Aspects involved in the (patho)physiology of the metabolic syndrome

Duivenvoorden, I.

Chapter 4

Response of apolipoprotein E*3-Leiden transgenic mice to dietary fatty acids: combining liver proteomics with physiological data

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Abstract

Dietary fatty acids have a profound impact on atherosclerosis, but mechanisms are not well understood. We studied the effects of a saturated fat diet supplemented with fish oil, *trans*10,*cis*12 CLA (CLA), or elaidic acid on lipid and glucose metabolism and liver protein levels of APOE*3Leiden transgenic mice, a model for lipid metabolism and atherosclerosis. Fish oil lowered plasma and liver cholesterol and triglycerides, plasma free fatty acids, and glucose, but increased plasma insulin. CLA lowered plasma cholesterol but increased plasma and liver triglycerides, β-hydroxybutyrate, and insulin. Elaidic acid lowered plasma and liver cholesterol. Proteomics identified significant regulation of 65 cytosolic and 8 membrane proteins. Many of these proteins were related to lipid and glucose metabolism, and to oxidative stress. Principle component analysis revealed that fish oil had a major impact on cytosolic proteins, and elaidic acid on membrane proteins. Correlation analysis between physiological and protein data revealed novel clusters of correlated variables, among which a metabolic syndrome cluster. The combination of proteomics and physiology gave new insights in mechanisms by which these dietary fatty acids regulate lipid metabolism and related pathways, for example, by altering protein levels of long-chain acyl-CoA thioester hydrolase and adipophilin in the liver.

Introduction

Coronary heart disease (CHD) is one of the major causes of mortality in industrialized countries, with diet believed to play a major role in disease development. Several dietary fatty acids (FA) may contribute to, or decrease the risk of CHD, primarily because of their detrimental, or beneficial, effects on the lipoprotein profile[1]. Numerous controlled feeding studies in humans have established that saturated FA increase and polyunsaturated FA decrease total and low density lipoprotein (LDL) cholesterol[2]. *Trans-FA* have been shown to raise LDL cholesterol and lower high density lipoprotein (HDL) cholesterol relative to *cis*-unsaturated FA[3,4] and to increase triglycerides (TG)[5]. Conjugated linoleic acids (CLA), which structurally may be classed as *trans-FA*, protect against the development of atherosclerosis in rabbits, hamsters, and transgenic mice[6-8]. Nevertheless, *in vivo* data on possible hypolipidemic effects of CLA are conflicting[9].

The mechanisms by which the different dietary FA affect lipid metabolism and the development of CHD are often not completely understood, although some studies are available. Omega-3 FA (present in fish and fish oil) decrease TG levels by inhibition of FA synthesis in the liver and up-regulation of oxidation in liver and skeletal muscle[10]. The effects of CLA on lipid metabolism appear to be produced largely by the *t*10,*c*12 isomer of CLA. This isomer significantly reduced apolipoprotein (apo) B secretion from HepG2 cells[11] as well as hepatic stearoyl-CoA desaturase expression[12] and activity[13]. Although these results suggest a TG-lowering effect of *trans*10,*cis*12 (*t*10,*c*12) CLA mediated by

falls in VLDL-TG secretion and FA synthesis, respectively, *in vivo* data on the hypolipidemic effects of CLA are inconsistent[9]. *Trans*-FA adversely affect essential FA metabolism and prostaglandin balance by inhibiting the enzyme delta-6 desaturase[14] and they may promote insulin resistance[15].

The differences in study design and the conflicting results from different animal models make it difficult to assess the physiological, biochemical and molecular mechanisms by which these dietary FA exert their effect. Thus, studies are needed in which the effects of different dietary FA are studied in a single model that is sensitive to relatively mild perturbations in the diet. The APOE*3Leiden mouse model responds well to modulators of lipoprotein metabolism and atherosclerosis, such as cafestol and plant stanols[16-18]. In addition, APOE*3Leiden mice have proven to be responsive to fish oil[19]. APOE*3Leiden mice express the human APOE*3Leiden gene, resulting in a lipoprotein profile similar to that of humans. Hence, these mice easily develop diet-induced hyperlipidemia and atherosclerosis[20,21] and are therefore suitable for a comparative study on the effects of dietary FA.

In this study, we have compared the impact of dietary fish oil, *t*10,*c*12 CLA, and elaidic acid on lipoprotein metabolism and insulin levels. Both CLA and fish oil are suggested to act as peroxisome proliferator-activated receptor (PPAR) agonists[10,22], and therefore we also included the TG-lowering drug fenofibrate as a positive control. Fibrates are PPAR agonists known to stimulate cellular FA uptake, conversion of FA to acyl-CoA derivatives, and catabolism of FA via the β-oxidation pathways. Combined with a reduction in FA and TG synthesis, this results in a decrease in very low density lipoprotein (VLDL) production[23], an observation confirmed in the APOE*3Leiden mouse. Effects on FA, lipoprotein, and glucose metabolism were assessed by measuring metabolite concentrations in plasma. Impact on liver physiology was studied by liver lipid analysis and by 2-dimensional (2D) gel electrophoresis on both cytosolic and membrane proteins. Proteomics was used to identify potential pathways through which dietary FA may exert their specific effects on physiology and CHD.

Materials and Methods

This study was approved by the animal care committee of TNO Quality of Life, Leiden, The Netherlands.

Animals and diet

Female APOE*3Leiden transgenic mice, aged 5-6 months, were kept in groups of 4 animals under standard conditions with free access to food and water. Forty animals consumed a semi-synthetic high-fat/cholesterol (HFC) diet (Hope Farms, The Netherlands) containing cocoa butter (15% w/w) and cholesterol (0.25% w/w) during a 3-week run-in period. They were then randomly assigned to 5 treatment groups of 8 animals each that were matched for body weight and total cholesterol. During the intervention period of 3

weeks, one group continued to be given the HFC diet (control group). The second group (fish oil group) received the HFC diet supplemented with 3% (w/w) of a mixture of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), (Marinol^{TM}, Loders Croklaan, The Netherlands). The third group (CLA group) received the HFC diet supplemented with1% (w/w) of the *t*10,*c*12 isomer of CLA (Loders Croklaan), the fourth group (elaidic group) received the HFC diet supplemented with 3% (w/w) of elaidic acid (Loders Croklaan). The fifth group (fenofibrate group) received the HFC diet supplemented with 0.04% (w/w) of fenofibrate (Sigma, Dorset, UK). All supplements were added at the expense of cacao butter, leading to equal fat and energy contents per gram food in the different diets (**Table1**). The percentage of energy provided by fish oil (EPA+DHA), *t*10,*c*12 CLA, and elaidic acid was 2.3%, 1.7%, and 4.1% respectively. Adequate portions of the diets were replaced every other day. Body weight and food intake were monitored throughout the experiment.

