ω-3 Fatty Acid Ethyl Esters Diminish Postprandial Lipemia in Familial Hypercholesterolemia

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Key words: Chylomicron metabolism, fish oil supplementation, familial hypercholesterolemia

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ABSTRACT

Context: Impaired postprandial chylomicron metabolism induces hypertriglyceridemia and may increase risk of atherosclerotic cardiovascular disease (ASCVD). Omega-3 fatty acid ethyl ester (ω-3 FAEE) supplementation decreases plasma triglycerides. However, its effect on postprandial chylomicron metabolism in familial hypercholesterolemia (FH) has not yet been investigated.

Objective: We aimed to examine the effect of ω-3 FAEE supplementation on postprandial responses in plasma triglycerides, very-low-density lipoprotein (VLDL)-apoB-100 and apoB-48 in FH patients receiving standard cholesterol-lowering treatment.

Design, Setting and Patients: We carried out an 8-week open-label, randomized, cross-over intervention trial to test the effect of oral supplementation with 4 g/day ω-3 FAEE (46% eicosapentaenoic acid and 38% docosahexaenoic acid) on postprandial triglyceride, VLDL-apoB-100 and apoB-48 responses in FH patients following ingestion of an oral fat load.

Outcomes Measures: Plasma total and incremental triglyceride, VLDL-apoB-100 and apoB-48 0-10 h area-under-the-curves (AUCs).

Results: ω-3 FAEE supplementation significantly (P<0.05 in all) reduced fasting plasma triglycerides (-20%), apoB (-8%), VLDL-apoB-100 (-26%), apoB-48 (-36%) concentration, systolic blood pressure (-6%) and diastolic blood pressure (-6%). Postprandial triglyceride and VLDL-apoB-100 total AUCs (-19% and -26%, respectively, P<0.01) and incremental AUCs (-18% and -35%, respectively P<0.05), as well as postprandial apoB-48 total AUC (-30%, P<0.02) were significantly reduced by ω-3 FAEE supplementation.

Conclusion: Supplementation with ω-3 FAEEs improves postprandial lipemia in FH patients receiving standard care; this may have implications for further reducing ASCVD in this high risk patient group.
INTRODUCTION

Familial hypercholesterolemia (FH) is the commonest monogenic cause of hypercholesterolemia and premature atherosclerotic cardiovascular disease (ASCVD), with a population frequency of at least 1 in 500 (1). FH results principally from mutations in the low-density lipoprotein (LDL) receptors that impair LDL catabolism and markedly increase plasma LDL-cholesterol concentrations (2). While FH markedly increases the onset of premature coronary heart disease (CHD), risk can be variable and this may relate to other cardiovascular risk factors or to hidden mechanisms, including postprandial lipemia (3-6). However, despite treatment of statin, with or without ezetimibe, a significant proportion of FH patients remain at increased residual risk of ASCVD (3).

Postprandial hypertriglyceridemia reflects prolonged accumulation of triglyceride-rich lipoproteins (TRLs), including very-low-density lipoprotein (VLDL)-apolipoprotein (apo) B-100 and apoB-48-containing chylomicrons and their remnants in the circulation (7). These lipoproteins are atherogenic, contributing to inflammation, oxidative stress, endothelial dysfunction and foam cell formation (3, 7, 8). Recent evidence from experimental and human studies suggests that deficiency in LDL receptor function in FH may disturb TRL metabolism by delaying remnant clearance (9, 10). Furthermore, there is evidence showing that apoB-48 concentrations are elevated in FH due to an increased production rate (11). We have recently demonstrated that elevated fasting triglyceride concentration is a significant predictor of CHD in FH (12). Patients with FH may accordingly have impaired triglyceride response to a fatty meal irrespective of the plasma triglyceride concentrations (4-6). This may contribute to increased CHD risk in FH patients.
Fish oils are a rich source of long-chain ω-3 fatty acids, primarily eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). There is some evidence that fish oil supplementation protects against cardiovascular disease and this may be partly mediated by improvement in hypertriglyceridemia (13, 14). However, data from several meta-analyses suggest that fish oil supplementation does not lower risk of major CVD events (15-17). It should be noted, however, most of these studies used low doses of fish oil supplementation (<2 g/day). We have previously reported that ω-3 fatty acid ethyl ester (ω-3 FAEE) supplementation (4 g/day) lowers fasting and postprandial triglyceride concentrations in obese subjects (18). To date, no study has examined the effect of ω-3 FAEE supplementation on postprandial TRL response in FH patients receiving standard treatment for lowering LDL-cholesterol.

