Effects of Elaidic Acid on Lipid Metabolism in HepG2 Cells, Investigated by an Integrated Approach of Lipidomics, Transcriptomics and Proteomics

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Abstract

Trans fatty acid consumption in the human diet can cause adverse health effects, such as cardiovascular disease, which is associated with higher total cholesterol, a higher low density lipoprotein-cholesterol level and a decreased high density lipoprotein-cholesterol level. The aim of the study was to elucidate the hepatic response to the most abundant trans fatty acid in the human diet, elaidic acid, to help explain clinical findings on the relationship between trans fatty acids and cardiovascular disease. The human HepG2 cell line was used as a model to investigate the hepatic response to elaidic acid in a combined proteomic, transcriptomic and lipidomic approach. We found many of the proteins responsible for cholesterol synthesis up-regulated together with several proteins involved in the esterification and hepatic import/export of cholesterol. Furthermore, a profound remodeling of the cellular membrane occurred at the phospholipid level. Our findings contribute to the explanation on how trans fatty acids from the diet can cause modifications in plasma cholesterol levels by inducing abundance changes in several hepatic proteins and the hepatic membrane composition.

Introduction

Despite increasing evidence of an adverse relationship between the human dietary intake of industrially produced trans fatty acid (IP-TFA) and cardiovascular disease [1], IP-TFAs are still common constituents of foodstuffs [2]. IP-TFA is in semi-solid fat produced by the partial hydrogenation of vegetable oils. The main isomer present is elaidic acid (EA), a monounsaturated C18 fatty acid containing a double bond at position 9 in the trans configuration [3,4]. A dietary intake of IP-TFA results in an adverse blood lipid profile with increased total cholesterol and low density lipoprotein-cholesterol level and a decreased high density lipoprotein-cholesterol (HDL-C) and a decreased level of high density lipoprotein-cholesterol (HDL-C) [5,6]. Meta-analyses of prospective cohort studies showed the incidence of coronary heart disease increased by 27% (95% CI=1.14-1.42) when 2 Energy% of monounsaturated fatty acids in the diets were replaced by IP-TFA [7].

Research aimed at elucidating the mechanisms behind the observed adverse effects of IP-TFA has focused on determining the levels and activities of single molecules involved in cholesterol trafficking between lipoproteins and cells [8,9]. However, the adverse health effects of IP-TFA intake is probably not caused by the altered activity of a single molecule, but rather the sum of changes in the levels and activities of many proteins. So far, omics technologies have only been applied to a limited extent, which include two studies of the hepatic response to EA. The first study used 2D-electrophoresis and investigated the hepatic response to EA in a hyperlipoproteinemic transgenic mouse, but only a low number of proteins were identified and even fewer were in response to the IP-TFA [10]. The second study, based on transcriptomics found that many genes were differentially expressed in hepatic tissue from mice fed on a high IP-TFA diet, but an appropriate high fat control group was absent,
making it difficult to discriminate between effects derived from high fat and IP-TFA [11].

In this study we have applied a parallel proteomic, transcriptomic and lipidomic approach to unravel the hepatic cellular response to EA. A hepatocyte cell line (HepG2-SF) optimized for serum free conditions was used, allowing complete control of free fatty acid (FFA) supplemented medium composition and unambiguous identification of HepG2 secreted proteins. Stable isotope labeling by amino acids in cell culture (SILAC) was employed to investigate the long-term effects of incubation in medium supplemented with common dietary non-polyunsaturated C18 fatty acids, such as EA (trans-Δ9-C18:1), oleic acid (cis-Δ9-C18:1) (OA) or stearic acid (C18: 0) (SA) on the HepG2-SF protein expression. Furthermore, gene expression microarray analysis (GEMA) was applied to investigate gene expression after supplementation with EA or OA. Proliferation of the HepG2-SF cells in the variously supplemented media, along with their content of various fatty acids (FA) in the phospholipid (PL) fraction was measured. Our work contributes to the explanation of the molecular mechanisms behind the clinical findings regarding TFA intake and adverse health effects.

Experimental Procedures

Cell culture

HepG2-SF cells (Cell Culture Service, cell passage 42) were propagated in serum-free medium composed of RPMI-1640 (Invitrogen), 10% SynQ (Cell Culture Service), 4 mM L-glutamine (Sigma-Aldrich), 20 U/mL penicillin (Invitrogen) and 20 μg/mL streptomycin (Invitrogen). Nunc culture flasks (75 cm²) were maintained at 37°C in a 5% CO₂ humidified incubator. The cell culture medium was changed every four days, and the cells were subcultured every two weeks.

SILAC adaptation of HepG2-SF cells

Medium was prepared using RPMI-1640 (Sigma) and SynQ (Cell Culture Service) without arginine, lysine and leucine. The medium was supplemented with leucine and lysine at final concentrations of 0.38 mM and 0.87 mM, respectively. From this medium, three different SILAC growth media were prepared containing 1.15 mM Arg (Sigma), 13C₆Arg or 15N₆Arg (Cambridge Isotope Laboratories). The cells were cultured for five doublings and tested by matrix-assisted laser desorption/ionization MS for full incorporation of 13C, 15N, 14N, and 15N, 14N Arg prior to FFA incubation.

Incubation of HepG2-SF cells in FFA-supplemented media for GEMA and SILAC experiments

FFA-supplemented medium was prepared as follows: FFAs in a 2:1 complex with human serum albumin (HSA) [12] were adjusted to a final concentration of 100 µM in serum free medium or SILAC medium. FFAs and lyophilic HSA were all obtained from Sigma-Aldrich.

Two 75 cm² culture flasks containing 90% confluent cells were trypsinized and seeded into 12 petri dishes representing the three different FFA-supplementations with four biological replicates. Cells were allowed to attach for 24 h before 3 mL FFA-supplemented medium was added. The medium was changed on days 3 and 6. Only 1 mL of FFA-supplemented medium was added on day 6. After 24 h, the cell supernatant was aspirated and centrifuged at 1000 x g for 5 min.

The cleared cell supernatant was kept on ice until further processing. Cells for GEMA were trypsinized, washed three times in PBS and stored at -80 °C before RNA extraction. Cells for SILAC were washed in PBS (Invitrogen) and lysed in lysis buffer (1% NP-40 (Sigma-Aldrich), 150 mM NaCl, 50 mM Tris-HCl, pH 7.4) before storage at -20 °C.

Assessment of HepG2-SF growth in FFA-supplemented medium by CyQuant proliferation assay

In a 96 well microtiter-plate, approximately 10000 cells were seeded per well and allowed to attach for 24 h before 300 μL of 100 μM FFA-supplemented medium was added (six replicas for each of the four experimental groups). On days 0, 2, 4 and 6, proliferation was assessed by adding 200 μL CyQuant dye in lysis buffer (Invitrogen) per well. After 5 min incubation, fluorescence (480 nm excitation, 520 nm emission) was measured using a FLUOstar Omega (BMG Labtech) until a plateau was obtained. Measurements significantly differing from Controls were determined using the unpaired t-test, two-tailed with a 95% confidence interval.

