Effects of dietary fish oil on serum lipids and VLDL kinetics in hyperlipidemic apolipoprotein E*3-Leiden transgenic mice

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Abstract **Studying the effects of dietary fish oil on VLDL metabolism in humans is subject to both large intra- and interindividual variability. In the present study we therefore used hyperlipidemic apolipoprotein (APO) E*3-Leiden mice, which have impaired chylomicron and very low density lipoprotein (VLDL) remnant metabolism, to study the effects of dietary fish oil on serum lipids and VLDL kinetics under highly standardized conditions. For this, female APOE*3-Leiden mice were fed a fat- and cholesterol-containing diet supplemented with either 0, 3 or 6% w/w (i.e. 0, 6, or 12% of total energy) of fish oil. Fish oil-fed mice showed a significant dose-dependent decrease in serum cholesterol (up to −43%) and triglyceride levels (up to −60%), mainly due to a reduction of VLDL (−80%). LDL and HDL cholesterol levels were not affected by fish oil feeding. VLDL-apoB kinetic studies showed that fish oil feeding resulted in a significant 2-fold increase in VLDL-apoB fractional catabolic rate (FCR). Hepatic VLDL-apoB production was, however, not affected by fish oil feeding. VLDL-triglyceride turnover studies revealed that fish oil significantly decreased hepatic VLDL-triglyceride production rate (−60%). A significant increase in VLDL-triglyceride FCR was observed (+70%), which was not related to increased lipolytic activity. We conclude that APOE*3-Leiden mice are highly responsive to dietary fish oil. The observed strong reduction in serum very low density lipoprotein (VLDL) is primarily due to an effect of fish oil to decrease hepatic VLDL triglyceride production rate and to increase VLDL-apoB fractional catabolic rate.—**van Vlijmen, B. J. M., R. P. Mensink, H. B. van 't Hof, R. F. G. Offermans, M. H. Hofker, and L. M. Havekes. **Effects of dietary fish oil on serm lipids and VLDL kinetics in hyperlipidemic apolipoprotein E*3-Leiden transgenic mice.** ***J. Lipid Res.*** **1998.** 39: **1181–1188.**

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Dietary fish oils, rich in long-chain n–3 fatty acids, lower plasma triglyceride and very low density lipoprotein (VLDL) levels in both normo- and hyperlipidemic subjects (for review, see ref. 1). VLDL-triglyceride kinetic studies have been performed in humans and they suggested that the hypotriglyceridemic effect of fish oil is primarily caused by inhibition of VLDL-triglyceride synthesis (2–5). However, effects on VLDL production and/or VLDL-triglyceride removal rate via increased lipoprotein lipolysis cannot be ruled out (2, 6). The increasing effects of fish oil supplementation on low density lipoprotein (LDL) cholesterol levels have been variable and somewhat confusing. In some studies, LDL levels decreased upon fish oil administration; in other studies levels increased (for review see ref. 1). It has been suggested that n–3 fish oils increase plasma levels of LDL by reducing the number of LDL receptors in liver and peripheral tissues (7–10) or by increasing the conversion of VLDL into LDL (11).

Many studies involving the metabolic consequences of dietary fish oil feeding were performed in humans. However, due to ethical and technical limitations, not all mechanistic aspects of VLDL metabolism can be studied in detail in humans. Recently, we described the generation of transgenic mice expressing the human APOE*3-Leiden gene (12). These mice have an impaired clearance of chylomicron and VLDL remnant lipoproteins from the blood circulation by the liver. As a consequence, these mice have elevated plasma cholesterol and triglyceride levels due to increases in levels of VLDL/LDL-sized lipoproteins. In addition, the plasma cholesterol and triglyceride levels in these mice are highly responsive to small changes in chylomicron and VLDL production and clearance (13, 14). The "human-like" lipoprotein profile and the extreme sensitivity of plasma lipid levels to changes in VLDL

Abbreviations: VLDL, very low density lipoprotein; IDL, intermediate low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; apo, apolipoprotein; FCR, fractional catabolic rate; SR, secretion rate; FA, fatty acids.

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metabolism suggested to us that APOE*3-Leiden mice may serve as an suitable animal model for studying in detail the metabolic consequences of fish oil feeding under highly standardized conditions.

The present study shows that the plasma lipid levels in APOE*3-Leiden transgenic mice are highly responsive to dietary fish oil. A strong reduction in the serum cholesterol and triglycerides was observed due to a reduction in serum levels of VLDL-sized lipoproteins. We found that dietary fish oil lowers serum VLDL levels primarily by decreasing hepatic VLDL triglyceride production rate, and secondly by increasing VLDL-apoB clearance rate.

MATERIALS AND METHODS

Animals

All studies were performed with female APOE*3-Leiden transgenic mice (line #2) (12). Transgenic mice were obtained by mating male transgene carriers with C57BL/6J females (The Broekman Institute bv, Someren, The Netherlands). Mice of the F10 generation were used for all studies. Transgenic mice were identified by sandwich ELISA for the presence of human apoE in the serum as described previously (14). At the time of the study, animals were 3–4 months of age.

