)	NS	P=0.0509	NS
5	100.7 (13)	106.3 (2)	97.6 (29)	104.7 (23)	NS	P=0.0052	NS
5.5	102.4 (11)	111.2 (5)	78.7 (27)	73.9 (24)	NS	NS	NS
6	102.0 (11)	104.3 (5)	99.9 (31)	103.7 (20)	NS	NS	NS
		Mean DBP[2] (mm Hg) (n)					
4	65.5 (11)	59.8 (2)	61.3 (28)	60.4(21)	NS	NS	NS
4.5	61.1 (11)	61 (3)	60.9 (27)	64.2 (23)	NS	NS	NS
5	62.9 (12)	58.8 (2)	61.1 (29)	63.5 (23)	NS	NS	NS
5.5	62 (11)	67.6 (4)	67.0 (27)	63.6 (23)	NS	NS	NS
6	65.6 (11)	68 (5)	62.5(32)	65.5 (19)	NS	NS	NS

[1]SBP: systolic blood pressure (n)
[2]DBP: diastolic blood pressure (n)

TABLE 6. GLM PROC Model to test the nested effects of weight status, age, and group (DHA vs control) on systolic blood pressure (mm Hg).

Source		DF	Sum of Squares	Mean Square	F Value	P > F
Model		6	1483.45752	247.24292	3.13	0.0054
Error		324	25580.02586	78.95070		
Corrected Total		330	27063.48338			
Type I SS	**Age**	4		97.474991	1.23	0.2960
	BMI	1		1069.531645	13.55	0.0003
	DHA	1		24.025909	0.30	0.5816
Type	**Age**	4		80.036217	1.01	0.4003
II, III, IV SS	**BMI**	1		1092.607259	13.84	0.0002
	DHA	1		24.025909	0.30	0.5816

TABLE 7. The least square estimates of systolic blood pressure 1 for each group of age, by BMI or DHA groups.

AGE (Years)	SBP[1] LS[2] Mean (mm Hg)
4	101.535246
4.5	101.886787
5	101.543506
5.5	104.172108
6	102.354683
BMI ≤ 85th percentile	100.394401
BMI > 85th percentile	104.202532
Control group	102.624028
DHA group	101.972904

[1]SBP: systolic blood pressure
[2]LS: least square

TABLE 8. GLM PROC Model to test the nested effects of weight status, age, and group (DHA vs control) on DBP (mm Hg).

Source		DF	Sum of Squares	Mean Square	F Value	P > F
Model		6	575.68375	95.94729	1.30	0.2581
Error		321	23749.83378	73.98702		
Corrected Total		327	24325.51753			
Type	**Age**	4		119.2092797	1.61	0.1711
I SS	**BMI**	1		48.0477272	0.65	0.4209
	DHA	1		50.7989088	0.69	0.4079
Type	**Age**	4		116.6367361	1.58	0.1803
II, III, IV	**BMI**	1		66.2761624	0.90	0.3446
SS	**DHA**	1		50.7989088	0.69	0.4079

TABLE 9. The least square estimates of DBP1 for each group of age, by BMI or DHA groups.

AGE (Years)	DBP LS[2] Mean (mm Hg)
4	62.0611379
4.5	62.7110149
5	62.5795355
5.5	65.0301124
6	64.6600517
BMI ≤ 85th percentile	62.9376011
BMI > 85th percentile	63.8791399
Control group	63.8877661
DHA group	62.9289749

[1]DBP: diastolic blood pressure
[2]LS: least square

FIGURE 1. Average Systolic Blood Pressure (SBP) (mm Hg) by DHA/control group and weight status at 4, 4.5, 5, 5.5, and 6 years of age.