In the present study, we investigated the responses of triglyceride and apoB-48 concentrations following ingestion of an oral fat load. We hypothesized that ω-3 FAEE would reduce postprandial responses for plasma triglycerides and apoB-48 in FH patients who were on statin and/or ezetimibe treatment.
METHODS

Subjects

Twenty-two patients (11 men and 11 women) with FH aged 18-70 years [body mass index (BMI) <40 kg/m2] were recruited from the Lipid Disorders Clinic at Royal Perth Hospital. Diagnosis of FH was defined by the Dutch Lipid Clinic Network criteria (DLNC) score >8 (definite FH) and/or the presence of a pathogenic mutation in the LDLR gene (19). However, one subject withdrew consent before completing the first intervention period, and another was withdrawn because of an adverse event related to atrial fibrillation during the isotopic infusion that resolved spontaneously. Homozygous and compound/double heterozygous FH, and subjects with APOB (i.e. familial defective apoB-100 or familial hypobetalipoproteinemia) and PCSK9 mutations were excluded. None of the subjects were APOE ε2 homozygotes, nor had proteinuria, creatininemia (>120 µmol/L), hypothyroidism, or abnormal liver enzymes (alanine aminotransferase >120 U/L for men and >90 U/L for women). None reported a cardiovascular event within six months prior to the study, or were taking ω-3 FAEE supplementation (within the last six months); or anti-diabetic medication. The study was approved by the Human Research Ethics Committee of the Royal Perth Hospital, and written informed consent was obtained from all subjects.

Clinical Protocol

This study was an open-label, randomized, cross-over intervention trial (Figure 1). The study protocol included infusion of a stable isotope to assess TRL kinetics, which will be reported separately. All eligible patients were on statins (or statin plus ezetimibe) at recruitment and throughout the duration of the study. All patients entered a 4-week run-in diet stabilizing period, at the end of which they were randomized to into one of the two groups, no treatment or a 8-week treatment period of 4 g/day ω-3 FAEE supplementation (Omacor® 46% EPA and 38% DHA in ethyl ester form, Abbott Products Pty Ltd) with a 8-week washout between each intervention.
period. Compliance with ω-3 FAEE supplementation was confirmed by capsule count at the end of ω-3 FAEE intervention. Three-day food, alcohol and exercise diaries were given to the participants to complete at the end of each treatment period. Diaries were assessed for energy and major nutrients completed during the lead-in period (week 4) and at the end of each treatment period (week 12 and 28) using FoodWorks 7 Pro (Xyris, Queensland Australia). Physical activity was also assessed by a seven day recall questionnaire at the end of each treatment period (20). All dietary assessments and recommendations were conducted by a registered dietitian. All subjects were reviewed fortnightly and requested to maintain their dietary intake and level of physical activity constant.

**Comparison with non-FH controls**

For comparison purpose, 10 healthy non-FH subjects (5 men and 5 women, age 59 ± 7 years, weight 83 ± 12 kg, BMI 28.9 ± 5.7 kg/m², systolic blood pressure 126 ± 12 mmHg, diastolic blood pressure 75 ± 7 mmHg, plasma triglycerides 1.20 ± 0.56 mmol/L and total cholesterol 5.29 ± 0.87 mmol/L on no drug treatment) were also recruited from the community in which the fat load test was performed once.

**Postprandial oral fat-load test**

All subjects were admitted to the metabolic ward in the morning after a 14-h fast. They were studied in a semi-recumbent position and allowed to drink only water after the test-meal. Arterial blood pressure was recorded after 3 min in the supine position using a Dinamap1846 SX/P monitor (Critikon Inc, Tampa, FL, USA). Following a baseline fasting blood sample, a liquid formulated high fat test meal was the consumed within 2 min (a total of 4800kJ, 130 g fat, 17 g protein and 21 g carbohydrate), following with blood samples were obtained after 30, 40 min, and 1, 2, 3, 4, 6, 8 and 10 h (18). Briefly, the test meal was a milkshake consisting of 100 mL of full cream milk, 150 mL of pure cream, 70 mL of corn oil, 90 g of whole egg (approximately 2 large
eggs) and 10 g of sugar. The meal provided 470mg cholesterol and 120g fat (polyunsaturated fat 27%, monounsaturated fat 28% and saturated fat 40%). Subjects were then given a snack and allowed to go home. All the procedures were repeated after 8-week intervention period.