Study design

The study had a parallel group design and included two experimental groups (10 animals per group, 2 dose levels) and one control group (12 animals). Animals were distributed over these groups, stratified for age. They were housed in shoebox cages (5–6 per cage) with hoppers that allowed feeding of a powdered diet. Three weeks prior to the experimental feeding, mice were fed a powdered semi-synthetic sucrose-rich diet containing cocoa butter (15% w/w) and cholesterol (0.25% w/w). The diet was composed essentially according to Nishina, Verstuyft, and Paigen (15), and described by us earlier (14) (Western-type or high fat/cholesterol (HFC) diet, Hope Farms, Woerden, The Netherlands). During the following 4 weeks mice were fed the same powdered diet to which 3% w/w or 6% w/w of fish oil (kindly provided by Hoffmann LaRoche GmbH, Basel, Switzerland) was added at the expense of cacao butter. The percentage of energy in the diets provided by eicosapentaenoic acid and docosahexaenoic acid was 1.3 and 0.9% versus 2.2 and 1.5%, for the 3% w/w and 6% w/w fish oil diet, respectively (for detailed fatty acid composition of experimental diets see **Table 1**). Experimental diets were stored at −20°C and mice were given a fresh aliquot daily at 5 pm. Animals had free access to food and water.

TABLE 1. Fatty acid composition of the experimental fish oil diets

	Fish Oil		
Fatty Acid (FA)	0% w/w	3% w/w	6% w/w
		g/100 g diet	
Saturated FA	8.9	8.2	7.4
Monounsaturated FA	5.1	4.8	4.7
Polyunsaturated FA	1.1	2.3	3.1
N–6 polyunsaturated FA	0.9	1.1	1.1
N–3 polyunsaturated FA	0.2	1.2	2.0
Eicosapentaenoic acid	0.1	0.6	1.0
Docosahexaenoic acid	0.0	0.4	0.7

The fatty acid composition of the experimental fish oil diets was analyzed as described in Methods. In addition to the fat, all diets contained 0.25% cholesterol, 40.5% sucrose, 10% corn starch, 5.95% cellulose, 20% casein, 1% choline chloride, 0.2% methionine, and 5.1% mineral mixture. All percentages are in weight/weight. To convert from % of weight to % of metabolic energy, multiply by 2.16. Total energy and metabolic energy content of the three diets were 4620 and 4160 kcal/kg, respectively.

At time 0, after 2 weeks, and at the end of the experimental period (4 weeks) mice were fasted from 8 am to 1 pm, weighed, and approximately 200 μl of blood was obtained through tail-bleeding under diethylether anesthesia. Some 70 μl of individual serum was immediately frozen in liquid nitrogen and stored at −80°C until lipid analysis. The remainder was used for lipoprotein analysis.

For determination of in vivo VLDL-apoB and VLDL-triglyceride turnover (methods see below), indicated parts of the experiment were repeated under identical conditions using mice with comparable genetic background and age.

Lipid and lipoprotein analysis

Total serum cholesterol, triglycerides (without measuring free glycerol), and free fatty acids were measured enzymatically using commercially available kits: #236691 (Boehringer Mannheim, Germany), #337-B (Sigma, Mo.), and #994-75409 (Wako Chemicals GmbH, Neuss, Germany), respectively. Serum β-hydroxybutyrate was determined as described (16).

Phospholipids from pooled serum samples and total lipids from diet aliquots were extracted according to standard procedures (17, 18). After saponification and methylation (19), the proportion of individual fatty acids in the diet and serum phospholipids were determined with an Autosystem Perkin Elmer gaschromatograph fitted with a 50 m CP Sil 88 capillary column with an inner diameter of 0.25 mm and 0.20 μm film thickness. (Chrompack, Middelburg, the Netherlands). Helium (130 kPa) was used as a carrier gas. The oven temperature was programmed to stay at 160°C for 10 min, then to rise to 190°C with a rate of 2.5°C/min, to keep this temperature for 20 min, to increase again at a rate of 4°C/min to 230°C, and then kept constant. The temperature of the injector and the flame ionization detector was set at 300°C, while a split ratio of 1:20 was used. A standard mixture was used to identify the fatty acid methylesters by means of the retention times. Results were expressed as a proportion of total identified fatty acids. Butylated hydroxy-toluene (0.005 %, w/v, Sigma B1378) was added to all organic solvents to prevent oxidation of the polyunsaturated fatty acids.

For determining the serum lipoprotein distribution, some 100 μl of pooled serum was subjected to density gradient ultracentrifugation according to Redgrave, Roberts, and West (20). After ultracentrifugation, the volume was fractionated in fractions of 0.5 ml and density was measured using a DMA 602M densitometer (Paar, Germany). Fractions with density d < 1.006, 1.006–1.019, 1.019–1.063, and 1.063–1.120 g/ml correspond to VLDL, IDL, LDL, and HDL, respectively. After dialysis against PBS, containing 1 mm EDTA (pH 7.4), lipoprotein fractions were analyzed enzymatically for cholesterol and triglyceride content, using kits #236691 and #701904 (Boehringer Mannheim, Germany), respectively.