PL extraction and characterization

A suspension (1 mL volume) containing at least 4 x 10⁷ cells (originating from the cell culture after thorough washings in PBS) was lysed by the addition of water and centrifuged at 14000 rpm for 40 min at 4°C. The membrane pellet was used to perform PL extraction as previously described [13]. The PL fraction was treated with 0.5 M KOH/MeOH for 10 min at room temperature, and the corresponding fatty acid methyl esters (FAMES) were formed, extracted with n-hexane, and examined by gas chromatography analyses. Geometrical TFAs were recognized by comparison with standard references obtained by synthesis, as already described [14]. The amounts of the individual FAMES were calculated as a percentage of the total measured FAME and their standard deviations calculated in Excel (Table S1). Measurements significantly differing from Controls were determined using the unpaired t-test, two-tailed with a 95% confidence interval. The data was normalized to the percentage of total measured FAME and plotted as histograms for the four series. Hierarchical clustering was done with the R-package made4 (1.30.0) using the function heatplot.

SILAC sample preparation

Cell medium was depleted of albumin by applying affinity chromatography on a column with a recombinant albumin-binding domain of streptococcal protein G [15]. The flow through was collected and the column regenerated using 20 mM sodium citrate, 150 mM NaCl, pH 2.5. The protein concentration of the depleted cell supernatant was determined using the Quick Start Bradford protein assay (Bio-Rad laboratories) and equal protein amounts from each replicate were pooled. The protein concentration for the cell lysates was...
SILAC Data analysis

Mass spectra were analyzed using MaxQuant software (version 1.0.13.13). The data were searched using Mascot (version 2.1.04, Matrix Science) against human international protein index protein sequence databases (version 3.52 or 3.69) supplemented with frequently observed contaminants and concatenated with reversed copies of all sequences. Quantification mode was set to triple encoding selecting Arg6 and Arg10. Enzyme specificity was set to trypsin. Propionamide was set as a variable modification. The maximum allowed mass deviation was initially set to 7 ppm for monoisotopic precursor ions and 0.6 Da for MS/MS peaks. A maximum of one missed cleavage and two labeled amino acids were allowed. The required false discovery rate was set to 1% at the peptide and protein level with the minimum peptide length set to six amino acids. A minimum of two unique peptides was required for protein identification. For quantification, two ratio counts were at least required. The MS datasets for the supernatant samples and the cell lysates were processed separately. The two datasets were merged in Excel and statistically significant regulated proteins were identified by a p-value < 0.01 (reported as “Significance (A)” by MaxQuant) and fold regulation >1.3. For redundant data resulting from the merging of datasets, the highest fold was reported and used for further analysis. Since the biological replicates were pooled, the reported p-values show the significance of a given protein regulation based on multiple peptides representing the same protein.

GEMA using Human OneArray™

GEMA analysis was performed as described previously [17]. The full dataset have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE34045.

Ingenuity pathway analysis

The SILAC data and the GEMA data was imported into Ingenuity pathway analysis software (IPA) by their RefSeq identifiers. IPA core analyses were performed with default settings and with p-value < 0.01 for fold changes greater than 1.3 for SILAC and BH p-value < 0.01 for fold changes greater than 1.5 for GEMA. Categories within “Molecular and Cellular Functions” and “Disease and Disorders” were investigated. The results were exported and perturbed IPA categories were plotted in Excel with a BH p-value calculated by IPA to indicate the statistical significance of the category perturbation. Functions represented by the three most perturbed categories were manually extracted and the functions commonly perturbed in SILAC and GEMA were plotted in Excel with a BH p-value calculated by IPA for the statistical significance of the function perturbation. All regulated proteins and gene transcripts reported in the IPA category “Lipid Metabolism” were extracted (Table 1).

Results

Terminology

The different groups of HepG2-SF cells incubated in media supplemented with EA, OA, SA or no added FFA, they will be denoted as Elaidic, Oleic, Stearic or Control, respectively. Comparisons of e.g. Elaidic and Oleic will be denoted EvsO and of Stearic and Control Svsc.

HepG2-SF proliferation in FFA supplemented medium

The viability of HepG2-SF cells in the presence of 100 µM FFA was measured by a CyQuant cell proliferation assay, where the level of fluorescence is proportional to the total amount of nucleic acid and thus the total number of cells. Stearic, Oleic and Controls have almost equal growth rates, while Elaidic was slightly impaired in their proliferation rate during an incubation period of six days, particularly after day 4 (Figure 1).

Fatty acid incorporation into the PL fraction of HepG2-SF cell membranes

The FA composition of HepG2-SF cell membrane PLs was analyzed by gas chromatography of the derived FAMEs. The most common FAs present are divided into high (Figure 2A) and low (Figure 2B) abundant FAs. For each of the three FFA groups, a profound change in the FA composition of the plasma membrane PL was observed. The addition of SA (red bars) or OA (green bars) supplemented medium induced a two-fold increase in the SA and OA content as compared to Control (blue bars), from 4.0% to 9.8% and from 21.1% to 43.7%, respectively. In Controls, the concentration of EA was null as expected, since the double bond trans geometry is not naturally produced in eukaryotes. The supplementation with EA (purple bars) resulted in a 27.2% level of EA thereby indicating the effective incorporation similar to that of its cis-isomer, OA. With the high incorporation of supplemented FAs, all other FAs would expectedly decrease due to simple dilution. This dilution is observed to various degrees for almost all of the measured FAs, whereas palmitoleic acid and arachidonic acid levels seem to counteract the dilution caused by high incorporation of EA by being increased. In Stearic there was a significant increase in OA content compared to Control, with hierarchical clustering analysis revealing Stearic and Oleic have similar PL profiles (Figure 2C). To assess a possible combined effect of the PL remodeling on membrane fluidity, the average weighted melting point based on the FA composition was calculated for the membrane PLs for each of the four groups: Oleic (25.1°C)
Table 1. IPA “Lipid Metabolism” genes and proteins regulated in EvsO.