**Biochemical measurements**

Fasting whole venous blood samples collected in EDTA were immediately centrifuged at 1500 x g for 15 min at 4°C. Plasma was collected and stored at -80°C for analysis. Plasma lipid and glucose concentrations were measured using enzymatic methods (Hitachi 917 Biochemical Analyser, Roche Diagnostics Australia Pty Limited, Castle Hill, NSW, Australia). LDL cholesterol was estimated by the Friedewald calculation. Lipoprotein(a) was measured with a turbidimetric method using immunoglobulin G anti-human Lp(a) (Quantia Lp(a) assay and standard). Fasting insulin was measured using chemiluminescent immunometric assay (Abbott Diagnostics, North Ryde, NSW, Australia), and insulin resistance was estimated using homeostasis model assessment (HOMA) score (21). Plasma apoB-48 levels were measured by enzyme immunoassay kit with an intra-assay CV of <6% (Fujirebio, Tokyo, Japan). VLDL fraction was isolated from 3.5 mL plasma by ultracentrifugation (Optima XL-100K, Beckman Coulter, Australia) at $d=1.006$ g/mL (40,000 rpm, 16 h, 4°C). VLDL-apoB-100 was measured using ELISA kit (Mabtech, Nacka, Sweden). This kit is specific for apoB-100 and does not recognize apoB-48. Postprandial metabolism was quantified by calculating the area- and incremental under-the-curve (AUC) and iAUC, respectively, for plasma triglyceride, VLDL-apoB-100 and apoB-48 (0-10 h) using the trapezium rule. The iAUC was estimated as the difference between the area defined below the baseline concentration and the area under the plasma curve between 0 and 10 h.
Statistical Analyses

Data are reported as mean ± SEM unless specified. Skewed variables were log-transformed. Statistical significance was defined at the 5% level using a two-tailed test. Our sample size for this study was based on a previous study that ω-3 FAEE supplementation decreased the AUCs for triglyceride and apoB-48 by 30% (18). Groups were compared using independent $t$-tests using the SPSS 21 (SPSS, Chicago, IL) software. Carry-over effects of the crossover design were estimated based on the Grizzle’s model using general linear modeling (PROC GLM, SAS 9.2 SAS Institute, Cary, North Carolina, USA). If the carryover effect was not statistically significant, then the data from the two periods was combined and analyzed using paired $t$-test to estimate the treatment effect of ω-3 FAEE supplementation in our FH patients.
RESULTS

Baseline characteristics

A total of twenty patients (10 men and 10 women) completed the study. Of these subjects, seventeen patients were genetically diagnosed with FH (i.e., pathogenic mutations in the \textit{LDLR} gene) and the other three had a DLNC score $>8$ (definite phenotypic FH). The \textit{LDLR} mutations were classified as follows: Class 1 (n=3), Class 2 (n=3), Class 4 (n=2), Class 5 (n=4) and intron splice site (n=5). Of the 20 FH patients, 13 of the FH patients men were \textit{APOE} $\varepsilon3\varepsilon3$ homozygotes, 2 were \textit{APOE} $\varepsilon2\varepsilon3$ heterozygotes, 2 were \textit{APOE} $\varepsilon3\varepsilon4$ heterozygotes, 2 were \textit{APOE} $\varepsilon2\varepsilon4$ heterozygote and 1 were \textit{APOE} $\varepsilon4\varepsilon4$ homozygotes. On average, they were middle-aged (53.3 ± 3.0 years), non-obese (BMI 27.0 ± 6.3 kg/m$^2$), normotensive and normolipidemic (Table 1). Nine patients were on rosuvastatin (12.5-40 mg/day), eight on atorvastatin (40-80 mg/day) and three on simvastatin (80 mg/day). Of these, thirteen patients were also on ezetimibe (10 mg/day). Nine patients were on aspirin, three on anti-hypertension medication and four had a history of CAD event. Baseline (lead-in) average daily energy and nutrient intake of the 20 FH studied was 7270 ± 1720 kJ, 33 ± 5% energy from fat, 44 ± 8% energy from carbohydrates, 20 ± 3% energy from protein and 3 ± 2% energy from alcohol; cholesterol intake 278 ± 122 mg. The percent contribution of total fat from dietary polyunsaturated, monounsaturated and saturated fat was 23 ± 9%, 38 ± 8% and 39 ± 8%, respectively.