VLDL-apoB turnover studies: isolation and labeling of VLDL

After a 5-h fasting period, mice were anesthetized by diethylether and blood was collected from the retroorbital plexus. VLDL was isolated from serum of 6 control fed or 12 fish oil-fed (3% w/w) female transgenic mice by density gradient ultracentrifugation. Total and free cholesterol, triglyceride (without glycerol), and phospholipid content of the VLDL were measured enzymatically, using commercially available kits (#236691 and #310328: Boehringer Mannheim, Mannheim, Germany; #337-B:

Sigma Chemicals Co., St. Louis, MO; and 990-54009: Wako Chemicals GmbH, Neuss, Germany, respectively). VLDL protein was determined using the method of Lowry et al. (21). To determine apoB(100 + 48) and apoE content of the VLDL, some 5 μg of VLDL protein was subjected to a 4-20% gradient SDS-PAGE. After staining with Coomassie Brilliant Blue R and destaining in 30% methanol/10% acetic acid the gels were scanned using a HP ScanJet Plus (Hewlett-Packard, Santa Clara, CA) and the amount of apoB(100 + 48) relative to total protein was calculated. For both VLDL samples (control and fish oil-fed mice), the percentage of total apoB was about 25% of total VLDL protein. VLDL was radiolabeled with ^{125}I by the iodine monochloride method (22). The fraction of ^{125}I-label present in apoB was determined by isopropanol precipitation (23, 24) and ranged from 30 to 40%.

VLDL-apoB turnover studies: in vivo removal of ^{125}I-labeled VLDL-apoB

Fasted mice were intravenously injected with 0.2 ml of 0.9% NaCl containing bovine serum albumin (1 mg/ml) and 10 μg of ^{125}I-labeled VLDL. Blood samples of approximately 25 μl were collected from the tail vein at t = 2, 5, 10, 30, 60, and 90 min after injection. The serum content of ^{125}I-labeled apoB(100 + 48) was measured by isopropanol precipitation followed by counting ^{125}I-label. A bi-exponential model was used to estimate the area under the ^{125}I-labeled apoB decay curve and subsequently to calculate VLDL-apoB kinetics. VLDL secretion rate was calculated from VLDL fractional catabolic rate (FCR) and serum VLDL-apoB pool. As more than 90% of serum triglycerides were associated with the d < 1.006 g/ml lipoprotein fraction, we calculated the serum VLDL-apoB pool by multiplying serum triglyceride levels at t = 2 min after injection with the apoB/triglyceride ratio in the isolated d < 1.006 g/ml lipoproteins (VLDL) of mice from the same group (autologous). The amount of ^{125}I-labeled VLDL apoB injected represented ≤5% of the total VLDL-apoB pool, for both control and fish oil-fed animals.

VLDL-triglyceride turnover studies: preparation of endogenously labeled VLDL.

[^{3}H-]palmitate dissolved in ethanol (Amersham International, Little Chalfont, UK) was evaporated under nitrogen and redissolved in 0.9% NaCl containing 2 mg/ml BSA. Fasted mice were injected intravenously via the tail vein with 100 μCi of the prepared [^{3}H-]palmitate. Twenty-five minutes after injection, mice were anesthetized by diethylether and bled from the retro-orbital plexus. Radiolabeled VLDL used for clearance studies was isolated from serum of 6 control fed and 10 fish oil (3% w/w)-fed mice by ultracentrifugation. Obtained VLDL samples were dialyzed against PBS, pH 7.4, at 4°C. In both VLDL samples used, more than 95% of the radioactive label was bound to triglycerides.

VLDL-triglyceride turnover studies: in vivo removal of [^{3}H-]triglyceride labeled VLDL

Fasted mice were intravenously injected with 0.2 ml of 0.9% NaCl containing bovine serum albumin (1 mg/ml) and 80,000 dpm of [^{3}H-]triglyceride labeled VLDL. Blood samples of approximately 25 μl were collected from the tail vein at t = 1, 2.5, 5, 7.5, 10, 12.5, 15, 30, and 60 min after injection. Total plasma radioactivity was used to represent VLDL-triglyceride radioactivity, as a pilot study showed that the disappearance of radioactivity as measured after lipid extraction followed by TLC analysis did not differ from disappearance of total plasma radioactivity (not shown). As for VLDL apoB-turnover, a bi-exponential model was used to estimate the area under the [^{3}H-]triglyceride decay curve and subsequent calculation of VLDL-triglyceride kinetics. VLDL secretion rate was calculated from VLDL fractional catabolic rate and serum triglyceride pool as measured in each mouse during the experiment. The amount of [^{3}H-]triglyceride labeled VLDL injected represented <1% of the total VLDL-triglyceride pool, for both control and fish oil-fed animals.

Statistical analysis

The data were analyzed with the General Linear Models procedure of the Statistical Analysis System (SAS; SAS Institute Inc. SAS User's guide: Statistics Version 5 Edition. Cary, NC: SAS Institute Inc., 1985.). The outcome variables consisted of the changes between the start and the end of the study. When the analysis indicated a significant effect of diet ($P < 0.05$), the Tukey method was used to adjust for multiple comparisons. As a result, only two-tailed *P*-values of less than 0.02 were considered significant. For the VLDL-kinetic studies, data were analyzed using non-parametric Mann-Whitney rank sum tests. *P*-values less than 0.05 were regarded as significant.