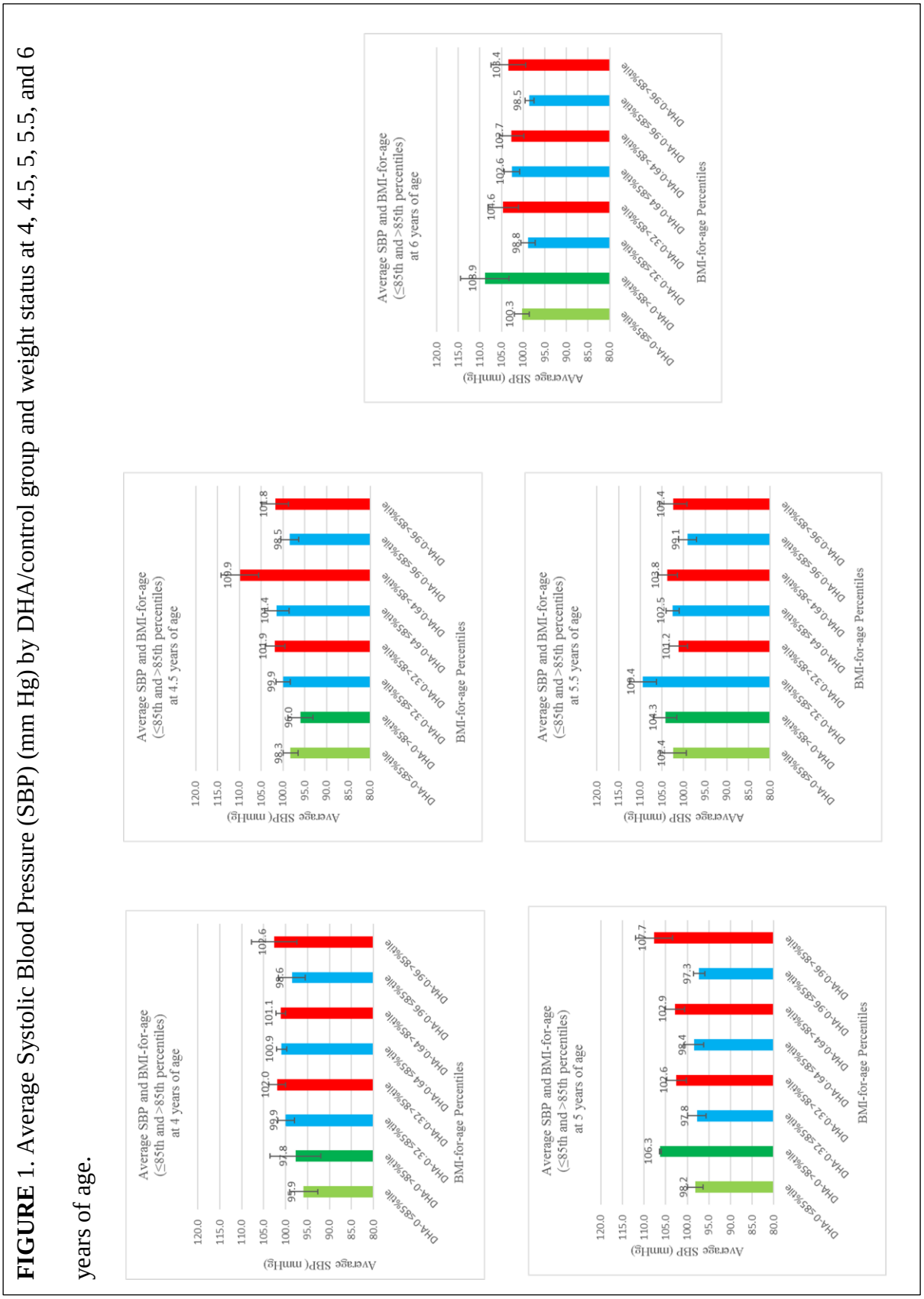
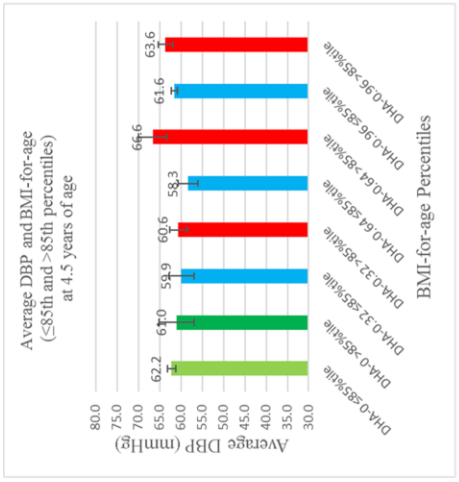
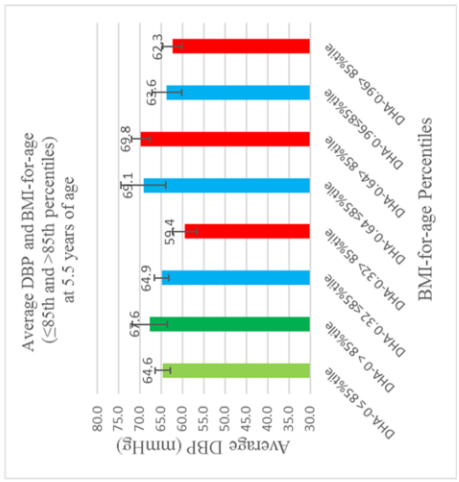