Table 1. Fatty acid composition of the experimental diets

Fatty acid (FA)	Control	Fish oil	CLA	Elaidic acid	Fenofibrate
			g/100 g diet		
Saturated FA	10.6	10.3	10.6	9.4	11.1
Monounsaturated FA	5.9	5.7	5.8	6.5	6.1
Elaidic acid	0.0	0.0	0.0	0.9	0.0
Polyunsaturated FA	1.9	2.4	2.2	1.6	1.6
n-6 polyunsaturated FA	1.1	1.0	1.7	1.0	1.1
*t*10,*c*12 CLA	0.0	0.0	0.7	0.0	0.0
n-3 polyunsaturated FA	0.8	1.4	0.5	0.6	0.5
Eicosapentaenoic acid	0.3	0.7	0.2	0.3	0.2
Docosahexaenoic acid	0.0	0.4	0.0	0.0	0.0

The fatty acid composition of the experimental diets was analyzed as described in materials and 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. The fenofibrate diet contained 0.04% (w/w) of fenofibrate. All percentages are in weight/weight.
CLA *trans*10,*cis*12 conjugated linoleic acid

Dietary lipid analysis

Total lipids from diet subsamples were extracted using the method of Folch[24] and total lipid was then converted to its methyl esters by direct *trans*-esterification using 1% sulphuric acid in methanol. The proportion of individual FA in the diet was determined with an Agilent 6890 gas chromatograph fitted with a 30 m DB23 capillary column (J&W Scientific, Folsom, CA, USA) with an inner diameter of 0.25 mm and 0.25 µm film thickness. Helium

(25 psi) was used as a carrier gas. The initial oven temperature was programmed at 800°C, then to rise to 1800°C at a rate of 250°C per min, then to rise to 2200°C at a rate of 10°C per min, and then kept constant. The temperature of the injector and the flame-ionization detector was set at 2500°C, while a split ratio of 50:1 was used. A standard mixture was used to identify the FA methyl esters by means of the retention times. Results were expressed as a proportion of total identified FA. Butylated hydroxy-toluene (0.005% w/v, Sigma) was added to all organic solvents to prevent oxidation of the polyunsaturated FA.

Plasma analyses

At week 0 and 3 of the intervention period, mice were fasted for 4 h, after which blood samples were obtained from the tail vain into chilled paraoxon-coated capillaries to prevent lipolysis[25]. Plasma was collected via centrifugation at 13000 rpm for 5 min for the measurement of plasma total cholesterol (Roche Diagnostics, Mannheim, Germany), TG without free glycerol (Triglyceride GPO-Trinder, Sigma Diagnostics, St. Louis, MO, USA), non-esterified fatty acids (Wako chemicals, Neuss, Germany), glucose (Trinder 500, Sigma Diagnostics), and β-hydroxybutyrate (Sigma Diagnostics) by standard commercial kits, according to the manufacturer's instructions. Plasma insulin was measured by radio-immunoassay, using rat insulin standards that have 100% cross-reaction with mouse and human insulin (sensitive rat insulin assay, Linco research, St. Charles, MO, USA).

For size fractionation of lipoproteins, 50 µl of pooled plasma was injected onto a Superose 6 column (3.2 x 300 mm, Äkta purifier, Amersham Pharmacia Biotech, Uppsala, Sweden) and eluted at a constant flow rate of 50 µl/min with phosphate buffered saline (PBS) (pH 7.4, containing 1 mmol/l EDTA). Fractions of 50 µl were collected and assayed for total cholesterol (as described above) and TG (Triglycerides GPO-PAP, Roche Diagnostics).

Liver lipid analyses

After the intervention period of 3 weeks, mice were fasted 4 h and then sacrificed to obtain liver tissue for lipid analyses and proteomics. The liver was perfused with ice-cold PBS, weighed and samples were snap-frozen in liquid nitrogen. Liver lipid content was analyzed by sample homogenization in PBS (+/-10% wet wt/vol). Protein content was determined by a Lowry assay[26], followed by extraction of lipids using the Bligh and Dyer method[27]. The lipids were separated using high-performance thin-layer chromatography (HPTLC) on silica gel plates as described before[28] and subsequent analysis was performed by TINA2.09 software[29] (Raytest Isotopen meβgeräte, Straubenhardt, Germany).

Proteomics

Preparation of cytoplasm protein fraction

A sample of frozen liver (± 125 mg) was added to 500 µl extraction buffer (pH 7.1) containing 50 mM Tris, 100 mM KCl, 20% glycerol, 1.4 µM pepstatin A, 1.0 mM PMSF and the protease inhibitor cocktail (Roche Diagnostics) Complete™ according to the manufacturer's instructions (Boehringer Mannheim). This sample was homogenized with an eppendorf homogenizer on ice for 30 sec, sonicated in ice water for 15 sec and again homogenized on ice for 30 sec. Thereafter, the homogenate was centrifuged (Beckman TL-100) for 30 min at 55000 rpm at 4°C. The resulting supernatant was withdrawn, and the pellet was weighed and re-homogenized in the extraction buffer as outlined above. After the second centrifugation step, the supernatant was added to the first fraction and the protein content of the combined supernatant fractions was measured using the Bradford assay[30].

Preparation of membrane protein fraction

The pellet was weighed and re-homogenized in CHAPS buffer (0.2 M KCl, 0.1 M sodium phosphate, 20% glycerol, 0.12M CHAPS and Complete™) according to the manufacturer's instructions at a ratio of 2 µl buffer per mg of pellet. The sample was homogenized and sonicated as for the preparation of cytoplasm proteins. Urea and DL-dithiothreitol (DTT) were added to obtain final concentrations of 9 M and 70 mM, respectively. When the additions were fully dissolved, the sample was centrifuged at 55000 rpm for 30 min at 20°C. The supernatant was carefully removed, after which 2% ampholytes 3-10 (Servalyt™, Serva Electrophoresis, Heidelberg, Germany) were added prior to 2D electrophoresis. The protein content of the membrane protein fraction was measured using the Bradford assay[30].