RESULTS

Effect of dietary fish oil on serum lipids and lipoprotein levels

The effects of dietary fish oil on serum cholesterol and triglyceride levels are presented in **Table 2**. Compared to control mice (no fish oil), fish oil-fed mice showed a significant dose-dependent decrease in serum total cholesterol levels (up to −43%). In addition, a strong significant decrease in serum triglyceride levels (up to −60%) was observed. Unlike cholesterol levels, plasma triglyceride levels did not further decrease when the amount of fish oil in the diet was increased from 3 to 6% w/w.

The distribution of serum cholesterol and triglycerides over the lipoproteins was determined by density gradient

TABLE 2. Effect of dietary fish oil on serum lipid levels in APOE*3-Leiden transgenic mice

	Serum Cholesterol			Serum Triglycerides		
Fish Oil	0 wk	4 wk	$\Delta_{0\rightarrow4\ wk}$	0 wk	4 wk	$\Delta_{0\rightarrow4\ wk}$
	mmol/l			*mmol/l*		
0% w/w	9.43 ± 1.51	10.34 ± 1.28	0.91 ± 1.61	1.42 ± 0.41	1.50 ± 0.59	0.08 ± 0.57
3% w/w	8.99 ± 2.00	6.26 ± 1.08	−2.73 ± 1.20[a]	1.28 ± 0.49	0.63 ± 0.20	−0.65 ± 0.45[a]
6% w/w	9.44 ± 1.11	5.78 ± 0.46	−3.66 ± 0.95[a]	1.33 ± 0.32	0.61 ± 0.13	−0.73 ± 0.35[a]

$\Delta_{0\rightarrow4\ wk}$, difference in serum levels between 0 and 4 wk data. Mice were fed for 4 weeks a Western (Table 1) diet to which was added the indicated amount of fish oil. Fasted mice were bled and serum triglycerides and cholesterol were determined. Values are the mean of 10–12 mice ± SD.

[a] Indicates a significant difference from control mice ($P < 0.020$).

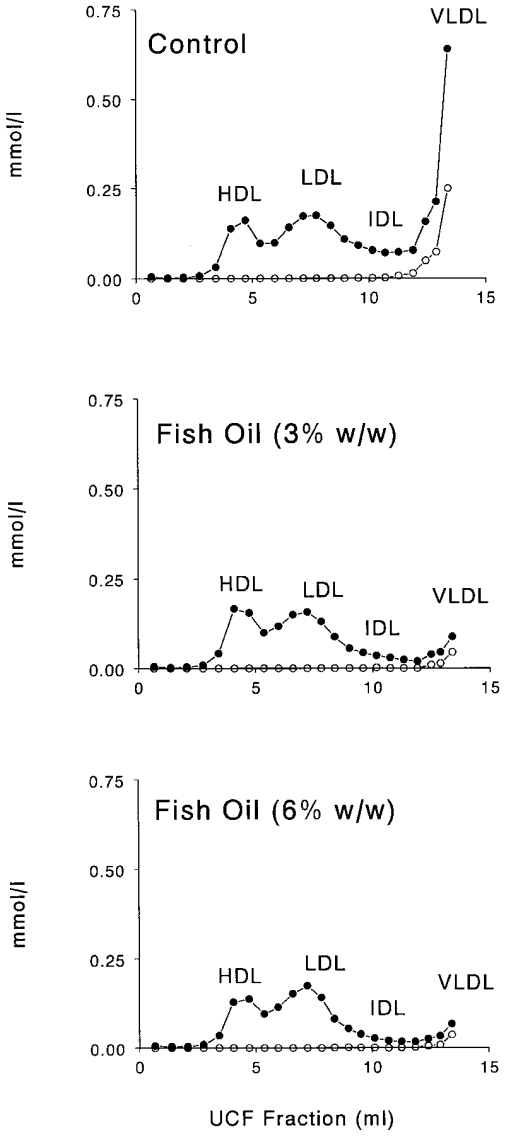

Fig. 1. Distribution of serum cholesterol and triglycerides among lipoprotein fractions. Lipoprotein fractions were separated by density gradient ultracentrifugation, and fractions were analyzed for cholesterol (●) and triglycerides (○). Lipoprotein profiles are shown for female APOE*3-Leiden mice fed a control diet (panel A) or a diet supplemented with 3% w/w fish oil (panel B) or 6% w/w dietary fish oil (panel C). Each run is performed with a fasted pool serum of at least 10 mice of the same group. Fractions with density d < 1.006, 1.006–1.019, 1.019–1.063, and 1.063–1.120 g/ml correspond to VLDL, IDL, LDL, and HDL, respectively.

ultracentrifugation (**Fig. 1**). The marked reduction in serum cholesterol on the fish oil diets was mainly confined to the VLDL fraction (−80%). However, a reduction in IDL cholesterol was also observed, while LDL and HDL cholesterol levels were not affected by fish oil feeding. As expected, fish oil only reduced triglyceride levels in the VLDL fraction (Fig. 1). As presented in **Table 3**, isolated particles (d < 1.006 g/ml lipoproteins) showed a decrease in cholesteryl ester (−60%) and triglyceride content (−13%), whereas phospholipid and free cholesterol content were not affected. Consequently, the ratio of core over shell lipids decreased (−41%), suggesting a decrease in particle size upon fish oil feeding.