Body weight, blood pressure and biochemical characteristics

Table 1 shows the effects of $\omega$-3 FAEE supplementation on clinical and biochemical characteristics in the 20 FH patients. Body weight, waist circumference and BMI did not alter significantly during the intervention (P>0.05 in all). $\omega$3-FAEE supplementation significantly (P<0.05 in all) lowered systolic blood pressure (-6%) and diastolic blood pressure (-6%), plasma triglycerides (-20%), apoB (-8%), VLDL-apoB-100 (-26%) and plasma apoB-48 (-36%) concentration. $\omega$-3 FAEE supplementation tended to lower total cholesterol and non-HDL-
cholesterol concentrations, but the difference was not statistically significant (P<0.1 for both). LDL-cholesterol and HDL-cholesterol concentrations were not significantly altered with ω-3 FAEE supplementation, nor were glucose, insulin concentrations and HOMA score. No significant changes in dietary and physical activity levels were reported during the study (data not shown). Capsule counts confirmed that compliance with randomization to ω-3 FAEE intervention was >95%. There were no side effects reported with ω-3 FAEE supplementation.

**Postprandial lipid responses**

**Case vs Controls** Age, body weight, BMI, systolic and diastolic blood pressures were not significantly different between the FH patients and non-FH controls (P>0.05 for all). While there were also no significant different in fasting plasma triglycerides and VLDL-apoB-100 between the groups (1.30 ± 0.63 mmol/L vs. 1.20 ± 0.56 mmol/L and 77 ± 28 mg/L vs. 89 ± 43 mg/L, P>0.05 in both), the FH patients showed a greater triglyceride responses, as estimated by the total (27.7 + 19.9 vs. 15.8 + 5.6 mmol/L•10 h, P<0.05) and incremental AUCs (14.5 + 10.2 mmol/L•10 h vs. 7.3 + 2.8 mmol/L•10 h, P<0.05), than the non-FH controls. Postprandial VLDL-apoB-100 AUC response was higher in the FH patients than the non-FH controls (952 + 340 mg/L•10 h vs 717 + 246 mg/L•10 h), which just failed to reach significance (P=0.08). Fasting apoB-48 concentration (8.8 ± 7.7 mg/L vs. 5.0 ± 1.7 mg/L, P<0.05) and postprandial apoB-48 total AUCs (189 ± 163 mg/L•10 h vs. 92.4 ± 24.4 mg/L•10 h, p<0.05) were also significantly higher in the FH patients compared with the control group. The foregoing postprandial responses were not dependent with presence of or class of LDLR mutations (data not shown).

**ω-3 FAEE intervention** The postprandial responses for plasma triglycerides, VLDL-apoB-100 and apoB-48 to the fat load are shown in Figure 2. As seen in Figure 3, ω-3 FAEE supplementation significantly reduced postprandial triglyceride and VLDL-apoB-100 total AUCs
(−19% and −26%, respectively, P<0.01) and incremental AUCs (−18% and −35%, respectively P<0.05). Postprandial apoB-48 total AUC (0-10 h) was also significantly reduced with ω-3 FAEE supplementation (−30%, P<0.02). ω-3 FAEE supplementation lowered postprandial apoB-48 incremental AUC (−25%), but this failed to reach statistical significance (P=0.09). The effects of ω-3 FAEE supplementation on postprandial triglyceride and VLDL-apoB-100 total and incremental AUCs, as well as postprandial apoB-48 total AUCs were comparable between FH men and women and were not dependent on the presence nor class of LDLR mutations nor APOE genotype (data not shown).
DISCUSSION

The major finding of this study was that FH patients with normal fasting plasma triglyceride concentrations had impaired postprandial triglyceride, VLDL-apoB-100 and apoB-48 responses to a fat load and these abnormalities were partially corrected by ω-3 FAEE supplementation. This was reflected by decreasing both fasting concentrations and total postprandial AUCs of triglyceride, VLDL-apoB-100 and apoB-48, against a background of cholesterol-lowering therapy (statin with and without ezetimibe).