| HUGO gene symbol | Protein name                                      | GEMA (n=4) | SILAC (n=4) | EvsO | EvsO | EvsS | Description                                                                 | Reference* |
|------------------|--------------------------------------------------|------------|-------------|------|------|------|------------------------------------------------------------------------------|------------|
|                  |                                                  | Fold change | Fold change  | Fold change  | Fold change |     | Description                                                                 |            |
| ACAT2            | Acetyl-CoA acetyltransferase                     | 2.3        | 1.37E-05    | 1.8         | 1.40E-07   | -1.1 | Synthesis of acetoacetyl-CoA from acetyl-CoA                                 | [1]        |
| CYP51A           | Lanosterol 14-alpha-demethylase                   | 3.4        | 5.95E-25    | -           | -          | -   | Removes the 14-alpha-methyl group from the lanosterol                         | [2]        |
| DHCR24           | 3-beta-hydroxysterol delta-24-reductase          | 3.0        | 1.27E-01    | 1.5         | 1.57E-04   | 1.7  | Reduces the δ24 double bond of sterol intermediates                           | [3]        |
| DHCR7            | Delta-7-sterol reductase                         | 3.4        | 1.05E-03    | 2.4         | 9.14E-14   | 2.1  | Reduces the δ7 double bond of sterol intermediates                            | [4]        |
| EBP              | 3-beta-hydroxysterol-\(\Delta^8,\Delta^7\)-isomerase | 2.2        | 5.85E-05    | -           | -          | -   | Converts 3-beta-Hydroxysteroid-\(\Delta^8,\Delta^7\) to lathosterol            | [5]        |
| FDFT1            | Farnesylpyrophosphate farnesyltransferase        | 4.1        | 6.63E-03    | 2.8         | 3.80E-18   | 2.5  | Converts trans-farnesylpyrophosphate to squalene                              | [6]        |
| FDP5             | Farnesylpyrophosphate synthetase                 | 1.4        | 1.21E-01    | 1.4         | 1.71E-03   | 1.4  | Converts isopentenylpyrophosphate and dimethylallyl pyrophosphate to both geranyl and farnesylpyrophosphate | [7]        |
| HMGCR            | 3-hydroxy-3-methylglutaryl-CoA reductase          | 2.9        | 7.32E-14    | -           | -          | -   | Synthesis of 3-hydroxy-3-methylglutaryl-CoA                                   | [8]        |
| HMGCS1           | 3-hydroxy-3-methylglutaryl-CoA synthase 1         | 3.8        | 5.27E-08    | -           | -          | -   | Synthesis of 3-hydroxy-3-methylglutaryl-CoA                                   | [9]        |
| HSD17B7          | 17-beta-hydroxysteroid dehydrogenase VII         | 2.3        | 7.58E-02    | 2.0         | 3.31E-09   | 3.8  | Synthesis of 4-alpha-methyl-\(\Delta^5\)-cholest-7-en-3-one                  | [10]       |
| IDI1             | Isopentenyl-diphosphate delta-isomerase          | 2.9        | 8.54E-03    | 1.7         | 6.10E-06   | 1.3  | Converts isopentenyl diphosphate to dimethylallyl diphosphate                | [11]       |
| LSS              | Lanosterol synthase                              | 1.7        | 7.94E-02    | 2.3         | 2.99E-13   | 2.2  | Cyclization of(3S)-\(\Delta^2\)-epoxy-3,3-dihydroxilane to lanosterol        | [12]       |
| MKV              | Mevalonate kinase                                | 2.2        | 3.86E-07    | -           | -          | -   | Converts mevalonic acid into mevalonate-5-phosphate                           | [13]       |
| NSDHL            | NAD(P)H steroid dehydrogenase-like protein       | 2.7        | 6.74E-23    | 1.8         | 4.07E-07   | 1.7  | Removal of two C-4 methyl groups in post-squalene cholesterol biosynth.       | [14]       |
| SQLE             | Squalene epoxidase                               | 3.3        | 8.24E-11    | 1.4         | 1.71E-03   | 1.4  | Epoxidation of C-C double bond of squalene to yield 2,3-oxidosqualene         | [15]       |
| ACLY             | ATP citrate lyase                                 | 1.3        | 7.50E-02    | 2.3         | 2.13E-03   | 1.9  | Synthesis of Acetyl-CoA from citrate                                          | [16]       |
| CYB5B            | Cytochrome b5, type B                            | 1.6        | 1.72E-02    | 1.6         | 4.15E-05   | 1.9  | Electron donor for de novo synthesis                                          | [17]       |
| DLAT             | Dihydropipecolic acid acetyltransferase          | -          | -           | 1.4         | 1.21E-03   | 1.5  | Synthesis of Acetyl-CoA from pyruvat                                          | [18]       |
| SC5DL            | Sterol C5-desaturase                             | 3.5        | 1.77E-10    | -           | -          | -   | Conversion of lathosterol into 7-dehydrocholesterol                           | [19]       |
| ACACA            | Acetyl-CoA carboxylase                           | -1.3       | 5.07E-01    | 1.4         | 2.70E-03   | 1.1  | Rate-limiting in long-chain fatty acid biosynthesis                           | [20]       |
| ACOT1            | Acyl-CoA thioesterase 1                          | 7.1        | 0.00E+00    | -           | -          | -   | Hydrolyze acyl-CoAs to the FFA and CoA                                        | [21]       |
| ACS1             | Acyl-CoA synthetase long-chain family, member 1  | 1.5        | 8.76E-02    | 1.7         | 1.12E-05   | 2.3  | Activation of long chain FAs                                                 | [22]       |
| ACS3             | Acyl-CoA synthetase long-chain family, member 3  | -          | -           | 1.8         | 1.15E-06   | 2.9  | Activation of long chain FAs                                                 | [23]       |
| ACS4             | Acyl-CoA synthetase long-chain family, member 4  | 2.6        | 3.16E-10    | 1.6         | 4.64E-05   | 1.8  | Activation of long chain FAs                                                 | [24]       |
| ELOVL2            | Elongation of very long chain fatty acids-like 2 | -2.7       | 1.40E-03    | -           | -          | -   | Elongation of C20 fatty acids                                                | [25]       |
| ELOVL6            | Elongation of very long chain fatty acids-like 6 | 3.2        | 1.17E-15    | -           | -          | -   | Elongation of PA to SA                                                       | [26]       |
| FADS1            | Fatty acid desaturase 1                          | 4.1        | 3.34E-04    | -           | -          | -   | \(\Delta^5\) desaturase for the synthesis of arachidonic and eicosapentanoic acid | [27]       |
| Protein name                                      | Fold change | Fold change | Fold change | Description                                                                                     | Reference* |
|--------------------------------------------------|-------------|-------------|-------------|-------------------------------------------------------------------------------------------------|------------|
| Fatty acid synthase                              | 1.2         | 4.23E-01    | 1.9         | Conversion of acetyl-CoA and malonyl-CoA, in the presence of NADPH, into long-chain saturated FAs | [28]       |
| 17-beta hydroxysteroid dehydrogenase-12          | 5.2         | 8.75E-05    | 1.7         | Reduce 3-ketoacyl-CoA to 3-hydroxyacyl-CoA during the second step of fatty acid elongation       | [29]       |
| Stearoyl-CoA desaturase                          | 7.2         | 5.36E-04    | -           | ∆9 desaturase for the synthesis of palmitoleic and OA                                           | [30]       |
| Low density lipoprotein receptor                 | 1.9         | 1.49E-09    | 1.0         | Internalizes LDL particles                                                                      | [31]       |