2D gel electrophoresis

For the cytoplasm protein fractions, four 2D electrophoresis gels were run per dietary treatment, each gel representing a pool of two randomly selected animals per group. For the membrane protein fraction, two 2D electrophoresis gels were run per treatment, each gel representing a pool of four randomly selected animals per group. BioRad immobilized pH gradient (IPG) strips (pI 5-8 for cytoplasm proteins and pI 3-10 for membrane proteins) were used for the separation of the proteins in the first dimension. Strips were rehydrated in 300 µg of protein sample at 20°C for one hour without applied voltage on a BioRad IEF cell. After 1 h, each lane was overlaid with mineral oil to prevent the strips from drying out. For another 16 h a voltage of 50 V per strip was applied, after which the strips were transferred to a clean tray and overlaid with mineral oil. The initial start up and ramping protocol were performed as recommended by BioRad. After 1 h, the strips were removed to a tray containing fresh wicks and were overlaid with mineral oil. The run proceeded until the

preset volthours had been reached, after which the voltage was held at 500 V total until the strips were ready to be transferred to the second dimension SDS-PAGE step.

SDS-PAGE

IPG strips were removed from the focusing tray and incubated in fresh equilibration buffer (6 M Urea; 2% SDS; 0.375 M Tris-HCl, pH 8.8: 20% glycerol; and 130 mM DTT) for 10-15 minutes at room temperature before transfer to a second equilibration buffer (6 M Urea; 2% SDS; 0.375 M Tris-HCl, pH 8.8; 20% glycerol; and 135 mM iodoacetamide) for 10-15 minutes at room temperature. The strip was then rinsed in tank buffer (24 mM Tris; 0.2 M glycine; and 0.1% SDS, pH 8.6) and applied to the top of an 18x18 cm gel cassette. The strip was fixed in position by overlaying with molten agarose (2% agarose in tank buffer with 2 mg/100 ml bromophenol blue). A 7.5 µl unstained BioRad precision standard was inserted in the well formed on the right of the cassette. Gels were run at 200 V for 9.5 h or until the bromophenol blue had reached the bottom of the gel. After the second dimension run, the gels were placed into a fixation solution of 50% ethanol, 2% ortho-phosphoric acid and 48% H_2O for a minimum of three hours. Gels were then washed for at least one hour with a couple of changes of H_2O after which they were shaken in a staining solution of 34% methanol, 2% ortho-phosphoric acid and 64% H_2O containing 17% $(NH_4)_2$ SO_4 and 1 mg/ml Coomassie blue sprinkled on top of the staining solution.

2D electrophoresis gel comparisons

2D electrophoresis gels were analyzed using PDQuest software (BioRad). Spots with densities that significantly differed between treatments were excised from the SDS-PAGE gels using the BioRad spot cutter. Gel plugs were directly placed into a 96-well V-bottomed plate with 100 µl of water that was removed immediately before the trypsination process. The proteins were trypsinized using the MassPrep Station (Waters, Micromass, Manchester, UK) protocol, which includes sequentially: destain steps for Coomassie Blue removal, reduction of the protein with DTT, alkylation of the protein with iodoacetamide, removal of DTT and iodoacetamide, dehydration of the gel plug, incubation with trypsin, and extraction of the peptides. Of the extracted peptides 1 µl was applied to the target area of a 96 x 2 teflon MALDI target plate (Applied Biosystems, Warrington, UK) and allowed to dry to ~ 50% of the original volume. At this point, 0.5 µl of an α-cyano-4-hydroxycinnamic acid matrix solution (5 mg/ml in 70% acetonitrile/H_2O, 0.1% TFA) was applied to the target. The samples were dried in a stream of air before matrix-assisted laser desorption/ionisation (MALDI) mass spectrometric analysis.

MALDI-TOF mass spectrometric analysis

MALDI – time of flight (TOF) mass spectrometry was performed using an Applied Biosystems Voyager-DE PRO in reflectron mode. Each spectrum was obtained using 500 shots of the appropriate laser power and, where appropriate, spectra were accumulated and filed. A macro was applied, which allowed baseline correction and de-isotoping of the peptide mass peaks. A peptide mass list of the most intense peaks was generated automati-

cally, and the list was pasted into Matrix Science Mascot by using the MSDB database during the search. We set the following search criteria: allowance of 0 or 1 missed cleavage, trypsin as digestion enzyme, carbamidomethyl modification of cystein, methione oxidation as partial modification, and charge state as MH^+.

Statistical analysis

The Mann-Whitney U test was used to determine differences in responses during the intervention period between the control group and the other treatment groups. Thus, the effects of dietary fish oil, *t*10,*c*12 CLA, elaidic acid, and fenofibrate were compared with the effects of saturated fat. All data are presented as mean ± SD. Analyses were performed using SPSS11.0 (SPSS, Chicago, IL, USA). Principle component analysis was performed using PLS toolbox (Version 3.0, Eigenvector Research, Manson, WA, USA) working under Matlab (Version 6.5, The MathWorks, Natick, MA, USA). Analysis of correlations was done with Pearson correlation coefficients. Proteins with very low expression, specifically occurring in the fish-oil treatment, were removed from the set, as they led to many false-positive correlations between those proteins. The analysis of multiple hypotheses testing for many combinations of variables was done with the QVALUE tool[31], running under the statistical software package R (http://www.r-project.org/). The resulting *q*-values estimate the probability that a correlation that is called significant, is false positive. For example, a *q*-value of 0.05 would mean, that we should expect that 5 out of 100 associations that were tested significant, are in fact false positive.

Results

Physiological studies

Food intake and body weight

Both food intake and body weight remained constant in all treatment groups throughout the intervention period (data not shown).