TABLE 3. Composition of the d < 1.006 g/ml lipoproteins

Fish Oil	TC	CE	FC	TG	PL	CE + TG/PL + FC	ApoE
	μmol/mg apoB						*mg/mg apoB*
0% w/w	68.9	44.7	24.2	31.6	19.8	1.7	2.3
3% w/w	42.9	17.8	25.1	27.5	20.5	1.0	1.6

TC, total cholesterol; CE, cholesteryl ester; FC, free cholesterol; TG, total triglycerides; PL, phospholipids. CE + TG/PL + FC, ratio of core and shell lipids. Control and 3% w/w fish oil-fed mice were fasted and bled via orbital puncture. d < 1.006 g/ml lipoproteins were isolated from pool serum by density gradient ultracentrifugation. d < 1.006 fraction was analyzed for total, free and esterified cholesterol, triglycerides and phospholipids. ApoB(100 + 48) and apoE content were determined by SDS/PAGE as described in Methods.

Serum cholesterol, triglycerides, and lipoprotein distributions were also determined after 2 weeks of feeding. Results were not significantly different from those of the 4-week data (data not shown).

Parallel to the decrease in serum triglyceride concentrations, fish oil significantly lowered serum free fatty acids levels by 30% (**Table 4**). Free glycerol levels in serum were not significantly different between the control and fish oil-fed groups. However, a slight but significant difference in serum free glycerol levels was observed between the 3 and 6% fish oil groups (Table 4). Fish oil lowered the level of serum β-hydroxybutyrate (Table 4). This effect was already maximal in animals on the 3% fish oil diet. Remarkably, serum β-hydroxybutyrate levels increased after 4 weeks of control feeding.

The fatty acid composition of the diets was reflected by the composition of the total serum lipids. Compared to control fed animals, the proportion of n-3 polyunsaturated fatty acids increased by 11.7% in the 3% fish oil group and by 13.2% in the 6% fish oil-fed group. The increase in n–3 polyunsaturated fatty acids was mainly compensated for by decreases in n–6 polyunsaturated fatty acids (−8.9 and −11.2%, respectively) and monounsaturated fatty acids (−4.3 and −5.3%, respectively).

During the 4-week experimental period, increases in body weight were higher in the fish oil groups as compared to the control group (3.1 ± 1.8 (3% fish oil) and 2.0 ± 0.8 (6% fish oil) versus 1.4 ± 1.4 gram (control)). However, only the difference in changes between the control and 3% fish oil groups reached statistical significance ($P = 0.007$).

Changes in serum cholesterol levels correlated positively with changes in triglyceride levels ($r = 0.64$, $P < 0.001$), which reflected the strong reduction in VLDL (cholesterol + triglyceride) levels (Fig 1). The changes in cholesterol and triglyceride levels also correlated positively with the changes in both free fatty acid levels ($r = 0.54$, $P = 0.0013$ and $r = 0.51$, $P = 0.003$) and β-hydroxybutyrate levels ($r = 0.70$, $P < 0.001$ and $r = 0.76$, $P < 0.001$). In addition, a positive correlation was found between the decrease in serum free fatty acid and β-hydroxybutyrate levels ($r = 0.49$, $P = 0.005$).

TABLE 4. Effect of dietary fish oil on serum free fatty acid, free glycerol, and β-hydroxybutyrate levels in APOE*3-Leiden transgenic mice

	Free Fatty Acids			Free Glycerol			β-Hydroxybutyrate		
Fish Oil	0 wk	4 wk	$\Delta_{0\rightarrow4\ wk}$	0 wk	4 wk	$\Delta_{0\rightarrow4\ wk}$	0 week	4 wk	$\Delta_{0\rightarrow4\ wk}$
	mmol/l			*mmol/l*			*mmol/l*		
0% w/w	1.72 ± 0.18	1.74 ± 0.49	0.03 ± 0.50	0.67 ± 0.09	0.55 ± 0.11	−0.11 ± 0.11	4.49 ± 1.71	8.47 ± 3.28	3.97 ± 2.93
3% w/w	1.71 ± 0.13	1.27 ± 0.22	−0.44 ± 0.24[a]	0.60 ± 0.08	0.40 ± 0.08	−0.19 ± 0.08	4.08 ± 1.44	2.74 ± 0.96	−1.34 ± 0.99[a]
6% w/w	1.62 ± 0.17	1.17 ± 0.12	−0.44 ± 0.17[a]	0.51 ± 0.08	0.43 ± 0.06	−0.07 ± 0.06[b]	3.53 ± 0.98	3.12 ± 1.30	−0.41 ± 1.20[a]

$\Delta_{0\rightarrow4\ wk}$, difference in serum levels between 0 and 4 wk data. Mice were fed for 4 weeks a Western diet supplemented with indicated amount of fish oil. Fasted mice were bled and serum free fatty acids, free glycerol, and β-hydroxybutyrate were determined. Values are the mean of 10–12 mice ± SD.