Previous studies This is the first demonstration the effect of ω-3 FAEE supplementation on postprandial TRL response in FH patients. Previous studies have only examined the postprandial effects of ω-3 FAEEs in non-FH subjects, with inconsistent results. Slivkoff-Clark et al. reported that in insulin resistant obese men, 4-week ω-3 FAEE supplementation (1.7 g/day) did not alter fasting apoB-48 concentration or postprandial apoB-48 incremental AUC (22). In non-obese healthy subjects, Park et al. reported that 4-week EPA or DHA supplementation (4 g/day) reduced total postprandial apoB-48 AUC (23). Tinker et al. found that high dose fish oil supplementation (5.2 g/day) for 6 weeks significantly reduced fasting apoB-48 concentration and total postprandial apoB-48 AUC in subjects with hypertriglyceridemia (24). We have recently reported that the addition of 16-week ω-3 FAEEs (4 g/day) to a moderate weight loss diet significantly reduced fasting and total postprandial AUCs of triglyceride and apoB-48 in obese subjects (18). The aforementioned discrepant findings might be explained by differences in subject characteristics, as well as the duration (4 to 16 weeks) and dose and composition of the fish oils employed (1.7 g to 5.2 g/day). We extend these studies by employing high dose ω-3 FAEE supplementation (4 g/day) and examining FH patients against a background of cholesterol-lowering treatment (i.e. statin with or without ezetimibe).
Postprandial lipemia in FH ApoB48-containing chylomicrons and their remnants are responsible for the transport of exogenous triglycerides from the intestine via the circulation to peripheral tissues. Remnant particles are then removed from the circulation by LDL receptor and/or LDL receptor-related protein-mediated mechanisms in the liver (25). However, the pathophysiology of postprandial hypertriglyceridemia in FH is not fully understood. It is well recognized that the classical metabolic defect in FH is hypocatabolism of LDL due to a decreased LDL receptor activity (1). In addition to reducing the catabolism of LDL, LDL receptor dysfunction can also increase the secretion of VLDLs in FH (26-28). Hence, disturbances in chylomicron metabolism in FH may collectively be due to an effect of the suppressed LDL receptor activity and, to a lesser extent, oversecretion of VLDLs. These effects potentially increase competition for hepatic uptake between chylomicron and VLDL remnants, thereby impairing the hepatic uptake of chylomicron remnants by the LDL receptor pathway. Consistent with the aforementioned mechanisms, we found that our FH patients had impaired postprandial responses for triglyceride, VLDL-apoB-100 and apoB-48 (total AUCs) following an oral fat load test. Whether FH patients exhibit oversecretion and/or impaired catabolism of TRLs (VLDL-apoB-100 and apoB48-containing lipoproteins) in the fasting and post-prandial conditions remains to be elucidated.

Mechanism of action of intervention The precise mechanism(s) of action of ω-3 FAEE supplementation lowering fasting concentrations and postprandial total AUCs of triglyceride, VLDL-apoB-100 and apoB-48 is not clear. Experimental data show that ω-3 FAEEs increase post-translational degradation of newly synthesized apoB-48 and decrease expression of apoB-48 m-RNA, and can decrease monoacylglycerol acyltransferase and diacylglycerol acyltransferase activities, resulting in a net reduction in triglyceride synthesis and apoB-48 secretion in the intestine (29-31). Hence, it is likely that ω-3 FAEE supplementation improves postprandial lipemia in FH by decreasing apoB-48 secretion. This speculation is supported by our previous report showing that in obese subjects, ω-3 FAEE supplementation lowered postprandial
plasma concentrations of triglycerides and apoB-48 chiefly by decreasing basal apoB-48 secretion without altering its catabolism (18). The lack of significant changes in apoB-48 incremental AUC suggests that ω-3 FAEEs have no significant impact on apoB-48 synthesis during the postprandial period. The precise reasons for the lack of an acute effect on apoB-48 metabolism remain to be elucidated. The reduction of fasting VLDL-apoB-100 concentration with ω-3 FAEE supplementation is consistent with studies in humans showing suppression of hepatic VLDL-apoB production with ω-3 FAEs (32, 33). This suppression is chiefly due to a decrease in triglyceride synthesis and an increase in fatty acid mitochondrial β-oxidation (34). Importantly, our findings also support the effect of ω-3 FAEE supplementation on VLDL-apoB metabolism during the postprandial state. Patsch et al found that postprandial triglyceride levels were 30% higher in patient with CAD than in non-CAD controls (35). Hence, it is possible that the 20-30% reduction in postprandial TRL responses with ω-3 FAEE supplementation in our study may be clinically important, but this requires investigation.

