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A Double-Blind Randomized Trial of Fish Oil to Lower Triglycerides and Improve Cardiometabolic Risk in Adolescents

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Abstract

Objectives: Obese children have hypertriglyceridemia and increased LDL particle number. We determined the efficacy of 4g/day fish oil to lower triglycerides and impact lipoprotein particles, inflammation, insulin resistance, coagulation, and thrombosis.

Study Design: Participants (n = 42, age 14 $\pm$ 2 yrs) with hypertriglyceridemia and LDL cholesterol < 160 mg/dl were enrolled in a randomized double blind cross over trial comparing 4 g fish oil daily with placebo. Treatment interval was 8 weeks with a 4 week wash out. Lipid profile, lipoprotein particle distribution and size, glucose, insulin, high sensitivity CRP, interleukin-6, fibrinogen, PA1-1, and thrombin generation were measured.

Results: Baseline lipid profile was total cholesterol 194 (5.4) mg/dl (mean (SE), triglycerides 272 (21) mg/dl, HDL cholesterol 39 (1) mg/dl, and LDL cholesterol 112 (3.7) mg/dl. LDL particle number was 1614 (60) nmol/L, LDL size was 19.9 (1.4) nm, and large VLDL/chylomicron particle number was 9.6 (1.4) nmol/L. Triglycerides decreased on fish oil treatment but the difference was not significant compared to placebo (-52 $\pm$ 16 mg/dl vs -16 $\pm$16 mg/dl). Large very low density lipoprotein particle number was reduced (-5.83 $\pm$ 1.29nmol/L vs. -0.96 $\pm$ 1.31nmol/L; $p < 0.0001$). There was no change in LDL particle number or size. There was a trend towards a lower pro-thrombotic state (lower fibrinogen and PAI-1; $0.10 > p > 0.05$); no other group defferences were seen.

Conclusions: In children, fish oil (4g/day) lowers triglycerides slightly and may have an anti-thrombotic effect but has no effect on LDL particles.

Clinical Trials Registration: www.clinicaltrials.gov; identifier NCT00915902

Key Words: lipids and lipoproteins, thrombosis, adolescence

Introduction

As a consequence of the obesity epidemic, the prevalence of dyslipidemia characterized by elevated levels of triglycerides and low levels of HDL cholesterol has increased, with an overall prevalence of dyslipidemia of 42% including elevated triglycerides present in obese adolescents who represent 16.9% of the pediatric population**.** [1] These dyslipidemic traits help characterize the metabolic syndrome, a phenotype associated with premature development of atherosclerosis, future type 2 diabetes mellitus, and premature cardiovascular disease in young adults. [2-4] Adolescents with this dyslipidemia may have a discordant distribution of LDL particles predicting risk higher than what would be predicted based on LDL cholesterol level alone. [5] In childhood, obesity-related metabolic derangements are associated with non-traditional risk factors leading to an insulin resistant, chronic inflammatory, and pro-thrombotic state. [6]

Fish oil (including docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)) has emerged as a controversial preventive treatment with potential benefit in the prevention of atherosclerotic vascular disease and sudden death, however the underlying therapeutic mechanisms remain unclear. While some adult clinical trials have shown a benefit of fish oil treatment on certain cardiovascular outcomes, recent meta-analyses, reviews, and clinical studies have questioned the benefit of 1g/day or lower supplementation.[7-10] Moderate doses of fish oil (4g/day) are known to lower triglycerides, however this effect alone may not be sufficient to explain clinical outcomes as the role of triglycerides per se in atherosclerosis progression has not been elucidated. [11] Recent reviews and clinical studies have suggested benefits of fish oil with regard to improvement in lipoprotein particle distribution, chronic inflammation, thrombotic potential, non-alcoholic steatohepatitis, and endothelial function that may explain the benefit of treatment. Fish oil does not appear to impact insulin resistance in adults.[12-14]