| Transfer RNA, mitochondrial, proline             | 1.7         | 9.60E-04    | 1.1         | Incorporate CE into ApoB lipoprotein                                                            | [32]       |
| Proprotein convertase, Subtilisin/kexin-type 9   | 3.6         | 1.59E-11    | 3.1         | Regulates amount of LDLR on surface                                                             | [33]       |
| Scavenger receptor, class B, member 1            | -1.7        | 1.80E-04    | 1.2         | Bidirectional transfer of C between cells and HDL                                                | [34,35]    |
| Sterol O-acyltransferase 1                       | 2.1         | 1.28E-08    | -           | Esterification of Cholesterol                                                                  | [36]       |
| Tissue factor pathway inhibitor                  | 2.3         | 3.22E-05    | -3.6        | Circulates associated with lipoproteins, regulates cholesterol levels, inhibit the extrinsic coagulation pathway, | [37,38]    |
| Angiopoietin-like 3                              | 4.1         | 1.62E-04    | -           | Inhibit LPL i.e. inhibit clearance of VLDL TAGs                                                | [39,40]    |
| Apolipoprotein A4                                | 2.6         | 2.06E-05    | 1.8         | Part of HDL, promotes C esterification and efflux in mice                                        | [41,42]    |
| Apolipoprotein E                                 | 1.8         | 1.61E-02    | 1.3         | Part of VLDL and chylomicrons, ligand for receptors causing clearance of remnants                | [43]       |
| Apolipoprotein H                                 | 2.2         | 3.33E-11    | -1.4        | Binds to phospholipid macromolecules, constituent of TAG rich lipoproteins, activates LPL       | [44]       |
| Fatty acid-binding protein 1                     | 4.6         | 9.18E-08    | -           | intracellular trafficking, lipid disposal (incl. bile synthesis and secretion)                  | [45,46]    |
| Fatty acid-binding protein 5                     | 2.0         | 3.24E-03    | -2.4        | Mediates arachidonoyl ethanolamide signaling                                                    | [47]       |
| Lipase A, lysosomal acid                         | 2.5         | 1.51E-06    | -1.4        | Release of C from CE                                                                           | [48]       |
| Organic solute transporter, Alpha                | 1.8         | 9.01E-04    | -           | Bil acid transporter                                                                            | [49]       |
| Serpin peptidase inhibitor, clade A, member 6    | 1.9         | 4.63E-07    | 1.0         | Major transport protein for glucocorticoids and progestins in the blood                        | [50]       |
| Serpin peptidase inhibitor, clade A, member 6    | 2.5         | 8.96E-04    | 1.9         | Constitutive apolipoprotein of HDL                                                              | [51]       |
| Apolipoprotein A1                                | 1.5         | 7.50E-04    | 1.1         | Catalyzes the terminal and only committed step in TAG synthesis by using DAG and fatty acyl CoA as substrates | [52]       |
| Apolipoprotein A4                                | 1.6         | 3.97E-06    | -           | Catalyzes the terminal and only committed step in TAG synthesis by using DAG and fatty acyl CoA as substrates | [53]       |
| Pancreatic lipase-related protein 2              | 1.5         | 1.10E-07    | -           | Hydrolysis of TAG into DAG and subsequently into MAG and FFA                                   | [54]       |
| Patatin-like phospholipase domain-containing protein 3 | 2.1         | 1.27E-05    | -           | Hydrolysis of TAG                                                                               | [55,56]    |
| Alkaline ceramidase 3                            | -1.7        | 2.19E-03    | -           | Hydrolysis of long-chain unsaturated ceramides to generate sphingosine                          | [57]       |
| HUGO gene symbol | Protein name                          | EvsO Fold change | EvsO p-value | EvsS Fold change | EvsS p-value | Description                                                                                   | Reference* |
|------------------|--------------------------------------|-----------------|--------------|-----------------|--------------|----------------------------------------------------------------------------------------------|------------|
| ALOX12           | 12-oxireductase                      | 1.8             | 5.49E-03     | -               | -            | Introduction of a molecular oxygen into the C-12 position of arachidonic acid to produce 12(S)-hydroperoxy-5,8,10,14-eicosatetraenoic acid | [58]       |
| GGT5             | gamma-glutamyltranspeptidase 5       | -1.7            | 5.48E-03     | -               | -            | Converst leukotriene C4 to leukotriene D4                                                                 | [59]       |
| GPX4             | Glutathione peroxidase 4             | 1.8             | 9.07E-03     | -               | -            | Reduces phospholipid hydroperoxides                                                                 | [60]       |
| UPH              | Lipase H                             | 1.7             | 1.52E-04     | -               | -            | Synthesis of 2-acyl lysophosphatidic acid                                                                 | [61]       |
| LPIN1            | Lpin1                                | 3.1             | 4.16E-07     | -               | -            | Catalyzes the dephosphorylation of PA to yield diacylglycerol and inorganic phosphate | [62]       |
| LPCAT2           | Lysophosphatidylcholine acyltransferase 2 | -1.7          | 3.37E-03     | -               | -            | Catalyzes the formation of CDP-ethanolamine from ethanolamine                                                                 | [63]       |
| PCYT2            | Phosphatidylinositol 3-kinase class III | 6.2            | 2.51E-03     | -               | -            | Catalyzes the formation of phosphatidylinositol 3-phosphate, Involved in the transport of lysosomal enzyme precursors to lysosomes | [64]       |
| SGPL1            | Sphingosine-1-phosphate lyase 1      | 2.0             | 1.25E-04     | -               | -            | Hydrolysis of sphingosine-1-phosphate to sphingosine                                                                                          | [66]       |
| AKR1C4           | Aldo-keto reductase family 1, member C4 | 3.1            | 1.78E-05     | -               | -            | Catalyzes an NAD(P)-dependent reversible oxidation of the 3-alpha-hydroxy group of various steroids and functions in the metabolism of steroid hormones and bile acids | [67,68]   |
| DBI              | Diazepam binding inhibitor           | 2.6             | 1.61E-03     | -               | -            | Catalyzes the formation of CDP-ethanolamine from ethanolamine                                                                 | [69]       |
| NPC2             | NPC2 gene                            | 2.8             | 8.41E-03     | -               | -            | Catalyzes the dephosphorylation of PA to yield diacylglycerol and inorganic phosphate                                                               | [70]       |
| SULF2A1          | Sulfotransferase family 2A           | 1.8             | 3.44E-05     | -               | -            | Catalyzes the formation of phosphatidylinositol 3-phosphate, Involved in the transport of lysosomal enzyme precursors to lysosomes | [71]       |