**Study limitations** Our study has limitations. The sample size was relatively small, but was based on a previous study that ω-3 FAEEs could decrease postprandial AUCs for triglyceride and apoB-48 by at least 20%. While the power was 85%, we might have underpowered to detect changes of small decrease in postprandial apoB-48 incremental AUC. As we employed an end-point blinded study design without a placebo control, there is the possibility that the increased performance with ω-3 FAEE intervention could be biased by our open, single-blind design. However, our findings are consistent with the postulated mechanisms of action of ω-3 FAEE on postprandial TRL metabolism. Our study did not investigate whether the higher total AUCs of triglycerides and TRLs were attributable to oversecretion and/or impaired catabolism of TRLs (VLDL-apoB and apoB-48 containing lipoproteins) in the fasting and postprandial conditions. Measurement of the kinetics of apoB-48, VLDL-apoB-100 and triglyceride may help to clarify the
mechanism of action of ω-3 FAEEs on apoB-48 metabolism. While we found that presence and class of LDLR mutation did not significantly influence postprandial triglyceride, VLDL-apoB-100 and apoB-48 responses, the individual number of patients in each class was small. A larger study selecting for class of LDLR mutation is required to examine the influence of specific gene variants on postprandial lipemia and its response to ω-3 FAEE supplementation in FH.

**Clinical implications** FH remains an under-appreciated condition with an extremely high risk of CHD, not all of which is explained by elevation in LDL-cholesterol (3). Elevated postprandial triglycerides and TRLs may play an important role in development of ASCVD in FH by contributing to endothelial dysfunction, coagulopathy and thrombosis, hepatic steatosis, inflammation and oxidative stress, and the development of atherosclerosis (9, 10) However, postprandial lipoproteins are not a focus of standard therapy. Our results demonstrate that our FH patients exhibit higher postprandial lipemia despite having normal fasting plasma triglyceride concentrations. To identify patients with FH who may benefit from triglyceride-lowering may require use of a standardized oral fat challenge. Our findings support the addition of ω-3 FAEE supplementation to cholesterol-lowering therapy to improve postprandial hypertriglyceridemia in patients with FH. Future studies should examine the effect of other triglyceride-lowering agents (such as peroxisome proliferator-activated receptor [PPAR] agonists and selective PPAR modulators) on TRL metabolism in FH (3, 8).

In conclusion, our data support the hypothesis that addition of ω-3 FAEEs to standard treatment of hypercholesterolemia significantly improves postprandial lipemia in FH subjects. Future studies should examine the additive effects of fibrates, anti-PCSK9 (proprotein convertase subtilisin/kexin type 9) therapies and other ω-3 FAEE preparations against background statin therapy on
postprandial lipid and lipoprotein metabolism. The full clinical significance of our findings remains to be formally demonstrated in a cardiovascular outcome trial of FH patient with postprandial lipemia and established ASCVD.

ACKNOWLEDGMENTS

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LEGENDS

Table 1. Effect of ω-3 FAEE supplementation on clinical and biochemical variables in the patients

Figure 1. Trial design for ω-3 FAEE supplementation in familial hypercholesterolemia

Figure 2. Plasma triglyceride (A), VLDL-apoB-100 (B) and apoB-48 (C) responses (mean ± SEM) to the fat load before and after ω-3 FAEE supplementation

Figure 3. Postprandial changes for plasma triglyceride (A), VLDL-apoB-100 (B) and apoB-48 (C) (mean ± SEM) following the oral fat load before and after ω-3 FAEE supplementation
Table 1. Effect of ω-3 FAEE supplementation on clinical and biochemical variables in the patients

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Values are Mean ± SEM