Dietary long-chain n-3 PUFAs increase LPL gene expression in adipose tissue of subjects with an atherogenic lipoprotein phenotype

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Abstract We sought to test the hypothesis that dietary long-chain n-3 PUFA (LC n-3 PUFA) in fish oil stimulate the gene expression of lipoprotein lipase (LPL) in human adipose tissue (AT). In a randomized, double blind, placebo-controlled, cross-over study, 51 male subjects expressing an atherogenic lipoprotein phenotype (ALP) had their diets supplemented with fish oil for 6 weeks. As we previously reported for this group, supplementation with LC n-3 PUFA produced a decrease in fasting plasma triglyceride (TG) (−35%, $P < 0.05$), attenuation of the postprandial TG response (area and incremental area under the curve; AUC and IAUC, $P < 0.05$), and a decrease in small, dense LDL. The present study extended these observations by showing that these changes were accompanied by a marked increase in the concentration of LPL mRNA in adipose tissue (AT-LPL mRNA, +55%, $P = 0.003$) and post-heparin LPL activity (PH-LPL, +31%, $P = 0.036$). There was also evidence of an association between LPL gene expression and polymorphism in the apolipoprotein E gene. We conclude that the favorable influence of dietary n-3 PUFA on the ALP may be mediated, in part, through an increase in the plasma activity and gene expression of lipoprotein lipase in human adipose tissue.—Khan, S., A-M. Minihane, P. J. Talmud, J. W. Wright, M. C. Murphy, C. M. Williams, and B. A. Griffin. **Dietary long-chain n-3 PUFAs increase lipoprotein lipase gene expression in adipose tissue of subjects with an atherogenic lipoprotein phenotype. *J. Lipid Res.* 2002.** 43: **979–985.**

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First described by Austin et al. (1), the atherogenic lipoprotein phenotype (ALP) defines a collection of abnormalities in plasma lipoproteins that confers a 3- to 6-fold increase in risk of coronary heart disease (CHD) and features moderately raised plasma triglyceride (TG), low HDL cholesterol (HDL-C), and predominance of small, dense LDL (2, 3). An ALP originates from defects in TG metabolism that include the impaired clearance of TG-rich lipoproteins in the postprandial period, coupled with an oversupply of lipid substrates for the production of TG and thus secretion of apolipoprotien B (apoB) as TG-rich VLDL in the liver (4). Although the underlying molecular basis for these defects is not yet understood, interplay between insulin resistance and nutrient-gene interactions is likely to be central to the development and, potentially, to the correction of this high-risk dyslipidemia.

Variation in response to different dietary fats is being increasingly ascribed to genetic heterogeneity and nutrient-gene interactions (5). While direct evidence for the role of dietary fatty acids and their derivatives as regulators of gene expression in humans is still lacking, it is likely that many of the potentially beneficial effects of long-chain n-3 PUFA (LC n-3 PUFA) on TG metabolism are mediated through the control of gene transcription and post-transcriptional events. Fish oil supplements have been shown to correct many of the metabolic sequelae associated with insulin resistance (6), which includes decreasing the concentration of plasma TG in the pre and postprandial periods (7). Fish oil may induce a reduction in VLDL by suppressing lipogenesis and by enhancing the oxidation of fatty acids, both of which are controlled at the level of gene transcription in the liver (8). While these events will indirectly increase the capacity to clear plasma TG in the postprandial period, as a result of reduced competition for lipoprotein lipase (LPL), LC n-3 PUFA may

Abbreviations: ALP, atherogenic lipoprotein phenotype; AT, adipose tissue; AUC, area under curve; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; IAUC, incremental area under the curve; LC, long-chain; PH-LPL, post-heparin lipoprotein lipase; TG, triglycerides.

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also exert direct effects on the removal of TG-rich lipoproteins through stimulation of LPL in peripheral tissues (9).

LPL activity is a rate-limiting determinant of the hydrolysis of TG in the plasma compartment. Although in quantitative terms the mass of muscle LPL far outweighs that of LPL in adipose tissue (AT-LPL), the latter removes relatively more TG in the postprandial period than skeletal muscle (10), making AT-LPL a prime target for metabolic regulation. While this is well known to occur acutely in response to insulin, considerably less is known about longer term regulation mediated by the effects of dietary fatty acids on the AT-LPL gene. There is evidence from animal studies (11, 12) and, more recently, from preliminary findings in small groups of normal (13) and diabetic humans (14) to suggest that dietary LC n-3 PUFAs increase post-heparin LPL (PH-LPL) activity and the expression of the AT-LPL gene in the longer term, though other studies have shown no effect on PH-LPL activity (15, 16).