In children and adolescents there is a paucity of clinical trial data on treatment of elevated triglycerides and on the cardio-metabolic effects of fish oil in particular. Prior trials have shown minimal or a modest effect of fish oil treatment on triglyceride levels and none have rigorously examined the impact of fish oil on the cardio-metabolic factors that may be associated with benefit including change in lipid particle distribution, inflammatory markers, liver enzymes, and thrombosis. [15, 16] Therefore, we conducted a multicenter, randomized, double-blind, placebo, crossover trial of 4g daily of fish oil in adolescents with elevated triglycerides (> 150 mg/dl). The primary end point was reduction in triglycerides with secondary aims of characterizing the LDL particle distribution of adolescents with elevated triglycerides and assessing impact of fish oil on lipid particle distribution, and markers of inflammation, insulin resistance, coagulation, and thrombosis.

Methods

This study was approved by the Institutional Review Boards at A. I. DuPont Hospital for Children, Thomas Jefferson University, and Johns Hopkins University Hospital. Parental permission and child assent were obtained prior to enrollment. The clinicaltrials.gov identifier is NCT00915902.

At A. I. DuPont Hospital for Children, Thomas Jefferson University, and Johns Hopkins University Hospital, adolescents seen in lipid referral clinics with the following characteristics were recruited: age 10-17 years, fasting triglyceride level of $\geq$ 150 mg/dl and < 750 mg/dl on 2 separate occasions, and LDL cholesterol level < 160 mg/dl. Exclusion criteria were any bleeding abnormality, diabetes mellitus, uncontrolled hypothyroidism, liver disease, allergy to fish/shellfish, chronic use of aspirin or anti-inflammatory agents, taking lipid lowering medication; LDL cholesterol levels > 160 mg/dl, smoking, alcohol use, pregnancy, or participation in another clinical trial. All 42 participants were Tanner 4 or greater. Racial/ethnic composition was Caucasian, non-Hispanic (36), Caucsasian Hispanic (3), African American (2) and other (1).

This randomized, double-blind, placebo, crossover trial consisted of two 8-week treatment periods, separated by a 4-week washout. Eligible patients were randomized to receive either fish oil 4 g daily or corn oil placebo during the first 8-week treatment period; patients received the alternate treatment during the next treatment period. GlaxoSmithKline supplied study drug (containing minimally 1.5 g DHA and 1.86 g EPA ethyl esters) and placebo.

Patients were evaluated t 6 time points: Visit 1/baseline (week 0), Visit 2/randomization (week 4), Visit 3/after treatment 1 (week 12) , Visit 4/after wash out (week 16), Visit 5/after

treatment 2 (week 24) and Visit 6/close out (week 28) . Patients were advised to maintain a stable diet and not alter baseline fish consumption . One participant took an oral contraceptive throughout the trial. Any fish oil supplements were discontinued. Advice on a heart healthy diet was provided. Blood pressure (right arm sitting with appropriate sized cuff, taken 3 times, last measurement used), height, and weight were measured at the beginning of the study, after the first wash out period, and close out. Participant phone contact was made during each treatment arm to assess diet stability. Fasting lipid profile was performed at every visit. Red blood cell fatty acid profile and secondary endpoints were performed at all visits except baseline. Secondary endpoints included parameters of thrombin generation, high sensitivity c reactive protein (CRP), interleukin-6 (IL-6), fibrinogen, and plasminogen activator inhibitor-1 (PAI-1). Lipid particle measurement and apolipoprotein B (apo B) measurement were performed at baseline and at the end of each treatment arm. Glucose, insulin, alanine aminotransferase (ALT), and aspartate-aminotransferase (AST) were obtained at baseline and at the end of each treatment arm. Adverse events were recorded at all study encounters. Adverse events were graded mild, moderate, or severe. An independent data and safety monitoring physician reviewed all events and safety data biannually.