Plasma lipids, glucose and insulin

After the 3-week intervention period, all diets significantly lowered plasma total cholesterol levels compared with the control diet (**Table 2**). Fish oil reduced plasma total cholesterol by 55%, *t*10,*c*12 CLA by 50%, elaidic acid by 25%, and fenofibrate by 55%, compared with the control group (all $P < 0.05$). Analysis of the lipoprotein profiles revealed that this decrease in cholesterol could be explained by a decrease in VLDL and IDL cholesterol levels compared with the control group. *t*10,*c*12 CLA appeared to cause a slight increase in LDL cholesterol compared with the control group (**Figure 1**).

Plasma levels of TG were significantly decreased by 67% in the fish oil group and by 82% in the fenofibrate group, as compared with the control group (both $P < 0.05$). Plasma levels of TG were significantly increased by 64% in the CLA group compared with the control group ($P < 0.05$). Plasma levels of TG in the elaidic acid group did not differ

significantly from the placebo group (**Table 2**). Changes in levels of total TG were due to either an increase or a decrease in the TG levels of predominantly VLDL and IDL lipoprotein particles (**Figure 1**).

Fish oil and fenofibrate treatment significantly decreased the amount of free FA in plasma to almost half the concentrations in the control group (both $P < 0.05$). *t*10,*c*12 CLA and elaidic acid did not affect plasma levels of free FA (**Table 2**). Plasma β-hydroxybutyrate, a keton body that is often used as an indicator of hepatic β-oxidation, was significantly increased by *t*10,*c*12 CLA, elaidic acid, and fenofibrate treatment as compared with the control group (**Table 2**; all $P < 0.05$). The fish oil group showed a trend toward a higher plasma level of β-hydroxybutyrate although this increase was not significant.

Plasma glucose was significantly lower in the fish oil group and in the fenofibrate group (both $P< 0.05$). Plasma insulin levels were increased 2.8 times in *t*10,*c*12 CLA-fed animals and also slightly increased (0.25 times) in fish oil-fed animals (**Table 2**; both $P < 0.05$).

Table 2. Plasma lipid, glucose, and insulin levels of APOE*3Leiden mice fed a high-fat/high-cholesterol diet (control), or this diet supplemented with fish oil, *trans*10,*cis*12 CLA, elaidic acid, or fenofibrate for 3 weeks

	Control	Fish oil	CLA	Elaidic acid	Fenofibrate
Total cholesterol (mmol/l)	13.74 ± 2.85	6.18 ± 0.53*	6.81 ± 0.85*	10.31 ± 1.93*	6.25 ± 0.70*
Triglycerides (mmol/l)	1.09 ± 0.33	0.36 ± 0.10*	1.79 ± 0.36*	1.32 ± 0.87	0.20 ± 0.05*
Free fatty acids (mmol/l)	1.31 ± 0.24	0.76 ± 0.12*	1.37 ± 0.23	1.06 ± 0.13	0.72 ± 0.06*
β-hydroxybutyrate (mmol/l)	0.36 ± 0.12	0.46 ± 0.21	0.75 ± 0.35*	0.56 ± 0.10*	0.75 ± 0.17*
Glucose (mmol/l)	11.41 ± 1.30	8.84 ± 1.00*	12.65 ± 1.57	10.9 ± 1.89	9.33 ± 1.94*
Insulin (pmol/l)	514 ± 41	639 ± 81*	1430 ± 653*	535 ± 82	582 ± 99

Blood samples were taken via tail-tip incision after a 4h-fasting period. Total cholesterol, triglycerides, free fatty acids, β-hydroxybutyrate, glucose, and insulin were measured as described in materials and methods. Values represent the mean ± SD for n=8 mice per group. *P < 0.05 vs. control group.
CLA *trans*10,*cis*12 conjugated linoleic acid

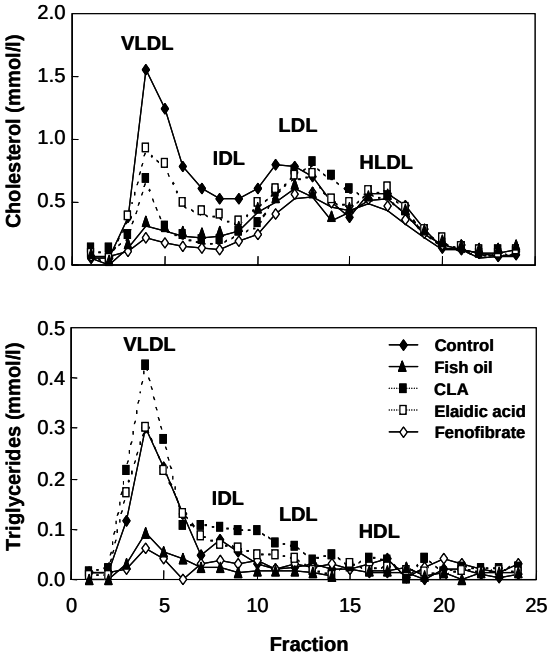

Figure 1. Cholesterol (upper panel) and triglyceride (lower panel) lipoprotein profiles
Plasmas of APOE*3Leiden mice, which had been fasting for 4 h, were pooled per dietary treatment group. Lipoprotein profiles were determined as described in materials and methods. CLA *trans*10,*cis*12 conjugated linoleic acid, HDL high density lipoprotein, IDL intermediate density lipoprotein, LDL low density lipoprotein, VLDL very low density lipoprotein

Liver weight and liver lipids

Consumption of fish oil led to a small but significant ($P < 0.05$) decrease in liver weight, whereas fenofibrate treatment caused a small but significant ($P < 0.05$) increase after 3 weeks of treatment (**Table 3**). Both treatments caused a significant ($P < 0.05$) decrease in the amount of free cholesterol, TG, and cholesteryl esters in the liver cells as compared with the control treatment. Consumption of *t*10,*c*12 CLA resulted in a two-fold increase in liver weight compared with the control group ($P< 0.05$). Liver cells contained significantly more TG and less cholesteryl esters after treatment with this dietary FA (**Table 3**).