The purpose of this study was to extend our previous observations on the effects of fish oil supplementation on the ALP (17) with the aim of determining whether these effects could be explained by an increase in AT-LPL gene expression. In view of our previous report of an influence of apoE polymorphism on the metabolic response to fish oil in this group (17), the data herein includes examination of AT-LPL gene expression in relation to apoE genotype.

MATERIALS AND METHODS

Subjects

Normal, healthy male subjects expressing the ALP (n = 51), moderately raised total plasma TG (1.5–4.0 mmol/l), low HDL-C (<1.1 mmol/l) and predominance of small, dense LDL, were recruited as previously described (17). The study protocol was approved by the University of Reading and West Berkshire Health Authority Ethics Committees, and each subject gave written consent prior to participating.

Study design

The study was a randomized, double blind, placebo-controlled crossover design with two 6-week intervention periods separated by a 12-week washout period. During the intervention periods, subjects supplemented their habitual diet with either 6 g of fish oil per day in the form of six 1 g capsules of a 50% eicosapentaenoic acid (EPA)-docosahexaenoic acid (DHA) TG concentrate (3 g long-chain n-3 PUFA) (Pikasol, EPAX 5500 TG, Pronova, Norway), or 6 g of encapsulated olive oil per day as a placebo (Pronova, Norway). Following the 12-week washout period, subjects were switched to the opposite regime. Blood samples (30 ml) were collected from fasted subjects at 0, 3, and 6 weeks of each intervention period. In a postprandial assessment, PH-LPL activity and adipose tissue biopsies for LPL gene expression were carried out at the end of each intervention period. The protocols for the postprandial assessment and measurement of plasma lipids, lipoproteins, LDL subclasses, and PH-LPL activity have been described in detail previously (17).

Adipose tissue biopsies for determination of LPL mRNA

Subcutaneous adipose tissue biopsies were taken under local anesthesia (2% lignocaine without adrenaline) from the gluteal region of the upper buttock between 4–5 h post-breakfast, and at exactly the same time on each of the two occasions. The choice of this specific tissue site was based on the finding that it minimized stress and subsequent inconvenience to the subjects. Approximately 200 mg of white subcutaneous adipose tissue was removed from a 2 cm incision. The incision was closed by a single exterior (prolene) stitch.

Assay of LPL mRNA levels

RNA extraction. White subcutaneous adipose tissue was weighed and immediately snap frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted from 50 mg of tissue using RNAgents Total RNA Isolation System (Promega, Southampton, UK). This commercially available kit employs a modification of the method of Chomczynski and Sacchi (18). Briefly, tissue samples were homogenized in a guanidine thiocyanate denaturing solution, extracted with phenol:chloroform:isoamyl alcohol and precipitated overnight in isopropanol. RNA pellets were washed in ethanol and re-suspended in nuclease-free water. RNA concentrations were quantified spectrophotometrically at 260 nm using the Genequant II microspectrophotometer (Pharmacia Biotech, St. Albans, UK).

Quantitative competitive RT-PCR. Specific mRNA for LPL was quantified by reverse transcription followed by competitive polymerase chain reaction (RT-PCR). This was performed using the multi-specific internal standard or competitor, published by Laville et al. (19), kindly donated by Professor Hubert Vidal. The production of this competitor from the vector has been previously described by Murphy et al. (13). RT-PCR utilized oligonucleotide primers that amplify both the competitor and the target RNA in the same reaction, producing cDNA products of different sizes, 267 bp and 227 bp, respectively. Reverse transcription and amplification were performed using the Access RT-PCR System Kit (Promega, Southampton, UK). First strand cDNA synthesis was performed at 48°C for 45 min and terminated at 94°C for 2 min. The cDNA products were amplified in the presence of *Tfl* DNA polymerase (supplied in the kit), annealing of primers at 60°C for 1 min and extension at 68°C for 2 min. Samples were subject to 27–30 cycles in an Omn-E thermal cycler (Hybaid, Middlesex, UK). In addition, β_2-microglobulin mRNA was amplified in parallel tubes as an internal standard or "house-keeping" gene, using β_2-microglobulin primers and under identical conditions. The amplification products were separated on 3% agarose gels pre-stained with ethidium bromide and photographed with a CCD video camera. Band intensities were evaluated from the negative film using gel scan software (Pharmacia Biotech Ltd, UK). The logarithm of the density ratio of the internal standard band (competitor) to the wild-type (target) cDNA band was plotted against the logarithm of the initial competitor concentration. The LPL mRNA results were normalized and expressed relative to the concentration of mRNA of β_2-microglobulin.