**Table 1 (continued).**

**Bile acid biosynthesis and secretion**

| HUGO gene symbol | Protein name                          | EvsO Fold change | EvsO p-value | EvsS Fold change | EvsS p-value | Description                                                                                   | Reference* |
|------------------|--------------------------------------|-----------------|--------------|-----------------|--------------|----------------------------------------------------------------------------------------------|------------|
| ADIPOR1          | Adiponectin receptor 1               | 1.6             | 4.58E-03     | -               | -            | Receptor for adiponectin, mediate FA oxidation, glucose uptake, PPARalpha ligand activities, | [72]       |
| AKT2             | V-akt murine thymoma viral oncogene homolog 2 | -1.9           | 2.03E-04     | -               | -            | Signaling, insulin responsive, FA oxidation                                                                                                         | [73]       |
| BRCA1            | Breast cancer 1 gene                 | 2.0             | 1.40E-03     | -               | -            | Binds to phosphorylated ACACA preventing enzyme activity                                                                                           | [74]       |
| CEBPA            | CCAAT/enhancer-binding protein alpha | 3.7             | 2.01E-03     | -               | -            | Binds to phosphorylated ACACA preventing enzyme activity                                                                                           | [75]       |
| DLK1             | Delta, Drosophila, Homolog-like 1    | 4.8             | 2.18E-12     | -               | -            | Catalyzes the formation of CDP-ethanolamine from ethanolamine                                                                                     | [76]       |
| IDE              | Insulin-degrading enzyme             | 1.6             | 9.23E-03     | 1.0             | 3.77E-01     | Regulates lipid catabolism through degradation of insulin                                                                                          | [77]       |
| NPPA             | Natriuretic Peptide Precursor A      | 1.8             | 5.09E-03     | -               | -            | Regulates lipid catabolism through degradation of insulin                                                                                          | [78]       |
| SCAP             | SREBP cleavage-activating protein    | -1.7            | 5.20E-03     | -               | -            | Catalyzes the dephosphorylation of PA to yield diacylglycerol and inorganic phosphate                                                               | [79]       |
| SREBF2           | Sterol regulatory element-binding protein-2 | 2.3            | 3.62E-03     | -               | -            | Catalyzes the dephosphorylation of PA to yield diacylglycerol and inorganic phosphate                                                               | [80]       |
| ZNF202           | Zinc finger protein 202              | 2.0             | 1.50E-03     | -               | -            | Transcriptional repressor of several proteins in lipid metabolism and efflux                                                                    | [81]       |
| HUGO gene symbol | Protein name | GEMA (n=4) | SILAC (n=4) | EvsO | EvsO | EvsS | Description | Reference* |
|------------------|--------------|------------|-------------|------|------|------|-------------|------------|
| ATP5A1           | ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1 | 1.6 | 3.19E-04 | 1.0  | 4.16E-01 | 1.1  | 3.96E-01 | Oxidative phosphorylation | [83] |
| ATP5B            | ATP synthase, H+ transporting, mitochondrial F1 complex, beta subunit 1 | 1.5 | 4.87E-04 | 1.0  | 4.65E-01 | 1.0  | 4.71E-01 | Oxidative phosphorylation | [84] |
| DCI              | Dodecenoyl-CoA delta isomerase | 1.5 | 2.55E-03 | 1.1  | 3.05E-01 | 1.6  | 6.28E-02 | Beta-oxidation of unsaturated FA with double bonds at odd positions | [85] |
| ALDH1A1          | Aldehyde dehydrogenase 1 family, member 1A | 1.7 | 6.33E-04 | 1.5  | 7.83E-02 | -2.0 | 4.66E-02 | Oxidation of both all-trans- and 9-cis-retinal. May play a vital role in the formation of retinoic acid. Detoxification of peroxidic aldehydes produced by ultraviolet light absorption | [86,87] |
| ALDH1A3          | Aldehyde dehydrogenase 1 family, member 3A | -1.7 | 1.64E-05 | -     | -     | -     | -     | Role in detoxification of peroxidic aldehydes. | [88] |
| AOX1             | Aldehyde oxidase 1 | 1.7 | 4.88E-03 | -     | -     | -     | -     | AOX1 is involved in retinoic acid synthesis | [89] |
| DHRS3            | Short-chain dehydrogenases/reductases family, member 3 | 1.9 | 1.76E-06 | -     | -     | -     | -     | Generating storage forms of retinol (retinal to retinol) | [90,91] |
| CD9              | CD9 antigen | 2.0 | 4.18E-04 | -     | -     | -     | -     | Undergoes acylation with palmitic acid | [92] |
| F2               | Coagulation factor II | 2.0 | 5.89E-03 | -1.7 | 1.20E-01 | -1.5 | 7.64E-02 | Converts fibrinogen to fibrin for blood clot formation | [93] |
| GNAQ             | Guanine nucleotide-binding protein, Q poly peptide. | -1.7 | 3.69E-03 | -     | -     | -     | -     | G-alpha-q is the alpha subunit of one of the heterotrimeric GTP-binding proteins that mediates stimulation of phospholipase C-beta | [94] |
| GNE              | UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase | 2.5 | 1.53E-04 | -     | -     | -     | -     | GNE is the rate-limiting enzyme in the sialic acid biosynthetic pathway | [95] |
| HPX              | Hemopexin | 2.0 | 2.11E-07 | -     | -     | -     | -     | Binds one heme with high affinity and transports it to hepatocytes for salvage of iron. Protects LDL from oxidation | [96,97] |
| HSD17B2          | 17-beta-hydroxysteroid dehydrogenase II | 1.9 | 1.90E-03 | -     | -     | -     | -     | Catalyzing the interconversion of testosterone and androstenedione, as well as estradiol and estrone | [98] |
| KCNMA1           | Potassium channel, calcium activated, large conductance, subfamily M, alpha member | -1.7 | 1.02E-06 | -     | -     | -     | -     | Genome wide association study identifies KCNMA1 contributing to human obesity | [99] |
| KNG1             | Kininogen 1 | 3.4 | 8.56E-28 | -     | -     | -     | -     | Contains the bradykinin peptide which upon release is a potent inflammatory mediator that causes vasodilation and enhanced capillary permeability, induces pain, and stimulates production of nitric oxide and prostacyclin | [100] |
| LPP              | Lipoma preferred partner | 2.4 | 2.28E-03 | -     | -     | -     | -     | Involved in lipoma | [101] |
| PHGDH            | Phosphoglycerate dehydrogenase | 1.8 | 1.67E-03 | -1.1 | 2.25E-01 | -1.4 | 1.98E-01 | transition of 3-phosphoglycerate into 3-phosphohydroxypyruvate in serine biosynthesis | [102] |
| PPP1R3C          | Protein phosphatase-1, regulatory subunit 3C | 3.0 | 8.78E-18 | -     | -     | -     | -     | glycocon-targeting subunit for PPI, Activates glycogen synthase, reduces glycogen phosphorylase activity and limits glycogen breakdown. | [103] |
| HUGO gene symbol | Protein name | Description | Reference* |
|------------------|--------------|-------------|------------|
| PPT1             | Palmitoyl-protein thioesterase 1 | Removes thioester-linked fatty acyl groups such as palmitate from modified cysteine residues in proteins or peptides during lysosomal degradation. Involved in neurodegenerative disease INCL through altered cholesterol metabolism. | [104] |
| PRPF19           | Pre-mRNA processing factor 19, homolog of S. Cerevisiae | Found associated with lipid droplets in mice. Plays a role in DNA double-strand break (DSB) repair and pre-mRNA splicing reaction | [105,106] |
| SCNN1A           | Sodium channel, non-voltage-gated 1 alpha subunit | association with fasting insulin levels, hypertension related | [107,108] |
| SERPINC1         | Serpin peptidase inhibitor, clade C, member 1 (Antitrombin) | regulates blood coagulation cascade | [109] |
| SERPINE1         | Serpin peptidase inhibitor, clade E, member 1 (Plasminogen activator inhibitor 1) | regulator of fibrinolysis. Binds to lipoprotein receptor-related protein (LRP) for degradation. | [110,111] |
| SMEK2            | Suppressor of MEK1, Homolog of Dictyostelium 2 | involved in responsiveness to dietary cholesterol and regulation of liver triglyceride (TG) levels in rats | [112] |
| SNTB1            | Syntaxin beta-1 | involved in responsiveness to dietary cholesterol and regulation of liver TG levels in rats | [113] |
| STX12            | Syntaxin-12 | Putative protein involved in the vesicular trafficking in insulin signaling | [114] |
| SUCLG2           | Succinate-CoA ligase, GDP-forming, beta subunit | Catalyzes the GTP-dependent ligation of succinate and CoA to form succinyl-CoA | [115] |
| TTR              | Transthyretin | Associates with HDL through ApoAI. Thyroid hormone-binding protein, serum and cerebrospinal fluid (CSF) protein that transports holo-retinol-binding protein (RBP; 180250) and thyroxine (T4). | [116,117] |
| WWDOX            | WW-domain-containing oxidoreductase | Influences HDL-C levels | [118] |
| YWHAH            | Tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein, eta isoform | regulator of PDPK1 | [119] |