Table 3. Liver weight and liver lipid levels determined in APOE*3Leiden mice fed a high-fat/high-cholesterol diet (control), or this diet supplemented with fish oil, *trans*10,*cis*12 CLA, elaidic acid, or fenofibrate for 3 weeks

Liver	Control	Fish oil	CLA	Elaidic acid	Fenofibrate
Weight (g)	1.15 ± 0.11	1.03 ± 0.10*	2.34 ± 0.09*	1.19 ± 0.13	1.70 ± 0.21*
Cholesteryl ester (µg /mg protein)	34.8 ± 3.2	25.2 ± 1.4*	22.9 ± 3.9*	37.0 ± 2.4	24.2 ± 2.3*
Free cholesterol (µg /mg protein)	13.4 ± 3.3	10.3 ± 1.1*	12.4 ± 1.0	13.8 ± 1.9	9.5 ± 1.2*
Triglycerides (µg /mg protein)	96.4 ± 18.0	67.6 ± 3.0*	144.6 ± 21.3*	109.0 ± 10.6	63.8 ± 4.4*

Hepatic levels of cholesteryl esters, free cholesterol, and triglycerides were determined as described in materials and methods. Values represent the mean ± SD for n=8 mice per group. *P < 0.05 vs. control. CLA *trans*10,*cis*12 conjugated linoleic acid

Proteomics

2D gel electrophoresis and MALDI-TOF mass spectrometry

When comparing the 2D gel electrophoresis gels, we found significant changes in the levels of 74 liver cytosolic proteins and 14 liver membrane proteins that were significantly up- or down-regulated by at least one of the dietary treatments compared with the control group. Of these, we could identify 65 liver cytosolic proteins and 8 liver membrane proteins using MALDI-TOF mass spectrometry (**Figure 2**). The cytosolic proteins were categorized according to their major functions to facilitate the study of pattern changes between treatments. These classifications are arbitrary to extent that they depend on currently recognized functions of proteins. However, clear effects could be seen on pathways that are involved with glucose and lipid metabolism, and in oxidation and aging. These pathways are also consistent with the physiological effects that were observed.

Principle component analysis

Liver cytosolic proteins

Principle component analysis of the log-transformed spot density values revealed that more than 47% of all variance in the dataset was accounted for by the first principle component (PC1) and nearly 21% of variance in the dataset was accounted for by the second principle component (PC2) (**Figure 3**, upper panel). The largest treatment effect on the first principle component (*i.e.*, the largest distance between the spots representing the saturated fat control group and the spots representing a dietary intervention group on the x-axis) was produced by fish oil. When considering the second principle component, fenofibrate treatment initiated a specific treatment effect, whereas all other treatments were situated much closer to the saturated fat control group. Treatment with elaidic acid produced the least treatment effect in relation to the saturated fat control group, both for PC1 and PC2. **Table 4a** summarizes the cytosolic proteins that provide the largest contribution to the dietary treatment effects in the principle component analysis.

Liver membrane proteins

Principle component analysis of the log-transformed spot density values revealed that more than 40% of all variance was accounted for by PC1 and more than 30% of variance was accounted for by PC2 (**Figure 3**, lower panel). The largest treatment effect was produced by elaidic acid, both on PC1 and PC2. The proteins that provided the largest contributions to the dietary treatment effects in the principle component analysis are outlined in **Table 4b**.

Pair-wise correlation analysis

In addition to PC analysis (PCA), we performed a pair-wise correlation analysis over the different treatments, including the physiological data on plasma lipid, glucose and insulin levels, and liver lipids (**Table 2** and **3**), as well as the data on protein levels. Such an analysis shows which parameters vary in a similar way throughout the different treatments. **Figure 4** shows a network of pair-wise interactions with a Pearson correlation higher than 0.8, a relating P-value of 0.0053 or lower, and a relating *q*-value 0.026 or lower, prepared using the software tool Cytoscape[32].

Figure 2 (below). Overview of cytoplasm and membrane proteins of which levels were significantly increased or decreased in one or more of the dietary intervention groups, as compared with the control group

Relative protein masses were calculated using the PDQuest software as described in materials and methods. The code indicates the percentage increase or decrease in protein mass in any dietary intervention group as compared with the control group. Proteins were identified using proteomics as described in materials and methods. CLA *trans*10,*cis*12 conjugated linoleic acid

Liver cytoplasm		Fish Oil	CLA	Elaidic acid	Fenofibrate
Glucose metabolism	Galactokinase				
	Ketohexokinase				
	Fructose bisphosphatase 1				
	Malate dehydrogenase				
	Alpha enolase				
	Alpha enolase				
	Thriosephophate isomerase				
	Phosphoglycerate mutase				
	Isocitrate dehydrogenase				
	Adenosine kinase				
Lipid metabolism	Adenosine kinase				
	Long chain acetyl-CoA dehydrogenase				
	Long chain acyl-CoA thioester hydrolase				
	Long chain acyl-CoA thioester hydrolase				
	Mitochondrial long-chain acyl-CoA thioester				
	Acyl-CoA thioester hydrolase				
	Acyl-CoA thioesterase				
	Apolipoprotein E				
	Apolipoprotein A1 precursor				
	Adipophilin				
	2-hydroxyphytanoyl-Coa lyase				
	CTP;phosphocholine cytidyltransferase b2				
	Isovaleryl CoA dehydrogenase				
	Phosphatidylethanolamine-binding protein				
	Hypothetical GDSL-like Lipase/ acylhydrolase				
Protein metabolism	Annexin A5				
	Formimimo cyclodeaminase				
	Ornithine aminotransferase				
	Glutamine synthetase				
	Glutamine synthetase				
	Histidine ammonia-lyase				
	Arginase-1liver				
	3-hydroxyanthranilate 3,4-dioxygenase				
	Cysteine sulfinic acid decarboxylase				

Liver Cytoplasm continued

		Fish Oil	CLA	Elaidic acid	Fenofibrate
Oxidation and aging	Senescence marker protein 30				
	Selenium binding protein 56kDa				
	Selenium binding protein 56kDa				
	T-complex protein 1, beta subunit				
	Peroxyredoxin 6				
	Glutathione S-transferase Mu 2				
	Gluthatione S-transferase Mu 1				
	Heat shock protein 74 kDa				
	Epoxide hydrolase				
	Catalase				
	Aldehyde dehydrogenase				
	Aldehyde dehydrogenase				
	Aldehyde dehydrogenase				
	L-gulonolactone oxidase				
Homocysteine metabolism	Thioether S-methyltransferase				
	Adenosylhomocysteinase				
	Glycine-N-methyltransferase				
BH4 metabolism	Sepiapterin reductase				
	Phenylanaline-4-hydroxylase				
Haem biosynthesis	Delta-aminoevulinate dehydratase				
	Hydroxymethylbiliane				
Energy metabolism	Pyrophosphatase				
	Nicotinate-nucleotide pyrophosphorylase				
Enzymes	N-sulfotransferase				
	Proteasome beta subunit				
	Nucleoside diphosphate kinase B				
	Guanidinoacetate N-methyltransferase				
	Purine nucleoside phosphorylase				
	Ubiquitin / ribosomal protein CEP52				
Carcinogenesis marker	Tetranectin				
Structural proteins	Tubulin alpha-2 chain				