ApoE genotyping

Blood was collected after an overnight fast into K_2EDTA. DNA was extracted from nucleated cells by the salting-out method (20). ApoE genotyping was performed by PCR and restriction digestion analysis by microplate array diagonal gel electrophoresis as previously described (21).

Statistical analysis

The results for plasma lipids and LPL gene expression are based on 51 subjects who provided tissue biopsies after both experimental periods. Data on post-heparin LPL (PH-LPL) activity was available on a subset of 36 subjects. Results are expressed as mean ± SD. Normality of data distribution was tested by normal

probability plots, and where appropriate, non-parametrically distributed data was $\log_{10}$ transformed for parametric testing (TG, PH-LPL, LPL mRNA). Differences between paired data (fish oil vs. control), were tested by Student's *t*-test for dependent samples. Differences between categorical variables (e.g., apoE genotypes) were tested by one-way ANOVA. Univariate associations between continuous variables were assessed by Pearson's correlation and product-moment coefficients and simple linear regression.

RESULTS

The baseline characteristics for the subjects have been described in our previous report (17). In accordance with the study entry criteria, all subjects expressed moderately raised plasma TG (>1.5 mmol/l), low HDL-C (<1.1 mmol/l), and a predominance of small, dense LDL (>40% or LDL subclass pattern B). Although 11 subjects (22%) could be classified as being clinically obese at baseline (BMI > 30 < 40), all subjects were normo-glycemic and normo-insulinemic on entry. This ALP group showed a significantly higher carrier frequency of the apoE4 polymorphism (41% carriers of the ε4 allele) as compared with the UK population (15–25%). Anthropometric variables including weight, BMI, and waist to hip ratio showed no significant change at the end of each intervention period (data not shown). The fish oil and control (olive oil) supplements were well tolerated, and compliance, as measured by capsule count and platelet fatty acid profile, was excellent. There was no evidence of any crossover effects or treatment-by-period interactions.

Effect of fish oil supplementation on plasma lipids and lipoproteins

The lipid and lipoprotein response to fish oil in this group has been reported elsewhere (17). All data shown herein, with the notable exception of the PH-LPL activities, relates to a cohort of 51 subjects. Fish oil supplementation produced a consistent decrease in fasting plasma TG (−26%, $P < 0.001$) and a marked decrease in the concentration of postprandial plasma TG (**Table 1**).

Platelet membrane fatty acid composition

Changes in platelet membrane fatty acid composition have been reported previously (17). Briefly, fish oil supplementation increased the concentration of LC n-3 PUFA [eicosapentaenoic (EPA C20:5) and docosahexaenoic (DHA C22:6)] acids in platelet membranes relative to the control. Total LC n-3 PUFA increased by 88% ($P < 0.001$), while the ratio of n-6 to LC n-3 PUFA in platelet membrane phospholipids decreased by 53% (9 to 4.2).

Post-heparin plasma LPL activity

Post-heparin plasma (PH) was obtained from a subgroup of 36 subjects for the measurement of LPL activity at the end of the fish oil and control phases. PH-LPL activity at 5 min showed an overall increase (+31%, $P < 0.036$) in response to fish oil supplementation relative to the control (Table 1). At 15 min, PH-LPL activities were higher than at 5 min, but the difference between treatment groups (13%) was no longer significant.

Adipose tissue LPL mRNA

The group showed an overall increase (+54%, $P = 0.003$) in the concentration of AT-LPL mRNA after fish oil relative to the control (Table 1). At the end of the control period, the concentration of AT-LPL mRNA was negatively correlated ($r = -0.41$, $P = 0.003$) with the extent of postprandial lipemia (IAUC) (**Fig. 1A**). There was a weak univariate correlation ($r = 0.33$, $P = 0.05$) between the changes (fish oil and control) in PH-LPL activity and concentration of AT-LPL mRNA (Fig. 1B). There was no rela-