[a] Indicates a significant difference from control fed mice ($P < 0.020$).

[b] Indicates a significant difference between 3 and 6% w/w fish oil-fed group ($P < 0.020$).

Effect of dietary fish oil on VLDL kinetics in APOE*3-Leiden mice

To investigate whether the strong reduction in VLDL cholesterol and triglycerides as a result of fish oil feeding was related to increased VLDL catabolism and/or decreased VLDL production rate, VLDL turnover studies were performed. The amount of fish oil in the diet was 3% (w/w), and the feeding period was 2 weeks, as effects were already maximal under these conditions.

As shown in **Fig. 2** (left panel) and presented in **Table 5**, fish oil-fed mice showed a significant more than 2-fold increase in VLDL-apoB clearance rate (VLDL-apoB FCR) as compared to control-fed animals. Feeding a fish oil diet did not affect the VLDL-apoB secretion rate, indicating that the increase in VLDL clearance rate is the sole factor contributing to the observed 2-fold reduction in the VLDL-apoB pool upon fish oil feeding.

To study VLDL-triglyceride turnover, autologous VLDL was in vivo labeled in the triglyceride core using [^{3}H]-palmitate. Fish oil-fed mice showed a significant 60% reduction in VLDL-triglyceride production rate (VLDL-TG SR) (Table 5). Combining the respective mean VLDL-apoB SR and mean VLDL-TG SR data, fish oil feeding resulted in the production of relatively triglyceride-poor VLDL particles as compared those from controls (0.04 versus 0.11 μmol TG/μg apoB), which is in line with the relative low triglyceride content of VLDL isolated from fish oil fed mice compared to control mice, under fasted conditions (0.027 versus 0.032 μmol TG/μg apoB).

As illustrated in Fig. 2 (right panel) and Table 5, the VLDL-triglyceride FCR was significantly higher (1.7×) in fish oil mice as compared to control mice. To study whether the increase of the FCR of VLDL-triglycerides was secondary to the secretion of triglyceride-poor VLDL and the consequent decrease in VLDL-triglyceride pool (−62%) or resulted from the stimulation of a VLDL-triglyceride clearance mechanism, i.e., lipoprotein lipase activity, we calculated the lipolytic ratio as described by Hultin et al. (25). As calculated from the mean VLDL-apoB FCR and VLDL-triglyceride FCR, the triglyceride label was cleared

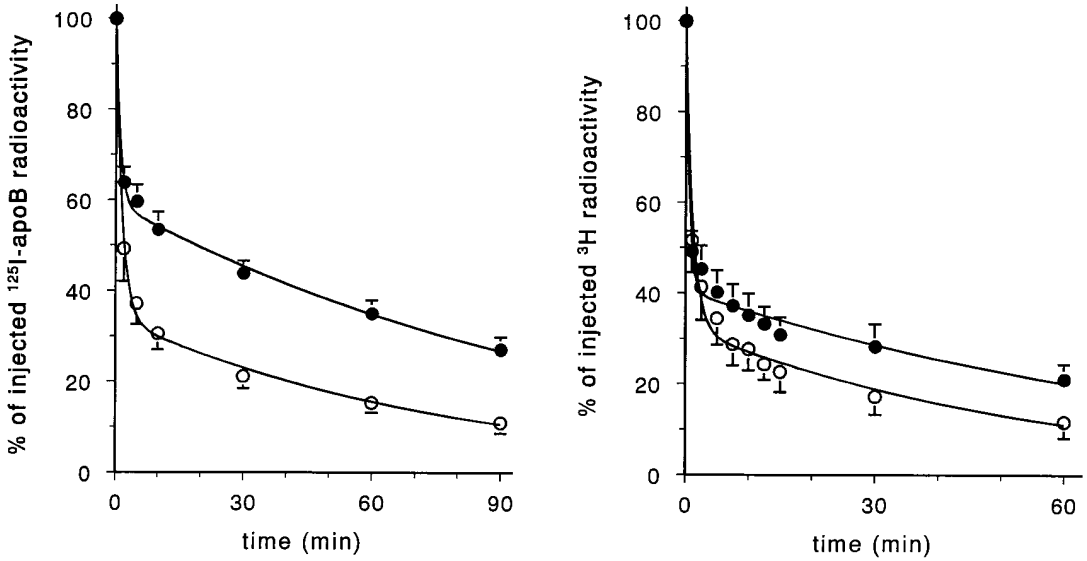

Fig. 2. VLDL-apoB and VLDL-triglyceride removal in control and fish oil-fed APOE*3-Leiden transgenic mice. After a 5-h fasting period, female APOE*3-Leiden transgenic mice fed for 2 weeks a control (●) or fish oil (3% w/w) diet (○) were injected with autologous ^{125}I-labeled VLDL (left panel) or [^{3}H]-palmitate-labeled VLDL (right panel). Twenty-five μl of blood was drawn at each time point and the respective ^{125}I-labeled apoB(100 + 48) or ^{3}H radioactivity of the serum sample was measured. Values are the mean ± SD of five mice. Curves were calculated from the mean data using a bi-exponential curve fit model.