Liver Membrane

Lipid metabolism	Carnitine palmitoyltransferase isoenzyme				
Protein metabolism	Fumarylacetoacetase				
Oxidation	Aldehyde dehydrogenase				
	Cytochrome P450 2B10				
	Cytochrome B5				
Pyrimidine biosynthesis	CTP synthase				
Carcinogenesis marker	Tetranectin precursor				
Structural protein	Vimentin				

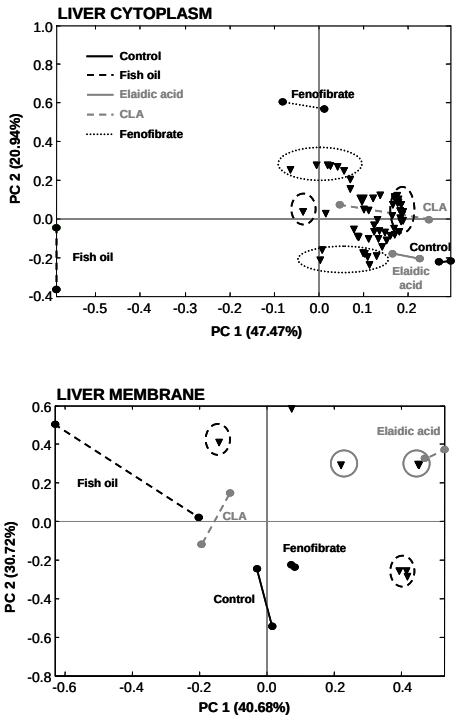

Figure 3. Scoreplot of an unsupervised principle component analyses of liver cytoplasm and membrane proteins that are significantly up- or down-regulated by fish oil, *trans*10,*cis*12 conjugated linoleic acid (CLA), elaidic acid and/or fenofibrate as compared with the control group

Table 4a. Liver cytoplasm proteins representing the highest positive and negative loadings towards the dietary treatment effects

Treatment	Proteins representing the highest positive loadings towards treatment	Proteins representing the highest negative loadings towards treatment
Fish oil	Annexin A5 Ornithine aminotransferase Ubiquitin/ribosomal protein CEP52 Selenium binding protein Triosephosphate isomerase Glutathione S-transferase Mu2 Epoxide hydrolase	Galactokinase Hydroxymethylbiliane synthase Isocitrate dehydrogenase Thioether S-methyltransferase Adipophilin GDSL-like lipase Nicotinate-nucleotide pyrophosphorylase T-complex protein 1, beta subunit Phosphatidyl ethanolamin-binding protein Apolipoprotein A1 precursor Guanidinoacetate N-methyltransferase Glutamine synthase Tubulin alpha 2 chain Purine nucleoside phosphorylase Glutatione S-transferase Mu1
CLA	Galactokinase Hydroxymethylbiliane synthase Isocitrate dehydrogenase Thioether S-methyltransferase Adipophilin Hypothetical GDSL-like lipase Nicotinate-nucleotide pyrophosphorylase T-complex protein 1, beta subunit Phosphatidyl ethanolamin-binding protein Apolipoprotein A1 precursor Guanidinoacetate N-methyltransferase Glutamine synthase Tubulin alpha 2 chain Purine nucleoside phosphorylase Glutatione S-transferase Mu1	Annexin A5 Ornithine aminotransferase Ubiquitin/ribosomal protein CEP52 Selenium binding protein Triosephosphate isomerase Glutathione S-transferase Mu2 Epoxide hydrolase
Fenofibrate	Acyl CoA thioester hydrolase Long chain acetyl-CoA dehydrogenase Long chain acyl-CoA thioester hydrolase Long chain acyl-CoA thioester hydrolase Acyl-CoA thioester hydrolase Cysteine sulfinic acid decarboxylase CTP:phosphocholine cytidyltransferase b2 Mitochondrial long chain acyl-CoA thioester hydrolase Catalase	Annexin A5 Ornithine aminotransferase Fructose biphophatase 1
Control + Elaidic acid	Adenosine kinase Phenylalanine-4-hydroxylase 3-Hydroxyanthranilate 3,4-dioxygenase Glutamine syntase Glycine-N-methyltransferase Fructose biphosphatase 1 Alpha enolase Senescence marker protein 30	Ubiquitin/ribosomal protein CEP52 Long chain acyl-CoA thioester hydrolase

Table 4b. Membrane proteins representing the highest positive and negative loadings towards the dietary treatment effects

Treatment	Proteins representing the highest positive loadings towards treatment	Proteins representing the highest negative loadings towards treatment
Fish oil		Fumarylacetoacetase Vimentin Cytochrome B5 precursor
CLA		Aldehyde dehydrogenase CTP synthase
Elaidic acid	Aldehyde dehydrogenase CTP synthase	
Fenofibrate +control		Carnitine palmitoyltransferase isoenzyme Cytochrome P450 2B10 Tetranectin precursor

Loadings were calculated using principle component analysis as described in materials and methods. CLA *trans*10,*cis*12 conjugated linoleic acid

Discussion

This study has been unique in two ways. First, it compared three important dietary FA known to regulate lipid, and possibly glucose metabolism, in a single well-validated animal model for lipid metabolism and atherosclerosis. Second, it combined physiological data on plasma and liver levels with a proteomic study of liver proteins. Such a combined approach allowed us to identify pathways and proteins that may underlie the changes in lipid and glucose metabolism under these dietary regimes. Conclusions from the results can first be derived by considering individual dietary treatments.