All genes/proteins were assigned manually to a function group based on the known literature. All genes and proteins were reported with the HUGO gene symbol, protein name, fold regulation, p-value and a description of the function according to a reference. Bold numbers indicates statistical significance of p < 0.01 for SILAC and BH p-value < 0.01 for GEMA. * Table references can be found in Table S4.

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Control (26.5°C) < Stearic (27.6°C) < Elaidic (30.7°C) (Table S1). Taken together, the PL profiles of supplemented cells suggest a profound PL remodeling in cells incubated in the presence of EA and a decreased fluidity of the membrane compartment.

Genes found regulated after FFA supplementation
A total of 15791 transcripts were quantified across all groups in the GEMA of the cellular response. Of these, 11534 transcripts were unique based on HUGO gene symbols. After BH correction of data, a cutoff p-value <0.01 was chosen together with a fold change cutoff of 1.5. The following comparisons were considered: OvsC, EvsC and EvsO. Fewest regulations were observed in the OvsC comparison where only 30 transcripts were differentially regulated, while EvsC showed 587 differentially regulated transcripts. The highest number of regulations was in the EvsO comparison with 793 transcripts differentially regulated in response to the supplemented FFAs. A list of the transcripts differentially regulated in the three comparisons (fold change greater than 1.5, Benjamini-Hochberg multiple testing corrected p-value (BH-p-value) < 0.01) appear in Table S2.

Proteins found regulated after FFA supplementation
SILAC was used to investigate the HepG2-SF cellular response to EA, OA and SA at the protein level. The analyses resulted in the quantification of 1273 non-redundant proteins, represented by unique HUGO gene symbols, across all three groups. Comparisons involving EvsO, EvsS and SvsO were selected with fold changes greater than 1.3 and p-value<0.01 chosen as cutoff values. The highest number of regulations was observed in EvsO with a total of 88 protein regulations, while EvsS showed 76 protein regulations and SvsO showed 79 protein regulations (Full MaxQuant output can be obtained by contacting corresponding author).

Comparison of SILAC and GEMA data
The combined search space for SILAC and GEMA contained 11887 unique gene transcripts and proteins with HUGO gene symbols. The overlapping search space for the two methods contained 920 quantified gene transcripts/proteins (Figure 3A). Based on this combined search space, 866 transcripts/proteins were found to be differentially regulated in the EvsO comparison by SILAC and/or GEMA (Figure 3B), where 15 of these were determined by both SILAC and GEMA to be up-regulated, while no entries were down-regulated mutually by SILAC and GEMA. Three transcripts/proteins were found down-regulated by SILAC but up-regulated by GEMA. From their respective search spaces of proteins and transcripts, both SILAC and GEMA found 7% of these to be regulated in Elaidic when compared to Oleic.
Figure 2. The FA composition of HepG2-SF phospholipids analyzed by gas chromatography. The composition of phospholipids from HepG2-SF cells incubated 6 days in 100 µM oleic, elaidic, or stearic acid supplemented medium were determined. Panel A and Panel B are the high and low abundant FAs present in the PLs, respectively. Each bar represents the average of three biological replicas. The y-axis is the percent of total FA methyl esters measured. The x-axis indicates the different PL FAs measured as their methyl esters. Color coding corresponds to the different supplemented FFAs. Panel C shows a heatmap representation of the distribution of FAs in PLs of the HepG2-SF cells after supplementation. The FA composition of HepG2-SF phospholipids change depending on the different supplemented fatty acids, Stearic and Oleic profiles are alike whereas the Elaidic FA PL profile differs from all the other groups. * marks measurements significantly differing from controls using the unpaired t-test, two-tailed with a 95% confidence interval.

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Ingenuity pathway analysis (IPA) on datasets obtained by SILAC and GEMA

The quantified proteins and gene transcripts found by SILAC and GEMA were subjected to IPA analysis. The IPA network eligible molecules included 1270 proteins quantified in SILAC and 10416 transcripts quantified in GEMA. Of the network eligible molecules, 85 proteins revealed by SILAC analysis were differentially regulated in the EvsO comparison, whereas the number of differentially regulated genes found by GEMA was 647.

Proteins and transcripts that were differentially regulated in the EvsO comparison were analyzed separately by IPA in the areas of “Molecular and Cellular functions” and “Disease and Disorders”. The top three categories significantly affected in Elaidic when compared to Oleic were “Lipid Metabolism”, “Small Molecule Biochemistry” and “Vitamin and Mineral Metabolism” (Figure 4A). The top 24 functions common to both GEMA and SILAC data included in the three categories contained 118 unique genes/proteins (Figure 4B). The affected functions were mainly synthesis/metabolism of cholesterol/sterols together with more general functions related to lipid metabolism, with the 104 regulated proteins/transcripts from the Lipid Metabolism category divided into functions based on manual assignment and described in detail (Table 1).

For the SILAC analysis we also cultured HepG2-SF cells in media supplemented with SA to enable EvsS and SvsO comparisons. The affected IPA categories revealed that EvsO (blue bars) and EvsS (yellow bars) largely perturbs the same categories, while the SvsO (red bars) comparison revealed relatively few affected IPA categories that also possessed –Log(BH p-value) higher than 2 (Table S3). From the 76 proteins significantly regulated in EvsS, 27 proteins were also found in EvsO. EvsS regulation data are shown in Table 1 for the proteins/gene transcripts already identified in the IPA category “Lipid Metabolism” for the EvsO comparison.