TABLE 1. Plasma triglyceride area and incremental area

	Total Group (n = 51)		Apoε4 (n = 21)		Non-ε4 (n = 30)	
	Control	Fish Oil	Control	Fish Oil	Control	Fish Oil
Plasma TG (mmol/l) (n = 51)	2.72 ± 0.82	2.02 ± 0.71[c]	2.82 ± 1.05	1.99 ± 0.89[b]	2.67 ± 0.69	2.01 ± 0.58[c]
AUC (mol/l/min) (n = 51)	1808 ± 483	1389 ± 448[c]	1868 ± 490	1413 ± 552[c]	1777 ± 500	1369 ± 384[c]
IAUC (mmol/l/min (n = 51)	522 ± 236	437 ± 193[b]	540 ± 203	469 ± 201	510 ± 262	424 ± 187[a]
Plasma LPL 5 min (μmols oleate/ml/h) (n = 36)	2.34 ± 1.66	3.06 ± 2.36[a]	2.74 ± 2.10	4.44 ± 2.77	2.05 ± 1.35	2.32 ± 1.86
Plasma LPL 15 min (μmols oleate/ml/h) (n = 36)	4.67 ± 2.60	5.26 ± 3.48	5.31 ± 3.13	6.42 ± 4.03	4.21 ± 2.24	4.48 ± 3.02
LPL mRNA (No. molecules mRNA LPL/β_2-microglobulin) (n = 51)	0.255 ± 0.346	0.396 ± 0.367[b]	0.127 ± 0.073	0.373 ± 0.313[c]	0.337 ± 0.439	0.399 ± 0.426

Plasma triglyceride (TG) (fasted) area and incremental area under the postprandial TG curve (AUC and IAUC), postheparin lipoprotein lipase activity (5 min and 15 min), and adipose tissue lipoprotein lipase mRNA in total group and carriers (apoε4) and non-carriers (non-ε4) of the apoε4 allele. Values are means ± SD.

[a] $P \leq 0.05$.

[b] $P \leq 0.01$.

[c] $P \leq 0.001$ (fish oil vs. control).

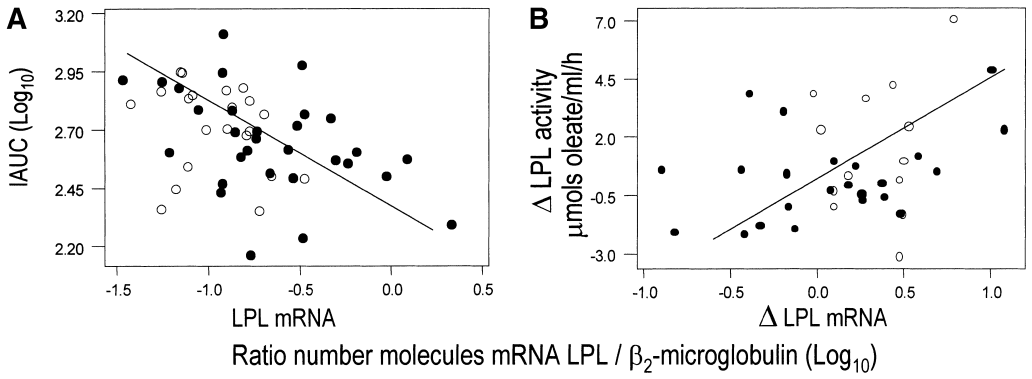

Fig. 1. Correlations between (A) adipose tissue lipoprotein lipase (LPL) mRNA and postprandial response as measured by incremental area under the postprandial TG curve (IAUC) after the control diet ($r = -0.41$, $P = 0.003$) and (B) the change [fish-oil − control (delta)] in the concentration of adipose tissue LPL versus the change (delta) in postheparin LPL activity ($r = 0.33$, $P = 0.05$). The open and closed circles denote carriers and non-carriers of the apo-ε4 allele, respectively.

tionship between PH-LPL activity and fasting TG, or any measure of the extent of postprandial lipemia after either intervention period.

Distribution of apoE genotypes

As shown previously (17), the distribution of individual alleles for the apoE polymorphism (ε2, ε3, ε4) in this group of ALP subjects was atypical for a free-living population, with 41% of the group being either heterozygous (ε3/ε4 or ε2/ε4) or homozygous (ε4/ε4) for the apoE4 genotype. There were no homozygotes for apoE2 genotype (ε2/ε2). Of the remaining 30 subjects, 22 were homozygous for the apoE3 genotype (ε3/ε3), with the remaining eight individuals being heterozygous for the apoE2 genotype (ε2/ε3). There was evidence that carriers of the apoε4 allele showed a different lipoprotein response to fish oil than non-ε4 carriers (17). For this reason, subjects were classified as either ε4 (n = 21) or non-ε4 (n = 30) carriers.