Fenofibrate and fish oil

In our mouse model, we used fenofibrate as a positive control for changes in FA catabolism, as this drug represents a validated agonist of PPARα[23]. Fenofibrate indeed changed proteins involved in FA oxidation (see **Figure 2** and **Table 4a**), lowered plasma and liver TG levels, reduced plasma levels of free FA, and increased β-hydroxybutyrate levels.

The modification of plasma lipoprotein metabolism by omega-3 FA represents a major anti-atherogenic mechanism of action[33]. Dietary polyunsaturated FA (PUFA) inhibit lipogenesis by suppressing the expression of a number of hepatic enzymes involved in glucose metabolism and FA biosynthesis[34-38] through a reduced expression of sterol regulatory element binding protein-1 (SREBP-1). At the same time, PUFA induce genes encoding proteins involved in FA oxidation and ketogenesis by activation of PPARα[10,34,39]. The latter mechanism is shared by both omega-3 FA and the hypotriglyceridemic drug fenofibrate[23]. Indeed, in this study both fish oil and fenofibrate exerted similar effects at a physiological level.

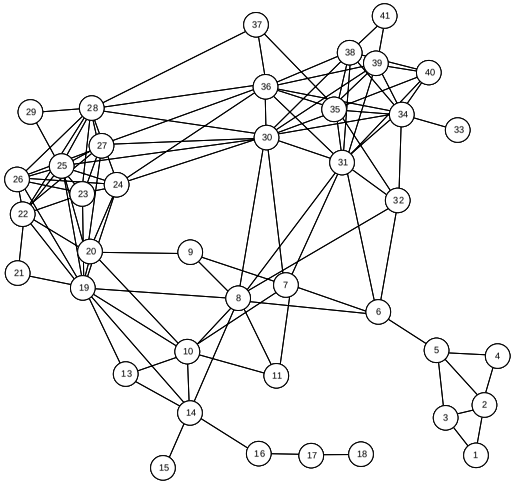

Figure 4. Network plot indicating all correlations between plasma lipid, β-hydroxybutyrate, glucose and insulin levels, liver lipid levels, and liver protein levels that had a Pearson correlation coefficient higher than 0.8, a relating P-value of 0.0053 or lower, and a relating q-value of 0.026 or lower

1 Glutathione S-transferase Mu 2
2 Long chain acyl-CoA thioester hydrolase B
3 Cysteine sulfinic acid decarboxylase
4 Long chain acyl-CoA thioester hydrolase A
5 Catalase
6 Liver Free Cholesterol
7 3-hydroxyanthranilate 3,4-dioxygenase
8 Adenosine kinase
9 Peroxyredoxin 6
10 Plasma Cholesterol
11 Senescence marker protein 30
13 Liver Cholesteryl Esters
14 Phenylanaline-4-hydroxylase
15 Glycine-N-methyltransferase
16 Ornithine aminotransferase
17 Liver weight
18 Tetranectin
19 Adenosylhomocysteinase
20 Aldehyde dehydrogenase
21 Pyrophosphatase
22 Tubulin alpha-2 chain
23 Delta-aminoevulinate dehydratase
24 Selenium binding protein 56kDa
25 Malate dehydrogenase
26 Thioether S-methyltransferase
27 Formimino cyclodeaminase
28 Nicotinate-nucleotide pyrophosphorylase
29 T-complex protein 1, beta subunit
30 Fructose biphosphatase 1
31 Plasma Free Fatty Acids
32 Glutamine synthase
33 Plasma Glucose
34 Ketohexokinase
35 Sepiapterin reductase
36 Alpha enolase
37 Phosphatidylethanolamine-binding protein
38 Plasma Triglycerides
39 Liver Triglycerides
40 Nucleoside diphosphate kinase B
41 Proteasome beta subunit

Both treatments significantly lowered plasma cholesterol and TG concentrations as compared with a saturated fat control diet, which coincided with a significant parallel reduction in levels of cholesteryl esters, free cholesterol, and TG in the liver. These effects are likely to be caused by an enhanced FA oxidation rate, as indicated by the level of β-hydroxybutyrate in plasma.

Proteome analyses of the mouse liver samples also revealed an increase in the rate of FA oxidation, as levels of catalase and long-chain acyl-CoA thioester hydrolase (both the cytosolic and the mitochondrial form) were significantly increased upon treatment with fish oil and fenofibrate. Till now, long-chain acyl-CoA thioester hydrolase has not been linked to specific dietary FA treatments. Acyl-CoA thioesterases hydrolyze CoA esters of various lengths to free FA and CoA-SH, and they are likely to play important roles in maintaining appropriate CoA-SH levels during periods of increased β-oxidation and FA overload[40]. The existence of selective acyl-CoA thioesterases could provide important control points in the oxidation of many peroxisomal substrates, and they may regulate intracellular levels of CoA esters and CoA-SH. To date, several thioesterase isoforms have been identified in peroxisomes, cytoplasm, and mitochondria, where they are thought to have distinct functions in lipid metabolism[40]. Treatment of mice with the peroxisome proliferator clofibrate also induced levels of long-chain cytosolic, mitochondrial, and peroxisomal acyl-CoA thioester activity in the liver in a previous study, although the cytosolic form was most strongly induced[41].

The lowering in liver lipids in the fish oil and fenofibrate groups matched a large reduction of the level of adipophilin in the liver on the 2D electrophoresis gels (**Figure 2**). Adipophilin is a protein associated with lipid storage droplets, which are dynamic structures that function as storage deposits for TG and cholesterol esters[42].

Although fish oil and fenofibrate are believed to share common modes of action, our PCA did show diverse treatment effects of both dietary interventions. The proteins responsible for the treatment effect of fish oil were involved in a range of metabolic functions (**Table 4a**), whereas the list of proteins responsible for the treatment effect of fenofibrate was dominated by those involved in β-oxidation of FA. This indicates that fish oil, unlike fenofibrate, triggers a more diverse range of mechanisms that could affect the physiological outcome.