Lipid, lipoprotein, and lipoprotein particle analysis was performed by a commercial laboratory (Liposcience Inc.; Raleigh, NC) as were glucose, insulin, ALT, and AST. Red blood cell fatty acid profile was obtained commercially (OmegaQuant, Sioux Falls, SD) to assess compliance and test the completeness of the washout between study arms. Thrombin generation was measured using a commercially available assay according to the guidelines of the manufacturer (Technothrombin-TGA, Technoclone). Evaluation of thrombin generation was done automatically using the manurfacturer's Technothrombin TGA evaluation software and

calculated as thrombin generation over time. For analysis, the peak height for thrombin generation, velocity index or peak rate of thrombin generation [peak thrombin/(peak time – lag time)] and the area under the curve which is also referred to as the endogenous thrombin potential (ETP) were used. Fibrinogen and CRP were measured by immuno-nephelometry (Siemens Healthcare Diagnostics, Deerfield IL). Enzyme-linked immunosorbent assays (ELISA) were used for measurement of IL-6 (R&D Systems, Minneapolis, MN) and PAI-1 (American Diagnostica, Greenwich, CT). All measurements were performed in replicate.

Data analysis: Study data were collected and managed using REDCAP electronic data capture tools hosted at A. I. DuPont Hospital for Children.[17] The study was powered to detect a difference in triglycerides of 50 $\pm$ 20 mg/dl between groups. Demographics and baseline characteristics were summarized by treatment sequence and visits. Quantitative variables were summarized using mean and SE, and categorical variables were summarized by frequencies and percentages. Two sample t-test was used to compare the mean baseline characteristics between treatment sequences and chi-square test was to compare the distribution of categorical variables between treatment sequences. Mean (SE) change in red cell membrane fatty acids from baseline (in period 1) and washout baseline (in period 2) were by placebo and fish oil. In addition, overall mean change were presented by placebo and fish oil groups. A mixed model repeated measures analysis of variance (ANOVA) was used to compare the mean change in red cell membrane fatty acid within as well as between Placebo and Fish Oil group. The change from baseline or washout baseline values were used as the response variables, while period (pre or post washout), treatment sequence, and their interactions were used as factors in the model. Each of the models was adjusted for baseline values of the corresponding response variable. Subjects were used as a random factor and an unstructured (natural structure of within subject correlation) within subject

correlation was used for the modeling purpose. The same analyses were performed for lipids, lipoprotein particles, thrombotic and inflammatory markers. In addition to baseline values of the response variables, other variables such as age and gender were used in the latter models if found significant with the change in corresponding response variables. Mean and SE of lipids, some of the lipoproteins and, inflammatory markers were presented in graphic form. All tests were two tailed at the level of significance of 0.05. Comparisons were repeated after BMI adjustment and results were similar, these results are not reported. The statistical software SAS (version 9.3, SAS Institute, Cary, NC, USA) and SPSS (version 18.0, SPSS IBM Corporation, Armonk, NY, USA) were used for data analysis.

Results

The cohort comprised 42 adolescents with a mean age of 14 $\pm$ 2 years, 13/42 were female. Three subjects did not complete the protocol; one patient was unable to adhere to the visit schedule and two patients had severe behavioral issues that led to subject withdrawal. Characteristics of the group for demographic variables, blood pressure, body mass index, and heart rate are shown in Table 1 stratified by randomization sequence. The cohort was obese with mean body mass index > 30 kg/m^2. All values were comparable at the beginning of each arm. At baseline both treatment sequences had comparable values for AST, ALT, glucose, and insulin. Table 1 also shows the lipid profile values stratified by randomization sequence. Again, the lipid values were comparable for each group with severely elevated triglycerides, low HDL cholesterol, and borderline levels of total and LDL cholesterol present.