TABLE 5. Effect of dietary fish oil on VLDL-apoB and VLDL-triglyceride turnover in APOE*3-Leiden transgenic mice

	VLDL-ApoB Turnover			VLDL-Triglyceride Turnover			
Fish Oil	VLDL-ApoB FCR	VLDL-ApoB Pool	VLDL-ApoB SR	VLDL-TG FCR	VLDL-TG pool	VLDL-TG SR	Lipolytic Ratio[b]
	pool/h	*μg/g mouse*	*μg/h/g mouse*	*pool/h*	*μmol/g mouse*	*μmol/h/g mouse*	
0% w/w	8.8 ± 1.3	3.4 ± 0.9	28.9 ± 5.7	36.1 ± 6.7	0.087 ± 0.037	3.2 ± 1.7	4.0
3% w/w	22.2 ± 4.6[a]	1.6 ± 0.9[a]	32.5 ± 17.1	60.4 ± 13.3[a]	0.022 ± 0.015[a]	1.3 ± 0.8[a]	2.7

FCR, fractional catabolic rate; SR, secretion rate; TG, triglyceride. VLDL kinetic studies were performed in female mice fed a control or a 3% w/w fish oil diet for 2 weeks (maximal reduction in serum VLDL levels were observed already under these conditions (not shown)). After a 5-h fasting period, female mice were injected with either 10 μg of autologous labeled ^{125}I-labeled VLDL protein of 10^5 dpm autologous [^{3}H-]TG-VLDL. ^{125}I-apoB and [^{3}H]TG-VLDL disappearance from circulation was determined and FCR and SR were calculated (see Methods). Values are the mean ± SD of five mice per group.

[a] $P < 0.05$, indicating significant difference between control and fish oil-fed mice, using non-parametric Mann-Whitney tests.

[b] Lipolytic ratio, as calculated from the mean VLDL-apoB FCR and VLDL-TG FCR.

4-times faster than the apoB-label in the control group, whereas in the fish oil group, the triglyceride label was cleared only 2.7-times faster than the apoB-label (lipolytic ratio of 4.0 and 2.7 for control and fish oil-fed group, respectively; Table 5). Thus, fish oil feeding decreased the rate of VLDL-triglyceride clearance relative to the apoB clearance, indicating that fish oil does not overstimulate the lipolytic rate.

DISCUSSION

In the present study, the effects of dietary fish oil on VLDL metabolism were investigated using hyperlipidemic APOE*3-Leiden transgenic mice. In addition to a genetic homogeneous background (inbred C57BL/6J), these mice have a "human-like" lipoprotein profile and are extremely sensitive to small changes in VLDL production and clearance rate (13, 14). Consequently, these mice may serve as a suitable animal model for studying the mechanisms underlying the effects of fish oil under highly standardized conditions.

Feeding a fish oil diet to APOE*3-Leiden transgenic mice resulted in a dramatic lowering of serum cholesterol and triglyceride levels, mainly due to a strong reduction in serum VLDL levels. This strong reduction in serum VLDL upon fish oil feeding was related to *i*) an increased clearance rate of the VLDL particles (Fig. 2, Table 5), and *ii*) a reduction in hepatic VLDL-triglyceride production (Table 5).

The effect of dietary fish oil to decrease hepatic VLDL triglyceride production observed in APOE*3-Leiden mice has been reported previously for humans (2–5). Fish oil reduces hepatic fatty acid synthesis and stimulates hepatic fatty acid catabolism thereby reducing the amount of fatty acids available for VLDL synthesis (5). In addition, a reduced flux of fatty acids to the liver, as reflected in the reduced serum free fatty acid levels observed in fish oil-fed APOE*3-Leiden mice (Table 4), may also contribute to the decrease in hepatic VLDL triglyceride production. Nestel et al. (2) in a study with five normolipidemic subjects and one hypertriglyceridemic subject showed that, in addition to VLDL triglyceride, VLDL apoB production also decreased after fish oil feeding. In our study, however, a concomitant reduction in hepatic VLDL-apoB production rate was not observed. Whether this differential effect of fish oil on VLDL-apoB production in our mouse study as compared to the human study of Nestel et al. (2) is the result of the species differences between mice and humans or the result of the large difference in the amount of fish oil supplemented to the diets (3% w/w versus 10–15% w/w, respectively) remains to be established. Our results indicate that fish oil feeding at low dose leads to production of normal amounts of VLDL particles, although the VLDL produced is triglyceride-poor. VLDL isolated from pooled serum of fish oil-fed mice indeed had a reduced triglyceride-to-apoB ratio when compared to particles isolated from control-fed mice (27.5 versus 31.6 μmol TG/mg apoB). This reduction in triglyceride-apoB ratio is rather small compared to the 2-fold difference in VLDL-triglyceride FCR and VLDL-triglyceride production rate. As this difference was determined using circulating VLDL particles, it can be expected that analysis of nascent non-lipolyzed VLDL (i.e. VLDL produced by a perfused liver) will yield a larger difference.