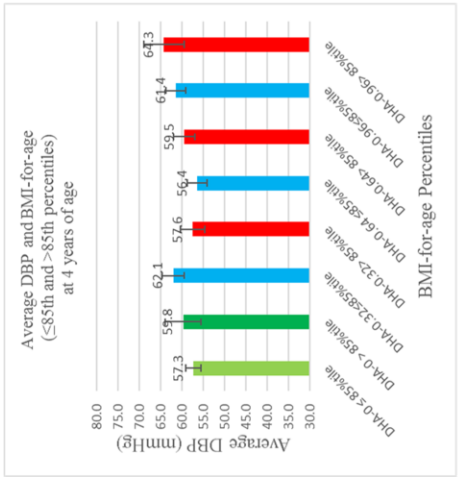
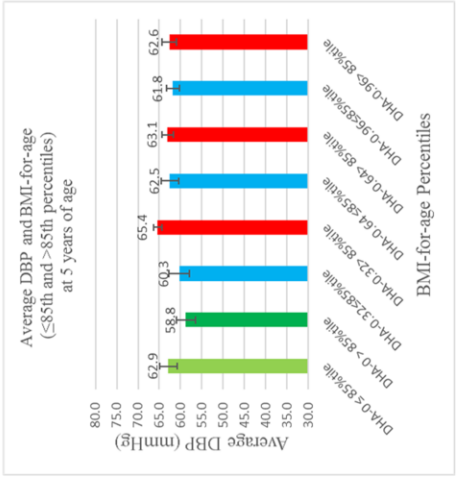