Table 2 (online only) presents the red cell fatty acid composition data for 5 key fatty acids: oleic acid (C18 1n9), linoleic acid (C18 2n6), eicosapentaenoic-n3 acid (EPA) (C20 5n3), docosapentaenoic-n3 acid (DPA) (C22 5n3), and docosahexaenoic acid (DHA) (C22 6n3). Values are the percent of total red cell fatty acids for each particular fatty acid. During both treatment periods, comparable rises in EPA, DPA, and DHA occurred in those treated with fish oil. Conversely, there were declines in oleic and linoleic acid in these groups. In those treated with placebo, there were no significant changes in oleic and linoleic acids. The washout after the first treatment arm was incomplete for those in the fish oil group and a further decline in EPA, DPA, and DHA occurred during the second treatment phase that was not observed in the first treatment phase. Compared to baseline, the group treated with fish oil first had EPA 0.64 (0.07)%, DPA 0.55 (0.08)%, and DHA 2.11 (0.21)% levels above baseline at the end of treatment arm 2, all $p < 0.0001$.

The results for the change in lipid levels after fish oil and placebo treatments are shown in Table 3 (adjusted for age, gender and baseline value) and Figure 1 (unadjusted). Overall, there was a significant decline in triglyceride levels compared to baseline levels of 52 $\pm$ 16 mg/dl (slightly < 20%) but this was not statistically significant compared to the placebo control group which had a small decline of 16 $\pm$ 15 mg/dl during the intervention (p=0.11). With log transformation of triglyceride values, this result became marginally significant ($p < 0.05$). The difference between groups achieved by fish oil was similar in the first and second treatment arms (29 or 35 mg/dl). Both HDL cholesterol (2.0 $\pm$ 0.9 mg/dl) and LDL cholesterol (7.9 $\pm$ 3.3 mg/dl) increased compared to baseline in the fish oil treatment groups but only the difference in LDL cholesterol was significant between groups. In the group that received fish oil in the first arm of the trial, despite incomplete washout after the first arm, there was no further rise in triglycerides during the placebo arm.

Lipid particle distribution and apo B levels were performed at baseline and at the close of each treatment arm (figure 2, Table 4 (online only)). The average LDL particle number in this cohort at 1613 $\pm$ 80 nmol/L which is more than twice the 50th percentile recently reported in 6th grade children and average LDL particle size was 19.2 nm, well below the 25th percentile. [5] Baseline large VLDL particle number was 9.5 nmol/L and the only significant difference between groups was a decline in large VLDL particles (-5.84 $\pm$ 1.30 (fish oil) vs. -0.96 $\pm$ 1.30, $p < 0.003$). Baseline apoB levels were 97 $\pm$ 2.9 mg/dl and there was a non-significant rise in apo B level (2.8 $\pm$ 2.1 (fish oil) vs. 0.2 $\pm$ 2.0 mg/dL (placebo). There were no differences in LDL particle number or distribution on HDL particle number or distribution related to fish oil treatment.

Secondary endpoints of the trial are presented at the bottom of Table 3. The most interesting impact of fish oil treatment was on the thrombosis-related measures. These included a significant decline in PAI-1 during treatment and a trend towards a decline in fibrinogen level. Parameters of thrombin generation, CRP, IL-6, glucose, and insulin were unaffected. There was a trend towards higher ALT in the fish oil treated patients; in both groups AST declined but more so in the placebo group.

Fish oil was well tolerated. Adverse events were reported in 27 of 42 enrolled subjects with six subjects experiencing an adverse event related to the study medication. Related adverse events included gastrointestinal symptoms, fishy taste, and frequent nosebleeds. These related events were mild to moderate in nature, expected with the study medication, resolved on their own, and did not result in discontinuation of study medication; however, the dose was reduced in two patients as a result of the adverse event.

Discussion

This study has shown, in adolescents with hypertriglyceridemia, that 4g/day treatment with fish oil for 8 weeks results in a modest decline in triglyceride levels of about 20% that was not significantly different than a corn oil placebo. This decline occurred as a result of a reduction in large VLDL particles without a comparable shift in HDL or LDL particle distribution. LDL cholesterol increased slightly and there was a non-significant rise in apo B levels. A trend towards a lower thrombotic state was observed using measures of PAI-1 and fibrinogen. There was no effect on glucose, insulin, thrombin generation or markers of inflammation (CRP and IL-6). Fish oil at a dose of 4g/day was well tolerated. Red blood cell fatty acid levels confirmed compliance with treatment.