Additional statistically significant protein regulations related to the functions of “Lipid Metabolism” were found in EvsS. These were: Glutathione S-transferase A2, GSTA2; Adipose differentiation-related protein, ADFP; Apolipoprotein B, APOB; apolipoprotein M, APOM; acyl-CoA synthase short-chain family member 3, ACS3 and prosaposin, PSAP. An integrated part of IPA is “Canonical Pathways” from the KEGG database, where the KEGG pathway “Biosynthesis of Steroids” was significantly perturbed (p-value < 0.05) in Elaidic when compared to Oleic. This was observed for both SILAC and GEMA.

Discussion

The aim of this study was to use transcriptomic, proteomic and lipidomic approaches to elucidate cellular responses that contribute to the observed clinical effects of TFA intake. It is important to unravel the mechanisms that contribute to the adverse effects of TFA. The intake of TFAs may increase the total cholesterol:HDL-C and LDL-C:HDL-C ratios [5,6] and increase the risk of cardiovascular diseases [1].

HepG2 SF cells show impaired proliferation rate when supplemented with EA, whereas cellular proliferation during OA supplementation does not differ from Controls. However, EA is not the only TFA present in human diet, several other TFAs are present at similar or lower amounts and these may also contribute to the negative health effects observed in relation to TFA containing diets. Analysis of platelets from patients with coronary artery disease showed a correlation of both EA and trans-10-C18:1 with disease risk [18], whereas our own...
Figure 4. IPA categories and functions specifically perturbed by EA as compared to OA, comparing GEMA and SILAC data. Panel A displays perturbed IPA categories. The y-axis reports the significances of the perturbation and is plotted as minus log of the BH multiple testing corrected p-value calculated by IPA based on the specific data set. Panel B displays the 24 significantly perturbed functions found in the top three IPA categories for both SILAC and GEMA. For both panels, the dotted line is the threshold BH-p-value 0.01, with orange bars representing the GEMA data and the blue bars representing the SILAC data.
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analysis of the HepG2 response to vaccenic acid (trans-11 C18: 1) shows less pronounced effect on cell proliferation than EA (data not shown). The effect of TFA mixtures on HepG2 cells and on humans will thus most likely depend on the composition and will be difficult to predict. OA have been reported to reverse stearic acid-induced inhibition of cell growth in human aortic endothelial cells [19] and to attenuate the effect of trans-10, cis-12 conjugated fatty acid mediated inflammatory gene expression in human adipocytes [20]. Thus, if a mixture of OA and EA had been used, both the proliferative, transcriptomic, lipidomic and proteomic response may be affected, and the adverse consequence of TFA would be more difficult to investigate. In this study, we have investigated the hepatocellular response to supplementation with single fatty acids to focus on specific responses and especially to compare EA to OA.

From the 11534 transcripts analyzed by GEMA and 1273 proteins analyzed by SILAC, we report 866 gene transcripts and/or proteins with statistically significant altered expression between EA and OA supplemented HepG2-SF cells after 7 days of incubation. The IPA software found that “Lipid Metabolism”, “Small Molecule Biochemistry” and “Vitamin and Mineral Metabolism” were the top ranked perturbed categories, but since the three categories contain an almost identical ensemble of genes/proteins, only the “Lipid metabolism” category was explored further (Figure 4A and Table 1). The molecular functions found perturbed within this category related to lipid metabolism in general and more specifically to cholesterol synthesis/metabolism and FA synthesis/metabolism (Figure 4B and Table 1).

SA was included in the experimental setup to investigate if the effect observed for EA could be attributed to the high FA melting point. An investigation of the regulated proteins in SvsO with IPA found only a few and weakly perturbed IPA categories (Table S3), indicating a common effect for SA and OA on known molecular functions. The common effects of SA and OA can be attributed to a possible conversion of SA to OA by SCD (all abbreviations for protein names used in the following text can be found in Table 1). From the IPA analysis it was also observed that the same categories were perturbed in both EvsO and EvsS comparisons, namely “Lipid Metabolism”, “Small Molecule Biochemistry” and “Vitamin and Mineral Metabolism”. Many of the enzymes responsible for the direct cholesterol synthesis were statistically significant regulated or trending similarly in EvsO and EvsS, indicating that EA is cholesterologenic compared to both SA and OA (Table 1). Selected proteins and possible implications of the response in Elaidic as compared with Oleic are discussed below.

The cholesterol sensing machinery may be affected

The expression of enzymes responsible for cholesterol synthesis depends on the transcription factor SREBP2 and to a lesser extent SREBP1a [21]. Mammalian cells sense the intracellular level of cholesterol through SCAP and Insig1/2. SREBPs interact with SCAP and at high cholesterol levels, the SREBP:SCAP complex is retained in the endoplasmic reticulum, ER, through its interaction with Insig1/2. At low levels of cholesterol, the SREBP:SCAP complex dissociates from Insig1/2 and translocates from the ER to the Golgi. After two proteolytic cleavages, the active transcription factor, nuclear SREBP, is formed. Upon further translocation to the nucleus nuclear SREBP activates transcription of the cholesterol synthesizing genes [22]. Upon EA supplementation, we observed SREBP2 to be statistically significant up-regulated and SCAP to be statistically significant down-regulated, while Insig2 showed a trend towards up-regulation (but was not found to be statistically significant). These changes indicate an alteration in the cholesterol sensing and synthesis inducing machinery (Table S2).

Essentially all proteins involved in cholesterol synthesis are up-regulated by EA

Besides the acetyl-CoA synthesizing enzymes ACLY and DLAT, 16 enzymes in the cholesterol synthesis pathway were found statistically significant up-regulated, ranging from 1.5 to 4.1 fold, including the rate-limiting enzyme HMGCR, which shows an up-regulation of 2.9 fold in GEMA. Other studies have reported increased cholesterol synthesis, export and total cholesterol in HepG2 cell supernatants as a response to long-term supplementation with EA compared to OA [23]. We observed that the expression of essentially all of the enzymes responsible for cholesterol synthesis was increased after seven days of incubation with EA, verifying a sustained effect on cholesterol synthesis.

FA synthesis is affected

High levels of cholesterol are cytotoxic for cells [24] and thus excess cholesterol has to be esterified with activated FAs for export or storage. ACACA is the rate-limiting enzyme in the synthesis of long chain FA [25] and both ACACA and FAS (which is responsible for the synthesis of palmitic acid (PA) from acetyl-CoA) were significantly up-regulated. ELOVL6 was also significantly up-regulated and is responsible for elongation of PA to SA. SCD desaturates both PA and SA to yield the monounsaturated fatty acids palmitoleic acid and OA, respectively, and was found significantly up-regulated. The up-regulation of these proteins in the FA synthesis indicates that supplementation with EA initiates synthesis of fundamental FAs like PA, palmitoleic acid, SA and OA.