FIGURE 2. Average Diastolic Blood Pressure (DBP) (mm Hg) and by DHA/control group and weight status at 4, 4.5, 5, 5.5, and 6 years of age.









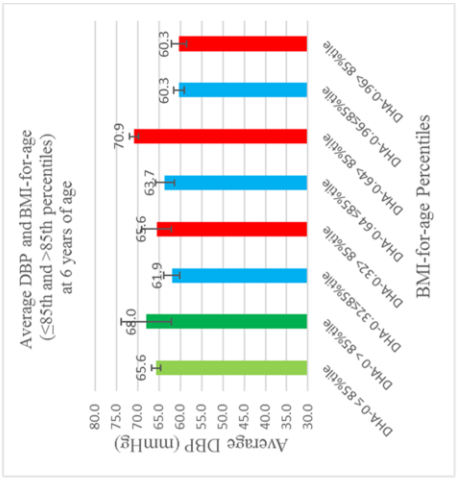


FIGURE 3. Interaction between weight status, DHA/control groups and age on systolic blood pressure (mm Hg)

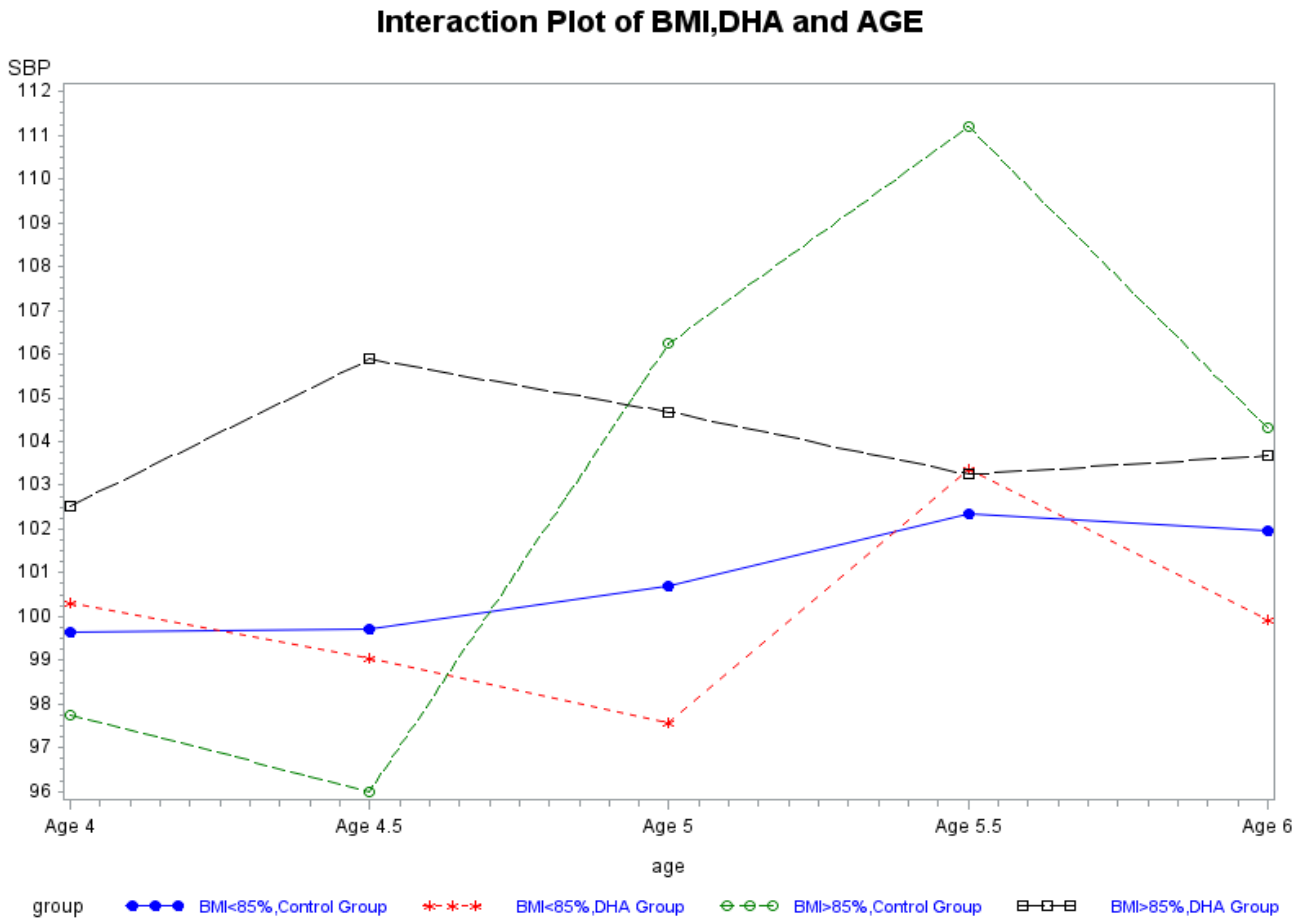
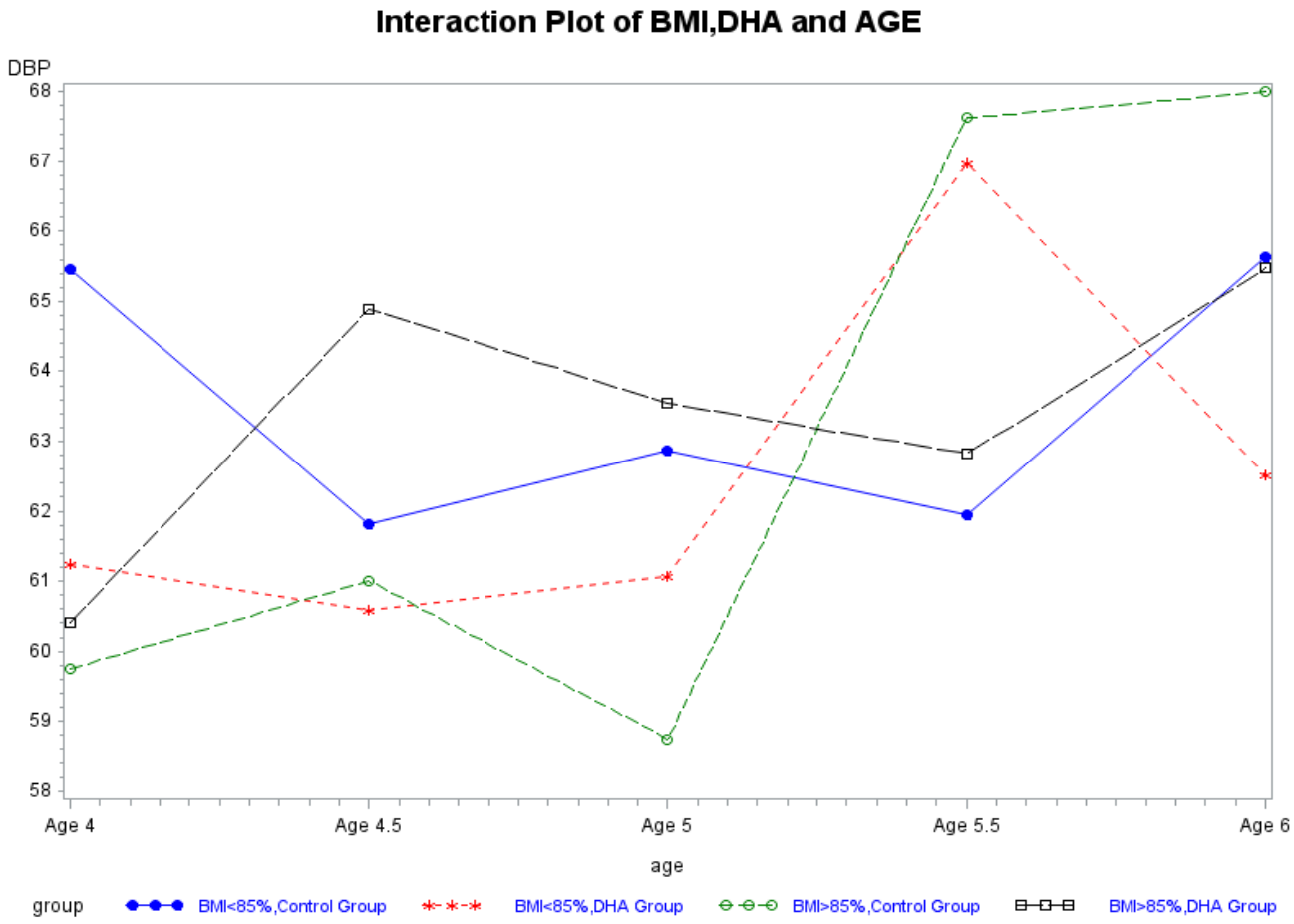