Additional findings in these obese children are the very high level of LDL particles and apo B. The Healthy Study, a recent cross-sectional study of 6th grade children, provided an LDL particle distribution; the median and 75th percentile values for LDL particle number were 667 and 885 nmol/L, substantially lower than the values in this cohort. [5] Mean LDL size was 19.2 nm in this cohort compared to a median of 21.5 nm and 25th percentile of 20.7nm in the Healthy study. The MESA study has shown the importance of LDL particle number in predicting carotid IMT (as opposed to LDL cholesterol level). LDL particle number of participants in this trial was one standard deviation above the mean in the MESA cohort, a difference that is associated with a clinically meaningful increase in carotid IMT. [18] Also in MESA, LDL particle number was more closely associated with cardiac events than LDL cholesterol. [19] Average apo B level in this cohort, 97 mg/dl and was in the borderline high range for United States children (between 90 and 110 mg/dl). [20]

There is limited clinical trial data on the use of fish oil for cardiovascular risk reduction in childhood. Engler et al showed in children with dyslipidemia that 1.2 g/day of DHA increased concentrations of larger LDL and HDL particles and decreased concentrations of smaller LDL particles. [16] Pedersen et al compared fish oil to vegetable oil contained in bread at a 1.5 g/day dose in 78 mildly overweight Danish boys. Fish oil lowered blood pressure slightly and had no effect on triglycerides, a slight rise in HDL and non HDL cholesterol was seen. [15] A 6 month trial of 250 or 500 mg/day dosing in children with hepatic steatosis improved triglycerides and insulin sensitivity as well as reducing liver fat. [21] Collectively, these studies show inconsistent results with regard to triglyceride lowering and at lower fish oil doses than the pharmacologic dose used in this study.

A recent review of adult clinical trials documented a dose-dependent effect with a lowering of about 40 mg/dl achieved with a 4g dose, an effect that is similar to the results seen in this study. [9] Particle distribution studies in adults from studies where fish oil is given as the only lipid lowering treatment generally suggest fish oil is associated with an increase in size in LDL particles, a response that is considered more favorable for atherosclerosis prevention. [22, 23] Reports on these studies also describe a significant reduction in large VLDL particle size, a finding that was also detected in our study. A recent dietary study in Alaskan Indians showed that higher intake of fish oil was associated with lower large VLDL particles, larger HDL particles, a trend towards larger LDL particles, but no change in LDL particle number. [24] After a single high fish oil content meal compared to a low omega 3 fatty acid meal, VLDL particles are significantly reduced in size with no impact on LDL or HDL particle composition. [25] Thus, our study is generally consistent with the adult literature on the magnitude of triglyceride lowering from a 4 g/day dose, on the reduction in large VLDL particle number, and the lack of change in total LDL particle number. We did not, however, detect an impact on LDL or HDL particle size distribution.

Omega-3 fatty acids are known to have a weak anti-thrombotic effect that is insufficient to cause significant bleeding as a complication of therapy. [26] In a study by Vanschoonbeek et al, 4 weeks of fish oil treatment resulted in reduced thrombin generation and fibrinogen levels in healthy volunteers however the effect was variable and more pronounced in those with a structural fibrinogen alpha-chain polymorphism. [27] Very recent studies have suggested that exogenous modification of platelet membranes by omega-3 fatty acids results in decreased platelet pro-coagulant activity and thrombus formation. [28] Platelets show less aggregation and activation in healthy subjects who are taking omega-3 supplements.[29] Population-based studies