Influence of apoE genotype on response to fish oil supplementation

The effect of apoE genotype on the lipid response to fish oil in this group has been reported previously (17). In the present study, despite there being no differences in plasma TG and the extent of postprandial lipemia between ε4 and non-ε4 groups, the overall LPL response to fish oil was due, in the main, to carriers of the ε4 allele who showed a mean increase in PH-LPL activity at 5 min of 62% ($P = 0.19$), ε4 carriers versus non-ε4 carriers (Table 1 and **Fig. 2A**). While the level of AT-LPL mRNA increased in all apoE genotypes on fish oil, ε4 carriers showed the most consistent response with nearly a 3-fold increase in gene expression (Table 1 and Fig. 2B, $P < 0.001$). Inverse correlations were demonstrated between LPL mRNA and the extent of postprandial lipemia in non-ε4 carriers after the control phase ($r = -0.44$, $P < 0.05$), and in ε4 carriers after the fish oil phase ($r = -0.58$, $P < 0.01$). Conversely, LPL mRNA was positively correlated with PH-LPL activity in ε4 carriers after fish oil ($r = 0.59$, $P < 0.05$).

DISCUSSION

The frequency distribution of an ALP in Western, industrialized populations may parallel that of insulin resistance (22). Although this dyslipidemia may have a mixed

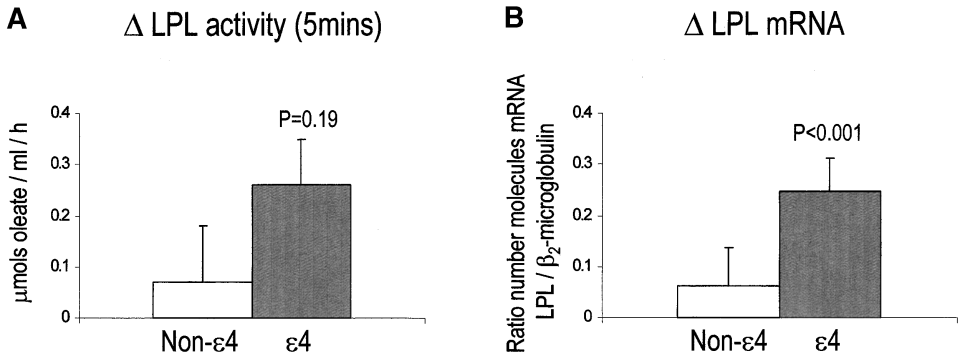

Fig. 2. Changes in (A) PH-LPL activity in plasma and (B) adipose tissue LPL mRNA in carriers and non-carriers of the apoε4 allele in response to fish-oil feeding (3 g eicosapentaenoic acid-docosahexaenoic acid per day). $P \leq 0.001$ (ε4 vs. non-ε4).

etiology, it is likely to represent the most common source of lipid-mediated CHD risk among free-living and otherwise healthy individuals. Fish oil can ameliorate the lipid abnormalities associated with this condition by suppressing the overproduction of TG in the liver and reducing the extent of postprandial lipemia (7). The present study sought to provide further evidence for the latter mechanism, and to ascertain the molecular basis of this effect with focus on the LPL pathway. Since adipose tissue represents an important depository site of postprandial lipid, this was chosen as a metabolically appropriate and practically accessible tissue for this investigation. Subnormal PH-LPL activity has been implicated as a possible cause of enhanced postprandial lipemia in insulin resistant states (23–25). Decreased PH-LPL levels have also been reported in a large cohort of individuals with coronary artery disease, and have been linked to adverse changes in plasma lipids and lipoproteins (26). On the strength of this evidence, it would seem reasonable to expect that activation of the LPL pathway would be beneficial by reversing this lipolytic deficit. However, studies on the effects of fish oil on PH-LPL activity in patients and healthy groups have yielded inconsistent results, due primarily to the indirect nature of the post-heparin measurement. While values for PH-LPL activity obtained in the present study were of a similar magnitude to those reported elsewhere, a direct comparison with published results to establish the relative normality of values in these ALP subjects was not possible due to variations in LPL assay conditions, experimental design (fed vs. fasted states), and the subjects under test. There was a heterogeneous, but overall significant, increase in PH-LPL activity in response to the fish oil, which was greater than that generally reported in the literature. The majority of studies report no effect of fish oil on PH–LPL, and conclude that the TG-lowering arises from a predominant influence in suppressing TG synthesis (27, 28). Nevertheless, increases in LPL activity in response to fish oil have been found in pre and post-heparin plasma (9, 29), though the exact relationship between these measurements is unclear. In view of the role of LPL as a rate-limiting determinant of TG removal in the plasma compartment, it was reasonable to expect an inverse relationship between PH-LPL activity and plasma TG, though the present data provide little evidence to support such a relationship.