*t*10,*c*12 CLA

Accumulating evidence indicates that CLA, in particular the *t*10,*c*12 isomer, may affect lipoprotein metabolism. We observed a significant increase in hepatic TG levels and a two-fold increase in liver weight upon treatment with *t*10,*c*12 CLA, and these findings were mirrored by a significant increase in hepatic levels of adipophilin. This protein also showed a high loading toward the treatment effect of *t*10,*c*12 CLA in the PCA. Increased expression of adipophilin has been associated with liver steatosis before[43], and recent attention has focused on the excessive accumulation of TG in the liver, or liver steatosis, as part of

the syndrome that involves visceral obesity and dyslipidemia, insulin resistance, and type 2 diabetes mellitus[44]. CLA-mediated liver steatosis has been observed in other studies in different strains of mice[45-53] but not in other animals. Several lines of evidence indicate that hepatic TG accumulation is also a causative factor involved in hepatic insulin resistance[44], and indeed, hyperinsulinemia in *t*10,*c*12 CLA-fed mice has been observed in several studies[50,52,54,55] as well as in our study, as evidenced by an almost three-fold increase in plasma insulin levels (**Table 2**). In a hyperinsulinemic state, a shift in fuel usage from carbohydrates to fat usually occurs, leading to an increase in the rate of β-oxidation of FA as well as an increase in ketogenesis. Indeed, we found a significant increase in levels of plasma keton bodies, as well as increased protein levels of carnitine palmitoyltransferase, catalase, and long-chain acyl-CoA thioester hydrolase upon treatment with *t*10,*c*12 CLA. Higher activity and mRNA expression of various mitochondrial and peroxisomal FA oxidation enzymes upon treatment with a CLA mixture has been described previously in C57Bl/6J mice[50].

The increased ratio of TG to cholesteryl esters in the liver upon feeding *t*10,*c*12 CLA were clearly reflected in the lipid composition of the lipoproteins. The converse effect of *t*10,*c*12 CLA on plasma TG and cholesterol suggests independent mechanisms by which CLA affects these levels. Furthermore, the effect of *t*10,*c*12 CLA on plasma TG levels in mice depends on the mouse strain used. Some studies report that CLA is effective in decreasing TG levels[48,51,56], others report no effect on plasma TG levels[50,55]. The decrease in plasma TG in previous studies has been attributed to an up-regulation of the LDL receptor. It appears therefore, that the overall effect of CLA on plasma TG is determined by two opposite actions of CLA: overproduction of VLDL and up-regulation of LDL receptors. In our APOE3*Leiden model with impaired clearance, the former action apparently dominated and resulted in hypertriglyceridemia.

Elaidic acid

Although studies investigating the mechanism of action of *trans*-FA are limited, several controlled metabolic studies have shown the unfavorable effects of *trans*-FA on lipoprotein metabolism and other biomarkers for CHD. *Trans*-fat has been shown to increase levels of LDL cholesterol and TG[1], and a high intake of *trans-fa*t has been associated with the development of insulin resistance and type 2 diabetes in humans[15,57]. However, in our mouse model, dietary elaidic acid decreased plasma levels of cholesterol and had no effect on plasma levels of TG, glucose, or insulin. However, this comparison was made against a saturated fat control diet, which might have masked the true negative effects of the elaidic acid.

Trans-FA are incorporated into membrane phospholipids and may therefore alter the packaging of the phospholipids and possibly influence the physical properties of the membrane or the activities of the membrane-associated enzymes[58]. We observed a very specific effect of elaidic acid treatment on protein levels in the liver membrane revealed by

principle component analysis. The proteins that provided the largest positive contribution to the differences between the elaidic acid treatment and the other dietary treatments were aldehyde dehydrogenase and CTP synthase. Levels of these proteins were up-regulated by more than 200% by elaidic acid. CTP synthase has been implicated in the regulation of phospholipid biosynthesis, at least in *Saccharomyces cerevisiae*[59].

Comparative analysis of all treatments

Principle component analysis was used to analyze the effects of the various treatments on the protein levels in the complete dataset. This approach visualizes the extent to which different treatments have similar or very different, effects on protein expression. Clearly, fish oil triggered a different treatment effect on cytosolic protein expression compared to all other treatments. Elaidic acid showed the strongest treatment effect on the liver membrane proteins studied.

The pair-wise correlation analysis revealed many associations, resulting in clustering of proteins that are related to each other (**Figure 4**). Some of these relations have been described before and are therefore consistent with previous studies, adding validity to the novel associations revealed by our study of the APOE*3Leiden mouse. For example, the associations within the cluster containing plasma and liver TG, plasma glucose, plasma free FA, and protein levels of hepatic fructokinase and fructose 1,6 bisphosphatase are all related to dyslipidemia and glucose intolerance, two important conditions related to the metabolic syndrome or Syndrome X. It is striking, that this cluster is observed already in a data set with relatively mild perturbations and at equal body weights but with clearly different liver weight and composition. The position of sepiapterin reductase in the middle of this cluster is unexpected. However, sepiapterin reductase is involved in the biosynthesis of tetrahydrobiopterin, an essential co-factor for eNOS activity[60], and may therefore play a role in the relationship between dyslipidemia, insulin resistance and endothelial dysfunction[61,62].

A second recognized cluster is that of the proteins catalase and two different forms of long-chain acyl CoA thioester hydrolases, which are related to the β-oxidation of FA. The addition of cysteine sulfinic acid decarboxylase to this cluster has, however, not been described before. Cysteine sulfinic acid decarboxylase is a rate-limiting enzyme for taurine biosynthesis, and taurine can be tissue-protective in many models of oxidant-induced injury[63]. Therefore, cysteine sulfinic acid decarboxylase, like catalase, might be involved in the protection of cells against oxidative stress generated by FA oxidation.

Proteomics of diet-induced changes in the liver of APOE*3Leiden mice revealed a wide array of proteins that were affected by the various dietary interventions. Our approach visualized the 500-800 most abundant proteins from a liver cell on a 2D electrophoresis gel, and this provided a detailed overview of novel and recognized alterations in lipid degradation and glycolysis pathways, reflecting changes in lipoprotein and glucose metabolism upon dietary treatment. We found, for example, that the consumption of

specific dietary FA induced a differential expression of long chain acyl-CoA thioester hydrolase protein (as an indicator of β-oxidation) and adipophilin (as an indicator of liver lipid content). Statistical analysis of our results revealed many associations, some of which are well known (like the metabolic syndrome), whereas others will be the basis of intriguing new leads for further studies.

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