suggest that for an association with lower fibrinogen levels to be seen, fish oil consumption must be high, achieving red blood cell levels similar to those seen on pharmacologic therapy. [30, 31] Early studies suggested that fish oil might increase PAI-1 activity but a meta-analysis of studies of fish oil supplementation did not identify an impact of fish oil on PAI-1 activity.[32] Weight reduction appears to have a more potent effect on PAI-1 levels than fish oil consumption in adults. [33] The findings from our study, including a trend towards lower levels of fibrinogen and a significant decrease in PAI-1 levels are consistent with the limited literature on the subtle, but potentially beneficial effects of fish oil supplementation, on coagulation and fibrinolysis. The enhanced efficiency of fibrinolysis through a reduction in PAI-1, the most important and rapidly acting physiological inhibitor of plasminogen, suggests that thrombin generation is secondary to enhanced fibrinolysis in the presence of fish oil. Fish oil has been proposed as a treatment for fatty liver disease and clinical trials have shown a reduction in liver fat after treatment without significant changes in ALT or AST levels similar to our study.[13] Despite occasional studies that suggest fish oil treatment has a beneficial effect on insulin resistance and markers of inflammation, review articles and meta-analyses indicate that fish oil does not have a significant impact on these measures, which is consistent with the results of our study.[14 34]

Strengths of this study include the double-blind cross-over design, the comprehensive assessment of not only changes in lipids but lipid particles, markers of inflammation, insulin resistance and thrombosis, the use of red cell fatty acid markers to evaluate compliance, the relatively large sample size compared to other pediatric trials of fish oil treatment and the use of a pharmacologic grade preparation that also did not have a "fishy aftertaste" to confound blinding.

There are a number of limitations. The study achieved the predicted significant lowering of triglycerides but statistical significance compared to the corn oil placebo was compromised by a small reduction of triglycerides in that group. Though it is unlikely, corn oil may have impacted levels, as polyunsaturated fats do have an effect on triglycerides. The intrinsic variability of triglycerides is large and this may have confounded analyses. This variability may have been impacted by pubertal changes in a subset of the cohort. DHA and EPA may have different effects on the outcomes in our study; this could not be assessed in this design.[3535] Our red cell fatty acid level data show incomplete wash out after one month, the wash out period could have been longer. However, the difference in triglyceride response between fish oil and placebo groups in the second arm of the trial was exactly the same as in the first arm of the trial.

In summary, we detected a small reduction of triglycerides with 4 g daily fish oil treatment in children with moderate hypertriglyceridemia. There was a trend towards lower thrombotic potential. Candidates for this study had very high LDL particle number, small LDL size, and elevated apoB levels consistent with elevated risk of early atherosclerosis; a risk that is higher than predicted by calculated LDL cholesterol concentration. If fish oil 4g daily is effective in prevention of early atherosclerosis, the mechanism is likely related to reducing the number of large VLDL particles or chylomicrons or on endothelial function (not measured in this study) and related anti-thrombotic effects rather than on affecting atherogenic LDL particles. This study suggests a need for further investigation in adolescents with elevated triglycerides regarding 1) the benefits of LDL particle number lowering as opposed to triglyceride lowering, 2) the atherogeneicity of large VLDL and chylomicron particles, and 3) the secondary benefits of fish oil on thrombosis and inflammation, particularly in relation to endothelial function and early atherosclerosis.

Abbreviations

ALT alanine aminotransferase

ANOVA analysis of variance

apoB apolipoprotein B

AST aspartate-amiontransferase

CRP c reactive protein

DHA docosahexaenoic acid

DPA docosapenttaenoic acid

EPA eicosapentaenoic acid

ETP endogenous thrombin potentisl

HDL high density lipoprotein

IL-6 interleukin 6

LDL low density lipoprotein

PAI-1 plasminogen activator inhibitor-1

VLDL very low density lipoprotein

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Figure Legends

Figure 1

Lipid values before and after placebo or fish oil treatment are shown in the graph. Triglycerides consistently decline in the fish oil treatment groups whereas they are essentially unchanged for the other lipids.

Figure 2

Lipid particle values, average particle size values, and apoB levels are shown compared to baseline values for each intervention interval. The only consistent change is a decline in large VLDL particles with fish oil treatment.