DGAT1 and 2 synthesize triacylglycerol (TAG) from diacylglycerol (DAG) and CoA-activated FAs, which is the terminal and only committed step in TAG synthesis. Transcripts for the two proteins were found up-regulated which could point to a cytosolic accumulation of FAs as TAGs in lipid droplets [26]. PNPLA3 was upregulated, and with new studies showing its physiological role involving the synthesis of TAG [27], it supports the regulations of DGAT1 and 2. In the PL analysis (Figure 2A), it was observed that the combined amount of PA and palmitoleic acid in Elaidic was higher compared to both Oleic and Stearic, which could be explained by an increased FA de novo synthesis. The observation of a lower PA level and a higher level of palmitoleic acid in Elaidic than in Oleic and Stearic could be explained by an increase in desaturase activity as a consequence of the observed increase in SCD expression. This could also explain the low level of SA and lower than expected dilution of OA in Elaidic PLs.
Cholesterol ester synthesis and Export seems increased

It has been shown in mice that SCD is essential for the synthesis of cholesterol esters (CE) because it provides the unsaturated FAs for the CE synthesis from cholesterol [28]. ACSL1, 3 and 4, which are needed for the activation of FFAs with CoA, were all found significantly up-regulated together with SOAT1, which esterifies free cholesterol with activated FFAs [29], and MTTP, which is capable of incorporating CE into ApoB lipoproteins. The accumulation of CE should decrease CE synthesis, but this can be counteracted by MTTP via incorporating CEs into ApoB100 containing very low density lipoprotein (VLDL) for secretion [30]. We did not find any ApoB100 expression increases in either GEMA or SILAC, while an earlier study reported no change in the ApoB content of cell supernatants from HepG2 cells treated with EA or OA [23]. The same study reported that the cholesterol content in secreted VLDL, LDL and HDL increased 43%, 70% and 34%, respectively, upon EA supplementation.

Bile synthesis

Another route of cholesterol export is in the form of bile acids. We found several up-regulated proteins which may suggest an increased export of cholesterol into the bile. These include SULT2A1 and NPC2, which increases bile hydrophilicity by sulfonation and mediates biliary cholesterol efflux through the ABCG5/G8 transporter [31] respectively.

Cholesterol import may be decreased by elaidic acid supplementation

Both LDL-receptor (LDLR) and PCSK9 levels increased in Elaidic (>3 fold in both SILAC and GEMA), most likely as a consequence of increased expression and/or activation of the transcription factor SREBP2 [32]. PCSK9 causes degradation of the LDLR upon binding, resulting in a decreased level of LDLR at the cell surface [33] and thus despite increased synthesis of LDLR, the overall level of the receptor at the cell surface may be decreased. LDLR is responsible for receptor mediated endocytosis of LDL and thus decreased LDLR would lead to decreased uptake of LDL from the surroundings. ApoB/CE containing LDL may therefore accumulate extracellularly and explain the findings by others that ApoB-LDL increases in plasma due to TFA intake [5].

SAA4 is a constitutive apolipoprotein of HDL [34] and ApoA4 is also found associated with HDL or free in plasma [35]. ApoA4 can promote cholesterol efflux from peripheral tissues and esterification into HDL via increased activity of lecithin:cholesterol acyltransferase, LCAT, extracellularly [36]. LCAT is trending towards up-regulation (1.4 fold down, BH-p-value= 0.0448), all together indicating a decreased level of phosphatidylcholine and increased levels of DAG, which could be used for the PE synthesis. LPCAT2 [43] is statistically significant down-regulated while LCAT is trending towards up-regulation (2 fold up, BH-p-value= 0.0448), which are key enzymes in the Land’s Cycle [44] of remodeling of PC. The collective regulation of these genes indicates an increased transfer of acyl chains from PC to cholesterol and thus also contributing to the formation and accumulation of extracellular CEs. It should also be mentioned that TFA in membranes increase the calcium influx into cells [45] and that increased calcium levels activate phospholipase A2, which is essential for lipid remodeling [46].

Another aspect of cholesterol and TFA in PLs is the linear configuration of TFA, which causes the TFA-PLs to pack tightly. TFA-containing artificial PL membranes also tend to bind more cholesterol than membranes without TFA [47]. The membrane cholesterol activity (amount of free cholesterol e.g. for export) is decreased, and the function and activity of integral membrane proteins and channels can be compromised due to the more rigid membrane [45,47,48]. Cell experiments have shown that the viscosity of cell membranes increase when the culture medium was supplemented with EA [41], which is supported by our calculations of relative membrane melting points based on the FA compositions, Oleic (25.1°C) <Stearic (27.6°C) <Elaidic (30.7°C). Studies on model lipid membranes containing TFA also show a reduced permeability [49].

EA has been shown to be rapidly taken up, oxidized for energy production or incorporated into the sn1-position of phospholipids and thereby displacing saturated FAs. This incorporation has been observed to be stable over time [50]. Furthermore, it has been shown ex vivo that the physical
Conclusion

FA remodeling in membrane phospholipids obtained from EA supplemented HepG2 cells was compared with saturated (SA) and monounsaturated (OA) supplementations. The results were integrated with transcriptomic and proteomic data, thus allowing for a comprehensive mapping of effects on lipid biosynthesis, metabolism and membrane status. In the presented study we have found that EA clearly induces an increase in cholesterol synthesis through significant up-regulation of essentially all proteins involved. The expression of proteins involved in FA synthesis, activation and esterification to cholesterol also increased and several proteins were found regulated as a means to export newly synthesized cholesterol and FA. Our finding on the regulation of several proteins involved in cholesterol and FA metabolism contributes to the unraveling of the mechanism behind the EA induced increase in cholesterol synthesis, export and extracellular levels observed by others and the distribution in HDL and LDL particles. The underlying causes of the observed effects warrant further investigations, but our findings has led us to the hypothesis that the cellular cholesterol sensing machinery including Insig1/2 and SCAP is perturbed by the presence of EA. It has been suggested that (i), the cholesterol is trapped by TFA-PLs in membranes [47] causing a lowered cholesterol activity and thus it is inaccessible for the sensory proteins [52], or (ii), the sensory proteins do not function optimally due to increased rigidity of the membrane [47]. As a consequence of lower cholesterol activity, the cell may increase cholesterol synthesis to restore membrane free cholesterol. Alternatively, we speculate that EA could affect the cholesterol sensing proteins directly by blocking the cholesterol binding site or indirectly by causing degradation of the sensory components. Due to the resulting cytotoxicity from cholesterol accumulation, the cell responds by increasing the export of cholesterol. Increased cholesterol synthesis and export in combination with decreased uptake in terms of altered hepatic protein expressions may be the reasons for the observations in the literature where IP-TFA alters total hepatic protein expressions may be the reasons for the observations in the literature where IP-TFA alters total hepatic protein expression.

Supporting Information

Table S1. FAME analysis.

Table S2. GEMA BH corrected COE.

Table S3. Categories graph SILAC.

Table S4. Table references.

Author Contributions

Conceived and designed the experiments: JJE LVN TPK. Performed the experiments: LVN TPK CY. Analyzed the data: ONJ CF CC JJE. Wrote the manuscript: LVN TPK JJE.

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