FIGURE 4. Interaction between weight status, DHA/control groups and age on diastolic blood pressure (mm Hg)



Chapter 5: Discussion

The data did not show that DHA supplementation during infancy is associated with protection against higher BP in children who became overweight or obese in the ages of 4 to 6 years compared to those who were fed the non-supplemented formula. An earlier RCT trial with a larger sample size, found infants fed LCPUFA- supplemented infant formula for 4 months had significantly lower DBP at 6 years of age compared to those who were fed a non-supplemented infant formula (2). Another controlled trial with teenage boys (13-15 years of age) found lower SBP and DBP following a 16-week dietary intervention with fish oil supplements (37), however, that trial is different in that the supplements containing n-3 LCPUFA were being consumed while the other historical trial and mine are related to possible early programming. My results are consistent with two previous studies that did not find any changes in BP; one study examined infant diet with DHA-supplemented infant formula and BP at 9 years of age (11), and the other looked at fish oil supplementation at time of weaning or introduction of solid and BP at 8 years of age (13).

There was also no suggestion of a dose-response effect for BP among various DHA concentrations compared to the control group. To our knowledge, no previous studies have looked at this factor, so we could not compare our results.

Timing of exposure to specific nutrients during fetal and child growth and development is believed to be a key factor in determining the relationship between early life exposure and later life health outcome. Researchers of the "Project Early Nutrition" (40) consortium assert that early nutrition programming effect is partly contributing to childhood obesity. In support of this project, Andersen et al. (41) found that offspring's BMI at 7 years of age was positively related

to mother's gestational weight gain during the first and second trimesters of pregnancy (weeks 12-32) but not the last trimester.

In relation to maternal DHA supplementation and BP, Hilton (42) showed that overweight/ obese (>85th BMI percentile) offspring of mothers who received DHA supplementation (600 mg/day) during pregnancy (2nd and 3rd trimesters), had lower SBP and DBP compared to overweight/obese children in the placebo group. In addition, these children had similar SBP and DBP as the underweight/normal (BMI ≤ 85th percentile) children in the placebo group. Therefore, improving maternal DHA status may be protective against BP in offspring who become overweight/obese at 5 years of age. However, our findings do not suggest any protection against BP when DHA supplementation is provided postnatally to term infants. One possible explanation for these contrasting results maybe that timing of exposure to DHA supplementation needs to occur at an earlier stage, prenatally rather than postnatally, to see desirable changes in childhood BP.

In addition to the time of DHA supplementation, this postnatally-supplemented cohort differed from the cohort studies by Hilton (42) in terms of racial make-up. Specifically, two-thirds of the children studied here were black whereas only 30% of the children whose mothers received DHA during pregnancy were black. Black race trended toward higher DBP and SBP in the study by Hilton (42); and black children were more likely to consume more than 1.9 g sodium/day. That said, mean blood pressures do not suggest major differences between the two populations except that children who were overweight and obese and who received DHA appear to have higher blood pressure in this cohort but not in the prenatally supplemented cohort.

Limitations

It was determined from this study that we can accept the null hypothesis that DHA supplementation has no effect on BP of young children who become overweight or obese, but these results should be interpreted with caution because of the small number of subjects in each group: we have as few as 10 and only as many as 31 children in each group with the number who are obese and overweight being as few as 2 in each group. The evaluation was done primarily to determine if any suggestion of an effect such as observed by Hilton (42) could be found. But, the study did not have a power analysis and it is highly likely that it is underpowered. Additionally, the small number did not permit to control for the effects of factors known to contribute to childhood BP such as sodium intake, maternal BMI, child race, and social or lifestyle factors. Also, the parent study was designed to evaluate the effect of 4 doses of DHA-supplemented formula intake on infant's visual acuity at one year of age. Therefore, this data may not have been optimal for looking at childhood BP as its primary outcome.

Had we had more children to evaluate, the longitudinal follow-up of the participants who received supplementation throughout infancy with repeated BP measurements from 4 to 6 years of age would have been a strength as similar studies provided much shorter duration of LCPUFA supplementation and did not include longitudinal follow up (2, 11, 12).

Implications

Unlike one previous study, I did not find any evidence that DHA-containing infant formulas fed during infancy influenced BP in young children who became overweight or obese between 4 and 6 years of age even though subjects in our study were fed the supplemented formulas for 12 instead of 4 months in infancy.

Conclusion

Intake of a LCPUFA-supplemented infant formula did not protect against higher BP levels seen in overweight/obese children when compared to intake of non LCPUFA-supplemented infant formula. This contrasts with another study from our population that showed a benefit for prenatal exposure to DHA.

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