Another reported effect of dietary fish oil is modulation of LDL receptor expression. Several in vitro (7, 9) and in vivo studies (8) have shown that n–3 fish oils reduce expression of LDL receptors in hepatic cells, suggesting a reduced plasma clearance of lipoproteins via the LDL receptor pathway upon fish oil feeding. In this study, we found that fish oil feeding increased the clearance rate of VLDL-apoB by 60%, suggesting that a possible decrease in hepatic LDL receptor expression does not affect VLDL-apoB clearance rate in APOE*3-Leiden mice. Moreover, the absence of an effect of fish oil on serum LDL levels in our study suggests no effect of fish oil on LDL receptor activity in these mice at all. The mechanism of how fish oil increases VLDL-apoB clearance rate is not clear. Possibly, the relative triglyceride-poor VLDL produced upon fish oil feeding forms a better substrate for uptake via hepatic receptors than normal VLDL. This can be either via the (possibly down-regulated) LDL receptor or LDL receptor-related protein (LRP), the second receptor involved in chylomicron and VLDL remnant uptake. In addition, a stimulatory effect of dietary fish oil on the expression level of this LRP cannot be excluded.

It has been suggested that fish oil feeding may lower plasma triglycerides and increase plasma LDL levels by in-

creasing lipolytic activity (6). The observation that VLDL-triglyceride fractional catabolic rate increases in fish oil-fed mice (Fig. 2, Table 4) may indicate a fish oil-mediated stimulation of lipolytic activity. However, in our study, the increase in VLDL triglyceride FCR may result, at least partly, from the observed decrease in the serum triglyceride pool which results primarily from the observed decrease in VLDL triglyceride production. The serum VLDL-triglyceride removal independent from that of the VLDL-particle (=VLDL-apoB) was not increased by fish oil feeding (Table 4), suggesting that fish oil had no extra stimulatory effect on lipolytic activity. This was supported by the fact that we did not observe an increase in serum free fatty acid levels upon fish oil feeding. Treatment of APOE*3-Leiden mice with gemfibrozil (B. J. M. van Vlijmen, unpublished results) resulted in a strong reduction in serum VLDL cholesterol and triglycerides and a concomitant increase in LDL cholesterol due to stimulation of the lipolytic activity by this drug. The absence of such an increase of plasma LDL in APOE*3-Leiden mice fed the fish oil diet also argues against a stimulatory effect of fish oil on lipolytic activity.

The tracer die-away curves for both VLDL-apoB and VLDL-triglyceride demonstrate a rapid and slow phase of clearance (Fig. 2). These data suggest that the clearance of VLDL is a multistep process and possibly involves the rapid conversion into smaller VLDL followed by a slower conversion into IDL and LDL. Because we performed overall kinetics of autologous VLDL, we can only speculate on the nature, size and residence time of the particles in the respective pools. It would be interesting to further study the kinetics of the various VLDL, IDL and LDL pools in control and fish oil-fed mice. Unfortunately, such studies are technically difficult due to the small size of the animals.

Fish oil feeding often results in increased ketogenesis due to induction of β-oxidation of fatty acids (26–28). Remarkably, feeding the APOE*3-Leiden mice a control diet resulted in increased levels of serum β-hydroxybutyrate and feeding fish oil resulted in decreased levels of serum β-hydroxybutyrate, suggesting a decrease in ketogenesis due to fish oil feeding. Our present data do not provide an explanation for this difference. Interestingly, however, the decrease in serum β-hydroxybutyrate levels strongly correlated with the decrease in serum cholesterol, triglycerides and free fatty acid levels. Future research focusing on the relationship between serum β-hydroxybutyrate levels and serum cholesterol, triglycerides and free fatty acid levels should clarify this.

The present study showed that the serum lipid levels of APOE*3-Leiden mice are highly responsive to dietary fish oil. The effects of fish oil observed for APOE*3-Leiden mice largely resembles the major effects observed in (hyperlipidemic) humans i.e. a strong reduction of serum VLDL levels, concomitant with a decreasing effect on hepatic VLDL-triglyceride production. APOE*3-Leiden mice, however, did not demonstrate the increasing effect of fish oil on serum LDL levels as found in (hypertriglyceridemic) humans (1). In addition, in our study, no reduction of VLDL-apoB production was observed after fish oil feeding, as was found in a human study at high intake (2). Instead, fish oil improved VLDL-apoB clearance. Furthermore, our results do not provide evidence for a stimulatory effect of dietary fish oil on lipolytic activity, as has been suggested (6).

The responsiveness of VLDL metabolism of APOE*3-Leiden mice to dietary fish oil feeding combined with the use of these mice as a sensitive atherosclerosis model, as reported elsewhere (13, 29), makes the APOE*3-Leiden mouse a suitable model for evaluating the effects of dietary fish oil on the development of atherosclerosis.

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