Post-heparin LPL activity in plasma at 5 min and 15 min is believed to represent a submaximal and maximal release of LPL respectively from the endothelium of skeletal muscle and adipose tissue, the relative activities of which will vary with time, previous nutrition, and the general physiological state of the individual. This variation may explain why metabolic relationships between PH-LPL and plasma lipids are not more apparent. Of the two tissue sites, skeletal muscle has received considerably more attention as the most abundant source of LPL and modulator of plasma TG and lipoprotein composition in response to exercise and TG-lowering drugs (30–33). Nonetheless, with few exceptions the majority of reported work has focused on lipid metabolism in the post-absorptive state, and takes no account of postprandial events in adipose tissue that may be of paramount importance to the impact of dietary fatty acids on the excursion of TG in the postprandial period. Although LPL activity specific to adipose tissue was not determined in the present study, it is reasonable to expect that this measure would be more closely associated with changes in postprandial lipemia and AT-LPL gene expression.

While dietary LC n-3 PUFA are perhaps the most favored nutrients to modulate pre and post-translational events (5), evidence for their effects on LPL gene expression in vivo in human tissues is lacking. This study set out to substantiate earlier findings from our laboratory that showed positive effects of fish oil on the pre-translational regulation of LPL gene expression in the epididymal fatpads of rats (12). In a follow-up study on a small group of normal, healthy male subjects (n = 6), the level of AT-LPL mRNA was shown to increase in four out of six subjects in response to fish oil (13). Furthermore, changes in the level of AT-LPL mRNA were inversely associated with changes in fasting plasma TG and postprandial lipemia (AUC), suggesting that LPL gene expression may control longer-term changes in the handling of exogenous and endogenous TG.

In the present study, the level of LPL mRNA increased by 54% on fish oil relative to the olive oil control. There are no other studies with a similar experimental design or with equivalent statistical power to match or corroborate these findings. In a recent study, Luo et al. (14) reported an increase in the level of LPL mRNA in abdominal subcutaneous adipose tissue in a small group of type II diabetics (n = 5) after 2 months of supplementation with fish oil relative to sunflower oil (6 g fish oil/day equivalent to 1.8 g LC n-3 PUFA). Beyond this, evidence for the effects of LC n-3 PUFA on AT-LPL mRNA levels derives entirely from cell culture studies and animal models, the results of which are equivocal, possibly because the effects are specific to intact adipose tissue (34). Experiments on isolated adipocytes indicate that while dietary fatty acids can influence pre-translational events, their effects on post-translational activation of LPL appear to be a more important mechanism of regulation in adipose tissue (35–37). In complete agreement with our earlier observations, the level of AT-LPL mRNA ex vivo from humans was inversely related to the incremental area under the postprandial curve after the control period (Fig. 1A). In addition, the change in gene expression (fish oil vs. control) was positively related to the change in PH-LPL activity (Fig. 1B). Although these relationships could be viewed as somewhat remarkable given that, under certain circumstances, up to 50% of newly synthesized LPL is not actually secreted but degraded in adipocytes (38), they do imply that dietary LC n-3 PUFA influences LPL activity and plasma TG in the longer term through regulation of pre-translational events.

In the present study, the response of both PH-LPL and AT-LPL mRNA to fish-oil was related to apoE genotype. The reasons why ε4 carriers should express relatively lower PH-LPL activity and levels of AT-LPL message than

non-ε4 carriers, and the mechanisms by which LC n-3 PUFA in fish oil may exert selective effects in this group are currently unknown and worthy of further investigation.

In conclusion, this study presents evidence to suggest that dietary LC n-3 PUFAs in fish-oil increase AT-LPL gene expression and PH-LPL activity. These effects may be modulated by the apoE polymorphism and could contribute to the correction of lipid abnormalities associated with the ALP.

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